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(54) **METHODS AND COMPOSITIONS FOR RNA INTERFERENCE**

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(57) **ABSTRACT**

The present invention provides methods for attenuating gene expression in a cell, especially in a mammalian cell, using gene-targeted double stranded RNA (dsRNA), such as a hairpin RNA. The dsRNA contains a nucleotide sequence that hybridizes under physiologic conditions of the cell to the nucleotide sequence of at least a portion of the gene to be inhibited (the "target" gene).

10 Claims, 68 Drawing Sheets

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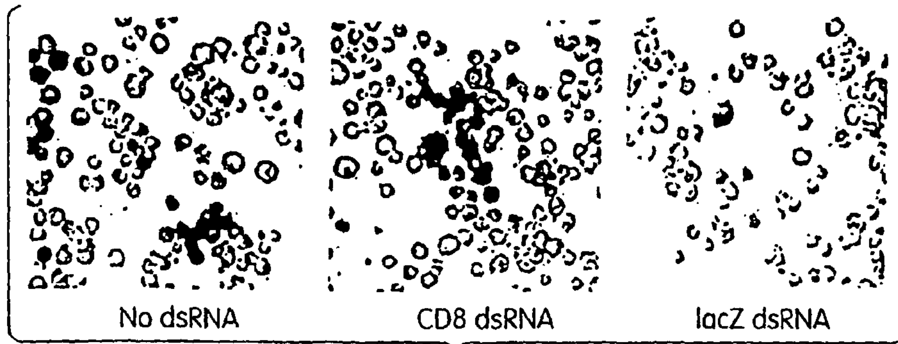
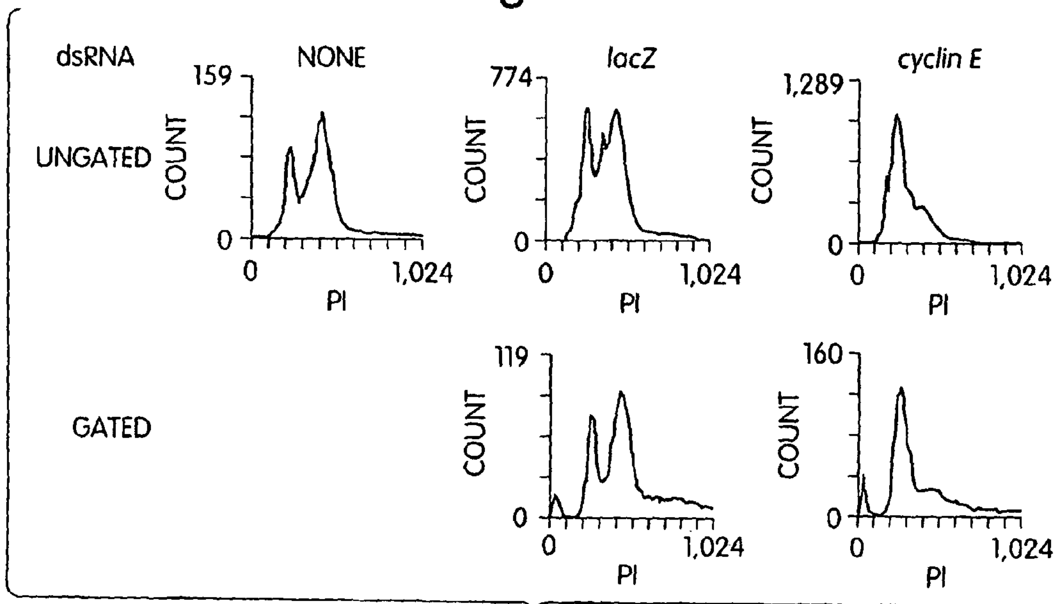


Fig. 1A



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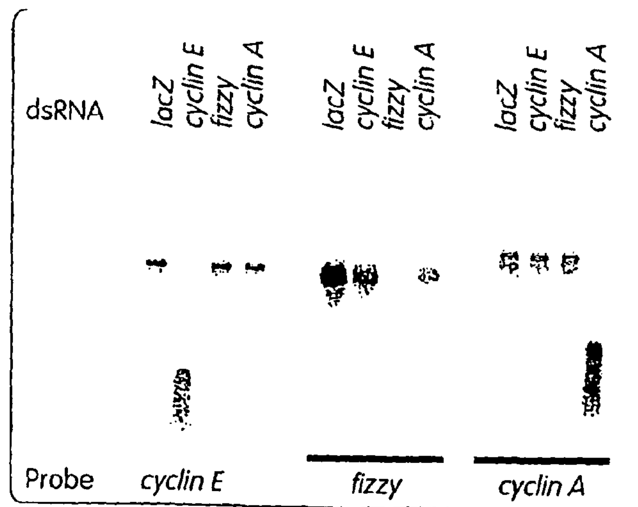
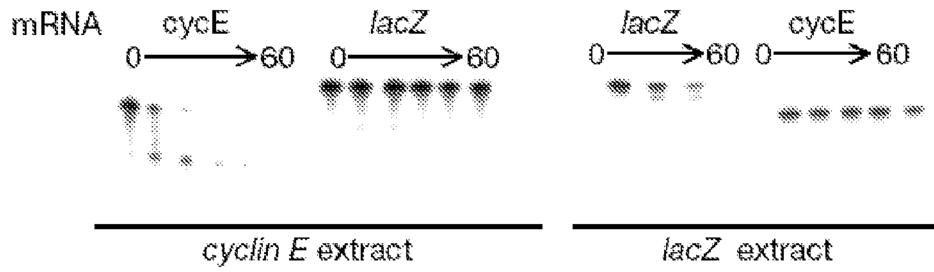


Fig. 1C



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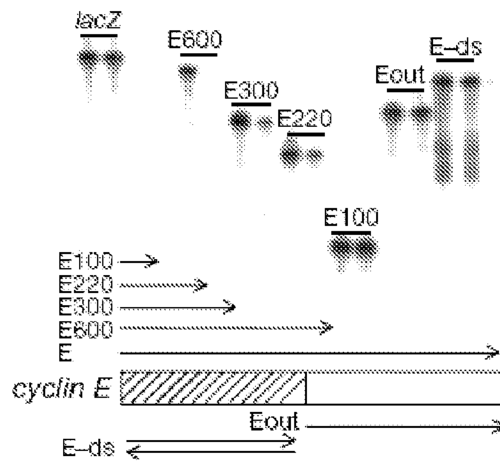

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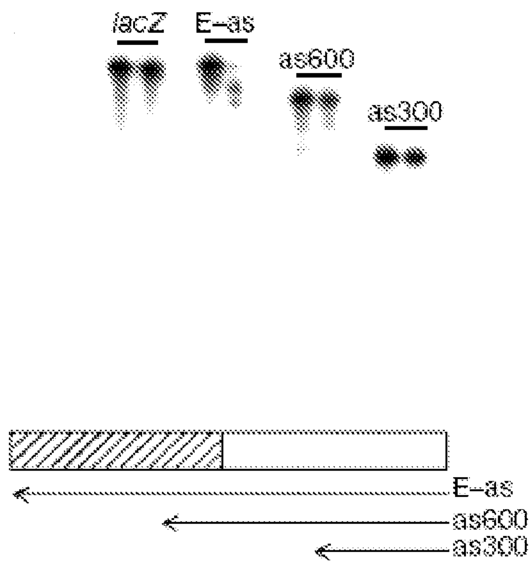

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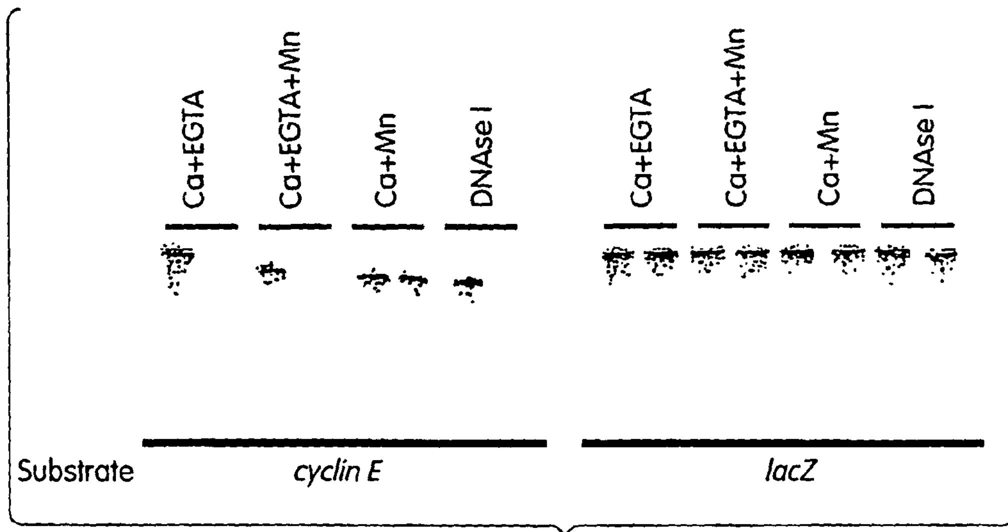
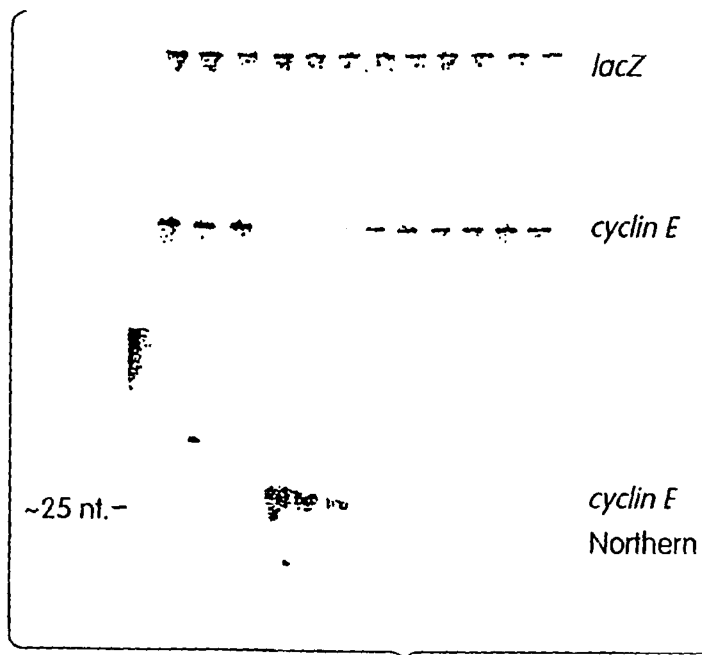


Fig. 3

Fig. 4A



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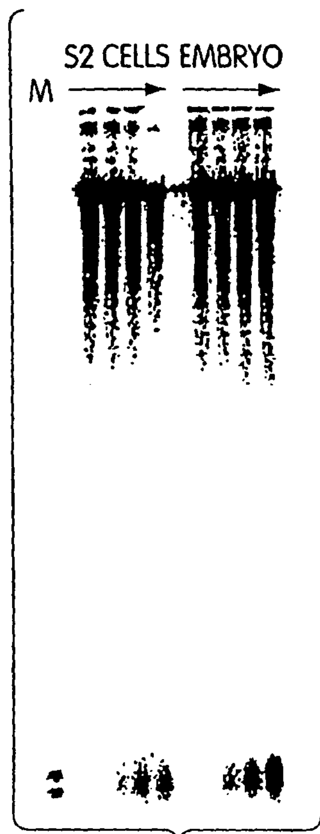

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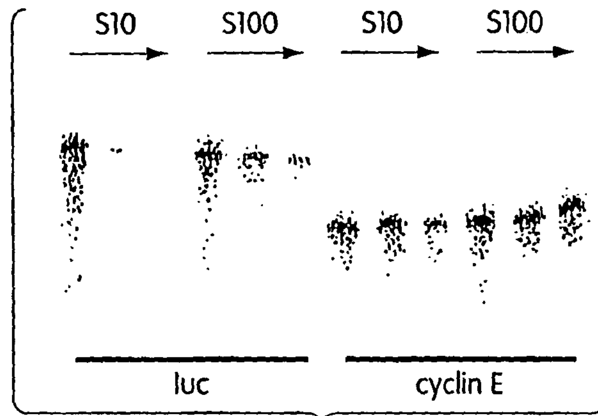

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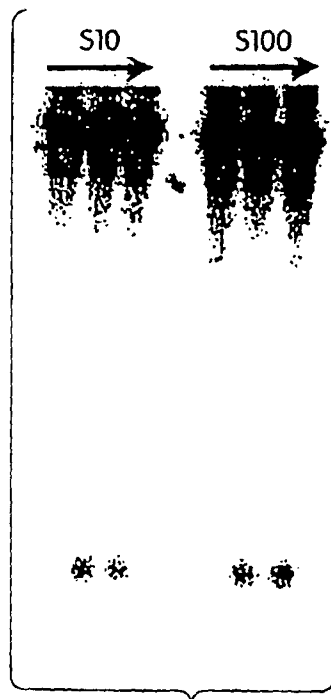

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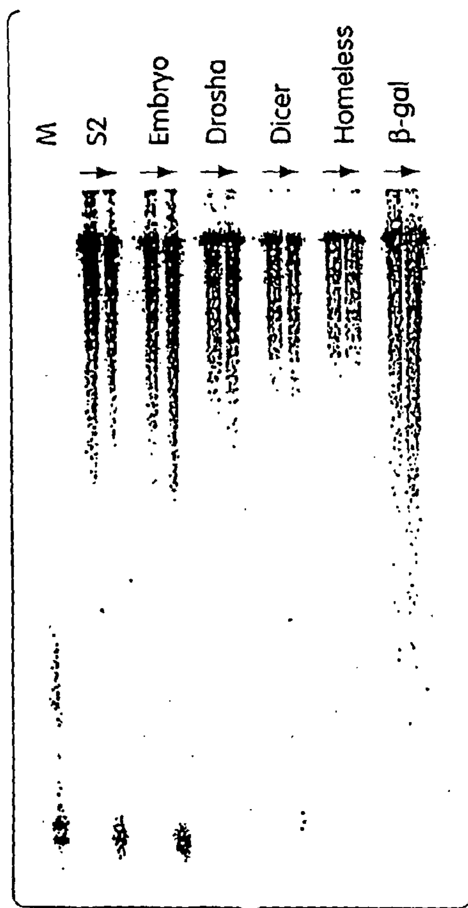

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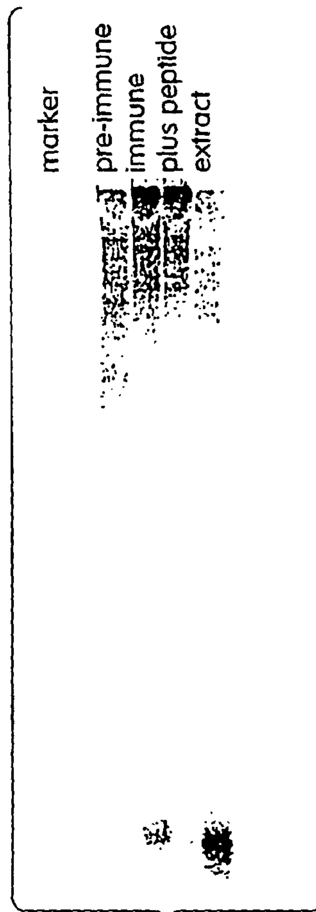

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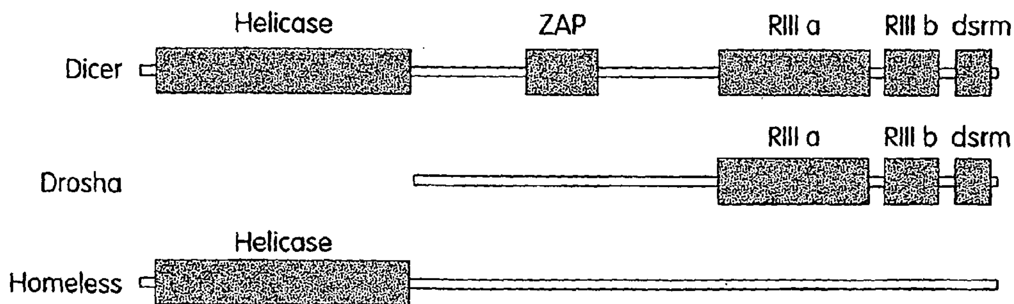

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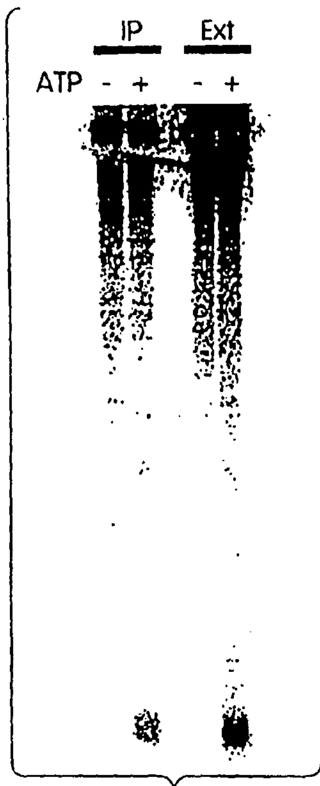

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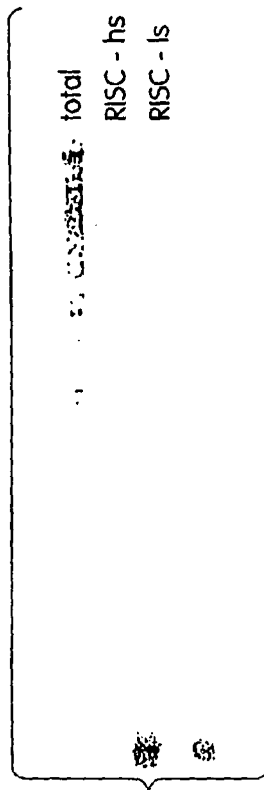

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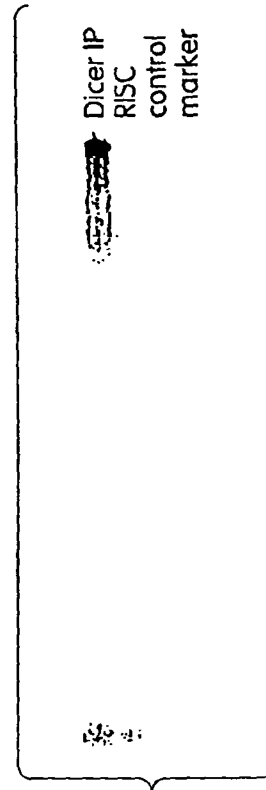

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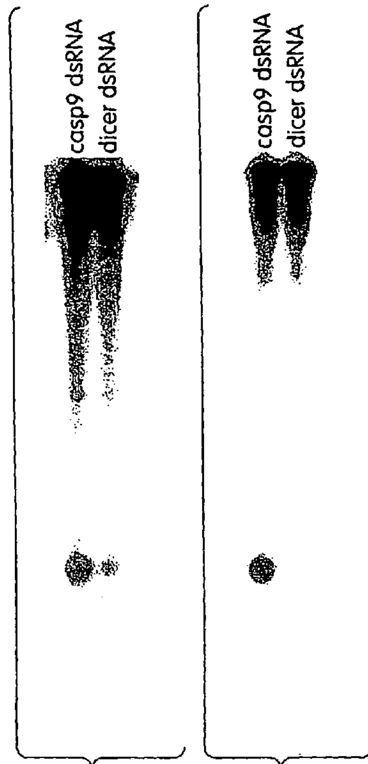


Fig. 7A Fig. 7B

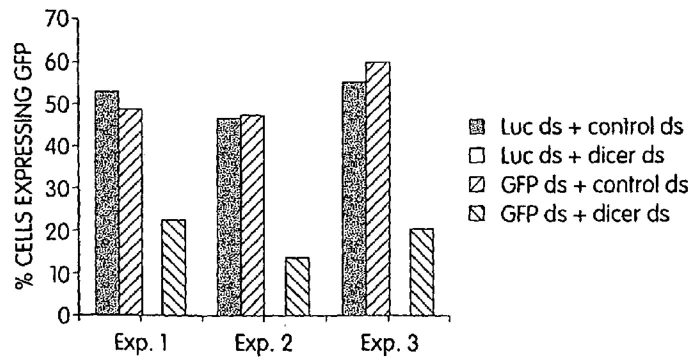


Fig. 7C

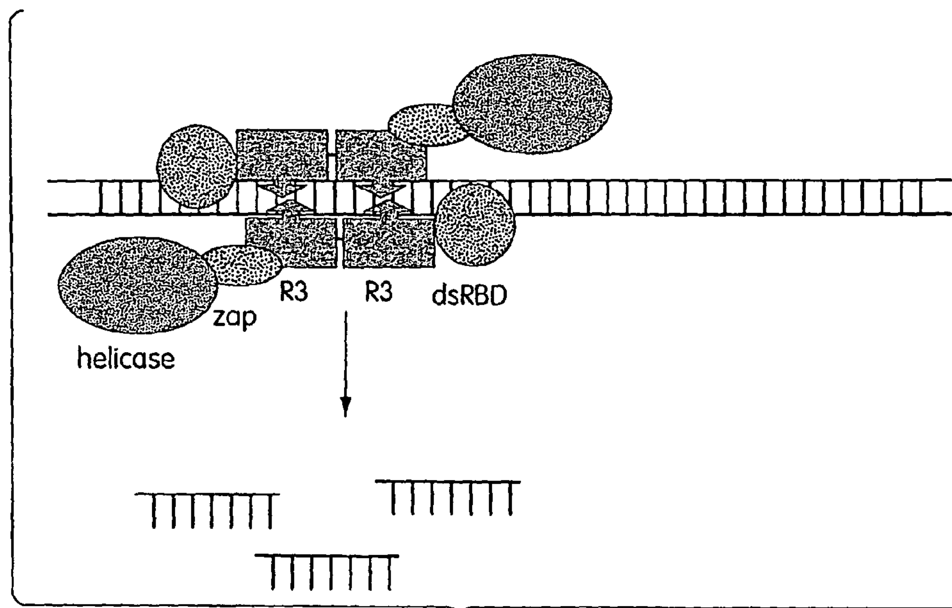


Fig. 8A

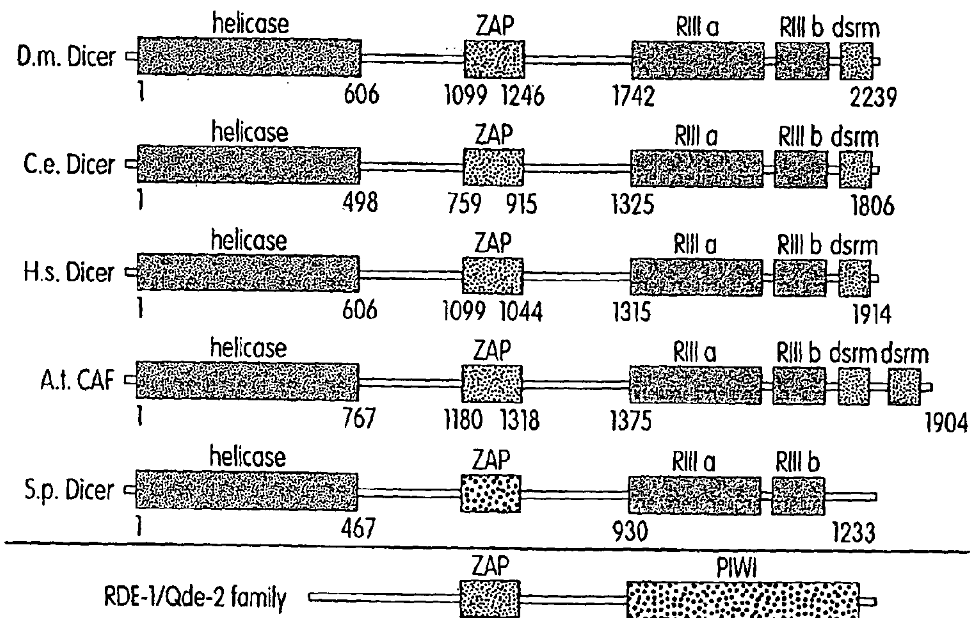


Fig. 8B

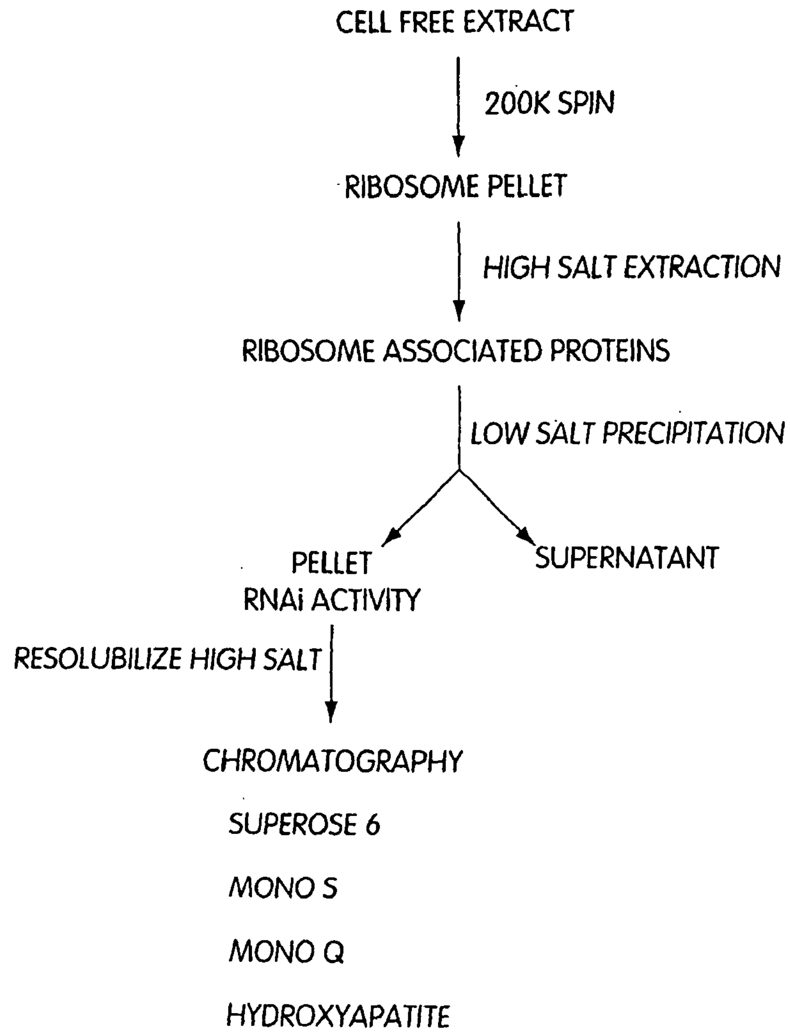
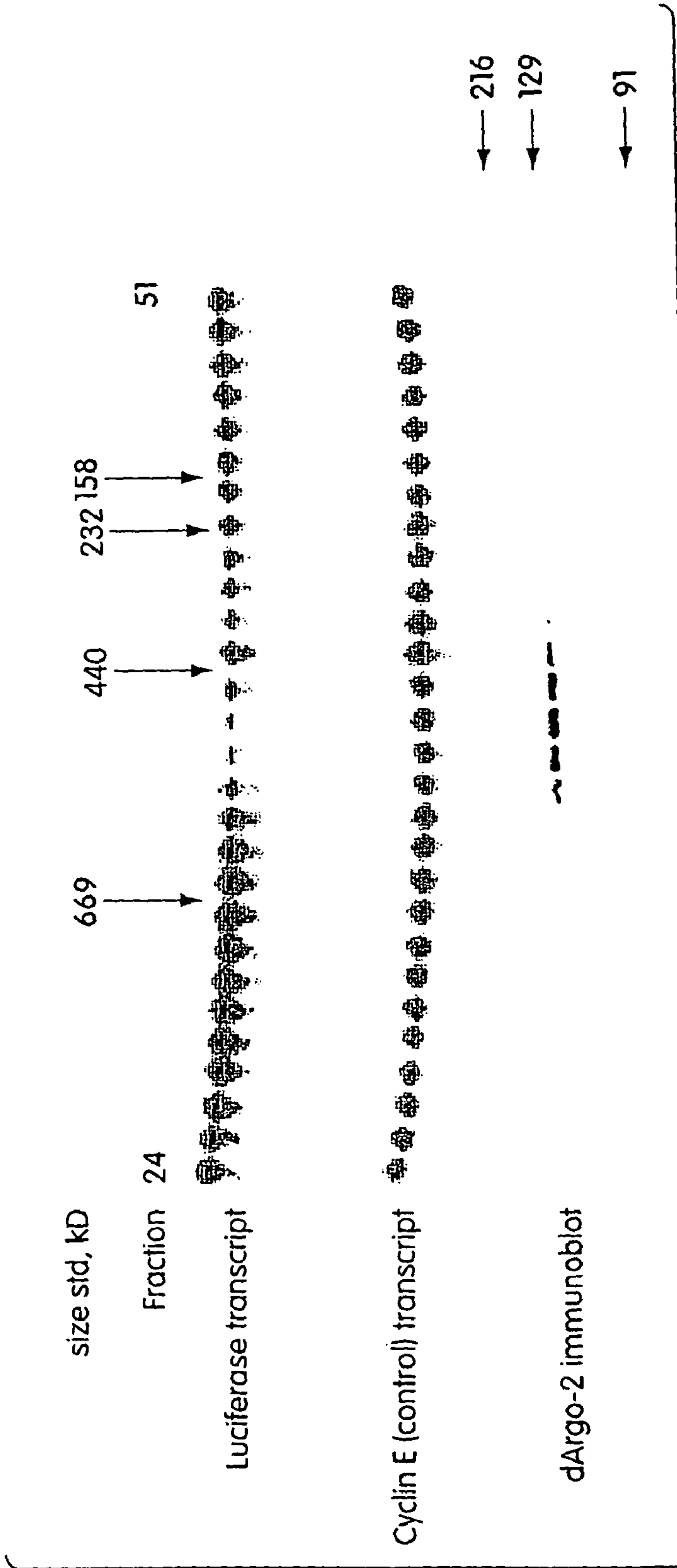


Fig. 9



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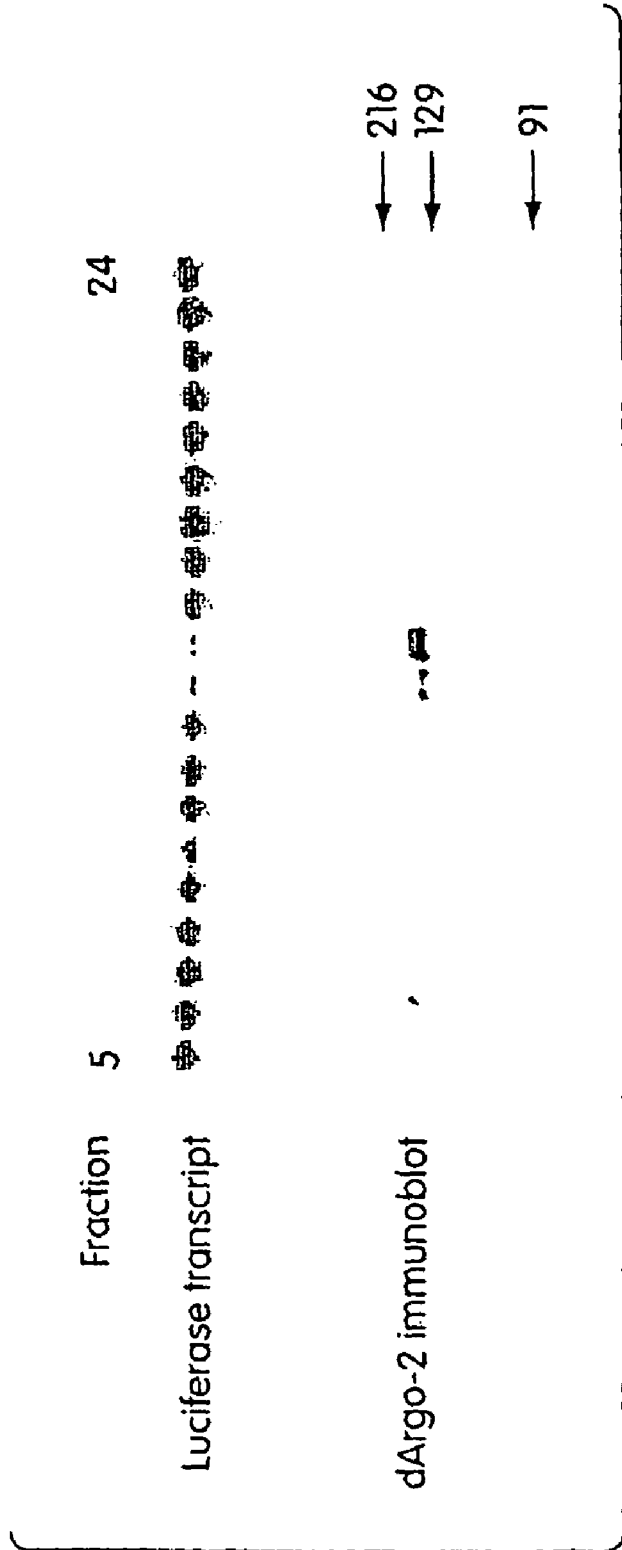

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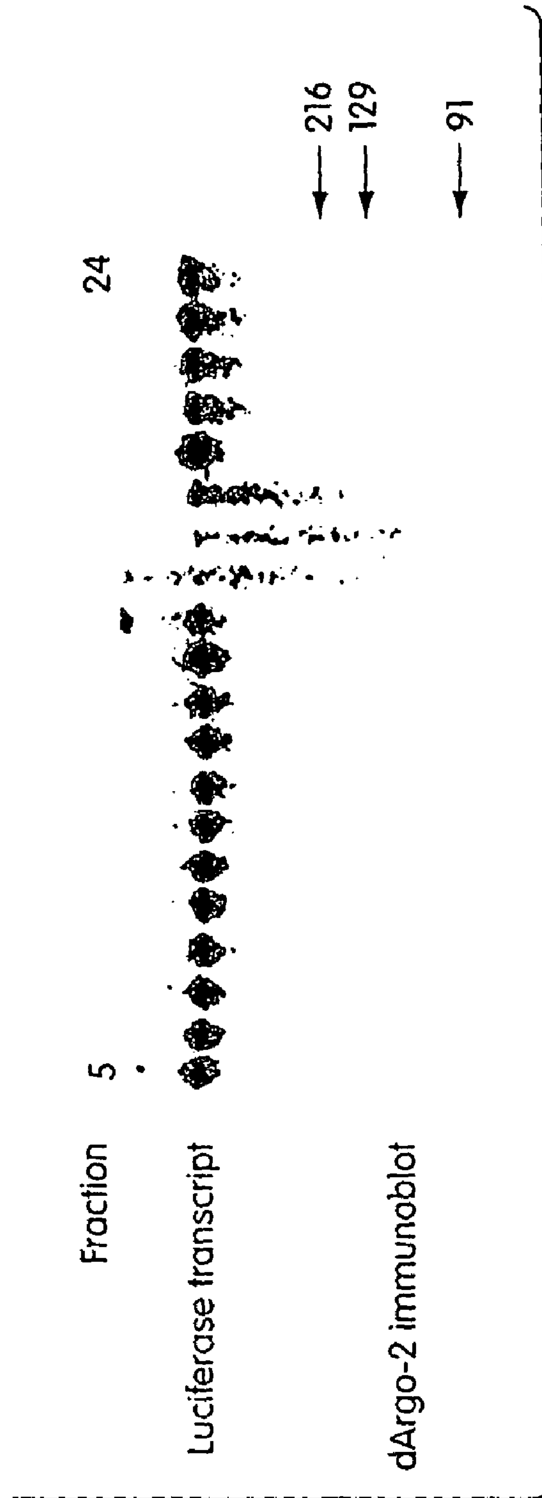

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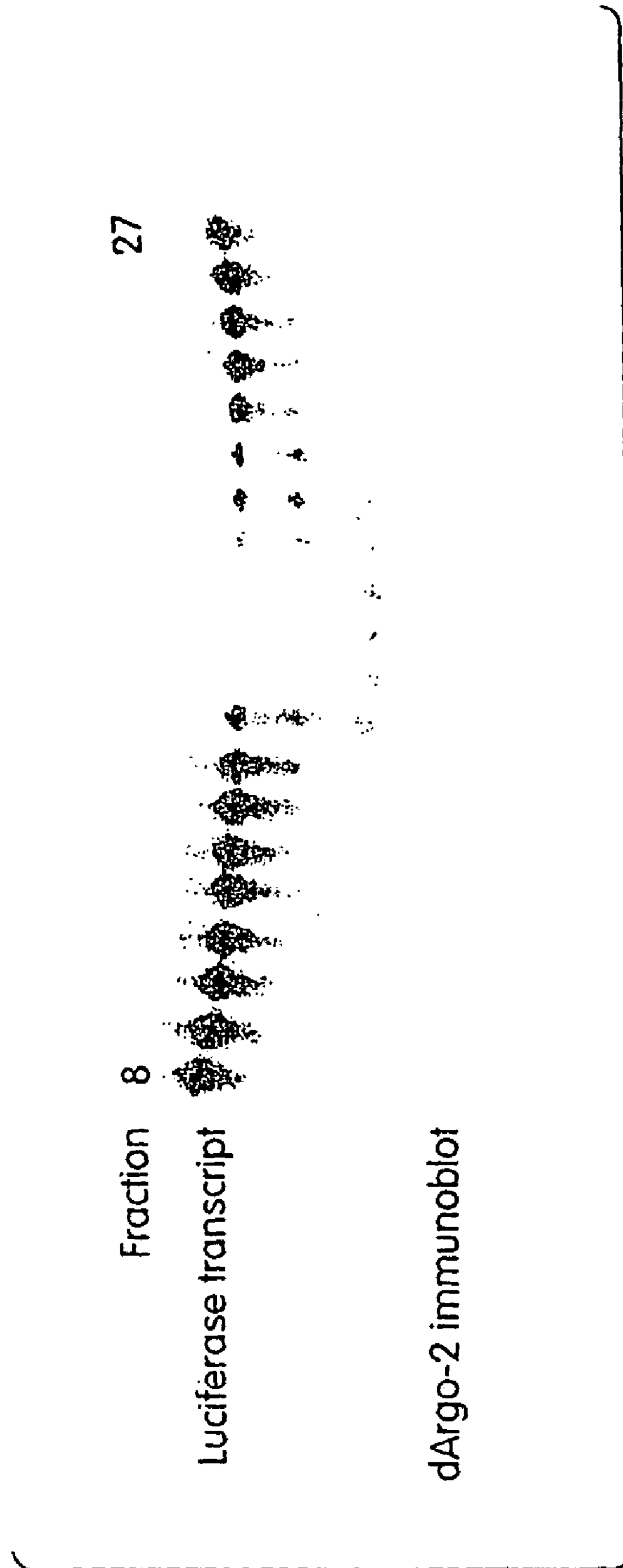

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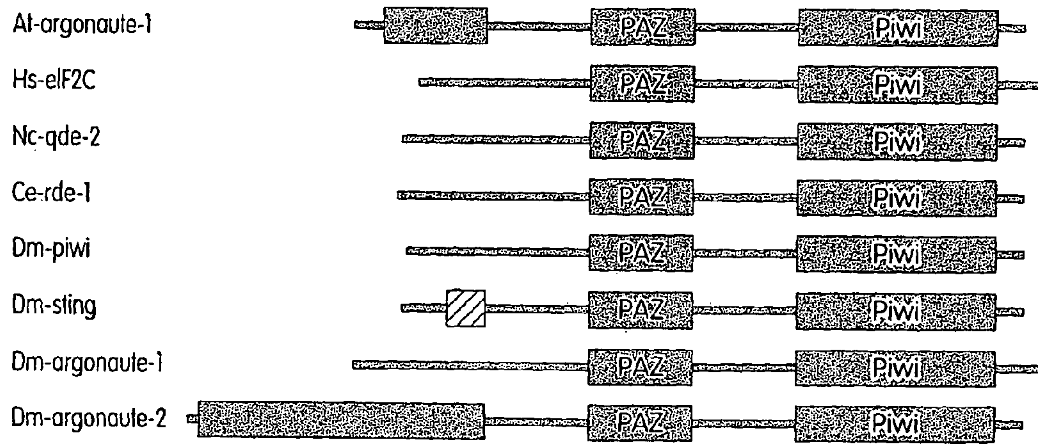

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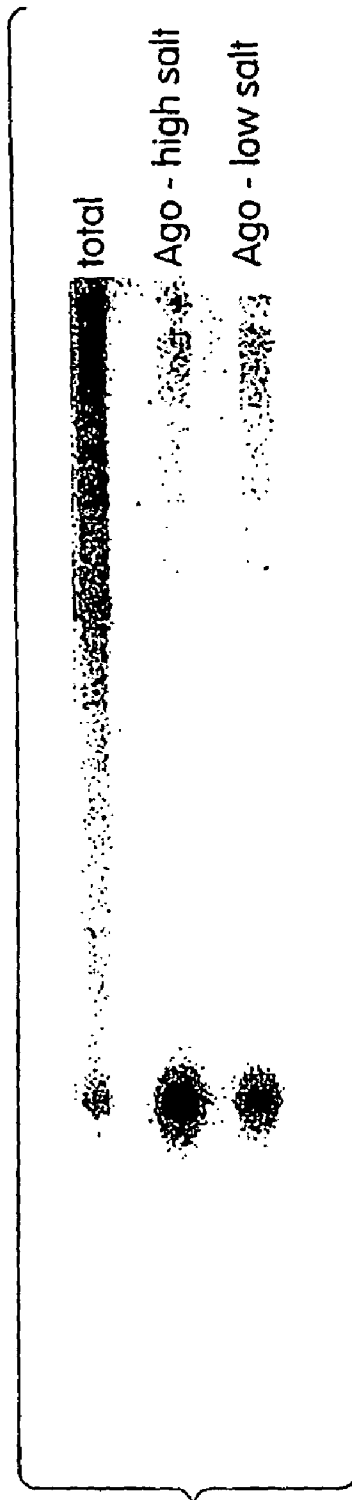

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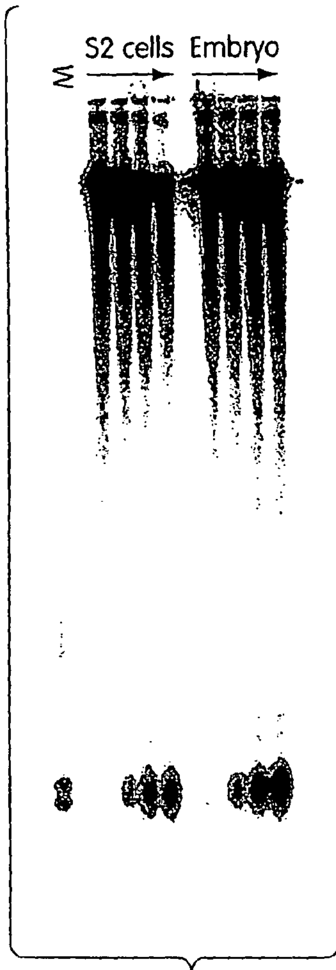
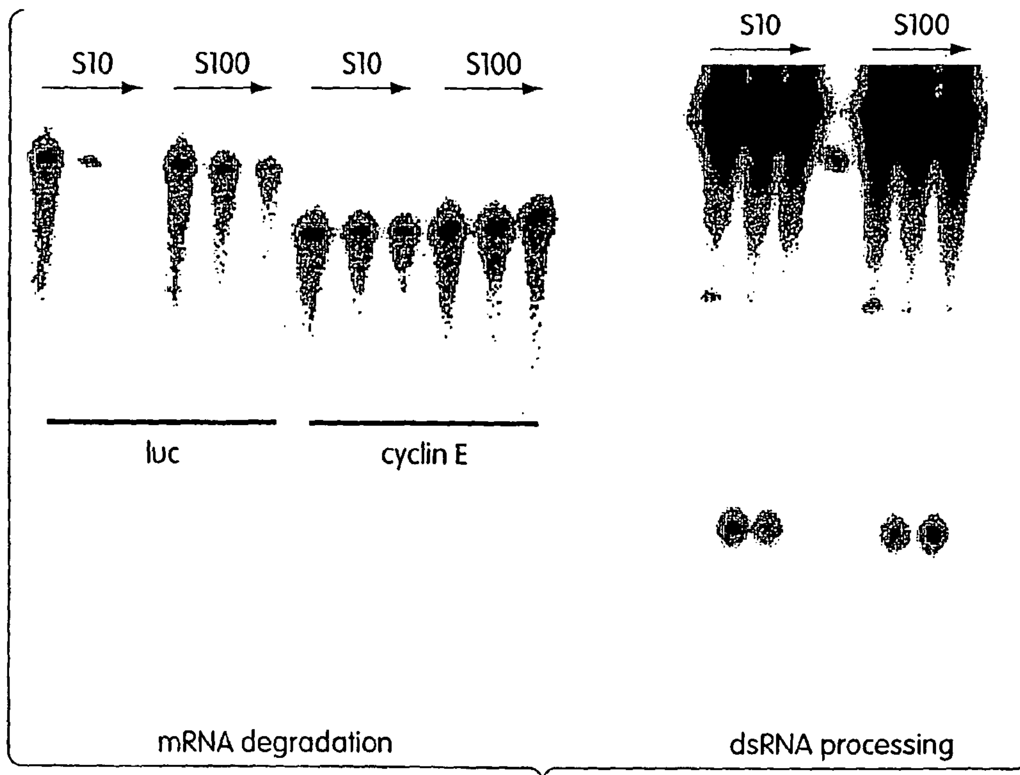


Fig. 16



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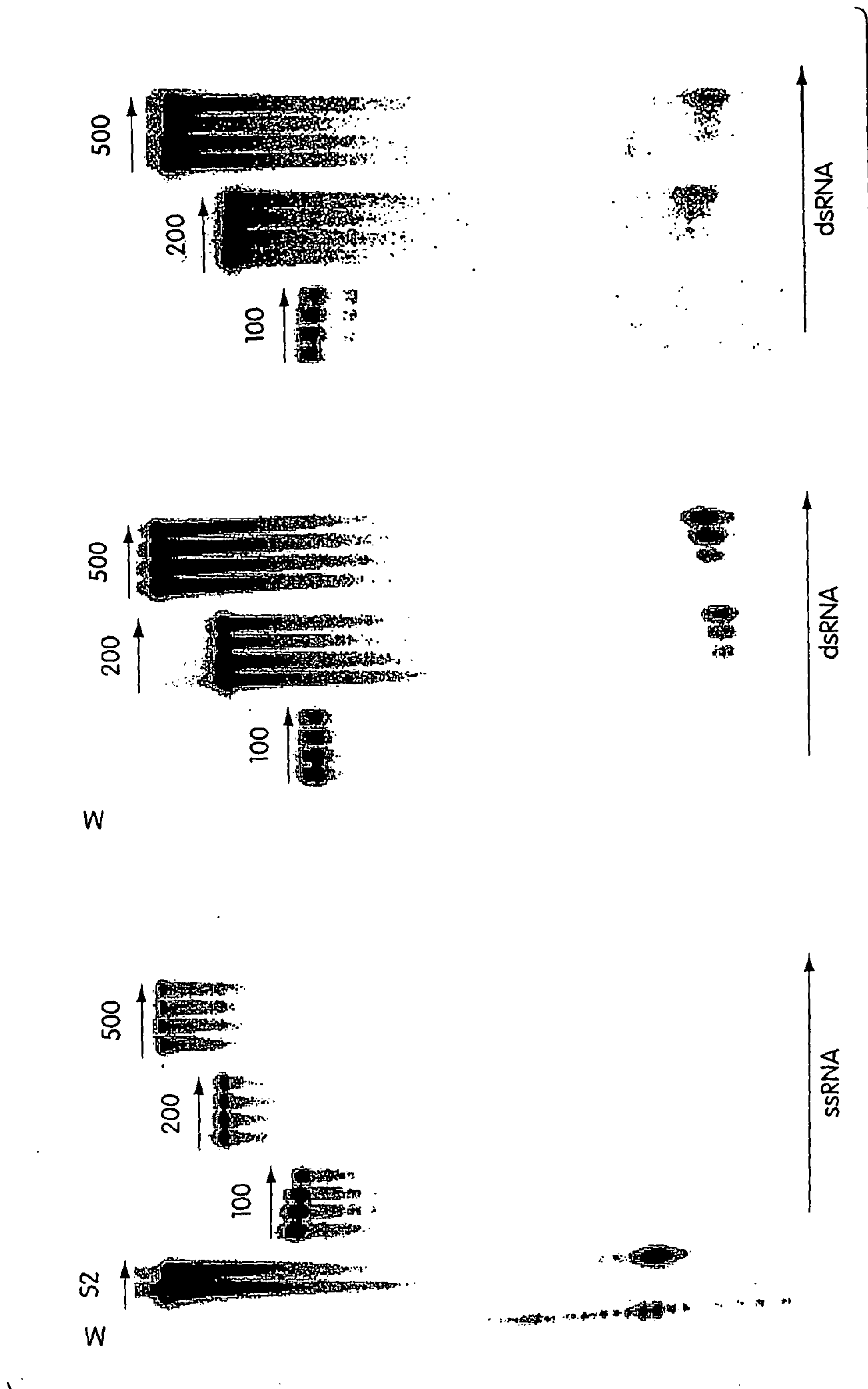
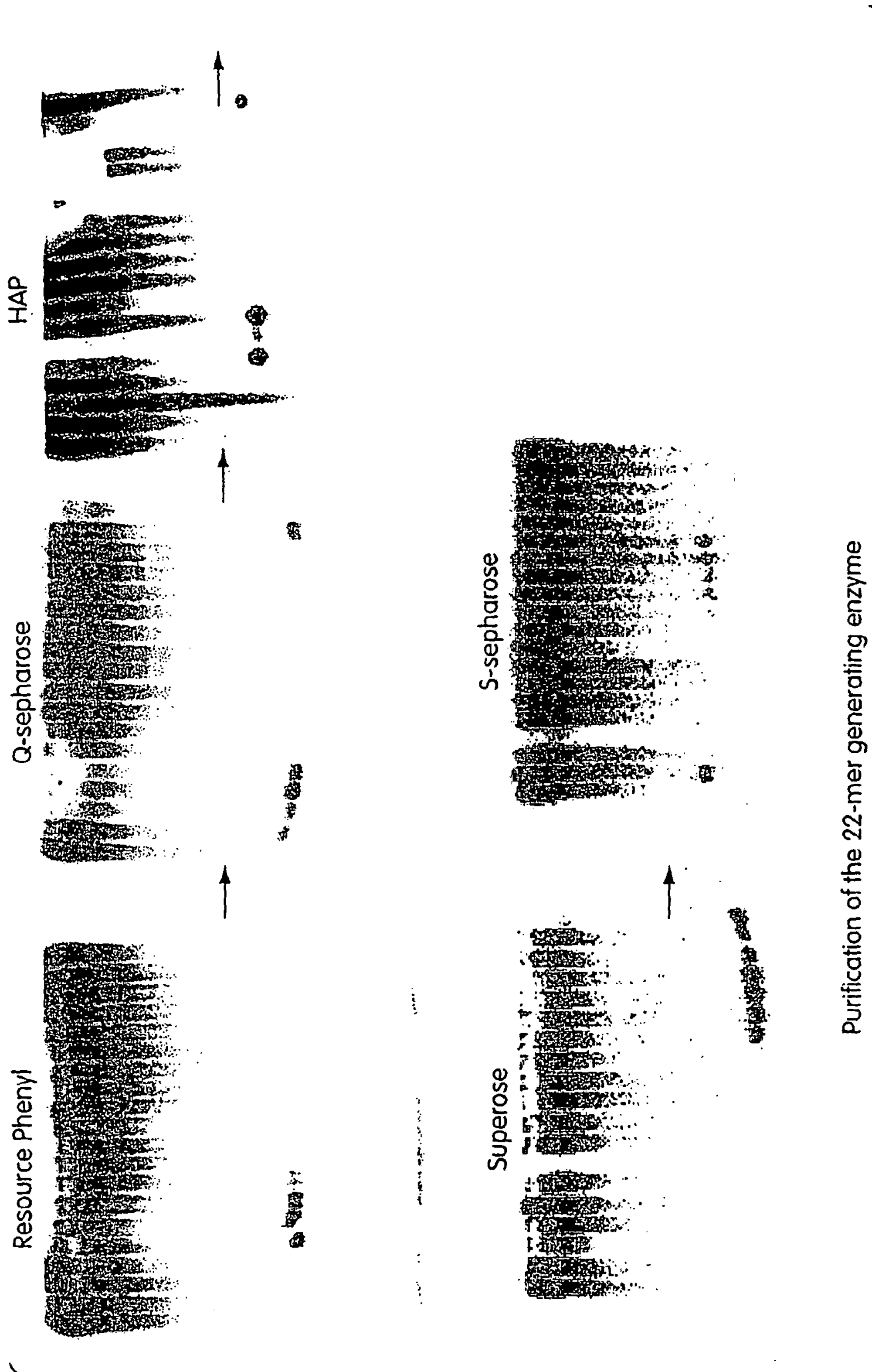


Fig. 18



Purification of the 22-mer generating enzyme

Fig. 19

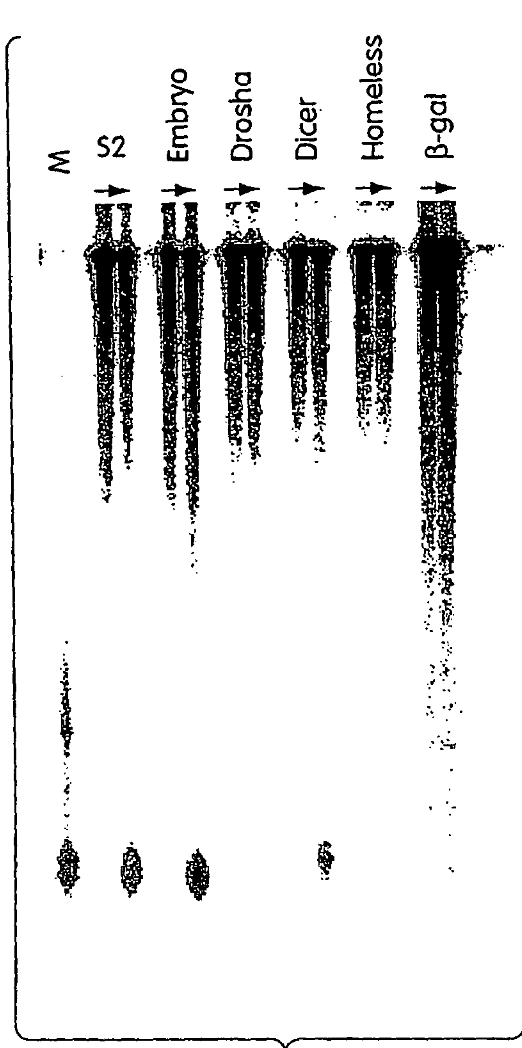


Fig. 20A

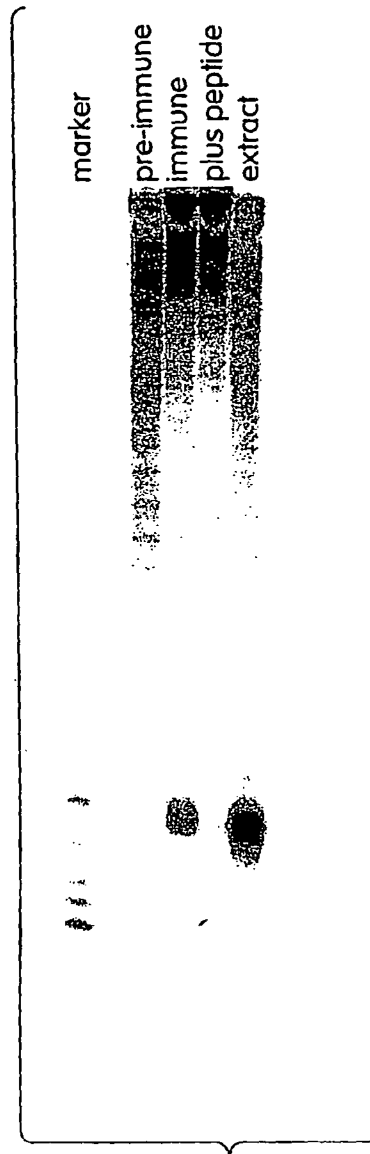


Fig. 20C

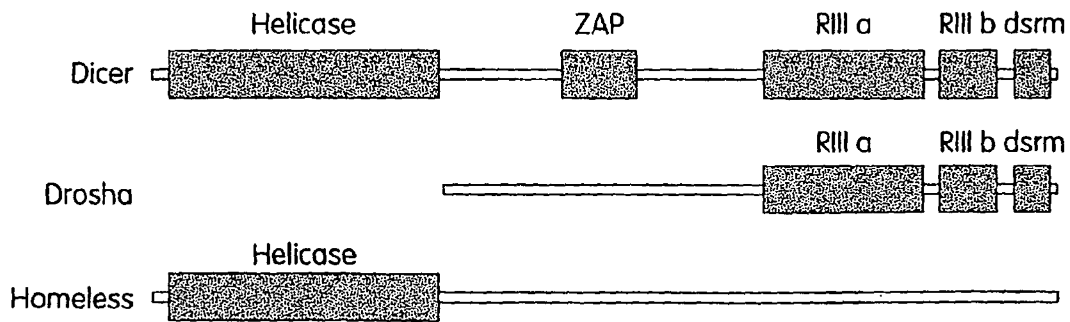


Fig. 20B

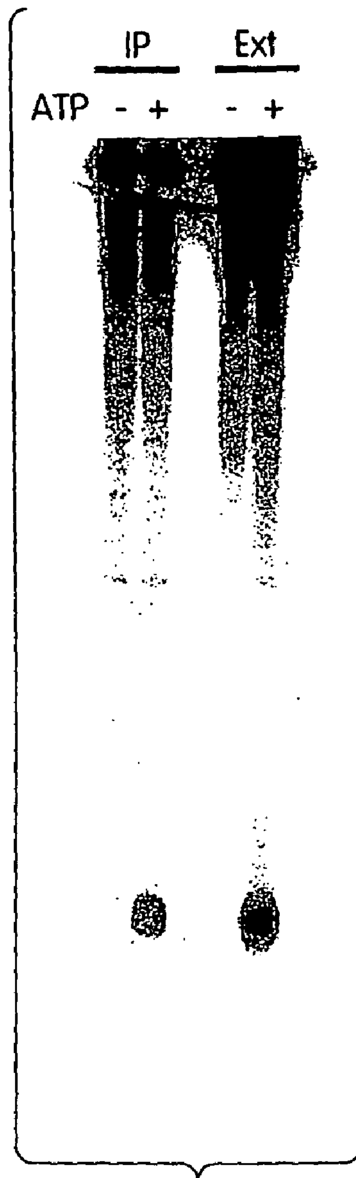


Fig. 21

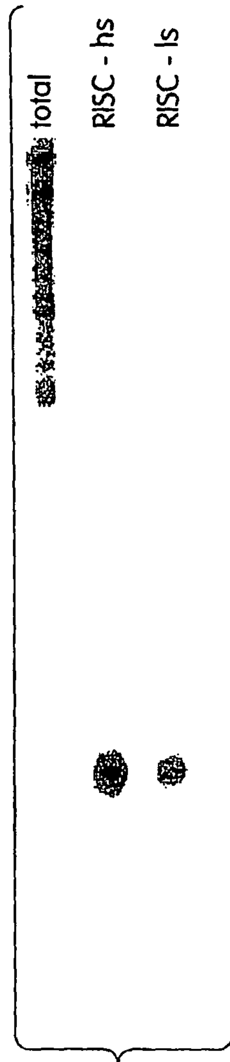


Fig. 22A

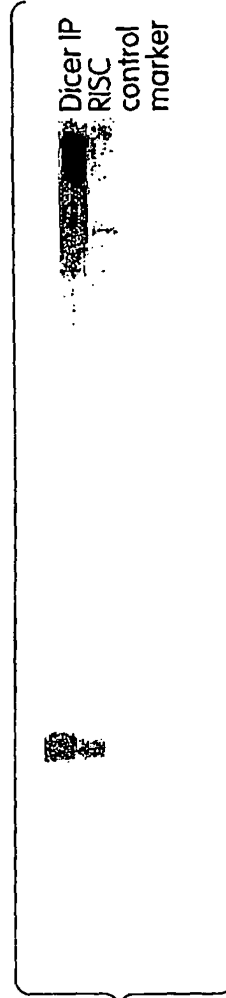


Fig. 22B

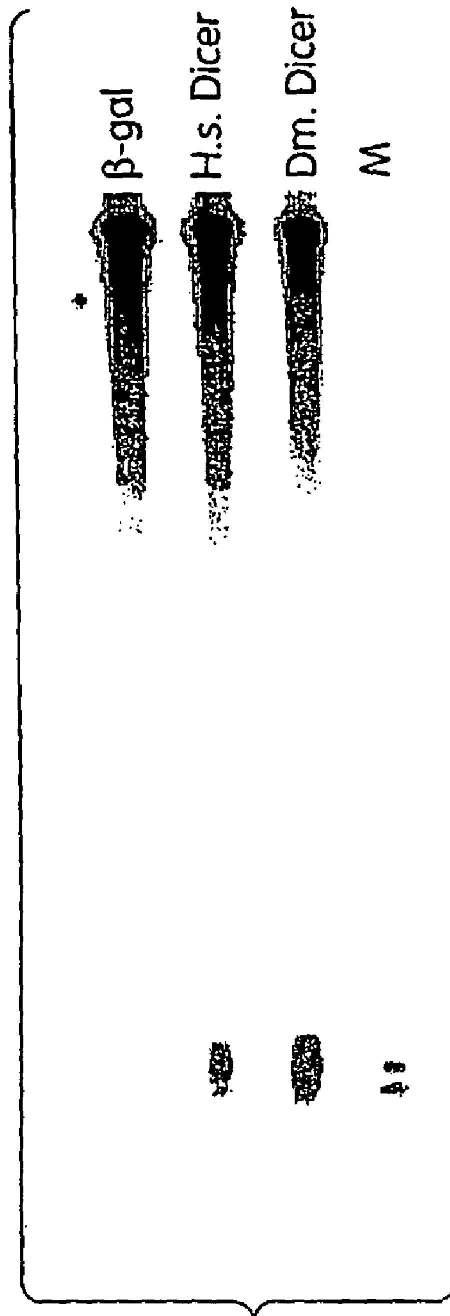


Fig. 23

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Fig. 24

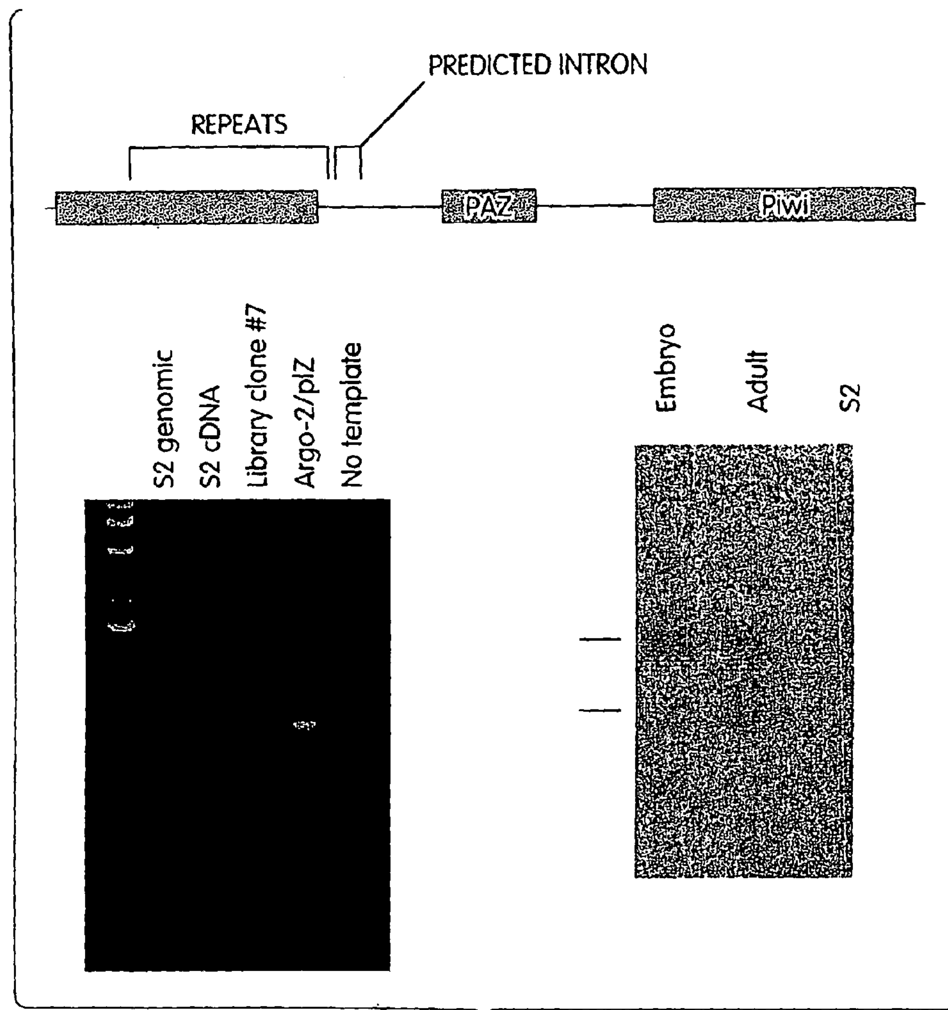


Fig. 25

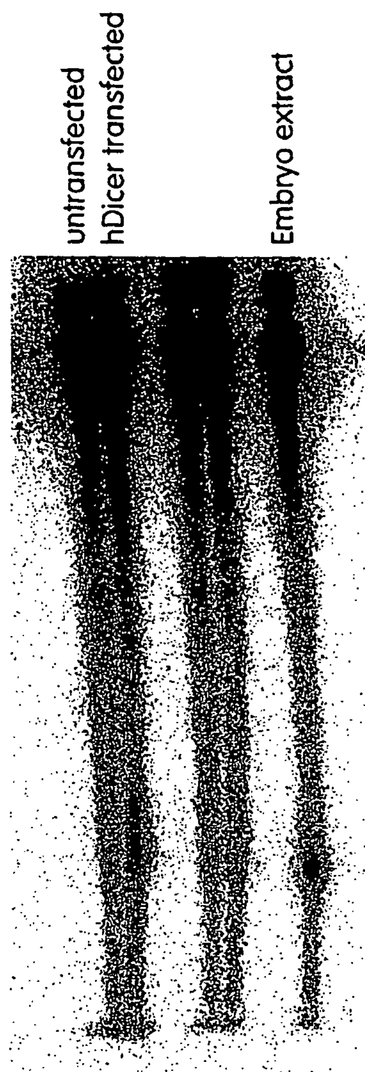
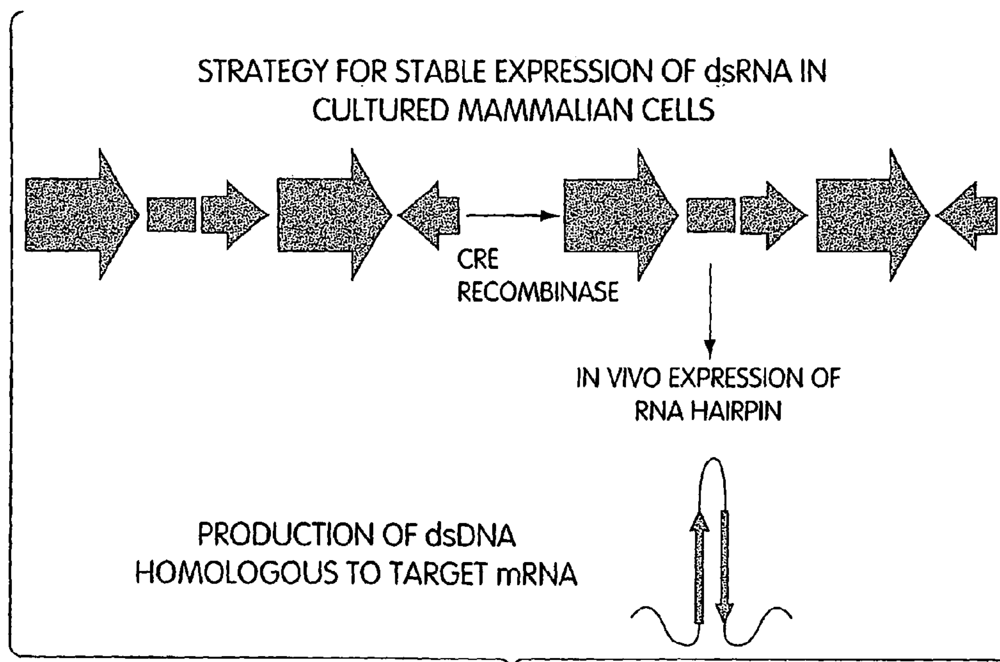


Fig. 26



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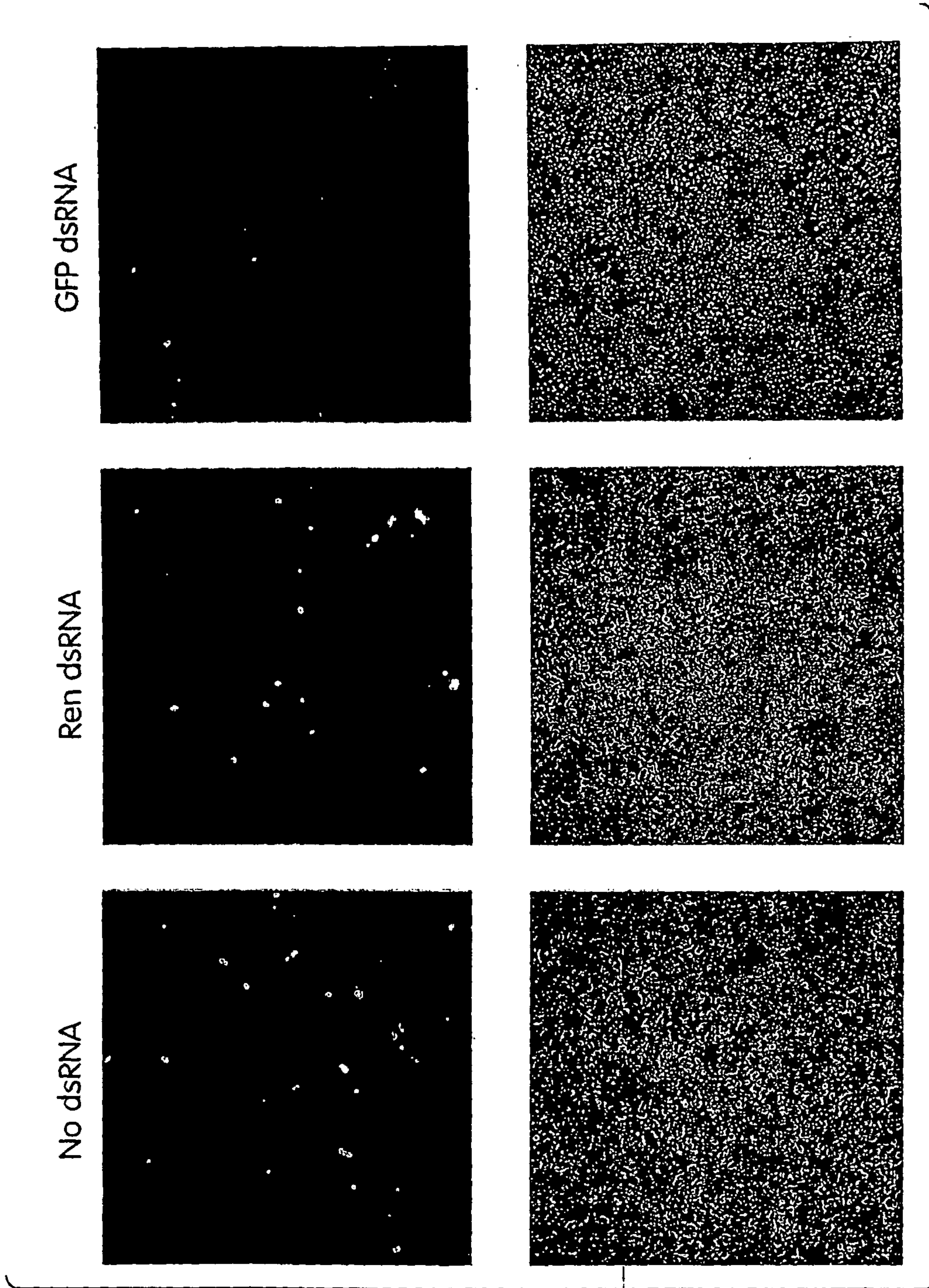

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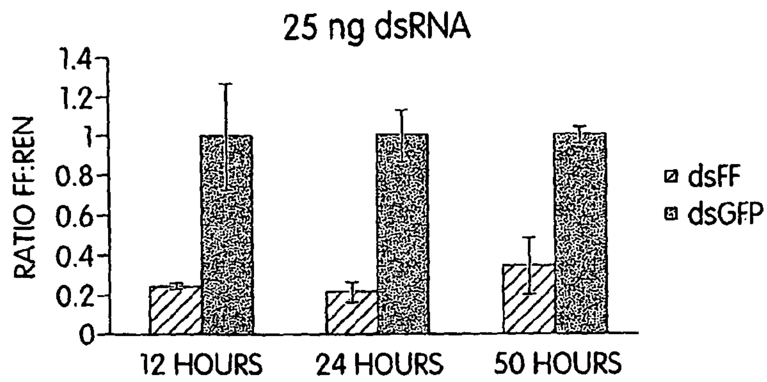
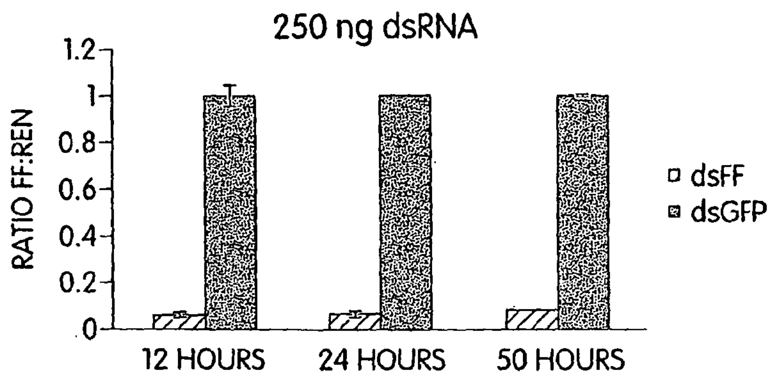


Fig. 29A



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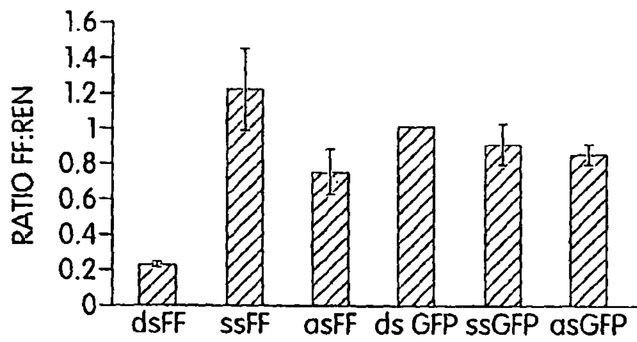

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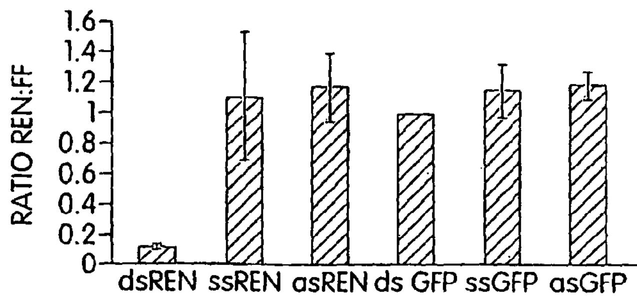


Fig. 29D

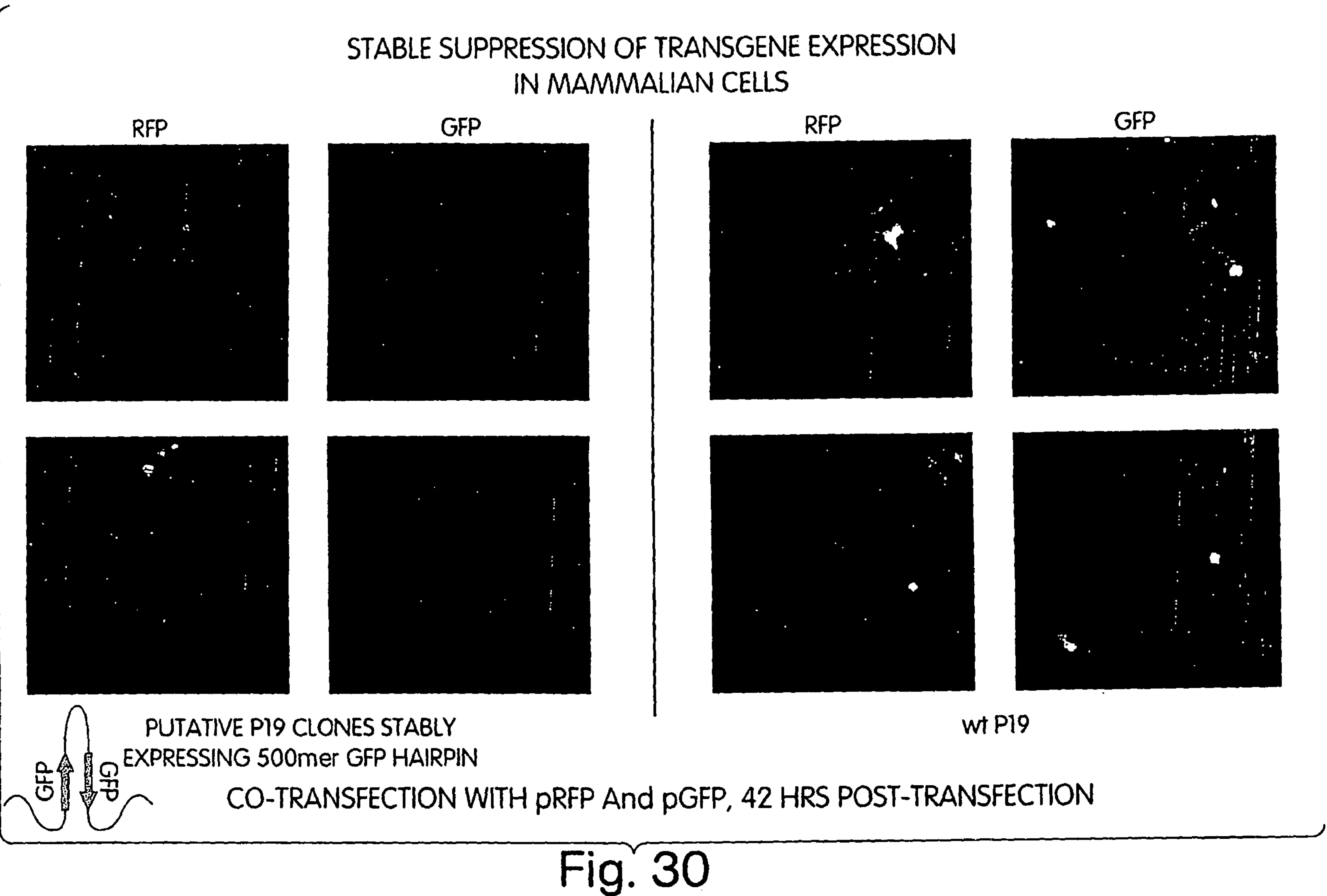


Fig. 30

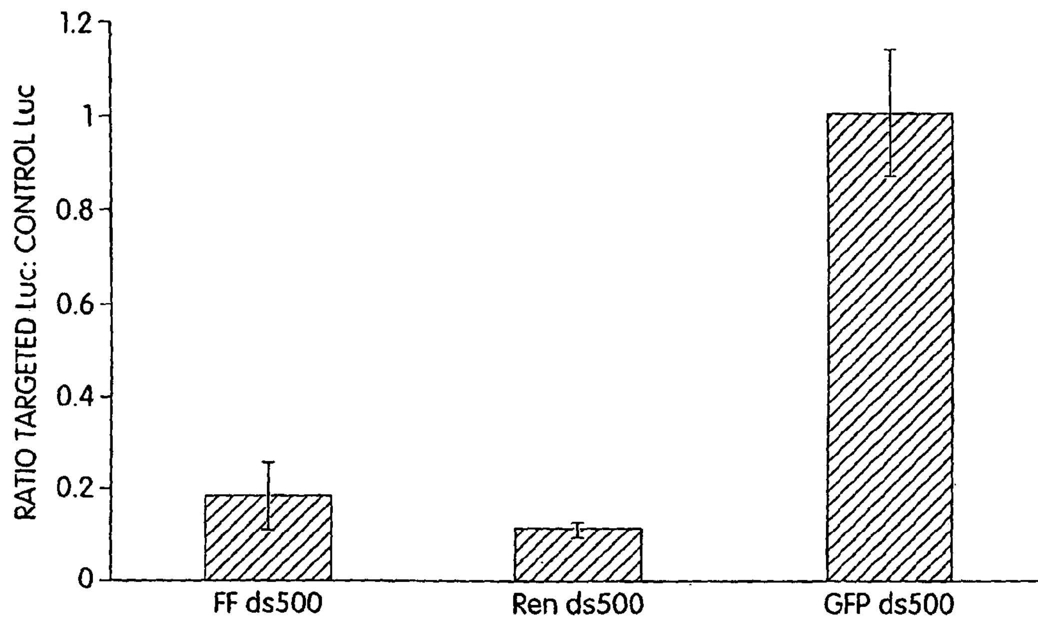


Fig. 31

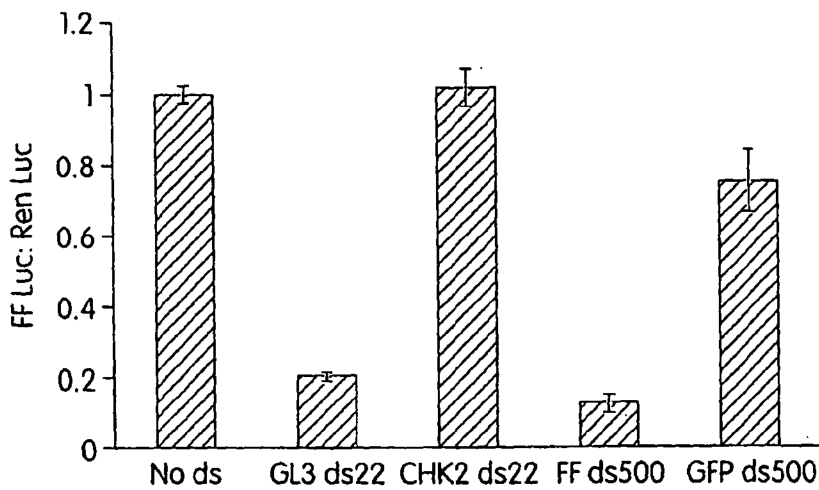


Fig. 32A

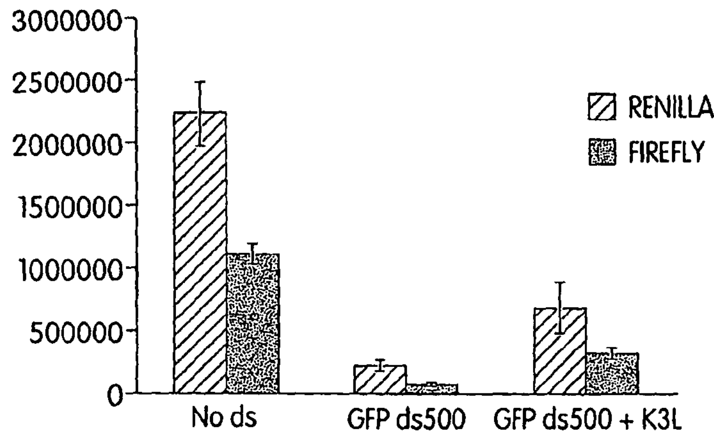


Fig. 32B

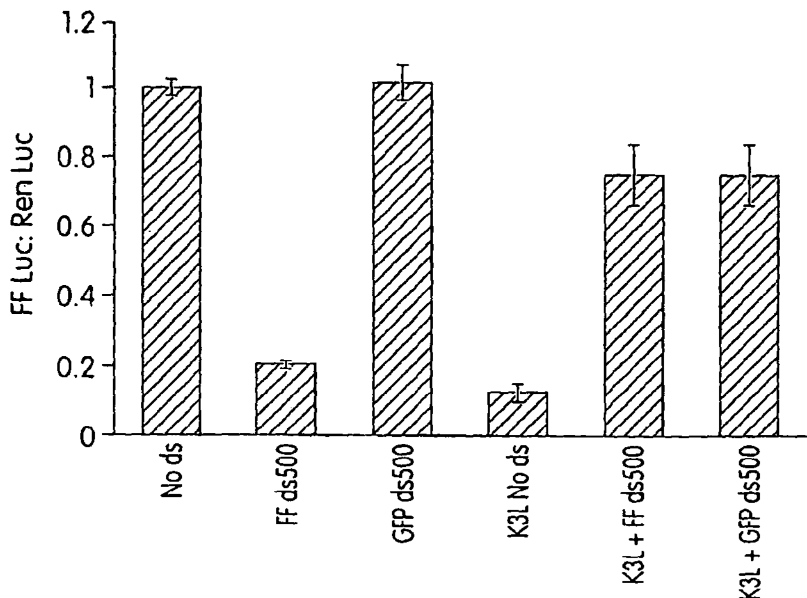


Fig. 32C

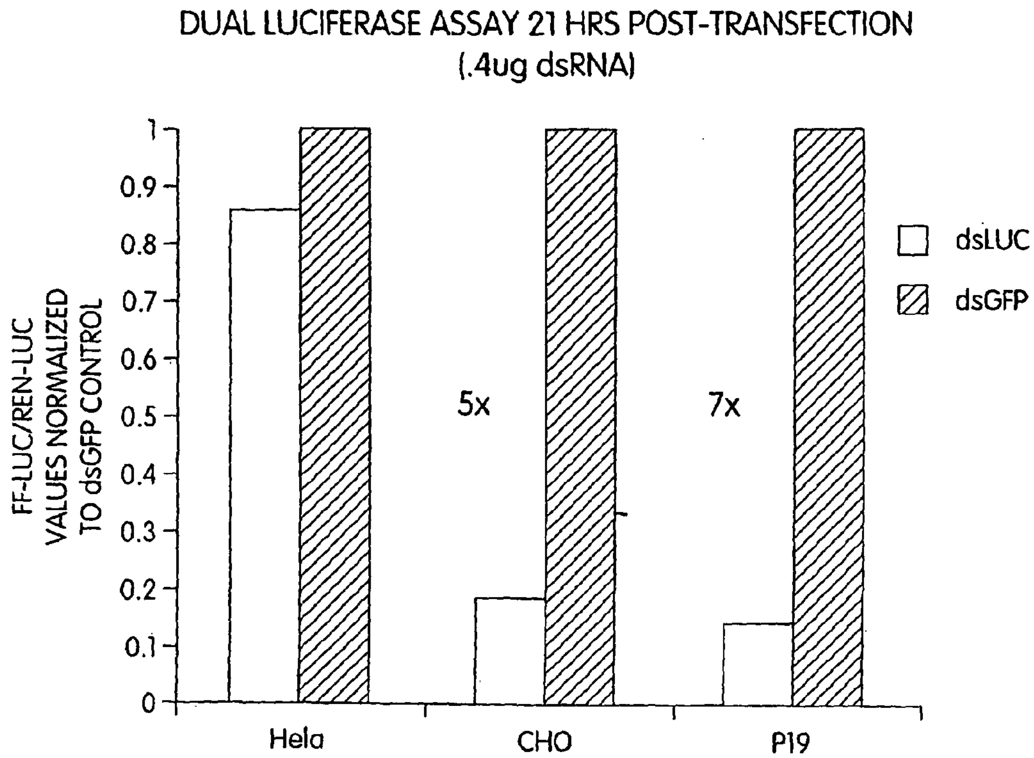


Fig. 33

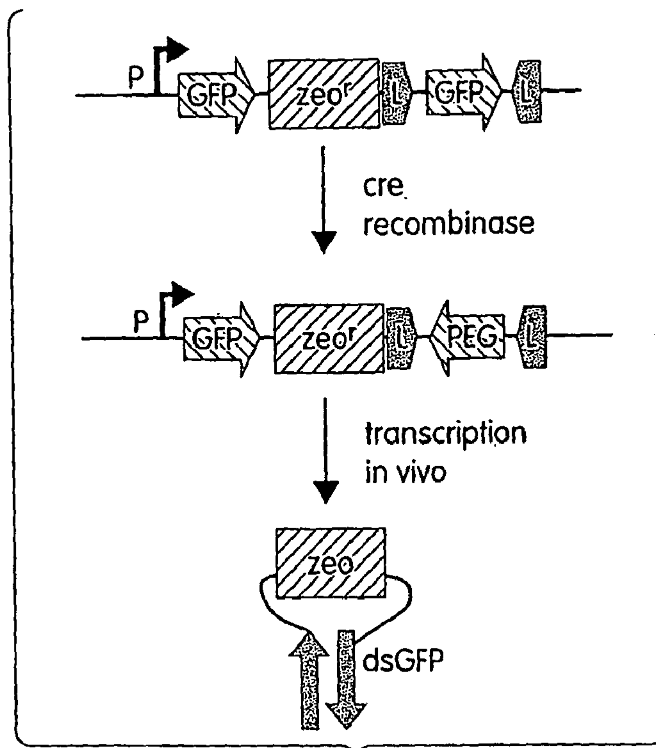


Fig. 34A

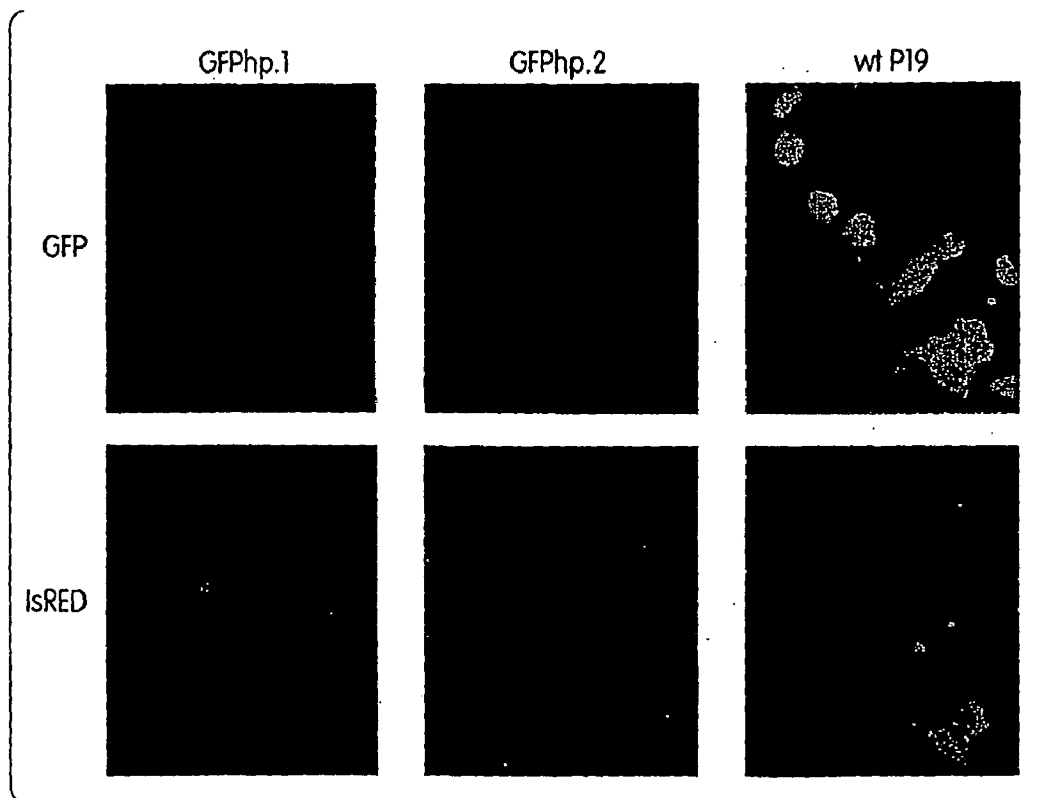
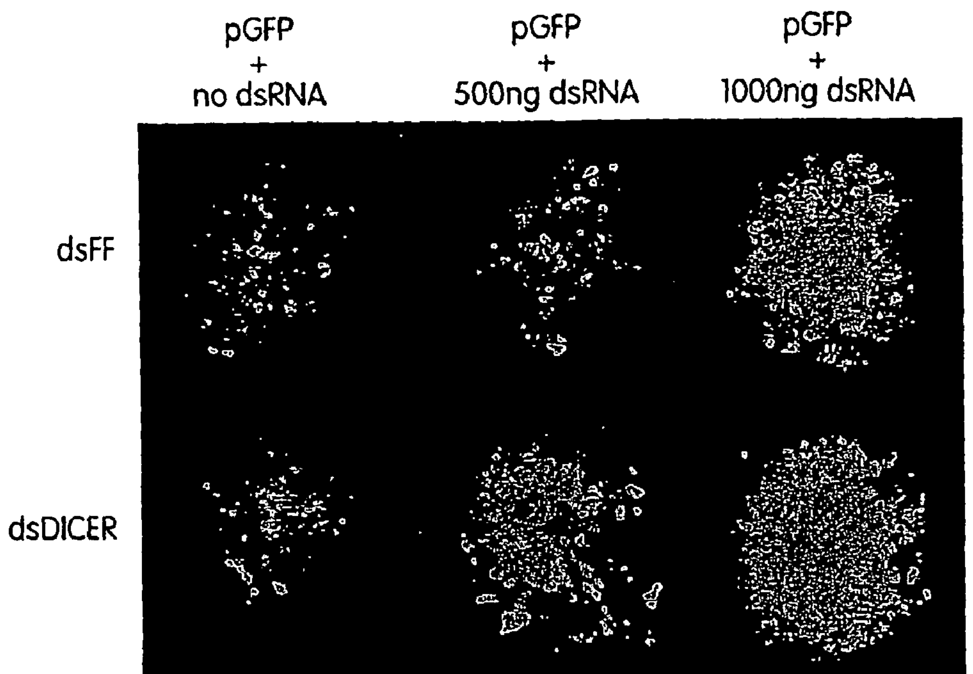


Fig. 34B



P19 GFP HAIRPIN CLONE NUMBER #10
48 HRS POST-TRANSFECTION
FLUORESCENT MICROSCOPY SUPERIMPOSED WITH BRIGHT FIELD

Fig. 34C

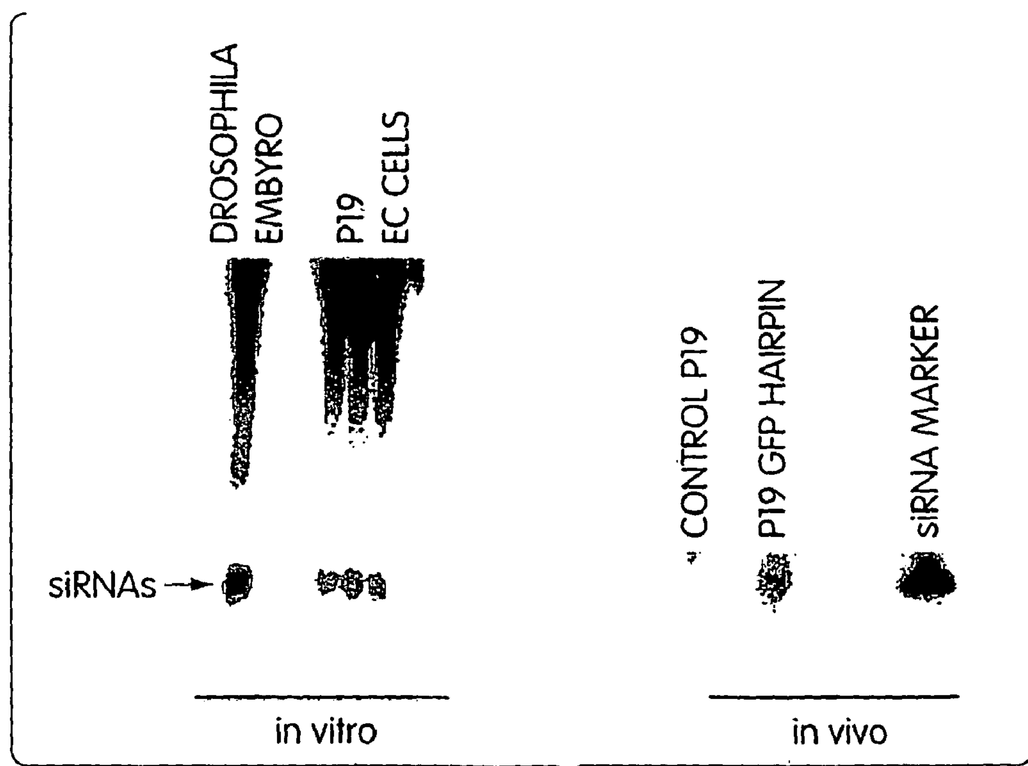


Fig. 34D

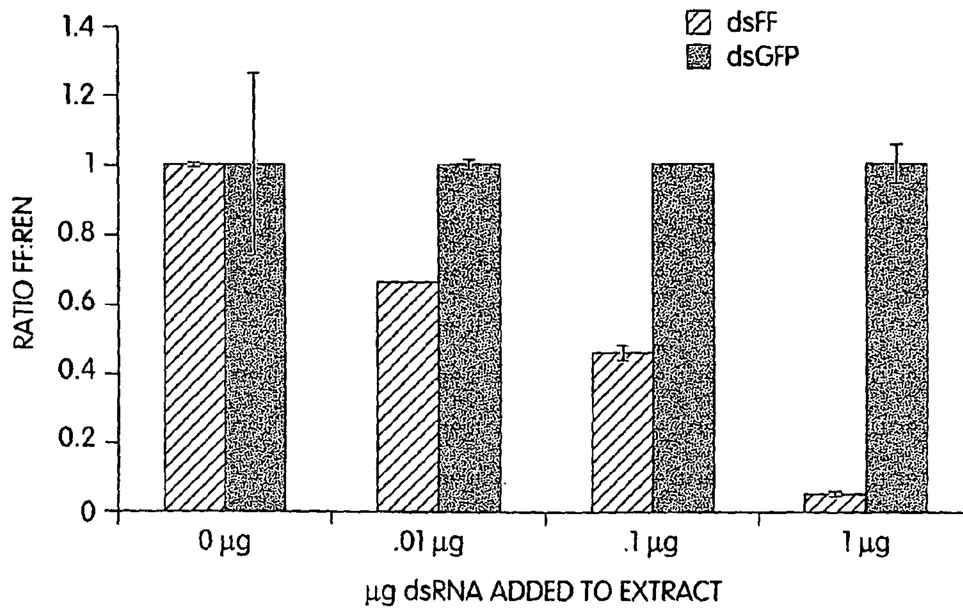


Fig. 35

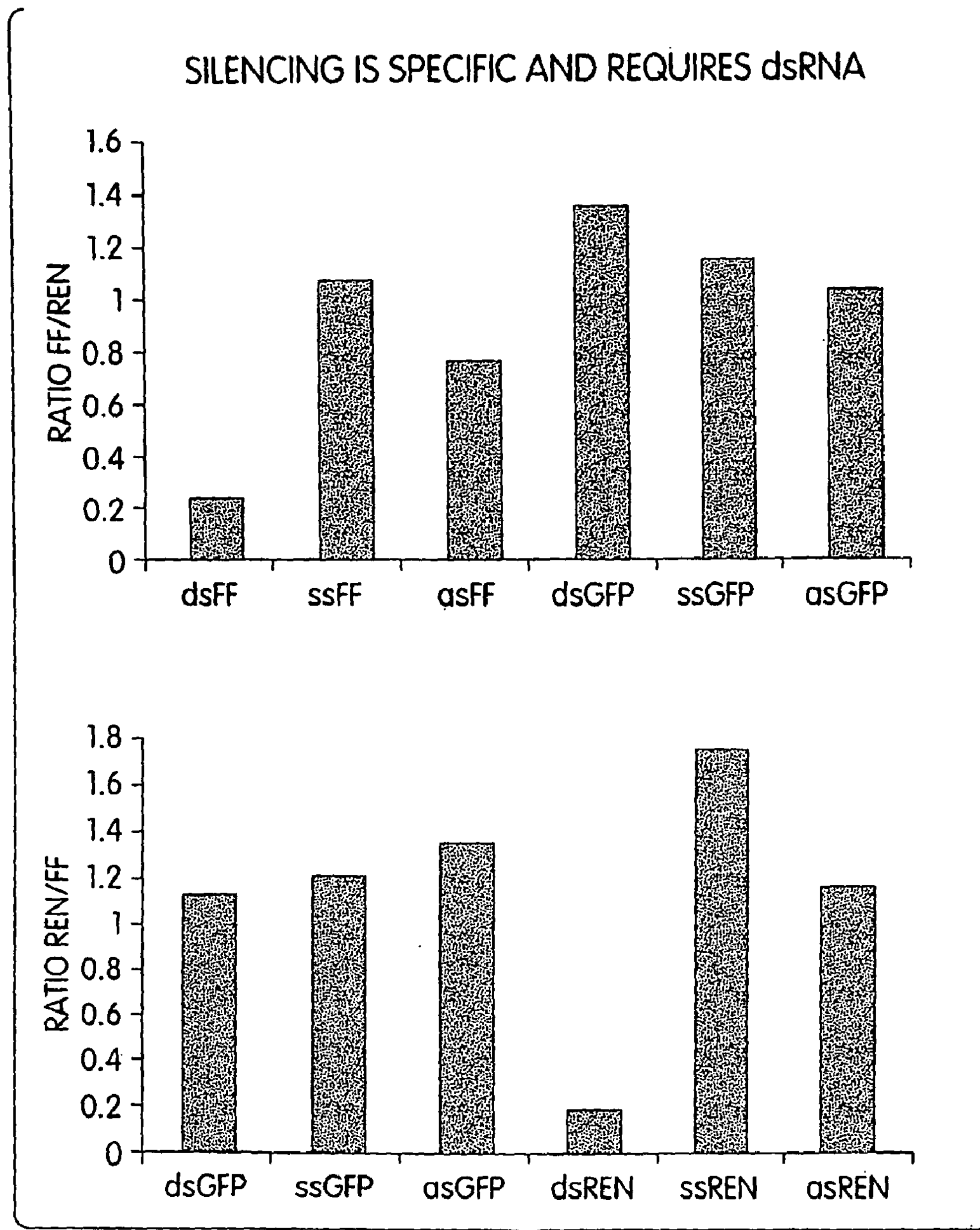


Fig. 36

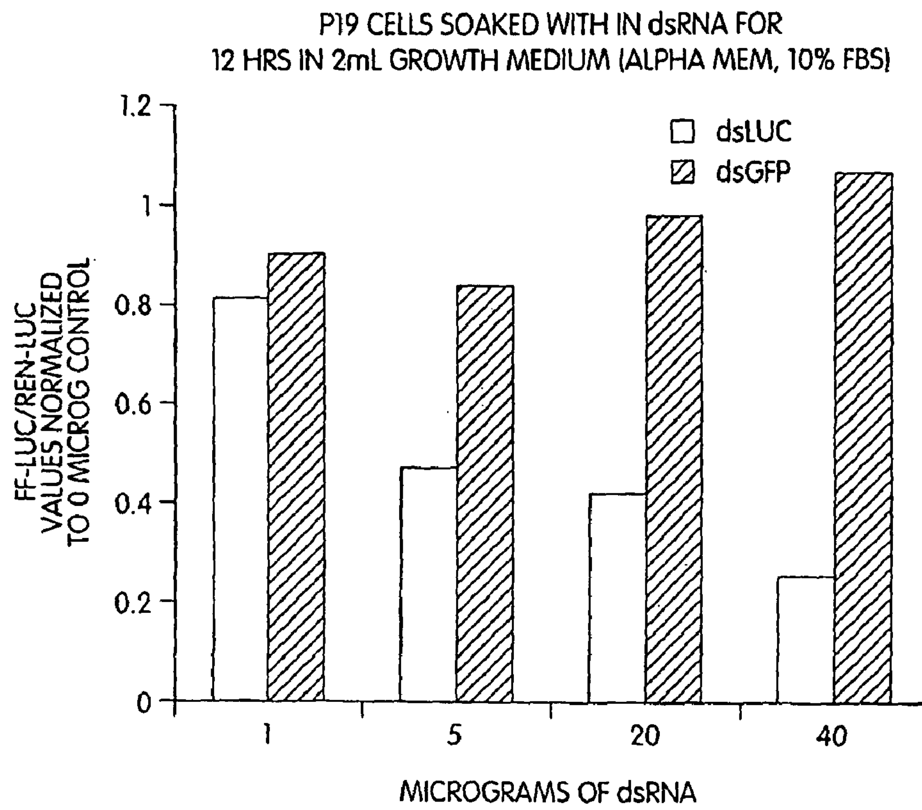


Fig. 37

IN VITRO SYNTHESIS OF siRNAs by T7 RNA POLYMERASE

a. T7

b. T7

TRANSCRIBE/ANNEAL

TREAT CELLS

ASSAY PHENOTYPE

DNA SYNTHESIS/RNA TRANSCRIPTION
~$16/siRNA VERSUS~$400/siRNA FOR CHEMICAL SYNTHESIS
BRINGS LARGE-SCALE PROJECTS WITHIN REASONABLE BUDGET RANGE

Fig. 38

siRNA

```
  UCGAAGUACUCAGCGUAAGUG
AAAGCUUCAUGAGUCGCAUUC
```

cshFf

```
                            U
CAUCGACUGAAAUCCCUGGUAAUCCGUUG U
GUAGCUGACUUUAGGGACCAUUAGGCAAC A
                            A
```

cshFf-L7

```
                              ----------      U
CAUCGACUGAAAUCCCUGGUAAUCCGUUU           GGGGC \
GUAGCUGAUUUUAGGGACUAUUAGGUAAA           UCCCG C
                              UAGGGUAUCG      U
```

cshFf-L7m

```
               GCC               ----------      U
CAUCGACUGAAAUCCC   GUAAUCCGUUU           GGGGC \
GUAGCUGAUUUUAGGG   UAUUAGGUAAA           UCCCG C
               AC-               UAGGGUAUCG      U
```

Fig. 39A

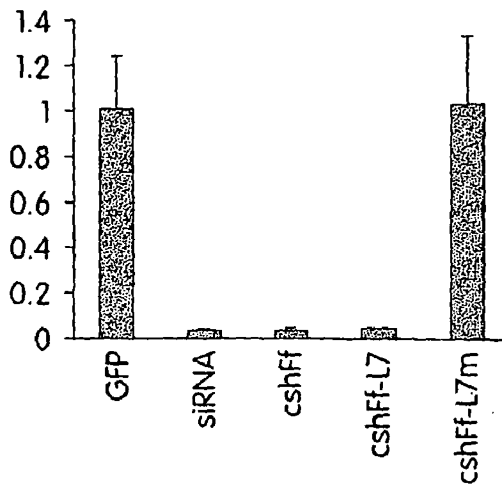


Fig. 39B

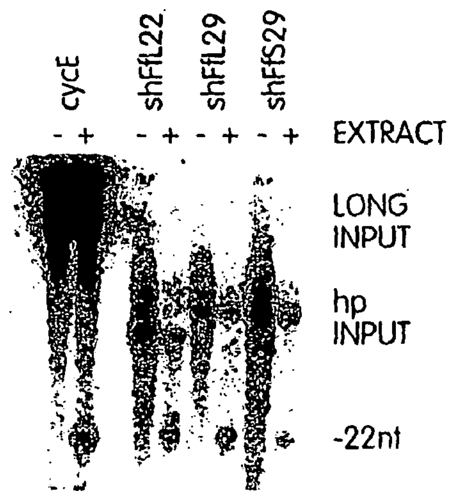


Fig. 39C

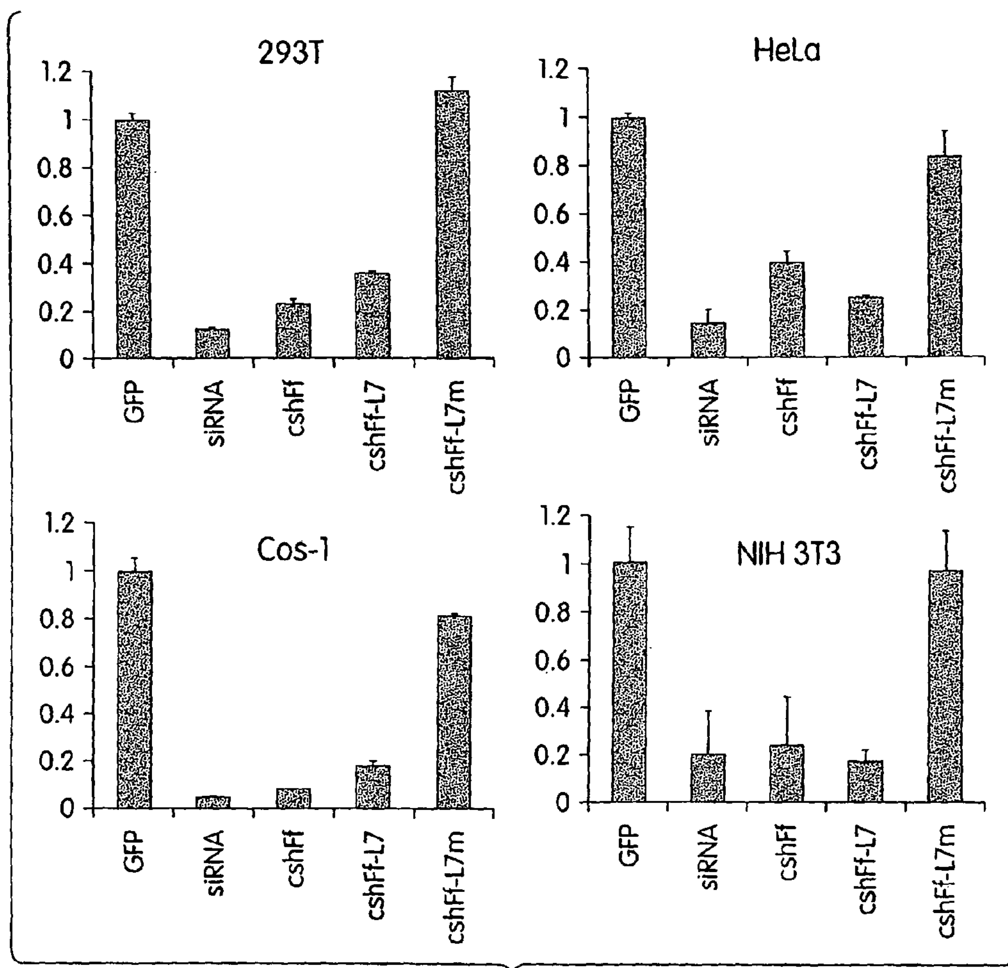


Fig. 40

siRNA
UCGAAGUACUCAGCGUAAGUG
AAAGCUUCAUGAGUCGCAUUC
T7siRNA
GGUCGAAGUACUCAGCGUAAGAA
AAAGCUUCATGAGUCGCAUUCGG
T7siFf-2
GGUUGUGGAUCUGGAUACCGG
UUCCAACACCUAGACCUAUGG
T7siFf-3
GGUGCCAACCCUAUUCUCCUU
GACCACGGUUGGGAUAAGAGG
T7siFf-8
GGCUAUGAAGAGAGUACGCCCU
UUCCGAUACUUCUCUCAUGCGG

Fig. 41A

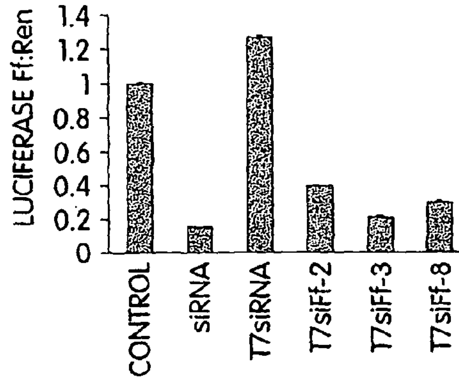


Fig. 41B

T7shFf29
```
GGU|                           U
   CGAAGUACUCAGCGUAAGUGAUGUCCAC U
   GUUUUGUGGGUUGUGUUUGUUGUGGGUG A
  G^                           A
```
T7shFf27
```
GGU|                         U
   CGAAGUACUCAGCGUAAGUGAUGUCC U
   GUUUUGUGGGUUGUGUUUGUUGUGGG A
  G^                         A
```
T7shFf25
```
GGU|                       U
   CGAAGUACUCAGCGUAAGUGAUGU U
   GUUUUGUGGGUUGUGUUUGUUGUG A
  G^                       A
```
T7shFf22
```
GGU|                    U
   CGAAGUACUCAGCGUAAGUGA U
   GUUUUGUGGGUUGUGUUUGUU A
  G^                    A
```
T7shFf29-5'T
```
GGCUCGAGU|                           U
         CGAAGUACUCAGCGUAAGUGAUGUCCAC U
         GUUUUGUGGGUUGUGUUUGUUGUGGGUG A
G--------^                           A
```
T7shFf29-3'T
```
-----G|                              U
      GUCGAAGUACUCAGCGUAAGUGAUGUCCAC U
      CGGUUUUGUGGGUUGUGUUUGUUGUGGGUG A
 GAGCU^                              A
```

Fig. 41C

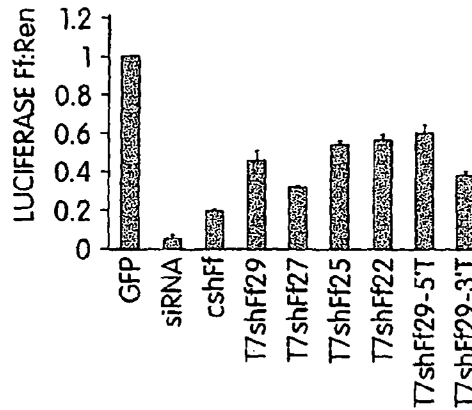


Fig. 41D

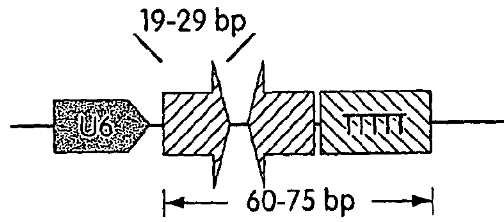


Fig. 42A

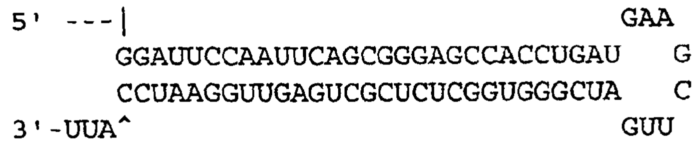


Fig. 42B

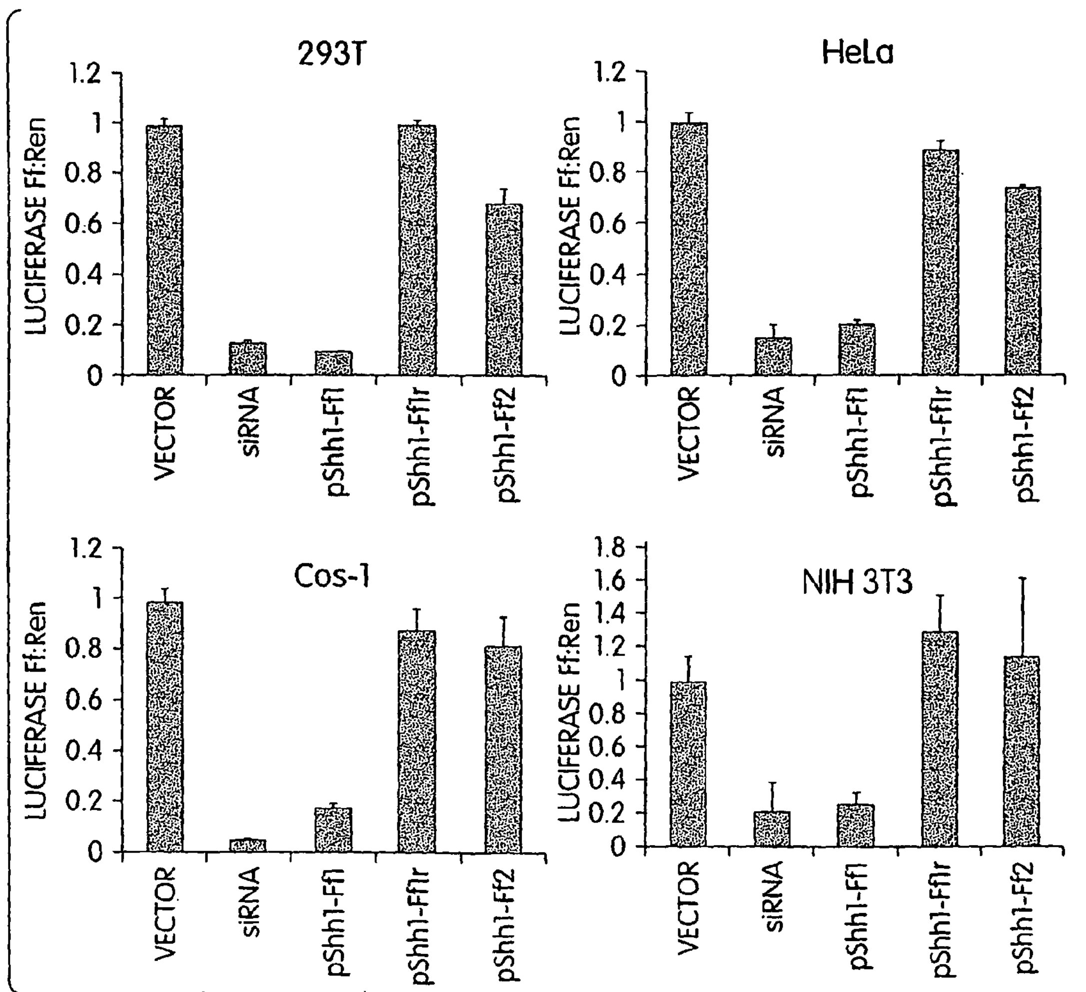


Fig. 42C

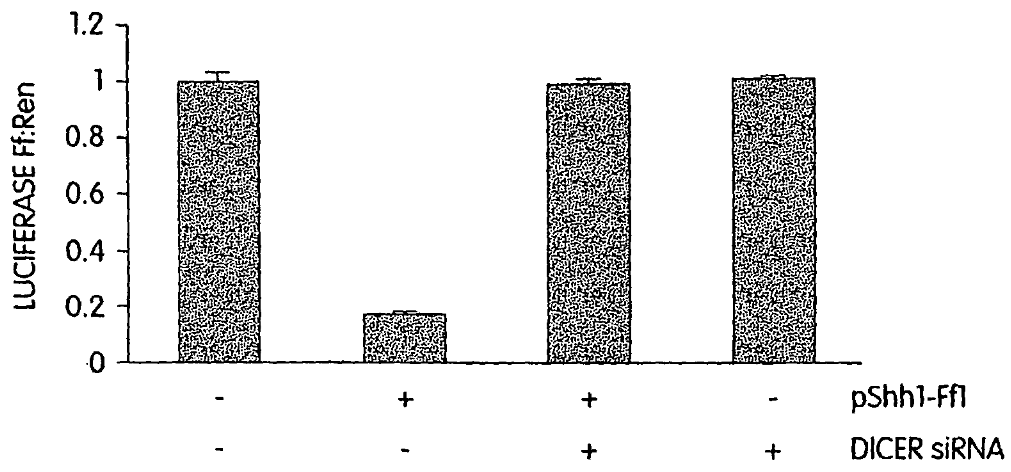


Fig. 43

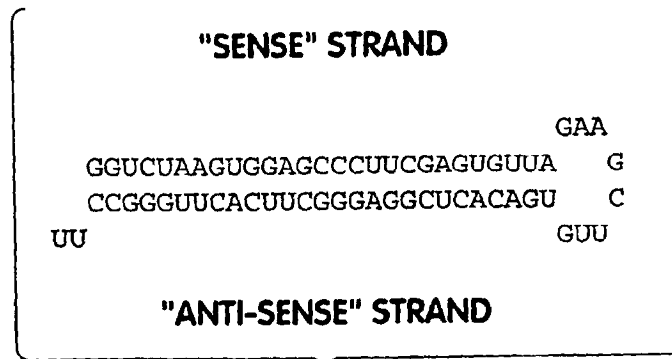


Fig. 44A

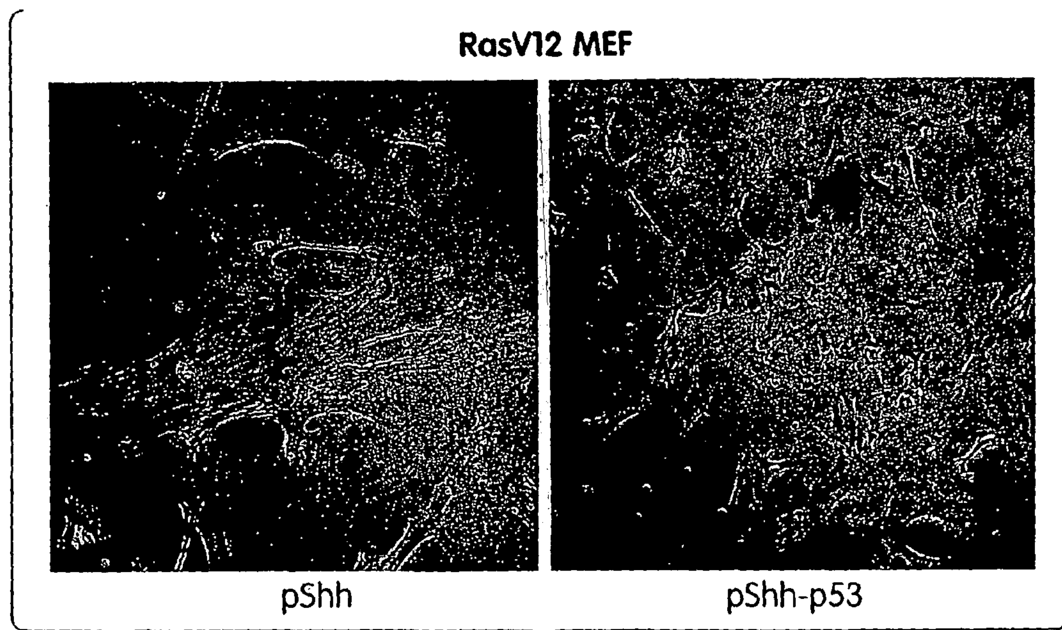


Fig. 44B

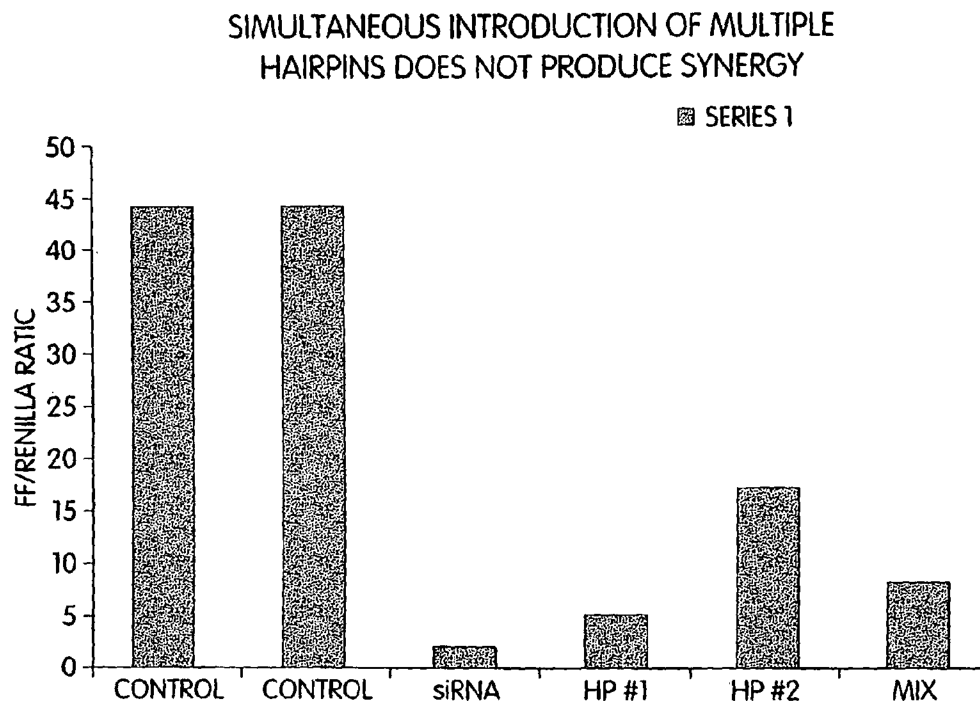


Fig. 45

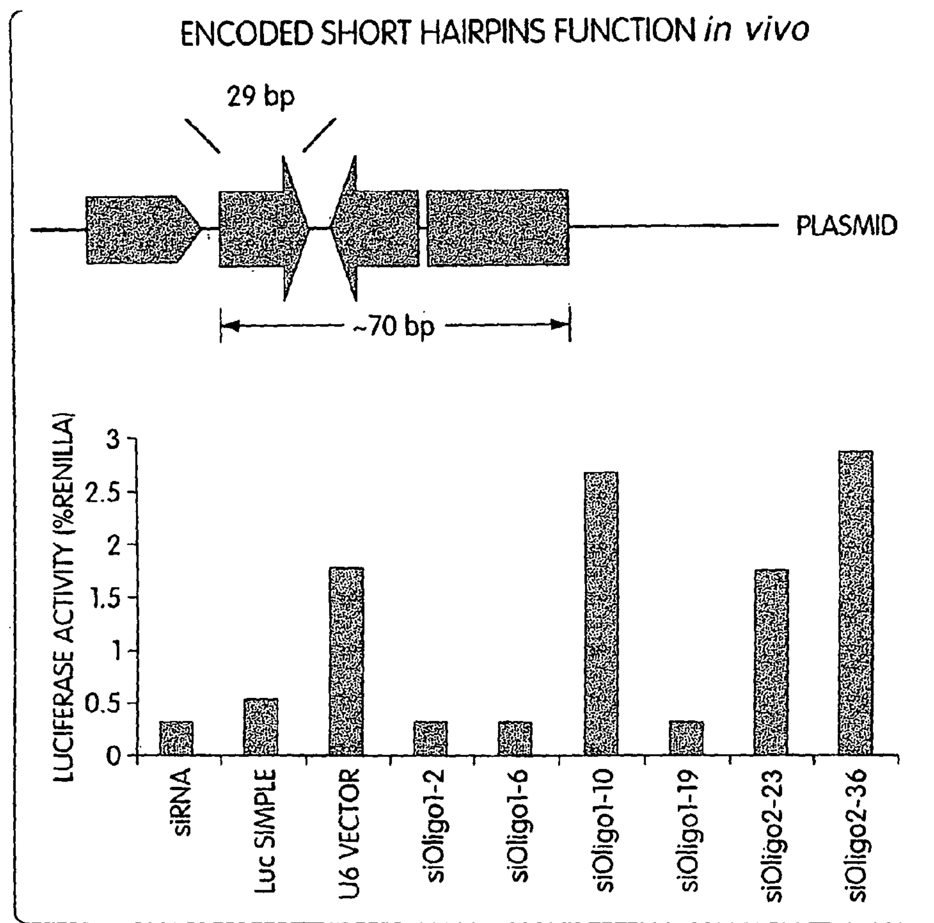


Fig. 46

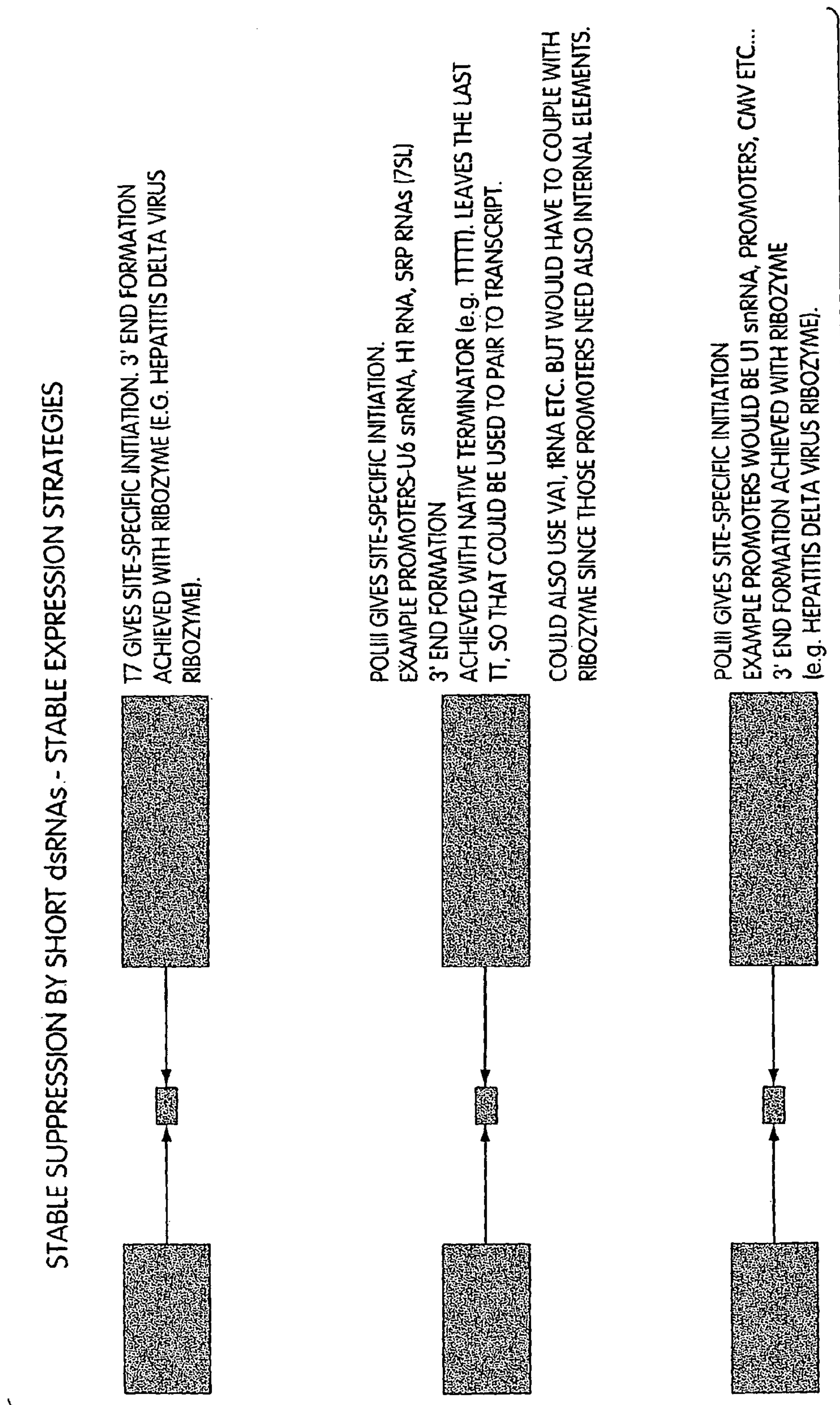


Fig. 47

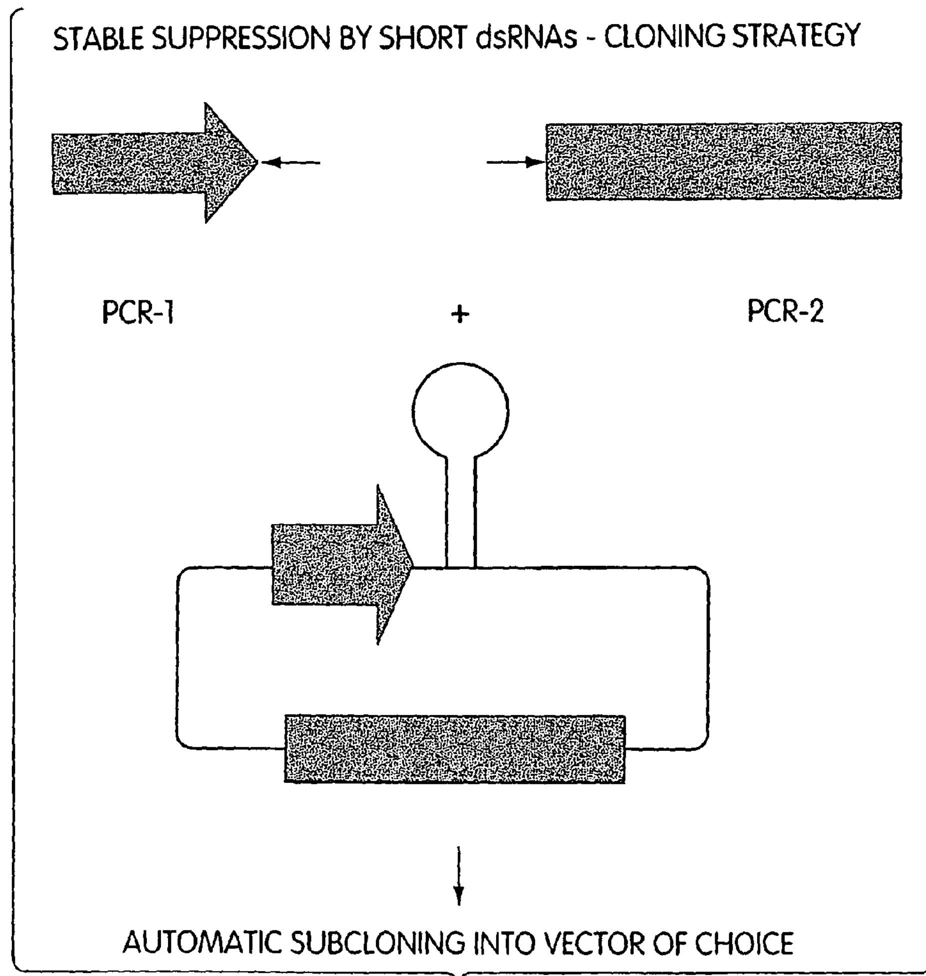


Fig. 48

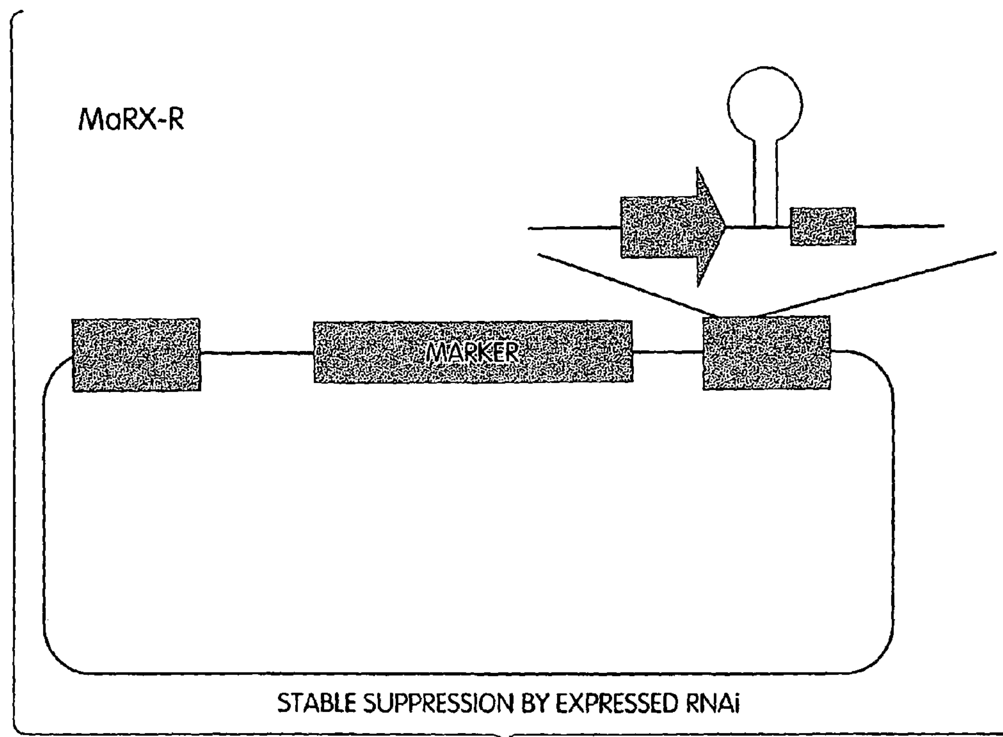


Fig. 49

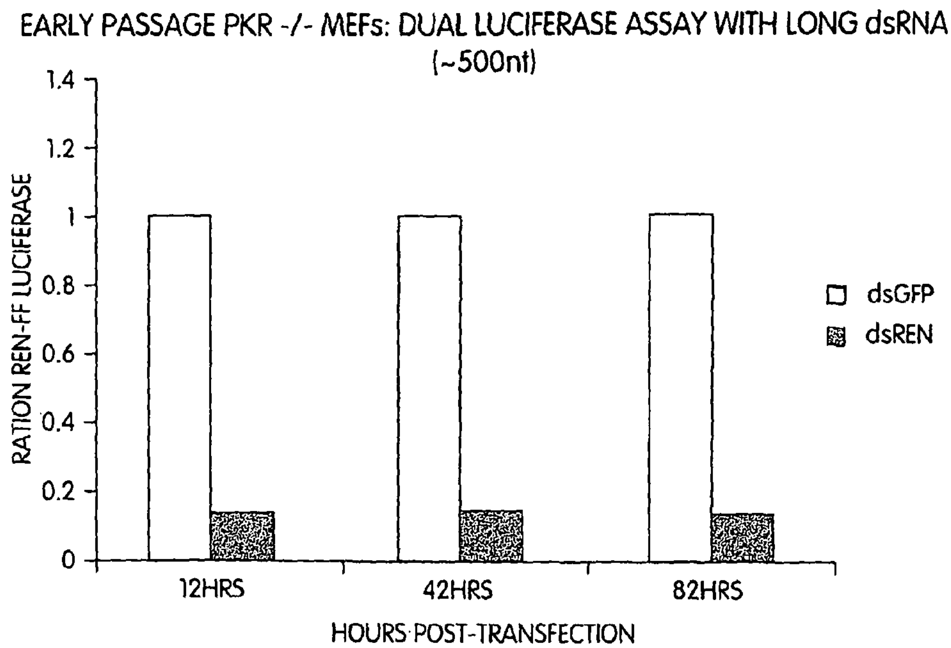


Fig. 50

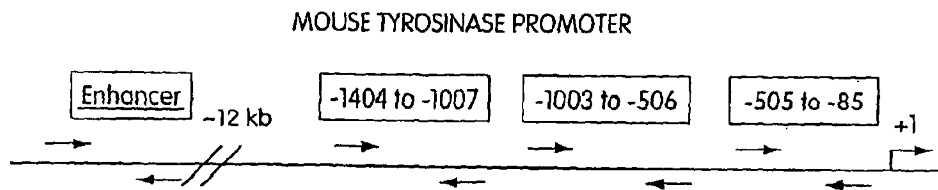


Fig. 51

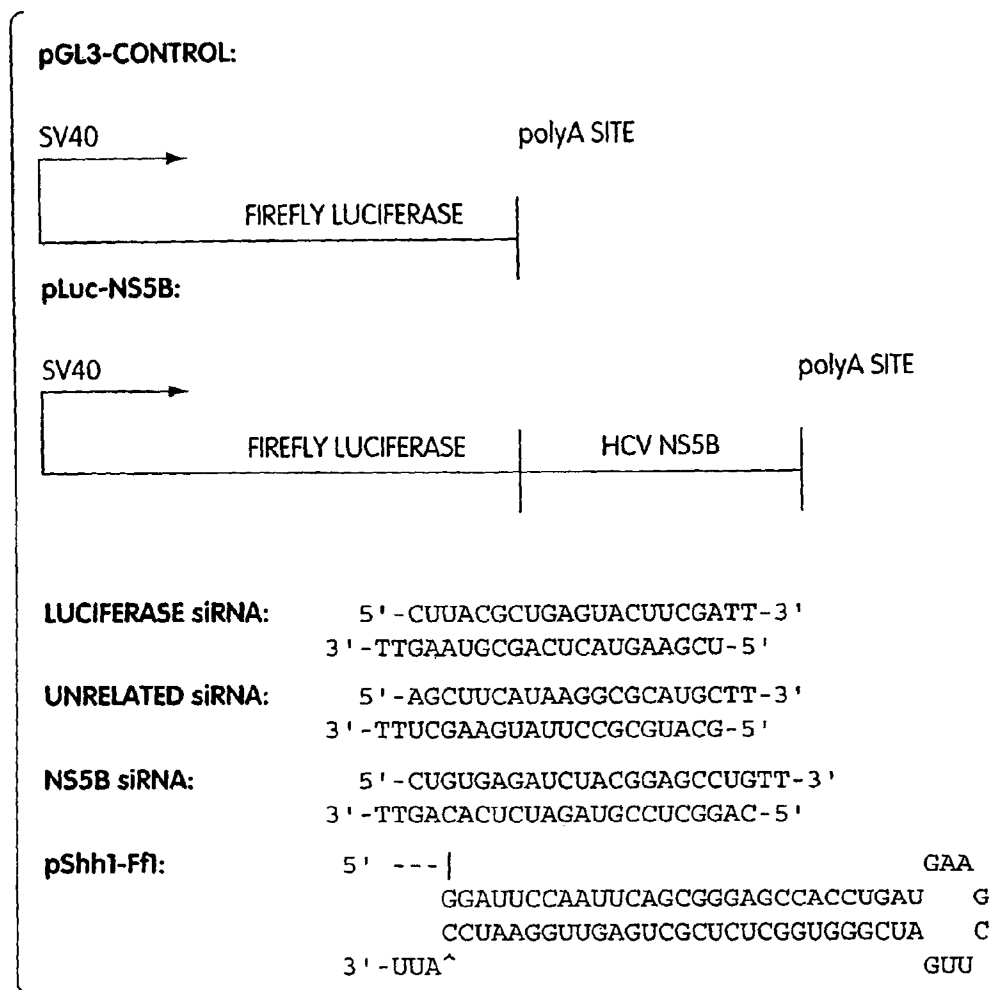


Fig. 52

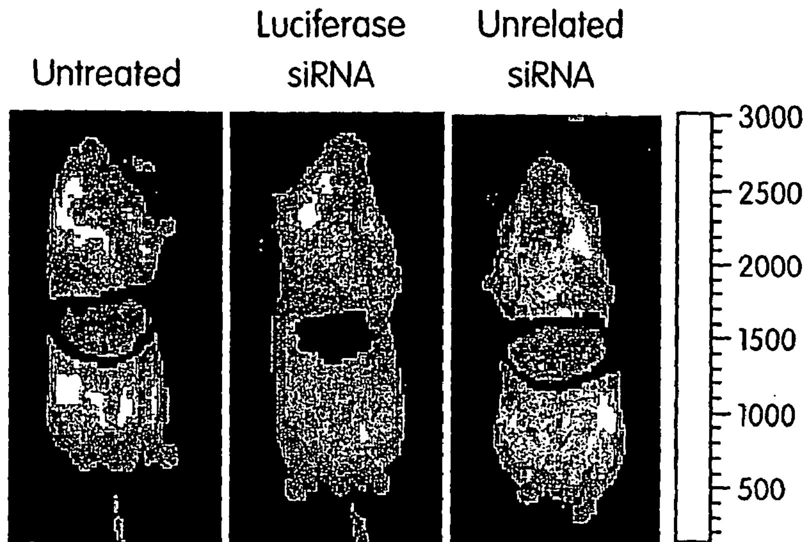


Fig. 53A

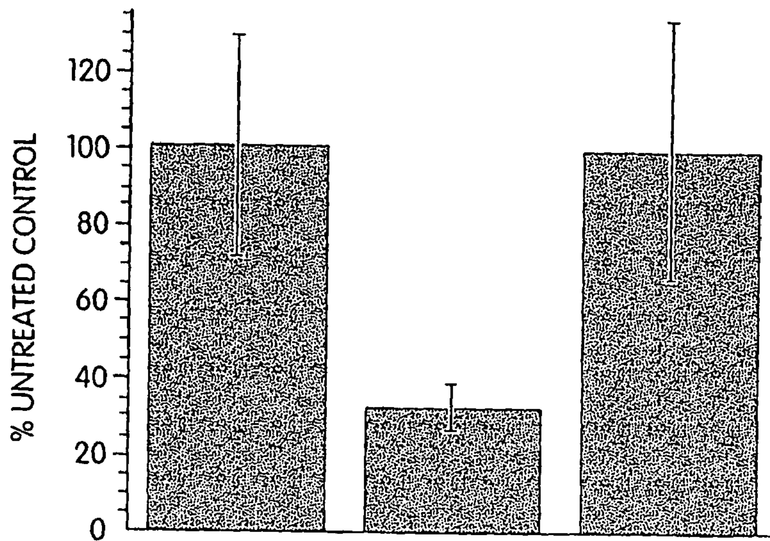


Fig. 53B

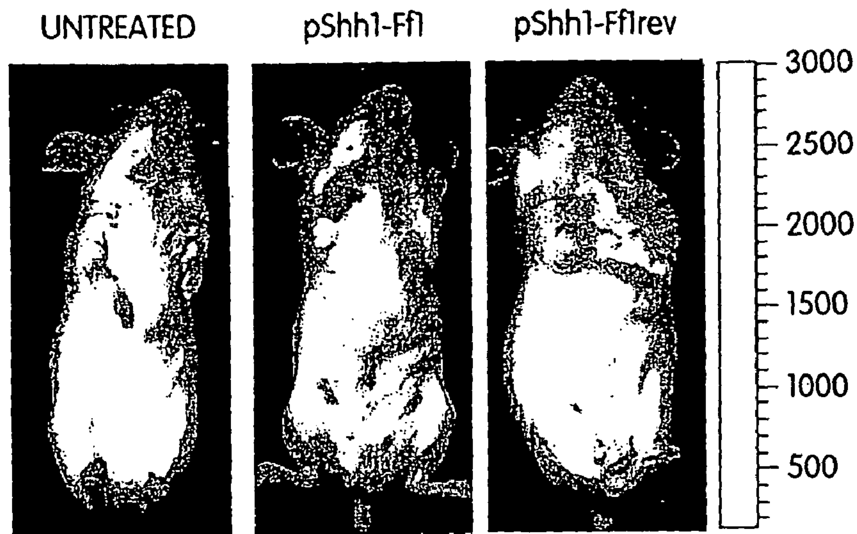


Fig. 54A

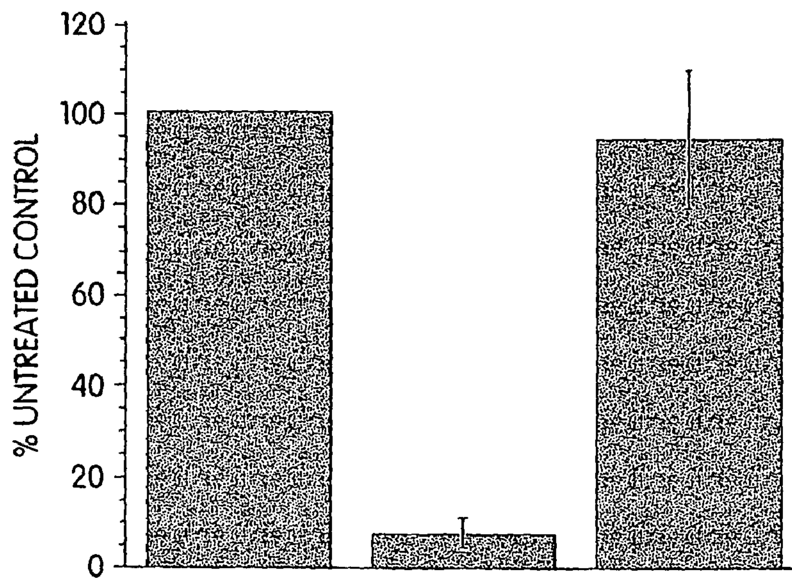


Fig. 54B

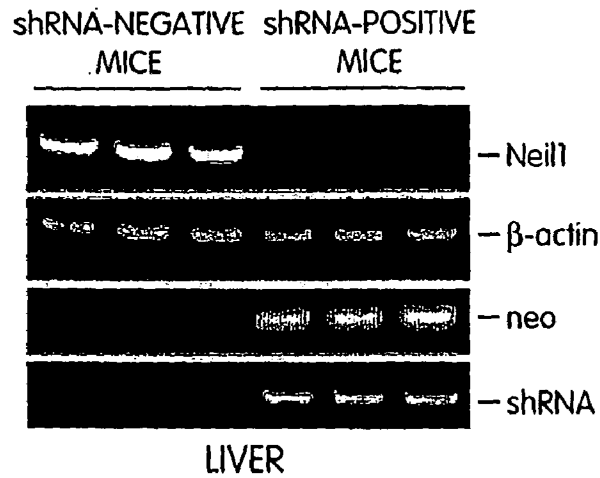


Fig. 55A

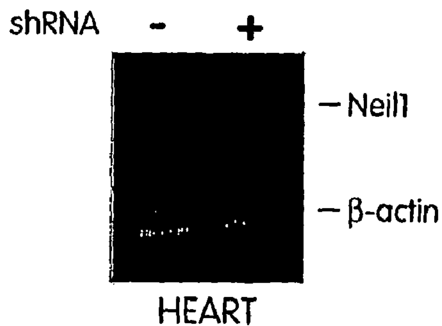


Fig. 55B

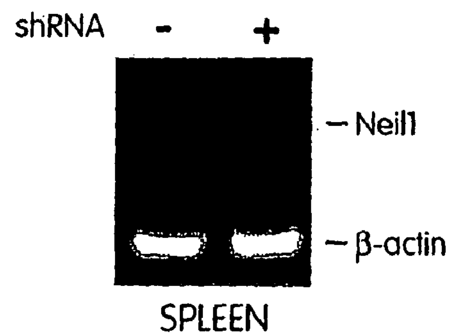


Fig. 55C

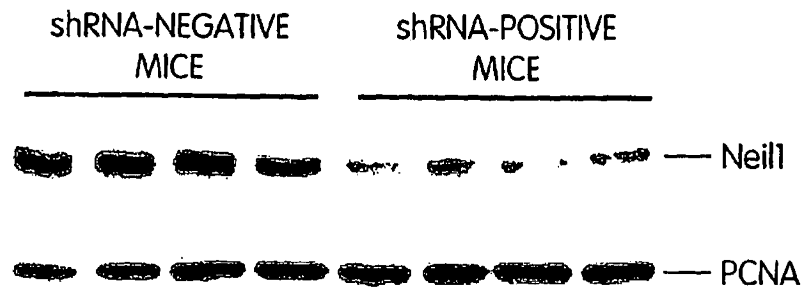


Fig. 56A

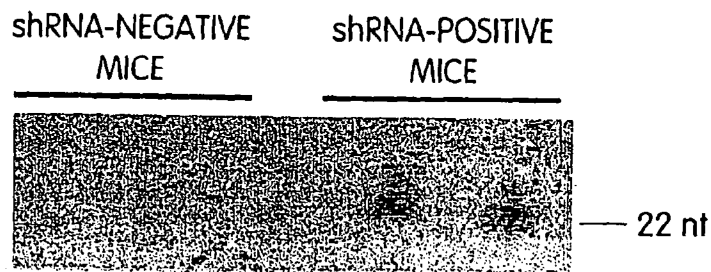


Fig. 56B

Fig. 57 A

29 nt. shRNA with overhang

NNNNNNNNNNNNNNNNNNNNNNNNNNNNN C C
UUNNNNNNNNNNNNNNNNNNNNNNNNNNNNN A A

29mer shRNA no overhang

NNNNNNNNNNNNNNNNNNNNNNNNNNNNN C C
NNNNNNNNNNNNNNNNNNNNNNNNNNNNN A A

19 nt. shRNA with overhang

NNNNNNNNNNNNNNNNNNN C C
UUNNNNNNNNNNNNNNNNNNN A A

19mer shRNA

NNNNNNNNNNNNNNNNNNN C C
NNNNNNNNNNNNNNNNNNN A A

Luciferase 29mer

AGUUGCGCCCGCGAAUGAUAUUUAUAAUG

Fig. 57 B

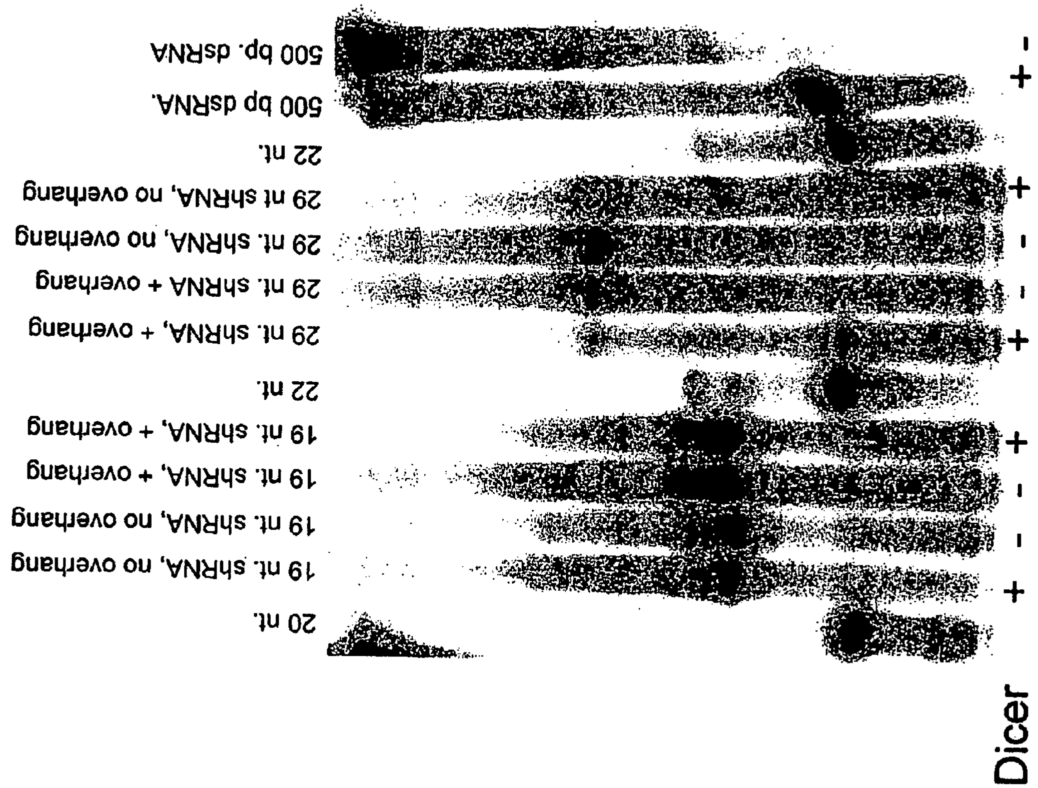


Fig. 57 C

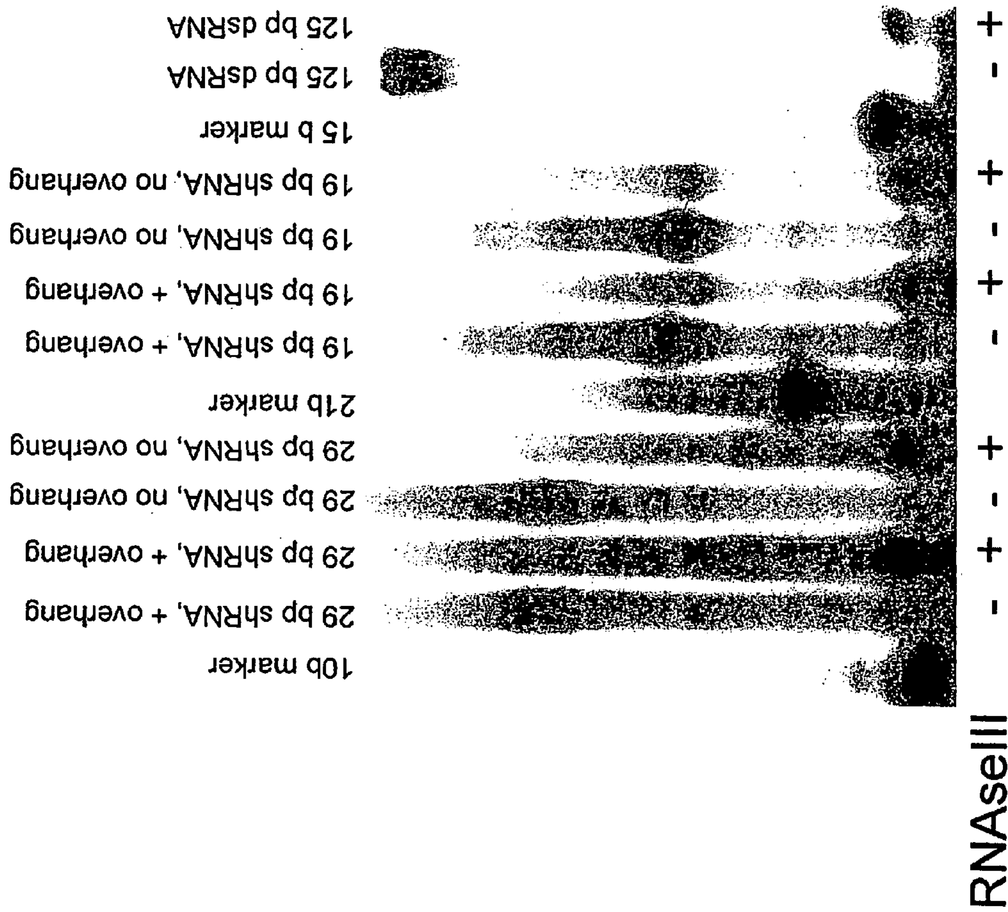


Fig. 58 A

Lane	Dicer	RT
25 b marker		
21 nt. siRNA	-	-
21 nt siRNA	-	+
22 nt. siRNA	-	-
22 nt. siRNA	-	+
20 b marker		
19 bp shRNA, + overhang	-	+
19 bp shRNA, + overhang	+	-
19 bp shRNA, + overhang	+	+
22 b marker		
19 bp shRNA, no overhang	-	+
19 bp shRNA, no overhang	+	-
19 bp shRNA, no overhang	+	+
primer only	-	+
15 b marker		

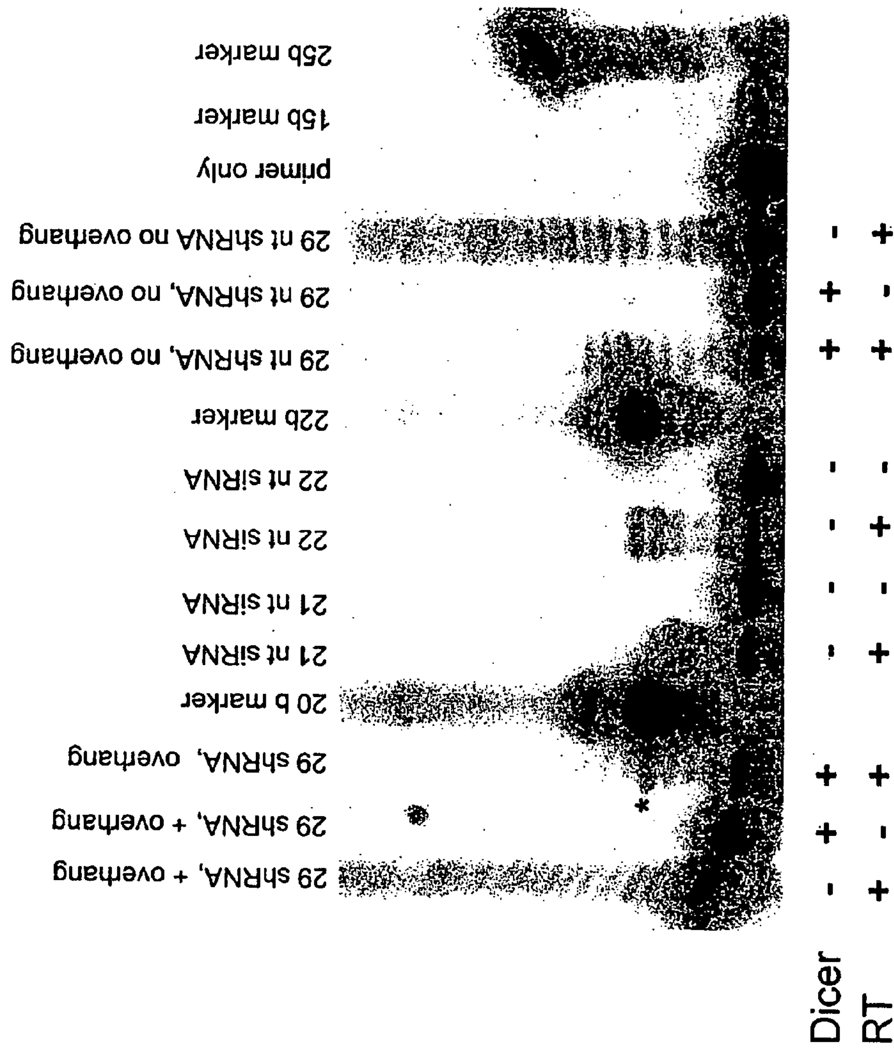


Fig. 58 B

Fig. 58 C

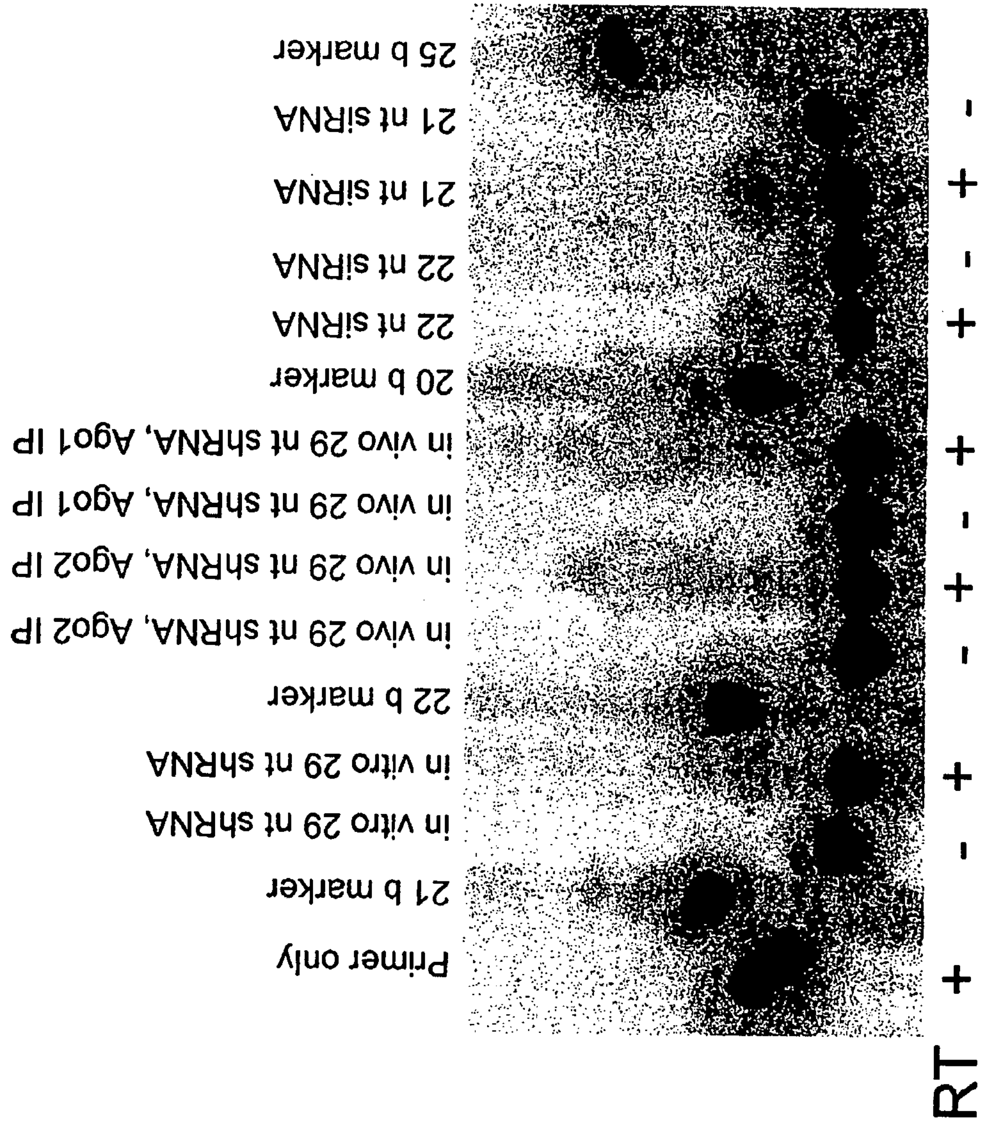


Fig. 59 A

siRNAs

19mer

NNNNNNNNNNNNNNNNNNNdTdT

dTdTNNNNNNNNNNNNNNNNNNN

Synthetic 19mer shRNAs

19mer of siRNA

NNNNNNNNNNNNNNNNNNN U U

UUNNNNNNNNNNNNNNNNNNN G G

Synthetic 29mer shRNAs

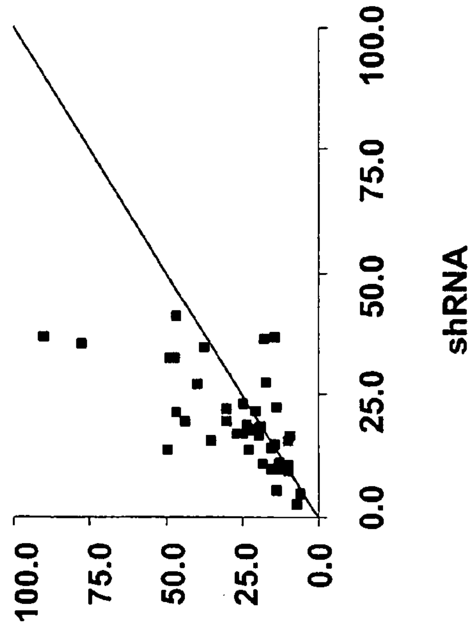
19mer of siRNA

XNNNNNNNNNNNNNNNNNNNXXXXXXXXX U U

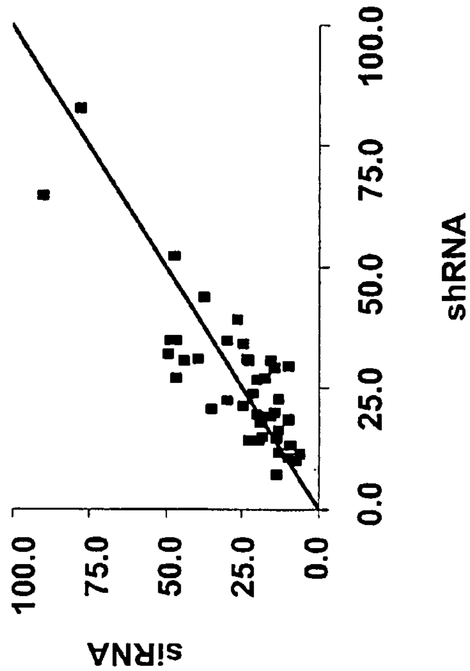
UUXNNNNNNNNNNNNNNNNNNNXXXXXXXXX G G

Fig. 59 B

29mer shRNAs vs. 19mer siRNAs



19mer shRNAs vs. siRNAs



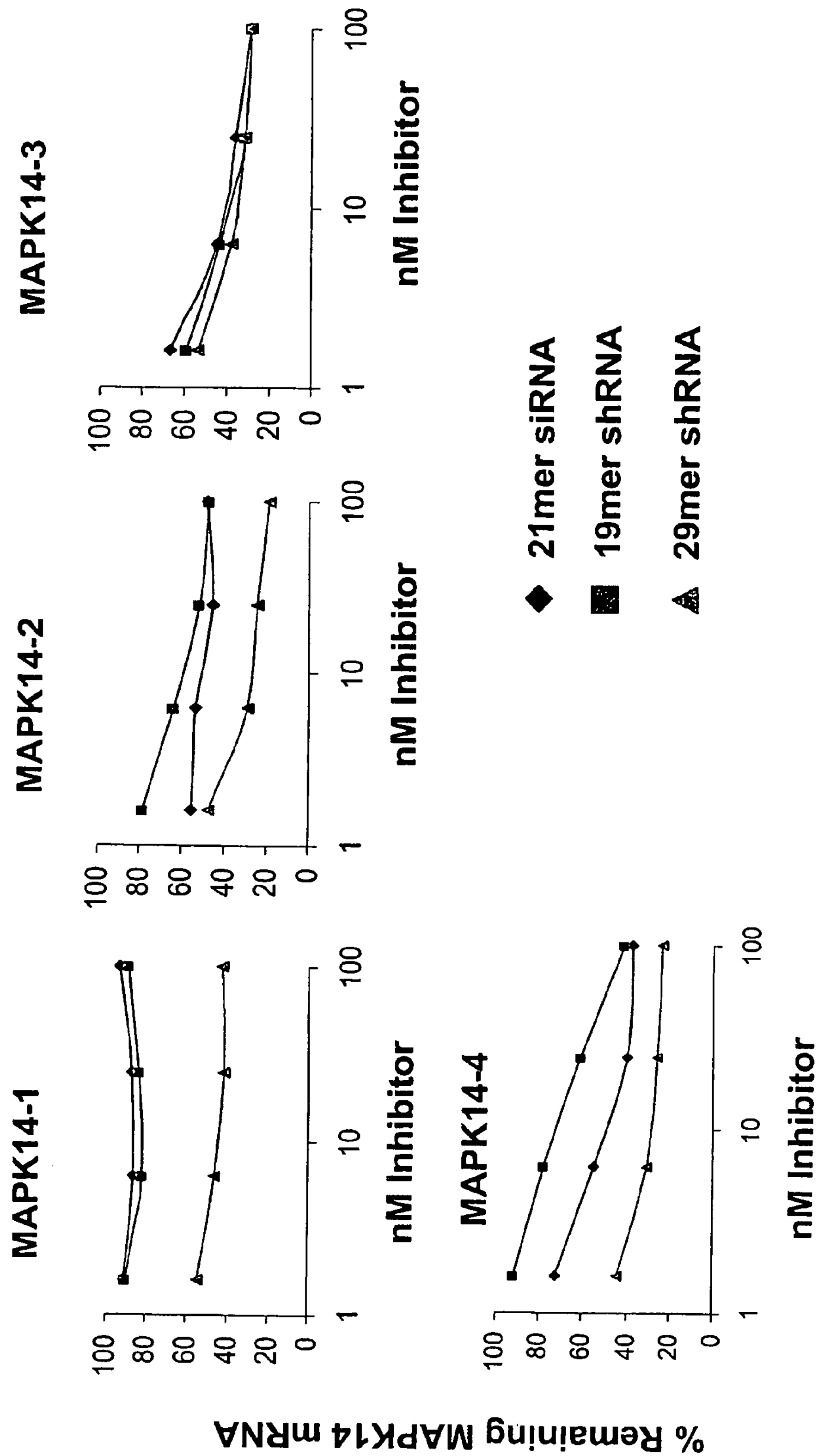


Fig. 59 C
MAPK14-1
MAPK14-2
MAPK14-3
MAPK14-4
% Remaining MAPK14 mRNA
100
80
60
40
20
0
1
10
100
nM Inhibitor
21mer siRNA
19mer shRNA
29mer shRNA

Fig. 60 A

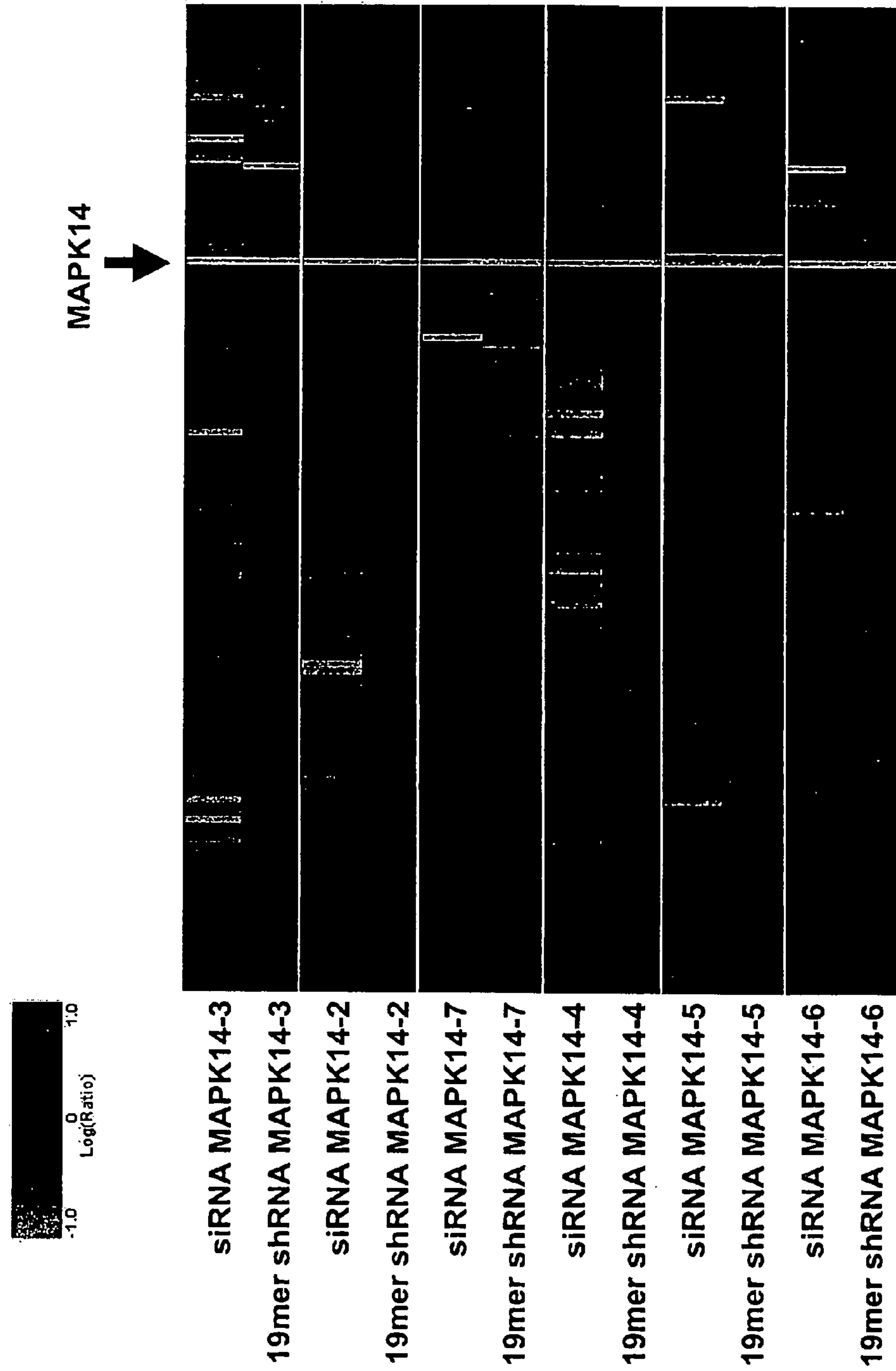
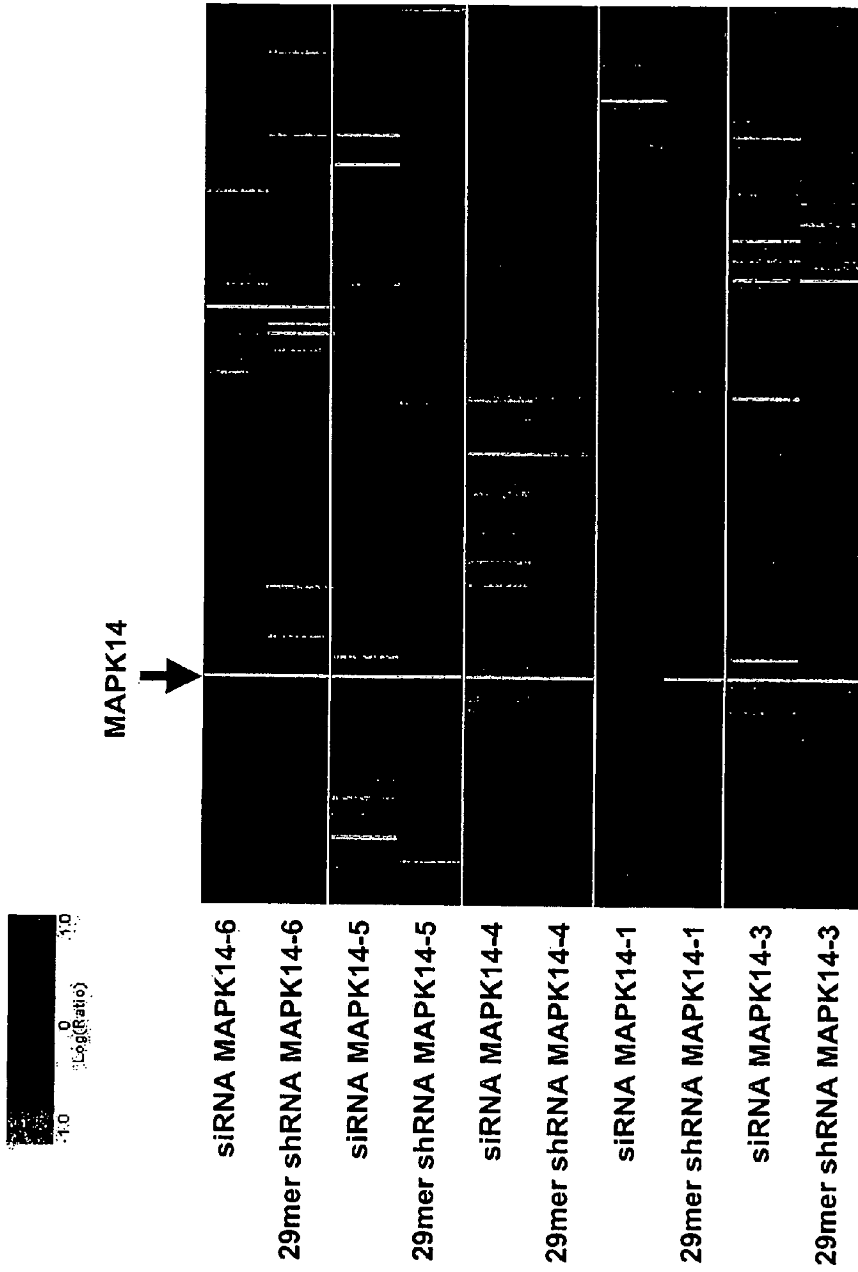


Fig. 60 B



METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

RELATED APPLICATIONS:

This application is a continuation application of U.S. Ser. No. 10/997,086, filed on Nov. 23, 2004, which is a continuation-in-part of U.S. Ser. No. 10/055,797, filed on Jan. 22, 2002, which is incorporated by reference herein.

GOVERNMENT SUPPORT

Work described herein was supported by National Institutes of Health Grant R01-GM62534. The United States Government may have certain rights in the invention.

BACKGROUND OF THE INVENTION

"RNA interference", "post-transcriptional gene silencing", "quelling"—these different names describe similar effects that result from the overexpression or misexpression of transgenes, or from the deliberate introduction of double-stranded RNA into cells (reviewed in Fire, *Trends Genet.* 15: 358-363, 1999; Sharp, *Genes Dev* 13: 139-141, 1999; Hunter, *Curr Biol* 9: R440-R442, 1999; Baulcombe, *Curr Biol* 9: R599-R601, 1999; Vaucheret et al., *Plant J* 16: 651-659, 1998). The injection of double-stranded RNA into the nematode *Caenorhabditis elegans*, for example, acts systemically to cause the post-transcriptional depletion of the homologous endogenous RNA (Fire et al., *Nature* 391: 806-811, 1998; and Montgomery et al., *PNAS* 95: 15502-15507, 1998). RNA interference, commonly referred to as RNAi, offers a way of specifically and potently inactivating a cloned gene, and is proving a powerful tool for investigating gene function. Although the phenomenon is interesting in its own right; the mechanism has been rather mysterious, but recent research—for example that recently reported by Smardon et al., *Curr Biol* 10: 169-178, 2000—is beginning to shed light on the nature and evolution of the biological processes that underlie RNAi.

RNAi was discovered when researchers attempting to use the antisense RNA approach to inactivate a *C. elegans* gene found that injection of sense-strand RNA was actually as effective as the antisense RNA at inhibiting gene function (Guo et al., *Cell* 81: 611-620, 1995). Further investigation revealed that the active agent was modest amounts of double-stranded RNA that contaminate in vitro RNA preparations. Researchers quickly determined the 'rules' and effects of RNAi which have become the paradigm for thinking about the mechanism which mediates this affect. Exon sequences are required, whereas introns and promoter sequences, while ineffective, do not appear to compromise RNAi (though there may be gene-specific exceptions to this rule). RNAi acts systemically—injection into one tissue inhibits gene function in cells throughout the animal. The results of a variety of experiments, in *C. elegans* and other organisms, indicate that RNAi acts to destabilize cellular RNA after RNA processing.

The potency of RNAi inspired Timmons and Fire (*Nature* 395: 854, 1998) to do a simple experiment that produced an astonishing result. They fed to nematodes bacteria that had been engineered to express double-stranded RNA corresponding to the *C. elegans* unc-22 gene. Amazingly, these nematodes developed a phenotype similar to that of unc-22 mutants that was dependent on their food source. The ability to conditionally expose large numbers of nematodes to gene-specific double-stranded RNA formed the basis for a very

powerful screen to select for RNAi-defective *C. elegans* mutants and then to identify the corresponding genes.

Double-stranded RNAs (dsRNAs) can provoke gene silencing in numerous in vitro contexts including *Drosophila, Caenorhabditis elegans*, planaria, hydra, trypanosomes, fungi and plants. However, the ability to recapitulate this phenomenon in higher eukaryotes, particularly mammalian cells, has not been accomplished in the art. Nor has the prior art demonstrated that this phenomena can be observed in cultured eukaryotic cells. Additionally, the 'rules' established by the prior art have taught that RNAi requires exon sequences, and thus constructs consisting of intronic or promoter sequences were not believed to be effective reagents in mediating RNAi. The present invention aims to address each of these deficiencies in the prior art and provides evidence both that RNAi can be observed in cultured eukaryotic cells and that RNAi constructs consisting of non-exon sequences can effectively repress gene expression.

SUMMARY OF THE INVENTION

One aspect of the present invention provides a method for attenuating expression of a target gene in cultured cells, comprising introducing double stranded RNA (dsRNA) into the cells in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene.

Another aspect of the present invention provides a method for attenuating expression of a target gene in a mammalian cell, comprising: (i) activating one or both of a Dicer activity or an Argonaut activity in the cell, and (ii) introducing into the cell a double stranded RNA (dsRNA) in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene.

In certain embodiments, the cell is suspended in culture; while in other embodiments the cell is in a whole animal, such as a non-human mammal.

In certain preferred embodiments, the cell is engineered with (i) a recombinant gene encoding a Dicer activity, (ii) a recombinant gene encoding an Argonaut activity, or (iii) both. For instance, the recombinant gene may encode, for a example, a protein which includes an amino acid sequence at least 50 percent identical to SEQ ID NO: 2 or 4; or be defined by a coding sequence which hybridizes under wash conditions of 2×SSC at 22° C. to SEQ ID NO: 1 or 3. In certain embodiments, the recombinant gene may encode, for a example, a protein which includes an amino acid sequence at least 50 percent identical to the Argonaut sequence shown in FIG. **24**. In certain embodiments, the recombinant gene may encode a protein which includes an amino acid sequence at least 60%, 70%, 80%, 85%, 90%, or 95% identical to SEQ ID NO: 2 or 4. In certain embodiments, the recombinant gene may be defined by a coding sequence which hybridizes under stringent conditions, including a wash step selected from 0.2-2.0×SSC at from 50° C.-65° C., to SEQ ID NO: 1 or 3.

In certain embodiments, rather than use a heterologous expression construct(s), an endogenous Dicer gene or Argonaut gene can be activated, e.g. by gene activation technology, expression of activated transcription factors or other signal transduction protein(s), which induces expression of the gene, or by treatment with an endogenous factor which upregulates the level of expression of the protein or inhibits the degradation of the protein.

In certain preferred embodiments, the target gene is an endogenous gene of the cell. In other embodiments, the target

gene is a heterologous gene relative to the genome of the cell, such as a pathogen gene, e.g., a viral gene.

In certain embodiments, the cell is treated with an agent that inhibits protein kinase RNA-activated (PKR) apoptosis, such as by treatment with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKR.

In certain preferred embodiments, the cell is a primate cell, such as a human cell.

In certain preferred embodiments, the length of the dsRNA is at least 20, 21 or 22 nucleotides in length, e.g., corresponding in size to RNA products produced by Dicer-dependent cleavage. In certain embodiments, the dsRNA construct is at least 25, 50, 100, 200, 300 or 400 bases. In certain embodiments, the dsRNA construct is 400-800 bases in length.

In certain preferred embodiments, expression of the target gene is attenuated by at least 5 fold, and more preferably at least 10, 20 or even 50 fold, e.g., relative to the untreated cell or a cell treated with a dsRNA construct which does not correspond to the target gene.

Yet another aspect of the present invention provides a method for attenuating expression of a target gene in cultured cells, comprising introducing an expression vector having a "coding sequence" which, when transcribed, produces double stranded RNA (dsRNA) in the cell in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene. In certain embodiments, the vector includes a single coding sequence for the dsRNA which is operably linked to (two) transcriptional regulatory sequences which cause transcription in both directions to form complementary transcripts of the coding sequence. In other embodiments, the vector includes two coding sequences which, respectively, give rise to the two complementary sequences which form the dsRNA when annealed. In still other embodiments, the vector includes a coding sequence which forms a hairpin. In certain embodiments, the vectors are episomal, e.g., and transfection is transient. In other embodiments, the vectors are chromosomally integrated, e.g., to produce a stably transfected cell line. Preferred vectors for forming such stable cell lines are described in U.S. Pat. No. 6,025,192 and PCT publication WO 98/12339, which are incorporated by reference herein.

Another aspect of the present invention provides a method for attenuating expression of a target gene in cultured cells, comprising introducing an expression vector having a "non-coding sequence" which, when transcribed, produces double stranded RNA (dsRNA) in the cell in an amount sufficient to attenuate expression of the target gene. The non-coding sequence may include intronic or promoter sequence of the target gene of interest, and the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the promoter or intron of the target gene. In certain embodiments, the vector includes a single sequence for the dsRNA which is operably linked to (two) transcriptional regulatory sequences which cause transcription in both directions to form complementary transcripts of the sequence. In other embodiments, the vector includes two sequences which, respectively, give rise to the two complementary sequences which form the dsRNA when annealed. In still other embodiments, the vector includes a coding sequence which forms a hairpin. In certain embodiments, the vectors are episomal, e.g., and transfection is transient. In other embodiments, the vectors are chromosomally integrated, e.g., to produce a stably transfected cell line. Preferred vectors for forming such stable cell lines are described in U.S. Pat. No. 6,025,192 and PCT publication WO 98/12339, which are incorporated by reference herein.

Another aspect the present invention provides a double stranded (ds) RNA for inhibiting expression of a mammalian gene. The dsRNA comprises a first nucleotide sequence that hybridizes under stringent conditions, including a wash step of 0.2×SSC at 65° C., to a nucleotide sequence of at least one mammalian gene and a second nucleotide sequence which is complementary to the first nucleotide sequence.

In one embodiment, the first nucleotide sequence of said double-stranded RNA is at least 20, 21, 22, 25, 50, 100, 200, 300, 400, 500, 800 nucleotides in length.

In another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to at least one mammalian gene. In another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to one mammalian gene. In yet another embodiment, the first nucleotide sequence of said double-stranded RNA hybridizes under stringent conditions to at least one human gene. In still another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to at least one human gene. In still another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to one human gene.

The double-stranded RNA may be an siRNA or a hairpin, and may be expressed transiently or stably. In one embodiment, the double-stranded RNA is a hairpin comprising a first nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of at least one mammalian gene, and a second nucleotide sequence which is a complementary inverted repeat of said first nucleotide sequence and hybridizes to said first nucleotide sequence to form a hairpin structure.

The first nucleotide sequence of said double-stranded RNA can hybridize to either coding or non-coding sequence of at least one mammalian gene. In one embodiment, the first nucleotide sequence of said double-stranded RNA hybridizes to a coding sequence of at least one mammalian gene. In another embodiment, the first nucleotide sequence of said double-stranded RNA hybridizes to a coding sequence of at least one human gene. In another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to a coding sequence of at least one mammalian gene. In still another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to a coding sequence of at least one human gene.

In another embodiment, the first nucleotide sequence of said double-stranded RNA is hybridizes to a non-coding sequence of at least one mammalian gene. In another embodiment, the first nucleotide sequence of said double-stranded RNA hybridizes to a non-coding sequence of at least one human gene. In another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to a non-coding sequence of at least one mammalian gene. In still another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to a non-coding sequence of at least one human gene. In any of the foregoing embodiments, the non-coding sequence may be a non-transcribed sequence.

Still another aspect of the present invention provides an assay for identifying nucleic acid sequences, either coding or non-coding sequences, responsible for conferring a particular phenotype in a cell, comprising: (i) constructing a variegated library of nucleic acid sequences from a cell in an orientation relative to a promoter to produce double stranded DNA; (ii) introducing the variegated dsRNA library into a culture of target cells; (iii) identifying members of the library which confer a particular phenotype on the cell, and identifying the

sequence from a cell which correspond, such as being identical or homologous, to the library member.

Yet another aspect of the present invention provides a method of conducting a drug discovery business comprising: (i) identifying, by the subject assay, a target gene which provides a phenotypically desirable response when inhibited by RNAi; (ii) identifying agents by their ability to inhibit expression of the target gene or the activity of an expression product of the target gene; (iii) conducting therapeutic profiling of agents identified in step (b), or further analogs thereof, for efficacy and toxicity in animals; and (iv) formulating a pharmaceutical preparation including one or more agents identified in step (iii) as having an acceptable therapeutic profile.

The method may include an additional step of establishing a distribution system for distributing the pharmaceutical preparation for sale, and may optionally include establishing a sales group for marketing the pharmaceutical preparation.

Another aspect of the present invention provides a method of conducting a target discovery business comprising: (i) identifying, by the subject assay, a target gene which provides a phenotypically desirable response when inhibited by RNAi; (ii) (optionally) conducting therapeutic profiling of the target gene for efficacy and toxicity in animals; and (iii) licensing, to a third party, the rights for further drug development of inhibitors of the target gene.

Another aspect of the invention provides a method for inhibiting RNAi by inhibiting the expression or activity of an RNAi enzyme. Thus, the subject method may include inhibiting the activity of Dicer and/or the 22-mer RNA.

Still another aspect relates to a method for altering the specificity of an RNAi by modifying the sequence of the RNA component of the RNAi enzyme.

In another aspect, gene expression in an undifferentiated stem cell, or the differentiated progeny thereof, is altered by introducing dsRNA of the present invention. In one embodiment, the stem cells are embryonic stem cells. Preferably, the embryonic stem cells are derived from mammals, more preferably from non-human primates, and most preferably from humans.

The embryonic stem cells may be isolated by methods known to one of skill in the art from the inner cell mass (ICM) of blastocyst stage embryos. In one embodiment the embryonic stem cells are obtained from previously established cell lines. In a second embodiment, the embryonic stem cells are derived de novo by standard methods.

In another aspect, the embryonic stem cells are the result of nuclear transfer. The donor nuclei are obtained from any adult, fetal, or embryonic tissue by methods well known in the art. In one embodiment, the donor nuclei is transferred to a recipient oocyte which had previously been modified. In one embodiment, the oocyte is modified using one or more dsRNAs. Exemplary modifications of the recipient oocyte include any changes in gene or protein expression that prevent an embryo derived from said modified oocyte from successfully implanting in the uterine wall. Since implantation in the uterine wall is essential for fertilized mammalian embryos to progress from beyond the blastocyst stage, embryos made from such modified oocytes could not give rise to viable organisms. Non-limiting examples of such modifications include those that decrease or eliminate expression of cell surface receptors (i.e., integrins) required for the recognition between the blastocyst and the uterine wall, modifications that decrease or eliminate expression of proteases (i.e., collagenase, stromelysin, and plasminogen activator) required to digest matrix in the uterine lining and thus allow proper implantation, and modifications that decrease or eliminate expression of proteases (i.e., trypsin) necessary for the blastocyst to hatch from the zona pellucida. Such hatching is required for implantation.

In another embodiment, embryonic stem cells, embryonic stem cells obtained from fertilization of modified oocytes, or the differentiated progeny thereof, can be modified or further modified with one or more dsRNAs. In a preferred embodiment, the modification decreases or eliminates MHC expression. Cells modified in this way will be tolerated by the recipient, thus avoiding complications arising from graft rejection. Such modified cells are suitable for transplantation into a related or unrelated patient to treat a condition characterized by cell damage or cell loss.

In another aspect of the invention, the undifferentiated stem cell is an adult stem cell. Exemplary adult stem cells include, but are not limited to, hematopoietic stem cells, mesenchymal stem cells, cardiac stem cells, pancreatic stem cells, and neural stem cells. Exemplary adult stem cells include any stem cell capable of forming differentiated ectodermal, mesodermal, or endodermal derivatives. Non-limiting examples of differentiated cell types which arise from adult stem cells include: blood, skeletal muscle, myocardium, endocardium, pericardium, bone, cartilage, tendon, ligament, connective tissue, adipose tissue, liver, pancreas, skin, neural tissue, lung, small intestine, large intestine, gall bladder, rectum, anus, bladder, female or male reproductive tract, genitals, and the linings of the body cavity.

In one embodiment, an undifferentiated adult stem cell, or the differentiated progeny thereof, is altered with one or more dsRNAs to decrease or eliminate MHC expression. Cells modified in this way will be tolerated by the recipient, thus avoiding complications arising from graft rejection. Such modified cells are suitable for transplantation into a related or unrelated patient to treat a condition characterized by cell damage or cell loss.

In another aspect of the invention, an embryonic stem cell, an undifferentiated adult stem cell, or the differentiated progeny of either an embryonic or adult stem cell is altered with one or more dsRNA to decrease or eliminate expression of genes required for HIV infection. In a preferred embodiment, the stem cell is one capable of giving rise to hematopoietic cells. Modified cells with hematopoietic potential can be transplanted into a patient as a preventative therapy or treatment for HIV or AIDS.

Another aspect of the invention relates to purified or semi-purified preparations of the RNAi enzyme or components thereof. In certain embodiments, the preparations are used for identifying compounds, especially small organic molecules, which inhibit or potentiate the RNAi activity. Small molecule inhibitors, for example, can be used to inhibit dsRNA responses in cells which are purposefully being transfected with a virus which produces double stranded RNA.

The dsRNA construct may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The dsRNA construct may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. In certain embodiments, dsRNA constructs containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition. RNA sequences with insertions, dele-

tions, and single point mutations relative to the target sequence (i.e., RNA sequences similar to the target sequence) have also been found to be effective for inhibition. Thus, sequence identity may be optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript. In another embodiment, dsRNA constructs containing nucleotide sequences identical to a non-coding portion of the target gene are preferred for inhibition. Exemplary non-coding regions include introns and the promoter region. Sequences with insertions, deletions, and single point mutations relative to the target non-coding sequence may also be used.

Yet another aspect of the invention pertains to transgenic non-human mammals which include a transgene encoding a dsRNA construct, wherein the dsRNA is identical or similar to either the coding or non-coding sequence of the target gene, preferably which is stably integrated into the genome of cells in which it occurs. The animals can be derived by oocyte microinjection, for example, in which case all of the nucleated cells of the animal will include the transgene, or can be derived using embryonic stem (ES) cells which have been transfected with the transgene, in which case the animal is a chimera and only a portion of its nucleated cells will include the transgene. In certain instances, the sequence-independent dsRNA response, e.g., the PKR response, is also inhibited in those cells including the transgene.

In still other embodiments, dsRNA itself can be introduced into an ES cell in order to effect gene silencing, and that phenotype will be carried for at least several rounds of division, e.g., into the progeny of that cell.

Another aspect of the invention provides a method for attenuating expression of a target gene in mammalian cells, comprising introducing into the mammalian cells a single-stranded hairpin ribonucleic acid (shRNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA: (i) is a substrate for cleavage by a RNaseIII enzyme to produce a double-stranded RNA product, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions. Preferably, the shRNA comprises a 3' overhang of about 14 nucleotides.

A related aspect of the invention provides a method for attenuating expression of a target gene in mammalian cells, comprising introducing into the mammalian cells a single-stranded hairpin ribonucleic acid (shRNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA: (i) is cleaved in the mammalian cells to produce an RNA guide sequence that enters an Argonaut-containing complex, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions. Preferably, the shRNA comprises a 3' overhang of about 1-4 nucleotides.

Yet another related aspect of the invention provides a method for attenuating expression of one or more target genes in mammalian cells, comprising introducing into the mammalian cells a variegated library of single-stranded hairpin ribonucleic acid (shRNA) species, each shRNA species comprising self complementary sequences of 19 to 100 nucleotides that form duplex regions and which hybridize under intracellular conditions to a target gene, wherein each of said hairpin RNA species: (i) is a substrate for cleavage by a RNaseIII enzyme to produce a double-stranded RNA product, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) if complementary to a target sequence, reduces expression of said target gene in a manner dependent on the sequence of said complementary regions. Preferably, the shRNA comprises a 3' overhang of about 1-4 nucleotides.

In one embodiment, the shRNA comprises a 3' overhang of 2 nucleotides.

In one embodiment, the shRNA comprises self-complementary sequences of 25 to 29 nucleotides that form duplex regions.

In one embodiment, the self-complementary sequences are 29 nucleotides in length.

In one embodiment, the shRNA is transfected or microinjected into said mammalian cells.

In one embodiment, the shRNA is a transcriptional product that is transcribed from an expression construct introduced into said mammalian cells, which expression construct comprises a coding sequence for transcribing said shRNA, operably linked to one or more transcriptional regulatory sequences. The transcriptional regulatory sequences may include a promoter for an RNA polymerase, such as a cellular RNA polymerase.

In one embodiment, the promoter is a U6 promoter, a T7 promoter, a T3 promoter, or an SP6 promoter.

In one embodiment, the transcriptional regulatory sequences includes an inducible promoter.

In one embodiment, the mammalian cells are stably transfected with said expression construct.

In one embodiment, the mammalian cells are primate cells, such as human cells.

In one embodiment, the shRNA is introduced into the mammalian cells in cell culture or in an animal.

In one embodiment, the expression of the target is attenuated by at least 33 percent relative expression in cells not treated said hairpin RNA.

In one embodiment, the target gene is an endogenous gene or a heterologous gene relative to the genome of the mammalian cell.

In one embodiment, the self complementary sequences hybridize under intracellular conditions to a non-coding sequence of the target gene selected from a promoter sequence, an enhancer sequence, or an intronic sequence.

In one embodiment, the shRNA includes one or more modifications to phosphate-sugar backbone or nucleosides residues.

In one embodiment, the variegated library of shRNA species are arrayed a solid substrate.

In one embodiment, the method includes the further step of identifying shRNA species of said variegated library which produce a detected phenotype in said mammalian cells.

In one embodiment, the shRNA is a chemically synthesized product or an in vitro transcription product.

Another aspect of the invention provides a method of enhancing the potency/activity of an RNAi therapeutic for a mammalian patient, said RNAi therapeutic comprising an siRNA of 19-22 paired polynucleotides, the method comprising replacing said siRNA with a single-stranded hairpin RNA (shRNA) of claim **1** or **2**, wherein said duplex region comprises the same 19-22 paired polynucleotides of said siRNA.

In one embodiment, the shRNA comprises a 3' overhang of 2 nucleotides.

In one embodiment, the half-maximum inhibition by said RNAi therapeutic is achieved by a concentration of said shRNA at least about 20% lower than that of said siRNA.

In one embodiment, the half-maximum inhibition by said RNAi therapeutic is achieved by a concentration of said shRNA at least about 100% lower than that of said siRNA.

In one embodiment, the end-point inhibition by said shRNA is at least about 40% higher than that of said siRNA.

In one embodiment, the end-point inhibition by said shRNA is at least about 2-6 fold higher than that of said siRNA.

Another aspect of the invention provides a method of designing a short hairpin RNA (shRNA) construct for RNAi, said shRNA comprising a 3' overhang of about 14 nucleotides, the method comprising selecting the nucleotide about 21 bases 5' to the most 3'-end nucleotide as the first paired nucleotide in a cognate doubled-stranded siRNA with the same 3' overhang.

In one embodiment, the shRNA comprises 25-29 paired polynucleotides.

In one embodiment, the shRNA, when cut by a Dicer enzyme, produces a product siRNA that is either identical to, or differ by a single basepair immediately 5' to the 3' overhang from, said cognate siRNA.

In one embodiment, the Dicer enzyme is a human Dicer.

In one embodiment, the 3' overhang has 2 nucleotides.

In one embodiment, the shRNA is for RNAi in mammalian cells.

All embodiments described above can be freely combined with one or more other embodiments whenever appropriate. Such combination also includes embodiments described under different aspects of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**: RNAi in S2 cells. (a) *Drosophila* S2 cells were transfected with a plasmid that directs lacZ expression from the copia promoter in combination with dsRNAs corresponding to either human CD8 or lacZ, or with no dsRNA, as indicated. (b) S2 cells were co-transfected with a plasmid that directs expression of a GFP-US9 fusion protein and dsRNAs of either lacZ or cyclin E, as indicated. Upper panels show FACS profiles of the bulk population. Lower panels show FACS profiles from GFP-positive cells. (c) Total RNA was extracted from cells transfected with lacZ, cyclin E, fizzy or cyclin A dsRNAs, as indicated. Northern blots were hybridized with sequences not present in the transfected dsRNAs.

FIG. **2**: RNAi in vitro. (a) Transcripts corresponding to either the first 600 nucleotides of *Drosophila cyclin* E (E600) or the first 800 nucleotides of lacZ (Z800) were incubated in lysates derived from cells that had been transfected with either lacZ or cyclin E (cycE) dsRNAs, as indicated. Time points were 0, 10, 20, 30, 40 and 60 min for cyclin E and 0, 10, 20, 30 and 60 min for lacZ. (b) Transcripts were incubated in an extract of S2 cells that had been transfected with cyclin E dsRNA (cross-hatched box, below). Transcripts corresponded to the first 800 nucleotides of lacZ or the first 600, 300, 220 or 100 nucleotides of cyclin E, as indicated. Eout is a transcript derived from the portion of the cyclin E cDNA not contained within the transfected dsRNA. E-ds is identical to the dsRNA that had been transfected into S2 cells. Time points were 0 and 30 min. (c) Synthetic transcripts complementary to the complete cyclin E cDNA (Eas) or the final 600 nucleotides (Eas600) or 300 nucleotides (Eas300) were incubated in extract for 0 or 30 min.

FIG. **3**: Substrate requirements of the RISC. Extracts were prepared from cells transfected with cyclin E dsRNA. Aliquots were incubated for 30 min at 30° C. before the addition of either the cyclin E (E600) or lacZ (Z800) substrate. Individual 20 μl aliquots, as indicated, were pre-incubated with 1 mM $CaCl_2$ and 5 mM EGTA, 1 mM $CaCl_2$, 5 mM EGTA and 60 U of micrococcal nuclease, 1 mM $CaCl_2$ and 60 U of micrococcal nuclease or 10 U of DNase I (Promega) and 5 mM EGTA. After the 30 min pre-incubation, EGTA was added to those samples that lacked it. Yeast tRNA (1 μg) was added to all samples. Time points were at 0 and 30 min.

FIG. **4**: The RISC contains a potential guide RNA. (a) Northern blots of RNA from either a crude lysate or the S100 fraction (containing the soluble nuclease activity, see Methods) were hybridized to a riboprobe derived from the sense strand of the cyclin E mRNA. (b) Soluble cyclin-E-specific nuclease activity was fractionated as described in Methods. Fractions from the anion-exchange resin were incubated with the lacZ, control substrate (upper panel) or the cyclin E substrate (centre panel). Lower panel, RNA from each fraction was analysed by northern blotting with a uniformly labeled transcript derived from sense strand of the cyclin E cDNA. DNA oligonucleotides were used as size markers.

FIG. **5**: Generation of 22mers and degradation of mRNA are carried out by distinct enzymatic complexes. (a) Extracts prepared either from 0-12 hour *Drosophila* embryos or *Drosophila* S2 cells (see Methods) were incubated for 0, 15, 30, or 60 minutes (left to right) with a uniformly-labeled double-stranded RNA corresponding to the first 500 nucleotides of the *Drosophila cyclin* E coding region. M indicates a marker prepared by in vitro transcription of a synthetic template. The template was designed to yield a 22 nucleotide transcript. The doublet most probably results from improper initiation at the +1 position. (b) Whole-cell extracts were prepared from S2 cells that had been transfected with a dsRNA corresponding to the first 500 nt. of the luciferase coding region. S10 extracts were spun at 30,000×g for 20 minutes which represents our standard RISC extract. S100 extracts were prepared by further centrifugation of S10 extracts for 60 minutes at 100,000×g. Assays for mRNA degradation were carried out as described previously for 0, 30 or 60 minutes (left to right in each set) with either a single-stranded luciferase mRNA or a single-stranded cyclin E mRNA, as indicated. (c) S10 or S100 extracts were incubated with cyclin E dsRNAs for 0, 60 or 120 minutes (L to R).

FIG. **6**: Production of 22mers by recombinant CG4792/Dicer. (a) *Drosophila* S2 cells were transfected with plasmids that direct the expression of T7-epitope tagged versions of Drosha, CG4792/Dicer-1 and Homeless. Tagged proteins were purified from cell lysates by immunoprecipitation and were incubated with cyclin E dsRNA. For comparison, reactions were also performed in *Drosophila* embryo and S2 cell extracts. As a negative control, immunoprecipitates were prepared from cells transfected with a β-galactosidase expression vector. Pairs of lanes show reactions performed for 0 or 60 minutes. The synthetic marker (M) is as described in the legend to FIG. **1**. (b) Diagrammatic representations of the domain structures of CG4792/Dicer-1, Drosha and Homeless are shown. (c) Immunoprecipitates were prepared from detergent lysates of S2 cells using an antiserum raised against the C-terminal 8 amino acids of *Drosophila* Dicer-1 (CG4792). As controls, similar preparations were made with a pre-immune serum and with an immune serum that had been pre-incubated with an excess of antigenic peptide. Cleavage reactions in which each of these precipitates was incubated with an ~500 nt. fragment of *Drosophila cyclin* E are shown. For comparison, an incubation of the substrate in *Drosophila* embryo extract was electrophoresed in parallel. (d) Dicer immunoprecipitates were incubated with dsRNA substrates

in the presence or absence of ATP. For comparison, the same substrate was incubated with S2 extracts that either contained added ATP or that were depleted of ATP using glucose and hexokinase (see methods). (e) *Drosophila* S2 cells were transfected with uniformly, ^{32}P-labelled dsRNA corresponding to the first 500 nt. of GFP. RISC complex was affinity purified using a histidine-tagged version of *Drosophila* Ago-2, a recently identified component of the RISC complex (Hammond et al., in prep). RISC was isolated either under conditions in which it remains ribosome associated (ls, low salt) or under conditions that extract it from the ribosome in a soluble form (hs, high salt). For comparison, the spectrum of labeled RNAs in the total lysate is shown. (f) Guide RNAs produced by incubation of dsRNA with a Dicer immunoprecipitate are compared to guide RNAs present in an affinity-purified RISC complex. These precisely co-migrate on a gel that has single-nucleotide resolution. The lane labeled control is an affinity selection for RISC from a cell that had been transfected with labeled dsRNA but not with the epitope-tagged *Drosophila* Ago-2.

FIG. **7**: Dicer participates in RNAi. (a) *Drosophila* S2 cells were transfected with dsRNAs corresponding to the two *Drosophila* Dicers (CG4792 and CG6493) or with a control dsRNA corresponding to murine caspase 9. Cytoplasmic extracts of these cells were tested for Dicer activity. Transfection with Dicer dsRNA reduced activity in lysates by 7.4-fold. (b) The Dicer-1 antiserum (CG4792) was used to prepare immunoprecipitates from S2 cells that had been treated as described above. Dicer dsRNA reduced the activity of Dicer-1 in this assay by 6.2-fold. (c) Cells that had been transfected two days previously with either mouse caspase 9 dsRNA or with Dicer dsRNA were cotransfected with a GFP expression plasmid and either control, luciferase dsRNA or GFP dsRNA. Three independent experiments were quantified by FACS. A comparison of the relative percentage of GFP-positive cells is shown for control (GFP plasmid plus luciferase dsRNA) or silenced (GFP plasmid plus GFP dsRNA) populations in cells that had previously been transfected with either control (caspase 9) or Dicer dsRNAs.

FIG. **8**: Dicer is an evolutionarily conserved ribonuclease. (a) A model for production of 22mers by Dicer. Based upon the proposed mechanism of action of Ribonuclease III, we propose that Dicer acts on its substrate as a dimer. The positioning of the two ribonuclease domains (RIIIa and RIIIb) within the enzyme would thus determine the size of the cleavage product. An equally plausible alternative model could be derived in which the RIIIa and RIIIb domains of each Dicer enzyme would cleave in concert at a single position. In this model, the size of the cleavage product would be determined by interaction between two neighboring Dicer enzymes. (b) Comparison of the domain structures of potential Dicer homologs in various organisms (Drosophila—CG4792, CG6493, *C. elegans*-K12H4.8, *Arabidopsis*—CARPEL FACTORY, T25K16.4, AC012328_1, human Helicase-MOI and *S. pombe*—YC9A_SCHPO). The ZAP domains were identified both by analysis of individual sequences with Pfam and by Psi-blast searches. The ZAP domain in the putative *S. pombe* Dicer is not detected by PFAM but is identified by Psi-Blast and is thus shown in a different color. For comparison, a domain structure of the RDE1/QDE2/ARGONAUTE family is shown. It should be noted that the ZAP domains are more similar within each of the Dicer and ARGONAUTE families than they are between the two groups. (c) An alignment of the ZAP domains in selected Dicer and Argonaute family members is shown. The alignment was produced using ClustalW.

FIG. **9**: Purification strategy for RISC. (second step in RNAi model).

FIG. **10**: Fractionation of RISC activity over sizing column. Activity fractionates as 500 KDa complex. Also, antibody to *Drosophila* argonaute 2 cofractionates with activity.

FIGS. **11**-**13**: Fractionation of RISC over monoS, monoQ, Hydroxyapatite columns. *Drosophila* argonaute 2 protein also cofactionates.

FIG. **14**: Alignment of *Drosophila* argonaute 2 with other family members.

FIG. **15**: Confirmation of *Drosophila* argonaute 2. S2 cells were transfected with labeled dsRNA and His tagged argonaute. Argonaute was isolated on nickel agarose and RNA component was identified on 15% acrylamide gel.

FIG. **16**: S2 cell and embryo extracts were assayed for 22-mer generating activity.

FIG. **17**: RISC can be separated from 22-mer generating activity (dicer). Spinning extracts (S100) can clear RISC activity from supernatant (left panel) however, S100 spins still contain dicer activity (right panel).

FIG. **18**: Dicer is specific for dsRNA and prefers longer substrates.

FIG. **19**: Dicer was fractionated over several columns.

FIG. **20**: Identification of dicer as enzyme which can process dsRNA into 22mers. Various RNaseIII family members were expressed with n terminal tags, immunoprecipitated, and assayed for 22-mer generating activity (left panel). In right panel, antibodies to dicer could also precipitate 22-mer generating activity.

FIG. **21**: Dicer requires ATP.

FIG. **22**: Dicer produces RNAs that are the same size as RNAs present in RISC.

FIG. **23**: Human dicer homolog when expressed and immunoprecipitated has 22-mer generating activity.

FIG. **24**: Sequence of *Drosophila* argonaute 2 (SEQ ID NO: 5). Peptides identified by microsequencing are shown in underline.

FIG. **25**: Molecular characterization of *Drosophila* argonaute 2. The presence of an intron in coding sequence was determined by northern blotting using intron probe. This results in a different 5' reading frame then the published genome sequence. Number of polyglutamine repeats was determined by genomic PCR.

FIG. **26**: Dicer activity can be created in human cells by expression of human dicer gene. Host cell was 293. Crude extracts had dicer activity, while activity was absent from untransfected cells. Activity is not dissimilar to that seen in *Drosophila* embryo extracts.

FIG. **27**: A ~500 nt. fragment of the gene that is to be silenced (X) is inserted into the modified vector as a stable direct repeat using standard cloning procedures. Treatment with commercially available cre recombinase reverses sequences within the loxP sites (L) to create an inverted repeat. This can be stably maintained and amplified in an sbc mutant bacterial strain (DL759). Transcription in vitro from the promoter of choice (P) yields a hairpin RNA that causes silencing. A zeocin resistance marker is included to insure maintenance of the direct and inverted repeat structures; however this is non-essential in vitro and could be removed by pre-mRNA splicing if desired. (Smith et al. (2000) *Nature* 407: 319-20).

FIG. **28**: RNAi in P19 embryonal carcinoma cells. Ten-centimeter plates of P19 cells were transfected by using 5 μg of GFP plasmid and 40 μg of the indicated dsRNA (or no RNA). Cells were photographed by fluorescent (tope panel)

and phase-contrast microscopy (bottom panel) at 72 h after transfection; silencing was also clearly evident at 48 h post-transfection.

FIG. **29**: RNAi of firefly and *Renilla* luciferase in P19 cells. (A and B) P19 cells were transfected with plasmids that direct the expression of firefly and *Renilla* luciferase and dsRNA 500 mers (25 or 250 ng, as indicated in A and B, respectively), that were either homologous to the firefly luciferase mRNA (dsFF) or nonhomologous (dsGFP). Luciferase activities were assayed at various times after transfection, as indicated. Ratios of firefly to *Renilla* activity are normalized to dsGFP controls. (C and D) P19 cells in 12-well culture dishes (2 ml of media) were transfected with 0.25 μg of a 9:1 mix of pGL3-Control and pRL-SV40 as well as 2 μg of the indicated RNA. Extracts were prepared 9 h after transfection. (C) Ratio of firefly to *Renilla* luciferase is shown. (D) Ratio of *Renilla* to firefly luciferase is shown. Values are normalized to dsGFP. The average of three independent experiments is shown; error bars indicate standard deviation.

FIG. **30**: The panels at the right show expression of either RFP or GFP following transient transfection into wild type P19 cells. The panels at the left demonstrate the specific suppression of GFP expression in P19 clones which stably express a 500 nt double stranded GFP hairpin. P19 clones which stably express the double stranded GFP hairpin were transiently transfected with RFP or GFP, and expression of RFP or GFP was assessed by visual inspection.

FIG. **31**: Specific silencing of luciferase expression by dsRNA in murine embryonic stem cells. Mouse embryonic stem cells in 12-well culture dishes (1 ml of media) were transfected with 1.5 μg of dsRNA along with 0.25 μg of a 10:1 mixture of the reporter plasmids pGL3-Control and pRL-SV40. Extracts were prepared and assayed 20 h after transfection. The ratio of firefly to *Renilla* luciferase expression is shown for FF ds500; the ratio of *Renilla* to firefly is shown for Ren ds500. Both are normalized to ratios from the dsGFP transfection. The average of three independent experiments is shown; error bars indicate standard deviation.

FIG. **32**: RNAi in C2C12 murine myoblast cells. (A) Mouse C2C12 cells in 12-well culture dishes (1 ml of media) were transfected with 1 μg of the indicated dsRNA along with 0.250 μg of the reporter plasmids pGL3-Control and pRL-SV40. Extracts were prepared and assayed 24 h after transfection. The ratio of firefly to *Renilla* luciferase expression is shown; values are normalized to ratios from the no dsRNA control. The average of three independent experiments is shown; error bars indicate standard deviation. (B) C2C12 cells cotransfected with 1 μg of either plasmid alone or a plasmid containing a hyperactive mutant of vaccinia virus K3L (Kawagishi-Kobayashi et al. 2000, Virology 276: 424-434). The absolute counts of *Renilla* and firefly luciferase activity are shown. (C) The ratios of firefly/*Renilla* activity from B, normalized to no dsRNA controls.

FIG. **33**: Hela, Chinese hamster ovary, and P19 (pluripotent, mouse embryonic carcinoma) cell lines transfected with plasmids expressing *Photinus* pyralis (firefly) and *Renilla* reniformis (sea pansy) luciferases and with dsRNA 500mers (400 ng), homologous to either firefly luciferase mRNA (dsLUC) or non-homologous (dsGFP). Dual luciferase assays were carried out using an Analytical Scientific Instruments model 3010 Luminometer. In this assay *Renilla* luciferase serves as an internal control for dsRNA-specific suppression of firefly luciferase activity. These data demonstrate that 500-mer dsRNA can specifically suppress cognate gene expression in vitro.

FIG. **34**: Expression of a hairpin RNA produces P19 EC cell lines that stably silence GFP. (A) A cartoon of the FLIP cassette used to construct the GFP hairpin. GFP represents the first 500 coding base pairs of EGFP. Zeo, zeocin resistance gene; L, Lox; P, the cytomegalovirus promoter in the expression plasmid pcDNA3. Homologous GFP fragments are first cloned as direct repeats into the FLIP cassette. To create inverted repeats for hairpin production, the second repeat is flipped by using Cre recombinase. When transcribed, the inverted repeat forms a GFP dsRNA with a hairpin loop. (B) P19 cell lines stably expressing the GFP hairpin plasmid, GFPhp.1 (clone 10) and GFPhp.2 (clone 12), along with wt P19 were transfected with 0.25 μg each of GFP and RFP reporter genes. Fluorescence micrographs were taken by using filters appropriate for GFP and RFP. Magnification is 200×. (C) P19 GFPhp.1 cells were transfected with pEGFP and 0, 0.5, or 1 μg of Dicer or firefly dsRNA. Fluorescence micrographs were taken at 48 h post-transfection and are superimposed with bright field images to reveal non-GFP expressing cells. Magnification is 100×. (D) In vitro and in vitro processing of dsRNA in P19 cells. In vitro Dicer assays were performed on S2 cells and three independently prepared P19 extracts by using ^{32}P-labeled dsRNA (30° C. for 30 min). A Northern blot of RNA extracted from control and GFPhp.1 P19-cells shows the production of ≈22-mer RNA species in hairpin-expressing cells but not in control cells. Blots were probed with a ^{32}P-labeled "sense" GFP transcript.

FIG. **35**: dsRNA induces silencing at the posttranscriptional level. P19 cell extracts were used for in vitro translation of firefly and *Renilla* luciferase mRNA (100 ng each). Translation reactions were programmed with various amounts of dsRNA 500mers, either homologous to firefly luciferase mRNA (dsLUC) or nonhomologous (dsGFP). Luciferase assays were carried out after a 1 h incubation at 30° C. Ratios of firefly to *Renilla* activity are normalized to no dsRNA controls. Standard deviations from the mean are shown.

FIG. **36**: S10 fractions from P19 cell lysates were used for in vitro translations of mRNA coding for *Photinus pyralis* (firefly) and *Renilla reniformis* (sea pansy) luciferases. Translation reactions were programmed with dsRNA, ssRNA, or asRNA 500mers, either complementary to firefly luciferase mRNA (dsFF, ssFF, or asFF), complementary to *Renilla* luciferase (dsREN, ssREN, or asREN) or non-complementary (dsGFP). Reactions were carried out at 30° C. for 1 hour, after a 30 min preincubation with dsRNA, ssRNA, or asRNA. Dual luciferase assays were carried out using an Analytical Scientific Instruments model 3010 Luminometer. On the left, *Renilla* luciferase serves as an internal control for dsRNA-specific suppression of firefly luciferase activity. On the right, firefly luciferase serves as an internal control for dsRNA-specific suppression of *Renilla* luciferase activity. These data demonstrate that 500-mer double-stranded RNA (dsRNA) but not single-stranded (ssRNA) or anti-sense RNA (asRNA) suppresses cognate gene expression in vitro in a manner consistent with post-transcriptional gene silencing.

FIG. **37**: P19 cells were grown in 6-well tissue culture plates to approximately 60% confluence. Various amounts of dsRNA, either homologous to firefly luciferase mRNA (dsLUC) or non-homologous (dsGFP), were added to each well and incubated for 12 hrs under normal tissue culture conditions. Cells were then transfected with plasmids expressing *Photinus pyralis* (firefly) and *Renilla reniformis* (sea pansy) luciferases and with dsRNA 500mers (500 ng). Dual luciferase assays were carried out 12 hrs post-transfection using an Analytical Scientific Instruments model 3010 Luminometer. In this assay *Renilla* luciferase serves as an internal control for dsRNA-specific suppression of firefly luciferase activity. These data show that 500-mer dsRNA can

specifically suppress cognate gene expression in vitro without transfection under normal tissue culture conditions.

FIG. **38**: Previous methods for generating siRNAs required costly chemical synthesis. The invention provides an in vitro method for synthesizing siRNAs using standard RNA transcription reactions.

FIG. **39**: Short hairpins suppress gene expression in *Drosophila* S2 cells. (A) Sequences and predicted secondary structure of representative chemically synthesized RNAs. Sequences correspond to positions 112-134 (siRNA) and 463-491 (shRNAs) of Firefly luciferase carried on pGL3-Control. An siRNA targeted to position 463-485 of the luciferase sequence was virtually identical to the 112-134 siRNA in suppressing expression, but is not shown. These sequences are represented by SEQ ID NOs: 6-10. (B) Exogenously supplied short hairpins suppress expression of the targeted Firefly luciferase gene in vitro. Six-well plates of S2 cells were transfected with 250 ng/well of plasmids that direct the expression of firefly and *Renilla* luciferase and 500 ng/well of the indicated RNA. Luciferase activities were assayed 48 h after transfection. Ratios of firefly to *Renilla* luciferase activity were normalized to a control transfected with an siRNA directed at the green fluorescent protein (GFP). The average of three independent experiments is shown; error bars indicate standard deviation. (C) Short hairpins are processed by the *Drosophila* Dicer enzyme. T7 transcribed hairpins shFfL22, shFfL29, and shFfS29 were incubated with (+) and without (–) 0-2-h *Drosophila* embryo extracts. Those incubated with extract produced ~22-nt siRNAs, consistent with the ability of these hairpins to induce RNA interference. A long dsRNA input (cyclin E 500-mer) was used as a control. Cleavage reactions were performed as described in Bernstein et al., 2001, Nature, 409:363-366.

FIG. **40**: Short hairpins function in mammalian cells. HEK 293T, HeLa, COS-1, and NIH 3T3 cells were transfected with plasmids and RNAs as in FIG. **1** and subjected to dual luciferase assays 48 h post-transfection. The ratios of firefly to *Renilla* luciferase activity are normalized to a control transfected with an siRNA directed at the green fluorescent protein (GFP). The average of three independent experiments is shown; error bars indicate standard deviation.

FIG. **41**: siRNAs and short hairpins transcribed in vitro suppress gene expression in mammalian cells. (A) Sequences and predicted secondary structure of representative in vitro transcribed siRNAs. Sequences correspond to positions 112-134 (siRNA) and 463-491 (shRNAs) of firefly luciferase carried on pGL3-Control. These sequences are represented by SEQ ID NOs: 11-20. (B) In vitro transcribed siRNAs suppress expression of the targeted firefly luciferase gene in vitro. HEK 293T cells were transfected with plasmids as in FIG. **2**. The presence of non-base-paired guanosine residues at the 5' end of siRNAs significantly alters the predicted end structure and abolishes siRNA activity. (C) Sequences and predicted secondary structure of representative in vitro transcribed shRNAs. Sequences correspond to positions 112-141 of firefly luciferase carried on pGL3-Control. These sequences are represented by SEQ ID NOs: 21-26. (D) Short hairpins transcribed in vitro suppress expression of the targeted firefly luciferase gene in vitro. HEK 293T cells were transfected with plasmids as in FIG. **2**.

FIG. **42**: Transcription of functional shRNAs in vitro. (A) Schematic of the pShh1 vector. Sequences encoding shRNAs with between 19 and 29 bases of homology to the targeted gene are synthesized as 60-75-bp double-stranded DNA oligonucleotides and ligated into an EcoRV site immediately downstream of the U6 promoter. This sequence is represented by SEQ ID NO: 27. (B) Sequence and predicted secondary structure of the Ff1 hairpin. (C) An shRNA expressed from the pShh1 vector suppresses luciferase expression in mammalian cells. HEK 293T, HeLa, COS-1, and NIH 3T3 cells were transfected with reporter plasmids as in FIG. **1**, and pShh1 vector, firefly siRNA, or pShh1 firefly shRNA constructs as indicated. The ratios of firefly to *Renilla* luciferase activity were determined 48 h after transfection and represent the average of three independent experiments; error bars indicate standard deviation.

FIG. **43**: Dicer is required for shRNA-mediated gene silencing. HEK 293T cells were transfected with luciferase reporter plasmids as well as pShh1-Ff1 and an siRNA targeting human Dicer either alone or in combination, as indicated. The Dicer siRNA sequence (TCAACCAGCCACTGCTGGA, SEQ ID NO: 37) corresponds to coordinates 3137-3155 of the human Dicer sequence. The ratios of firefly to *Renilla* luciferase activity were determined 26 h after transfection and represent the average of three independent experiments; error bars indicate standard deviation.

FIG. **44**: Stable shRNA-mediated gene silencing of an endogenous gene. (A) Sequence and predicted secondary structure of the p53 hairpin. The 5' shRNA stem contains a 27-nt sequence derived from mouse p53 (nucleotides 166-192), whereas the 3' stem harbors the complimentary antisense sequence. This sequence is represented by SEQ ID NO: 28. (B) Senescence bypass in primary mouse embryo fibroblasts (MEFs) expressing an shRNA targeted at p53. Wild-type MEFs, passage 5, were transfected with pBabe-RasV12 with control plasmid or with p53hp (5 μg each with FuGENE; Roche). Two days after transfection, cells were trypsinized, counted, and plated at a density of 1×10^5/10-cm plate in media containing 2.0 μg/mL of puromycin. Control cells cease proliferation and show a senescent morphology (left panel). Cells expressing the p53 hairpin continue to grow (right panel). Photos were taken 14 d post-transfection.

FIG. **45**: A mixture of two short hairpins, both corresponding to firefly luciferase, does not result in a synergistic suppression of gene expression. Suppression of firefly luciferase gene expression resulting from transfection of a mixture of two different short hairpins (HP #1 and HP #2) was examined. The mixture of HP #1 and HP #2 did not have a more robust effect on the suppression of firefly luciferase gene expression than expression of HP#1 alone.

FIG. **46**: Encoded short hairpins specifically suppress gene expression in vitro. DNA oligonucleotides encoding 29 nucleotide hairpins corresponding to firefly luciferase were inserted into a vector containing the U6 promoter. Three independent constructs were examined for their ability to specifically suppress firefly luciferase gene expression in 293T cells. siOligo1-2, siOligo1-6, and siOligo1-19 (construct in the correct orientation) each suppressed gene expression as effectively as siRNA. In contrast, siOligo1-10 (construct in the incorrect orientation) did not suppress gene expression. An independent construct targeted to a different portion of the firefly luciferase gene did not effectively suppress gene expression in either orientation (SiOligo2-23, SiOligo2-36).

FIGS. **47-49**: Strategies for stable expression of short dsRNAs.

FIG. **50**: Dual luciferase assays were performed as described in detail in FIGS. **28-35**, however the cells used in these experiments were $PKR^{-/-}$ murine embryonic fibroblasts (MEFs). Briefly, RNAi using long dsRNAs typically envokes a non-specific response in MEFs (due to PKR activity). To evaluate the effect of long dsRNA constructs to specifically inhibit gene expression in MEFs, RNAi was examined in $PKR^{-/-}$ MEFs. Such cells do not respond to dsRNA

with a non-specific response. The data summarized in this figure demonstrates that in the absence of the non-specific PKR response, long dsRNA constructs specifically suppress gene expression in MEFs.

FIG. **51**: Is a schematic representation of the mouse tyrosinase promoter. Primers were used to amplify three separate regions in the proximal promoter, or to amplify sequence corresponding to an enhancer located approximately 12 kb upstream.

FIG. **52**: Reporter expression plasmids and siRNA sequences used in Figures X and Y. PGL-3-Control and Pluc-NS5B are the expression plasmids used for transfection into mouse liver. The nucleotide sequences of the siRNAs used in the study are shown underneath. These sequences are represented by SEQ ID NOs: 29-35.

FIG. **53**: RNA interference in adult mice using siRNAs. (a) Representative images of light emitted from mice co-transfected with the luciferase plasmid pGL3-control and either no siRNA, luciferase siRNA or unrelated siRNA. A pseudocolour image representing intensity of emitted light (red, most intense; blue, least intense) superimposed on a greyscale reference image (for orientation) shows that RNAi functions in adult mice. Annealed 21-nucleotide siRNAs (40 μg; Dharmacon) were co-injected into the livers of mice with 2 μg pGL3-control DNA (Promega) and 800 units of RNasin (Promega) in 1.8 ml PBS buffer in 5-7 s. After 72 h, mice were anaesthetized and given 3 mg luciferin intraperitoneally 15 min before imaging. (b) siRNA results (six mice per group) from a representative experiment. Mice receiving luciferase siRNA emitted significantly less light than reporter-alone controls (one-way ANOVA with post hoc Fisher's test). Results for reporter alone and unrelated siRNA were statistically similar. Animals were treated according to the US National Institutes of Health's guidelines for animal care and the guidelines of Stanford University.

FIG. **54**: RNA interference in adult mice using shRNAs. (a) Representative images of light emitted from mice co-transfected with the luciferase plasmid control, pShh1-Ff1, and pShh1-Ff1rev. pShh1-Ff1, but not pShh1-Ff1rev, reduced luciferase expression in mice relative to the reporter-alone control. pShh1-Ff1 or pShh1-rev (10 μg) were co-injected with 2 μg pGL3-control in 1.8 ml PBS buffer. (b) Average of three independent shRNA experiments (n=5). Average values for the reporter-alone group are designated as 100% in each of the three experiments. Animals were treated according to the US National Institutes of Health's guidelines for animal care and the guidelines of Stanford University.

FIG. **55**: Heritable repression of Neil1 expression by RNAi in several tissues. (a) Expression of Neil1 mRNA in the livers of three mice containing the Neil1 shRNA transgene (shRNA-positive) or three siblings lacking the transgene (shRNA-negative) was assayed by RT-PCR (top row is Neil1). An RT-PCR of β-actin was done to ensure that equal quantities of mRNAs were tested for each mouse (second row). Expression of the neomycin resistance gene (neo), carried on the shRNA vector, was tested similarly (third row). Finally, the mice were genotyped using genomic DNA that was PCR-amplified with vector-specific primers (bottom row). (b) Similar studies were performed in the heart. (c) Similar studies were performed in the spleen. Animal procedures have been approved by the SUNY, Stony Brook Institutional Animal Care and Use Committee (IACUC).

FIG. **56**: Reduction in Neil1 protein correlates with the presence of siRNAs. (a) Expression of Neil1 protein was examined in protein extracts from the livers of mice carrying the shRNA transgene (shRNA-positive) or siblings lacking the transgene (shRNA-negative) by western blotting with Neil1-specific antiserum. A western blot for PCNA was used to standardize loading. (b) The presence of siRNAs in RNA derived from the livers of transgenic mice as assayed by northern blotting using a 300 nt probe, part of which was complementary to the shRNA sequence. We note siRNAs only in mice transgenic for the shRNA expression cassette.

FIG. **57**: In vitro processing of 29 nt. shRNAs by Dicer generates a single siRNA from the end of each short hairpin. a) The set of shRNAs containing 19 or 29 nt stems and either bearing or lacking a 2 nucleotide 3'overhang is depicted schematically. For reference the 29 nt sequence from luciferase (top, blue) strand is given. The presumed cleavage sites are indicated in green and by the arrows. b) In vitro Dicer processing of shRNAs. Substrates as depicted in a) were incubated either in the presence or absence of recombinant human Dicer (as indicated). Processing of a 500 bp. blunt-ended dsRNA is shown for comparison. Markers are end-labeled, single-stranded, synthetic RNA oligonucleotides. c) All shRNA substrates were incubated with bacterial RNase III to verify their double-stranded nature. This sequence is represented by SEQ ID NO: 36.

FIG. **58**: Primer extension analysis reveal a single siRNA generated from Dicer processing of shRNA both in vitro and in vivo. a) 19 nt. shRNAs, as indicated (see FIG. **57***a*), were processed by Dicer in vitro. Reacted RNAs were extended with a specific primer that yields a 20 base product if cleavage occurs 22 bases from the 3' end of the overhung RNA (see FIG. **57***a*). Lanes labeled siRNA are extensions of synthetic RNAs corresponding to predicted siRNAs that would be released by cleavage 21 or 22 nucleotides from the 3' end of the overhung precursor. Observation of extension products dependents entirely on the inclusion of RT (indicated). Markers are phosphorylated, synthetic DNA oligonucleotides. b) Analysis as described in a) for 29 nt. shRNAs. The * indicates the specific extension product from the overhung shRNA species. c) Primer extension were used to analyze products from processing of overhung 29 nt. shRNAs in vivo. For comparison, extensions of in vitro processed material are also shown. Again, the * indicates the specific extension product.

FIG. **59**: Gene suppression by shRNAs is comparable to or more effective than that achieved by siRNAs targeting the same sequences. a) Structures of synthetic RNAs used for these studies. b) mRNA suppression levels achieved by 43 siRNAs targeting 6 different genes compared with levels achieved by 19-mer (left) or 29-mer (right) shRNAs derived from the same target sequences. All RNAs were transfected at a final concentration of 100 nM. Values indicated on the X and Y axes reflect the percentage of mRNA remaining in HeLa cells 24 hours after RNA transfection compared with cells treated with transfection reagent alone. c) Titration analysis comparing efficacies of four siRNA/shRNA sets targeting MAPK14. Curves are graphed from data derived from transfections at 1.56, 6.25, 25, and 100 nM final concentrations of RNA. (diamonds: 21-mer siRNAs; squares: 19-mer shRNAs; triangles: 29-mer shRNAs).

FIG. **60**: Microarray profiling reveals sequence-specific gene expression profiles and more similarity between 29-mer shRNAs and cognate siRNAs than observed for 19-mer shRNAs. Each row of the heat maps reports the gene expression signature resulting from transfection of an individual RNA. Data shown represent genes that display at least a 2-fold change in expression level (P value <0.01 and log 10 intensity >1) relative to mock-transfected cells. Green indicates decreased expression relative to mock transfection whereas red indicates elevated expression. a) 19-mer shRNAs and siRNAs designed for six different target sequences within the coding region of the MAPK14 gene were tested for gene

silencing after 24 hours in HeLa cells. b) A similar experiment to that described in a) but carried out with five 29-mer shRNAs targeting MAPK14.

DETAILED DESCRIPTION OF CERTAIN PREFERRED EMBODIMENTS

I. Overview

The present invention provides methods for attenuating gene expression in a cell using gene-targeted double stranded RNA (dsRNA). The dsRNA contains a nucleotide sequence that hybridizes under physiologic conditions of the cell to the nucleotide sequence of at least a portion of the gene to be inhibited (the "target" gene). The nucleotide sequence can hybridize to either coding or non-coding sequence of the target gene.

A significant aspect to certain embodiments of the present invention relates to the demonstration in the present application that RNAi can in fact be accomplished both in cultured mammalian cells and in whole organisms. This had not been previously described in the art.

Another salient feature of the present invention concerns the ability to carry out RNAi in higher eukaryotes, particularly in non-oocytic cells of mammals, e.g., cells from adult mammals as an example.

Furthermore, in contrast to the teachings of the prior art, we demonstrate that RNAi in mammalian systems can be mediated with dsRNA identical or similar to non-coding sequence of a target gene. It was previously believed that although dsRNA identical or similar to non-coding sequences (i.e., promoter, enhancer, or intronic sequences) did not inhibit RNAi, such dsRNAs were not thought to mediate RNAi.

In addition, the instant invention also demonstrates that short hairpin RNA (shRNA) may effectively be used in the subject RNAi methods. In certain embodiments, shRNAs specifically designed as Dicer substrates can be used as more potent inducers of RNAi than siRNAs. Not only is maximal inhibition achieved at much lower levels of transfected RNA, but also endpoint inhibition is often greater. In certain other embodiments, mimicking natural pre-miRNAs by inclusion of a 1-5 nucleotide(s), especially a 2 nucleotide 3' overhang, enhances the efficiency of Dicer cleavage and directs cleavage to a specific position in the precursor. The presence of this specific processing site further permits the application of rules for siRNA design to shRNAs, both for chemical synthesis and vector-based delivery of such shRNA constructs. These teachings provide improved methods for evoking RNAi in mammalian cells, and thus improved ability to produce highly potent silencing triggers in therapeutic application of RNAi.

As described in further detail below, the present invention(s) are based on the discovery that the RNAi phenomenon is mediated by a set of enzyme activities, including an essential RNA component, that are evolutionarily conserved in eukaryotes ranging from plants to mammals.

One enzyme contains an essential RNA component. After partial purification, a multi-component nuclease (herein "RISC nuclease") co-fractionates with a discrete, 22-nucleotide RNA species which may confer specificity to the nuclease through homology to the substrate mRNAs. The short RNA molecules are generated by a processing reaction from the longer input dsRNA. Without wishing to be bound by any particular theory, these 22-mer guide RNAs may serve as guide sequences that instruct the RISC nuclease to destroy specific mRNAs corresponding to the dsRNA sequences.

As illustrated, double stranded forms of the 22-mer guide RNA can be sufficient in length to induce sequence-dependent dsRNA inhibition of gene expression. In the illustrated example, dsRNA constructs are administered to cells having a recombinant luciferase reporter gene. In the control cell, e.g., no exogenously added RNA, the level of expression of the luciferase reporter is normalized to be the value of "1". As illustrated, both long (500-mer) and short (22-mer) dsRNA constructs complementary to the luciferase gene could inhibit expression of that gene product relative to the control cell. On the other hand, similarly sized dsRNA complementary to the coding sequence for another protein, green fluorescence protein (GFP), did not significantly effect the expression of luciferase—indicating that the inhibitory phenomena was in each case sequence-dependent. Likewise, single stranded 22-mers of luciferase did not inhibit expression of that gene—indicating that the inhibitory phenomena is double stranded-dependent.

The appended examples also identify an enzyme, Dicer, that can produce the putative guide RNAs. Dicer is a member of the RNAse III family of nucleases that specifically cleave dsRNA and is evolutionarily conserved in worms, flies, plants, fungi and, as described herein, mammals. The enzyme has a distinctive structure which includes a helicase domain and dual RNAse III motifs. Dicer also contains a region of homology to the RDE1/QDE2/ARGONAUTE family, which have been genetically linked to RNAi in lower eukaryotes. Indeed, activation of, or overexpression of Dicer may be sufficient in many cases to permit RNA interference in otherwise non-receptive cells, such as cultured eukaryotic cells, or mammalian (non-oocytic) cells in culture or in whole organisms.

In certain embodiments, the cells can be treated with an agent(s) that inhibits the general double-stranded RNA response(s) by the host cells, such as may give rise to sequence-independent apoptosis. For instance, the cells can be treated with agents that inhibit the dsRNA-dependent protein kinase known as PKR (protein kinase RNA-activated). Double stranded RNAs in mammalian cells typically activate protein kinase PKR and lead to apoptosis. The mechanism of action of PKR includes phosphorylation and inactivation of eIF2α (Fire, *Trends Genet.* 15: 358, 1999). It has also been reported that induction of NF-κB by PKR is involved in apoptosis commitment and this process is mediated through activation of the IKK complex. This sequence-independent response may reflect a form of primitive immune response, since the presence of dsRNA is a common feature of many viral lifecycles.

As described herein, Applicants have demonstrated that the PKR response can be overcome in favor of the sequence-specific RNAi response. However, in certain instances, it may be desirable to treat the cells with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKR, and such methods are specifically contemplated for use in the present invention. Likewise, overexpression of agents which ectopically activate eIF2α can be used. Other agents Which can be used to suppress the PKR response include inhibitors of IKK phosphorylation of IκCB, inhibitors of IκB ubiquitination, inhibitors of IκB degradation, inhibitors of NF-κB nuclear translocation, and inhibitors of NF-κB interaction with κB response elements.

Other inhibitors of sequence-independent dsRNA response in cells include the gene product of the vaccinia virus E3L. The E3L gene product contains two distinct domains. A conserved carboxy-terminal domain has been shown to bind double-stranded RNA (dsRNA) and inhibit the antiviral dsRNA response by cells. Expression of at least that portion of the E3L gene in the host cell, or the use of polypeptide or peptidomimetics thereof, can be used to suppress the

general dsRNA response. Caspase inhibitors sensitize cells to killing by double-stranded RNA. Accordingly, ectopic expression or activation of caspases in the host cell can be used to suppress the general dsRNA response.

In other embodiments, the subject method is carried out in cells which have little or no general response to double stranded RNA, e.g., have no PKR-dependent dsRNA response, at least under the culture conditions. As illustrated in FIGS. **28-32**, CHO and P19 cells can be used without having to inhibit PKR or other general dsRNA responses.

Also as described in further detail below, the present invention(s) are partially based on the discovery that short hairpin RNA specifically designed as Dicer substrates are more potent inducers of RNAi than siRNAs. In certain embodiments, shRNA constructs with 1-5, preferably two 3' overhang nucleotides are substrates particularly well-adapted for Dicer-mediated cleavage, and are more potent inhibitors of target genes then their siRNA counterparts with identical complementary sequences. Such shRNA can be formed either in vitro or in vivo by, for example, sequence-specific pairing after chemical synthesis, or transcription from a promoter operatively-linked to a DNA encoding such hairpin structure.

Thus, the present invention provides a process and compositions for inhibiting expression of a target gene in a cell, especially a mammalian cell. In certain embodiments, the process comprises introduction of RNA (the "dsRNA construct") with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific in that a nucleotide sequence from a portion of the target gene is chosen to produce the dsRNA construct. The dsRNA may be identical or similar to coding or non-coding sequence of the target gene. In preferred embodiments, the method utilizes a cell in which Dicer and/or Argonaute activities are recombinantly expressed or otherwise ectopically activated. This process can be (1) effective in attenuating gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a genomic integrated vector, or "integrated vector", which can become integrated into the chromosomal DNA of the host cell. Another type of vector is an episomal vector, i.e., a nucleic acid capable of extra-chromosomal replication. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In the present specification, "plasmid" and "vector" are used interchangeably unless otherwise clear from the context.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide of the present invention, including both exon and (optionally) intron sequences. The nucleic acid may also optionally include non-coding sequences such as promoter or enhancer sequences. A "recombinant gene" refers to nucleic acid encoding such regulatory polypeptides, that may optionally include intron sequences that are derived from chromosomal DNA. The term "intron" refers to a DNA sequence present in a given gene that is not translated into protein and is generally found between exons.

A "protein coding sequence" or a sequence that "encodes" a particular polypeptide or peptide, is a nucleic acid sequence that is transcribed (in the case of DNA) and is translated (in the case of mRNA) into a polypeptide in vitro or in vitro when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from procaryotic or eukaryotic mRNA, genomic DNA sequences from procaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

Likewise, "encodes", unless evident from its context, will be meant to include DNA sequences that encode a polypeptide, as the term is typically used, as well as DNA sequences that are transcribed into inhibitory antisense molecules.

The term "loss-of-function", as it refers to genes inhibited by the subject RNAi method, refers to a diminishment in the level of expression of a gene(s) in the presence of one or more dsRNA construct(s) when compared to the level in the absence of such dsRNA construct(s).

The term "expression" with respect to a gene sequence refers to transcription of the is gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of, a protein coding sequence results from transcription and translation of the coding sequence.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

The term "cultured cells" refers to cells suspended in culture, e.g., dispersed in culture or in the form tissue. It does not, however, include oocytes or whole embryos (including blastocysts and the like) which may be provided in culture. In certain embodiments, the cultured cells are adults cells, e.g., non-embryonic.

By "recombinant virus" is meant a virus that has been genetically altered, e.g., by the addition or insertion of a heterologous nucleic acid construct into the particle.

As used herein, the terms "transduction" and "transfection" are art recognized and mean the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a dsRNA construct.

"Transient transfection" refers to cases where exogenous DNA does not integrate into the genome of a transfected cell, e.g., where episomal DNA is transcribed into mRNA and translated into protein.

A cell has been "stably transfected" with a nucleic acid construct when the nucleic acid construct is capable of being inherited by daughter cells.

As used herein, a "reporter gene construct" is a nucleic acid that includes a "reporter gene" operatively linked to at least one transcriptional regulatory sequence. Transcription of the reporter gene is controlled by these sequences to which they are linked. The activity of at least one or more of these control

sequences can be directly or indirectly regulated by the target receptor protein. Exemplary transcriptional control sequences are promoter sequences. A reporter gene is meant to include a promoter-reporter gene construct that is heterologously expressed in a cell.

As used herein, "transformed cells" refers to cells that have spontaneously converted to a state of unrestrained growth, i.e., they have acquired the ability to grow through an indefinite number of divisions in culture. Transformed cells may be characterized by such terms as neoplastic, anaplastic and/or hyperplastic, with respect to their loss of growth control. For purposes of this invention, the terms "transformed phenotype of malignant mammalian cells" and "transformed phenotype" are intended to encompass, but not be limited to, any of the following phenotypic traits associated with cellular transformation of mammalian cells: immortalization, morphological or growth transformation, and tumorigenicity, as detected by prolonged growth in cell culture, growth in semi-solid media, or tumorigenic growth in immuno-incompetent or syngeneic animals.

As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

As used herein, "immortalized cells" refers to cells that have been altered via chemical, genetic, and/or recombinant means such that the cells have the ability to grow through an indefinite number of divisions in culture.

The "growth state" of a cell refers to the rate of proliferation of the cell and the state of differentiation of the cell.

"MHC antigen", as used herein, refers to a protein product of one or more MHC genes; the term includes fragments or analogs of products of MHC genes which can evoke an immune response in a recipient organism. Examples of MHC antigens include the products (and fragments or analogs thereof) of the human MHC genes, i.e., the HLA genes.

The term "histocompatibility" refers to the similarity of tissue between different individuals. The level of histocompatibility describes how well matched the patient and donor are. The major histocompatibility determinants are the human leukocyte antigens (HLA). HLA typing is performed between the potential marrow donor and the potential transplant recipient to determine how close a HLA match the two are. The closer the match the less the donated marrow and the patient's body will react against each other.

The term "human leukocyte antigens" or "HLA", refers to proteins (antigens) found on the surface of white blood cells and other tissues that are used to match donor and patient. For instances, a patient and potential donor may have their white blood cells tested for such HLA antigens as, HLA-A, B and DR. Each individual has two sets of these antigens, one set inherited from each parent. For this reason, it is much more likely for a brother or sister to match the patient than an unrelated individual, and much more likely for persons of the same racial and ethnic backgrounds to match each other.

III. Exemplary Embodiments of Isolation Method

One aspect of the invention provides a method for potentiating RNAi by induction or ectopic activation of an RNAi enzyme in a cell (in vitro or in vitro) or cell-free mixtures. In preferred embodiments, the RNAi activity is activated or added to a mammalian cell, e.g., a human cell, which cell may be provided in vitro or as part of a whole organism. In other embodiments, the subject method is carried out using eukaryotic cells generally (except for oocytes) in culture. For instance, the Dicer enzyme may be activated by virtue of being recombinantly expressed or it may be activated by use of an agent which (i) induces expression of the endogenous gene, (ii) stabilizes the protein from degradation, and/or (iii) allosterically modifies the enzyme to increase its activity (by altering its k_{cat}, K_m or both).

A. Dicer and Argonaut Activities

In certain embodiments, at least one of the activated RNAi enzymes is Dicer, or a homolog thereof. In certain preferred embodiments, the present method provides for ectopic activation of Dicer. As used herein, the term "Dicer" refers to a protein which (a) mediates an RNAi response and (b) has an amino acid sequence at least 50 percent identical, and more preferably at least 75, 85, 90 or 95 percent identical to SEQ ID NO: 2 or 4, and/or which can be encoded by a nucleic acid which hybridizes under wash conditions of 2×SSC at 22° C., and more preferably 0.2×SSC at 65° C., to a nucleotide represented by SEQ ID NO: 1 or 3. Accordingly, the method may comprise introducing a dsRNA construct into a cell in which Dicer has been recombinantly expressed or otherwise ectopically activated.

In certain embodiment, at least one of the activated RNAi enzymes is Argonaut, or a homolog thereof. In certain preferred embodiments, the present method provides for ectopic activation of Argonaut. As used herein, the term "Argonaut" refers to a protein which (a) mediates an RNAi response and (b) has an amino acid sequence at least 50 percent identical, and more preferably at least 75, 85, 90 or 95 percent identical to the amino acid sequence shown in FIG. **24**. Accordingly, the method may comprise introducing a dsRNA construct into a cell in which Argonaut has been recombinantly expressed or otherwise ectopically activated.

This invention also provides expression vectors containing a nucleic acid encoding a Dicer or Argonaut polypeptide, operably linked to at least one transcriptional regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of the subject is Dicer or Argonaut proteins. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel, *Gene Expression Technology*: Methods in Enzymology 185, Academic Press, San Diego, Calif., 1990. For instance, any of a wide variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding Dicer or Argonaut polypeptides of this invention. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage λ, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed.

Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

The recombinant Dicer or Argonaut genes can be produced by ligating a nucleic acid encoding a Dicer or Argonaut polypeptide into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vectors for production of recombinant forms of the subject Dicer or Argonaut polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a Dicer or Argonaut polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli.*

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach et al. (1983) in Experimental Manipulation of Gene Expression, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as Ampicillin can be used. In an illustrative embodiment, a Dicer or Argonaut polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding sequence of a Dicer or Argonaut gene.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual,* 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

In yet another embodiment, the subject invention provides a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous Dicer or Argonaut gene. For instance, the gene activation construct can replace the endogenous promoter of a Dicer or Argonaut gene with a heterologous promoter, e.g., one which causes constitutive expression of the Dicer or Argonaut gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of Dicer or Argonaut. A variety of different formats for the gene activation constructs are available. See, for example, the Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

In preferred embodiments, the nucleotide sequence used as the gene activation construct can be comprised of (1) DNA from some portion of the endogenous Dicer or Argonaut gene (exon sequence, intron sequence, promoter sequences, etc.) which direct recombination and (2) heterologous transcriptional regulatory sequence(s) which is to be operably linked to the coding sequence for the genomic Dicer or Argonaut gene upon recombination of the gene activation construct. For use in generating cultures of Dicer or Argonaut producing cells, the construct may further include a reporter gene to detect the presence of the knockout construct in the cell.

The gene activation construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to provide the heterologous regulatory sequences in operative association with the native Dicer or Argonaut gene. Such insertion occurs by homologous recombination, i.e., recombination regions of the activation construct that are homologous to the endogenous Dicer or Argonaut gene sequence hybridize to the genomic DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding position of the genomic DNA.

The terms "recombination region" or "targeting sequence" refer to a segment (i.e., a portion) of a gene activation construct having a sequence that is substantially identical to or substantially complementary to a genomic gene sequence, e.g., including 5' flanking sequences of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a recombination region and a genomic sequence.

The heterologous regulatory sequences, e.g., which are provided in the replacement region, can include one or more of a variety of elements, including: promoters (such as constitutive or inducible promoters), enhancers, negative regulatory elements, locus control regions, transcription factor binding sites, or combinations thereof.

Promoters/enhancers which may be used to control the expression of the targeted gene in vitro include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., *J. Exp. Med.* 169: 13, 1989), the human β-actin promoter (Gunning et al., PNAS 84: 48314835, 1987), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al., *Mol. Cell. Biol.* 4: 1354-1362, 1984), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), the SV40 early or late region promoter (Bemoist et al., *Nature* 290: 304-310, 1981; Templeton et al., *Mol. Cell. Biol.* 4: 817, 1984; and Sprague et al., *J. Virol.* 45: 773, 1983), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., *Cell* 22: 787-797, 1980), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al., *PNAS* 82: 3567-71, 1981), and the herpes simplex virus LAT promoter (Wolfe et al., *Nature Genetics* 1: 379-384, 1992).

In still other embodiments, the replacement region merely deletes a negative transcriptional control element of the native gene, e.g., to activate expression, or ablates a positive control element, e.g., to inhibit expression of the targeted gene.

B. Cell/Organism

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus). The dsRNA construct may be synthesized either in vitro or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vitro, or cloned RNA polymerase can be used for transcription in vitro or in vitro. For generating double stranded transcripts from a transgene in vitro, a regulatory region may be used to tran-

scribe the RNA strand (or strands). Furthermore, dsRNA can be generated by transcribing an RNA strand which forms a hairpin, thus producing a dsRNA.

Genetic manipulation becomes possible in organisms that are not classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease models in which the target gene are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

The cell with the target gene may be derived from or contained in any organism. The organism may be a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

Plants include *arabidopsis*; field crops (e.g., alfalfa, barley, bean, com, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, faJoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (e.g., alder, ash, aspen, azalea, birch, boxwood, camellia, carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human.

Invertebrate animals include nematodes, other worms, *Drosophila*, and other insects. Representative generae of nematodes include those that infect animals (e.g., *Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tflichonema, Toxocara, Uncinaria*) and those that infect plants (e.g., *Bursaphalenchus, Criconerriella, Diiylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynchus, Tylenchus*, and *Xiphinerna*). Representative orders of insects include *Coleoptera, Diptera, Lepidoptera*, and *Homoptera*.

The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

C. Targeted Genes

The target gene may be a gene derived from the cell, an endogenous gene, a transgene, or a gene of a pathogen which is present in the cell after infection thereof. Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein.

"Inhibition of gene expression" refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. "Specificity" refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxy acid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin.

Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

As disclosed herein, the present invention is not limited to any type of target gene or nucleotide sequence. But the following classes of possible target genes are listed for illustrative purposes: developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Writ family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABLI, BCLI, BCL2, BCL6, CBFA2, CBL, CSFIR, ERBA, ERBB, EBRB2, ETSI, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCLI, MYCN, NRAS, PIM 1, PML, RET, SRC, TALI, TCL3, and YES); tumor suppressor genes (e.g., APC, BRCA 1, BRCA2, MADH4, MCC, NF 1, NF2, RB 1, TP53, and

WTI); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

D. dsRNA constructs

The dsRNA construct may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. The dsRNA construct may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by in vitro enzymatic or organic synthesis.

The dsRNA construct may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection of an RNA solution directly into the cell or extracellular injection into the organism.

The double-stranded structure may be formed by a single self-complementary RNA strand (such as in the form of shRNA) or two complementary RNA strands (such as in the form of siRNA). RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

dsRNA constructs containing a nucleotide sequences identical to a portion, of either coding or non-coding sequence, of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence (ds RNA similar to the target gene) have also been found to be effective for inhibition. Thus, sequence identity may be optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50° C. or 70° C. hybridization for 12-16 hours; followed by washing). In certain preferred embodiments, the length of the dsRNA is at least 20, 21 or 22 nucleotides in length, e.g., corresponding in size to RNA products produced by Dicer-dependent cleavage. In certain embodiments, the dsRNA construct is at least 25, 50, 100, 200, 300 or 400 bases. In certain embodiments, the dsRNA construct is 400-800 bases in length.

In one embodiment, the dsRNA is a single-stranded hairpin ribonucleic acid (shRNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA: (i) is a substrate for cleavage by a RNaseIII enzyme to produce a double-stranded RNA product, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions. In a preferred embodiment, the shRNA comprises a 3' overhang of about 14 nucleotides.

In a related embodiment, he dsRNA is a single-stranded hairpin ribonucleic acid (shRNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA: (i) is cleaved in the mammalian cells to produce an RNA guide sequence that enters an Argonaut-containing complex, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions. In a preferred embodiment, the shRNA comprises a 3' overhang of about 1-4 nucleotides.

The size of the duplex region of the subject shRNA may be longer (e.g., anywhere between 19 to about 1000 nucleotides, or 19-about 500 nt, or 19-about 250 nt, etc.), but in many applications, about 29 nucleotides is sufficient. In certain embodiments, the duplex region is any where between about 25-29 nt. In other embodiments, the duplex region is any where between about 19-25 nt.

The size of the 3' overhang may be 1-5 nucleotides, preferably 2-4 nucleotides. In one embodiment, the 3' overhang is 2 nucleotides. The specific sequences of the 3' overhang nucleotides are less important. In one embodiment, the overhang nucleotides can be any nucleotides, including "non-standard" or modified nucleotides. In other embodiments, the overhang sequences are mostly pyrimidines, such as U, C, or T. In one embodiment, the 2-nucleotide overhang is UU.

In certain embodiments, the 5' of the shRNA may have 1-5 nt overhang that does not pair with the 3' overhang.

The size of the "loop" between the paired duplex region may vary, but preferably contains at least about 3-8 nucleotides, such as 4 nucleotides.

100% sequence identity between the RNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

The dsRNA construct may be synthesized either in vitro or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vitro, or cloned RNA polymerase can be used for transcription in vitro or in vitro. For transcription

from a transgene in vitro or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the dsRNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. The dsRNA construct may be chemically or enzymatically synthesized by manual or automated reactions. The dsRNA construct may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and production of an expression construct are known in the art (see also WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by in vitro enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography or a combination thereof. Alternatively, the dsRNA construct may be used with no or a minimum of purification to avoid losses due to sample processing. The dsRNA construct may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

Physical methods of introducing nucleic acids include injection of a solution containing the dsRNA construct, bombardment by particles covered by the dsRNA construct, soaking the cell or organism in a solution of the RNA, microinjected into the target (e.g., mammalian target) cells, or electroporation of cell membranes in the presence of the dsRNA construct. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of dsRNA construct encoded by the expression construct. In one embodiment, the shRNA is a transcriptional product that is transcribed from an expression construct introduced into the target (e.g., mammalian target) cells, which expression construct comprises a coding sequence for transcribing said shRNA, operably linked to one or more transcriptional regulatory sequences. Such transcriptional regulatory sequences may include a promoter for an RNA polymerase, such as a cellular RNA polymerase. Exemplary but not limiting promoters include: a U6 promoter, a T7 promoter, a T3 promoter, or an SP6 promoter. In certain embodiments, the transcriptional regulatory sequences includes an inducible promoter.

The dsRNA constructs may be integrated into the host genome, such that the target cells are stably transfected with the dsRNA expression constructs. The constructs may be suitable for stable integration into either cells in culture or in an animal. For example, the constructs may be integrated into embryonic cells, such as a mouse ES cell, to generate a transgenic animal. The constructs may also be integrated into adult somatic cells, either primary cell or established cell line.

In certain embodiments, the expression of a target gene (either endogenous or heterologous gene) is attenuated by at least about 33%, or about 50%, about 60%, 70%, 80%, 90%, 95%, or 99% or more, relative to expression in cells not treated with the dsRNA (e.g., shRNA).

The shRNA may be chemically synthesized, or in vitro transcripted, and may further include one or more modifications to phosphate-sugar backbone or nucleosides residues.

Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical mediated transport, such as calcium phosphate, and the like. Thus the dsRNA construct may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

E. Illustrative Uses

One utility of the present invention is as a method of identifying gene function in an organism, especially higher eukaryotes, comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceuticals, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for mammalian genomes, can be coupled with the invention to determine gene function in a cell or in a whole organism. The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The ease with which the dsRNA construct can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from the target cell or organism. Inserts may be derived from genomic DNA or mRNA (e.g., cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (e.g., a 96 well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process.

In an exemplary embodiment, the subject invention provides an arrayed library of RNAi constructs. The array may be in the form of solutions, such as multi-well plates, or may be "printed" on solid substrates upon which cells can be grown. To illustrate, solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity.

In one embodiment, the subject method uses an arrayed library of RNAi constructs to screen for combinations of RNAi that are lethal to host cells. Synthetic lethality is a bedrock principle of experimental genetics. A synthetic lethality describes the properties of two mutations which, individually, are tolerated by the organism but which, in com-

bination, are lethal. The subject arrays can be used to identify loss-of-function mutations that are lethal in combination with alterations in other genes, such as activated oncogenes or loss-of-function mutations to tumor suppressors. To achieve this, one can create "phenotype arrays" using cultured cells. Expression of each of a set of genes, such as the host cell's genome, can be individually systematically disrupted using RNA interference. Combination with alterations in oncogene and tumor suppressor pathways can be used to identify synthetic lethal interactions that may identify novel therapeutic targets.

In certain embodiments, the RNAi constructs can be fed directly to, or injected into, the cell/organism containing the target gene. Alternatively, the duplex RNA can be produced by in vitro or in vitro transcription from an expression construct used to produce the library. The construct can be replicated as individual clones of the library and transcribed to produce the RNA; each clone can then be fed to, injected into, or delivered by another method known in the art to, the cell/organism containing the target gene. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. This screening could be amenable to small subjects that can be processed in large number, for example, tissue culture cells derived from mammals, especially primates, and most preferably humans.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, the duplex RNA can be introduced to the organism or cell, and whether an alteration in the characteristic is correlated with inhibition can be determined. Of course, there may be trivial explanations for negative results with this type of assay, for example: inhibition of the target gene causes lethality, inhibition of the target gene may not result in any observable alteration, the fragment contains nucleotide sequences that are not capable of inhibiting the target gene, or the target gene's activity is redundant.

The present invention may be useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or only in specific cellular compartments or tissues. The functional equivalent of conditional mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the target genome.

The present invention may be useful in allowing the inhibition of genes that have been difficult to inhibit using other methods due to gene redundancy. Since the present methods may be used to deliver more than one dsRNA to a cell or organism, dsRNA identical or similar to more than one gene, wherein the genes have a redundant function during normal development, may be delivered.

If alternative splicing produced a family of transcripts that were distinguished by usage of characteristic exons, the present invention can target inhibition through the appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a protein factor that contained an alternatively spliced transmembrane domain may be expressed in both membrane bound and secreted forms. Instead of isolating a nonsense mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone. That is, the subject method can be used for selected ablation of splicing variants.

The present invention may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the in vitro or in vitro introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

Alternatively, an organism may be engineered to produce dsRNA which produces commercially or medically beneficial results, for example, resistance to a pathogen or its pathogenic effects, improved growth, or novel developmental patterns.

Another aspect of the invention provides a method for attenuating expression of a target gene in mammalian cells, comprising introducing into the mammalian cells a single-stranded hairpin ribonucleic acid (shRNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA: (i) is a substrate for cleavage by a RNaseIII enzyme to produce a double-stranded RNA product, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions. In a preferred embodiment, the shRNA comprises a 3' overhang of about 14 nucleotides.

In a related aspect, the invention provides a method for attenuating expression of a target gene in mammalian cells, comprising introducing into the mammalian cells a single-stranded hairpin ribonucleic acid (shRNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA: (i) is cleaved in the mammalian cells to produce an RNA guide sequence that enters an Argonaut-containing complex, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions. In a preferred embodiment, the shRNA comprises a 3' overhang of about 14 nucleotides.

In yet another embodiment, the invention provides a method for attenuating expression of one or more target genes in mammalian cells, comprising introducing into the mammalian cells a variegated library of single-stranded hairpin ribonucleic acid (shRNA) species, each shRNA species comprising self complementary sequences of 19 to 100 nucleotides that form duplex regions and which hybridize under intracellular conditions to a target gene, wherein each of said hairpin RNA species: (i) is a substrate for cleavage by a RNaseIII enzyme to produce a double-stranded RNA product, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) if complementary to a target sequence, reduces expression of said target gene in a manner dependent on the sequence of said complementary regions. In a preferred embodiment, the shRNA comprises a 3' overhang of about 1-4 nucleotides.

In certain embodiments, the variegated library of shRNA species are arrayed a solid substrate.

In another embodiment, the method includes the further step of identifying shRNA species of said variegated library which produce a detected phenotype in the mammalian cells.

Yet another aspect of the invention provide a method of enhancing the potency/activity of an RNAi therapeutic for a mammalian patient, the RNAi therapeutic comprising an siRNA of 19-22 paired polynucleotides, the method comprising replacing the siRNA with a single-stranded hairpin RNA (shRNA) of the subject invention, wherein said duplex region comprises the same 19-22 paired polynucleotides of the siRNA. This aspect of the invention is partly based on the surprising discovery that shRNA constructs designed as Dicer substrates perform at least as well as, and in most cases much better/potent than the corresponding siRNA form of dsRNA (e.g., with the same eventual target guide sequence of about 22 nucleotides).

In certain embodiments, the half-maximum inhibition by the RNAi therapeutic is achieved by a concentration of the shRNA at least about 20%, or about 30%, 40%, 50%, 60%, 70%, 80%, 90% lower than that of the corresponding siRNA.

In another embodiment, the end-point inhibition by the shRNA is at least about 40%, or about 50%, 75%, 100%, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, or 10-fold higher than that of the siRNA.

Another aspect of the invention provides a method of designing a short hairpin RNA (shRNA) construct for RNAi, the shRNA comprising a 3' overhang of about 14 nucleotides, the method comprising selecting the nucleotide about 21 bases 5' to the most 3'-end nucleotide as the first paired nucleotide in a cognate doubled-stranded siRNA with the same 3' overhang. Such shRNA can be used, for example, for RNAi in mammalian cells.

In one embodiment, the shRNA comprises about 15-45, preferably about 25-29 paired polynucleotides.

In one embodiment, the 3' overhang has 2 nucleotides.

In one embodiment, the shRNA, when cut by a Dicer enzyme (e.g., a human Dicer enzyme), produces a product siRNA that is either identical to, or differ by a single basepair immediately 5' to the 3' overhang from the cognate siRNA.

In one embodiment, the shRNA construct has substantially the same profiles of off-target gene inhibition effects as compared to the cognate siRNA construct with substantially identical target sequences.

IV. Exemplification

The invention, now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention and are not intended to limit the invention.

EXAMPLE 1

An RNA-Directed Nuclease Mediates RNAi Gene Silencing

In a diverse group of organisms that includes *Caenorhabditis elegans, Drosophila*, planaria, hydra, trypanosomes, fungi and plants, the introduction of double-stranded RNAs inhibits gene expression in a sequence-specific manner (Sharp, *Genes and Development* 13: 139-141, 1999; Sanchez-Alvarado and Newmark, *PNAS* 96: 5049-5054, 1999; Lohman et al., *Developmental Biology* 214: 211-214, 1999; Cogoni and Macino, *Nature* 399: 166-169, 1999; Waterhouse et al., *PNAS* 95: 13959-13964, 1998; Montgomery and Fire, *Trends Genet.* 14: 225-228, 1998; Ngo et al., *PNAS* 95: 14687-14692, 1998). These responses, called RNA interference or post-transcriptional gene silencing, may provide anti-viral defense, modulate transposition or regulate gene expression (Sharp, *Genes and Development* 13: 139-141, 1999; Montgomery and Fire, *Trends Genet.* 14: 225-228, 1998; Tabara et al., *Cell* 99: 123-132, 1999; Ketting et al., *Cell* 99: 133-141, 1999; Ratcliff et al., *Science* 276: 1558-1560, 1997). We have taken a biochemical approach towards elucidating the mechanisms underlying this genetic phenomenon. Here we show that 'loss-of-function' phenotypes can be created in cultured *Drosophila* cells by transfection with specific double-stranded RNAs. This coincides with a marked reduction in the level of cognate cellular messenger RNAs. Extracts of transfected cells contain a nuclease activity that specifically degrades exogenous transcripts homologous to transfected double-stranded RNA. This enzyme contains an essential RNA component. After partial purification, the sequence-specific nuclease co-fractionates with a discrete, ~25-nucleotide RNA species which may confer specificity to the enzyme through homology to the substrate mRNAs.

Although double-stranded RNAs (dsRNAs) can provoke gene silencing in numerous biological contexts including *Drosophila* (Kennerdell et al., *Cell* 95: 1017-1026, 1998; Misquitta and Paterson, *PNAS* 96: 1451-1456, 1999), the mechanisms underlying this phenomenon have remained mostly unknown. We therefore wanted to establish a biochemically tractable model in which such mechanisms could be investigated.

Transient transfection of cultured, *Drosophila* S2 cells with a lacZ expression vector resulted in β-galactosidase activity that was easily detectable by an in situ assay (FIG. **1***a*). This activity was greatly reduced by co-transfection with a dsRNA corresponding to the first 300 nucleotides of the lacZ sequence, whereas co-transfection with a control dsRNA (CD8) (FIG. **1***a*) or with single-stranded RNAs of either sense or antisense orientation (data not shown) had little or no effect. This indicated that dsRNAs could interfere, in a sequence-specific fashion, with gene expression in cultured cells.

To determine whether RNA interference (RNAi) could be used to target endogenous genes, we transfected S2 cells with a dsRNA corresponding to the first 540 nucleotides of *Drosophila cyclin* E, a gene that is essential for progression into S phase of the cell cycle.

During log-phase growth, untreated S2 cells reside primarily in G2/M (FIG. **1***b*). Transfection with lacZ dsRNA had no effect on cell-cycle distribution, but transfection with the cyclin E dsRNA caused a G1-phase cell-cycle arrest (FIG. **1***b*). The ability of cyclin E dsRNA to provoke this response was length-dependent. Double-stranded RNAs of 540 and 400 nucleotides were quite effective, whereas dsRNAs of 200 and 300 nucleotides were less potent. Double-stranded cyclin E RNAs of 50 or 100 nucleotides were inert in our assay, and transfection with a single-stranded, antisense cyclin E RNA had virtually no effect.

One hallmark of RNAi is a reduction in the level of mRNAs that are homologous to the dsRNA. Cells transfected with the cyclin E dsRNA (bulk population) showed diminished endogenous cyclin E mRNA as compared with control cells (FIG. **1***c*). Similarly, transfection of cells with dsRNAs homologous to fizzy, a component of the anaphase-promoting complex (APC) or cyclin A, a cyclin that acts in S, G2 and M, also caused reduction of their cognate mRNAs (FIG. **1***c*). The modest reduction in fizzy mRNA levels in cells transfected with cyclin A dsRNA probably resulted from arrest at a point in the division cycle at which fizzy transcription is low (Wolf and Jackson, *Current Biology* 8: R637-R639, 1998; Kramer et al., *Current Biology* 8: 1207-1210, 1998). These

results indicate that RNAi may be a generally applicable method for probing gene function in cultured *Drosophila* cells.

The decrease in mRNA levels observed upon transfection of specific dsRNAs into *Drosophila* cells could be explained by effects at transcriptional or post-transcriptional levels. Data from other systems have indicated that some elements of the dsRNA response may affect mRNA directly (reviewed in Sharp, *Genes and Development* 13: 139-141, 1999; Montgomery and Fire, *Trends Genet.* 14: 225-228, 1998). We therefore sought to develop a cell-free assay that reflected, at least in part, RNAi.

S2 cells were transfected with dsRNAs corresponding to either cyclin E or lacZ. Cellular extracts were incubated with synthetic mRNAs of lacZ or cyclin E. Extracts prepared from cells transfected with the 540-nucleotide cyclin E dsRNA efficiently degraded the cyclin E transcript; however, the lacZ transcript was stable in these lysates (FIG. **2***a*). Conversely, lysates from cells transfected with the lacZ dsRNA degraded the lacZ transcript but left the cyclin E mRNA intact. These results indicate that RNAi ablates target mRNAs through the generation of a sequence-specific nuclease activity. We have termed this enzyme RISC (RNA-induced silencing complex). Although we occasionally observed possible intermediates in the degradation process (see FIG. **2**), the absence of stable cleavage end-products indicates an exonuclease (perhaps coupled to an endonuclease). However, it is possible that the RNAi nuclease makes an initial endonucleolytic cut and that non-specific exonucleases in the extract complete the degradation process (Shuttleworth and Colman, *EMBO J.* 7: 427-434, 1988). In addition, our ability to create an extract that targets lacZ in vitro indicates that the presence of an endogenous gene is not required for the RNAi response.

To examine the substrate requirements for the dsRNA-induced, sequence-specific nuclease activity, we incubated a variety of cyclin-E-derived transcripts with an extract derived from cells that had been transfected with the 540-nucleotide cyclin E dsRNA (FIGS. **2***b, c*). Just as a length requirement was observed for the transfected dsRNA, the RNAi nuclease activity showed a dependence on the size of the RNA substrate. Both a 600-nucleotide transcript that extends slightly beyond the targeted region (FIG. **2***b*) and an ~1-kilobase (kb) transcript that contains the entire coding sequence (data not shown) were completely destroyed by the extract. Surprisingly, shorter substrates were not degraded as efficiently. Reduced activity was observed against either a 300- or a 220-nucleotide transcript, and a 100-nucleotide transcript was resistant to nuclease in our assay. This was not due solely to position effects because ~100-nucleotide transcripts derived from other portions of the transfected dsRNA behaved similarly (data not shown). As expected, the nuclease activity (or activities) present in the extract could also recognize the antisense strand of the cyclin E mRNA. Again, substrates that contained a substantial portion of the targeted region were degraded efficiently whereas those that contained a shorter stretch of homologous sequence (~130 nucleotides) were recognized inefficiently (FIG. **2***c*, as600). For both the sense and antisense strands, transcripts that had no homology with the transfected dsRNA (FIG. **2***b*, Eout; FIG. **2***c*, as300) were not degraded. Although we cannot exclude the possibility that nuclease specificity could have migrated beyond the targeted region, the resistance of transcripts that do not contain homology to the dsRNA is consistent with data from *C. elegans*. Double-stranded RNAs homologous to an upstream cistron have little or no effect on a linked downstream cistron, despite the fact that unprocessed, polycistronic mRNAs can be readily detected (Tabara et al., *Science* 282: 430-432, 1998; Bosher et al., *Genetics* 153: 1245-1256, 1999). Furthermore, the nuclease was inactive against a dsRNA identical to that used to provoke the RNAi response in vitro (FIG. **2***b*). In the in vitro system, neither a 5' cap nor a poly(A) tail was required, as such transcripts were degraded as efficiently as uncapped and non-polyadenylated RNAs.

Gene silencing provoked by dsRNA is sequence specific. A plausible mechanism for determining specificity would be incorporation of nucleic-acid guide sequences into the complexes that accomplish silencing (Hamilton and Baulcombe, *Science* 286: 950-952, 1999). In accord with this idea, pretreatment of extracts with a Ca^{2+}-dependent nuclease (micrococcal nuclease) abolished the ability of these extracts to degrade cognate mRNAs (FIG. **3**). Activity could not be rescued by addition of non-specific RNAs such as yeast transfer RNA. Although micrococcal nuclease can degrade both DNA and RNA, treatment of the extract with DNAse I had no effect (FIG. **3**). Sequence-specific nuclease activity, however, did require protein (data not shown). Together, our results support the possibility that the RNAi nuclease is a ribonucleoprotein, requiring both RNA and protein components. Biochemical fractionation (see below) is consistent with these components being associated in extract rather than being assembled on the target mRNA after its addition.

In plants, the phenomenon of co-suppression has been associated with the existence of small (~25-nucleotide) RNAs that correspond to the gene that is being silenced (Hamilton and Baulcombe, *Science* 286: 950-952, 1999). To address the possibility that a similar RNA might exist in *Drosophila* and guide the sequence-specific nuclease in the choice of substrate, we partially purified our activity through several fractionation steps. Crude extracts contained both sequence-specific nuclease activity and abundant, heterogeneous RNAs homologous to the transfected dsRNA (FIGS. **2** and **4***a*). The RNAi nuclease fractionated with ribosomes in a high-speed centrifugation step. Activity could be extracted by treatment with high salt, and ribosomes could be removed by an additional centrifugation step. Chromatography of soluble nuclease over an anion-exchange column resulted in a discrete peak of activity (FIG. **4***b*, cyclin E). This retained specificity as it was inactive against a heterologous mRNA (FIG. **4***b*, lacZ). Active fractions also contained an RNA species of 25 nucleotides that is homologous to the cyclin E target (FIG. **4***b*, northern). The band observed on northern blots may represent a family of discrete RNAs because it could be detected with probes specific for both the sense and antisense cyclin E sequences and with probes derived from distinct segments of the dsRNA (data not shown). At present, we cannot determine whether the 25-nucleotide RNA is present in the nuclease complex in a double-stranded or single-stranded form.

RNA interference allows an adaptive defense against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. Our data, and that of others (Hamilton and Baulcombe, *Science* 286: 950-952, 1999), is consistent with a model in which dsRNAs present in a cell are converted, either through processing or replication, into small specificity determinants of discrete size in a manner analogous to antigen processing. Our results suggest that the post-transcriptional component of dsRNA-dependent gene silencing is accomplished by a sequence-specific nuclease that incorporates these small RNAs as guides that target specific messages based upon sequence recognition. The identical size of putative specificity determinants in plants (Hamilton and Baulcombe, supra) and animals predicts a conservation of both the mechanisms and the components of dsRNA-induced, post-transcriptional gene silencing

in diverse organisms. In plants, dsRNAs provoke not only post-transcriptional gene silencing but also chromatin remodeling and transcriptional repression (Jones et al., *EMBO J.* 17: 6385-6393, 1998; Jones et al., *Plant Cell* 11: 2291-2301, 1999). It is now critical to determine whether conservation of gene-silencing mechanisms also exists at the transcriptional level and whether chromatin remodeling can be directed in a sequence-specific fashion by these same dsRNA-derived guide sequences.

Methods:

Cell culture and RNA methods S2 cells (Schneider, *J Embryol Exp Morpho* 27: 353-365, 1972) were cultured at 27° C. in 90% Schneider's insect media (Sigma), 10% heat inactivated fetal bovine serum (FBS). Cells were transfected with dsRNA and plasmid DNA by calcium phosphate co-precipitation (DiNocera and Dawid, PNAS 80: 7095-7098, 1983). Identical results were observed when cells were transfected using lipid reagents (for example, Superfect, Qiagen). For FACS analysis, cells were additionally transfected with a vector that directs expression of a green fluorescent protein (GFP)-US9 fusion protein (Kalejta et al., *Exp Cell Res.* 248: 322-328, 1999). These cells were fixed in 90% ice-cold ethanol and stained with propidium iodide at 25 μg/ml. FACS was performed on an Elite flow cytometer (Coulter). For northern blotting, equal loading was ensured by over-probing blots with a control complementary DNA (RP49). For the production of dsRNA, transcription templates were generated by polymerase chain reaction such that they contained T7 promoter sequences on each end of the template. RNA was prepared using the RiboMax kit (Promega). Confirmation that RNAs were double stranded came from their complete sensitivity to RNAse III. Target mRNA transcripts were synthesized using the Riboprobe kit (Promega) and were gel purified before use.

Extract preparation Log-phase S2 cells were plated on 15-cm tissue culture dishes and transfected with 30 μg dsRNA and 30 μg carrier plasmid DNA. Seventy-two hours after transfection, cells were harvested in PBS containing 5 mM EGTA, washed twice in PBS and once in hypotonic buffer (10 mM HEPES pH 7.3, 6 mM α-mercaptoethanol). Cells were suspended in 0.7 packed-cell volumes of hypotonic buffer containing Complete protease inhibitors (Boehringer) and 0.5 units/ml of RNasin (Promega). Cells were disrupted in a dounce homogenizer with a type B pestle, and lysates were centrifuged at 30,000 g for 20 min. Supernatants were used in an in vitro assay containing 20 mM HEPES pH 7.3, 110 mM KOAc, 1 mM $Mg(OAc)_2$, 3 mM EGTA, 2 mM $CaCl_2$, 1 mM DTT. Typically, 5 μl extract was used in a 10 μl assay that contained also 10,000 c.p.m. synthetic mRNA substrate.

Extract fractionation Extracts were centrifuged at 200,000 g for 3 h and the resulting pellet (containing ribosomes) was extracted in hypotonic buffer containing also 1 mM $MgCl_2$ and 300 mM KOAc. The extracted material was spun at 100,000 g for 1 h and the resulting supernatant was fractionated on Source 15% column (Pharmacia) using a KCl gradient in buffer A (20 mM HEPES pH 7.0, 1 mM dithiothreitol, 1 mM $MgCl_2$). Fractions were assayed for nuclease activity as described above. For northern blotting, fractions were proteinase K/SDS treated, phenol extracted, and resolved on 15% acrylamide 8M urea gels. RNA was electroblotted onto Hybond N+ and probed with strand-specific riboprobes derived from cyclin E mRNA. Hybridization was carried out in 500 mM $NaPO_4$ pH 7.0, 15% formamide, 7% SDS, 1% BSA. Blots were washed in 1×SSC at 37-45° C.

EXAMPLE 2

Role for a Bidentate Ribonuclease in the Initiation Step of RNA Interference

Genetic approaches in worms, fungi and plants have identified a group of proteins that are essential for double-stranded RNA-induced gene silencing. Among these are ARGONAUTE family members (e.g. RDE1, QDE2) (Tabara et al., *Cell* 99: 123-132, 1999; Catalanotto et al., *Nature* 404: 245, 2000; Fagard et al., *PNAS* 97: 11650-11654, 2000), recQ-family helicases (MUT-7, QDE3) (Ketting et al., *Cell* 99: 133-141, 1999; Cogoni and Macino, *Science* 286: 2342-2344, 1999), and RNA-dependent RNA polymerases (e.g., EGO-1, QDE1, SGS2/SDE1) (Cogoni and Macino, *Nature* 399: 166-169, 1999; Smardon et al., *Current Biology* 10: 169-178, 2000; Mourrain et al., *Cell* 101: 533-542, 2000; Dalmay et al., *Cell* 101: 543-553, 2000). While potential roles have been proposed, none of these genes has been assigned a definitive function in the silencing process. Biochemical studies have suggested that PTGS is accomplished by a multicomponent nuclease that targets mRNAs for degradation (Hammond et al., *Nature* 404: 293-296, 2000; Zamore et al., *Cell* 101: 25-33, 2000; Tuschl et al., *Genes and Development* 13: 3191-3197, 1999). We have shown that the specificity of this complex may derive from the incorporation of a small guide sequence that is homologous to the mRNA substrate (Hammond et al., *Nature* 404: 293-296, 2000). Originally identified in plants that were actively silencing transgenes (Hamilton and Baulcombe, *Science* 286: 950-952, 1999), these ~22 nt. RNAs have been produced during RNAi in vitro using an extract prepared from *Drosophila* embryos (Zamore et al., *Cell* 101: 25-33, 2000). Putative guide RNAs can also be produced in extracts from *Drosophila* S2 cells (FIG. **5***a*). With the goal of understanding the mechanism of post-transcriptional gene silencing, we have undertaken both biochemical fractionation and candidate gene approaches to identify the enzymes that execute each step of RNAi.

Our previous studies resulted in the partial purification of a nuclease, RISC, that is an effector of RNA interference. See Example 1. This enzyme was isolated from *Drosophila* S2 cells in which RNAi had been initiated in vitro by transfection with dsRNA. We first sought to determine whether the RISC enzyme and the enzyme that initiates RNAi via processing of dsRNA into 22mers are distinct activities. RISC activity could be largely cleared from extracts by high-speed centrifugation (100,000×g for 60 min.) while the activity that produces 22mers remained in the supernatant (FIGS. **5***b,c*). This simple fractionation indicated that RISC and the 22mer-generating activity are separable and thus distinct enzymes. However, it seems likely that they might interact at some point during the silencing process.

RNAse III family members are among the few nucleases that show specificity for double-stranded RNA (Nicholson, *FEMS Microbiol Rev* 23: 371-390, 1999). Analysis of the *Drosophila* and *C. elegans* genomes reveals several types of RNAse III enzymes. First is the canonical RNAse III which contains a single RNAse III signature motif and a double-stranded RNA binding domain (dsRBD; e.g. RNC_CAEEL). Second is a class represented by Drosha (Filippov et al., *Gene* 245: 213-221, 2000), a *Drosophila* enzyme that contains two RNAse III motifs and a dsRBD (CeDrosha in *C. elegans*). A third class contains two RNAse III signatures and an amino terminal helicase domain (e.g. *Drosophila* CG4792, CG6493, *C. elegans* K12H4.8), and these had previously been proposed by Bass as candidate RNAi nucleases (Bass,

Cell 101: 235-238, 2000). Representatives of all three classes were tested for the ability to produce discrete, ~22 nt. RNAs from dsRNA substrates.

Partial digestion of a 500 nt. cyclin E dsRNA with purified, bacterial RNAse III produced a smear of products while nearly complete digestion produced a heterogeneous group of ~11-17 nucleotide RNAs (not shown). In order to test the dual-RNAse III enzymes, we prepared T7 epitope-tagged versions of Drosha and CG4792. These were expressed in transfected S2 cells and isolated by immunoprecipitation using antibody-agarose conjugates. Treatment of the dsRNA with the CG4792 immunoprecipitate yielded ~22 nt. fragments similar to those produced in either S2 or embryo extracts (FIG. **6***a*). Neither activity in extract nor activity in immunoprecipitates depended on the sequence of the RNA substrate since dsRNAs derived from several genes were processed equivalently (see Supplement 1). Negative results were obtained with Drosha and with immunoprecipitates of a DE×H box helicase (Homeless (Gillespie et al., *Genes and Development* 9: 2495-2508, 1995); see FIGS. **6***a,b*). Western blotting confirmed that each of the tagged proteins was expressed and immunoprecipitated similarly (see Supplement 2). Thus, we conclude that CG4792 may carry out the initiation step of RNA interference by producing ~22 nt. guide sequences from dsRNAs. Because of its ability to digest dsRNA into uniformly sized, small RNAs, we have named this enzyme Dicer (Dcr). Dicer mRNA is expressed in embryos, in S2 cells, and in adult flies, consistent with the presence of functional RNAi machinery in all of these contexts (see Supplement 3).

The possibility that Dicer might be the nuclease responsible for the production of guide RNAs from dsRNAs prompted us to raise an antiserum directed against the carboxy-terminus of the Dicer protein (Dicer-1, CG4792). This antiserum could immunoprecipitate a nuclease activity from either *Drosophila* embryo extracts or from S2 cell lysates that produced ~22 nt. RNAs from dsRNA substrates (FIG. **6**C). The putative guide RNAs that are produced by the Dicer-1 enzyme precisely co-migrate with 22mers that are produced in extract and with 22mers that are associated with the RISC enzyme (FIG. **6** D,F). It had previously been shown that the enzyme that produced guide RNAs in *Drosophila* embryo extracts was ATP-dependent (Zamore et al., *Cell* 101: 25-33, 2000). Depletion of this cofactor resulted in an ~6-fold lower rate of dsRNA cleavage and in the production of RNAs with a slightly lower mobility. Of interest was the fact that both Dicer-1 immunoprecipitates and extracts from S2 cells require ATP for the production of ~22mers (FIG. **6**D). We do not observe the accumulation of lower mobility products in these cases, although we do routinely observe these in ATP-depleted embryo extracts. The requirement of this nuclease for ATP is a quite unusual property. We hypothesize that this requirement could indicate that the enzyme may act processively on the dsRNA, with the helicase domain harnessing the energy of ATP hydrolysis both for unwinding guide RNAs and for translocation along the substrate.

Efficient induction of RNA interference in *C. elegans* and in *Drosophila* has several requirements. For example, the initiating RNA must be double-stranded, and it must be several hundred nucleotides in length. To determine whether these requirements are dictated by Dicer, we characterized the ability of extracts and of immunoprecipitated enzyme to digest various RNA substrates. Dicer was inactive against single stranded RNAs regardless of length (see Supplement 4). The enzyme could digest both 200 and 500 nucleotide dsRNAs but was significantly less active with shorter substrates (see Supplement 4). Double-stranded RNAs as short as 35 nucleotides could be cut by the enzyme, albeit very inefficiently (data not shown). In contrast, *E. coli* RNAse III could digest to completion dsRNAs of 35 or 22 nucleotides (not shown). This suggests that the substrate preferences of the Dicer enzyme may contribute to but not wholly determine the size dependence of RNAi.

To determine whether the Dicer enzyme indeed played a role in RNAi in vitro, we sought to deplete Dicer activity from S2 cells and test the effect on dsRNA-induced gene silencing. Transfection of S2 cells with a mixture of dsRNAs homologous to the two *Drosophila* Dicer genes (CG4792 and CG6493) resulted in an ~6-7 fold reduction of Dicer activity either in whole cell lysates or in Dicer-1 immunoprecipitates (FIG. **7**A,B). Transfection with a control dsRNA (murine caspase 9) had no effect. Qualitatively similar results were seen if Dicer was examined by Northern blotting (not shown). Depletion of Dicer in this manner substantially compromised the ability of cells to silence subsequently an exogenous, GFP transgene by RNAi (FIG. **7**C). These results indicate that Dicer is involved in RNAi in vitro. The lack of complete inhibition of silencing could result from an incomplete suppression of Dicer (which is itself required for RNAi) or could indicate that in vitro, guide RNAs can be produced by more than one mechanism (e.g. through the action of RNA-dependent RNA polymerases).

Our results indicate that the process of RNA interference can be divided into at least two distinct steps. According to this model, initiation of PTGS would occur upon processing of a double-stranded RNA by Dicer into ~22 nucleotide guide sequences, although we cannot formally exclude the possibility that another, Dicer-associated nuclease may participate in this process. These guide RNAs would be incorporated into a distinct nuclease complex (RISC) that targets single-stranded mRNAs for degradation. An implication of this model is that guide sequences are themselves derived directly from the dsRNA that triggers the response. In accord with this model, we have demonstrated that ^{32}P-labeled, exogenous dsRNAs that have been introduced into S2 cells by transfection are incorporated into the RISC enzyme as 22 mers (FIG. **7**E). However, we cannot exclude the possibility that RNA-dependent RNA polymerases might amplify 22mers once they have been generated or provide an alternative method for producing guide RNAs.

The structure of the Dicer enzyme provokes speculation on the mechanism by which the enzyme might produce discretely sized fragments irrespective of the sequence of the dsRNA (see Supplement 1, FIG. **8***a*). It has been established that bacterial RNAse III acts on its substrate as a dimer (Nicholson, *FEMS Microbiol Rev* 23: 371-390, 1999; Robertson et al., *J Biol Chem* 243: 82-91, 1968; Dunn, *J Biol Chem* 251: 3807-3814, 1976). Similarly, a dimer of Dicer enzymes may be required for cleavage of dsRNAs into ~22 nt. pieces. According to one model, the cleavage interval would be determined by the physical arrangement of the two RNAse III domains within Dicer enzyme (FIG. **8***a*). A plausible alternative model would dictate that cleavage was directed at a single position by the two RIII domains in a single Dicer protein. The 22 nucleotide interval could be dictated by interaction of neighboring Dicer enzymes or by translocation along the mRNA substrate. The presence of an integral helicase domain suggests that the products of Dicer cleavage might be single-stranded 22 mers that are incorporated into the RISC enzyme as such.

A notable feature of the Dicer family is its evolutionary conservation. Homologs are found in *C. elegans* (K12H4.8), *Arabidopsis* (e.g., CARPEL FACTORY (Jacobson et al., Development 126: 5231-5243, 1999), T25K16.4,

AC012328_1), mammals (Helicase-MOI (Matsuda et al., *Biochim Biophys Acta* 1490: 163-169, 2000) and *S. pombe* (YC9A_SCHPO) (FIG. **8***b*, see Supplements 6, 7 for sequence comparisons). In fact, the human Dicer family member is capable of generating ~22 nt. RNAs from dsRNA substrates (Supplement 5) suggesting that these structurally similar proteins may all share similar biochemical functions. It has been demonstrated that exogenous dsRNAs can affect gene function in early mouse embryos (Wianny et al., *Nature Cell Biology* 2: 70-75, 2000), and our results suggest that this regulation may be accomplished by an evolutionarily conserved RNAi machinery.

In addition to RNaseIII and helicase motifs, searches of the PFAM database indicate that each Dicer family member also contains a ZAP domain (FIG. **8***c*) (Sonnhammer et al., *Proteins* 28: 405-420, 1997). This sequence was defined based solely upon its conservation in the Zwille/ARGONAUTE/Piwi family that has been implicated in RNAi by mutations in *C. elegans* (Rde-1) and *Neurospora* (Qde-2) (Tabara et al., *Cell* 99: 123-132, 1999; Catalanotto et al., *Nature* 404: 245, 2000). Although the function of this domain is unknown, it is intriguing that this region of homology is restricted to two gene families that participate in dsRNA-dependent silencing. Both the ARGONAUTE and Dicer families have also been implicated in common biological processes, namely the determination of stem-cell fates. A hypomorphic allele of carpel factory, a member of the Dicer family in *Arabidopsis*, is characterized by increased proliferation in floral meristems (Jacobsen et al., *Development* 126: 5231-5243, 1999). This phenotype and a number of other characteristic features are also shared by *Arabidopsis* ARGONAUTE (agol-1) mutants (Bohmert et al., *EMBO J.* 17: 170-180, 1998; C. Kidner and R. Martiennsen, pers. comm.). These genetic analyses begin to provide evidence that RNAi may be more than a defensive response to unusual RNAs but may also play important roles in the regulation of endogenous genes.

With the identification of Dicer as a catalyst of the initiation step of RNAi, we have begun to unravel the biochemical basis of this unusual mechanism of gene regulation. It will be of critical importance to determine whether the conserved family members from other organisms, particularly mammals, also play a role in dsRNA-mediated gene regulation.

Methods:

Plasmid constructs. A full-length cDNA encoding Drosha was obtained by PCR from an EST sequenced by the Berkeley *Drosophila* genome project. The Homeless clone was a gift from Gillespie and Berg (Univ. Washington). The T7 epitope-tag was added to the amino terminus of each by PCR, and the tagged cDNAs were cloned into pRIP, a retroviral vector designed specifically for expression in insect cells (E. Bernstein, unpublished). In this vector, expression is driven by the *Orgyia* pseudotsugata IE2 promoter (Invitrogen). Since no cDNA was available for CG4792/Dicer, a genomic clone was amplified from a bacmid (BACR23F10; obtained from the BACPAC Resource Center in the Dept. of Human Genetics at the Roswell Park Cancer Institute). Again, during amplification, a T7 epitope tag was added at the amino terminus of the coding sequence. The human Dicer gene was isolated from a cDNA library prepared from HaCaT cells (GJH, unpublished). A T7-tagged version of the complete coding sequence was cloned into pcDNA3 (Invitrogen) for expression in human cells (LinX-A).

Cell culture and extract preparation. S2 and embryo culture. S2 cells were cultured at 27° C. in 5% CO_2 in Schneider's insect media supplemented with 10% heat inactivated fetal bovine serum (Gemini) and 1% antibiotic-antimycotic solution (GIBCO BRL). Cells were harvested for extract preparation at 10×10^6 cells/ml. The cells were washed 1× in PBS and were resuspended in a hypotonic buffer (10 mM HEPES pH 7.0, 2 mM $MgCl_2$, 6 mM βME) and dounced. Cell lysates were spun 20,000×g for 20 minutes. Extracts were stored at −80° C. *Drosophila* embryos were reared in fly cages by standard methodologies and were collected every 12 hours. The embryos were dechorionated in 50% chlorox bleach and washed thoroughly with distilled water. Lysis buffer (10 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 10 mM P-glycerophosphate, 1 mM DTT, 0.2 mM PMSF) was added to the embryos, and extracts were prepared by homogenization in a tissue grinder. Lysates were spun for two hours at 200,000×g and were frozen at −80° C. LinX-A cells, a highly-transfectable derivative of human 293 cells, (Lin Xie and GJH, unpublished) were maintained in DMEM/10% FCS.

Transfections and immunoprecipitations S2 cells were transfected using a calcium phosphate procedure essentially as previously described (Hammond et al., Nature 404: 293-296, 2000). Transfection rates were ~90% as monitored in controls using an in situ D-galactosidase assay. LinX-A cells were also transfected by calcium phosphate co-precipitation. For immunoprecipitations, cells (~5×10^6 per IP) were transfected with various clones and lysed three days later in IP buffer (125 mM KOAc, 1 mM MgOAc, 1 mM $CaCl_2$, 5 mM EGTA, 20 mM Hepes pH 7.0, 1 mM DTT, 1% NP-40 plus Complete protease inhibitors, Roche). Lysates were spun for 10 minutes at 14,000×g and supernatants were added to T7 antibody-agarose beads (Novagen). Antibody binding proceeded for 4 hours at 4° C. Beads were centrifuged and washed in lysis buffer three times, and once in reaction buffer. The Dicer antiserum was raised in rabbits using a KLH-conjugated peptide corresponding to the C-terminal 8 amino acids of *Drosophila* Dicer-1 (CG4792).

Cleavage reactions. RNA preparation. Templates to be transcribed into dsRNA were generated by PCR with forward and reverse primers, each containing a T7 promoter sequence. RNAs were produced using Riboprobe (Promega) kits and were uniformly labeling during the transcription reaction with ^{32}P-UTP. Single-stranded RNAs were purified from 1% agarose gels. dsRNA cleavage. Five microliters of embryo or S2 extracts were incubated for one hour at 30° C. with dsRNA in a reaction containing 20 mM Hepes pH 7.0, 2 mM MgOAc, 2 mM DTT, 1 mM ATP and 5% Superasin (Ambion). Immunoprecipitates were treated similarly except that a minimal volume of reaction buffer (including ATP and Superasin) and dsRNA were added to beads that had been washed in reaction buffer (see above). For ATP depletion, *Drosophila* embryo extracts were incubated for 20 minutes at 30° C. with 2 mM glucose and 0.375 U of hexokinase (Roche) prior to the addition of dsRNA.

Northern and Western analysis. Total RNA was prepared from *Drosophila* embryos (0-12 hour), from adult flies, and from S2 cells using Trizol (Lifetech). Messenger RNA was isolated by affinity selection using magnetic oligo-dT beads (Dynal). RNAs were electrophoresed on denaturing formaldehyde/agarose gels, blotted and probed with randomly primed DNAs corresponding to Dicer. For Western analysis, T7-tagged proteins were immunoprecipitated from whole cell lysates in IP buffer using anti-T7-antibody-agarose conjugates. Proteins were released from the beads by boiling in Laemmli buffer and were separated by electrophoresis on 8% SDS PAGE. Following transfer to nitrocellulose, proteins were visualized using an HRP-conjugated anti-T7 antibody (Novagen) and chemiluminescent detection (Supersignal, Pierce).

RNAi of Dicer. *Drosophila* S2 cells were transfected either with a dsRNA corresponding to mouse caspase 9 or with a mixture of two dsRNAs corresponding to *Drosophila* Dicer-1 and Dicer-2 (CG4792 and CG6493). Two days after the initial transfection, cells were again transfected with a mixture containing a GFP expression plasmid and either luciferase dsRNA or GFP dsRNA as previously described (Hammond et al., *Nature* 404: 293-296, 2000). Cells were assayed for Dicer activity or fluorescence three days after the second transfection. Quantification of fluorescent cells was done on a Coulter EPICS cell sorter after fixation. Control transfections indicated that Dicer activity was not affected by the introduction of caspase 9 dsRNA.

EXAMPLE 3

A Simplified Method for the Creation of Hairpin Constructs for RNA Interference

In numerous model organisms, double stranded RNAs have been shown to cause effective and specific suppression of gene function (Bosher and Labouesse, *Nature Cell Biology* 2: E31-E36, 2000). This response, termed RNA interference or post-transcriptional gene silencing, has evolved into a highly effective reverse genetic tool in *C. elegans, Drosophila*, plants and numerous other systems. In these cases, double-stranded RNAs can be introduced by injection, transfection or feeding; however, in all cases, the response is both transient and systemic. Recently, stable interference with gene expression has been achieved by expression of RNAs that form snap-back or hairpin structures (Fortier and Belote, *Genesis* 26: 240-244, 2000; Kennerdell and Carthew, *Nature Biotechnology* 18: 896-898, 2000; Lam and Thummel, *Current Biology* 10: 957-963, 2000; Shi et al., RNA 6: 1069-1076, 2000; Smith et al., *Nature* 407: 319-320, 2000; Tavernarakis et al., *Nature Genetics* 24: 180-183, 2000). This has the potential not only to allow stable silencing of gene expression but also inducible silencing as has been observed in trypanosomes and adult *Drosophila* (Fortier and Belote, *Genesis* 26: 240-244, 2000; Lam and Thummel, *Current Biology* 10: 957-963, 2000; Shi et al., *RNA* 6: 1069-1076, 2000). The utility of this approach is somewhat hampered by the difficulties that arise in the construction of bacterial plasmids containing the long inverted repeats that are necessary to provoke silencing. In a recent report, it was stated that more than 1,000 putative clones were screened to identify the desired construct (Tavemarakis et al., *Nature Genetics* 24: 180-183, 2000).

The presence of hairpin structures often induces plasmid rearrangement, in part due to the *E. coli* sbc proteins that recognize and cleave cruciform DNA structures (Connelly et al., *Genes Cell* 1: 285-291, 1996). We have developed a method for the construction of hairpins that does not require cloning of inverted repeats, per se. Instead, the fragment of the gene that is to be silenced is cloned as a direct repeat, and the inversion is accomplished by treatment with a site-specific recombinase, either in vitro (or potentially in vitro) (see FIG. **27**). Following recombination, the inverted repeat structure is stable in a bacterial strain that lacks an intact SBC system (DL759). We have successfully used this strategy to construct numerous hairpin expression constructs that have been successfully used to provoke gene silencing in *Drosophila* cells.

In the following examples, we use this method to express long dsRNAs in a variety of mammalian cell types. We show that such long dsRNAs mediate RNAi in a variety of cell types. Additionally, since the vector described in FIG. **27** contains a selectable marker, dsRNAs produced in this manner can be stably expressed in cells. Accordingly, this method allows not only the examination of transient effects of RNA suppression in a cell, but also the effects of stable and prolonged RNA suppression.

Methods:

Plasmids expressing hairpin RNAs were constructed by cloning the first 500 bps of the GFP coding region into the FLIP cassette of pRIP-FLIP as a direct repeat. The FLIP cassette contains two directional cloning sites, the second of which is flanked by LoxP sites. The Zeocin gene, present between the cloning sites, maintains selection and stability. To create an inverted repeat for hairpin production, the direct repeat clones were exposed to Cre recombinase (Stratagene) in vitro and, afterwards, transformed into DL759 *E. coli*. These bacteria permit the replication of DNA containing cruciform structures, which tend to form inverted repeats.

EXAMPLE 4

Long dsRNAs Suppress Gene Expression in Mammalian Cells

Previous experiments have demonstrated that dsRNA, produced using a variety of methods including via the construction of hairpins, can suppress gene expression in *Drosophila* cells. We now demonstrate that dsRNA can also suppress gene expression in mammalian cells in culture. Additionally, the power of RNAi as a genetic tool would be greatly enhanced by the ability to engineer stable silencing of gene expression. We therefore undertook an effort to identify mammalian cells in which long dsRNAs could be used as RNAi triggers in the hope that these same cell lines would provide a platform upon which to develop stable silencing strategies. We demonstrate that RNA suppression can be mediated by stably expressing a long hairpin in a mammalian cell line. The ability to engineer stable silencing of gene expression in cultured mammalian cells, in addition to the ability to transiently silence gene expression, has many important applications.

A. RNAi in Pluripotent Murine P19 Cells.

We first sought to determine whether long dsRNA triggers could induce sequence-specific silencing in cultured murine cells, both to develop this approach as a tool for probing gene function and to allow mechanistic studies of dsRNA-induced silencing to be propagated to mammalian systems. We, therefore, attempted to extend previous studies in mouse embryos (Wianny et al., *Nat. Cell Biol.* 2: 70-75, 2000; Svoboda et al., Development 127: 4147-4156, 2000) by searching for RNAi-like mechanisms in pluripotent, embryonic cell types. We surveyed a number of cell lines of embryonic origin for the degree to which generalized suppression of gene expression occurred upon introduction of dsRNA. As an assay, we tested the effects of dsRNA on the expression of GFP as measured in situ by counting fluorescent cells. As expected, in both human embryonic kidney cells (293) and mouse embryo fibroblasts, GFP expression was virtually eliminated irrespective of the sequence of the cotransfected dsRNA. In some pluripotent teratocarcinoma and teratoma cell lines (e.g., N-Teral, F9), the PKR response was attenuated but still evident; however, in contrast, transfection of nonhomologous dsRNAs had no effect on the expression of reporter genes (e.g., GFP or luciferase) either in mouse embryonic stem cells or in p19 embryonal carcinoma cells (FIG. **28**).

Transfection of P19 embryonal carcinoma cells with GFP in the presence of cognate dsRNA corresponding to the first ≈500 nts of the GFP coding sequence had a strikingly differ-

ent effect. GFP expression was eliminated in the vast majority of cotransfected cells (FIG. **28**), suggesting that these cultured murine cells might respond to dsRNA in a manner similar to that which we had previously demonstrated in cultured, *Drosophila* S2 cells (Hammond et al., *Nature* 404: 293-296, 2000).

To quantify the extent to which dsRNA could induce sequence-specific gene silencing, we used a dual luciferase reporter assay similar to that which had first been used to demonstrate RNAi in *Drosophila* embryo extracts (Tuscbl et al., *Genes Dev.* 13: 3191-3197, 1999). P19 EC cells were transfected with a mixture of two plasmids that individually direct the expression of firefly luciferase and *Renilla* luciferase. These were cotransfected with no dsRNA, with dsRNA that corresponds to the first ≈500 nts of the firefly luciferase, or with dsRNA corresponding to the first ~500 nts of GFP as a control. Cotransfection with GFP dsRNA gave luciferase activities that were similar to the no-dsRNA control, both in the firefly/*Renilla* activity ratio and in the absolute values of both activities. In contrast, in cells that received the firefly luciferase dsRNA, the ratio of firefly to *Renilla* luciferase activity was reduced by up to 30-fold (250 ng, FIG. **29**B). For comparison, we carried out an identical set of experiments in *Drosophila* S2 cells. Although qualitatively similar results were obtained, the silencing response was more potent. At equivalent levels of dsRNA, S2 cells suppressed firefly luciferase activity to virtually background levels.

The complementary experiment, in which dsRNA was homologous to *Renilla* luciferase, was also performed. Again, in this case, suppression of the expression of the *Renilla* enzyme was ≈10-fold (FIG. **29**D). Thus, the dsRNA response in P19 cells was flexible, and the silencing machinery was able to adapt to dsRNAs directed against any of the reporters that were tested.

We took two approaches to test whether this response was specific for dsRNA. Pretreatment of the trigger with purified RNase III, a dsRNA-specific ribonuclease, before transfection greatly reduced its ability to provoke silencing. Furthermore, transfection of cells with single-stranded antisense RNAs directed against either firefly or *Renilla* luciferase had little or no effect on expression of the reporters (FIGS. **29**C and **29**D). Considered together, these results provided a strong indication that double-stranded RNAs provoke a potent and specific silencing response in P19 embryonal carcinoma cells. Efficient silencing could be provoked with relatively low concentrations of dsRNA (25 ng/ml culture media; see FIG. **29**A). The response was concentration-dependent, with maximal suppression of ≈20-fold being achieved at a dose of 1.5 μg/ml culture media. Silencing was established rapidly and was evident by 9 h post-transfection (the earliest time point examined). Furthermore, the response persisted without significant changes in the degree of suppression for up to 72 h following a single dose of dsRNA.

FIG. **30** further shows wild-type P19 cells which have been co-transfected with either RFP or GFP (right panel). Note the robust expression of RFP or GFR respectively approximately 42 hours post-transfection. We isolated P19 clones which stably express a 500 nt. GFP hairpin. Such clones were then transfected with either RFP or GFP, and expression of RFP or GFP was assessed by visual inspection of the cells. The left panel demonstrates that a 500 nt GFP hairpin specifically suppresses expression of GFP in P19 cells.

B. RNAi in Embryonic Stem Cells.

To assess whether the presence of a sequence-specific response to dsRNA was a peculiarity of P19 cells or whether it also extended to normal murine embryonic cells, we performed similar silencing assays in mouse embryonic stem cells. Cotransfection of embryonic stem cells with noncognate dsRNAs (e.g., GFP), again, had no dramatic effect on either the absolute values or the ratios of *Renilla* and firefly luciferase activity (FIG. **31**). However, transfection with either firefly or *Renilla* luciferase dsRNA dramatically and specifically reduced the activity of the targeted enzyme (FIG. **31**).

This result suggests that RNAi can operate in multiple murine cell types of embryonic origin, including normal embryonic stem cells. The ability to provoke silencing in a cell type that is normally used for the generation of genetic, mosaic animals suggests the possibility of eventually testing the biological effects of silencing both in culture and in reconstituted animal models. Our ability to successfully manipulate ES cell via RNAi allows the use of RNAi in the generation of transgenic and knock-out mice.

C. RNAi in Murine Somatic Cells.

RNAi effector pathways are likely to be present in mammalian somatic cells, based on the ability of siRNAs to induce transient silencing (Elbashir et al., *Nature* 411: 494-498, 2001). Furthermore, we have shown that RNAi initiator and effector pathways clearly exist in embryonic cells that can enforce silencing in response to long dsRNA triggers. We therefore sought to test whether the RNAi machinery might exist intact in some somatic cell lines.

Transfection of HeLa cells with luciferase reporters in combination with long dsRNA triggers caused a nearly complete suppression of activity, irrespective of the RNA sequence. In a murine myoblast cell line, C2C12, we noted a mixture of two responses. dsRNAs homologous to firefly luciferase provoked a sequence-specific effect, producing a degree of suppression that was slightly more potent than was observed upon transfection with cognate ≈21-nt siRNA (Elbashir et al., *Nature* 411: 494-498, 2001) (see FIG. **32**A). However, with long dsRNA triggers, the specific effect was superimposed upon a generalized suppression of reporter gene expression that was presumably because of PKR activation (FIG. **32**B).

Numerous mammalian viruses have evolved the ability to block PKR as an aid to efficient infection. For example, adenoviruses express VA RNAs, which mimic dsRNA with respect to binding but not to activation of PKR (Clarke et al., *RNA* 1: 7-20, 1995). Vaccinia virus uses two strategies to evade PKR. The first is expression of E3L, which binds and masks dsRNAs (Kawagishi-Kobayashi et al., *Virology* 276: 424-434, 2000). The second is expression of K3L, which binds and inhibits PKR via its ability to mimic the natural substrate of this enzyme, eIF2α (Kawagishi-Kobayashi et al. 2000, supra).

Transfection of C2C12 cells with a vector that directs K3L expression attenuates the generalized repression of reporter genes in response to dsRNA. However, this protein had no effect on the magnitude of specific inhibition by RNAi (FIG. **32**C).

FIG. **33** further shows the results of a transient co-transfection assay performed in Hela cells, CHO cells, and P19 cells. The cell lines were each transfected with plasmids expressing *Photinus pyralis* (firefly) and *Renila reniformis* (sea pansy) luciferases. The cells lines were additionally transfected with 400 ng of 500 nt dsRNAs corresponding to either firefly luciferase (dsLUC) or dsGFP. The results demonstrate that dsRNA can specifically mediate suppression in a multiple mammalian cells types in culture.

These results raise the possibility that, at least in some cell lines and/or cell types, blocking nonspecific responses to dsRNA will enable the use of long dsRNAs for the study of

gene function. This might be accomplished through the use of viral inhibitors, as described here, or through the use of cells isolated from animals that are genetically modified to lack undesirable responses.

D. Stable Suppression of Gene Expression Using RNAi.

To date, dsRNAs have been used to induce sequence-specific gene silencing in either cultured mammalian cells or in embryos only in a transient fashion. However, the most powerful applications of genetic manipulation are realized only with the creation of stable mutants. The ability to induce silencing by using long dsRNAs offers the opportunity to translate into mammalian cells work from model systems such as *Drosophila*, plants, and *C. elegans* wherein stable silencing has been achieved by enforced expression of hairpin RNAs (Kennerdell et al., *Nat. Biotechnol.* 18: 896-898, 2000; Smith et al., *Nature* 407: 319-320, 2000; Tavernarakis et al., *Nat. Genet.* 24:180-183, 2000).

P19 EC cells were transfected with a control vector or with an expression vector that directs expression of a ≈500-nt GFP hairpin RNA from an RNA polymerase II promoter (cytomegalovirus). Colonies arising from cells that had stably integrated either construct were selected and expanded into clonal cell lines. Each cell line was assayed for persistent RNAi by transient co-transfection with a mixture of two reporter genes, dsRED to mark transfected cells and GFP to test for stable silencing.

Transfection of clonal P19 EC cells that had stably integrated the control vector produced equal numbers of red and green cells, as would be expected in the absence of any specific silencing response (FIG. **34**B), whereas cells that express the GFP hairpin RNA gave a very different result. These cells expressed the dsRED protein with an efficiency comparable to that observed in cells containing the control vector. However, the cells failed to express the cotransfected GFP reporter (FIG. **34**B). These data provide a strong indication that continuous expression of a hairpin dsRNA can provoke stable, sequence-specific silencing of a target gene.

In *Drosophila* S2 cells and *C. elegans*, RNAi is initiated by the Dicer enzyme, which processes dsRNA into 22-nt siRNAs (Bernstein et al., *Nature* 409: 363-366, 2001; Grishok et al., *Cell* 106: 23-34, 2001; Hutvagner et al., *Science* 293: 834-838, 2001; Ketting et al., *Genes Dev.* 15: 2654-2659, 2001; Knight et al., *Science* 293: 2269-2271, 2001). In both, S2 cells and *C. elegans* experiments by using dsRNA to target Dicer suppress the RNAi response. Whether Dicer plays a central role in hairpin-induced gene silencing in P19 cells was tested by transfecting P19 cells stably transfected with GFP hairpin constructs with mouse Dicer dsRNA. Treatment with Dicer dsRNA, but not control dsRNA, resulted in depression of GFP (FIG. **34**C).

E. dsRNA Induces Posttranscriptional Silencing.

A key feature of RNAi is that it exerts its effect at the posttranscriptional level by destruction of targeted mRNAs (Hammond et al., *Nat. Rev. Genet.* 2: 110-119, 2001). To test whether dsRNAs induced silencing in mouse cells via posttranscriptional mechanisms, we used an assay identical to that, used initially to characterize RNAi responses in *Drosophila* embryo extracts (Tuschl et al., *Genes Dev.* 13: 3191-3197, 1999). We prepared lysates from P19 EC cells that were competent for in vitro translation of capped mRNAs corresponding to *Renilla* and firefly luciferase. Addition of nonspecific dsRNAs to these extracts had no substantial effect on either the absolute amount of luciferase expression or on the ratio of firefly to *Renilla* luciferase (FIG. **35**). In contrast, addition of dsRNA homologous to the firefly luciferase induced a dramatic and dose-dependent suppression of activity. Addition of RNA corresponding to only the antisense strand of the dsRNA had little effect, comparable to a nonspecific dsRNA control, and pretreatment of the dsRNA silencing trigger with RNase III greatly reduced its potential to induce silencing in vitro. A second hallmark of RNAi is the production of small, ≈22-nt siRNAs, which determine the specificity of silencing. We found that such RNA species were generated from dsRNA in P19 cell extracts (FIG. **34**D, in vitro), indicative of the presence of a mouse Dicer activity. These species were also produced in cells that stably express GFP hairpin RNAs (FIG. **34**D, in vitro). Considered together, the posttranscriptional nature of dsRNA-induced silencing, the association of silencing with the production of ~22-nt siRNAs, and the dependence of this response on Dicer, a key player in the RNAi pathway, strongly suggests that dsRNA suppresses gene expression in murine cells via a conventional RNAi mechanism.

F. RNAi-Mediated Gene Silencing is Specific and Requires dsRNAs.

We carried out experiments to verify that the suppressive effects observed in the in vitro system were specific to double stranded RNA. Briefly, experiments were performed in accordance with the methods outlined above. Either dsRNA (ds), single-stranded RNA (ss), or antisense-RNA (as) corresponding to firefly (FF) or *Renilla* (Ren) luciferase was added to the translation reaction. Following reactions performed at 30° C. for 1 hour, dual luciferase assays were performed using an Analytical Scientific Instruments model 3010 Luminometer.

FIG. **36** summarizes the results of these experiments which demonstrate that the suppression of gene expression observed in this in vitro assay is specific for dsRNA. These results further support the conclusion that dsRNA suppresses gene expression in this mammalian in vitro system in a manner consistent with post-transcriptional silencing.

G. Mammalian Cells Soaked with dsRNAs Results in Gene Silencing.

Studies of post-transcriptional silencing in invertebrates have demonstrated that transfection or injection of the dsRNA is not necessary to achieve the suppressive affects. For example, dsRNA suppression in *C. elegans* can be observed by either soaking the worms in dsRNA, or by feeding the worms bacteria expressing the dsRNA of interest. We addressed whether dsRNA suppression in mammalian cells could be observed without transfection of the dsRNA. Such a result would present additional potential for easily using dsRNA suppression in mammalian cells, and would also allow the use of dsRNA to suppress gene expression in cell types which have been difficult to transfect (i.e., cell types with a low transfection efficiency, or cell types which have proven difficult to transfect at all).

P19 cells were grown in 6-well tissue culture plates to approximately 60% confluency in growth media (αMEM/10% FBS). Varying concentrations of firefly dsRNA were added to the cultures, and cells were cultured for 12 hours in growth media+dsRNA. Cells were then transfected with plasmids expressing firefly or sea pansy luciferase, as described in detail above. Dual luciferase assays were carried out 12 hours post-transfection using an Analytical Scientific Instruments model 3010 Luminometer.

FIG. **37** summarizes these results which demonstrate that dsRNA can suppress gene expression in mammalian cells without transfection. Culturing cells in the presence of dsRNA resulted in a dose dependent suppression of firefly luciferase gene expression.

Methods:

Cell Culture. P19 mouse embryonic carcinoma cells (American Type Culture Collection, CRL-1825) were cultured in α-MEM (GIBCO/BRL) supplemented with 10%

heat-inactivated FBS and 1% antibiotic/antimycotic solution (GIBCO/BRL). Mouse embryo stem cells (J1, provided by S. Kim, Cold Spring Harbor Laboratory) were cultured in DMEM containing ESgro (Chemicon) according to the manufacturer's instructions. C2C12 murine myoblast cells (gift of N. Tonks, Cold Spring Harbor Laboratory) were cultured in DMEM (GIBCO/BRL) supplemented with 10% beat-inactivated FBS and 1% antibiotic/antimycotic solution (GIBCO/BRL).

RNA Preparation. For the production of dsRNA, transcription templates were generated by PCR; they contained T7 promoter sequences on each end of the template (see Hammond et al. 2000, Nature 404: 293-296). dsRNAs were prepared by using the RiboMax kit (Ambion, Austin, Tex.). Firefly and *Renilla* luciferase mRNA transcripts were synthesized by using the Riboprobe kit (Promega) and were gel purified before use.

Transfection and Gene Silencing Assays. Cells were transfected with indicated amounts of dsRNA and plasmid DNA by using FuGENE6 (Roche Biochemicals) according to the manufacturer's instructions. Cells were transfected at 50-70% confluence in 12-well plates containing either 1 or 2 ml of medium per well. Dual luciferase assays (Promega) were carried out by co-transfecting cells with plasmids contain firefly luciferase under the control of SV40 promoter (pGL3-Control, Promega) and *Renilla* luciferase under the control of the SV40 early enhancer/promoter region (pSV40, Promega). These plasmids were cotransfected by using a 1:1 or 10:1 ratio of pGL3-control (250 ng/well) to pRL-SV40. Both ratios yielded similar results. For some experiments, cells were transfected with vectors that direct expression of enhanced green fluorescent protein (EGFP)-US9 fusion protein (Kalejta et al., *Exp. Cell Res.* 248: 322-328, 1999) or red fluorescent protein (RFP) (pDsRed N1, CLONTECH). RNAi in S2 cells was performed as described (Hammond et al., *Nature* 404: 293-296, 2000).

Plasmids expressing hairpin RNAs (RNAs with a self-complimentary stem loop) were constructed by cloning the first 500 bp of the EGFP coding region (CLONTECH) into the FLIP cassette of pRIP-FLIP as a direct repeat. The FLIP cassette contains two directional cloning sites, the second of which sports flanking LoxP sites (see FIG. **35**A). The Zeocin gene (Stratagene), present between the cloning sites, maintains selection and, thus, stability of the FLIP cassette. The FLIP cassette containing EGFP direct repeats was subcloned into pcDNA3 (Invitrogen). To create an inverted repeat for hairpin production, EGFP direct repeat clones were exposed to Cre recombinase (Stratagene) in vitro and, afterward, transformed into DL759 *Escherichia coli* (Connelly et al., *Genes Cells* 1: 285-291, 1996). These bacteria permit the replication of DNA containing cruciform structures, which tend to form from inverted repeats. DL759 transformants were screened for plasmids containing inverted repeats (≈50%).

Silencing of Dicer was accomplished by using a dsRNA comprising exon 25 of the mouse Dicer gene and corresponding to nucleotides 5284-5552 of the human Dicer cDNA.

In vitro Translation and in vitro Dicer Assays. Logarithmically growing cells were harvested in PBS containing 5 mM EGTA washed twice in PBS and once in hypotonic buffer (10 mM Hepes, pH 7.3/6 mM β-mercaptoethanol). Cells were suspended in 0.7 packed-cell volumes of hypotonic buffer containing Complete protease inhibitors (Roche Molecular Biochemicals) and 0.5 units/ml of RNasin (Promega). Cells were disrupted in a Dounce homogenizer with a type B pestle, and lysates were centrifuged at 30,000×g for 20 min. Supernatants were used in an in vitro translation assay containing capped m7G(5')pppG firefly and *Renilla* luciferase mRNA or in in vitro Dicer assays containing ^{32}P-labeled dsRNA. For in vitro translation assays, 5 μl of extract were mixed with 100 ng of firefly and *Renilla* mRNA along with 1 μg of dsRNA (or buffer)/10 mM DTT/0.5 mM spermidine/200 mM Hepes, 3.3 mM MgOAc/800 mM KOAc/1 mM ATP/1 mM GTP/4 units of Rnasin/215 μg of creatine phosphate/1 μg of creatine phosphate kinase/1 mM amino acids (Promega). Reactions were carried out for 1 h at 30° C. and quenched by adding 1× passive lysis buffer (Promega). Extracts were then assayed for luciferase activity. In vitro assays for Dicer activity were performed as described (Bernstein et al., *Nature* 409: 363-366, 2001).

Construction of Stable Silencing Lines. Ten-centimeter plates of P19 cells were transfected with 5 μg of GFP hairpin expression plasmid and selected for stable integrants by using G-418 (300 ng/ml) for 14 days. Clones were selected and screened for silencing of GFP.

EXAMPLE 5

Compositions and Methods for Synthesizing siRNAs

Previous results have indicated that short synthetic RNAs (siRNAs) can efficiently induce RNA suppression. Since short RNAs do not activate the non-specific PKR response, they offer a means for efficiently silencing gene expression in a range of cell types. However, the current state of the art with respect to siRNAs has several limitations. Firstly, siRNAs are currently chemically synthesized at great cost (approx. $400/siRNA). Such high costs make siRNAs impractical for either small laboratories or for use in large scale screening efforts. Accordingly, there is a need in the art for methods for generating siRNAs at reduced cost.

We provide compositions and methods for synthesizing siRNAs by T7 polymerase. This approach allows for the efficient synthesis of siRNAs at a cost consistent with standard RNA transcription reactions (approx. $16/siRNA). This greatly reduced cost makes the use of siRNA a reasonable approach for small laboratories, and also will facilitate their use in large-scale screening projects.

FIG. **38** shows the method for producing siRNAs using T7 polymerase. Briefly, T7 polymerase is used to transcribe both a sense and antisense transcript. The transcripts are then annealed to provide an siRNA. One of skill in the art will recognize that any one of the available RNA polymerases can be readily substituted for T7 to practice the invention (i.e., T3, Sp6, etc.).

This approach is amenable to the generation of a single siRNA species, as well as to the generation of a library of siRNAs. Such a library of siRNAs can be used in any number of high-throughput screens including cell based phenotypic screens and gene array based screens.

EXAMPLE 6

Generation of Short Hairpin dsRNA and Suppression of Gene Expression Using Such Short Hairpins

Since the realization that small, endogenously encoded hairpin RNAs could regulate gene expression via elements of the RNAi machinery, we have sought to exploit this biological mechanism for the regulation of desired target genes. Here we show that short hairpin RNAs (shRNAs) can induce sequence-specific gene silencing in mammalian cells. As is normally done with siRNAs, silencing can be provoked by transfecting exogenously synthesized hairpins into cells.

However, silencing can also be triggered by endogenous expression of shRNAs. This observation opens the door to the production of continuous cells lines in which RNAi is used to stably suppress gene expression in mammalian cells. Furthermore, similar approaches should prove efficacious in the creation of transgenic animals and potentially in therapeutic strategies in which long-term suppression of gene function is essential to produce a desired effect.

Several groups (Grishok et al., *Cell* 106: 23-34, 2001; Ketting et al., *Genes & Dev.* 15: 2654-2659, 2001; Knight et al., *Science* 293: 2269-2271, 2001; Hutvagner et al., *Science* 293: 834-838, 2001) have shown that endogenous triggers of gene silencing, specifically small temporal RNAs (stRNAs) let-7 and lin-4, function at least in part through RNAi pathways. Specifically, these small RNAs are encoded by hairpin precursors that are processed by Dicer into mature, ~21-nt forms. Moreover, genetic studies in *C. elegans* have shown a requirement for Argonaute-family proteins in stRNA function. Specifically, alg-1 and alg-2, members of the EIF2c subfamily, are implicated both in stRNA processing and in their downstream effector functions (Grishok et al., 2001, supra). We have recently shown that a component of RISC, the effector nuclease of RNAi, is a member of the Argonaute family, prompting a model in which stRNAs may function through RISC-like complexes, which regulate mRNA translation rather than mRNA stability (Hammond et al., *Science* 293: 1146-1150, 2001).

A. Short Hairpin RNAs Triggeedr Gene Silencing in *Drosophila* Cells.

We wished to test the possibility that we might retarget these small, endogenously encoded hairpin RNAs to regulate genes of choice with the ultimate goal of subverting this regulatory system for manipulating gene expression stably in mammalian cell lines and in transgenic animals. Whether triggered by long dsRNAs or by siRNAs, RNAi is generally more potent in the suppression of gene expression in *Drosophila* S2 cells than in mammalian cells. We therefore chose this model system in which to test the efficacy of short hairpin RNAs (shRNAs) as inducers of gene silencing.

Neither stRNAs nor the broader group of miRNAs that has recently been discovered form perfect hairpin structures. Indeed, each of these RNAs is predicted to contain several bulged nucleotides within their rather short (~30-nt) stem structures. Because the position and character of these bulged nucleotides have been conserved throughout evolution and among at least a subset of miRNAs, we sought to design retargeted miRNA mimics to conserve these predicted structural features. Only the let-7 and lin-4 miRNAs have known mRNA targets (Wightman et al., *Cell* 75: 855-862, 1993; Slack et al., *Mol. Cell.* 5: 659-669, 2000). In both cases, pairing to binding sites within the regulated transcripts is imperfect, and in the case of lin-4, the presence of a bulged nucleotide is critical to suppression (Ha et al., *Genes & Dev.* 10: 3041-3050, 1996). We therefore also designed shRNAs that paired imperfectly with their target substrates. A subset of these shRNAs is depicted in FIG. **39**A.

To permit rapid testing of large numbers of shRNA variants and quantitative comparison of the efficacy of suppression, we chose to use a dual-luciferase reporter system, as previously described for assays of RNAi in both *Drosophila* extracts (Tuschl et al., *Genes & Dev.* 13: 3191-3197, 1999) and mammalian cells (Caplen et al., *Proc. Natl. Acad. Sci.* 98: 9742-9747, 2001; Elbashir et al., Nature 411: 494-498, 2001). Cotransfection of firefly and *Renilla* luciferase reporter plasmids with either long dsRNAs or with siRNAs homologous to the firefly luciferase gene yielded an ~95% suppression of firefly luciferase without effect on *Renilla* luciferase (FIG. **39**B; data not shown). Firefly luciferase could also be specifically silenced by co-transfection with homologous shRNAs. The most potent inhibitors were those composed of simple hairpin structures with complete homology to the substrate. Introduction of G-U basepairs either within the stem or within the substrate recognition sequence had little or no effect (FIGS. **39**A and **39**B; data not shown).

These results show that short hairpin RNAs can induce gene silencing in *Drosophila* S2 cells with potency similar to that of siRNAs (FIG. **39**B). However, in our initial observation of RNA interference in *Drosophila* S2 cells, we noted a profound dependence of the efficiency of silencing on the length of the dsRNA trigger (Hammond et al., *Nature* 404: 293-296, 2000). Indeed, dsRNAs of fewer than ~200 nt triggered silencing very inefficiently. Silencing is initiated by an RNase III family nuclease, Dicer, that processes long dsRNAs into 22-nt siRNAs. In accord with their varying potency as initiators of silencing, long dsRNAs are processed much more readily than short RNAs by the Dicer enzyme (Bernstein et al., Nature 409: 363-366, 2001). We therefore tested whether shRNAs were substrates for the Dicer enzyme.

We had noted previously that let-7 (Ketting et al., *Genes & Dev.* 15: 2654-2659, 2001) and other miRNAs (E. Bernstein, unpublished data) are processed by Dicer with an unexpectedly high efficiency as compared with short, nonhairpin dsRNAs. Similarly, Dicer efficiently processed shRNAs that targeted firefly luciferase, irrespective of whether they were designed to mimic a natural Dicer substrate (let-7) or whether they were simple hairpin structures (FIG. **39**C). These data suggest that recombinant shRNAs can be processed by Dicer into siRNAs and are consistent with the idea that these short hairpins trigger gene silencing via an RNAi pathway.

B. Short Hairpin RNAs Activated Gene Silencing in Mammalian Cells.

Mammalian cells contain several endogenous systems that were predicted to hamper the application of RNAi. Chief among these is a dsRNA-activated protein kinase, PKR, which effects a general suppression of translation via phosphorylation of EIF-2α (Williams, *Biochem. Soc. Trans.* 25: 509-513, 1997; Gil et al., *Apoptosis* 5: 107-114, 2000). Activation of these, and other dsRNA-responsive pathways, generally requires duplexes exceeding 30 bp in length, possibly to permit dimerization of the enzyme on its allosteric activator (e.g., Clarke et al., RNA 1: 7-20, 1995). Small RNAs that mimic Dicer products, siRNAs, presumably escape this limit and trigger specific silencing, in part because of their size. However, short duplex RNAs that lack signature features of siRNAs can efficiently induce silencing in *Drosophila* S2 cells but not in mammalian cells (A. A. Caudy, unpublished data). Endogenously encoded miRNAs may also escape PKR surveillance because of their size but perhaps also because of the discontinuity of their duplex structure. Given that shRNAs of <30 bp were effective inducers of RNAi in *Drosophila* S2 cells, we tested whether these RNAs could also induce sequence-specific silencing in mammalian cells.

Human embryonic kidney (HEK293T) cells were cotransfected with chemically synthesized shRNAs and with a mixture of firefly and *Renilla* luciferase reporter plasmids. As had been observed in S2 cells, shRNAs were effective inducers of gene silencing. Once again, hairpins designed to mimic let-7 were consistently less effective than were simple hairpin RNAs, and the introduction of mismatches between the antisense strand of the shRNA and the mRNA target abolished silencing (FIG. **40**A; data not shown). Overall, shRNAs were somewhat less potent silencing triggers than were siRNAs. Whereas siRNAs homologous to firefly luciferase routinely yielded ~90%-95% suppression of gene expression, suppres-

sion levels achieved with shRNAs ranged from 80%-90% on average. As we also observe with siRNAs, the most important determinant of the potency of the silencing trigger is its sequence. We find that roughly 50% of both siRNAs and shRNAs are competent for suppressing gene expression. However, neither analysis of the predicted structures of the target mRNA nor analysis of alternative structures in siRNA duplexes or shRNA hairpins has proved of predictive value for choosing effective inhibitors of gene expression.

We have adopted as a standard, shRNA duplexes containing 29 bp. However, the size of the helix can be reduced to ~25 nt without significant loss of potency. Duplexes as short as 22 bp can still provoke detectable silencing, but do so less efficiently than do longer duplexes. In no case did we observe a reduction in the internal control reporter (*Renilla* luciferase) that would be consistent with an induction of nonspecific dsRNA responses.

The ability of shRNAs to induce gene silencing was not confined to 293T cells. Similar results were also obtained in a variety of other mammalian cell lines, including human cancer cells (HeLa), transformed monkey epithelial cells (COS-1), murine fibroblasts (NIH 3T3), and diploid human fibroblasts (IMR90; FIG. **40**; data not shown).

C. Synthesis of Effective Inhibitors of Gene Expression Using T7 RNA Polymerse.

The use of siRNAs to provoke gene silencing is developing into a standard methodology for investigating gene function in mammalian cells. To date, siRNAs have been produced exclusively by chemical synthesis (e.g., Caplen et al., *Proc. Natl. Acad. Sci.* 98: 9742-9747, 2001; Elbashir et al., *Nature* 411: 494-498, 2001). However, the costs associated with this approach are significant, limiting its potential utility as a tool for investigating in parallel the functions of large numbers of genes. Short hairpin RNAs are presumably processed into active siRNAs in vitro by Dicer. Thus, these may be more tolerant of terminal structures, both with respect to nucleotide overhangs and with respect to phosphate termini. We therefore tested whether shRNAs could be prepared by in vitro transcription with T7 RNA polymerase.

Transcription templates that were predicted to generate siRNAs and shRNAs similar to those prepared by chemical RNA synthesis were prepared by DNA synthesis (FIG. **41**A, C). These were tested for efficacy both in S2 cells (data not shown) and in human 293 cells (FIG. **41**B,D). Overall, the performance of the T7-synthesized hairpin or siRNAs closely matched the performance of either produced by chemical synthesis, both with respect to the magnitude of inhibition and with respect to the relative efficiency of differing sequences. Because T7 polymerase prefers to initiate at twin guanosine residues, however, it was critical to consider initiation context when designing in vitro transcribed siRNAs (FIG. **41**B). In contrast, shRNAs, which are processed by Dicer (see FIG. **39**C), tolerate the addition of these bases at the 5' end of the transcript.

Studies in *Drosophila* embryo extracts indicate that siRNAs possess 5' phosphorylated termini, consistent with their production by an RNase III family nuclease. In vitro, this terminus is critical to the induction of RNAi by synthetic RNA oligonucleotides (Elbashir et al., EMBO J. 20: 6877-6888, 2001; Nykanen et al., *Cell* 107: 309-321, 2001). Chemically synthesized siRNAs are nonphosphorylated, and enzymatic addition of a 5' phosphate group in vitro prior to transfection does not increase the potency of the silencing effect (A. A. Caudy, unpublished data). This suggests either that the requirement for phosphorylated termini is less stringent in mammalian cells or that a kinase efficiently phosphorylates siRNAs in vitro. RNAs synthesized with T7 RNA polymerase, however, possess 5' triphosphate termini. We therefore explored the possibility of synthesizing siRNAs with T7 polymerase followed by treatment in vitro with pyrophosphatase to modify the termini to resemble those of siRNAs. Surprisingly, monophosphorylated siRNAs (data not shown) were as potent in inducing gene silencing as transcription products bearing triphosphate termini (FIG. **41**B). This may suggest either that the requirement for monophosphorylated termini is less stringent in mammalian cells or that siRNAs are modified in vitro to achieve an appropriate terminal structure.

Considered together, our data suggest that both shRNAs and siRNA duplexes can be prepared by synthesis with T7 RNA polymerase in vitro. This significantly reduces the cost of RNAi in mammalian cells and paves the way for application of RNAi on a whole-genome scale.

D. Transcription of Small Hairpin RNAs In vitro by RNA Polymerase III.

Although siRNAs are an undeniably effective tool for probing gene function in mammalian cells, their suppressive effects are by definition of limited duration. Delivery of siRNAs can be accomplished by any of a number of transient transfection methodologies, and both the timing of peak suppression and the recovery of protein levels as silencing decays can vary with both the cell type and the target gene. Therefore, one limitation on siRNAs is the development of continuous cell lines in which the expression of a desired target is stably silenced.

Hairpin RNAs, consisting of long duplex structures, have been proved as effective triggers of stable gene silencing in plants, in *C. elegans*, and in *Drosophila* (Kennerdell et al., *Nat. Biotechnol.* 18: 896-898, 2000; Smith et al., *Nature* 407: 319-320, 2000; Tavernarakis et al., *Nat. Genet.* 24: 180-183, 2000). We have recently shown stable suppression of gene expression in cultured mammalian cells by continuous expression of a long hairpin RNA (Paddison et al., *Proc. Natl. Acad. Sci.* 99: 1443-1448, 2002). However, the scope of this approach was limited by the necessity of expressing such hairpins only in cells that lack a detectable PKR response. In principle, shRNAs could bypass such limitations and provide a tool for evoking stable suppression by RNA in mammalian somatic cells.

To test this possibility, we initially cloned sequences encoding a firefly luciferase shRNA into a CMV-based expression plasmid. This was predicted to generate a capped, polyadenylated RNA polymerase II transcript in which the hairpin was extended on both the 5' and 3' ends by vector sequences and poly(A). This construct was completely inert in silencing assays in 293T cells.

During our studies on chemically and T7-synthesized shRNAs, we noted that the presence of significant single-stranded extensions (either 5' or 3' of the duplex) reduced the efficacy of shRNAs. We therefore explored the use of alternative promoter strategies in an effort to produce more defined hairpin RNAs. In particular, RNA polymerase III promoters have well-defined initiation and termination sites and naturally produce a variety of small, stable RNA species. Although many Pol III promoters contain essential elements within the transcribed region, limiting their utility for our purposes; class III promoters use exclusively nontranscribed promoter sequences. Of these, the U6 snRNA promoter and the H1 RNA promoter have been well studied (Lobo et al., *Nucleic Acids Res.* 18: 2891-2899, 1990; Hannon et al., *J. Biol. Chem.* 266: 22796-22799, 1991; Chong et al., *J. Biol. Chem.* 276: 20727-20734, 2001).

By placing a convenient cloning site immediately behind the U6 snRNA promoter, we have constructed pShh-1, an

expression vector in which short hairpins are harnessed for gene silencing. Into this vector either of two shRNA sequences derived from firefly luciferase were cloned from synthetic oligonucleotides. These were cotransfected with firefly and *Renilla* luciferase expression plasmids into 293T cells. One of the two encoded shRNAs provoked effective silencing of firefly luciferase without altering the expression of the internal control (FIG. **42**C). The second encoded shRNA also produced detectable, albeit weak, repression. In both cases, silencing was dependent on insertion of the shRNA in the correct orientation with respect to the promoter (FIG. **42**C; data not shown). Although the shRNA itself is bilaterally symmetric, insertion in the incorrect orientation would affect Pol III termination and is predicted to produce a hairpin with both 5' and 3' single-stranded extensions. Similar results were also obtained in a number of other mammalian cell lines including HeLa, COS-1, NIH 3T3, and IMR90 (FIG. **42**; data not shown). pShh1-Ff1 was, however, incapable of effecting suppression of the luciferase reporter in *Drosophila* cells, in which the human U6 promoter is inactive.

E. Dicer is Required for shRNA-Mediated Gene Silencing.

As a definitive test of whether the plasmid-encoded shRNAs brought about gene silencing via the mammalian RNAi pathway, we assessed the dependence of suppression on an essential component of the RNAi pathway. We transfected pShh1-Ff1 along with an siRNA homologous to human Dicer. FIG. **43** shows that treatment of cells with Dicer siRNAs is able to completely depress the silencing induced by pShh1-Ff1. Addition of an unrelated siRNA had no effect on the magnitude of suppression by pShh1-Ff1. Importantly, Dicer siRNAs had no effect on siRNA-induced silencing of firefly luciferase. These results are consistent with shRNAs operating via an RNAi pathway similar to those provoked by stRNAs and long dsRNAs. Furthermore, it suggests that siRNA-mediated silencing is less sensitive to depletion of the Dicer enzyme.

F. Stable shRNA-Mediated Gene Silencing of an Endogenous Gene.

The ultimate utility of encoded short hairpins will be in the creation of stable mutants that permit the study of the resulting phenotypes. We therefore tested whether we could create a cellular phenotype through stable suppression. Expression of activated alleles of the ras oncogene in primary mouse embryo fibroblasts (MEFs) induces a stable growth arrest that resembles, as a terminal phenotype, replicative senescence (Serrano et al., *Cell* 88: 593-602, 1997). Cells cease dividing and assume a typical large, flattened morphology. Senescence can be countered by mutations that inactivate the p53 tumor suppressor pathway (Serrano et al. 1997, supra). As a test of the ability of vector-encoded shRNAs to stably suppress an endogenous cellular gene, we generated a hairpin that was targeted to the mouse p53 gene. As shown in FIG. **44**, MEFs transfected with pBabe-RasV12 fail to proliferate and show a senescent morphology when cotransfected with an empty control vector. As noted previously by Serrano et al., the terminally arrested state is achieved in 100% of drug-selected cells in culture by 8 d post-transfection. However, upon cotransfection of an activated ras expression construct with the pShh-p53, cells emerged from drug selection that not only fail to adopt a senescent morphology but also maintain the ability to proliferate for a minimum of several weeks in culture (FIG. **44**). These data strongly suggest that shRNA expression constructs can be used for the creation of continuous mammalian cell lines in which selected target genes are stably suppressed.

G. Simultaneous Introduction of Multiple Hairpin RNAs Does Not Produce Synergy.

In an attempt to further understand the mechanisms by which short hairpins suppress gene expression, we examined the effects of transfecting cells with a mixture of two different short hairpins corresponding to firefly luciferase. FIG. **45** summarizes the results of experiments which suggest that there is no synergistic affects on suppression of firefly luciferase gene expression obtained when cells are exposed to a mixture of such short hairpins.

Methods:

Cell culture. HEK 293T, HeLa, COS-1, MEF, and IMR90 cells were cultured in DMEM (GIBCO BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (GIBCO BRL). NIH 3T3 cells were cultured in DMEM supplemented with 10% heat-inactivated calf serum and 1% antibiotic/antimycotic solution.

RNA preparation. Both shRNAs and siRNAs were produced in vitro using chemically synthesized DNA oligonucleotide templates (Sigma) and the T7 Megashortscript kit (Ambion). Transcription templates were designed such that they contained T7 promoter sequences at the 5' end. shRNA transcripts subjected to in vitro Dicer processing were synthesized using a Riboprobe kit (Promega). Chemically synthesized RNAs were obtained from Dharmacon, Inc.

Transfection and gene silencing assays. Cells were transfected with indicated amounts of siRNA, shRNA, and plasmid DNA using standard calcium phosphate procedures at 50%-70% confluence in 6-well plates. Dual luciferase assays (Promega) were carried out by cotransfecting cells with plasmids containing firefly luciferase under the control of the is SV40 promoter (pGL3-Control, Promega) and *Renilla* luciferase under the control of the SV40 early enhancer/promoter region (pSV40, Promega). Plasmids were cotransfected using a 1:1 ratio of pGL3-Control (250 ng/well) to pRL-SV40. RNAi in S2 cells was performed as previously described (Hammond et al., *Nature* 404: 293-296, 2000). For stable silencing, primary MEFs (a gift from S. Lowe, Cold Spring Harbor Laboratory, NY) were cotransfected using Fugene 6 with pBabe-Ha-rasV12 and pShh-p53 (no resistance marker), according to the manufacturer's recommendations. Selection was for the presence of the activated Ha-rasV12 plasmid, which carries a puromycin-resistance marker. The pShh-p53 plasmid was present in excess, as is standard in a cotransfection experiment. We have now generated a version of the U6 promoter vector (pSHAG-1) that is compatible with the GATEWAY system (Invitrogen), and this can be used to transport the shRNA expression cassette into a variety of recipient vectors that carry cis-linked selectable markers. Furthermore, we have validated delivery of shRNAs using retroviral vectors. Updated plasmid information can be obtained at:

http://www.cshl.org/public/science/hannon.html.

Plasmids expressing hairpin RNAs. The U6 promoter region from −265 to +1 was amplified by PCR, adding 5' KpnI and 3' EcoRV sites for cloning into pBSSK$^+$. A linker/terminator oligonucleotide set bearing the U6 terminator sequence and linker ends of 5' EcoRV and 3' NotI was cloned into the promoter construct, resulting in a U6 cassette with an EcoRV site for insertion of new sequences. This vector has been named pShh1. Blunt-ended, double-stranded DNA oligonucleotides encoding shRNAs with between 19 and 29 bases of homology to the targeted gene were ligated into the EcoRV site to produce expression constructs. The oligonucleotide sequence used to construct Ff1 was: TCCAATTCAGCGG-GAGCCACCTGATGAAGCTTGATCGGGTG-

GCTCTCGCTGAGTTGGAATCCATTTTTTTT (SEQ ID NO: 38). This sequence is preceded by the sequence GGAT, which is supplied by the vector, and contains a tract of more than five Ts as a Pol III terminator.

In vitro Dicer assays. In vitro assays for Dicer activity were performed as described (Bernstein et al., *Nature* 409: 363-366, 2001).

EXAMPLE 7

Encoded Short Hairpins Function In vitro

An object of the present invention is to improve methods for generating siRNAs and short hairpins for use in specifically suppressing gene expression. Example 6 demonstrates that siRNAs and short hairpins are highly effective in specifically suppressing gene expression. Accordingly, it would be advantageous to combine the efficient suppression of gene expression attainable using short hairpins and siRNAs with a method to encode such RNA on a plasmid and express it either transiently or stably.

FIG. **46** demonstrates that short hairpins encoded on a plasmid are effective in suppressing gene expression. DNA oligonucleotides encoding 29 nucleotide hairpins corresponding to firefly luciferase were inserted into a vector containing the U6 promoter. Three independent constructs were examined for their ability to specifically suppress firefly luciferase gene expression in 293T cells. siOligo1-2, siOligo1-6, and siOligo1-19 (construct in the correct orientation) each suppressed gene expression as effectively as siRNA. In contrast, siOligo1-10 (construct in the incorrect orientation) did not suppress gene expression. Additionally, an independent construct targeted to a different portion of the firefly luciferase gene did not effectively suppress gene expression in either orientation (SiOligo2-23, SiOligo2-36).

The results summarized in FIG. **46** demonstrate that transient expression of siRNAs and short hairpins encoded on a plasmid can efficiently suppress gene expression. One of skill can choose from amongst a range of vectors to either transiently or stably express an siRNA or short hairpin. Non-limiting examples of vectors and strategies to stably express short dsRNAs are presented in FIGS. **47-49**.

EXAMPLE 8

dsRNA Suppression in the Absence of a PKR Response

One potential impediment to the use of RNAi to suppress gene expression in some cell types, is the non-specific PKR response that can be triggered by long dsRNAs. Numerous mammalian viruses have evolved the ability to block PKR in order to aid in the infection of potential host cells. For example, adenoviruses express RNAs which mimic dsRNA but do not activate the PKR response. Vaccinia virus uses two strategies to evade PKR: the expression of E3L which binds and masks dsRNA; the expression of K3L to mimic the natural PKR substrate eIF2α.

Our understanding of the mechanisms by which viruses avoid the PKR response allows us to design approaches to circumvent the PKR response in cell types in which in might be advantageous to suppression gene expression with long dsRNAs. Possible approaches include treating cells with an agent that inhibits protein kinase RNA-activated (PKR) apoptosis, such as by treatment with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKR. Accordingly, RNAi suppression of gene expression in such cell types could involve first inhibiting the PKR response, and then delivering a dsRNA identical or similar to a target gene.

A. In a murine myoblast cell line, C2C12, we noted that the cells responded to long dsRNAs with a mixture of specific and non-specific (presumably PKR) responses. In order to attenuate the non-specific PKR response while maintaining the robust and specific suppression due to the long dsRNA, C2C12 cells were transfected with a vector that directs K3L expression. This additional step successfully attenuated the PKR response, however expression of K3L protein had no effect on the magnitude of specific inhibition.

B. However, since the efficacy of such a two step approach had not been previously demonstrated, it was formerly possible that dsRNA suppression would not be possible in cells with a PKR response. FIG. **50** summarizes results which demonstrate that such a two step approach is possible, and that robust and specific dsRNA mediated suppression is possible in cells which had formerly possessed a robust PKR response.

Briefly, dual luciferase assay were carried out as described in detail above. The experiments were carried out using $PKR^{-/-}$ MEFs harvested from E13.5 $PKR^{-/-}$ mouse embryos. MEFs typically have a robust PKR response, and thus treatment with long dsRNAs typically results in non-specific suppression of gene expression and apoptosis. However, in $PKR^{-/-}$ cells examined 12, 42, and 82 hours after transfection, expression of ds*Renilla* luciferase RNA specifically suppresses expression *Renilla reniformis* (sea pansy) luciferase. This suppression is stable over time.

These results demonstrate that the non-specific PKR response can be blocked without affecting specific suppression of gene expression mediated by dsRNA. This allows the use of long dsRNAs to suppress gene expression in a diverse range of cell types, including those that would be previously intractable due to the confounding influences of the non-specific PKR response to long dsRNA.

EXAMPLE 9

Suppression of Gene Expression using dsRNA which Corresponds to Non-Coding Sequence

Current models for the mechanisms which drive RNAi have suggested that the dsRNA construct must contain coding sequence corresponding to the gene of interest. Although evidence has demonstrated that such coding sequence need not be a perfect match to the endogenous coding sequence (i.e., it may be similar), it has been widely held that the dsRNA construct must correspond to coding sequence. We present evidence that contradicts the teachings of the prior art, and demonstrate that dsRNA corresponding to non-coding regions of a gene can suppress gene function in vitro. These results are significant not only because they demonstrate that dsRNA identical or similar to non-coding sequences (i.e., promoter sequences, enhancer sequences, or intronic sequences) can mediate suppression, but also because we demonstrate the in vitro suppression of gene expression using dsRNA technology in a mouse model.

We generated doubled stranded RNA corresponding to four segments of the mouse tyrosinase gene promoter. Three of these segments correspond to the proximal promoter and one corresponds to an enhancer (FIG. **51**). The tyrosinase gene encodes the rate limiting enzyme involved in the melanin biosynthetic pathway (Bilodeau et al., *Pigment Cell Research* 14: 328-336, 2001). Accordingly, suppression of the tyrosinase gene is expected to inhibit pigmentation.

Double stranded RNA corresponding to each of the above promoter segments was injected into the pronuclei of fertilized eggs. Pups were born after 19 days. In total 42/136 (31%) of the embryos were carried to term. This number is within the expected range for transgenesis (30-40%). Two pups out of 42 (5%) appear totally unpigmented at birth, consistent with suppression of tyrosinase function.

Methods:

dsRNA from non-coding promoter region of tyrosinase gene. Four segments of the mouse tyrosinase gene promoter were amplified by PCR using primers which incorporated T7 RNA polymerase promoters into the PCR products (shown in bold—FIG. **51**). Sequences of the mouse tyrosinase gene 5' flanking regions were obtained from GenBank (accession number D00439 and X51743). The sequence of the tyrosinase enhancer, located approximately 12 kb upstream of the transcriptional start site, was also obtained from GenBank (accession number X76647).

The sequences of the primers used were as follows: note the sequence of the T7 RNA polymerase promoter is shown in bold:

(a) Tyrosinase enhancer (~12 kb upstream):

(a) Tyrosinase enhancer (~12 kb upstream):

(SEQ ID NO: 39)
5' **TAATACGACTCACTATAGGG**CAAGGTCATAGTTCCTGCCAGCTG 3'

(SEQ ID NO: 40)
5' **TAATACGACTCACTATAGGG**CAGATATTTTCTTACCACCCACCC 3'

(b) -1404 to -1007:

(SEQ ID NO: 41)
5' **TAATACGACTCACTATAGGG**TTAAGTTTAACAGGAGAAGCTGGA 3'

(SEQ ID NO: 42)
5' **TAATACGACTCACTATAGGG**AAATCATTGCTTTCCTGATAATGC 3'

(c) -1003 to -506:

(SEQ ID NO: 43)
5' **TAATACGACTCACTATAGGG**TAGATTTCCGCAGCCCCAGTGTTC 3'

(SEQ ID NO: 44)
5' **TAATACGACTCACTATAGGG**GTTGCCTCTCATTTTTCCTTGATT 3'

(d) -505 to -85:

(SEQ ID NO: 45)
5' **TAATACGACTCACTATAGGG**TATTTTAGACTGATTACTTTTATA

A 3'

(SEQ ID NO: 46)
5' **TAATACGACTCACTATAGGG**TCACATGTTTTGGCTAAGACCTAT 3'

PCR products were gel purified from 1% TAE agarose gels using QiaExII Gel Extraction Kit (Qiagen). Double stranded RNA was produced from these templates using T7-Megashortscript Kit (Ambion). Enzymes and unincorporated nucleotides were removed using Qiaquick MinElute PCR Purification Kit. RNA was phenol/chloroform extracted twice, and ethanol precipitated. Pellets were resuspended in injection buffer ((10 mM Tris (pH 7.5), 0.15 nM EDTA (pH 8.0)) at a concentration of 20 ng/ul and run on a 1% TAE agarose gel to confirm integrity.

Generation of mice: An equal mixture of double stranded RNA from each of the above primer sets was injected into the pronuclei of fertilized eggs from C57BL6J mice. A total of 136 injections was performed, and 34 embryos were implanted into each of 4 pseudopregnant CD-1 females. Pups were born after 19 days. In total, 42/136 (31%) of the embryos were carried to term. 2/42 pups (5%) appear totally unpigmented at birth.

It is not clear whether the RNAi mediated by dsRNA identical or similar to non-coding sequence works via the same mechanism as PTGS observed in the presence of dsRNA identical or similar to coding sequence. However, whether these results ultimately reveal similar or differing mechanisms does not diminish the tremendous utility of the compositions and methods of the present invention to suppress expression of one or more genes in vitro or in vitro.

The present invention demonstrates that dsRNA ranging in length from 20-500 nt can readily suppress expression of target genes both in vitro and in vitro. Furthermore, the present invention demonstrates that the dsRNAs can be generated using a variety of methods including the formation of hairpins, and that these dsRNAs can be expressed either stably or transiently. Finally, the present invention demonstrates that dsRNA identical or similar to non-coding sequences can suppress target gene expression.

EXAMPLE 10

RNA interference in Adult Mice

RNA interference is an evolutionarily conserved surveillance mechanism that responds to double-stranded RNA by sequence-specific silencing of homologous genes. Here we show that transgene expression can be suppressed in adult mice by synthetic small interfering RNAs and by small-hairpin RNAs transcribed in vitro from DNA templates. We also show the therapeutic potential of this technique by demonstrating effective targeting of a sequence from hepatitis C virus by RNA interference in vitro.

Small interfering RNAs (siRNAs) mimic intermediates in the RNA-interference (RNAi) pathway and can silence genes in somatic cells without activating non-specific suppression by double-stranded RNA-dependent protein kinase (Elbashir et al., Nature 411: 494-498, 2001). To investigate whether siRNAs also inhibit gene expression in vitro, we used a modification of hydrodynamic transfection methods (Zhang et al., *Hum. Gene Therapy* 10: 1735-1737, 1999; Liu et al., *Gene Therapy* 6: 1258-1266, 1999; Chang et al., *J. Virol.* 75: 3469-3473, 2001) to deliver naked siRNAs to the livers of adult mice. Either an siRNA derived from firefly luciferase or an unrelated siRNA was co-injected with a luciferase-expression plasmid (for construct description and sequences, see FIG. **52**). We monitored luciferase expression in living animals using quantitative whole-body imaging (Contag, et al., *Photochem. Photobiol.* 66: 523-531, 1997) (see FIG. **53***a*, **54***a*), and found that it was dependent on reporter-plasmid dose.

In each experiment, serum measurements of a co-injected human α-1 antitrypsin (hAAT) plasmid (Yant et al., *Nature Genet.* 25: 3541, 2000) served to normalize transfection efficiency and to monitor non-specific translational inhibition. Average serum concentrations of hAAT after 74 h were similar in all groups.

Our results indicate that there was specific, siRNA-mediated inhibition of luciferase expression in adult mice (P<0.0115) and that unrelated siRNAs had no effect (P<0.864; FIG. **53***a*, **53***b*). In 11 independent experiments, luciferase siRNAs reduced luciferase expression (as judged by emitted light) by an average of 81% (±2.2%). These findings indicate that RNAi can downregulate gene expression in adult mice.

As RNAi degrades respiratory syncitial virus RNAs in culture (Bitko et al. 2001, BMC Microbiol. 1: 34), we investigated whether RNAi could be directed against a human pathogenic RNA expressed in a mouse, namely that of hepa-

titis C virus (HCV). Infection by HCV (an RNA virus that infects 1 in 40 people worldwide) is the most common reason for liver transplantation in the United States and Europe. We fused the NS5B region (non-structural protein 5B, viral-polymerase-encoding region) of this virus with luciferase RNA and monitored RNAi by co-transfection in vitro. An siRNA targeting the NS5B region reduced luciferase expression from the chimaeric HCV NS5B protein-luciferase fusion by 75% (±6.8%; 6 animals per group). This result suggests that it may be feasible to use RNAi as a therapy against other important human pathogens.

Although our results show that siRNAs are functional in mice, delivery remains a major obstacle. Unlike siRNAs, functional small-hairpin RNAs (shRNAs) can be expressed in vitro from DNA templates using RNA polymerase III promoters (Paddison et al., *Genes Dev.* 16: 948-958, 2002; Tuschl, *Nature Biotechnol.* 20: 446-448, 2002); they are as effective as siRNAs in inducing gene suppression. Expression of a cognate shRNA (pShh1-Ff1) inhibited luciferase expression by up to 98% (+0.6%), with an average suppression of 92.8% (+3.39%) in three independent experiments (see FIG. **54***a*, **54***b*). An empty shRNA-expression vector had no effect; reversing the orientation of the shRNA (pShh1-Ff1 rev) insert prevents gene silencing because it alters the termination by RNA polymerase III and generates an improperly structured shRNA. These findings indicate that plasmid-encoded shRNAs can induce a potent and specific RNAi response in adult mice.

RNAi may find application in functional genomics or in identifying targets for designer drugs. It is a more promising system than gene-knockout mice because groups of genes can be simultaneously rendered ineffective without the need for time-consuming crosses. Gene therapy currently depends on the ectopic expression of exogenous proteins; however, RNAi may eventually complement this gain-of-function approach by silencing disease-related genes with DNA constructs that direct the expression of shRNAs. Our method of RNAi delivery could also be tailored to take advantage of developing viral and non-viral gene-transfer vectors in a clinical context.

EXAMPLE 11

Germ-line transmission of RNAi in mice

MicroRNA molecules (miRNAs) are small, noncoding RNA molecules that have been found in a diverse array of eukaryotes, including mammals. miRNA precursors share a characteristic secondary structure, forming short 'hairpin' RNAs. Genetic and biochemical studies have indicated that miRNAs are processed to their mature forms by Dicer, an RNAse III family nuclease, and function through RNA-mediated interference (RNAi) and related pathways to regulate the expression of target genes (Hannon, *Nature* 418: 244-251, 2002; Pasquinelli et al., *Ann. Rev. Cell. Dev. Biol.* 18: 495-513, 2002). Recently, we and others have remodeled miRNAs to permit experimental manipulation of gene expression in mammalian cells and have dubbed these synthetic silencing triggers 'short hairpin RNAs' (shRNAs) (Paddison et al., *Cancer Cell* 2: 17-23, 2002). Silencing by shRNAs requires the RNAi machinery and correlates with the production of small interfering RNAs (siRNAs), which are a signature of RNAi.

Expression of shRNAs can elicit either transient or stable silencing, depending upon whether the expression cassette is integrated into the genome of the recipient cultured cell (Paddison et al., *Cancer Cell* 2: 17-23, 2002). shRNA expression vectors also induce gene silencing in adult mice following transient delivery (Lewis et al., *Nat. Genet.* 32: 107-108, 2002; McCaffrey et al., *Nature* 418: 38-39, 2002). However, for shRNAs to be a viable genetic tool in mice, stable manipulation of gene expression is essential. Hemann and colleagues have demonstrated long-term suppression of gene expression in vitro following retroviral delivery of shRNA-expression cassettes to hematopoietic stem cells (Hemann et al., *Nat. Genet.* in the press, 2003). Here we sought to test whether shRNA-expression cassettes that were passed through the mouse germ-line could enforce heritable gene silencing.

We began by taking standard transgenesis approaches (Gordon et al., *Methods Enzymol.* 225: 747-771, 1993) using shRNAs directed against a variety of targets with expected phenotypes, including the genes encoding tyrosinase (albino), myosin VIIIa (shaker), Bmp-5 (crinkled ears), Hox a-10 (limb defects), homogentisate 1,2,-dioxygenase (urine turns black upon exposure to air), Hairless (hair loss) and melanocortin 1 receptor (yellow). Three constructs per gene were linearized and injected into pronuclei to produce transgenic founder animals. Although we noted the presence of the transgene in some animals, virtually none showed a distinct or reproducible phenotype that was expected for a hypomorphic allele of the targeted gene.

Therefore, we decided to take another approach: verifying the presence of the shRNA and its activity toward a target gene in cultured embryonic stem (ES) cells and then asking whether those cells retained suppression in a chimeric animal in vitro. We also planned to test whether such cells could pass a functional RNAi-inducing construct through the mouse germ-line. For these studies, we chose to examine a novel gene, Neil1, which is proposed to have a role in DNA repair. Oxidative damage accounts for 10,000 DNA lesions per cell per day in humans and is thought to contribute to carcinogenesis, aging and tissue damage following ischemia (Ames et al., *Proc. Natl. Acad. Sci. USA* 90: 7915-7922, 1993; Jackson et al., *Mutat. Res.* 477: 7-21, 2001). Oxidative DNA damage includes abasic sites, strand breaks and at least 20 oxidized bases, many of which are cytotoxic or pro-mutagenic (Dizdaroglu et al., *Free Radic. Biol. Med.* 32: 1102-1115, 2002). DNA N-glycosylases initiate the base excision repair pathway by recognizing specific bases in DNA and cleaving the sugar base bond to release the damaged base (David et al., *Chem. Rev.* 98: 1221-1262, 1998).

The Neil genes are a newly discovered family of mammalian DNA N-glycosylases related to the Fpg/Nei family of proteins from *Escherichia coli* (Hazra et al., *Proc. Natl. Acad. Sci. USA* 99: 3523-3528, 2002; Bandaru et al., *DNA Repair* 1: 517-529, 2002). Neil1 recognizes and removes a wide spectrum of oxidized pyrimidines and ring-opened purines from DNA, including thymine glycol (Tg), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 4,6-diamino-5-formidopyrimidine (FapyA). Tg, FapyG and FapyA are among the most prevalent oxidized bases produced by ionizing radiation (Dizdaroglu et al. *Free Radic. Biol. Med.* 32: 1102-1115, 2002) and can block replicative DNA polymerases, which can, in turn, cause cell death (Asagoshi et al. *J. Biol. Chem.* 277: 14589-14597, 2002; Clark et al., Biochemistry 28: 775-779, 1989).

The Nth1 and Ogg1 glycosylases each remove subsets of oxidized DNA bases that overlap with substrates of Neil1 (Nishimura, *Free Radic. Biol. Med.* 32: 813-821, 2002; Asagoshi et al., *Biochemistry* 39: 11389-11398, 2000; Dizdaroglu et al., *Biochemistry* 38: 243-246, 1999). However, mice with null mutations in either Nthl (Ocampo et al., *Mol. Cell. Biol.* 22: 6111-6121, 2002; Takao et al., *EMBO J.* 21: 3486-3493, 2002) or Oggl (Klungland et al., *Proc. Natl. Acad. Sci. USA* 96: 13300-13305, 1999; Minowa et al., *Proc. Natl. Acad.*

Sci. USA 97: 4156-4161, 2000) are viable, raising the possibility that Neil1 activity tempers the loss of Nth1 or Ogg1. Recently, a residual Tg-DNA glycosylase activity in $Nthl^{-/-}$ mice has been identified as Neil1 (Takao et al., *J. Biol. Chem.* 277: 4220542213, 2002).

We constructed a single shRNA expression vector targeting a sequence near the 5' end of the Neil1 coding region. This vector was introduced into mouse embryonic stem cells by electroporation, and individual stable integrants were tested for expression of the Neil1 protein (see the weblink: http://www.cshl.edu/public/SCIENCE/hannon.btml for detailed procedures). The majority of cell lines showed an –80% reduction in Neil1 protein, which correlated with a similar change in levels of Neil1 mRNA. These cells showed an approximately two-fold increase in their sensitivity to ionizing radiation, consistent with a role for Neil1 in DNA repair. Two independent ES cell lines were injected into BL/6 blastocysts, and several high-percentage chimeras were obtained. These chimeras were out-crossed, and germ-line transmission of the shRNA-expression construct was noted in numerous F_1 progeny (13/27 for one line and 12/26 for the other).

To determine whether the silencing of Neil1 that had been observed in ES cells was transmitted faithfully, we examined Neil1 mRNA and protein levels. Both were reduced by approximately the same extent that had been observed in the engineered ES cells (FIGS. **55**, **56**). Consistent with this having occurred through the RNAi pathway, we detected the presence of siRNAs corresponding to the shRNA sequence in F_1 animals that carry the shRNA expression vector but not in those that lack the vector (FIG. **56***b*).

The aforementioned data demonstrate that shRNAs can be used to create germ-line transgenic mice in which RNAi has silenced a target gene. These observations open the door to using of RNAi as a complement to standard knock-out methodologies and provide a means to rapidly assess the consequences of suppressing a gene of interest in a living animal. Coupled with activator-dependent U6 promoters, the use of shRNAs will ultimately provide methods for tissue-specific, inducible and reversible suppression of gene expression in mice.

EXAMPLE 12

Dicer Cleaves a Single siRNA From the End of Each shRNA

We performed the following experiments in order to understand how Dicer processes shRNAs, and in order to permit comparison of the efficiency of different silencing triggers.

We began by producing ~70 chemically synthesized shRNAs, targeting various endogenous genes and reporters. We initially focused on a detailed analysis of one set of four shRNAs that target firefly luciferase (FIG. **57***a*). The individual species differed in two distinct ways. First, the stems of the shRNAs were either 19 or 29 nucleotides in length. Second, each shRNA either contained or lacked a 2 nucleotide 3' overhang, identical to that produced by processing of pri-miRNAs by Drosha. Each species was end-labeled by enzymatic phosphorylation and incubated with recombinant human Dicer. The 29 nt. shRNA bearing the 3' overhang was converted almost quantitatively into a 22 nt product by Dicer (FIG. **57***b*). In contrast, the 29 nt shRNA that lacked the overhang generated very little 22 nt labeled product, although there was a substantial depletion of the starting material. Neither 19 nt shRNA was cleaved to a significant extent by the Dicer enzyme. This result was not due to the lack of dsRNA in the 19 nt shRNAs as all shRNA substrates were efficiently cleaved by bacterial RNAseIII (FIG. **57***c*). Parallel analysis of identical shRNA substrates that were produced by in vitro transcription with T7 polymerase and uniformly labeled clarified the results obtained with end-labeled substrates (not shown). Specifically, 19 nt shRNAs were not cleaved. However, both the overhung and the blunt 29 nucleotide shRNAs gave rise to 22 nt products, albeit at reduced levels in the latter case. These results suggest that Dicer requires a minimum stem length for productive cleavage. Furthermore, they are consistent with a hypothesis that the presence of a correct 3' overhang enhances the efficiency and specificity of cleavage, directing Dicer to cut ~22 nucleotides from the end of the substrate.

A number of previous studies have suggested that Dicer might function as an end-recognizing endonuclease, without positing a role for the 3' overhang. Processive Dicer cleavage was first implied by in vitro analysis of RISC cleavage (Zamore et al., *Cell* 101: 25-33, 2000). In *Drosophila* embryo extracts programmed for RISC assembly using a long dsRNA, phased cleavage sites occurred at approximately 22 nucleotide intervals along an mRNA substrate. Similarly, analysis of *C. elegans* Dicer in whole cell extracts (Ketting et al., *Genes Dev* 15: 2654-9, 2001) or purified human Dicer in vitro (Zhang et al., EMBO J. 21: 5875-85, 2002) showed accumulation of discretely sized cleavage intermediates. Blocking of the ends of dsRNAs using either fold-back structures or chimeric RNA-DNA hybrids attenuated, but did not abolish, the ability of human Dicer to generate siRNAs (Zhang et al., *EMBO J.* 21: 5875-85, 2002). Finally, Lund and colleagues suggested that Dicer cleaved ~22 nt from the blunt end of an extended pre-miRNA, designed in part to mimic a pri-miRNA (see Lund et al., *Science* 303: 95-8, 2004).

Our results suggest that while the overhang is not obligate for Dicer processing of its substrates (see Zhang et al., *EMBO J* 21: 5875-85, 2002, and FIG. **57***b*), this structure does aid in determining the specificity of cleavage. Furthermore, time courses of processing of blunt and overhung 29 nt shRNAs do show a more rapid processing of the overhung substrate if reactions are performed in the linear range for the enzyme (not shown).

To map more precisely the position of Dicer cleavage in the shRNA, we used primer extension analysis. The shRNAs described in FIG. **57***a* were reacted with recombinant human Dicer as shown in FIG. **57***b*. Total RNA was recovered from the processing reactions and used in primer extension assays. Consistent with direct analysis of the RNA, shRNAs with 19 nt stems failed to yield discrete extension products. The extension products that would be predicted from the unreacted substrate are not seen due to secondary structure of the uncleaved precursor (FIG. **58***a*). Both of the 29 nt shRNAs give rise to extension products with the overhung precursor giving a relatively discrete product of 20 nucleotides, as predicted for a cleavage precisely 22 nt from the 3' end of the substrate (FIG. **58***b*). The blunt-ended precursor gave a distribution of products, as was predicted from the analysis of uniformly and end-labeled RNAs.

In *Drosophila, Dicer*2 acts in a complex with a double-stranded RNA binding protein, R2D2 (Liu et al., *Science* 301: 1921-5, 2003). Similarly, biochemical evidence from *C. elegans* suggests that its Dicer binds RDE-1, RDE-4 and DRH-1 (Tabara et al., *Cell* 109: 861-71, 2002). These results suggest that the human enzyme might also function as part of a larger complex, which could show altered cleavage specificities. Therefore, we also mapped the cleavage of our shRNAs in vitro. Precursors were transfected into cells, and the processed form of each was isolated by virtue of its co-immunoprecipitation with human Argonaute proteins, Ago1

and Ago2. Primer extension suggested identical cleavage specificities upon exposure of shRNAs to Dicer in vitro and in living cells (FIG. **58***c*).

EXAMPLE 13

shRNAs are Generally More Effective Than siRNAs

Since each shRNA gave rise to a single, predictable 22 nt sequence in RISC, we compared the efficacy of shRNAs and siRNAs. Toward this goal, we selected 43 sequences targeting a total of 6 genes (3-9 sequences per gene). For each sequence, we synthesized a 21 nt siRNA (19 base stem) and 19 and 29 nt shRNAs that were predicted to give Dicer products that were either identical to the siRNAs or that differed by the addition of one 3' nucleotide (FIG. **59***a*). Each RNA species was transfected into HeLa cells at a relatively high concentration (100 nM). The level of suppression was determined by semi-quantitative RT-PCR and the performance of each shRNA compared to the performance of the corresponding siRNA (FIG. **59***b*). Comparison of 19 nt shRNAs with siRNAs revealed that there was little difference in endpoint inhibition with these species (left panel). A comparison of siRNAs with 29 nt shRNAs gave a different result. Clustering of the comparison data points above the diagonal indicated consistently better endpoint inhibition with the 29 nt shRNAs (right panel).

The generally better endpoint inhibition observed with 29 nt shRNAs led us to investigate in more detail the performance of these silencing triggers as compared to siRNAs. Seventeen complete sets comprising an siRNA, a 19 nt shRNA and a 29 nt shRNA were examined for suppression in titration experiments. In all cases, the 19 nt shRNAs performed as well as or worse than the corresponding siRNAs. In contrast, 29 nt shRNAs exceeded the performance of siRNAs in the majority of cases. Four representative examples, targeting MAPK-14 are shown in FIG. **59***c*. Several 29 nt shRNAs (e.g., see MAPK14-1) showed both significantly greater endpoint inhibition and efficacy at lower concentrations than the corresponding siRNA. In other cases (e.g., see MAPK14-2 and MAPK-14-4), the maximal level of suppression for the 29 nt. shRNA was approximately two-fold greater than the maximal level of suppression for the corresponding siRNA. Finally, in a minority of cases, exemplified by MAPK14-3, the performance of the three types of silencing triggers was similar. Importantly, in only one case out of 17 did we note that the 29 nt shRNA with a 2 nt. 3' overhang performed less effectively than the corresponding siRNA (data not shown).

EXAMPLE 14

siRNAs and shRNAs Give Similar Profiles of Off-Target Effects at Saturation

Sequence specificity is a critical parameter in RNAi experiments. Microarray analysis has revealed down-regulation of many non-targeted transcripts following transfection of siRNAs into HeLa cells (Jackson et al., *Nat Biotechnol* 21: 635-7, 2003). Notably, these gene expression signatures differed between different siRNAs targeting the same gene. Many of the "off target" transcripts contained sites of partial identity to the individual siRNA, possibly explaining the source of the effects. To examine potential off-target effects of synthetic shRNAs, we compared shRNA signatures with those of siRNAs derived from the same target sequence. Using microarray gene expression profiling, we obtained a genome-wide view of transcript suppression in response to siRNA and shRNA transfection. FIG. **60** (*a* and *b*) shows heat maps of signatures produced in HeLa cells 24 hours after transfection of 19 nt and 29 nt shRNAs compared with those generated by corresponding siRNAs. 19 nt shRNAs produced signatures that resembled, but were not identical to, those of corresponding siRNAs. In contrast, the signatures of the 29 nt shRNAs (FIG. **60***a*) were nearly identical to those of the siRNAs.

These results indicate that off target effects may be inherent to the use of synthetic RNAs for eliciting RNAi and cannot be ameliorated by intracellular processing of an upstream precursor in the RNAi pathway. Furthermore, the agreement between the signatures of 29 nt shRNAs and siRNAs is consistent with precise intracellular processing of the shRNA to generate a single siRNA rather than a random sampling of the hairpin stem by Dicer. The basis of the divergence between the signature of the 19 nt shRNA and the corresponding siRNA is presently unclear.

Considered together, our results indicate that chemically synthesized, 29 nt shRNAs are often substantially more effective triggers of RNAi than are siRNAs. While not wishing to be bound by any particular theory, a possible mechanistic explanation for this finding may lie in the fact that 29 nt shRNAs are substrates for Dicer processing both in vitro and in vitro. We originally suggested that siRNAs might be passed from Dicer to RISC in a solid state reaction on the basis of an interaction between Dicer and Argonaute2 in *Drosophila* S2 cell extracts (Hammond et al., *Science* 293: 1146-50, 2001). More recently, results from several laboratories have strongly suggested a model for assembly of the RNAi effector complex in which a multi-protein assembly containing Dicer and accessory proteins interacts with an Argonaute protein and actively loads one strand of the siRNA or miRNA into RISC (Lee et al., *Cell* 117: 69-81, 2004; Pham et al., *Cell* 117: 83-94, 2004; Tomari et al., *Cell* 116: 831-41, 2004). Our result is consistent with a model where Dicer substrates, derived from nuclear processing of pri-miRNAs or cytoplasmic delivery of pre-miRNA mimetics, are loaded into RISC more effectively than siRNAs. Our data support such a model, since it is not the hairpin structure of the synthetic RNA that determines its increased efficacy but the fact that the shRNA is a Dicer substrate that correlates with enhanced potency. Again, not wishing to be bound by any particular theory, it is possible that even siRNAs enter RISC via a Dicer-mediated assembly pathway. Our data may also reflect an increased affinity of Dicer for longer duplexes substrates. Alternatively, hairpin RNAs, such as miRNA precursors, might interact with specific cellular proteins that facilitate delivery of these substrates to Dicer, whereas siRNAs might not benefit from such chaperones.

Overall, our results provide an improved method for triggering RNAi in mammalian cells that uses higher potency RNAi triggers. Mapping the single 22 nt sequence that appears in RISC from each of these shRNAs now permits the combination of this more effective triggering method with rules for effective siRNA design.

Methods

RNA Sequence Design

Each set of RNAs began with the choice of a single 19-mer sequence. These 19mers were used directly to create siRNAs. To create shRNAs with 19-mer stems, we appended a 4-base loop (either CCAA or UUGG) to the end of the 19-mer sense strand target sequence followed by the 9-mer complementary sequence and a UU overhang. To create 29-mer stems, we increased the length of the 19-mer target sequence by adding 1 base upstream and 9 bases downstream from the target

region and used the same loop sequence and UU overhang. All synthetic RNA molecules used in this study were purchased from Dharmacon.

Dicer Processing

RNA hairpins corresponding to luciferase were end-labeled with [γ-^{32}P] ATP and T4 Polynucleotide kinase. 0.1 pmoles of RNA were then processed with 2 units of Dicer (Stratagene) at 37° C. for 2 hours. Reaction products were trizol extracted, isopropanol precipitated, run on an 18% polyacrylamide, 8M urea denaturing gel. For RNaseIII digestion, 0.1 pmoles were digested with 1 unit of *E. coli* RNase III (NEB) for 30 minutes at 37° C. and analyzed as described above. For primer extension analysis, hairpins were processed with Dicer at 37° C. for 2 hours, followed by heat inactivation of the enzyme. DNA primers were 5' labeled with PNK and annealed to 0.05 pmole of RNA as follows: 95° C. for one minute, 10 minutes at 50° C. and then 1 min on ice. Extensions were carried out at 42° C. for 1 hour using MoMLV reverse transcriptase. Products were analyzed by electrophoresis on a 8M Urea/20% polyacrylamide gel. For analysis of in vitro processing, LinxA cells were transfected in 10 cm plates using Mirus TKO (10 μg hairpin RNA) or Mirus LT4 reagent for DNA transfection (12 μg of tagged Ago1/Ago 2 DNA; J. Liu, unpublished). Cells were lysed and immunoprecipitated after 48 hours using with myc Antibody (9E14) Antibody. Immuno-precipitations were washed 3× in lysis buffer and treated with DNase for 15 minutes. Immunoprecipitates were then primer extended as described above.

siRNA and shRNA Transfections and mRNA Quantitation

HeLa cells were transfected in 96-well plates by use of Oligofectamine (Invitrogen) with the final nanomolar concentrations of each synthetic RNA indicated in the graphs. RNA quantitation was performed by Real-time PCR, using appropriate Applied Biosystems TaqMan™ primer probe sets. The primer probe set used for MAPK14 was Hs00176247_m__1. RNA values were normalized to RNA for HGUS (probe 4310888E).

Microarray Gene Expression Profiling

HeLa cells were transfected in 6-well plates by use of Oligofectamine. RNA from transfected cells was hybridized competitively with RNA from mock-transfected cells (treated with transfection reagent in the absence of synthetic RNA). Total RNA was purified by Qiagen RNeasy kit, and processed as described previously (Hughes et al., *Nat Biotechnol* 19: 342-7, 2001) for hybridization to microarrays containing oligonucleotides corresponding to approximately 21,000 human genes. Ratio hybridizations were performed with fluorescent label reversal to eliminate dye bias. Microarrays were purchased from Agilent Technologies. Error models have been described previously (Hughes et al., *Nat Biotechnol* 19: 342-7, 2001). Data were analyzed using Rosetta Resolver™ software.

SUPPLEMENTARY TABLE 1

Sequences of the siRNAs used in this study

Gene	Accession number	Target sequence ID	Target sequence
IGF1R	NM_000875	IGF1R-1	GGAUGCACCAUCUUCAAGG (SEQ ID NO: 47)
IGF1R	NM_000875	IGF1R-2	GACAAAAUCCCCAUCAGGA (SEQ ID NO: 48)
IGF1R	NM_000875	IGF1R-3	ACCGCAAAGUCUUUGAGAA (SEQ ID NO: 49)
IGF1R	NM_000875	IGF1R-4	GUCCUGACAUGCUGUUUGA (SEQ ID NO: 50)
IGF1R	NM_000875	IGF1R-5	GACCACCAUCAACAAUGAG (SEQ ID NO: 51)
IGF1R	NM_000875	IGF1R-6	CAAAUUAUGUGUUUCCGAA (SEQ ID NO: 52)
IGF1R	NM_000875	IGF1R-7	CGCAUGUGCUGGCAGUAUA (SEQ ID NO: 53)
IGF1R	NM_000875	IGF1R-8	CCGAAGAUUUCACAGUCAA (SEQ ID NO: 54)
IGF1R	NM_000875	IGF1R-9	ACCAUUGAUUCUGUUACUU (SEQ ID NO: 55)
KIF11	NM_004523	KIF11-1	CUGACAAGAGCUCAAGGAA (SEQ ID NO: 56)
KIF11	NM_004523	KIF11-2	CGUUCUGGAGCUGUUGAUA (SEQ ID NO: 57)
KIF11	NM_004523	KIF11-3	GAGCCCAGAUCAACCUUUA (SEQ ID NO: 58)
KIF11	NM_004523	KIF11-4	GGCAUUAACACACUGGAGA (SEQ ID NO: 59)
KIF11	NM_004523	KIF11-5	GAUGGCAGCUCAAAGCAAA (SEQ ID NO: 60)
KIF11	NM_004523	KIF11-6	CAGCAGAAAUCUAAGGAUA (SEQ ID NO: 61)
KIF14	NM_014875	KIF14-1	CAGGGAUGCUGUUUGGAUA (SEQ ID NO: 62)
KIF14	NM_014875	KIF14-2	ACUGACAACAAAGUGCAGC (SEQ ID NO: 63)
KIF14	NM_014875	KIF14-3	AAACUGGGAGGCUACUUAC (SEQ ID NO: 64)
KIF14	NM_014875	KIF14-4	CACUGAAUGUGGGAGGUGA (SEQ ID NO: 65)
KIF14	NM_014875	KIF14-5	GUCUGGGUGGAAAUUCAAA (SEQ ID NO: 66)
KIF14	NM_014875	KIF14-6	CAUCUUUGCUGAAUCGAAA (SEQ ID NO: 67)

SUPPLEMENTARY TABLE 1-continued

Sequences of the siRNAs used in this study

Gene	Accession number	Target sequence ID	Target sequence
KIF14	NM_014875	KIF14-7	GGGAUUGACGGCAGUAAGA (SEQ ID NO: 68)
KIF14	NM_014875	KIF14-8	CAGGUAAAGUCAGAGACAU (SEQ ID NO: 69)
KIF14	NM_014875	KIF14-9	CUCACAUUGUCCACCAGGA (SEQ ID NO: 70)
KNSL1	NM_004523	KNSL1-1	GACCUGUGCCUUUUAGAGA (SEQ ID NO: 71)
KNSL1	NM_004523	KNSL1-2	AAAGGACAACUGCAGCUAC (SEQ ID NO: 72)
KNSL1	NM_004523	KNSL1-3	GACUUCAUUGACAGUGGCC (SEQ ID NO: 73)
MAPK14	NM_139012	MAPK14-1	AAUAUCCUCAGGGGUGGAG (SEQ ID NO: 74)
MAPK14	NM_139012	MAPK14-2	GUGCCUCUUGUUGCAGAGA (SEQ ID NO: 75)
MAPK14	NM_139012	MAPK14-3	GAAGCUCUCCAGACCAUUU (SEQ ID NO: 76)
MAPK14	NM_001315	MAPK14-4	CUCCUGAGAUCAUGCUGAA (SEQ ID NO: 77)
MAPK14	NM_001315	MAPK14-5	GCUGUUGACUGGAAGAACA (SEQ ID NO: 78)
MAPK14	NM_001315	MAPK14-6	GGAAUUCAAUGAUGUGUAU (SEQ ID NO: 79)
MAPK14	NM_001315	MAPK14-7	CCAUUUCAGUCCAUCAUUC (SEQ ID NO: 80)
PLK	NM_005030	PLK-1	CCCUGUGUGGGACUCCUAA (SEQ ID NO: 81)
PLK	NM_005030	PLK-2	CCGAGUUAUUCAUCGAGAC (SEQ ID NO: 82)
PLK	NM_005030	PLK-3	GUUCUUUACUUCUGGCUAU (SEQ ID NO: 83)
PLK	NM_005030	PLK-4	CGCCUCAUCCUCUACAAUG (SEQ ID NO: 84)
PLK	NM_005030	PLK-5	AAGAGACCUACCUCCGGAU (SEQ ID NO: 85)
PLK	NM_005030	PLK-6	GGUGUUCGCGGGCAAGAUU (SEQ ID NO: 86)
PLK	NM_005030	PLK-7	CUCCUUAAAUAUUUCCGCA (SEQ ID NO: 87)
PLK	NM_005030	PLK-8	AAGAAGAACCAGUGGUUCG (SEQ ID NO: 88)
PLK	NM_005030	PLK-9	CUGAGCCUGAGGCCCGAUA (SEQ ID NO: 89)

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V. Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. All of the above-cited references and publications are hereby incorporated by reference.

SEQUENCE LISTING

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Met Thr Pro Ala Ser Ser Pro Met Gly Pro Phe Phe Gly Leu Pro Trp
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caa caa gaa gca att cat gat aac att tat acg cca aga aaa tat cag      144
Gln Gln Glu Ala Ile His Asp Asn Ile Tyr Thr Pro Arg Lys Tyr Gln
        35                  40                  45

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Val Glu Leu Leu Glu Ala Ala Leu Asp His Asn Thr Ile Val Cys Leu
    50                  55                  60

aac act ggc tca ggg aag aca ttt att gct agt act act cta cta aag      240
Asn Thr Gly Ser Gly Lys Thr Phe Ile Ala Ser Thr Thr Leu Leu Lys
65                  70                  75                  80

agc tgt ctc tat cta gat cta ggg gag act tca gct aga aat gga aaa      288
Ser Cys Leu Tyr Leu Asp Leu Gly Glu Thr Ser Ala Arg Asn Gly Lys
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agg acg gtg ttc ttg gtc aac tct gca aac cag gtt gct caa caa gtg      336
Arg Thr Val Phe Leu Val Asn Ser Ala Asn Gln Val Ala Gln Gln Val
            100                 105                 110

tca gct gtc aga act cat tca gat ctc aag gtt ggg gaa tac tca aac      384
Ser Ala Val Arg Thr His Ser Asp Leu Lys Val Gly Glu Tyr Ser Asn
        115                 120                 125

cta gaa gta aat gca tct tgg aca aaa gag aga tgg aac caa gag ttt      432
Leu Glu Val Asn Ala Ser Trp Thr Lys Glu Arg Trp Asn Gln Glu Phe
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act aag cac cag gtt ctc att atg act tgc tat gtc gcc ttg aat gtt      480
Thr Lys His Gln Val Leu Ile Met Thr Cys Tyr Val Ala Leu Asn Val
145                 150                 155                 160

ttg aaa aat ggt tac tta tca ctg tca gac att aac ctt ttg gtg ttt      528
Leu Lys Asn Gly Tyr Leu Ser Leu Ser Asp Ile Asn Leu Leu Val Phe
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Asp Glu Cys His Leu Ala Ile Leu Asp His Pro Tyr Arg Glu Phe Met
            180                 185                 190

aag ctc tgt gaa att tgt cca tca tgt cct cgc att ttg gga cta act      624
Lys Leu Cys Glu Ile Cys Pro Ser Cys Pro Arg Ile Leu Gly Leu Thr
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gct tcc att tta aat ggg aaa tgg gat cca gag gat ttg gaa gaa aag      672
Ala Ser Ile Leu Asn Gly Lys Trp Asp Pro Glu Asp Leu Glu Glu Lys
    210                 215                 220

ttt cag aaa cta gag aaa att ctt aag agt aat gct gaa act gca act      720
Phe Gln Lys Leu Glu Lys Ile Leu Lys Ser Asn Ala Glu Thr Ala Thr
225                 230                 235                 240

gac ctg gtg gtc tta gac agg tat act tct cag cca tgt gag att gtg      768
Asp Leu Val Val Leu Asp Arg Tyr Thr Ser Gln Pro Cys Glu Ile Val
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gtg gat tgt gga cca ttt act gac aga agt ggg ctt tat gaa aga ctg      816
Val Asp Cys Gly Pro Phe Thr Asp Arg Ser Gly Leu Tyr Glu Arg Leu
            260                 265                 270

ctg atg gaa tta gaa gaa gca ctt aat ttt atc aat gat tgt aat ata      864
Leu Met Glu Leu Glu Glu Ala Leu Asn Phe Ile Asn Asp Cys Asn Ile
        275                 280                 285

tct gta cat tca aaa gaa aga gat tct act tta att tcg aaa cag ata      912
Ser Val His Ser Lys Glu Arg Asp Ser Thr Leu Ile Ser Lys Gln Ile
    290                 295                 300

cta tca gac tgt cgt gcc gta ttg gta gtt ctg gga ccc tgg tgt gca      960
Leu Ser Asp Cys Arg Ala Val Leu Val Val Leu Gly Pro Trp Cys Ala
305                 310                 315                 320

gat aaa gta gct gga atg atg gta aga gaa cta cag aaa tac atc aaa     1008
Asp Lys Val Ala Gly Met Met Val Arg Glu Leu Gln Lys Tyr Ile Lys
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cat gag caa gag gag ctg cac agg aaa ttt tta ttg ttt aca gac act     1056
His Glu Gln Glu Glu Leu His Arg Lys Phe Leu Leu Phe Thr Asp Thr
            340                 345                 350

ttc cta agg aaa ata cat gca cta tgt gaa gag cac ttc tca cct gcc     1104
Phe Leu Arg Lys Ile His Ala Leu Cys Glu Glu His Phe Ser Pro Ala
        355                 360                 365

tca ctt gac ctg aaa ttt gta act cct aaa gta atc aaa ctg ctc gaa     1152
Ser Leu Asp Leu Lys Phe Val Thr Pro Lys Val Ile Lys Leu Leu Glu
    370                 375                 380

atc tta cgc aaa tat aaa cca tat gag cga cac agt ttt gaa agc gtt     1200
Ile Leu Arg Lys Tyr Lys Pro Tyr Glu Arg His Ser Phe Glu Ser Val
385                 390                 395                 400

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Glu Trp Tyr Asn Asn Arg Asn Gln Asp Asn Tyr Val Ser Trp Ser Asp
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tct gag gat gat gat gag gat gaa gaa att gaa gaa aaa gag aag cca     1296
Ser Glu Asp Asp Asp Glu Asp Glu Glu Ile Glu Glu Lys Glu Lys Pro
            420                 425                 430

gag aca aat ttt cct tct cct ttt acc aac att ttg tgc gga att att     1344
Glu Thr Asn Phe Pro Ser Pro Phe Thr Asn Ile Leu Cys Gly Ile Ile
        435                 440                 445

ttt gtg gaa aga aga tac aca gca gtt gtc tta aac aga ttg ata aag     1392
Phe Val Glu Arg Arg Tyr Thr Ala Val Val Leu Asn Arg Leu Ile Lys
    450                 455                 460

gaa gct ggc aaa caa gat cca gag ctg gct tat atc agt agc aat ttc     1440
Glu Ala Gly Lys Gln Asp Pro Glu Leu Ala Tyr Ile Ser Ser Asn Phe
465                 470                 475                 480

ata act gga cat ggc att ggg aag aat cag cct cgc aac aac acg atg     1488
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cat gag acc aac ctg ctt att gca aca agt att gta gaa gag ggt gtt      1584
His Glu Thr Asn Leu Leu Ile Ala Thr Ser Ile Val Glu Glu Gly Val
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gat ata cca aaa tgc aac ttg gtg gtt cgt ttt gat ttg ccc aca gaa      1632
Asp Ile Pro Lys Cys Asn Leu Val Val Arg Phe Asp Leu Pro Thr Glu
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tat cga tcc tat gtt caa tct aaa gga aga gca agg gca ccc atc tct      1680
Tyr Arg Ser Tyr Val Gln Ser Lys Gly Arg Ala Arg Ala Pro Ile Ser
545                 550                 555                 560

aat tat ata atg tta gcg gat aca gac aaa ata aaa agt ttt gaa gaa      1728
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tcc aag tcg gtt gat act ggt gag act gac att gat cct gtc atg gat      1824
Ser Lys Ser Val Asp Thr Gly Glu Thr Asp Ile Asp Pro Val Met Asp
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gat gat cac gtt ttc cca cca tat gtg ttg agg cct gac gat ggt ggt      1872
Asp Asp His Val Phe Pro Pro Tyr Val Leu Arg Pro Asp Asp Gly Gly
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cca cga gtc aca atc aac acg gcc att gga cac atc aat aga tac tgt      1920
Pro Arg Val Thr Ile Asn Thr Ala Ile Gly His Ile Asn Arg Tyr Cys
625                 630                 635                 640

gct aga tta cca agt gat ccg ttt act cat cta gct cct aaa tgc aga      1968
Ala Arg Leu Pro Ser Asp Pro Phe Thr His Leu Ala Pro Lys Cys Arg
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acc cga gag ttg cct gat ggt aca ttt tat tca act ctt tat ctg cca      2016
Thr Arg Glu Leu Pro Asp Gly Thr Phe Tyr Ser Thr Leu Tyr Leu Pro
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att aac tca cct ctt cga gcc tcc att gtt ggt cca cca atg agc tgt      2064
Ile Asn Ser Pro Leu Arg Ala Ser Ile Val Gly Pro Pro Met Ser Cys
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gta cga ttg gct gaa aga gtt gtc gct ctc att tgc tgt gag aaa ctg      2112
Val Arg Leu Ala Glu Arg Val Val Ala Leu Ile Cys Cys Glu Lys Leu
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cac aaa att ggc gaa ctg gat gac cat ttg atg cca gtt ggg aaa gag      2160
His Lys Ile Gly Glu Leu Asp Asp His Leu Met Pro Val Gly Lys Glu
705                 710                 715                 720

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Thr Val Lys Tyr Glu Glu Glu Leu Asp Leu His Asp Glu Glu Glu Thr
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Ser Val Pro Gly Arg Pro Gly Ser Thr Lys Arg Arg Gln Cys Tyr Pro
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Lys Ala Ile Pro Glu Cys Leu Arg Asp Ser Tyr Pro Arg Pro Asp Gln
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ccc tgt tac ctg tat gtg ata gga atg gtt tta act aca cct tta cct      2352
Pro Cys Tyr Leu Tyr Val Ile Gly Met Val Leu Thr Thr Pro Leu Pro
    770                 775                 780

gat gaa ctc aac ttt aga agg cgg aag ctc tat cct cct gaa gat acc      2400
Asp Glu Leu Asn Phe Arg Arg Arg Lys Leu Tyr Pro Pro Glu Asp Thr
785                 790                 795                 800

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Leu Lys Lys Ser Gly Phe Met Leu Ser Leu Gln Met Leu Glu Leu Ile
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Thr Arg Leu His Gln Tyr Ile Phe Ser His Ile Leu Arg Leu Glu Lys
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Pro Ala Leu Glu Phe Lys Pro Thr Asp Ala Asp Ser Ala Tyr Cys Val
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Lys Phe Met Glu Asp Ile Glu Lys Ser Glu Ala Arg Ile Gly Ile Pro
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agt aca aag tat aca aaa gaa aca ccc ttt gtt ttt aaa tta gaa gat     2784
Ser Thr Lys Tyr Thr Lys Glu Thr Pro Phe Val Phe Lys Leu Glu Asp
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Tyr Gln Asp Ala Val Ile Ile Pro Arg Tyr Arg Asn Phe Asp Gln Pro
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cat cga ttt tat gta gct gat gtg tac act gat ctt acc cca ctc agt     2880
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Lys Tyr Asn Leu Asp Leu Thr Asn Leu Asn Gln Pro Leu Leu Asp Val
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Gln Lys Gly Lys Ala Leu Pro Leu Ser Ser Ala Glu Lys Arg Lys Ala
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aaa tgg gaa agt ctg cag aat aaa cag ata ctg gtt cca gaa ctc tgt     3120
Lys Trp Glu Ser Leu Gln Asn Lys Gln Ile Leu Val Pro Glu Leu Cys
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Ala Ile His Pro Ile Pro Ala Ser Leu Trp Arg Lys Ala Val Cys Leu
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ccc agc ata ctt tat cgc ctt cac tgc ctt ttg act gca gag gag cta     3216
Pro Ser Ile Leu Tyr Arg Leu His Cys Leu Leu Thr Ala Glu Glu Leu
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aga gcc cag act gcc agc gat gct ggc gtg gga gtc aga tca ctt cct     3264
Arg Ala Gln Thr Ala Ser Asp Ala Gly Val Gly Val Arg Ser Leu Pro
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gcg gat ttt aga tac cct aac tta gac ttc ggg tgg aaa aaa tct att     3312
Ala Asp Phe Arg Tyr Pro Asn Leu Asp Phe Gly Trp Lys Lys Ser Ile
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Val Asn Cys Arg Thr Leu Leu Ser Glu Ser Pro Gly Lys Leu His Val
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gga aat cag cta aat tac tac aag cag gaa ata ccc gtg caa cca act      3648
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Ala Asn Lys Ser Thr Ser Asp Gly Ser Pro Val Met Ala Val Met Pro
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ggt acg aca gac act att caa gtg ctc aag ggc agg atg gat tct gag      3840
Gly Thr Thr Asp Thr Ile Gln Val Leu Lys Gly Arg Met Asp Ser Glu
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Gln Ser Pro Ser Ile Gly Tyr Ser Ser Arg Thr Leu Gly Pro Asn Pro
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Ile Thr Thr Tyr Leu Phe Cys Thr Tyr Pro Asp Ala His Glu Gly Arg
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ctt tca tat atg aga agc aaa aag gtc agc aac tgt aat ctg tat cgc      4080
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gat ccc cct gtg aat tgg ctt cct cct ggt tat gta gta aat caa gac      4176
Asp Pro Pro Val Asn Trp Leu Pro Pro Gly Tyr Val Val Asn Gln Asp
        1380                1385                1390

aaa agc aac aca gat aaa tgg gaa aaa gat gaa atg aca aaa gac tgc      4224
Lys Ser Asn Thr Asp Lys Trp Glu Lys Asp Glu Met Thr Lys Asp Cys
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atg ctg gcg aat ggc aaa ctg gat gag gat tac gag gag gag gat gag      4272
Met Leu Ala Asn Gly Lys Leu Asp Glu Asp Tyr Glu Glu Glu Asp Glu
   1410                1415                1420

gag gag gag agc ctg atg tgg agg gct ccg aag gaa gag gct gac tat      4320
Glu Glu Glu Ser Leu Met Trp Arg Ala Pro Lys Glu Glu Ala Asp Tyr
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cct ttt tca acc act gat tct gca tat gaa tgg aaa atg ccc aaa aaa     4464
Pro Phe Ser Thr Thr Asp Ser Ala Tyr Glu Trp Lys Met Pro Lys Lys
        1475                1480                1485

tcc tcc tta ggt agt atg cca ttt tca tca gat ttt gag gat ttt gac     4512
Ser Ser Leu Gly Ser Met Pro Phe Ser Ser Asp Phe Glu Asp Phe Asp
    1490                1495                1500

tac agc tct tgg gat gca atg tgc tat ctg gat cct agc aaa gct gtt     4560
Tyr Ser Ser Trp Asp Ala Met Cys Tyr Leu Asp Pro Ser Lys Ala Val
1505                1510                1515                1520

gaa gaa gat gac ttt gtg gtg ggg ttc tgg aat cca tca gaa gaa aac     4608
Glu Glu Asp Asp Phe Val Val Gly Phe Trp Asn Pro Ser Glu Glu Asn
                1525                1530                1535

tgt ggt gtt gac acg gga aag cag tcc att tct tac gac ttg cac act     4656
Cys Gly Val Asp Thr Gly Lys Gln Ser Ile Ser Tyr Asp Leu His Thr
            1540                1545                1550

gag cag tgt att gct gac aaa agc ata gcg gac tgt gtg gaa gcc ctg     4704
Glu Gln Cys Ile Ala Asp Lys Ser Ile Ala Asp Cys Val Glu Ala Leu
        1555                1560                1565

ctg ggc tgc tat tta acc agc tgt ggg gag agg gct gct cag ctt ttc     4752
Leu Gly Cys Tyr Leu Thr Ser Cys Gly Glu Arg Ala Ala Gln Leu Phe
    1570                1575                1580

ctc tgt tca ctg ggg ctg aag gtg ctc ccg gta att aaa agg act gat     4800
Leu Cys Ser Leu Gly Leu Lys Val Leu Pro Val Ile Lys Arg Thr Asp
1585                1590                1595                1600

cgg gaa aag gcc ctg tgc cct act cgg gag aat ttc aac agc caa caa     4848
Arg Glu Lys Ala Leu Cys Pro Thr Arg Glu Asn Phe Asn Ser Gln Gln
                1605                1610                1615

aag aac ctt tca gtg agc tgt gct gct gct tct gtg gcc agt tca cgc     4896
Lys Asn Leu Ser Val Ser Cys Ala Ala Ala Ser Val Ala Ser Ser Arg
            1620                1625                1630

tct tct gta ttg aaa gac tcg gaa tat ggt tgt ttg aag att cca cca     4944
Ser Ser Val Leu Lys Asp Ser Glu Tyr Gly Cys Leu Lys Ile Pro Pro
        1635                1640                1645

aga tgt atg ttt gat cat cca gat gca gat aaa aca ctg aat cac ctt     4992
Arg Cys Met Phe Asp His Pro Asp Ala Asp Lys Thr Leu Asn His Leu
    1650                1655                1660

ata tcg ggg ttt gaa aat ttt gaa aag aaa atc aac tac aga ttc aag     5040
Ile Ser Gly Phe Glu Asn Phe Glu Lys Lys Ile Asn Tyr Arg Phe Lys
1665                1670                1675                1680

aat aag gct tac ctt ctc cag gct ttt aca cat gcc tcc tac cac tac     5088
Asn Lys Ala Tyr Leu Leu Gln Ala Phe Thr His Ala Ser Tyr His Tyr
                1685                1690                1695

aat act atc act gat tgt tac cag cgc tta gaa ttc ctg gga gat gcg     5136
Asn Thr Ile Thr Asp Cys Tyr Gln Arg Leu Glu Phe Leu Gly Asp Ala
            1700                1705                1710

att ttg gac tac ctc ata acc aag cac ctt tat gaa gac ccg cgg cag     5184
Ile Leu Asp Tyr Leu Ile Thr Lys His Leu Tyr Glu Asp Pro Arg Gln
        1715                1720                1725

cac tcc ccg ggg gtc ctg aca gac ctg cgg tct gcc ctg gtc aac aac     5232
His Ser Pro Gly Val Leu Thr Asp Leu Arg Ser Ala Leu Val Asn Asn
    1730                1735                1740

acc atc ttt gca tcg ctg gct gta aag tac gac tac cac aag tac ttc     5280
Thr Ile Phe Ala Ser Leu Ala Val Lys Tyr Asp Tyr His Lys Tyr Phe
1745                1750                1755                1760

aaa gct gtc tct cct gag ctc ttc cat gtc att gat gac ttt gtg cag     5328
Lys Ala Val Ser Pro Glu Leu Phe His Val Ile Asp Asp Phe Val Gln
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            1765                1770                1775

ttt cag ctt gag aag aat gaa atg caa gga atg gat tct gag ctt agg     5376
Phe Gln Leu Glu Lys Asn Glu Met Gln Gly Met Asp Ser Glu Leu Arg
            1780                1785                1790

aga tct gag gag gat gaa gag aaa gaa gag gat att gaa gtt cca aag     5424
Arg Ser Glu Glu Asp Glu Glu Lys Glu Glu Asp Ile Glu Val Pro Lys
        1795                1800                1805

gcc atg ggg gat att ttt gag tcg ctt gct ggt gcc att tac atg gat     5472
Ala Met Gly Asp Ile Phe Glu Ser Leu Ala Gly Ala Ile Tyr Met Asp
    1810                1815                1820

agt ggg atg tca ctg gag aca gtc tgg cag gtg tac tat ccc atg atg     5520
Ser Gly Met Ser Leu Glu Thr Val Trp Gln Val Tyr Tyr Pro Met Met
1825                1830                1835                1840

cgg cca cta ata gaa aag ttt tct gca aat gta ccc cgt tcc cct gtg     5568
Arg Pro Leu Ile Glu Lys Phe Ser Ala Asn Val Pro Arg Ser Pro Val
                1845                1850                1855

cga gaa ttg ctt gaa atg gaa cca gaa act gcc aaa ttt agc ccg gct     5616
Arg Glu Leu Leu Glu Met Glu Pro Glu Thr Ala Lys Phe Ser Pro Ala
            1860                1865                1870

gag aga act tac gac ggg aag gtc aga gtc act gtg gaa gta gta gga     5664
Glu Arg Thr Tyr Asp Gly Lys Val Arg Val Thr Val Glu Val Val Gly
        1875                1880                1885

aag ggg aaa ttt aaa ggt gtt ggt cga agt tac agg att gcc aaa tct     5712
Lys Gly Lys Phe Lys Gly Val Gly Arg Ser Tyr Arg Ile Ala Lys Ser
    1890                1895                1900

gca gca gca aga aga gcc ctc cga agc ctc aaa gct aat caa cct cag     5760
Ala Ala Ala Arg Arg Ala Leu Arg Ser Leu Lys Ala Asn Gln Pro Gln
1905                1910                1915                1920

gtt ccc aat agc tga                                                 5775
Val Pro Asn Ser  *


<210> SEQ ID NO 2
<211> LENGTH: 1924
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

Met Lys Ser Pro Ala Leu Gln Pro Leu Ser Met Ala Gly Leu Gln Leu
1               5                   10                  15

Met Thr Pro Ala Ser Ser Pro Met Gly Pro Phe Phe Gly Leu Pro Trp
            20                  25                  30

Gln Gln Glu Ala Ile His Asp Asn Ile Tyr Thr Pro Arg Lys Tyr Gln
        35                  40                  45

Val Glu Leu Leu Glu Ala Ala Leu Asp His Asn Thr Ile Val Cys Leu
    50                  55                  60

Asn Thr Gly Ser Gly Lys Thr Phe Ile Ala Ser Thr Thr Leu Leu Lys
65                  70                  75                  80

Ser Cys Leu Tyr Leu Asp Leu Gly Glu Thr Ser Ala Arg Asn Gly Lys
                85                  90                  95

Arg Thr Val Phe Leu Val Asn Ser Ala Asn Gln Val Ala Gln Gln Val
            100                 105                 110

Ser Ala Val Arg Thr His Ser Asp Leu Lys Val Gly Glu Tyr Ser Asn
        115                 120                 125

Leu Glu Val Asn Ala Ser Trp Thr Lys Glu Arg Trp Asn Gln Glu Phe
    130                 135                 140

Thr Lys His Gln Val Leu Ile Met Thr Cys Tyr Val Ala Leu Asn Val
145                 150                 155                 160

Leu Lys Asn Gly Tyr Leu Ser Leu Ser Asp Ile Asn Leu Leu Val Phe
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                165                 170                 175

Asp Glu Cys His Leu Ala Ile Leu Asp His Pro Tyr Arg Glu Phe Met
            180                 185                 190

Lys Leu Cys Glu Ile Cys Pro Ser Cys Pro Arg Ile Leu Gly Leu Thr
        195                 200                 205

Ala Ser Ile Leu Asn Gly Lys Trp Asp Pro Glu Asp Leu Glu Glu Lys
    210                 215                 220

Phe Gln Lys Leu Glu Lys Ile Leu Lys Ser Asn Ala Glu Thr Ala Thr
225                 230                 235                 240

Asp Leu Val Val Leu Asp Arg Tyr Thr Ser Gln Pro Cys Glu Ile Val
                245                 250                 255

Val Asp Cys Gly Pro Phe Thr Asp Arg Ser Gly Leu Tyr Glu Arg Leu
            260                 265                 270

Leu Met Glu Leu Glu Glu Ala Leu Asn Phe Ile Asn Asp Cys Asn Ile
        275                 280                 285

Ser Val His Ser Lys Glu Arg Asp Ser Thr Leu Ile Ser Lys Gln Ile
    290                 295                 300

Leu Ser Asp Cys Arg Ala Val Leu Val Val Leu Gly Pro Trp Cys Ala
305                 310                 315                 320

Asp Lys Val Ala Gly Met Met Val Arg Glu Leu Gln Lys Tyr Ile Lys
                325                 330                 335

His Glu Gln Glu Glu Leu His Arg Lys Phe Leu Leu Phe Thr Asp Thr
            340                 345                 350

Phe Leu Arg Lys Ile His Ala Leu Cys Glu Glu His Phe Ser Pro Ala
        355                 360                 365

Ser Leu Asp Leu Lys Phe Val Thr Pro Lys Val Ile Lys Leu Leu Glu
    370                 375                 380

Ile Leu Arg Lys Tyr Lys Pro Tyr Glu Arg His Ser Phe Glu Ser Val
385                 390                 395                 400

Glu Trp Tyr Asn Asn Arg Asn Gln Asp Asn Tyr Val Ser Trp Ser Asp
                405                 410                 415

Ser Glu Asp Asp Asp Glu Asp Glu Glu Ile Glu Glu Lys Glu Lys Pro
            420                 425                 430

Glu Thr Asn Phe Pro Ser Pro Phe Thr Asn Ile Leu Cys Gly Ile Ile
        435                 440                 445

Phe Val Glu Arg Arg Tyr Thr Ala Val Val Leu Asn Arg Leu Ile Lys
    450                 455                 460

Glu Ala Gly Lys Gln Asp Pro Glu Leu Ala Tyr Ile Ser Ser Asn Phe
465                 470                 475                 480

Ile Thr Gly His Gly Ile Gly Lys Asn Gln Pro Arg Asn Asn Thr Met
                485                 490                 495

Glu Ala Glu Phe Arg Lys Gln Glu Glu Val Leu Arg Lys Phe Arg Ala
            500                 505                 510

His Glu Thr Asn Leu Leu Ile Ala Thr Ser Ile Val Glu Glu Gly Val
        515                 520                 525

Asp Ile Pro Lys Cys Asn Leu Val Val Arg Phe Asp Leu Pro Thr Glu
    530                 535                 540

Tyr Arg Ser Tyr Val Gln Ser Lys Gly Arg Ala Arg Ala Pro Ile Ser
545                 550                 555                 560

Asn Tyr Ile Met Leu Ala Asp Thr Asp Lys Ile Lys Ser Phe Glu Glu
                565                 570                 575

Asp Leu Lys Thr Tyr Lys Ala Ile Glu Lys Ile Leu Arg Asn Lys Cys
            580                 585                 590
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Ser Lys Ser Val Asp Thr Gly Glu Thr Asp Ile Asp Pro Val Met Asp
        595                 600                 605

Asp Asp His Val Phe Pro Pro Tyr Val Leu Arg Pro Asp Asp Gly Gly
    610                 615                 620

Pro Arg Val Thr Ile Asn Thr Ala Ile Gly His Ile Asn Arg Tyr Cys
625                 630                 635                 640

Ala Arg Leu Pro Ser Asp Pro Phe Thr His Leu Ala Pro Lys Cys Arg
                645                 650                 655

Thr Arg Glu Leu Pro Asp Gly Thr Phe Tyr Ser Thr Leu Tyr Leu Pro
            660                 665                 670

Ile Asn Ser Pro Leu Arg Ala Ser Ile Val Gly Pro Pro Met Ser Cys
        675                 680                 685

Val Arg Leu Ala Glu Arg Val Val Ala Leu Ile Cys Cys Glu Lys Leu
    690                 695                 700

His Lys Ile Gly Glu Leu Asp Asp His Leu Met Pro Val Gly Lys Glu
705                 710                 715                 720

Thr Val Lys Tyr Glu Glu Glu Leu Asp Leu His Asp Glu Glu Glu Thr
                725                 730                 735

Ser Val Pro Gly Arg Pro Gly Ser Thr Lys Arg Arg Gln Cys Tyr Pro
            740                 745                 750

Lys Ala Ile Pro Glu Cys Leu Arg Asp Ser Tyr Pro Arg Pro Asp Gln
        755                 760                 765

Pro Cys Tyr Leu Tyr Val Ile Gly Met Val Leu Thr Thr Pro Leu Pro
    770                 775                 780

Asp Glu Leu Asn Phe Arg Arg Arg Lys Leu Tyr Pro Pro Glu Asp Thr
785                 790                 795                 800

Thr Arg Cys Phe Gly Ile Leu Thr Ala Lys Pro Ile Pro Gln Ile Pro
                805                 810                 815

His Phe Pro Val Tyr Thr Arg Ser Gly Glu Val Thr Ile Ser Ile Glu
            820                 825                 830

Leu Lys Lys Ser Gly Phe Met Leu Ser Leu Gln Met Leu Glu Leu Ile
        835                 840                 845

Thr Arg Leu His Gln Tyr Ile Phe Ser His Ile Leu Arg Leu Glu Lys
    850                 855                 860

Pro Ala Leu Glu Phe Lys Pro Thr Asp Ala Asp Ser Ala Tyr Cys Val
865                 870                 875                 880

Leu Pro Leu Asn Val Val Asn Asp Ser Ser Thr Leu Asp Ile Asp Phe
                885                 890                 895

Lys Phe Met Glu Asp Ile Glu Lys Ser Glu Ala Arg Ile Gly Ile Pro
            900                 905                 910

Ser Thr Lys Tyr Thr Lys Glu Thr Pro Phe Val Phe Lys Leu Glu Asp
        915                 920                 925

Tyr Gln Asp Ala Val Ile Ile Pro Arg Tyr Arg Asn Phe Asp Gln Pro
    930                 935                 940

His Arg Phe Tyr Val Ala Asp Val Tyr Thr Asp Leu Thr Pro Leu Ser
945                 950                 955                 960

Lys Phe Pro Ser Pro Glu Tyr Glu Thr Phe Ala Glu Tyr Tyr Lys Thr
                965                 970                 975

Lys Tyr Asn Leu Asp Leu Thr Asn Leu Asn Gln Pro Leu Leu Asp Val
            980                 985                 990

Asp His Thr Ser Ser Arg Leu Asn Leu Leu Thr Pro Arg His Leu Asn
        995                 1000                1005

Gln Lys Gly Lys Ala Leu Pro Leu Ser Ser Ala Glu Lys Arg Lys Ala
    1010                1015                1020
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Lys Trp Glu Ser Leu Gln Asn Lys Gln Ile Leu Val Pro Glu Leu Cys
1025                1030                1035                1040

Ala Ile His Pro Ile Pro Ala Ser Leu Trp Arg Lys Ala Val Cys Leu
                1045                1050                1055

Pro Ser Ile Leu Tyr Arg Leu His Cys Leu Leu Thr Ala Glu Glu Leu
            1060                1065                1070

Arg Ala Gln Thr Ala Ser Asp Ala Gly Val Gly Val Arg Ser Leu Pro
        1075                1080                1085

Ala Asp Phe Arg Tyr Pro Asn Leu Asp Phe Gly Trp Lys Lys Ser Ile
    1090                1095                1100

Asp Ser Lys Ser Phe Ile Ser Ile Ser Asn Ser Ser Ser Ala Glu Asn
1105                1110                1115                1120

Asp Asn Tyr Cys Lys His Ser Thr Ile Val Pro Glu Asn Ala Ala His
                1125                1130                1135

Gln Gly Ala Asn Arg Thr Ser Ser Leu Glu Asn His Asp Gln Met Ser
            1140                1145                1150

Val Asn Cys Arg Thr Leu Leu Ser Glu Ser Pro Gly Lys Leu His Val
        1155                1160                1165

Glu Val Ser Ala Asp Leu Thr Ala Ile Asn Gly Leu Ser Tyr Asn Gln
    1170                1175                1180

Asn Leu Ala Asn Gly Ser Tyr Asp Leu Ala Asn Arg Asp Phe Cys Gln
1185                1190                1195                1200

Gly Asn Gln Leu Asn Tyr Tyr Lys Gln Glu Ile Pro Val Gln Pro Thr
                1205                1210                1215

Thr Ser Tyr Ser Ile Gln Asn Leu Tyr Ser Tyr Glu Asn Gln Pro Gln
            1220                1225                1230

Pro Ser Asp Glu Cys Thr Leu Leu Ser Asn Lys Tyr Leu Asp Gly Asn
        1235                1240                1245

Ala Asn Lys Ser Thr Ser Asp Gly Ser Pro Val Met Ala Val Met Pro
    1250                1255                1260

Gly Thr Thr Asp Thr Ile Gln Val Leu Lys Gly Arg Met Asp Ser Glu
1265                1270                1275                1280

Gln Ser Pro Ser Ile Gly Tyr Ser Ser Arg Thr Leu Gly Pro Asn Pro
                1285                1290                1295

Gly Leu Ile Leu Gln Ala Leu Thr Leu Ser Asn Ala Ser Asp Gly Phe
            1300                1305                1310

Asn Leu Glu Arg Leu Glu Met Leu Gly Asp Ser Phe Leu Lys His Ala
        1315                1320                1325

Ile Thr Thr Tyr Leu Phe Cys Thr Tyr Pro Asp Ala His Glu Gly Arg
    1330                1335                1340

Leu Ser Tyr Met Arg Ser Lys Lys Val Ser Asn Cys Asn Leu Tyr Arg
1345                1350                1355                1360

Leu Gly Lys Lys Lys Gly Leu Pro Ser Arg Met Val Val Ser Ile Phe
                1365                1370                1375

Asp Pro Pro Val Asn Trp Leu Pro Pro Gly Tyr Val Val Asn Gln Asp
            1380                1385                1390

Lys Ser Asn Thr Asp Lys Trp Glu Lys Asp Glu Met Thr Lys Asp Cys
        1395                1400                1405

Met Leu Ala Asn Gly Lys Leu Asp Glu Asp Tyr Glu Glu Glu Asp Glu
    1410                1415                1420

Glu Glu Glu Ser Leu Met Trp Arg Ala Pro Lys Glu Glu Ala Asp Tyr
1425                1430                1435                1440

Glu Asp Asp Phe Leu Glu Tyr Asp Gln Glu His Ile Arg Phe Ile Asp
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            1445                1450                1455

Asn Met Leu Met Gly Ser Gly Ala Phe Val Lys Lys Ile Ser Leu Ser
        1460                1465                1470

Pro Phe Ser Thr Thr Asp Ser Ala Tyr Glu Trp Lys Met Pro Lys Lys
    1475                1480                1485

Ser Ser Leu Gly Ser Met Pro Phe Ser Ser Asp Phe Glu Asp Phe Asp
    1490                1495                1500

Tyr Ser Ser Trp Asp Ala Met Cys Tyr Leu Asp Pro Ser Lys Ala Val
1505                1510                1515                1520

Glu Glu Asp Asp Phe Val Val Gly Phe Trp Asn Pro Ser Glu Glu Asn
            1525                1530                1535

Cys Gly Val Asp Thr Gly Lys Gln Ser Ile Ser Tyr Asp Leu His Thr
        1540                1545                1550

Glu Gln Cys Ile Ala Asp Lys Ser Ile Ala Asp Cys Val Glu Ala Leu
    1555                1560                1565

Leu Gly Cys Tyr Leu Thr Ser Cys Gly Glu Arg Ala Ala Gln Leu Phe
    1570                1575                1580

Leu Cys Ser Leu Gly Leu Lys Val Leu Pro Val Ile Lys Arg Thr Asp
1585                1590                1595                1600

Arg Glu Lys Ala Leu Cys Pro Thr Arg Glu Asn Phe Asn Ser Gln Gln
            1605                1610                1615

Lys Asn Leu Ser Val Ser Cys Ala Ala Ala Ser Val Ala Ser Ser Arg
        1620                1625                1630

Ser Ser Val Leu Lys Asp Ser Glu Tyr Gly Cys Leu Lys Ile Pro Pro
    1635                1640                1645

Arg Cys Met Phe Asp His Pro Asp Ala Asp Lys Thr Leu Asn His Leu
    1650                1655                1660

Ile Ser Gly Phe Glu Asn Phe Glu Lys Lys Ile Asn Tyr Arg Phe Lys
1665                1670                1675                1680

Asn Lys Ala Tyr Leu Leu Gln Ala Phe Thr His Ala Ser Tyr His Tyr
            1685                1690                1695

Asn Thr Ile Thr Asp Cys Tyr Gln Arg Leu Glu Phe Leu Gly Asp Ala
        1700                1705                1710

Ile Leu Asp Tyr Leu Ile Thr Lys His Leu Tyr Glu Asp Pro Arg Gln
    1715                1720                1725

His Ser Pro Gly Val Leu Thr Asp Leu Arg Ser Ala Leu Val Asn Asn
    1730                1735                1740

Thr Ile Phe Ala Ser Leu Ala Val Lys Tyr Asp Tyr His Lys Tyr Phe
1745                1750                1755                1760

Lys Ala Val Ser Pro Glu Leu Phe His Val Ile Asp Asp Phe Val Gln
            1765                1770                1775

Phe Gln Leu Glu Lys Asn Glu Met Gln Gly Met Asp Ser Glu Leu Arg
        1780                1785                1790

Arg Ser Glu Glu Asp Glu Glu Lys Glu Glu Asp Ile Glu Val Pro Lys
    1795                1800                1805

Ala Met Gly Asp Ile Phe Glu Ser Leu Ala Gly Ala Ile Tyr Met Asp
    1810                1815                1820

Ser Gly Met Ser Leu Glu Thr Val Trp Gln Val Tyr Tyr Pro Met Met
1825                1830                1835                1840

Arg Pro Leu Ile Glu Lys Phe Ser Ala Asn Val Pro Arg Ser Pro Val
            1845                1850                1855

Arg Glu Leu Leu Glu Met Glu Pro Glu Thr Ala Lys Phe Ser Pro Ala
        1860                1865                1870
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Glu Arg Thr Tyr Asp Gly Lys Val Arg Val Thr Val Glu Val Val Gly
        1875                1880                1885

Lys Gly Lys Phe Lys Gly Val Gly Arg Ser Tyr Arg Ile Ala Lys Ser
    1890                1895                1900

Ala Ala Ala Arg Arg Ala Leu Arg Ser Leu Lys Ala Asn Gln Pro Gln
1905                1910                1915                1920

Val Pro Asn Ser


<210> SEQ ID NO 3
<211> LENGTH: 6750
<212> TYPE: DNA
<213> ORGANISM: Drosophila melanogaster
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)...(6750)

<400> SEQUENCE: 3

atg gcg ttc cac tgg tgc gac aac aat ctg cac acc acc gtg ttc acg       48
Met Ala Phe His Trp Cys Asp Asn Asn Leu His Thr Thr Val Phe Thr
1               5                   10                  15

ccg cgc gac ttt cag gtg gag cta ctg gcc acc gcc tac gag cgg aac       96
Pro Arg Asp Phe Gln Val Glu Leu Leu Ala Thr Ala Tyr Glu Arg Asn
            20                  25                  30

acg att att tgc ctg ggc cat cga agt tcc aag gag ttt ata gcc ctc      144
Thr Ile Ile Cys Leu Gly His Arg Ser Ser Lys Glu Phe Ile Ala Leu
        35                  40                  45

aag ctg ctc cag gag ctg tcg cgt cga gca cgc cga cat ggt cgt gtc      192
Lys Leu Leu Gln Glu Leu Ser Arg Arg Ala Arg Arg His Gly Arg Val
    50                  55                  60

agt gtc tat ctc agt tgc gag gtt ggc acc agc acg gaa cca tgc tcc      240
Ser Val Tyr Leu Ser Cys Glu Val Gly Thr Ser Thr Glu Pro Cys Ser
65                  70                  75                  80

atc tac acg atg ctc acc cac ttg act gac ctg cgg gtg tgg cag gag      288
Ile Tyr Thr Met Leu Thr His Leu Thr Asp Leu Arg Val Trp Gln Glu
                85                  90                  95

cag ccg gat atg caa att ccc ttt gat cat tgc tgg acg gac tat cac      336
Gln Pro Asp Met Gln Ile Pro Phe Asp His Cys Trp Thr Asp Tyr His
            100                 105                 110

gtt tcc atc cta cgg cca gag gga ttt ctt tat ctg ctc gaa act cgc      384
Val Ser Ile Leu Arg Pro Glu Gly Phe Leu Tyr Leu Leu Glu Thr Arg
        115                 120                 125

gag ctg ctg ctg agc agc gtc gaa ctg atc gtg ctg gaa gat tgt cat      432
Glu Leu Leu Leu Ser Ser Val Glu Leu Ile Val Leu Glu Asp Cys His
    130                 135                 140

gac agc gcc gtt tat cag agg ata agg cct ctg ttc gag aat cac att      480
Asp Ser Ala Val Tyr Gln Arg Ile Arg Pro Leu Phe Glu Asn His Ile
145                 150                 155                 160

atg cca gcg cca ccg gcg gac agg cca cgg att ctc gga ctc gct gga      528
Met Pro Ala Pro Pro Ala Asp Arg Pro Arg Ile Leu Gly Leu Ala Gly
                165                 170                 175

ccg ctg cac agc gcc gga tgt gag ctg cag caa ctg agc gcc atg ctg      576
Pro Leu His Ser Ala Gly Cys Glu Leu Gln Gln Leu Ser Ala Met Leu
            180                 185                 190

gcc acc ctg gag cag agt gtg ctt tgc cag atc gag acg gcc agt gat      624
Ala Thr Leu Glu Gln Ser Val Leu Cys Gln Ile Glu Thr Ala Ser Asp
        195                 200                 205

att gtc acc gtg ttg cgt tac tgt tcc cga ccg cac gaa tac atc gta      672
Ile Val Thr Val Leu Arg Tyr Cys Ser Arg Pro His Glu Tyr Ile Val
    210                 215                 220

cag tgc gcc ccc ttc gag atg gac gaa ctg tcc ctg gtg ctt gcc gat      720
Gln Cys Ala Pro Phe Glu Met Asp Glu Leu Ser Leu Val Leu Ala Asp
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225                 230                 235                 240

gtg ctc aac aca cac aag tcc ttt tta ttg gac cac cgc tac gat ccc      768
Val Leu Asn Thr His Lys Ser Phe Leu Leu Asp His Arg Tyr Asp Pro
                245                 250                 255

tac gaa atc tac ggc aca gac cag ttt atg gac gaa ctg aaa gac ata      816
Tyr Glu Ile Tyr Gly Thr Asp Gln Phe Met Asp Glu Leu Lys Asp Ile
            260                 265                 270

ccc gat ccc aag gtg gac ccc ctg aac gtc atc aac tca cta ctg gtc      864
Pro Asp Pro Lys Val Asp Pro Leu Asn Val Ile Asn Ser Leu Leu Val
        275                 280                 285

gtg ctg cac gag atg ggt cct tgg tgc acg cag cgg gct gca cat cac      912
Val Leu His Glu Met Gly Pro Trp Cys Thr Gln Arg Ala Ala His His
    290                 295                 300

ttt tac caa tgc aat gag aag tta aag gtg aag acg ccg cac gaa cgt      960
Phe Tyr Gln Cys Asn Glu Lys Leu Lys Val Lys Thr Pro His Glu Arg
305                 310                 315                 320

cac tac ttg ctg tac tgc cta gtg agc acg gcc ctt atc caa ctg tac     1008
His Tyr Leu Leu Tyr Cys Leu Val Ser Thr Ala Leu Ile Gln Leu Tyr
                325                 330                 335

tcc ctc tgc gaa cac gca ttc cat cga cat tta gga agt ggc agc gat     1056
Ser Leu Cys Glu His Ala Phe His Arg His Leu Gly Ser Gly Ser Asp
            340                 345                 350

tca cgc cag acc atc gaa cgc tat tcc agc ccc aag gtg cga cgt ctg     1104
Ser Arg Gln Thr Ile Glu Arg Tyr Ser Ser Pro Lys Val Arg Arg Leu
        355                 360                 365

ttg cag aca ctg agg tgc ttc aag ccg gaa gag gtg cac acc caa gcg     1152
Leu Gln Thr Leu Arg Cys Phe Lys Pro Glu Glu Val His Thr Gln Ala
    370                 375                 380

gac gga ctg cgc aga atg cgg cat cag gtg gat cag gcg gac ttc aat     1200
Asp Gly Leu Arg Arg Met Arg His Gln Val Asp Gln Ala Asp Phe Asn
385                 390                 395                 400

cgg tta tct cat acg ctg gaa agc aag tgc cga atg gtg gat caa atg     1248
Arg Leu Ser His Thr Leu Glu Ser Lys Cys Arg Met Val Asp Gln Met
                405                 410                 415

gac caa ccg ccg acg gag aca cga gcc ctg gtg gcc act ctt gag cag     1296
Asp Gln Pro Pro Thr Glu Thr Arg Ala Leu Val Ala Thr Leu Glu Gln
            420                 425                 430

att ctg cac acg aca gag gac agg cag acg aac aga agc gcc gct cgg     1344
Ile Leu His Thr Thr Glu Asp Arg Gln Thr Asn Arg Ser Ala Ala Arg
        435                 440                 445

gtg act cct act cct act ccc gct cat gcg aag ccg aaa cct agc tct     1392
Val Thr Pro Thr Pro Thr Pro Ala His Ala Lys Pro Lys Pro Ser Ser
    450                 455                 460

ggt gcc aac act gca caa cca cga act cgt aga cgt gtg tac acc agg     1440
Gly Ala Asn Thr Ala Gln Pro Arg Thr Arg Arg Arg Val Tyr Thr Arg
465                 470                 475                 480

cgc cac cac cgg gat cac aat gat ggc agc gac acg ctc tgc gca ctg     1488
Arg His His Arg Asp His Asn Asp Gly Ser Asp Thr Leu Cys Ala Leu
                485                 490                 495

att tac tgc aac cag aac cac acg gct cgc gtg ctc ttt gag ctt cta     1536
Ile Tyr Cys Asn Gln Asn His Thr Ala Arg Val Leu Phe Glu Leu Leu
            500                 505                 510

gcg gag att agc aga cgt gat ccc gat ctc aag ttc cta cgc tgc cag     1584
Ala Glu Ile Ser Arg Arg Asp Pro Asp Leu Lys Phe Leu Arg Cys Gln
        515                 520                 525

tac acc acg gac cgg gtg gca gat ccc acc acg gag ccc aaa gag gct     1632
Tyr Thr Thr Asp Arg Val Ala Asp Pro Thr Thr Glu Pro Lys Glu Ala
    530                 535                 540

gag ttg gag cac cgg cgg cag gaa gag gtg cta aag cgc ttc cgc atg     1680
Glu Leu Glu His Arg Arg Gln Glu Glu Val Leu Lys Arg Phe Arg Met
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545                 550                 555                 560

cat gac tgc aat gtc ctg atc ggt act tcg gtg ctg gaa gag ggc atc      1728
His Asp Cys Asn Val Leu Ile Gly Thr Ser Val Leu Glu Glu Gly Ile
                565                 570                 575

gat gtg ccc aag tgc aat ttg gtt gtg cgc tgg gat ccg cca acc aca      1776
Asp Val Pro Lys Cys Asn Leu Val Val Arg Trp Asp Pro Pro Thr Thr
            580                 585                 590

tat cgc agt tac gtt cag tgc aaa ggt cga gcc cgt gct gct cca gcc      1824
Tyr Arg Ser Tyr Val Gln Cys Lys Gly Arg Ala Arg Ala Ala Pro Ala
        595                 600                 605

tat cat gtc att ctc gtc gct ccg agt tat aaa agc cca act gtg ggg      1872
Tyr His Val Ile Leu Val Ala Pro Ser Tyr Lys Ser Pro Thr Val Gly
    610                 615                 620

tca gtg cag ctg acc gat cgg agt cat cgg tat att tgc gcg act ggt      1920
Ser Val Gln Leu Thr Asp Arg Ser His Arg Tyr Ile Cys Ala Thr Gly
625                 630                 635                 640

gat act aca gag gcg gac agc gac tct gat gat tca gcg atg cca aac      1968
Asp Thr Thr Glu Ala Asp Ser Asp Ser Asp Asp Ser Ala Met Pro Asn
                645                 650                 655

tcg tcc ggc tcg gat ccc tat act ttt ggc acg gca cgc gga acc gtg      2016
Ser Ser Gly Ser Asp Pro Tyr Thr Phe Gly Thr Ala Arg Gly Thr Val
            660                 665                 670

aag atc ctc aac ccc gaa gtg ttc agt aaa caa cca ccg aca gcg tgc      2064
Lys Ile Leu Asn Pro Glu Val Phe Ser Lys Gln Pro Pro Thr Ala Cys
        675                 680                 685

gac att aag ctg cag gag atc cag gac gaa ttg cca gcc gca gcg cag      2112
Asp Ile Lys Leu Gln Glu Ile Gln Asp Glu Leu Pro Ala Ala Ala Gln
    690                 695                 700

ctg gat acg agc aac tcc agc gac gaa gcc gtc agc atg agt aac acg      2160
Leu Asp Thr Ser Asn Ser Ser Asp Glu Ala Val Ser Met Ser Asn Thr
705                 710                 715                 720

tct cca agc gag agc agt aca gaa caa aaa tcc aga cgc ttc cag tgc      2208
Ser Pro Ser Glu Ser Ser Thr Glu Gln Lys Ser Arg Arg Phe Gln Cys
                725                 730                 735

gag ctg agc tct tta acg gag cca gaa gac aca agt gat act aca gcc      2256
Glu Leu Ser Ser Leu Thr Glu Pro Glu Asp Thr Ser Asp Thr Thr Ala
            740                 745                 750

gaa atc gat act gct cat agt tta gcc agc acc acg aag gac ttg gtg      2304
Glu Ile Asp Thr Ala His Ser Leu Ala Ser Thr Thr Lys Asp Leu Val
        755                 760                 765

cat caa atg gca cag tat cgc gaa atc gag cag atg ctg cta tcc aag      2352
His Gln Met Ala Gln Tyr Arg Glu Ile Glu Gln Met Leu Leu Ser Lys
    770                 775                 780

tgc gcc aac aca gag ccg ccg gag cag gag cag agt gag gcg gaa cgt      2400
Cys Ala Asn Thr Glu Pro Pro Glu Gln Glu Gln Ser Glu Ala Glu Arg
785                 790                 795                 800

ttt agt gcc tgc ctg gcc gca tac cga ccc aag ccg cac ctg cta aca      2448
Phe Ser Ala Cys Leu Ala Ala Tyr Arg Pro Lys Pro His Leu Leu Thr
                805                 810                 815

ggc gcc tcc gtg gat ctg ggt tct gct ata gct ttg gtc aac aag tac      2496
Gly Ala Ser Val Asp Leu Gly Ser Ala Ile Ala Leu Val Asn Lys Tyr
            820                 825                 830

tgc gcc cga ctg cca agc gac acg ttc acc aag ttg acg gcg ttg tgg      2544
Cys Ala Arg Leu Pro Ser Asp Thr Phe Thr Lys Leu Thr Ala Leu Trp
        835                 840                 845

cgc tgc acc cga aac gaa agg gct gga gtg acc ctg ttt cag tac aca      2592
Arg Cys Thr Arg Asn Glu Arg Ala Gly Val Thr Leu Phe Gln Tyr Thr
    850                 855                 860

ctc cgt ctg ccc atc aac tcg cca ttg aag cat gac att gtg ggt ctt      2640
Leu Arg Leu Pro Ile Asn Ser Pro Leu Lys His Asp Ile Val Gly Leu
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865                 870                 875                 880

ccg atg cca act caa aca ttg gcc cgc cga ctg gct gcc ttg cag gct      2688
Pro Met Pro Thr Gln Thr Leu Ala Arg Arg Leu Ala Ala Leu Gln Ala
                885                 890                 895

tgc gtg gaa ctg cac agg atc ggt gag tta gac gat cag ttg cag cct      2736
Cys Val Glu Leu His Arg Ile Gly Glu Leu Asp Asp Gln Leu Gln Pro
            900                 905                 910

atc ggc aag gag gga ttt cgt gcc ctg gag ccg gac tgg gag tgc ttt      2784
Ile Gly Lys Glu Gly Phe Arg Ala Leu Glu Pro Asp Trp Glu Cys Phe
        915                 920                 925

gaa ctg gag cca gag gac gaa cag att gtg cag cta agc gat gaa cca      2832
Glu Leu Glu Pro Glu Asp Glu Gln Ile Val Gln Leu Ser Asp Glu Pro
    930                 935                 940

cgt ccg gga aca acg aag cgt cgt cag tac tat tac aaa cgc att gca      2880
Arg Pro Gly Thr Thr Lys Arg Arg Gln Tyr Tyr Tyr Lys Arg Ile Ala
945                 950                 955                 960

tcc gaa ttt tgc gat tgc cgt ccc gtt gcc gga gcg cca tgc tat ttg      2928
Ser Glu Phe Cys Asp Cys Arg Pro Val Ala Gly Ala Pro Cys Tyr Leu
                965                 970                 975

tac ttt atc caa ctg acg ctc caa tgt ccg att ccc gaa gag caa aac      2976
Tyr Phe Ile Gln Leu Thr Leu Gln Cys Pro Ile Pro Glu Glu Gln Asn
            980                 985                 990

acg cgg gga cgc aag att tat ccg ccc gaa gat gcg cag cag gga ttc      3024
Thr Arg Gly Arg Lys Ile Tyr Pro Pro Glu Asp Ala Gln Gln Gly Phe
        995                 1000                1005

ggc att cta acc acc aaa cgg ata ccc aag ctg agt gct ttc tcg ata      3072
Gly Ile Leu Thr Thr Lys Arg Ile Pro Lys Leu Ser Ala Phe Ser Ile
    1010                1015                1020

ttc acg cgt tcc ggt gag gtg aag gtt tcc ctg gag tta gct aag gaa      3120
Phe Thr Arg Ser Gly Glu Val Lys Val Ser Leu Glu Leu Ala Lys Glu
1025                1030                1035                1040

cgc gtg att cta act agc gaa caa ata gtc tgc atc aac gga ttt tta      3168
Arg Val Ile Leu Thr Ser Glu Gln Ile Val Cys Ile Asn Gly Phe Leu
                1045                1050                1055

aac tac acg ttc acc aat gta ctg cgt ttg caa aag ttt ctg atg ctc      3216
Asn Tyr Thr Phe Thr Asn Val Leu Arg Leu Gln Lys Phe Leu Met Leu
            1060                1065                1070

ttc gat ccg gac tcc acg gaa aat tgt gta ttc att gtg ccc acc gtg      3264
Phe Asp Pro Asp Ser Thr Glu Asn Cys Val Phe Ile Val Pro Thr Val
        1075                1080                1085

aag gca cca gct ggc ggc aag cac atc gac tgg cag ttt ctg gag ctg      3312
Lys Ala Pro Ala Gly Gly Lys His Ile Asp Trp Gln Phe Leu Glu Leu
    1090                1095                1100

atc caa gcg aat gga aat aca atg cca cgg gca gtg ccc gat gag gag      3360
Ile Gln Ala Asn Gly Asn Thr Met Pro Arg Ala Val Pro Asp Glu Glu
1105                1110                1115                1120

cgc cag gcg cag ccg ttt gat ccg caa cgc ttc cag gat gcc gtc gtt      3408
Arg Gln Ala Gln Pro Phe Asp Pro Gln Arg Phe Gln Asp Ala Val Val
                1125                1130                1135

atg ccg tgg tat cgc aac cag gat caa ccg cag tat ttc tat gtg gcg      3456
Met Pro Trp Tyr Arg Asn Gln Asp Gln Pro Gln Tyr Phe Tyr Val Ala
            1140                1145                1150

gag ata tgt cca cat cta tcc cca ctc agc tgc ttc cct ggt gac aac      3504
Glu Ile Cys Pro His Leu Ser Pro Leu Ser Cys Phe Pro Gly Asp Asn
        1155                1160                1165

tac cgc acg ttc aag cac tac tac ctc gtc aag tat ggt ctg acc ata      3552
Tyr Arg Thr Phe Lys His Tyr Tyr Leu Val Lys Tyr Gly Leu Thr Ile
    1170                1175                1180

cag aat acc tcg cag ccg cta ttg gac gtg gat cac acc agt gcg cgg      3600
Gln Asn Thr Ser Gln Pro Leu Leu Asp Val Asp His Thr Ser Ala Arg
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1185                1190                1195                1200

tta aac ttc ctc acg cca cga tac gtt aat cgc aag ggc gtt gct ctg      3648
Leu Asn Phe Leu Thr Pro Arg Tyr Val Asn Arg Lys Gly Val Ala Leu
                1205                1210                1215

ccc act agt tcg gag gag aca aag cgg gca aag cgc gag aat ctc gaa      3696
Pro Thr Ser Ser Glu Glu Thr Lys Arg Ala Lys Arg Glu Asn Leu Glu
            1220                1225                1230

cag aag cag atc ctt gtg cca gag ctc tgc act gtg cat cca ttc ccc      3744
Gln Lys Gln Ile Leu Val Pro Glu Leu Cys Thr Val His Pro Phe Pro
        1235                1240                1245

gcc tcc ttg tgg cga act gcc gtg tgc ctg ccc tgc atc ctg tac cgc      3792
Ala Ser Leu Trp Arg Thr Ala Val Cys Leu Pro Cys Ile Leu Tyr Arg
    1250                1255                1260

ata aat ggt ctt cta ttg gcc gac gat att cgg aaa cag gtt tct gcg      3840
Ile Asn Gly Leu Leu Leu Ala Asp Asp Ile Arg Lys Gln Val Ser Ala
1265                1270                1275                1280

gat ctg ggg ctg gga agg caa cag atc gaa gat gag gat ttc gag tgg      3888
Asp Leu Gly Leu Gly Arg Gln Gln Ile Glu Asp Glu Asp Phe Glu Trp
                1285                1290                1295

ccc atg ctg gac ttt ggg tgg agt cta tcg gag gtg ctc aag aaa tcg      3936
Pro Met Leu Asp Phe Gly Trp Ser Leu Ser Glu Val Leu Lys Lys Ser
            1300                1305                1310

cgg gag tcc aaa caa aag gag tcc ctt aag gat gat act att aat ggc      3984
Arg Glu Ser Lys Gln Lys Glu Ser Leu Lys Asp Asp Thr Ile Asn Gly
        1315                1320                1325

aaa gac tta gct gat gtt gaa aag aaa ccg act agc gag gag acc caa      4032
Lys Asp Leu Ala Asp Val Glu Lys Lys Pro Thr Ser Glu Glu Thr Gln
    1330                1335                1340

cta gat aag gat tca aaa gac gat aag gtt gag aaa agt gct att gaa      4080
Leu Asp Lys Asp Ser Lys Asp Asp Lys Val Glu Lys Ser Ala Ile Glu
1345                1350                1355                1360

cta atc att gag gga gag gag aag ctg caa gag gct gat gac ttc att      4128
Leu Ile Ile Glu Gly Glu Glu Lys Leu Gln Glu Ala Asp Asp Phe Ile
                1365                1370                1375

gag ata ggc act tgg tca aac gat atg gcc gac gat ata gct agt ttt      4176
Glu Ile Gly Thr Trp Ser Asn Asp Met Ala Asp Asp Ile Ala Ser Phe
            1380                1385                1390

aac caa gaa gac gac gac gag gat gac gcc ttc cat ctc cca gtt tta      4224
Asn Gln Glu Asp Asp Asp Glu Asp Asp Ala Phe His Leu Pro Val Leu
        1395                1400                1405

ccg gca aac gtt aag ttc tgt gat cag caa acg cgc tac ggt tcg ccc      4272
Pro Ala Asn Val Lys Phe Cys Asp Gln Gln Thr Arg Tyr Gly Ser Pro
    1410                1415                1420

aca ttt tgg gat gtg agc aat ggc gaa agc ggc ttc aag ggt cca aag      4320
Thr Phe Trp Asp Val Ser Asn Gly Glu Ser Gly Phe Lys Gly Pro Lys
1425                1430                1435                1440

agc agt cag aat aag cag ggt ggc aag ggc aaa gca aag ggt ccg gca      4368
Ser Ser Gln Asn Lys Gln Gly Gly Lys Gly Lys Ala Lys Gly Pro Ala
                1445                1450                1455

aag ccc aca ttt aac tat tat gac tcg gac aat tcg ctg ggt tcc agc      4416
Lys Pro Thr Phe Asn Tyr Tyr Asp Ser Asp Asn Ser Leu Gly Ser Ser
            1460                1465                1470

tac gat gac gac gat aac gca ggt ccg ctc aat tac atg cat cac aac      4464
Tyr Asp Asp Asp Asp Asn Ala Gly Pro Leu Asn Tyr Met His His Asn
        1475                1480                1485

tac agt tcg gat gac gac gat gtg gca gat gat atc gat gcg gga cgc      4512
Tyr Ser Ser Asp Asp Asp Asp Val Ala Asp Asp Ile Asp Ala Gly Arg
    1490                1495                1500

att gcg ttc acc tcc aag aat gaa gcg gag act att gaa acc gca cag      4560
Ile Ala Phe Thr Ser Lys Asn Glu Ala Glu Thr Ile Glu Thr Ala Gln
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1505                1510                1515                1520

gaa gtg gaa aag cgc cag aag cag ctg tcc atc atc cag gcg acc aat      4608
Glu Val Glu Lys Arg Gln Lys Gln Leu Ser Ile Ile Gln Ala Thr Asn
                1525                1530                1535

gct aac gag cgg cag tat cag cag aca aag aac ctg ctc att gga ttc      4656
Ala Asn Glu Arg Gln Tyr Gln Gln Thr Lys Asn Leu Leu Ile Gly Phe
            1540                1545                1550

aat ttt aag cat gag gac cag aag gaa cct gcc act ata aga tat gaa      4704
Asn Phe Lys His Glu Asp Gln Lys Glu Pro Ala Thr Ile Arg Tyr Glu
        1555                1560                1565

gaa tcc ata gct aag ctc aaa acg gaa ata gaa tcc ggc ggc atg ttg      4752
Glu Ser Ile Ala Lys Leu Lys Thr Glu Ile Glu Ser Gly Gly Met Leu
    1570                1575                1580

gtg ccg cac gac cag cag ttg gtt cta aaa aga agt gat gcc gct gag      4800
Val Pro His Asp Gln Gln Leu Val Leu Lys Arg Ser Asp Ala Ala Glu
1585                1590                1595                1600

gct cag gtt gca aag gta tcg atg atg gag cta ttg aag cag ctg ctg      4848
Ala Gln Val Ala Lys Val Ser Met Met Glu Leu Leu Lys Gln Leu Leu
                1605                1610                1615

ccg tat gta aat gaa gat gtg ctg gcc aaa aag ctg ggt gat agg cgc      4896
Pro Tyr Val Asn Glu Asp Val Leu Ala Lys Lys Leu Gly Asp Arg Arg
            1620                1625                1630

gag ctt ctg ctg tcg gat ttg gta gag cta aat gca gat tgg gta gcg      4944
Glu Leu Leu Leu Ser Asp Leu Val Glu Leu Asn Ala Asp Trp Val Ala
        1635                1640                1645

cga cat gag cag gag acc tac aat gta atg gga tgc gga gat agt ttt      4992
Arg His Glu Gln Glu Thr Tyr Asn Val Met Gly Cys Gly Asp Ser Phe
    1650                1655                1660

gac aac tat aac gat cat cat cgg ctg aac ttg gat gaa aag caa ctg      5040
Asp Asn Tyr Asn Asp His His Arg Leu Asn Leu Asp Glu Lys Gln Leu
1665                1670                1675                1680

aaa ctg caa tac gaa cga att gaa att gag cca cct act tcc acg aag      5088
Lys Leu Gln Tyr Glu Arg Ile Glu Ile Glu Pro Pro Thr Ser Thr Lys
                1685                1690                1695

gcc ata acc tca gcc ata tta cca gct ggc ttc agt ttc gat cga caa      5136
Ala Ile Thr Ser Ala Ile Leu Pro Ala Gly Phe Ser Phe Asp Arg Gln
            1700                1705                1710

ccg gat cta gtg ggc cat cca gga ccc agt ccc agc atc att ttg caa      5184
Pro Asp Leu Val Gly His Pro Gly Pro Ser Pro Ser Ile Ile Leu Gln
        1715                1720                1725

gcc ctc aca atg tcc aat gct aac gat ggc atc aat ctg gag cga ctg      5232
Ala Leu Thr Met Ser Asn Ala Asn Asp Gly Ile Asn Leu Glu Arg Leu
    1730                1735                1740

gag aca att gga gat tcc ttt cta aag tat gcc att acc acc tac ttg      5280
Glu Thr Ile Gly Asp Ser Phe Leu Lys Tyr Ala Ile Thr Thr Tyr Leu
1745                1750                1755                1760

tac atc acc tac gag aat gtg cac gag gga aaa cta agt cac ctg cgc      5328
Tyr Ile Thr Tyr Glu Asn Val His Glu Gly Lys Leu Ser His Leu Arg
                1765                1770                1775

tcc aag cag gtt gcc aat ctc aat ctc tat cgt ctg ggc aga cgt aag      5376
Ser Lys Gln Val Ala Asn Leu Asn Leu Tyr Arg Leu Gly Arg Arg Lys
            1780                1785                1790

aga ctg ggt gaa tat atg ata gcc act aaa ttc gag ccg cac gac aat      5424
Arg Leu Gly Glu Tyr Met Ile Ala Thr Lys Phe Glu Pro His Asp Asn
        1795                1800                1805

tgg ctg cca ccc tgc tac tac gtg cca aag gag cta gag aag gcg ctc      5472
Trp Leu Pro Pro Cys Tyr Tyr Val Pro Lys Glu Leu Glu Lys Ala Leu
    1810                1815                1820

atc gag gcg aag atc ccc act cac cat tgg aag ctg gcc gat ctg cta      5520
Ile Glu Ala Lys Ile Pro Thr His His Trp Lys Leu Ala Asp Leu Leu
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1825                1830                1835                1840

gac att aag aac cta agc agt gtg caa atc tgc gag atg gtt cgc gaa      5568
Asp Ile Lys Asn Leu Ser Ser Val Gln Ile Cys Glu Met Val Arg Glu
                1845                1850                1855

aaa gcc gat gcc ctg ggc ttg gag cag aat ggg ggt gcc caa aat gga      5616
Lys Ala Asp Ala Leu Gly Leu Glu Gln Asn Gly Gly Ala Gln Asn Gly
            1860                1865                1870

caa ctt gac gac tcc aat gat agc tgc aat gat ttt agc tgt ttt att      5664
Gln Leu Asp Asp Ser Asn Asp Ser Cys Asn Asp Phe Ser Cys Phe Ile
        1875                1880                1885

ccc tac aac ctt gtt tcg caa cac agc att ccg gat aag tct att gcc      5712
Pro Tyr Asn Leu Val Ser Gln His Ser Ile Pro Asp Lys Ser Ile Ala
    1890                1895                1900

gat tgc gtc gaa gcc ctc att gga gcc tat ctc att gag tgc gga ccc      5760
Asp Cys Val Glu Ala Leu Ile Gly Ala Tyr Leu Ile Glu Cys Gly Pro
1905                1910                1915                1920

cga ggg gct tta ctc ttt atg gcc tgg ctg ggc gtg aga gtg ctc cct      5808
Arg Gly Ala Leu Leu Phe Met Ala Trp Leu Gly Val Arg Val Leu Pro
                1925                1930                1935

atc aca agg cag ttg gac ggg ggt aac cag gag caa cga ata ccc ggt      5856
Ile Thr Arg Gln Leu Asp Gly Gly Asn Gln Glu Gln Arg Ile Pro Gly
            1940                1945                1950

agc aca aaa ccg aat gcc gaa aat gtg gtc acc gtt tac ggt gca tgg      5904
Ser Thr Lys Pro Asn Ala Glu Asn Val Val Thr Val Tyr Gly Ala Trp
        1955                1960                1965

ccc acg ccg cgt agt cca ctg ctg cac ttt gct cca aat gct acg gag      5952
Pro Thr Pro Arg Ser Pro Leu Leu His Phe Ala Pro Asn Ala Thr Glu
    1970                1975                1980

gag ctg gac cag tta cta agc ggc ttt gag gag ttt gag gag agt ttg      6000
Glu Leu Asp Gln Leu Leu Ser Gly Phe Glu Glu Phe Glu Glu Ser Leu
1985                1990                1995                2000

gga tac aag ttc cgg gat cgg tcg tac ctg ttg caa gcc atg aca cat      6048
Gly Tyr Lys Phe Arg Asp Arg Ser Tyr Leu Leu Gln Ala Met Thr His
                2005                2010                2015

gcc agt tac acg ccc aat cga ttg acg gat tgc tat cag cgt ctg gag      6096
Ala Ser Tyr Thr Pro Asn Arg Leu Thr Asp Cys Tyr Gln Arg Leu Glu
            2020                2025                2030

ttc ctg ggc gat gct gtt cta gat tac ctc att acg cgg cat tta tac      6144
Phe Leu Gly Asp Ala Val Leu Asp Tyr Leu Ile Thr Arg His Leu Tyr
        2035                2040                2045

gaa gat ccc cgc cag cat tct cca ggc gca tta acg gat ttg cgg tca      6192
Glu Asp Pro Arg Gln His Ser Pro Gly Ala Leu Thr Asp Leu Arg Ser
    2050                2055                2060

gca ctg gtg aat aat aca ata ttc gcc tcc ctg gct gtt cgc cat ggc      6240
Ala Leu Val Asn Asn Thr Ile Phe Ala Ser Leu Ala Val Arg His Gly
2065                2070                2075                2080

ttc cac aag ttc ttc cgg cac ctc tcg ccg ggc ctt aac gat gtg att      6288
Phe His Lys Phe Phe Arg His Leu Ser Pro Gly Leu Asn Asp Val Ile
                2085                2090                2095

gac cgt ttt gtg cgg atc cag cag gag aat gga cac tgc atc agt gag      6336
Asp Arg Phe Val Arg Ile Gln Gln Glu Asn Gly His Cys Ile Ser Glu
            2100                2105                2110

gag tac tac tta ttg tcc gag gag gag tgc gat gac gcc gag gac gtt      6384
Glu Tyr Tyr Leu Leu Ser Glu Glu Glu Cys Asp Asp Ala Glu Asp Val
        2115                2120                2125

gag gtg ccc aag gca ttg ggc gac gtt ttc gag tcg atc gca ggt gcc      6432
Glu Val Pro Lys Ala Leu Gly Asp Val Phe Glu Ser Ile Ala Gly Ala
    2130                2135                2140

att ttt ctc gac tca aac atg tcg ctg gac gtg gtt tgg cac gta tat      6480
Ile Phe Leu Asp Ser Asn Met Ser Leu Asp Val Val Trp His Val Tyr
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2145                2150                2155                2160

agc aac atg atg agc ccg gag atc gag cag ttc agc aac tca gtg cca      6528
Ser Asn Met Met Ser Pro Glu Ile Glu Gln Phe Ser Asn Ser Val Pro
                2165                2170                2175

aaa tcg ccc att cgg gag ctc ctc gag ctg gag ccg gaa acc gcc aag      6576
Lys Ser Pro Ile Arg Glu Leu Leu Glu Leu Glu Pro Glu Thr Ala Lys
            2180                2185                2190

ttc ggc aag ccc gag aag ctg gcg gat ggg cga cgg gtg cgc gtt acc      6624
Phe Gly Lys Pro Glu Lys Leu Ala Asp Gly Arg Arg Val Arg Val Thr
        2195                2200                2205

gtg gat gtc ttc tgc aaa gga acc ttc cgt ggc atc gga cgc aac tat      6672
Val Asp Val Phe Cys Lys Gly Thr Phe Arg Gly Ile Gly Arg Asn Tyr
    2210                2215                2220

cgc att gcc aag tgc acg gcg gcc aaa tgc gca ttg cgc caa ctc aaa      6720
Arg Ile Ala Lys Cys Thr Ala Ala Lys Cys Ala Leu Arg Gln Leu Lys
2225                2230                2235                2240

aag cag ggc ttg ata gcc aaa aaa gac taa                              6750
Lys Gln Gly Leu Ile Ala Lys Lys Asp  *
                2245


<210> SEQ ID NO 4
<211> LENGTH: 2249
<212> TYPE: PRT
<213> ORGANISM: Drosophila melanogaster

<400> SEQUENCE: 4

Met Ala Phe His Trp Cys Asp Asn Asn Leu His Thr Thr Val Phe Thr
1               5                   10                  15

Pro Arg Asp Phe Gln Val Glu Leu Leu Ala Thr Ala Tyr Glu Arg Asn
            20                  25                  30

Thr Ile Ile Cys Leu Gly His Arg Ser Ser Lys Glu Phe Ile Ala Leu
        35                  40                  45

Lys Leu Leu Gln Glu Leu Ser Arg Arg Ala Arg Arg His Gly Arg Val
    50                  55                  60

Ser Val Tyr Leu Ser Cys Glu Val Gly Thr Ser Thr Glu Pro Cys Ser
65                  70                  75                  80

Ile Tyr Thr Met Leu Thr His Leu Thr Asp Leu Arg Val Trp Gln Glu
                85                  90                  95

Gln Pro Asp Met Gln Ile Pro Phe Asp His Cys Trp Thr Asp Tyr His
            100                 105                 110

Val Ser Ile Leu Arg Pro Glu Gly Phe Leu Tyr Leu Leu Glu Thr Arg
        115                 120                 125

Glu Leu Leu Leu Ser Ser Val Glu Leu Ile Val Leu Glu Asp Cys His
    130                 135                 140

Asp Ser Ala Val Tyr Gln Arg Ile Arg Pro Leu Phe Glu Asn His Ile
145                 150                 155                 160

Met Pro Ala Pro Pro Ala Asp Arg Pro Arg Ile Leu Gly Leu Ala Gly
                165                 170                 175

Pro Leu His Ser Ala Gly Cys Glu Leu Gln Gln Leu Ser Ala Met Leu
            180                 185                 190

Ala Thr Leu Glu Gln Ser Val Leu Cys Gln Ile Glu Thr Ala Ser Asp
        195                 200                 205

Ile Val Thr Val Leu Arg Tyr Cys Ser Arg Pro His Glu Tyr Ile Val
    210                 215                 220

Gln Cys Ala Pro Phe Glu Met Asp Glu Leu Ser Leu Val Leu Ala Asp
225                 230                 235                 240

Val Leu Asn Thr His Lys Ser Phe Leu Leu Asp His Arg Tyr Asp Pro
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                245                 250                 255

Tyr Glu Ile Tyr Gly Thr Asp Gln Phe Met Asp Glu Leu Lys Asp Ile
            260                 265                 270

Pro Asp Pro Lys Val Asp Pro Leu Asn Val Ile Asn Ser Leu Leu Val
        275                 280                 285

Val Leu His Glu Met Gly Pro Trp Cys Thr Gln Arg Ala Ala His His
    290                 295                 300

Phe Tyr Gln Cys Asn Glu Lys Leu Lys Val Lys Thr Pro His Glu Arg
305                 310                 315                 320

His Tyr Leu Leu Tyr Cys Leu Val Ser Thr Ala Leu Ile Gln Leu Tyr
                325                 330                 335

Ser Leu Cys Glu His Ala Phe His Arg His Leu Gly Ser Gly Ser Asp
            340                 345                 350

Ser Arg Gln Thr Ile Glu Arg Tyr Ser Ser Pro Lys Val Arg Arg Leu
        355                 360                 365

Leu Gln Thr Leu Arg Cys Phe Lys Pro Glu Glu Val His Thr Gln Ala
    370                 375                 380

Asp Gly Leu Arg Arg Met Arg His Gln Val Asp Gln Ala Asp Phe Asn
385                 390                 395                 400

Arg Leu Ser His Thr Leu Glu Ser Lys Cys Arg Met Val Asp Gln Met
                405                 410                 415

Asp Gln Pro Pro Thr Glu Thr Arg Ala Leu Val Ala Thr Leu Glu Gln
            420                 425                 430

Ile Leu His Thr Thr Glu Asp Arg Gln Thr Asn Arg Ser Ala Ala Arg
        435                 440                 445

Val Thr Pro Thr Pro Thr Pro Ala His Ala Lys Pro Lys Pro Ser Ser
    450                 455                 460

Gly Ala Asn Thr Ala Gln Pro Arg Thr Arg Arg Arg Val Tyr Thr Arg
465                 470                 475                 480

Arg His His Arg Asp His Asn Asp Gly Ser Asp Thr Leu Cys Ala Leu
                485                 490                 495

Ile Tyr Cys Asn Gln Asn His Thr Ala Arg Val Leu Phe Glu Leu Leu
            500                 505                 510

Ala Glu Ile Ser Arg Arg Asp Pro Asp Leu Lys Phe Leu Arg Cys Gln
        515                 520                 525

Tyr Thr Thr Asp Arg Val Ala Asp Pro Thr Thr Glu Pro Lys Glu Ala
    530                 535                 540

Glu Leu Glu His Arg Arg Gln Glu Glu Val Leu Lys Arg Phe Arg Met
545                 550                 555                 560

His Asp Cys Asn Val Leu Ile Gly Thr Ser Val Leu Glu Glu Gly Ile
                565                 570                 575

Asp Val Pro Lys Cys Asn Leu Val Val Arg Trp Asp Pro Pro Thr Thr
            580                 585                 590

Tyr Arg Ser Tyr Val Gln Cys Lys Gly Arg Ala Arg Ala Ala Pro Ala
        595                 600                 605

Tyr His Val Ile Leu Val Ala Pro Ser Tyr Lys Ser Pro Thr Val Gly
    610                 615                 620

Ser Val Gln Leu Thr Asp Arg Ser His Arg Tyr Ile Cys Ala Thr Gly
625                 630                 635                 640

Asp Thr Thr Glu Ala Asp Ser Asp Ser Asp Asp Ser Ala Met Pro Asn
                645                 650                 655

Ser Ser Gly Ser Asp Pro Tyr Thr Phe Gly Thr Ala Arg Gly Thr Val
            660                 665                 670
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Lys Ile Leu Asn Pro Glu Val Phe Ser Lys Gln Pro Pro Thr Ala Cys
        675                 680                 685

Asp Ile Lys Leu Gln Glu Ile Gln Asp Glu Leu Pro Ala Ala Ala Gln
    690                 695                 700

Leu Asp Thr Ser Asn Ser Ser Asp Glu Ala Val Ser Met Ser Asn Thr
705                 710                 715                 720

Ser Pro Ser Glu Ser Ser Thr Glu Gln Lys Ser Arg Arg Phe Gln Cys
                725                 730                 735

Glu Leu Ser Ser Leu Thr Glu Pro Glu Asp Thr Ser Asp Thr Thr Ala
            740                 745                 750

Glu Ile Asp Thr Ala His Ser Leu Ala Ser Thr Thr Lys Asp Leu Val
        755                 760                 765

His Gln Met Ala Gln Tyr Arg Glu Ile Glu Gln Met Leu Leu Ser Lys
    770                 775                 780

Cys Ala Asn Thr Glu Pro Pro Glu Gln Glu Gln Ser Glu Ala Glu Arg
785                 790                 795                 800

Phe Ser Ala Cys Leu Ala Ala Tyr Arg Pro Lys Pro His Leu Leu Thr
                805                 810                 815

Gly Ala Ser Val Asp Leu Gly Ser Ala Ile Ala Leu Val Asn Lys Tyr
            820                 825                 830

Cys Ala Arg Leu Pro Ser Asp Thr Phe Thr Lys Leu Thr Ala Leu Trp
        835                 840                 845

Arg Cys Thr Arg Asn Glu Arg Ala Gly Val Thr Leu Phe Gln Tyr Thr
    850                 855                 860

Leu Arg Leu Pro Ile Asn Ser Pro Leu Lys His Asp Ile Val Gly Leu
865                 870                 875                 880

Pro Met Pro Thr Gln Thr Leu Ala Arg Arg Leu Ala Ala Leu Gln Ala
                885                 890                 895

Cys Val Glu Leu His Arg Ile Gly Glu Leu Asp Asp Gln Leu Gln Pro
            900                 905                 910

Ile Gly Lys Glu Gly Phe Arg Ala Leu Glu Pro Asp Trp Glu Cys Phe
        915                 920                 925

Glu Leu Glu Pro Glu Asp Glu Gln Ile Val Gln Leu Ser Asp Glu Pro
    930                 935                 940

Arg Pro Gly Thr Thr Lys Arg Arg Gln Tyr Tyr Tyr Lys Arg Ile Ala
945                 950                 955                 960

Ser Glu Phe Cys Asp Cys Arg Pro Val Ala Gly Ala Pro Cys Tyr Leu
                965                 970                 975

Tyr Phe Ile Gln Leu Thr Leu Gln Cys Pro Ile Pro Glu Glu Gln Asn
            980                 985                 990

Thr Arg Gly Arg Lys Ile Tyr Pro Pro Glu Asp Ala Gln Gln Gly Phe
        995                 1000                1005

Gly Ile Leu Thr Thr Lys Arg Ile Pro Lys Leu Ser Ala Phe Ser Ile
    1010                1015                1020

Phe Thr Arg Ser Gly Glu Val Lys Val Ser Leu Glu Leu Ala Lys Glu
1025                1030                1035                1040

Arg Val Ile Leu Thr Ser Glu Gln Ile Val Cys Ile Asn Gly Phe Leu
                1045                1050                1055

Asn Tyr Thr Phe Thr Asn Val Leu Arg Leu Gln Lys Phe Leu Met Leu
            1060                1065                1070

Phe Asp Pro Asp Ser Thr Glu Asn Cys Val Phe Ile Val Pro Thr Val
        1075                1080                1085

Lys Ala Pro Ala Gly Gly Lys His Ile Asp Trp Gln Phe Leu Glu Leu
    1090                1095                1100
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Ile Gln Ala Asn Gly Asn Thr Met Pro Arg Ala Val Pro Asp Glu Glu
1105                1110                1115                1120

Arg Gln Ala Gln Pro Phe Asp Pro Gln Arg Phe Gln Asp Ala Val Val
                1125                1130                1135

Met Pro Trp Tyr Arg Asn Gln Asp Gln Pro Gln Tyr Phe Tyr Val Ala
            1140                1145                1150

Glu Ile Cys Pro His Leu Ser Pro Leu Ser Cys Phe Pro Gly Asp Asn
        1155                1160                1165

Tyr Arg Thr Phe Lys His Tyr Tyr Leu Val Lys Tyr Gly Leu Thr Ile
    1170                1175                1180

Gln Asn Thr Ser Gln Pro Leu Leu Asp Val Asp His Thr Ser Ala Arg
1185                1190                1195                1200

Leu Asn Phe Leu Thr Pro Arg Tyr Val Asn Arg Lys Gly Val Ala Leu
                1205                1210                1215

Pro Thr Ser Ser Glu Glu Thr Lys Arg Ala Lys Arg Glu Asn Leu Glu
            1220                1225                1230

Gln Lys Gln Ile Leu Val Pro Glu Leu Cys Thr Val His Pro Phe Pro
        1235                1240                1245

Ala Ser Leu Trp Arg Thr Ala Val Cys Leu Pro Cys Ile Leu Tyr Arg
    1250                1255                1260

Ile Asn Gly Leu Leu Leu Ala Asp Asp Ile Arg Lys Gln Val Ser Ala
1265                1270                1275                1280

Asp Leu Gly Leu Gly Arg Gln Gln Ile Glu Asp Glu Asp Phe Glu Trp
                1285                1290                1295

Pro Met Leu Asp Phe Gly Trp Ser Leu Ser Glu Val Leu Lys Lys Ser
            1300                1305                1310

Arg Glu Ser Lys Gln Lys Glu Ser Leu Lys Asp Asp Thr Ile Asn Gly
        1315                1320                1325

Lys Asp Leu Ala Asp Val Glu Lys Lys Pro Thr Ser Glu Glu Thr Gln
    1330                1335                1340

Leu Asp Lys Asp Ser Lys Asp Asp Lys Val Glu Lys Ser Ala Ile Glu
1345                1350                1355                1360

Leu Ile Ile Glu Gly Glu Glu Lys Leu Gln Glu Ala Asp Asp Phe Ile
                1365                1370                1375

Glu Ile Gly Thr Trp Ser Asn Asp Met Ala Asp Asp Ile Ala Ser Phe
            1380                1385                1390

Asn Gln Glu Asp Asp Asp Glu Asp Asp Ala Phe His Leu Pro Val Leu
        1395                1400                1405

Pro Ala Asn Val Lys Phe Cys Asp Gln Gln Thr Arg Tyr Gly Ser Pro
    1410                1415                1420

Thr Phe Trp Asp Val Ser Asn Gly Glu Ser Gly Phe Lys Gly Pro Lys
1425                1430                1435                1440

Ser Ser Gln Asn Lys Gln Gly Gly Lys Gly Lys Ala Lys Gly Pro Ala
                1445                1450                1455

Lys Pro Thr Phe Asn Tyr Tyr Asp Ser Asp Asn Ser Leu Gly Ser Ser
            1460                1465                1470

Tyr Asp Asp Asp Asp Asn Ala Gly Pro Leu Asn Tyr Met His His Asn
        1475                1480                1485

Tyr Ser Ser Asp Asp Asp Asp Val Ala Asp Asp Ile Asp Ala Gly Arg
    1490                1495                1500

Ile Ala Phe Thr Ser Lys Asn Glu Ala Glu Thr Ile Glu Thr Ala Gln
1505                1510                1515                1520

Glu Val Glu Lys Arg Gln Lys Gln Leu Ser Ile Ile Gln Ala Thr Asn
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            1525                1530                1535

Ala Asn Glu Arg Gln Tyr Gln Gln Thr Lys Asn Leu Leu Ile Gly Phe
        1540                1545                1550

Asn Phe Lys His Glu Asp Gln Lys Glu Pro Ala Thr Ile Arg Tyr Glu
    1555                1560                1565

Glu Ser Ile Ala Lys Leu Lys Thr Glu Ile Glu Ser Gly Gly Met Leu
    1570                1575                1580

Val Pro His Asp Gln Gln Leu Val Leu Lys Arg Ser Asp Ala Ala Glu
1585                1590                1595                1600

Ala Gln Val Ala Lys Val Ser Met Met Glu Leu Leu Lys Gln Leu Leu
            1605                1610                1615

Pro Tyr Val Asn Glu Asp Val Leu Ala Lys Lys Leu Gly Asp Arg Arg
        1620                1625                1630

Glu Leu Leu Leu Ser Asp Leu Val Glu Leu Asn Ala Asp Trp Val Ala
    1635                1640                1645

Arg His Glu Gln Glu Thr Tyr Asn Val Met Gly Cys Gly Asp Ser Phe
    1650                1655                1660

Asp Asn Tyr Asn Asp His His Arg Leu Asn Leu Asp Glu Lys Gln Leu
1665                1670                1675                1680

Lys Leu Gln Tyr Glu Arg Ile Glu Ile Glu Pro Pro Thr Ser Thr Lys
            1685                1690                1695

Ala Ile Thr Ser Ala Ile Leu Pro Ala Gly Phe Ser Phe Asp Arg Gln
        1700                1705                1710

Pro Asp Leu Val Gly His Pro Gly Pro Ser Pro Ser Ile Ile Leu Gln
    1715                1720                1725

Ala Leu Thr Met Ser Asn Ala Asn Asp Gly Ile Asn Leu Glu Arg Leu
    1730                1735                1740

Glu Thr Ile Gly Asp Ser Phe Leu Lys Tyr Ala Ile Thr Thr Tyr Leu
1745                1750                1755                1760

Tyr Ile Thr Tyr Glu Asn Val His Glu Gly Lys Leu Ser His Leu Arg
            1765                1770                1775

Ser Lys Gln Val Ala Asn Leu Asn Leu Tyr Arg Leu Gly Arg Arg Lys
        1780                1785                1790

Arg Leu Gly Glu Tyr Met Ile Ala Thr Lys Phe Glu Pro His Asp Asn
    1795                1800                1805

Trp Leu Pro Pro Cys Tyr Tyr Val Pro Lys Glu Leu Glu Lys Ala Leu
    1810                1815                1820

Ile Glu Ala Lys Ile Pro Thr His His Trp Lys Leu Ala Asp Leu Leu
1825                1830                1835                1840

Asp Ile Lys Asn Leu Ser Ser Val Gln Ile Cys Glu Met Val Arg Glu
            1845                1850                1855

Lys Ala Asp Ala Leu Gly Leu Glu Gln Asn Gly Gly Ala Gln Asn Gly
        1860                1865                1870

Gln Leu Asp Asp Ser Asn Asp Ser Cys Asn Asp Phe Ser Cys Phe Ile
    1875                1880                1885

Pro Tyr Asn Leu Val Ser Gln His Ser Ile Pro Asp Lys Ser Ile Ala
    1890                1895                1900

Asp Cys Val Glu Ala Leu Ile Gly Ala Tyr Leu Ile Glu Cys Gly Pro
1905                1910                1915                1920

Arg Gly Ala Leu Leu Phe Met Ala Trp Leu Gly Val Arg Val Leu Pro
            1925                1930                1935

Ile Thr Arg Gln Leu Asp Gly Gly Asn Gln Glu Gln Arg Ile Pro Gly
        1940                1945                1950
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Ser Thr Lys Pro Asn Ala Glu Asn Val Val Thr Val Tyr Gly Ala Trp
        1955                1960                1965

Pro Thr Pro Arg Ser Pro Leu Leu His Phe Ala Pro Asn Ala Thr Glu
    1970                1975                1980

Glu Leu Asp Gln Leu Leu Ser Gly Phe Glu Glu Phe Glu Glu Ser Leu
1985                1990                1995                2000

Gly Tyr Lys Phe Arg Asp Arg Ser Tyr Leu Leu Gln Ala Met Thr His
                2005                2010                2015

Ala Ser Tyr Thr Pro Asn Arg Leu Thr Asp Cys Tyr Gln Arg Leu Glu
            2020                2025                2030

Phe Leu Gly Asp Ala Val Leu Asp Tyr Leu Ile Thr Arg His Leu Tyr
        2035                2040                2045

Glu Asp Pro Arg Gln His Ser Pro Gly Ala Leu Thr Asp Leu Arg Ser
    2050                2055                2060

Ala Leu Val Asn Asn Thr Ile Phe Ala Ser Leu Ala Val Arg His Gly
2065                2070                2075                2080

Phe His Lys Phe Phe Arg His Leu Ser Pro Gly Leu Asn Asp Val Ile
                2085                2090                2095

Asp Arg Phe Val Arg Ile Gln Gln Glu Asn Gly His Cys Ile Ser Glu
            2100                2105                2110

Glu Tyr Tyr Leu Leu Ser Glu Glu Glu Cys Asp Asp Ala Glu Asp Val
        2115                2120                2125

Glu Val Pro Lys Ala Leu Gly Asp Val Phe Glu Ser Ile Ala Gly Ala
    2130                2135                2140

Ile Phe Leu Asp Ser Asn Met Ser Leu Asp Val Val Trp His Val Tyr
2145                2150                2155                2160

Ser Asn Met Met Ser Pro Glu Ile Glu Gln Phe Ser Asn Ser Val Pro
                2165                2170                2175

Lys Ser Pro Ile Arg Glu Leu Leu Glu Leu Glu Pro Glu Thr Ala Lys
            2180                2185                2190

Phe Gly Lys Pro Glu Lys Leu Ala Asp Gly Arg Arg Val Arg Val Thr
        2195                2200                2205

Val Asp Val Phe Cys Lys Gly Thr Phe Arg Gly Ile Gly Arg Asn Tyr
    2210                2215                2220

Arg Ile Ala Lys Cys Thr Ala Ala Lys Cys Ala Leu Arg Gln Leu Lys
2225                2230                2235                2240

Lys Gln Gly Leu Ile Ala Lys Lys Asp
                2245


<210> SEQ ID NO 5
<211> LENGTH: 1145
<212> TYPE: PRT
<213> ORGANISM: Drosophila melanogaster

<400> SEQUENCE: 5

Met Gly Lys Lys Asp Lys Asn Lys Lys Gly Gly Gln Asp Ser Ala Ala
1               5                   10                  15

Ala Pro Gln Pro Gln Gln Gln Gln Lys Gln Gln Gln Gln Arg Gln Gln
            20                  25                  30

Gln Pro Gln Gln Leu Gln Gln Pro Gln Gln Leu Gln Gln Pro Gln Gln
        35                  40                  45

Leu Gln Gln Pro Gln Gln Gln Gln Gln Gln Gln Pro His Gln Gln Gln
    50                  55                  60

Gln Gln Ser Ser Arg Gln Gln Pro Ser Thr Ser Ser Gly Gly Ser Arg
65                  70                  75                  80
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Ala Ser Gly Phe Gln Gln Gly Gly Gln Gln Gln Lys Ser Gln Asp Ala
                85                  90                  95

Glu Gly Trp Thr Ala Gln Lys Lys Gln Gly Lys Gln Gln Val Gln Gly
            100                 105                 110

Trp Thr Lys Gln Gly Gln Gln Gly Gly His Gln Gln Gly Arg Gln Gly
        115                 120                 125

Gln Asp Gly Gly Tyr Gln Gln Arg Pro Pro Gly Gln Gln Gln Gly Gly
    130                 135                 140

His Gln Gln Gly Arg Gln Gly Gln Glu Gly Gly Tyr Gln Gln Arg Pro
145                 150                 155                 160

Pro Gly Gln Gln Gln Gly Gly His Gln Gln Gly Arg Gln Gly Gln Glu
                165                 170                 175

Gly Gly Tyr Gln Gln Arg Pro Ser Gly Gln Gln Gln Gly Gly His Gln
            180                 185                 190

Gln Gly Arg Gln Gly Gln Glu Gly Gly Tyr Gln Gln Arg Pro Pro Gly
        195                 200                 205

Gln Gln Gln Gly Gly His Gln Gln Gly Arg Gln Gly Gln Glu Gly Gly
    210                 215                 220

Tyr Gln Gln Arg Pro Ser Gly Gln Gln Gln Gly Gly His Gln Gln Gly
225                 230                 235                 240

Arg Gln Gly Gln Glu Gly Gly Tyr Gln Gln Arg Pro Ser Gly Gln Gln
                245                 250                 255

Gln Gly Gly His Gln Gln Gly Arg Gln Gly Gln Glu Gly Gly Tyr Gln
            260                 265                 270

Gln Arg Pro Ser Gly Gln Gln Gln Gly Gly His Gln Gln Gly Arg Gln
        275                 280                 285

Gly Gln Glu Gly Gly Tyr Gln Gln Arg Pro Pro Gly Gln Gln Pro Asn
    290                 295                 300

Gln Thr Gln Ser Gln Gly Gln Tyr Gln Ser Arg Gly Pro Pro Gln Gln
305                 310                 315                 320

Gln Gln Ala Ala Pro Leu Pro Leu Pro Pro Gln Pro Ala Gly Ser Ile
                325                 330                 335

Lys Arg Gly Thr Ile Gly Lys Pro Gly Gln Val Gly Ile Asn Tyr Leu
            340                 345                 350

Asp Leu Asp Leu Ser Lys Met Pro Ser Val Ala Tyr His Tyr Asp Val
        355                 360                 365

Lys Ile Met Pro Glu Arg Pro Lys Lys Phe Tyr Arg Gln Ala Phe Glu
    370                 375                 380

Gln Phe Arg Val Asp Gln Leu Gly Gly Ala Val Leu Ala Tyr Asp Gly
385                 390                 395                 400

Lys Ala Ser Cys Tyr Ser Val Asp Lys Leu Pro Leu Asn Ser Gln Asn
                405                 410                 415

Pro Glu Val Thr Val Thr Asp Arg Asn Gly Arg Thr Leu Arg Tyr Thr
            420                 425                 430

Ile Glu Ile Lys Glu Thr Gly Asp Ser Thr Ile Asp Leu Lys Ser Leu
        435                 440                 445

Thr Thr Tyr Met Asn Asp Arg Ile Phe Asp Lys Pro Met Arg Ala Met
    450                 455                 460

Gln Cys Val Glu Val Val Leu Ala Ser Pro Cys His Asn Lys Ala Ile
465                 470                 475                 480

Arg Val Gly Arg Ser Phe Phe Lys Met Ser Asp Pro Asn Asn Arg His
                485                 490                 495

Glu Leu Asp Asp Gly Tyr Glu Ala Leu Val Gly Leu Tyr Gln Ala Phe
            500                 505                 510
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Met Leu Gly Asp Arg Pro Phe Leu Asn Val Asp Ile Ser His Lys Ser
        515                 520                 525

Phe Pro Ile Ser Met Pro Met Ile Glu Tyr Leu Glu Arg Phe Ser Leu
    530                 535                 540

Lys Ala Lys Ile Asn Asn Thr Thr Asn Leu Asp Tyr Ser Arg Arg Phe
545                 550                 555                 560

Leu Glu Pro Phe Leu Arg Gly Ile Asn Val Val Tyr Thr Pro Pro Gln
                565                 570                 575

Ser Phe Gln Ser Ala Pro Arg Val Tyr Arg Val Asn Gly Leu Ser Arg
            580                 585                 590

Ala Pro Ala Ser Ser Glu Thr Phe Glu His Asp Gly Lys Lys Val Thr
        595                 600                 605

Ile Ala Ser Tyr Phe His Ser Arg Asn Tyr Pro Leu Lys Phe Pro Gln
    610                 615                 620

Leu His Cys Leu Asn Val Gly Ser Ser Ile Lys Ser Ile Leu Leu Pro
625                 630                 635                 640

Ile Glu Leu Cys Ser Ile Glu Glu Gly Gln Ala Leu Asn Arg Lys Asp
                645                 650                 655

Gly Ala Thr Gln Val Ala Asn Met Ile Lys Tyr Ala Ala Thr Ser Thr
            660                 665                 670

Asn Val Arg Lys Arg Lys Ile Met Asn Leu Leu Gln Tyr Phe Gln His
        675                 680                 685

Asn Leu Asp Pro Thr Ile Ser Arg Phe Gly Ile Arg Ile Ala Asn Asp
    690                 695                 700

Phe Ile Val Val Ser Thr Arg Val Leu Ser Pro Pro Gln Val Glu Tyr
705                 710                 715                 720

His Ser Lys Arg Phe Thr Met Val Lys Asn Gly Ser Trp Arg Met Asp
                725                 730                 735

Gly Met Lys Phe Leu Glu Pro Lys Pro Lys Ala His Lys Cys Ala Val
            740                 745                 750

Leu Tyr Cys Asp Pro Arg Ser Gly Arg Lys Met Asn Tyr Thr Gln Leu
        755                 760                 765

Asn Asp Phe Gly Asn Leu Ile Ile Ser Gln Gly Lys Ala Val Asn Ile
    770                 775                 780

Ser Leu Asp Ser Asp Val Thr Tyr Arg Pro Phe Thr Asp Asp Glu Arg
785                 790                 795                 800

Ser Leu Asp Thr Ile Phe Ala Asp Leu Lys Arg Ser Gln His Asp Leu
                805                 810                 815

Ala Ile Val Ile Ile Pro Gln Phe Arg Ile Ser Tyr Asp Thr Ile Lys
            820                 825                 830

Gln Lys Ala Glu Leu Gln His Gly Ile Leu Thr Gln Cys Ile Lys Gln
        835                 840                 845

Phe Thr Val Glu Arg Lys Cys Asn Asn Gln Thr Ile Gly Asn Ile Leu
    850                 855                 860

Leu Lys Ile Asn Ser Lys Leu Asn Gly Ile Asn His Lys Ile Lys Asp
865                 870                 875                 880

Asp Pro Arg Leu Pro Met Met Lys Asn Thr Met Tyr Ile Gly Ala Asp
                885                 890                 895

Val Thr His Pro Ser Pro Asp Gln Arg Glu Ile Pro Ser Val Val Gly
            900                 905                 910

Val Ala Ala Ser His Asp Pro Tyr Gly Ala Ser Tyr Asn Met Gln Tyr
        915                 920                 925

Arg Leu Gln Arg Gly Ala Leu Glu Glu Ile Glu Asp Met Phe Ser Ile
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    930                 935                 940

Thr Leu Glu His Leu Arg Val Tyr Lys Glu Tyr Arg Asn Ala Tyr Pro
945                 950                 955                 960

Asp His Ile Ile Tyr Tyr Arg Asp Gly Val Ser Asp Gly Gln Phe Pro
                965                 970                 975

Lys Ile Lys Asn Glu Glu Leu Arg Cys Ile Lys Gln Ala Cys Asp Lys
            980                 985                 990

Val Gly Cys Lys Pro Lys Ile Cys Cys Val Ile Val Val Lys Arg His
        995                 1000                1005

His Thr Arg Phe Phe Pro Ser Gly Asp Val Thr Thr Ser Asn Lys Phe
    1010                1015                1020

Asn Asn Val Asp Pro Gly Thr Val Val Asp Arg Thr Ile Val His Pro
1025                1030                1035                1040

Asn Glu Met Gln Phe Phe Met Val Ser Gly Gln Ala Ile Gln Gly Thr
                1045                1050                1055

Ala Lys Pro Thr Arg Tyr Asn Val Ile Glu Asn Thr Gly Asn Leu Asp
            1060                1065                1070

Ile Asp Leu Leu Gln Gln Leu Thr Tyr Asn Leu Cys His Met Phe Pro
        1075                1080                1085

Arg Cys Asn Arg Ser Val Ser Tyr Pro Ala Pro Ala Tyr Leu Ala His
    1090                1095                1100

Leu Val Ala Ala Arg Gly Arg Val Tyr Leu Thr Gly Thr Asn Arg Phe
1105                1110                1115                1120

Leu Asp Leu Lys Lys Glu Tyr Ala Lys Arg Thr Ile Val Pro Glu Phe
                1125                1130                1135

Met Lys Lys Asn Pro Met Tyr Phe Val
            1140                1145


<210> SEQ ID NO 6
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 6

ucgaaguacu cagcguaagu g                                              21


<210> SEQ ID NO 7
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 7

cuuacgcuga guacuucgaa a                                              21


<210> SEQ ID NO 8
<211> LENGTH: 62
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 8

caucgacuga aaucccuggu aauccguugu uaacaacgga uuaccaggga uuucagucga     60

ug                                                                   62


<210> SEQ ID NO 9
<211> LENGTH: 81
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 9
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caucgacuga aaucccuggu aauccguuug gggcucugcc cugcuauggg auaaauggau     60

uaucagggau uuuagucgau g                                               81


<210> SEQ ID NO 10
<211> LENGTH: 82
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 10

caucgacuga aaucccgccg uaauccguuu ggggcucugc ccugcuaugg gauaaaugga     60

uuaucaggga uuuuagucga ug                                              82


<210> SEQ ID NO 11
<211> LENGTH: 64
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,
      17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 34, 35,
      36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49,
      50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62
<223> OTHER INFORMATION: n = A,T,C or G
<220> FEATURE:
<223> OTHER INFORMATION: generic structure for 29 nt. shRNA with 3'
      overhang

<400> SEQUENCE: 11

nnnnnnnnnn nnnnnnnnnn nnnnnnnnnc caannnnnnn nnnnnnnnnn nnnnnnnnnn     60

nnuu                                                                  64


<210> SEQ ID NO 12
<211> LENGTH: 62
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,
      17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 34, 35,
      36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49,
      50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62
<223> OTHER INFORMATION: n = A,T,C or G
<220> FEATURE:
<223> OTHER INFORMATION: generic structure for 29 nt. shRNA without 3'
      overhang

<400> SEQUENCE: 12

nnnnnnnnnn nnnnnnnnnn nnnnnnnnnc caannnnnnn nnnnnnnnnn nnnnnnnnnn     60

nn                                                                    62


<210> SEQ ID NO 13
<211> LENGTH: 23
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 13

ggucgaagua cucagcguaa gaa                                             23


<210> SEQ ID NO 14
<211> LENGTH: 23
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 14

ggcuuacgcu gaguacuucg aaa                                             23
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<210> SEQ ID NO 15
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 15

gguuguggau cuggauaccg g 21

<210> SEQ ID NO 16
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 16

gguauccaga uccacaaccu u 21

<210> SEQ ID NO 17
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 17

ggugccaacc cuauucuccu u 21

<210> SEQ ID NO 18
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 18

ggagaauagg guuggcacca g 21

<210> SEQ ID NO 19
<211> LENGTH: 22
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 19

ggcuaugaag agaguacgcc cu 22

<210> SEQ ID NO 20
<211> LENGTH: 22
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 20

ggcguacucu cuucauagcc uu 22

<210> SEQ ID NO 21
<211> LENGTH: 64
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 21

ggucgaagua cucagcguaa gugaugucca cuuaaguggg uguuguuugu guuggguguu 60

uugg 64

<210> SEQ ID NO 22
<211> LENGTH: 60
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 22

ggucgaagua cucagcguaa gugauguccu uaagggguguu guuuguguug gguguuuugg 60

<210> SEQ ID NO 23
<211> LENGTH: 56
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 23

ggucgaagua cucagcguaa gugauguuua aguguuguuu guguugggug uuuugg 56

<210> SEQ ID NO 24
<211> LENGTH: 50
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 24

ggucgaagua cucagcguaa gugauuaauu guuuguguug gguguuuugg 50

<210> SEQ ID NO 25
<211> LENGTH: 70
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 25

ggcucgaguc gaaguacuca gcguaaguga uguccacuua agugggguguu guuuguguug 60

gguguuuugg 70

<210> SEQ ID NO 26
<211> LENGTH: 70
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 26

ggucgaagua cucagcguaa gugaugucca cuuaaguggg uguuguuugu guugggguguu 60

uuggcucgag 70

<210> SEQ ID NO 27
<211> LENGTH: 69
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 27

ggauuccaau ucagcgggag ccaccugaug aagcuugauc gggugggcucu cgcugaguug 60

gaauccauu 69

<210> SEQ ID NO 28
<211> LENGTH: 64
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: shRNA targeting mouse p53

<400> SEQUENCE: 28

ggucuaagug gagcccuucg aguguuagaa gcuugugaca cucggagggc uucacuuggg 60

ccuu 64

<210> SEQ ID NO 29
<211> LENGTH: 21
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<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 29

cuuacgcuga guacuucgau u 21

<210> SEQ ID NO 30
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 30

ucgaaguacu cagcguaagu u 21

<210> SEQ ID NO 31
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 31

agcuucauaa ggcgcaugcu u 21

<210> SEQ ID NO 32
<211> LENGTH: 21
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<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 32

gcaugcgccu uaugaagcuu u 21

<210> SEQ ID NO 33
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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: siRNA targeting HCV NS5B

<400> SEQUENCE: 33

cugugagauc uacggagccu guu 23

<210> SEQ ID NO 34
<211> LENGTH: 23
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: siRNA targeting HCV NS5B

<400> SEQUENCE: 34

caggcuccgu agaucucaca guu 23

<210> SEQ ID NO 35
<211> LENGTH: 69
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 35

ggauuccaau ucagcgggag ccaccugaug aagcuugauc ggguggcucu cgcugaguug 60

gaauccauu 69

<210> SEQ ID NO 36
<211> LENGTH: 29
<212> TYPE: RNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 36

aguugcgccc gcgaaugaua uuuauaaug 29

<210> SEQ ID NO 37
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 37

tcaaccagcc actgctgga 19

<210> SEQ ID NO 38
<211> LENGTH: 71
<212> TYPE: DNA
<213> ORGANISM: Photinus pyralis

<400> SEQUENCE: 38

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ccattttttt t 71

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<211> LENGTH: 44
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<220> FEATURE:
<223> OTHER INFORMATION: Tyrosinase enhancer PCR primer

<400> SEQUENCE: 39

taatacgact cactataggg caaggtcata gttcctgcca gctg 44

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Tyrosinase enhancer PCR primer

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taatacgact cactataggg cagatatttt cttaccaccc accc 44

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<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Tyrosinase enhancer PCR primer

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taatacgact cactataggg ttaagtttaa caggagaagc tgga 44

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 42

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<210> SEQ ID NO 43
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 43

taatacgact cactataggg tagatttccg cagccccagt gttc 44

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taatacgact cactataggg gttgcctctc atttttcctt gatt 44

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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taatacgact cactataggg tattttagac tgattacttt tataa 45

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<220> FEATURE:
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<400> SEQUENCE: 46

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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 47

ggaugcacca ucuucaagg 19

<210> SEQ ID NO 48
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gacaaaaucc ccaucagga 19

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<211> LENGTH: 19
<212> TYPE: RNA
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accgcaaagu cuuugagaa 19

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guccugacau gcuguuuga 19

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<212> TYPE: RNA
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<400> SEQUENCE: 51

gaccaccauc aacaaugag 19

<210> SEQ ID NO 52
<211> LENGTH: 19
<212> TYPE: RNA
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<400> SEQUENCE: 52

caaauuaugu guuuccgaa 19

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<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 53

cgcaugugcu ggcaguaua 19

<210> SEQ ID NO 54
<211> LENGTH: 19
<212> TYPE: RNA
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<400> SEQUENCE: 54

ccgaagauuu cacagucaa 19

<210> SEQ ID NO 55
<211> LENGTH: 19
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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 55

accauugauu cuguuacuu 19

<210> SEQ ID NO 56
<211> LENGTH: 19
<212> TYPE: RNA
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<400> SEQUENCE: 56

cugacaagag cucaaggaa 19

<210> SEQ ID NO 57
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 57

cguucuggag cuguugaua 19

<210> SEQ ID NO 58
<211> LENGTH: 19
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<400> SEQUENCE: 58

gagcccagau caaccuuua 19

<210> SEQ ID NO 59
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<400> SEQUENCE: 59

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gauggcagcu caaagcaaa                                                    19


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<212> TYPE: RNA
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<400> SEQUENCE: 61

cagcagaaau cuaaggaua                                                    19


<210> SEQ ID NO 62
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<400> SEQUENCE: 62

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<210> SEQ ID NO 63
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<400> SEQUENCE: 63

acugacaaca aagugcagc                                                    19


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<400> SEQUENCE: 65

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<210> SEQ ID NO 66
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<400> SEQUENCE: 66

gucugggugg aaauucaaa                                                    19


<210> SEQ ID NO 67
<211> LENGTH: 19
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<212> TYPE: RNA
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<400> SEQUENCE: 67

caucuuugcu gaaucgaaa 19

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<400> SEQUENCE: 68

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<400> SEQUENCE: 69

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<400> SEQUENCE: 70

cucacauugu ccaccagga 19

<210> SEQ ID NO 71
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<400> SEQUENCE: 71

gaccugugcc uuuuagaga 19

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<211> LENGTH: 19
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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 72

aaaggacaac ugcagcuac 19

<210> SEQ ID NO 73
<211> LENGTH: 19
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<400> SEQUENCE: 73

gacuucauug acaguggcc 19

<210> SEQ ID NO 74
<211> LENGTH: 19
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<400> SEQUENCE: 74

aauauccuca ggggguggag 19

<210> SEQ ID NO 75
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<212> TYPE: RNA
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<400> SEQUENCE: 75

gugccucuug uugcagaga 19

<210> SEQ ID NO 76
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 76

gaagcucucc agaccauuu 19

<210> SEQ ID NO 77
<211> LENGTH: 19
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cuccugagau caugcugaa 19

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gcuguugacu ggaagaaca 19

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<400> SEQUENCE: 79

ggaauucaau gauguguau 19

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<400> SEQUENCE: 80

ccauuucagu ccaucauuc 19

<210> SEQ ID NO 81
<211> LENGTH: 19
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cccugugugg gacuccuaa 19

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ccgaguuauu caucgagac 19

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guucuuuacu ucuggcuau 19

<210> SEQ ID NO 84
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cgccucaucc ucuacaaug 19

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aagagaccua ccuccggau 19

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gguguucgcg ggcaagauu 19

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cuccuuaaau auuuccgca 19

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aagaagaacc agugguucg 19

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cugagccuga ggcccgaua 19

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<223> OTHER INFORMATION: generic structure for 19 nt. shRNA with 3'
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<220> FEATURE:
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17, 18, 19, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35,
36, 37, 38, 39, 40, 41, 42

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<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 90

nnnnnnnnnn nnnnnnnnnc caannnnnnn nnnnnnnnnn nnuu                    44


<210> SEQ ID NO 91
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      36, 37, 38, 39, 40, 41, 42
<223> OTHER INFORMATION: n = A,T,C or G

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nnnnnnnnnn nnnnnnnnnc caannnnnnn nnnnnnnnnn nn                      42


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<212> TYPE: RNA
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      17, 18, 19, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35,
      36, 37, 38, 39, 40, 41, 42
<223> OTHER INFORMATION: n = A,T,C or G

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nnnnnnnnnn nnnnnnnnnu uggnnnnnnn nnnnnnnnnn nnuu                    44


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<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: generic structure for synthetic 29 nt. shRNA
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<220> FEATURE:
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      17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 34, 35,
      36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49,
      50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 93

nnnnnnnnnn nnnnnnnnnn nnnnnnnnnu uggnnnnnnn nnnnnnnnnn nnnnnnnnnn    60

nnuu                                                                 64
```

We claim:

1. A method for attenuating expression of a target gene in a mammalian cell, the method comprising

introducing into mammalian cells a library of RNA expression constructs, each expression construct comprising:

(i) an RNA polymerase promoter, and

(ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides,

wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell,

wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and

wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence

specific manner, and is expressed in the cell without use of a PK inhibitor, whereby expression of the target gene is inhibited.

2. The method of claim **1**, wherein the expression construct further comprises LTR sequences located 5' and 3' of the sequence encoding the short hairpin RNA molecule.

3. The method of claim **1**, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 21 nucleotides.

4. The method of claim **1**, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 22 nucleotides.

5. The method of claim **1**, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 25 nucleotides.

6. The method of claim **1**, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of 29 nucleotides.

7. The method of claim **1**, wherein the short hairpin RNA molecule has a total length of 70 nucleotides.

8. The method of claim **1**, wherein the RNA polymerase promoter comprises a pol II promoter or a pol III promoter.

9. The method of claim **8**, wherein the pol III promoter comprises a U6, an H1, or an SRP promoter.

10. The method of claim **8**, wherein the pol II promoter comprises a U1 or a CMV promoter.

* * * * *

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08/20/2007

PTO/SB/05 (09-04)
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UTILITY PATENT APPLICATION TRANSMITTAL

(ONLY FOR NEW NONPROVISIONAL APPLICATIONS UNDER 37 CFR 1.53(B))

Attorney Docket No.	CSHL-P08-010
First Inventor	Gregory J. Hannon
Title	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE
Express Mail Label No.	EV543610569US

APPLICATION ELEMENTS
See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

1. [ ] **Fee Transmittal Form** (e.g., PTO/SB/17) *(Submit an original and a duplicate for fee processing)*
2. [X] **Applicant claims small entity status.** See 37 CFR 1.27.
3. [X] **Specification** [*Total Pages* 103]
   Both the claims and abstract must start on a new page
   (For information on the preferred arrangement, see MPEP 608.01(a))
4. [X] **Drawing(s)** *(35 U.S.C. 113)* [*Total Sheets* 67]
5. **Oath or Declaration** [*Total Sheets*]
   a. [ ] Newly executed (original or copy)
   b. [ ] A copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional with Box 18 completed)*
      i. [ ] DELETION OF INVENTOR(S)
      SIGNED STATEMENT ATTACHED DELETING INVENTOR(S) NAMED IN THE PRIOR APPLICATION, SEE 37 CFR 1.63(D)(2) AND 1.33(B).
6. [X] **Application Data Sheet**. See 37 CFR 1.76
7. [ ] **CD-ROM or CD-R** in duplicate, large table or Computer Program *(Appendix)*
   [ ] Landscape Table on CD
8. **Nucleotide and/or Amino Acid Sequence Submission** *(if applicable, items a. – c. are required)*
   a. [ ] Computer Readable Form (CRF)
   b. Specification Sequence Listing on:
      i. [ ] CD-ROM or CD-R (2 copies); or ii. [ ] Paper
   c. [ ] Statements verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

9. [ ] **Assignment Papers** (cover sheet & document(s))
   Name of Assignee: **Cold Spring Harbor Laboratory**
10. [ ] **37 CFR 3.73(b) Statement** *(when there is an assignee)* [ ] **Power of Attorney**
11. [ ] **English Translation Document** *(if applicable)*
12. [ ] **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    [ ] Copies of citations attached
13. [X] **Preliminary Amendment**
14. [X] **Return Receipt Postcard** (MPEP 503) *(Should be specifically itemized)*
15. [ ] **Certified Copy of Priority Document(s)** *(if foreign priority is claimed)*
16. [ ] **Nonpublication Request** under 35 U.S.C.122 (b)(2)(B)(i). Applicant must attach form PTO/SB/35 or its equivalent.
17. [ ] Other:

18. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below and in the first sentence of the specification following the title, or in an Application Data Sheet under 37 CFR 1.76:*

[X] Continuation [ ] Divisional [ ] Continuation-in-part (CIP) of prior application No.: 11/791,554

Prior application information: Examiner Not Yet Assigned *Art Unit:* 1656

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Name (Print/Type)	Yu Lu	Registration No. (Attorney/Agent)	50,306

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EV543610569US, in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Dated: August 20, 2007 Signature: (Scott Whittemore)

17607 U.S. PTO
082007

EV543610569 US

Attorney Docket No.: CSHL-P08-010

Methods and Compositions for RNA Interference

Government Support

Work described herein was supported by National Institutes of Health Grant R01-
5 GM62534. The United States Government may have certain rights in the invention.

Related Applications

This application is a continuation-in-part of U.S. Application No. 10/350,798, filed on January 24, 2003, which is a continuation-in-part of U.S. Application No. 10/055,797, filed on January 22, 2002, which is a continuation-in-part of International Application No. 10 PCT/US01/08435, filed on March 16, 2001, which claims the benefit of priority from U.S. Provisional Application Nos. 60/189,739, filed on March 16, 2000, and 60/243,097, filed on October 24, 2000. U.S. Application No. 10/350,798 is also a continuation-in-part of U.S. Application No. 09/866,557, filed on May 24, 2001, which is a continuation-in-part of International Application No. PCT/US01/08435, filed on March 16, 2001, which claims the 15 benefit of priority from U.S. Provisional Application Nos. 60/189,739, filed on March 16, 2000, and 60/243,097, filed on October 24, 2000. U.S. Application No. 10/350,798 is also a continuation-in-part of U.S. Application No. 09/858,862, filed on May 16, 2001, which is a continuation-in-part of International Application No. PCT/US01/08435, filed on March 16, 2001, which claims the benefit of priority from U.S. Provisional Application Nos. 20 60/189,739, filed on March 16, 2000, and 60/243,097, filed on October 24, 2000. The specifications of such applications are incorporated by reference herein. International Application PCT/US01/08435 was published under PCT Article 21(2) in English.

Background of the Invention

"RNA interference", "post-transcriptional gene silencing", "quelling" — these 25 different names describe similar effects that result from the overexpression or misexpression of transgenes, or from the deliberate introduction of double-stranded RNA into cells (reviewed in Fire, *Trends Genet* **15**: 358–363, 1999; Sharp, *Genes Dev* **13**: 139–141, 1999; Hunter, *Curr Biol* **9**: R440–R442, 1999; Baulcombe, *Curr Biol* **9**: R599–R601, 1999; Vaucheret *et al.*, *Plant J* **16**: 651–659, 1998). The injection of double-stranded RNA into the 30 nematode *Caenorhabditis elegans*, for example, acts systemically to cause the post-transcriptional depletion of the homologous endogenous RNA (Fire *et al.*, *Nature* **391**: 806–

811, 1998; and Montgomery *et al.*, *PNAS* **95**: 15502–15507, 1998). RNA interference, commonly referred to as RNAi, offers a way of specifically and potently inactivating a cloned gene, and is proving a powerful tool for investigating gene function. Although the phenomenon is interesting in its own right; the mechanism has been rather mysterious, but 5 recent research - for example that recently reported by Smardon *et al.*, *Curr Biol* **10**: 169–178, 2000 - is beginning to shed light on the nature and evolution of the biological processes that underlie RNAi.

RNAi was discovered when researchers attempting to use the antisense RNA approach to inactivate a *C. elegans* gene found that injection of sense-strand RNA was 10 actually as effective as the antisense RNA at inhibiting gene function (Guo *et al.*, *Cell* **81**: 611–620, 1995). Further investigation revealed that the active agent was modest amounts of double-stranded RNA that contaminate *in vitro* RNA preparations. Researchers quickly determined the 'rules' and effects of RNAi which have become the paradigm for thinking about the mechanism which mediates this affect. Exon sequences are required, whereas 15 introns and promoter sequences, while ineffective, do not appear to compromise RNAi (though there may be gene-specific exceptions to this rule). RNAi acts systemically — injection into one tissue inhibits gene function in cells throughout the animal. The results of a variety of experiments, in *C. elegans* and other organisms, indicate that RNAi acts to destabilize cellular RNA after RNA processing.

20 The potency of RNAi inspired Timmons and Fire (*Nature* **395**: 854, 1998) to do a simple experiment that produced an astonishing result. They fed to nematodes bacteria that had been engineered to express double-stranded RNA corresponding to the *C. elegans unc-22* gene. Amazingly, these nematodes developed a phenotype similar to that of *unc-22* mutants that was dependent on their food source. The ability to conditionally expose large numbers of 25 nematodes to gene-specific double-stranded RNA formed the basis for a very powerful screen to select for RNAi-defective *C. elegans* mutants and then to identify the corresponding genes.

Double-stranded RNAs (dsRNAs) can provoke gene silencing in numerous *in vitro* contexts including *Drosophila*, *Caenorhabditis elegans*, planaria, hydra, trypanosomes, fungi and plants. However, the ability to recapitulate this phenomenon in higher eukaryotes, 30 particularly mammalian cells, has not been accomplished in the art. Nor has the prior art demonstrated that this phenomena can be observed in cultured eukaryotic cells. Additionally, the 'rules' established by the prior art have taught that RNAi requires exon sequences, and thus constructs consisting of intronic or promoter sequences were not believed to be effective reagents in mediating RNAi. The present invention aims to address each of these deficiencies

in the prior art and provides evidence both that RNAi can be observed in cultured eukaryotic cells and that RNAi constructs consisting of non-exon sequences can effectively repress gene expression.

Summary of the Invention

5 One aspect of the present invention provides a method for attenuating expression of a target gene in cultured cells, comprising introducing double stranded RNA (dsRNA) into the cells in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene.

10 Another aspect of the present invention provides a method for attenuating expression of a target gene in a mammalian cell, comprising: (i) activating one or both of a Dicer activity or an Argonaut activity in the cell, and (ii) introducing into the cell a double stranded RNA (dsRNA) in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a 15 nucleotide sequence of the target gene.

In certain embodiments, the cell is suspended in culture; while in other embodiments the cell is in a whole animal, such as a non-human mammal.

In certain preferred embodiments, the cell is engineered with (i) a recombinant gene encoding a Dicer activity, (ii) a recombinant gene encoding an Argonaut activity, or (iii) 20 both. For instance, the recombinant gene may encode, for a example, a protein which includes an amino acid sequence at least 50 percent identical to SEQ ID NO: 2 or 4; or be defined by a coding sequence which hybridizes under wash conditions of 2 x SSC at 22°C to SEQ ID NO: 1 or 3. In certain embodiments, the recombinant gene may encode, for a example, a protein which includes an amino acid sequence at least 50 percent identical to the 25 Argonaut sequence shown in Figure 24. In certain embodiments, the recombinant gene may encode a protein which includes an amino acid sequence at least 60%, 70%, 80%, 85%, 90%, or 95% identical to SEQ ID NO: 2 or 4. In certain embodiments, the recombinant gene may be defined by a coding sequence which hybridizes under stringent conditions, including a wash step selected from 0.2 - 2.0 × SSC at from 50°C-65°C, to SEQ ID NO: 1 or 3.

30 In certain embodiments, rather than use a heterologous expression construct(s), an endogenous Dicer gene or Argonaut gene can be activated, *e.g.*, by gene activation technology, expression of activated transcription factors or other signal transduction protein(s), which induces expression of the gene, or by treatment with an endogenous factor

which upregulates the level of expression of the protein or inhibits the degradation of the protein.

In certain preferred embodiments, the target gene is an endogenous gene of the cell. In other embodiments, the target gene is a heterologous gene relative to the genome of the cell, 5 such as a pathogen gene, *e.g.*, a viral gene.

In certain embodiments, the cell is treated with an agent that inhibits protein kinase RNA-activated (PKR) apoptosis, such as by treatment with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKR.

In certain preferred embodiments, the cell is a primate cell, such as a human cell.

10 In certain preferred embodiments, the length of the dsRNA is at least 20, 21 or 22 nucleotides in length, *e.g.*, corresponding in size to RNA products produced by Dicer-dependent cleavage. In certain embodiments, the dsRNA construct is at least 25, 50, 100, 200, 300 or 400 bases. In certain embodiments, the dsRNA construct is 400-800 bases in length.

15 In certain preferred embodiments, expression of the target gene is attenuated by at least 5 fold, and more preferably at least 10, 20 or even 50 fold, *e.g.*, relative to the untreated cell or a cell treated with a dsRNA construct which does not correspond to the target gene.

Yet another aspect of the present invention provides a method for attenuating expression of a target gene in cultured cells, comprising introducing an expression vector 20 having a "coding sequence" which, when transcribed, produces double stranded RNA (dsRNA) in the cell in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene. In certain embodiments, the vector includes a single coding sequence for the dsRNA which is operably linked to (two) 25 transcriptional regulatory sequences which cause transcription in both directions to form complementary transcripts of the coding sequence. In other embodiments, the vector includes two coding sequences which, respectively, give rise to the two complementary sequences which form the dsRNA when annealed. In still other embodiments, the vector includes a coding sequence which forms a hairpin. In certain embodiments, the vectors are episomal, 30 *e.g.*, and transfection is transient. In other embodiments, the vectors are chromosomally integrated, *e.g.*, to produce a stably transfected cell line. Preferred vectors for forming such stable cell lines are described in US Patent 6,025,192 and PCT publication WO 98/12339, which are incorporated by reference herein.

Another aspect of the present invention provides a method for attenuating expression

of a target gene in cultured cells, comprising introducing an expression vector having a "non-coding sequence" which, when transcribed, produces double stranded RNA (dsRNA) in the cell in an amount sufficient to attenuate expression of the target gene. The non-coding sequence may include intronic or promoter sequence of the target gene of interest, and the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the promoter or intron of the target gene. In certain embodiments, the vector includes a single sequence for the dsRNA which is operably linked to (two) transcriptional regulatory sequences which cause transcription in both directions to form complementary transcripts of the sequence. In other embodiments, the vector includes two sequences which, respectively, give rise to the two complementary sequences which form the dsRNA when annealed. In still other embodiments, the vector includes a coding sequence which forms a hairpin. In certain embodiments, the vectors are episomal, *e.g.*, and transfection is transient. In other embodiments, the vectors are chromosomally integrated, *e.g.*, to produce a stably transfected cell line. Preferred vectors for forming such stable cell lines are described in US Patent 6,025,192 and PCT publication WO 98/12339, which are incorporated by reference herein.

Another aspect the present invention provides a double stranded (ds) RNA for inhibiting expression of a mammalian gene. The dsRNA comprises a first nucleotide sequence that hybridizes under stringent conditions, including a wash step of 0.2 × SSC at 65°C, to a nucleotide sequence of at least one mammalian gene and a second nucleotide sequence which is complementary to the first nucleotide sequence.

In one embodiment, the first nucleotide sequence of said double-stranded RNA is at least 20, 21, 22, 25, 50, 100, 200, 300, 400, 500, 800 nucleotides in length.

In another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to at least one mammalian gene. In another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to one mammalian gene. In yet another embodiment, the first nucleotide sequence of said double-stranded RNA hybridizes under stringent conditions to at least one human gene. In still another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to at least one human gene. In still another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to one human gene.

The double-stranded RNA may be an siRNA or a hairpin, and may be expressed transiently or stably. In one embodiment, the double-stranded RNA is a hairpin comprising a

first nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of at least one mammalian gene, and a second nucleotide sequence which is a complementary inverted repeat of said first nucleotide sequence and hybridizes to said first nucleotide sequence to form a hairpin structure.

5 The first nucleotide sequence of said double-stranded RNA can hybridize to either coding or non-coding sequence of at least one mammalian gene. In one embodiment, the first nucleotide sequence of said double-stranded RNA hybridizes to a coding sequence of at least one mammalian gene. In another embodiment, the first nucleotide sequence of said double-stranded RNA hybridizes to a coding sequence of at least one human gene. In another 10 embodiment, the first nucleotide sequence of said double-stranded RNA is identical to a coding sequence of at least one mammalian gene. In still another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to a coding sequence of at least one human gene.

In another embodiment, the first nucleotide sequence of said double-stranded RNA 15 hybridizes to a non-coding sequence of at least one mammalian gene. In another embodiment, the first nucleotide sequence of said double-stranded RNA hybridizes to a non-coding sequence of at least one human gene. In another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to a non-coding sequence of at least one mammalian gene. In still another embodiment, the first nucleotide sequence of said double- 20 stranded RNA is identical to a non-coding sequence of at least one human gene. In any of the foregoing embodiments, the non-coding sequence may be a non-transcribed sequence.

Still another aspect of the present invention provides an assay for identifying nucleic acid sequences, either coding or non-coding sequences, responsible for conferring a particular phenotype in a cell, comprising: (i) constructing a variegated library of nucleic acid 25 sequences from a cell in an orientation relative to a promoter to produce double stranded DNA; (ii) introducing the variegated dsRNA library into a culture of target cells; (iii) identifying members of the library which confer a particular phenotype on the cell, and identifying the sequence from a cell which correspond, such as being identical or homologous, to the library member.

30 Yet another aspect of the present invention provides a method of conducting a drug discovery business comprising: (i) identifying, by the subject assay, a target gene which provides a phenotypically desirable response when inhibited by RNAi; (ii) identifying agents by their ability to inhibit expression of the target gene or the activity of an expression product of the target gene; (iii) conducting therapeutic profiling of agents identified in step (b), or

further analogs thereof, for efficacy and toxicity in animals; and (iv) formulating a pharmaceutical preparation including one or more agents identified in step (iii) as having an acceptable therapeutic profile.

The method may include an additional step of establishing a distribution system for
5 distributing the pharmaceutical preparation for sale, and may optionally include establishing a sales group for marketing the pharmaceutical preparation.

Another aspect of the present invention provides a method of conducting a target discovery business comprising: (i) identifying, by the subject assay, a target gene which provides a phenotypically desirable response when inhibited by RNAi; (ii) (optionally)
10 conducting therapeutic profiling of the target gene for efficacy and toxicity in animals; and (iii) licensing, to a third party, the rights for further drug development of inhibitors of the target gene.

Another aspect of the invention provides a method for inhibiting RNAi by inhibiting the expression or activity of an RNAi enzyme. Thus, the subject method may include
15 inhibiting the activity of Dicer and/or the 22-mer RNA.

Still another aspect relates to a method for altering the specificity of an RNAi by modifying the sequence of the RNA component of the RNAi enzyme.

In another aspect, gene expression in an undifferentiated stem cell, or the differentiated progeny thereof, is altered by introducing dsRNA of the present invention. In
20 one embodiment, the stem cells are embryonic stem cells. Preferably, the embryonic stem cells are derived from mammals, more preferably from non-human primates, and most preferably from humans.

The embryonic stem cells may be isolated by methods known to one of skill in the art from the inner cell mass (ICM) of blastocyst stage embryos. In one embodiment the
25 embryonic stem cells are obtained from previously established cell lines. In a second embodiment, the embryonic stem cells are derived *de novo* by standard methods.

In another aspect, the embryonic stem cells are the result of nuclear transfer. The donor nuclei are obtained from any adult, fetal, or embryonic tissue by methods well known in the art. In one embodiment, the donor nuclei is transferred to a recipient oocyte which had
30 previously been modified. In one embodiment, the oocyte is modified using one or more dsRNAs. Exemplary modifications of the recipient oocyte include any changes in gene or protein expression that prevent an embryo derived from said modified oocyte from successfully implanting in the uterine wall. Since implantation in the uterine wall is essential for fertilized mammalian embryos to progress from beyond the blastocyst stage, embryos

made from such modified oocytes could not give rise to viable organisms. Non-limiting examples of such modifications include those that decrease or eliminate expression of cell surface receptors (*i.e.*, integrins) required for the recognition between the blastocyst and the uterine wall, modifications that decrease or eliminate expression of proteases (*i.e.*, collagenase, stromelysin, and plasminogen activator) required to digest matrix in the uterine lining and thus allow proper implantation, and modifications that decrease or eliminate expression of proteases (*i.e.*, trypsin) necessary for the blastocyst to hatch from the zona pellucida. Such hatching is required for implantation.

In another embodiment, embryonic stem cells, embryonic stem cells obtained from fertilization of modified oocytes, or the differentiated progeny thereof, can be modified or further modified with one or more dsRNAs. In a preferred embodiment, the modification decreases or eliminates MHC expression. Cells modified in this way will be tolerated by the recipient, thus avoiding complications arising from graft rejection. Such modified cells are suitable for transplantation into a related or unrelated patient to treat a condition characterized by cell damage or cell loss.

In another aspect of the invention, the undifferentiated stem cell is an adult stem cell. Exemplary adult stem cells include, but are not limited to, hematopoietic stem cells, mesenchymal stem cells, cardiac stem cells, pancreatic stem cells, and neural stem cells. Exemplary adult stem cells include any stem cell capable of forming differentiated ectodermal, mesodermal, or endodermal derivatives. Non-limiting examples of differentiated cell types which arise from adult stem cells include: blood, skeletal muscle, myocardium, endocardium, pericardium, bone, cartilage, tendon, ligament, connective tissue, adipose tissue, liver, pancreas, skin, neural tissue, lung, small intestine, large intestine, gall bladder, rectum, anus, bladder, female or male reproductive tract, genitals, and the linings of the body cavity.

In one embodiment, an undifferentiated adult stem cell, or the differentiated progeny thereof, is altered with one or more dsRNAs to decrease or eliminate MHC expression. Cells modified in this way will be tolerated by the recipient, thus avoiding complications arising from graft rejection. Such modified cells are suitable for transplantation into a related or unrelated patient to treat a condition characterized by cell damage or cell loss.

In another aspect of the invention, an embryonic stem cell, an undifferentiated adult stem cell, or the differentiated progeny of either an embryonic or adult stem cell is altered with one or more dsRNA to decrease or eliminate expression of genes required for HIV infection. In a preferred embodiment, the stem cell is one capable of giving rise to

hematopoietic cells. Modified cells with hematopoietic potential can be transplanted into a patient as a preventative therapy or treatment for HIV or AIDS.

Another aspect of the invention relates to purified or semi-purified preparations of the RNAi enzyme or components thereof. In certain embodiments, the preparations are used for 5 identifying compounds, especially small organic molecules, which inhibit or potentiate the RNAi activity. Small molecule inhibitors, for example, can be used to inhibit dsRNA responses in cells which are purposefully being transfected with a virus which produces double stranded RNA.

The dsRNA construct may comprise one or more strands of polymerized 10 ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The dsRNA construct may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may 15 yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. In certain embodiments, dsRNA constructs containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence (*i.e.*, RNA sequences similar to the 20 target sequence) have also been found to be effective for inhibition. Thus, sequence identity may be optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript. In another embodiment, dsRNA constructs containing 25 nucleotide sequences identical to a non-coding portion of the target gene are preferred for inhibition. Exemplary non-coding regions include introns and the promoter region. Sequences with insertions, deletions, and single point mutations relative to the target non-coding sequence may also be used.

Yet another aspect of the invention pertains to transgenic non-human mammals which 30 include a transgene encoding a dsRNA construct, wherein the dsRNA is identical or similar to either the coding or non-coding sequence of the target gene, preferably which is stably integrated into the genome of cells in which it occurs. The animals can be derived by oocyte microinjection, for example, in which case all of the nucleated cells of the animal will include the transgene, or can be derived using embryonic stem (ES) cells which have been transfected

with the transgene, in which case the animal is a chimera and only a portion of its nucleated cells will include the transgene. In certain instances, the sequence-independent dsRNA response, *e.g.*, the PKR response, is also inhibited in those cells including the transgene.

In still other embodiments, dsRNA itself can be introduced into an ES cell in order to effect gene silencing, and that phenotype will be carried for at least several rounds of division, *e.g.*, into the progeny of that cell.

Another aspect of the invention provides a method for attenuating expression of a target gene in mammalian cells, comprising introducing into the mammalian cells a single-stranded hairpin ribonucleic acid (shRNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA: (i) is a substrate for cleavage by a RNaseIII enzyme to produce a double-stranded RNA product, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions. Preferably, the shRNA comprises a 3' overhang of about 1-4 nucleotides.

A related aspect of the invention provides a method for attenuating expression of a target gene in mammalian cells, comprising introducing into the mammalian cells a single-stranded hairpin ribonucleic acid (shRNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA: (i) is cleaved in the mammalian cells to produce an RNA guide sequence that enters an Argonaut-containing complex, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions. Preferably, the shRNA comprises a 3' overhang of about 1-4 nucleotides.

Yet another related aspect of the invention provides a method for attenuating expression of one or more target genes in mammalian cells, comprising introducing into the mammalian cells a variegated library of single-stranded hairpin ribonucleic acid (shRNA) species, each shRNA species comprising self complementary sequences of 19 to 100 nucleotides that form duplex regions and which hybridize under intracellular conditions to a target gene, wherein each of said hairpin RNA species: (i) is a substrate for cleavage by a RNaseIII enzyme to produce a double-stranded RNA product, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) if complementary to a target sequence, reduces expression of said target gene in a manner dependent on the sequence of

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said complementary regions. Preferably, the shRNA comprises a 3' overhang of about 1-4 nucleotides.

In one embodiment, the shRNA comprises a 3' overhang of 2 nucleotides.

In one embodiment, the shRNA comprises self-complementary sequences of 25 to 29
5 nucleotides that form duplex regions.

In one embodiment, the self-complementary sequences are 29 nucleotides in length.

In one embodiment, the shRNA is transfected or microinjected into said mammalian cells.

In one embodiment, the shRNA is a transcriptional product that is transcribed from an
10 expression construct introduced into said mammalian cells, which expression construct comprises a coding sequence for transcribing said shRNA, operably linked to one or more transcriptional regulatory sequences. The transcriptional regulatory sequences may include a promoter for an RNA polymerase, such as a cellular RNA polymerase.

In one embodiment, the promoter is a U6 promoter, a T7 promoter, a T3 promoter, or
15 an SP6 promoter.

In one embodiment, the transcriptional regulatory sequences includes an inducible promoter.

In one embodiment, the mammalian cells are stably transfected with said expression construct.

20 In one embodiment, the mammalian cells are primate cells, such as human cells.

In one embodiment, the shRNA is introduced into the mammalian cells in cell culture or in an animal.

In one embodiment, the expression of the target is attenuated by at least 33 percent relative expression in cells not treated said hairpin RNA.

25 In one embodiment, the target gene is an endogenous gene or a heterologous gene relative to the genome of the mammalian cell.

In one embodiment, the self complementary sequences hybridize under intracellular conditions to a non-coding sequence of the target gene selected from a promoter sequence, an enhancer sequence, or an intronic sequence.

30 In one embodiment, the shRNA includes one or more modifications to phosphate-sugar backbone or nucleosides residues.

In one embodiment, the variegated library of shRNA species are arrayed a solid substrate.

In one embodiment, the method includes the further step of identifying shRNA

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species of said variegated library which produce a detected phenotype in said mammalian cells.

In one embodiment, the shRNA is a chemically synthesized product or an in vitro transcription product.

5 Another aspect of the invention provides a method of enhancing the potency / activity of an RNAi therapeutic for a mammalian patient, said RNAi therapeutic comprising an siRNA of 19-22 paired polynucleotides, the method comprising replacing said siRNA with a single-stranded hairpin RNA (shRNA) of claim 1 or 2, wherein said duplex region comprises the same 19-22 paired polynucleotides of said siRNA.

10 In one embodiment, the shRNA comprises a 3' overhang of 2 nucleotides.

In one embodiment, the half-maximum inhibition by said RNAi therapeutic is achieved by a concentration of said shRNA at least about 20% lower than that of said siRNA.

In one embodiment, the half-maximum inhibition by said RNAi therapeutic is achieved by a concentration of said shRNA at least about 100% lower than that of said 15 siRNA.

In one embodiment, the end-point inhibition by said shRNA is at least about 40% higher than that of said siRNA.

In one embodiment, the end-point inhibition by said shRNA is at least about 2-6 fold higher than that of said siRNA.

20 Another aspect of the invention provides a method of designing a short hairpin RNA (shRNA) construct for RNAi, said shRNA comprising a 3' overhang of about 1-4 nucleotides, the method comprising selecting the nucleotide about 21 bases 5' to the most 3'-end nucleotide as the first paired nucleotide in a cognate doubled-stranded siRNA with the same 3' overhang.

25 In one embodiment, the shRNA comprises 25-29 paired polynucleotides.

In one embodiment, the shRNA, when cut by a Dicer enzyme, produces a product siRNA that is either identical to, or differ by a single basepair immediately 5' to the 3' overhang from, said cognate siRNA.

In one embodiment, the Dicer enzyme is a human Dicer.

30 In one embodiment, the 3' overhang has 2 nucleotides.

In one embodiment, the shRNA is for RNAi in mammalian cells.

All embodiments described above can be freely combined with one or more other embodiments whenever appropriate. Such combination also includes embodiments described under different aspects of the invention.

Brief Description of the Drawings

Figure 1: RNAi in S2 cells. (**a**) *Drosophila* S2 cells were transfected with a plasmid that directs lacZ expression from the copia promoter in combination with dsRNAs corresponding to either human CD8 or lacZ, or with no dsRNA, as indicated. (**b**) S2 cells were co-transfected with a plasmid that directs expression of a GFP–US9 fusion protein and dsRNAs of either *lacZ* or *cyclin E*, as indicated. Upper panels show FACS profiles of the bulk population. Lower panels show FACS profiles from GFP-positive cells. (**c**) Total RNA was extracted from cells transfected with *lacZ*, *cyclin E*, *fizzy* or *cyclin A* dsRNAs, as indicated. Northern blots were hybridized with sequences not present in the transfected dsRNAs.

Figure 2: RNAi *in vitro*. (**a**) Transcripts corresponding to either the first 600 nucleotides of *Drosophila cyclin E* (E600) or the first 800 nucleotides of *lacZ* (Z800) were incubated in lysates derived from cells that had been transfected with either *lacZ* or *cyclin E* (cycE) dsRNAs, as indicated. Time points were 0, 10, 20, 30, 40 and 60 min for *cyclin E* and 0, 10, 20, 30 and 60 min for *lacZ*. (**b**) Transcripts were incubated in an extract of S2 cells that had been transfected with *cyclin E* dsRNA (cross-hatched box, below). Transcripts corresponded to the first 800 nucleotides of *lacZ* or the first 600, 300, 220 or 100 nucleotides of *cyclin E*, as indicated. Eout is a transcript derived from the portion of the *cyclin E* cDNA not contained within the transfected dsRNA. E-ds is identical to the dsRNA that had been transfected into S2 cells. Time points were 0 and 30 min. (**c**) Synthetic transcripts complementary to the complete *cyclin E* cDNA (Eas) or the final 600 nucleotides (Eas600) or 300 nucleotides (Eas300) were incubated in extract for 0 or 30 min.

Figure 3: Substrate requirements of the RISC. Extracts were prepared from cells transfected with *cyclin E* dsRNA. Aliquots were incubated for 30 min at 30 °C before the addition of either the *cyclin E* (E600) or *lacZ* (Z800) substrate. Individual 20 µl aliquots, as indicated, were pre-incubated with 1 mM $CaCl_2$ and 5 mM EGTA, 1 mM $CaCl_2$, 5 mM EGTA and 60 U of micrococcal nuclease, 1 mM $CaCl_2$ and 60 U of micrococcal nuclease or 10 U of DNase I (Promega) and 5 mM EGTA. After the 30 min pre-incubation, EGTA was added to those samples that lacked it. Yeast tRNA (1 µg) was added to all samples. Time points were at 0 and 30 min.

Figure 4: The RISC contains a potential guide RNA. (**a**) Northern blots of RNA from either a crude lysate or the S100 fraction (containing the soluble nuclease activity, see Methods) were hybridized to a riboprobe derived from the sense strand of the *cyclin E*

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mRNA. (**b**) Soluble *cyclin-E*-specific nuclease activity was fractionated as described in Methods. Fractions from the anion-exchange resin were incubated with the lacZ, control substrate (upper panel) or the cyclin E substrate (centre panel). Lower panel, RNA from each fraction was analysed by northern blotting with a uniformly labeled transcript derived from
5 sense strand of the *cyclin E* cDNA. DNA oligonucleotides were used as size markers.

Figure 5: Generation of 22mers and degradation of mRNA are carried out by distinct enzymatic complexes. (**a**) Extracts prepared either from 0-12 hour *Drosophila* embryos or *Drosophila* S2 cells (see Methods) were incubated for 0, 15, 30, or 60 minutes (left to right) with a uniformly-labeled double-stranded RNA corresponding to the first 500 nucleotides of
10 the *Drosophila cyclin E* coding region. M indicates a marker prepared by *in vitro* transcription of a synthetic template. The template was designed to yield a 22 nucleotide transcript. The doublet most probably results from improper initiation at the +1 position. (**b**) Whole-cell extracts were prepared from S2 cells that had been transfected with a dsRNA corresponding to the first 500 nt. of the luciferase coding region. S10 extracts were spun at
15 30,000xg for 20 minutes which represents our standard RISC extract. S100 extracts were prepared by further centrifugation of S10 extracts for 60 minutes at 100,000xg. Assays for mRNA degradation were carried out as described previously for 0, 30 or 60 minutes (left to right in each set) with either a single-stranded luciferase mRNA or a single-stranded cyclin E mRNA, as indicated. (**c**) S10 or S100 extracts were incubated with cyclin E dsRNAs for 0, 60
20 or 120 minutes (L to R).

Figure 6: Production of 22mers by recombinant CG4792/Dicer. (**a**) *Drosophila* S2 cells were transfected with plasmids that direct the expression of T7-epitope tagged versions of Drosha, CG4792/Dicer-1 and Homeless. Tagged proteins were purified from cell lysates by immunoprecipitation and were incubated with *cyclin E* dsRNA. For comparison, reactions
25 were also performed in *Drosophila* embryo and S2 cell extracts. As a negative control, immunoprecipitates were prepared from cells transfected with a β-galactosidase expression vector. Pairs of lanes show reactions performed for 0 or 60 minutes. The synthetic marker (M) is as described in the legend to Figure 1. (**b**) Diagrammatic representations of the domain structures of CG4792/Dicer-1, Drosha and Homeless are shown. (**c**) Immunoprecipitates were
30 prepared from detergent lysates of S2 cells using an antiserum raised against the C-terminal 8 amino acids of *Drosophila* Dicer-1 (CG4792). As controls, similar preparations were made with a pre-immune serum and with an immune serum that had been pre-incubated with an excess of antigenic peptide. Cleavage reactions in which each of these precipitates was

incubated with an ~500 nt. fragment of *Drosophila* cyclin E are shown. For comparison, an incubation of the substrate in *Drosophila* embryo extract was electrophoresed in parallel. **(d)** Dicer immunoprecipitates were incubated with dsRNA substrates in the presence or absence of ATP. For comparison, the same substrate was incubated with S2 extracts that either
5 contained added ATP or that were depleted of ATP using glucose and hexokinase (see methods). (e) *Drosophila* S2 cells were transfected with uniformly, ^{32}P-labelled dsRNA corresponding to the first 500 nt. of GFP. RISC complex was affinity purified using a histidine-tagged version of *Drosophila* Ago-2, a recently identified component of the RISC complex (Hammond *et al.*, in prep). RISC was isolated either under conditions in which it
10 remains ribosome associated (ls, low salt) or under conditions that extract it from the ribosome in a soluble form (hs, high salt). For comparison, the spectrum of labeled RNAs in the total lysate is shown. (f) Guide RNAs produced by incubation of dsRNA with a Dicer immunoprecipitate are compared to guide RNAs present in an affinity-purified RISC complex. These precisely co-migrate on a gel that has single-nucleotide resolution. The lane
15 labeled control is an affinity selection for RISC from a cell that had been transfected with labeled dsRNA but not with the epitope-tagged *Drosophila* Ago-2.

Figure 7: Dicer participates in RNAi. **(a)** *Drosophila* S2 cells were transfected with dsRNAs corresponding to the two *Drosophila* Dicers (CG4792 and CG6493) or with a control dsRNA corresponding to murine caspase 9. Cytoplasmic extracts of these cells were
20 tested for Dicer activity. Transfection with Dicer dsRNA reduced activity in lysates by 7.4-fold. **(b)** The Dicer-1 antiserum (CG4792) was used to prepare immunoprecipitates from S2 cells that had been treated as described above. Dicer dsRNA reduced the activity of Dicer-1 in this assay by 6.2-fold. **(c)** Cells that had been transfected two days previously with either mouse caspase 9 dsRNA or with Dicer dsRNA were cotransfected with a GFP expression
25 plasmid and either control, luciferase dsRNA or GFP dsRNA. Three independent experiments were quantified by FACS. A comparison of the relative percentage of GFP-positive cells is shown for control (GFP plasmid plus luciferase dsRNA) or silenced (GFP plasmid plus GFP dsRNA) populations in cells that had previously been transfected with either control (caspase 9) or Dicer dsRNAs.

30 Figure 8: Dicer is an evolutionarily conserved ribonuclease. **(a)** A model for production of 22mers by Dicer. Based upon the proposed mechanism of action of Ribonuclease III, we propose that Dicer acts on its substrate as a dimer. The positioning of the two ribonuclease domains (RIIIa and RIIIb) within the enzyme would thus determine the size of the cleavage product. An equally plausible alternative model could be derived in

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which the RIIIa and RIIIb domains of each Dicer enzyme would cleave in concert at a single position. In this model, the size of the cleavage product would be determined by interaction between two neighboring Dicer enzymes. (**b**) Comparison of the domain structures of potential Dicer homologs in various organisms (*Drosophila* - CG4792, CG6493, *C. elegans* -
5 K12H4.8, *Arabidopsis* – CARPEL FACTORY, T25K16.4, AC012328_1, human Helicase-MOI and *S. pombe* - YC9A_SCHPO). The ZAP domains were identified both by analysis of individual sequences with Pfam and by Psi-blast searches. The ZAP domain in the putative *S. pombe* Dicer is not detected by PFAM but is identified by Psi-Blast and is thus shown in a different color. For comparison, a domain structure of the RDE1/QDE2/ARGONAUTE
10 family is shown. It should be noted that the ZAP domains are more similar within each of the Dicer and ARGONAUTE families than they are between the two groups. (**c**) An alignment of the ZAP domains in selected Dicer and Argonaute family members is shown. The alignment was produced using ClustalW.

Figure 9: Purification strategy for RISC. (second step in RNAi model).

15 Figure 10: Fractionation of RISC activity over sizing column. Activity fractionates as 500 KDa complex. Also, antibody to *Drosophila* argonaute 2 cofractionates with activity.

Figures 11-13: Fractionation of RISC over monoS, monoQ, Hydroxyapatite columns. *Drosophila* argonaute 2 protein also cofactionates.

Figure 14: Alignment of *Drosophila* argonaute 2 with other family members.

20 Figure 15: Confirmation of *Drosophila* argonaute 2. S2 cells were transfected with labeled dsRNA and His tagged argonaute. Argonaute was isolated on nickel agarose and RNA component was identified on 15% acrylamide gel.

Figure 16: S2 cell and embryo extracts were assayed for 22-mer generating activity.

Figure 17: RISC can be separated from 22-mer generating activity (dicer). Spinning
25 extracts (S100) can clear RISC activity from supernatant (left panel) however, S100 spins still contain dicer activity (right panel).

Figure 18: Dicer is specific for dsRNA and prefers longer substrates.

Figure 19: Dicer was fractionated over several columns.

Figure 20: Identification of dicer as enzyme which can process dsRNA into 22mers.
30 Various RNaseIII family members were expressed with n terminal tags, immunoprecipitated, and assayed for 22-mer generating activity (left panel). In right panel, antibodies to dicer could also precipitate 22-mer generating activity.

Figure 21: Dicer requires ATP.

Figure 22: Dicer produces RNAs that are the same size as RNAs present in RISC.

Figure 23: Human dicer homolog when expressed and immunoprecipitated has 22-mer generating activity.

Figure 24: Sequence of *Drosophila* argonaute 2 (SEQ ID NO: 5). Peptides identified by microsequencing are shown in underline.

5 Figure 25: Molecular characterization of *Drosophila* argonaute 2. The presence of an intron in coding sequence was determined by northern blotting using intron probe. This results in a different 5' reading frame then the published genome sequence. Number of polyglutamine repeats was determined by genomic PCR.

Figure 26: Dicer activity can be created in human cells by expression of human dicer 10 gene. Host cell was 293. Crude extracts had dicer activity, while activity was absent from untransfected cells. Activity is not dissimilar to that seen in *Drosophila* embryo extracts.

Figure 27: A ~500 nt. fragment of the gene that is to be silenced (X) is inserted into the modified vector as a stable direct repeat using standard cloning procedures. Treatment with commercially available cre recombinase reverses sequences within the loxP sites (L) to 15 create an inverted repeat. This can be stably maintained and amplified in an sbc mutant bacterial strain (DL759). Transcription *in vitro* from the promoter of choice (P) yields a hairpin RNA that causes silencing. A zeocin resistance marker is included to insure maintenance of the direct and inverted repeat structures; however this is non-essential *in vitro* and could be removed by pre-mRNA splicing if desired. (Smith *et al.* (2000) Nature 407: 20 319-20).

Figure 28: RNAi in P19 embryonal carcinoma cells. Ten-centimeter plates of P19 cells were transfected by using 5 μg of GFP plasmid and 40 μg of the indicated dsRNA (or no RNA). Cells were photographed by fluorescent (tope panel) and phase-contrast microscopy (bottom panel) at 72 h after transfection; silencing was also clearly evident at 25 48 h post-transfection.

Figure 29: RNAi of firefly and *Renilla* luciferase in P19 cells. (**A** and **B**) P19 cells were transfected with plasmids that direct the expression of firefly and *Renilla* luciferase and dsRNA 500 mers (25 or 250 ng, as indicated in A and B, respectively), that were either homologous to the firefly luciferase mRNA (dsFF) or nonhomologous (dsGFP). Luciferase 30 activities were assayed at various times after transfection, as indicated. Ratios of firefly to *Renilla* activity are normalized to dsGFP controls. (**C** and **D**) P19 cells in 12-well culture dishes (2 ml of media) were transfected with 0.25 μg of a 9:1 mix of pGL3-Control and pRL-SV40 as well as 2 μg of the indicated RNA. Extracts were prepared 9 h after transfection. (**C**) Ratio of firefly to *Renilla* luciferase is shown. (**D**) Ratio of *Renilla* to firefly luciferase is

shown. Values are normalized to dsGFP. The average of three independent experiments is shown; error bars indicate standard deviation.

Figure 30: The panels at the right show expression of either RFP or GFP following transient transfection into wild type P19 cells. The panels at the left demonstrate the specific suppression of GFP expression in P19 clones which stably express a 500 nt double stranded GFP hairpin. P19 clones which stably express the double stranded GFP hairpin were transiently transfected with RFP or GFP, and expression of RFP or GFP was assessed by visual inspection.

Figure 31: Specific silencing of luciferase expression by dsRNA in murine embryonic stem cells. Mouse embryonic stem cells in 12-well culture dishes (1 ml of media) were transfected with 1.5 µg of dsRNA along with 0.25 µg of a 10:1 mixture of the reporter plasmids pGL3-Control and pRL-SV40. Extracts were prepared and assayed 20 h after transfection. The ratio of firefly to *Renilla* luciferase expression is shown for FF ds500; the ratio of *Renilla* to firefly is shown for Ren ds500. Both are normalized to ratios from the dsGFP transfection. The average of three independent experiments is shown; error bars indicate standard deviation.

Figure 32: RNAi in C2C12 murine myoblast cells. (A) Mouse C2C12 cells in 12-well culture dishes (1 ml of media) were transfected with 1 µg of the indicated dsRNA along with 0.250 µg of the reporter plasmids pGL3-Control and pRL-SV40. Extracts were prepared and assayed 24 h after transfection. The ratio of firefly to *Renilla* luciferase expression is shown; values are normalized to ratios from the no dsRNA control. The average of three independent experiments is shown; error bars indicate standard deviation. (**B**) C2C12 cells cotransfected with 1 µg of either plasmid alone or a plasmid containing a hyperactive mutant of vaccinia virus K3L (Kawagishi-Kobayashi *et al.* 2000, Virology 276: 424-434). The absolute counts of *Renilla* and firefly luciferase activity are shown. (**C**) The ratios of firefly/*Renilla* activity from **B**, normalized to no dsRNA controls.

Figure 33: Hela, Chinese hamster ovary, and P19 (pluripotent, mouse embryonic carcinoma) cell lines transfected with plasmids expressing Photinus pyralis (firefly) and Renilla reniformis (sea pansy) luciferases and with dsRNA 500mers (400ng), homologous to either firefly luciferase mRNA (dsLUC) or non-homologous (dsGFP). Dual luciferase assays were carried out using an Analytical Scientific Instruments model 3010 Luminometer. In this assay Renilla luciferase serves as an internal control for dsRNA-specific suppression of firefly luciferase activity. These data demonstrate that 500-mer dsRNA can specifically suppress cognate gene expression *in vitro*.

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Figure 34: Expression of a hairpin RNA produces P19 EC cell lines that stably silence GFP. (**A**) A cartoon of the FLIP cassette used to construct the GFP hairpin. GFP represents the first 500 coding base pairs of EGFP. Zeo, zeocin resistance gene; L, Lox; P, the cytomegalovirus promoter in the expression plasmid pcDNA3. Homologous GFP fragments are first cloned as direct repeats into the FLIP cassette. To create inverted repeats for hairpin production, the second repeat is flipped by using Cre recombinase. When transcribed, the inverted repeat forms a GFP dsRNA with a hairpin loop. (**B**) P19 cell lines stably expressing the GFP hairpin plasmid, GFPhp.1 (clone 10) and GFPhp.2 (clone 12), along with wt P19 were transfected with 0.25 μg each of GFP and RFP reporter genes. Fluorescence micrographs were taken by using filters appropriate for GFP and RFP. Magnification is 200×. (**C**) P19 GFPhp.1 cells were transfected with pEGFP and 0, 0.5, or 1 μg of Dicer or firefly dsRNA. Fluorescence micrographs were taken at 48 h post-transfection and are superimposed with bright field images to reveal non-GFP expressing cells. Magnification is 100×. (**D**) *In vitro* and *in vitro* processing of dsRNA in P19 cells. *In vitro* Dicer assays were performed on S2 cells and three independently prepared P19 extracts by using ^{32}P-labeled dsRNA (30 °C for 30 min). A Northern blot of RNA extracted from control and GFPhp.1 P19 cells shows the production of ≈22-mer RNA species in hairpin-expressing cells but not in control cells. Blots were probed with a ^{32}P-labeled "sense" GFP transcript.

Figure 35: dsRNA induces silencing at the posttranscriptional level. P19 cell extracts were used for *in vitro* translation of firefly and *Renilla* luciferase mRNA (100 ng each). Translation reactions were programmed with various amounts of dsRNA 500mers, either homologous to firefly luciferase mRNA (dsLUC) or nonhomologous (dsGFP). Luciferase assays were carried out after a 1 h incubation at 30 °C. Ratios of firefly to *Renilla* activity are normalized to no dsRNA controls. Standard deviations from the mean are shown.

Figure 36: S10 fractions from P19 cell lysates were used for *in vitro* translations of mRNA coding for Photinus pyralis (firefly) and Renilla reniformis (sea pansy) luciferases. Translation reactions were programmed with dsRNA, ssRNA, or asRNA 500mers, either complementary to firefly luciferase mRNA (dsFF, ssFF, or asFF), complementary to Renilla luciferase (dsREN, ssREN, or asREN) or non-complementary (dsGFP). Reactions were carried out at 30 °C for 1 hour, after a 30 min preincubation with dsRNA, ssRNA, or asRNA. Dual luciferase assays were carried out using an Analytical Scientific Instruments model 3010 Luminometer. On the left, Renilla luciferase serves as an internal control for dsRNA-specific suppression of firefly luciferase activity. On the right, firefly luciferase serves as an internal control for dsRNA-specific suppression of Renilla luciferase activity. These data

demonstrate that 500-mer double-stranded RNA (dsRNA) but not single-stranded (ssRNA) or anti-sense RNA (asRNA) suppresses cognate gene expression *in vitro* in a manner consistent with post-transcriptional gene silencing.

Figure 37: P19 cells were grown in 6-well tissue culture plates to approximately 60% confluence. Various amounts of dsRNA, either homologous to firefly luciferase mRNA (dsLUC) or non-homologous (dsGFP), were added to each well and incubated for 12hrs under normal tissue culture conditions. Cells were then transfected with plasmids expressing Photinus pyralis (firefly) and Renilla reniformis (sea pansy) luciferases and with dsRNA 500mers (500ng). Dual luciferase assays were carried out 12 hrs post-transfection using an Analytical Scientific Instruments model 3010 Luminometer. In this assay Renilla luciferase serves as an internal control for dsRNA-specific suppression of firefly luciferase activity. These data show that 500-mer dsRNA can specifically suppress cognate gene expression *in vitro* without transfection under normal tissue culture conditions.

Figure 38: Previous methods for generating siRNAs required costly chemical synthesis. The invention provides an *in vitro* method for synthesizing siRNAs using standard RNA transcription reactions.

Figure 39: Short hairpins suppress gene expression in *Drosophila* S2 cells. **(A)** Sequences and predicted secondary structure of representative chemically synthesized RNAs. Sequences correspond to positions 112-134 (siRNA) and 463-491 (shRNAs) of Firefly luciferase carried on pGL3-Control. An siRNA targeted to position 463-485 of the luciferase sequence was virtually identical to the 112-134 siRNA in suppressing expression, but is not shown. These sequences are represented by SEQ ID NOs: 6-10. **(B)** Exogenously supplied short hairpins suppress expression of the targeted Firefly luciferase gene *in vitro*. Six-well plates of S2 cells were transfected with 250 ng/well of plasmids that direct the expression of firefly and *Renilla* luciferase and 500 ng/well of the indicated RNA. Luciferase activities were assayed 48 h after transfection. Ratios of firefly to *Renilla* luciferase activity were normalized to a control transfected with an siRNA directed at the green fluorescent protein (GFP). The average of three independent experiments is shown; error bars indicate standard deviation. **(C)** Short hairpins are processed by the *Drosophila* Dicer enzyme. T7 transcribed hairpins shFfL22, shFfL29, and shFfS29 were incubated with (+) and without (-) 0-2-h *Drosophila* embryo extracts. Those incubated with extract produced ~22-nt siRNAs, consistent with the ability of these hairpins to induce RNA interference. A long dsRNA input (cyclin E 500-mer) was used as a control. Cleavage reactions were performed as described in Bernstein *et al.*, 2001, Nature, 409:363-366.

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Figure 40: Short hairpins function in mammalian cells. HEK 293T, HeLa, COS-1, and NIH 3T3 cells were transfected with plasmids and RNAs as in Figure 1 and subjected to dual luciferase assays 48 h post-transfection. The ratios of firefly to *Renilla* luciferase activity are normalized to a control transfected with an siRNA directed at the green fluorescent protein (GFP). The average of three independent experiments is shown; error bars indicate standard deviation.

Figure 41: siRNAs and short hairpins transcribed *in vitro* suppress gene expression in mammalian cells. **(A)** Sequences and predicted secondary structure of representative *in vitro* transcribed siRNAs. Sequences correspond to positions 112-134 (siRNA) and 463-491 (shRNAs) of firefly luciferase carried on pGL3-Control. These sequences are represented by SEQ ID NOs: 11-20. **(B)** *In vitro* transcribed siRNAs suppress expression of the targeted firefly luciferase gene *in vitro*. HEK 293T cells were transfected with plasmids as in Figure 2. The presence of non-base-paired guanosine residues at the 5' end of siRNAs significantly alters the predicted end structure and abolishes siRNA activity. **(C)** Sequences and predicted secondary structure of representative *in vitro* transcribed shRNAs. Sequences correspond to positions 112-141 of firefly luciferase carried on pGL3-Control. These sequences are represented by SEQ ID NOs: 21-26. **(D)** Short hairpins transcribed *in vitro* suppress expression of the targeted firefly luciferase gene *in vitro*. HEK 293T cells were transfected with plasmids as in Figure 2.

Figure 42: Transcription of functional shRNAs *in vitro*. **(A)** Schematic of the pShh1 vector. Sequences encoding shRNAs with between 19 and 29 bases of homology to the targeted gene are synthesized as 60-75-bp double-stranded DNA oligonucleotides and ligated into an *Eco*RV site immediately downstream of the U6 promoter. This sequence is represented by SEQ ID NO: 27. **(B)** Sequence and predicted secondary structure of the Ff1 hairpin. **(C)** An shRNA expressed from the pShh1 vector suppresses luciferase expression in mammalian cells. HEK 293T, HeLa, COS-1, and NIH 3T3 cells were transfected with reporter plasmids as in Figure 1, and pShh1 vector, firefly siRNA, or pShh1 firefly shRNA constructs as indicated. The ratios of firefly to *Renilla* luciferase activity were determined 48 h after transfection and represent the average of three independent experiments; error bars indicate standard deviation.

Figure 43: Dicer is required for shRNA-mediated gene silencing. HEK 293T cells were transfected with luciferase reporter plasmids as well as pShh1-Ff1 and an siRNA targeting human Dicer either alone or in combination, as indicated. The Dicer siRNA sequence (TCAACCAGCCACTGCTGGA, SEQ ID NO: 37) corresponds to coordinates

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3137-3155 of the human *Dicer* sequence. The ratios of firefly to *Renilla* luciferase activity were determined 26 h after transfection and represent the average of three independent experiments; error bars indicate standard deviation.

Figure 44: Stable shRNA-mediated gene silencing of an endogenous gene. **(A)** Sequence and predicted secondary structure of the *p53* hairpin. The 5' shRNA stem contains a 27-nt sequence derived from mouse *p53* (nucleotides 166-192), whereas the 3' stem harbors the complimentary antisense sequence. This sequence is represented by SEQ ID NO: 28. **(B)** Senescence bypass in primary mouse embryo fibroblasts (MEFs) expressing an shRNA targeted at *p53*. Wild-type MEFs, passage 5, were transfected with pBabe-RasV12 with control plasmid or with p53hp (5 µg each with FuGENE; Roche). Two days after transfection, cells were trypsinized, counted, and plated at a density of 1×10^5/10-cm plate in media containing 2.0 µg/mL of puromycin. Control cells cease proliferation and show a senescent morphology (*left* panel). Cells expressing the *p53* hairpin continue to grow (*right* panel). Photos were taken 14 d post-transfection.

Figure 45: A mixture of two short hairpins, both corresponding to firefly luciferase, does not result in a synergistic suppression of gene expression. Suppression of firefly luciferase gene expression resulting from transfection of a mixture of two different short hairpins (HP #1 and HP #2) was examined. The mixture of HP #1 and HP #2 did not have a more robust effect on the suppression of firefly luciferase gene expression than expression of HP #1 alone.

Figure 46: Encoded short hairpins specifically suppress gene expression *in vitro*. DNA oligonucleotides encoding 29 nucleotide hairpins corresponding to firefly luciferase were inserted into a vector containing the U6 promoter. Three independent constructs were examined for their ability to specifically suppress firefly luciferase gene expression in 293T cells. siOligo1-2, siOligo1-6, and siOligo1-19 (construct in the correct orientation) each suppressed gene expression as effectively as siRNA. In contrast, siOligo1-10 (construct in the incorrect orientation) did not suppress gene expression. An independent construct targeted to a different portion of the firefly luciferase gene did not effectively suppress gene expression in either orientation (siOligo2-23, siOligo2-36).

Figures 47-49: Strategies for stable expression of short dsRNAs.

Figure 50: Dual luciferase assays were performed as described in detail in figures 28-35, however the cells used in these experiments were $PKR^{-/-}$ murine embryonic fibroblasts (MEFs). Briefly, RNAi using long dsRNAs typically envokes a non-specific response in MEFs (due to PKR activity). To evaluate the effect of long dsRNA constructs to specifically

inhibit gene expression in MEFs, RNAi was examined in PKR$^{-/-}$ MEFs. Such cells do not respond to dsRNA with a non-specific response. The data summarized in this figure demonstrates that in the absence of the non-specific PKR response, long dsRNA constructs specifically suppress gene expression in MEFs.

5 Figure 51: Is a schematic representation of the mouse tyrosinase promoter. Primers were used to amplify three separate regions in the proximal promoter, or to amplify sequence corresponding to an enhancer located approximately 12 kb upstream.

Figure 52: Reporter expression plasmids and siRNA sequences used in Figures X and Y. PGL-3-Control and Pluc -NS5B are the expression plasmids used for transfection into 10 mouse liver. The nucleotide sequences of the siRNAs used in the study are shown underneath. These sequences are represented by SEQ ID NOs: 29-35.

Figure 53: RNA interference in adult mice using siRNAs. **(a)** Representative images of light emitted from mice co-transfected with the luciferase plasmid pGL3-control and either no siRNA, luciferase siRNA or unrelated siRNA. A pseudocolour image representing 15 intensity of emitted light (red, most intense; blue, least intense) superimposed on a greyscale reference image (for orientation) shows that RNAi functions in adult mice. Annealed 21-nucleotide siRNAs (40 μg; Dharmacon) were co-injected into the livers of mice with 2 μg pGL3-control DNA (Promega) and 800 units of RNasin (Promega) in 1.8 ml PBS buffer in 5–7 s. After 72 h, mice were anaesthetized and given 3 mg luciferin intraperitoneally 15 min 20 before imaging. **(b)** siRNA results (six mice per group) from a representative experiment. Mice receiving luciferase siRNA emitted significantly less light than reporter-alone controls (one-way ANOVA with post hoc Fisher's test). Results for reporter alone and unrelated siRNA were statistically similar. Animals were treated according to the US National Institutes of Health's guidelines for animal care and the guidelines of Stanford University.

25 Figure 54: RNA interference in adult mice using shRNAs. **(a)** Representative images of light emitted from mice co-transfected with the luciferase plasmid control, pShh1-Ff1, and pShh1-Ff1rev. pShh1-Ff1, but not pShh1-Ff1rev, reduced luciferase expression in mice relative to the reporter-alone control. pShh1-Ff1 or pShh1-rev (10 μg) were co-injected with 2 μg pGL3-control in 1.8 ml PBS buffer. **(b)** Average of three independent shRNA 30 experiments (n = 5). Average values for the reporter-alone group are designated as 100% in each of the three experiments. Animals were treated according to the US National Institutes of Health's guidelines for animal care and the guidelines of Stanford University.

Figure 55: Heritable repression of Neil1 expression by RNAi in several tissues. **(a)** Expression of Neil1 mRNA in the livers of three mice containing the Neil1 shRNA transgene

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(shRNA-positive) or three siblings lacking the transgene (shRNA-negative) was assayed by RT-PCR (top row is Neil1). An RT-PCR of β-actin was done to ensure that equal quantities of mRNAs were tested for each mouse (second row). Expression of the neomycin resistance gene (neo), carried on the shRNA vector, was tested similarly (third row). Finally, the mice
5 were genotyped using genomic DNA that was PCR-amplified with vector-specific primers (bottom row). (**b**) Similar studies were performed in the heart. (**c**) Similar studies were performed in the spleen. Animal procedures have been approved by the SUNY, Stony Brook Institutional Animal Care and Use Committee (IACUC).

Figure 56: Reduction in Neil1 protein correlates with the presence of siRNAs. (**a**)
10 Expression of Neil1 protein was examined in protein extracts from the livers of mice carrying the shRNA transgene (shRNA-positive) or siblings lacking the transgene (shRNA-negative) by western blotting with Neil1-specific antiserum. A western blot for PCNA was used to standardize loading. (**b**) The presence of siRNAs in RNA derived from the livers of transgenic mice as assayed by northern blotting using a 300 nt probe, part of which was
15 complementary to the shRNA sequence. We note siRNAs only in mice transgenic for the shRNA expression cassette.

Figure 57: *In vitro* processing of 29 nt. shRNAs by Dicer generates a single siRNA from the end of each short hairpin. a) The set of shRNAs containing 19 or 29 nt stems and either bearing or lacking a 2 nucleotide 3'overhang is depicted schematically. For reference
20 the 29 nt sequence from luciferase (top, blue) strand is given. The presumed cleavage sites are indicated in green and by the arrows. b) *In vitro* Dicer processing of shRNAs. Substrates as depicted in a) were incubated either in the presence or absence of recombinant human Dicer (as indicated). Processing of a 500 bp. blunt-ended dsRNA is shown for comparison. Markers are end-labeled, single-stranded, synthetic RNA oligonucleotides. c) All shRNA
25 substrates were incubated with bacterial RNase III to verify their double-stranded nature. This sequence is represented by SEQ ID NO: 36.

Figure 58: Primer extension analysis reveal a single siRNA generated from Dicer processing of shRNA both *in vitro* and *in vivo.* a) 19 nt. shRNAs, as indicated (see Fig. 57a), were processed by Dicer *in vitro*. Reacted RNAs were extended with a specific primer that
30 yields a 20 base product if cleavage occurs 22 bases from the 3' end of the overhung RNA (see Fig. 57a). Lanes labeled siRNA are extensions of synthetic RNAs corresponding to predicted siRNAs that would be released by cleavage 21 or 22 nucleotides from the 3' end of the overhung precursor. Observation of extension products dependents entirely on the inclusion of RT (indicated). Markers are phosphorylated, synthetic DNA oligonucleotides. b)

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Analysis as described in a) for 29 nt. shRNAs. The * indicates the specific extension product from the overhung shRNA species. c) Primer extension were used to analyze products from processing of overhung 29 nt. shRNAs *in vivo*. For comparison, extensions of *in vitro* processed material are also shown. Again, the * indicates the specific extension product.

5 Figure 59: Gene suppression by shRNAs is comparable to or more effective than that achieved by siRNAs targeting the same sequences. a) Structures of synthetic RNAs used for these studies. b) mRNA suppression levels achieved by 43 siRNAs targeting 6 different genes compared with levels achieved by 19-mer (left) or 29-mer (right) shRNAs derived from the same target sequences. All RNAs were transfected at a final concentration of 100 nM. Values 10 indicated on the X and Y axes reflect the percentage of mRNA remaining in HeLa cells 24 hours after RNA transfection compared with cells treated with transfection reagent alone. c) Titration analysis comparing efficacies of four siRNA/shRNA sets targeting MAPK14. Curves are graphed from data derived from transfections at 1.56, 6.25, 25, and 100 nM final concentrations of RNA. (diamonds: 21-mer siRNAs; squares: 19-mer shRNAs; triangles: 29- 15 mer shRNAs).

Figure 60: Microarray profiling reveals sequence-specific gene expression profiles and more similarity between 29-mer shRNAs and cognate siRNAs than observed for 19-mer shRNAs. Each row of the heat maps reports the gene expression signature resulting from transfection of an individual RNA. Data shown represent genes that display at least a 2-fold 20 change in expression level (P value < 0.01 and log10 intensity > 1) relative to mock-transfected cells. Green indicates decreased expression relative to mock transfection whereas red indicates elevated expression. a) 19-mer shRNAs and siRNAs designed for six different target sequences within the coding region of the MAPK14 gene were tested for gene silencing after 24 hours in HeLa cells. b) A similar experiment to that described in a) but 25 carried out with five 29-mer shRNAs targeting MAPK14.

Detailed Description of Certain Preferred Embodiments

I. Overview

The present invention provides methods for attenuating gene expression in a cell 30 using gene-targeted double stranded RNA (dsRNA). The dsRNA contains a nucleotide sequence that hybridizes under physiologic conditions of the cell to the nucleotide sequence of at least a portion of the gene to be inhibited (the "target" gene). The nucleotide sequence

can hybridize to either coding or non-coding sequence of the target gene.

A significant aspect to certain embodiments of the present invention relates to the demonstration in the present application that RNAi can in fact be accomplished both in cultured mammalian cells and in whole organisms. This had not been previously described in 5 the art.

Another salient feature of the present invention concerns the ability to carry out RNAi in higher eukaryotes, particularly in non-oocytic cells of mammals, *e.g.*, cells from adult mammals as an example.

Furthermore, in contrast to the teachings of the prior art, we demonstrate that RNAi in 10 mammalian systems can be mediated with dsRNA identical or similar to non-coding sequence of a target gene. It was previously believed that although dsRNA identical or similar to non-coding sequences (*i.e.*, promoter, enhancer, or intronic sequences) did not inhibit RNAi, such dsRNAs were not thought to mediate RNAi.

In addition, the instant invention also demonstrates that short hairpin RNA (shRNA) 15 may effectively be used in the subject RNAi methods. In certain embodiments, shRNAs specifically designed as Dicer substrates can be used as more potent inducers of RNAi than siRNAs. Not only is maximal inhibition achieved at much lower levels of transfected RNA, but also endpoint inhibition is often greater. In certain other embodiments, mimicking natural pre-miRNAs by inclusion of a 1-5 nucleotide(s), especially a 2 nucleotide 3' overhang, 20 enhances the efficiency of Dicer cleavage and directs cleavage to a specific position in the precursor. The presence of this specific processing site further permits the application of rules for siRNA design to shRNAs, both for chemical synthesis and vector-based delivery of such shRNA constructs. These teachings provide improved methods for evoking RNAi in mammalian cells, and thus improved ability to produce highly potent silencing triggers in 25 therapeutic application of RNAi.

As described in further detail below, the present invention(s) are based on the discovery that the RNAi phenomenon is mediated by a set of enzyme activities, including an essential RNA component, that are evolutionarily conserved in eukaryotes ranging from plants to mammals.

30 One enzyme contains an essential RNA component. After partial purification, a multi-component nuclease (herein "RISC nuclease") co-fractionates with a discrete, 22-nucleotide RNA species which may confer specificity to the nuclease through homology to the substrate mRNAs. The short RNA molecules are generated by a processing reaction from the longer input dsRNA. Without wishing to be bound by any particular theory, these 22-mer guide

RNAs may serve as guide sequences that instruct the RISC nuclease to destroy specific mRNAs corresponding to the dsRNA sequences.

As illustrated, double stranded forms of the 22-mer guide RNA can be sufficient in length to induce sequence-dependent dsRNA inhibition of gene expression. In the illustrated example, dsRNA constructs are administered to cells having a recombinant luciferase reporter gene. In the control cell, *e.g.*, no exogeneously added RNA, the level of expression of the luciferase reporter is normalized to be the value of "1". As illustrated, both long (500-mer) and short (22-mer) dsRNA constructs complementary to the luciferase gene could inhibit expression of that gene product relative to the control cell. On the other hand, similarly sized dsRNA complementary to the coding sequence for another protein, green fluorescence protein (GFP), did not significantly effect the expression of luciferase – indicating that the inhibitory phenomena was in each case sequence-dependent. Likewise, single stranded 22-mers of luciferase did not inhibit expression of that gene – indicating that the inhibitory phenomena is double stranded-dependent.

The appended examples also identify an enzyme, Dicer, that can produce the putative guide RNAs. Dicer is a member of the RNAse III family of nucleases that specifically cleave dsRNA and is evolutionarily conserved in worms, flies, plants, fungi and, as described herein, mammals. The enzyme has a distinctive structure which includes a helicase domain and dual RNAse III motifs. Dicer also contains a region of homology to the RDE1 / QDE2 / ARGONAUTE family, which have been genetically linked to RNAi in lower eukaryotes. Indeed, activation of, or overexpression of Dicer may be sufficient in many cases to permit RNA interference in otherwise non-receptive cells, such as cultured eukaryotic cells, or mammalian (non-oocytic) cells in culture or in whole organisms.

In certain embodiments, the cells can be treated with an agent(s) that inhibits the general double-stranded RNA response(s) by the host cells, such as may give rise to sequence-independent apoptosis. For instance, the cells can be treated with agents that inhibit the dsRNA-dependent protein kinase known as PKR (protein kinase RNA-activated). Double stranded RNAs in mammalian cells typically activate protein kinase PKR and lead to apoptosis. The mechanism of action of PKR includes phosphorylation and inactivation of eIF2α (Fire, *Trends Genet* **15**: 358, 1999). It has also been reported that induction of NF-κB by PKR is involved in apoptosis commitment and this process is mediated through activation of the IKK complex. This sequence-independent response may reflect a form of primitive immune response, since the presence of dsRNA is a common feature of many viral lifecycles.

As described herein, Applicants have demonstrated that the PKR response can be overcome in favor of the sequence-specific RNAi response. However, in certain instances, it may be desirable to treat the cells with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKR, and such methods are specifically
5 contemplated for use in the present invention. Likewise, overexpression of agents which ectopically activate eIF2α can be used. Other agents which can be used to suppress the PKR response include inhibitors of IKK phosphorylation of IκB, inhibitors of IκB ubiquitination, inhibitors of IκB degradation, inhibitors of NF-κB nuclear translocation, and inhibitors of NF-κB interaction with κB response elements.

10 Other inhibitors of sequence-independent dsRNA response in cells include the gene product of the vaccinia virus E3L. The E3L gene product contains two distinct domains. A conserved carboxy-terminal domain has been shown to bind double-stranded RNA (dsRNA) and inhibit the antiviral dsRNA response by cells. Expression of at least that portion of the E3L gene in the host cell, or the use of polypeptide or peptidomimetics thereof, can be used
15 to suppress the general dsRNA response. Caspase inhibitors sensitize cells to killing by double-stranded RNA. Accordingly, ectopic expression or activation of caspases in the host cell can be used to suppress the general dsRNA response.

In other embodiments, the subject method is carried out in cells which have little or no general response to double stranded RNA, *e.g.*, have no PKR-dependent dsRNA response,
20 at least under the culture conditions. As illustrated in Figures 28-32, CHO and P19 cells can be used without having to inhibit PKR or other general dsRNA responses.

Also as described in further detail below, the present invention(s) are partially based on the discovery that short hairpin RNA specifically designed as Dicer substrates are more potent inducers of RNAi than siRNAs. In certain embodiments, shRNA constructs with 1-5,
25 preferably two 3' overhang nucleotides are substrates particulary well-adpated for Dicer-mediated cleavage, and are more potent inhibitors of target genes then their siRNA counterparts with identical complementary sequences. Such shRNA can be formed either *in vitro* or *in vivo* by, for example, sequence-specific pairing after chemical synthesis, or transcription from a promoter operatively-linked to a DNA encoding such hairpin structure.

30 Thus, the present invention provides a process and compositions for inhibiting expression of a target gene in a cell, especially a mammalian cell. In certain embodiments, the process comprises introduction of RNA (the "dsRNA construct") with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is

specific in that a nucleotide sequence from a portion of the target gene is chosen to produce the dsRNA construct. The dsRNA may be identical or similar to coding or non-coding sequence of the target gene. In preferred embodiments, the method utilizes a cell in which Dicer and/or Argonaute activities are recombinantly expressed or otherwise ectopically
5 activated. This process can be (1) effective in attenuating gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

II. Definitions

For convenience, certain terms employed in the specification, examples, and
10 appended claims are collected here.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a genomic integrated vector, or "integrated vector", which can become integrated into the chromosomal DNA of the host cell. Another type of vector is an episomal vector, *i.e.*, a nucleic acid
15 capable of extra-chromosomal replication. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In the present specification, "plasmid" and "vector" are used interchangeably unless otherwise clear from the context.

As used herein, the term "nucleic acid" refers to polynucleotides such as
20 deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide of the present invention, including
25 both exon and (optionally) intron sequences. The nucleic acid may also optionally include non-coding sequences such as promoter or enhancer sequences. A "recombinant gene" refers to nucleic acid encoding such regulatory polypeptides, that may optionally include intron sequences that are derived from chromosomal DNA. The term "intron" refers to a DNA sequence present in a given gene that is not translated into protein and is generally found
30 between exons.

A "protein coding sequence" or a sequence that "encodes" a particular polypeptide or peptide, is a nucleic acid sequence that is transcribed (in the case of DNA) and is translated (in the case of mRNA) into a polypeptide *in vitro* or *in vitro* when placed under the control of

appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from procaryotic or eukaryotic mRNA, genomic DNA sequences from procaryotic or eukaryotic DNA, and even
5 synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

Likewise, "encodes", unless evident from its context, will be meant to include DNA sequences that encode a polypeptide, as the term is typically used, as well as DNA sequences that are transcribed into inhibitory antisense molecules.

10 The term "loss-of-function", as it refers to genes inhibited by the subject RNAi method, refers to a diminishment in the level of expression of a gene(s) in the presence of one or more dsRNA construct(s) when compared to the level in the absence of such dsRNA construct(s).

The term "expression" with respect to a gene sequence refers to transcription of the
15 gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein coding sequence results from transcription and translation of the coding sequence.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the
20 progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

The term "cultured cells" refers to cells suspended in culture, *e.g.*, dispersed in culture
25 or in the form tissue. It does not, however, include oocytes or whole embryos (including blastocysts and the like) which may be provided in culture. In certain embodiments, the cultured cells are adults cells, *e.g.*, non-embryonic.

By "recombinant virus" is meant a virus that has been genetically altered, *e.g.*, by the addition or insertion of a heterologous nucleic acid construct into the particle.

30 As used herein, the terms "transduction" and "transfection" are art recognized and mean the introduction of a nucleic acid, *e.g.*, an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a dsRNA construct.

"Transient transfection" refers to cases where exogenous DNA does not integrate into the genome of a transfected cell, *e.g.*, where episomal DNA is transcribed into mRNA and translated into protein.

A cell has been "stably transfected" with a nucleic acid construct when the nucleic
5 acid construct is capable of being inherited by daughter cells.

As used herein, a "reporter gene construct" is a nucleic acid that includes a "reporter gene" operatively linked to at least one transcriptional regulatory sequence. Transcription of the reporter gene is controlled by these sequences to which they are linked. The activity of at least one or more of these control sequences can be directly or indirectly regulated by the
10 target receptor protein. Exemplary transcriptional control sequences are promoter sequences. A reporter gene is meant to include a promoter-reporter gene construct that is heterologously expressed in a cell.

As used herein, "transformed cells" refers to cells that have spontaneously converted to a state of unrestrained growth, *i.e.*, they have acquired the ability to grow through an
15 indefinite number of divisions in culture. Transformed cells may be characterized by such terms as neoplastic, anaplastic and/or hyperplastic, with respect to their loss of growth control. For purposes of this invention, the terms "transformed phenotype of malignant mammalian cells" and "transformed phenotype" are intended to encompass, but not be limited to, any of the following phenotypic traits associated with cellular transformation of
20 mammalian cells: immortalization, morphological or growth transformation, and tumorigenicity, as detected by prolonged growth in cell culture, growth in semi-solid media, or tumorigenic growth in immuno-incompetent or syngeneic animals.

As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

As used herein, "immortalized cells" refers to cells that have been altered via
25 chemical, genetic, and/or recombinant means such that the cells have the ability to grow through an indefinite number of divisions in culture.

The "growth state" of a cell refers to the rate of proliferation of the cell and the state of differentiation of the cell.

"MHC antigen", as used herein, refers to a protein product of one or more MHC
30 genes; the term includes fragments or analogs of products of MHC genes which can evoke an immune response in a recipient organism. Examples of MHC antigens include the products (and fragments or analogs thereof) of the human MHC genes, *i.e.*, the HLA genes.

The term "histocompatibility" refers to the similarity of tissue between different individuals. The level of histocompatibility describes how well matched the patient and donor

are. The major histocompatibility determinants are the human leukocyte antigens (HLA). HLA typing is performed between the potential marrow donor and the potential transplant recipient to determine how close a HLA match the two are. The closer the match the less the donated marrow and the patient's body will react against each other.

5 The term "human leukocyte antigens" or "HLA", refers to proteins (antigens) found on the surface of white blood cells and other tissues that are used to match donor and patient. For instances, a patient and potential donor may have their white blood cells tested for such HLA antigens as, HLA-A, B and DR. Each individual has two sets of these antigens, one set inherited from each parent. For this reason, it is much more likely for a brother or sister to 10 match the patient than an unrelated individual, and much more likely for persons of the same racial and ethnic backgrounds to match each other.

III. Exemplary Embodiments of Isolation Method

One aspect of the invention provides a method for potentiating RNAi by induction or 15 ectopic activation of an RNAi enzyme in a cell (*in vitro* or *in vitro*) or cell-free mixtures. In preferred embodiments, the RNAi activity is activated or added to a mammalian cell, *e.g.*, a human cell, which cell may be provided *in vitro* or as part of a whole organism. In other embodiments, the subject method is carried out using eukaryotic cells generally (except for oocytes) in culture. For instance, the Dicer enzyme may be activated by virtue of being 20 recombinantly expressed or it may be activated by use of an agent which (i) induces expression of the endogenous gene, (ii) stabilizes the protein from degradation, and/or (iii) allosterically modifies the enzyme to increase its activity (by altering its k_{cat}, K_m or both).

A. Dicer and Argonaut Activities

25 In certain embodiments, at least one of the activated RNAi enzymes is Dicer, or a homolog thereof. In certain preferred embodiments, the present method provides for ectopic activation of Dicer. As used herein, the term "Dicer" refers to a protein which (a) mediates an RNAi response and (b) has an amino acid sequence at least 50 percent identical, and more preferably at least 75, 85, 90 or 95 percent identical to SEQ ID NO: 2 or 4, and/or which can 30 be encoded by a nucleic acid which hybridizes under wash conditions of 2 × SSC at 22°C, and more preferably 0.2 × SSC at 65°C, to a nucleotide represented by SEQ ID NO: 1 or 3. Accordingly, the method may comprise introducing a dsRNA construct into a cell in which

Dicer has been recombinantly expressed or otherwise ectopically activated.

In certain embodiment, at least one of the activated RNAi enzymes is Argonaut, or a homolog thereof. In certain preferred embodiments, the present method provides for ectopic activation of Argonaut. As used herein, the term "Argonaut" refers to a protein which (a) mediates an RNAi response and (b) has an amino acid sequence at least 50 percent identical, and more preferably at least 75, 85, 90 or 95 percent identical to the amino acid sequence shown in Figure 24. Accordingly, the method may comprise introducing a dsRNA construct into a cell in which Argonaut has been recombinantly expressed or otherwise ectopically activated.

This invention also provides expression vectors containing a nucleic acid encoding a Dicer or Argonaut polypeptide, operably linked to at least one transcriptional regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of the subject Dicer or Argonaut proteins. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel, *Gene Expression Technology*: Methods in Enzymology **185**, Academic Press, San Diego, CA, 1990. For instance, any of a wide variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding Dicer or Argonaut polypeptides of this invention. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage λ, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, *e.g.*, Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed.

Moreover, the vector's copy number, the ability to control that copy number and the

expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

The recombinant Dicer or Argonaut genes can be produced by ligating a nucleic acid encoding a Dicer or Argonaut polypeptide into a vector suitable for expression in either 5 prokaryotic cells, eukaryotic cells, or both. Expression vectors for production of recombinant forms of the subject Dicer or Argonaut polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a Dicer or Argonaut polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic 10 cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach *et al.* (1983) in Experimental Manipulation of Gene Expression, ed. M. Inouye 15 Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as Ampicillin can be used. In an illustrative embodiment, a Dicer or Argonaut polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding 20 sequence of a Dicer or Argonaut gene.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived 25 vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient 30 expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16

and 17.

In yet another embodiment, the subject invention provides a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous Dicer or Argonaut gene. For instance, 5 the gene activation construct can replace the endogenous promoter of a Dicer or Argonaut gene with a heterologous promoter, *e.g.*, one which causes constitutive expression of the Dicer or Argonaut gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of Dicer or Argonaut. A variety of different formats for the gene activation constructs are available. See, for example, the Transkaryotic 10 Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

In preferred embodiments, the nucleotide sequence used as the gene activation construct can be comprised of (1) DNA from some portion of the endogenous Dicer or Argonaut gene (exon sequence, intron sequence, promoter sequences, etc.) which direct 15 recombination and (2) heterologous transcriptional regulatory sequence(s) which is to be operably linked to the coding sequence for the genomic Dicer or Argonaut gene upon recombination of the gene activation construct. For use in generating cultures of Dicer or Argonaut producing cells, the construct may further include a reporter gene to detect the presence of the knockout construct in the cell.

20 The gene activation construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to provide the heterologous regulatory sequences in operative association with the native Dicer or Argonaut gene. Such insertion occurs by homologous recombination, *i.e.*, recombination regions of the activation construct that are homologous to the endogenous Dicer or Argonaut gene sequence hybridize to the genomic 25 DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding position of the genomic DNA.

The terms "recombination region" or "targeting sequence" refer to a segment (*i.e.*, a portion) of a gene activation construct having a sequence that is substantially identical to or substantially complementary to a genomic gene sequence, *e.g.*, including 5' flanking 30 sequences of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a recombination region and a genomic sequence.

The heterologous regulatory sequences, *e.g.*, which are provided in the replacement region, can include one or more of a variety of elements, including: promoters (such as constitutive or inducible promoters), enhancers, negative regulatory elements, locus control regions, transcription factor binding sites, or combinations thereof.

5 Promoters/enhancers which may be used to control the expression of the targeted gene *in vitro* include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama *et al.*, *J. Exp. Med* **169**: 13, 1989), the human β-actin promoter (Gunning *et al.*, *PNAS* **84**: 4831-4835, 1987), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig *et al.*, *Mol. Cell Biol.* **4**:
10 1354-1362, 1984), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss *et al.* (1985) *RNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist *et al.*, *Nature* **290**: 304-310, 1981; Templeton *et al.*, *Mol. Cell Biol.* **4**: 817, 1984; and Sprague *et al.*, *J. Virol.* 45: 773, 1983), the promoter contained in the 3' long terminal repeat of Rous sarcoma
15 virus (RSV) (Yamamoto *et al.*, *Cell* 22: 787-797, 1980), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner *et al.*, *PNAS* **82**: 3567-71, 1981), and the herpes simplex virus LAT promoter (Wolfe *et al.*, *Nature Genetics* **1**: 379-384, 1992).

In still other embodiments, the replacement region merely deletes a negative transcriptional control element of the native gene, *e.g.*, to activate expression, or ablates a
20 positive control element, *e.g.*, to inhibit expression of the targeted gene.

B. Cell/Organism

The cell with the target gene may be derived from or contained in any organism (*e.g.*, plant, animal, protozoan, virus, bacterium, or fungus). The dsRNA construct may be
25 synthesized either *in vitro* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vitro*, or cloned RNA polymerase can be used for transcription *in vitro* or *in vitro*. For generating double stranded transcripts from a transgene *in vitro*, a regulatory region may be used to transcribe the RNA strand (or strands). Furthermore, dsRNA can be generated by transcribing an RNA strand which forms a hairpin, thus producing a dsRNA.

30 Genetic manipulation becomes possible in organisms that are not classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease models in which the target gene

are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

The cell with the target gene may be derived from or contained in any organism. The organism may be a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a 5 monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

Plants include arabidopsis; field crops (*e.g.*, alfalfa, barley, bean, com, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable 10 crops (*e.g.*, asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); fruit and nut crops (*e.g.*, almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, faJoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, 15 pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (*e.g.*, alder, ash, aspen, azalea, birch, boxwood, camellia, carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human.

20 Invertebrate animals include nematodes, other worms, *Drosophila*, and other insects. Representative generae of nematodes include those that infect animals (*e.g.*, Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tflichonema, 25 Toxocara, Uncinaria) and those that infect plants (*e.g.*, Bursaphalenchus, Criconerriella, Diiylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynchus, Tylenchus, and Xiphinema). Representative orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

30 The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils,

eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

C. Targeted Genes

The target gene may be a gene derived from the cell, an endogenous gene, a transgene, or a gene of a pathogen which is present in the cell after infection thereof. Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein.

"Inhibition of gene expression" refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. "Specificity" refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxy acid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin.

Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected

material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells (*e.g.*, at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the
5 efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

As disclosed herein, the present invention is not limited to any type of target gene or
10 nucleotide sequence. But the following classes of possible target genes are listed for illustrative purposes: developmental genes (*e.g.*, adhesion molecules, cyclin kinase inhibitors, Writ family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors); oncogenes (*e.g.*, ABLI, BCLI, BCL2,
15 BCL6, CBFA2, CBL, CSFIR, ERBA, ERBB, EBRB2, ETSI, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCLI, MYCN, NRAS, PIM 1, PML, RET, SRC, TALI, TCL3, and YES); tumor suppressor genes (*e.g.*, APC, BRCA 1, BRCA2, MADH4, MCC, NF 1, NF2, RB 1, TP53, and WTI); and enzymes (*e.g.*, ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose
20 pyrophorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine
25 synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

30 D. <u>dsRNA constructs</u>

The dsRNA construct may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to

include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. The dsRNA construct may be produced enzymatically or by 5 partial/total organic synthesis, any modified ribonucleotide can be introduced by *in vitro* enzymatic or organic synthesis.

The dsRNA construct may be directly introduced into the cell (*i.e.*, intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing 10 RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection of an RNA solution directly into the cell or extracellular injection into the organism.

15 The double-stranded structure may be formed by a single self-complementary RNA strand (such as in the form of shRNA) or two complementary RNA strands (such as in the form of siRNA). RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (*e.g.*, at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded 20 material may yield more effective inhibition; lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

dsRNA constructs containing a nucleotide sequences identical to a portion, of either coding or non-coding sequence, of the target gene are preferred for inhibition. RNA 25 sequences with insertions, deletions, and single point mutations relative to the target sequence (ds RNA similar to the target gene) have also been found to be effective for inhibition. Thus, sequence identity may be optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the 30 nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (*e.g.*, University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is

capable of hybridizing with a portion of the target gene transcript (*e.g.*, 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C hybridization for 12-16 hours; followed by washing). In certain preferred embodiments, the length of the dsRNA is at least 20, 21 or 22 nucleotides in length, *e.g.*, corresponding in size to RNA products produced by Dicer-
5 dependent cleavage. In certain embodiments, the dsRNA construct is at least 25, 50, 100, 200, 300 or 400 bases. In certain embodiments, the dsRNA construct is 400-800 bases in length.

In one embodiment, the dsRNA is a single-stranded hairpin ribonucleic acid (shRNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region,
10 which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA: (i) is a substrate for cleavage by a RNaseIII enzyme to produce a double-stranded RNA product, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions. In a preferred embodiment, the
15 shRNA comprises a 3' overhang of about 1-4 nucleotides.

In a related embodiment, he dsRNA is a single-stranded hairpin ribonucleic acid (shRNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA: (i) is cleaved in the mammalian cells to produce
20 an RNA guide sequence that enters an Argonaut-containing complex, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions. In a preferred embodiment, the shRNA comprises a 3' overhang of about 1-4 nucleotides.

The size of the duplex region of the subject shRNA may be longer (e.g., anywhere
25 between 19 to about 1000 nucleotides, or 19 – about 500 nt, or 19 – about 250 nt, etc.), but in many applications, about 29 nucleotides is sufficient. In certain embodiments, the duplex region is any where between about 25 – 29 nt. In other embodiments, the duplex region is any where between about 19 – 25 nt.

The size of the 3' overhang may be 1-5 nucleotides, preferably 2-4 nucleotides. In one
30 embodiment, the 3' overhang is 2 nucleotides. The specific sequences of the 3' overhang nucleotides are less important. In one embodiment, the overhang nucleotides can be any nucleotides, including "non-standard" or modified nucleotides. In other embodiments, the overhang sequences are mostly pyramidines, such as U, C, or T. In one embodiment, the 2-nucleotide ovehang is UU.

In certain embodiments, the 5' of the shRNA may have 1-5 nt overhang that does not pair with the 3' overhang.

The size of the "loop" between the paired duplex region may vary, but preferably contains at least about 3-8 nucleotides, such as 4 nucleotides.

5 100% sequence identity between the RNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

The dsRNA construct may be synthesized either *in vitro* or *in vitro*. Endogenous RNA 10 polymerase of the cell may mediate transcription *in vitro*, or cloned RNA polymerase can be used for transcription *in vitro* or *in vitro*. For transcription from a transgene *in vitro* or an expression construct, a regulatory region (*e.g.*, promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the dsRNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; 15 stimulation of an environmental condition (*e.g.*, infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. The dsRNA construct may be chemically or enzymatically synthesized by manual or automated reactions. The dsRNA 20 construct may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (*e.g.*, T3, T7, SP6). The use and production of an expression construct are known in the art (see also WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by *in vitro* enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For 25 example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography or a combination thereof. Alternatively, the dsRNA construct may be used with no or a minimum of purification to avoid losses due to sample processing. The dsRNA construct may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization 30 of the duplex strands.

Physical methods of introducing nucleic acids include injection of a solution containing the dsRNA construct, bombardment by particles covered by the dsRNA construct, soaking the cell or organism in a solution of the RNA, microinjected into the target (e.g., mammalian target) cells, or electroporation of cell membranes in the presence of the dsRNA

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construct. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of dsRNA construct encoded by the expression construct. In one embodiment, the shRNA is a transcriptional product that is transcribed from an expression construct introduced into the target (e.g., mammalian target) cells, which expression construct comprises a coding sequence for transcribing said shRNA, operably linked to one or more transcriptional regulatory sequences. Such transcriptional regulatory sequences may include a promoter for an RNA polymerase, such as a cellular RNA polymerase. Examplery but not limiting promoters include: a U6 promoter, a T7 promoter, a T3 promoter, or an SP6 promoter. In certain embodiments, the transcriptional regulatory sequences includes an inducible promoter.

The dsRNA constructs may be integrated into the host genome, such that the target cells are stably transfected with the dsRNA expression constructs. The constructs may be suitable for stable integration into either cells in culture or in an animal. For example, the constructs may be integrated into embryonic cells, such as a mouse ES cell, to generate a transgenic animal. The constructs may also be integrated into adult somatic cells, either primary cell or established cell line.

In certain embodiments, the expression of a target gene (either endogenous or heterologous gene) is attenuated by at least about 33%, or about 50%, about 60%, 70%, 80%, 90%, 95%, or 99% or more, relative to expression in cells not treated with the dsRNA (e.g., shRNA).

The shRNA may be chemically synthesized, or *in vitro* transcripted, and may further include one or more modifications to phosphate-sugar backbone or nucleosides residues.

Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical mediated transport, such as calcium phosphate, and the like. Thus the dsRNA construct may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

E. Illustrative Uses

One utility of the present invention is as a method of identifying gene function in an organism, especially higher eukaryotes, comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time

consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceuticals, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for mammalian genomes, can be coupled with the invention to determine gene function in a cell or in a whole organism. The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The ease with which the dsRNA construct can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from the target cell or organism. Inserts may be derived from genomic DNA or mRNA (*e.g.*, cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (*e.g.*, a 96 well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process.

In an exemplary embodiment, the subject invention provides an arrayed library of RNAi constructs. The array may be in the form of solutions, such as multi-well plates, or may be "printed" on solid substrates upon which cells can be grown. To illustrate, solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity.

In one embodiment, the subject method uses an arrayed library of RNAi constructs to

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screen for combinations of RNAi that are lethal to host cells. Synthetic lethality is a bedrock principle of experimental genetics. A synthetic lethality describes the properties of two mutations which, individually, are tolerated by the organism but which, in combination, are lethal. The subject arrays can be used to identify loss-of-function mutations that are lethal in combination with alterations in other genes, such as activated oncogenes or loss-of-function mutations to tumor suppressors. To achieve this, one can create "phenotype arrays" using cultured cells. Expression of each of a set of genes, such as the host cell's genome, can be individually systematically disrupted using RNA interference. Combination with alterations in oncogene and tumor suppressor pathways can be used to identify synthetic lethal interactions that may identify novel therapeutic targets.

In certain embodiments, the RNAi constructs can be fed directly to, or injected into, the cell/organism containing the target gene. Alternatively, the duplex RNA can be produced by *in vitro* or *in vitro* transcription from an expression construct used to produce the library. The construct can be replicated as individual clones of the library and transcribed to produce the RNA; each clone can then be fed to, injected into, or delivered by another method known in the art to, the cell/organism containing the target gene. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. This screening could be amenable to small subjects that can be processed in large number, for example, tissue culture cells derived from mammals, especially primates, and most preferably humans.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, the duplex RNA can be introduced to the organism or cell, and whether an alteration in the characteristic is correlated with inhibition can be determined. Of course, there may be trivial explanations for negative results with this type of assay, for example: inhibition of the target gene causes lethality, inhibition of the target gene may not result in any observable alteration, the fragment contains nucleotide sequences that are not capable of inhibiting the target gene, or the target gene's activity is redundant.

The present invention may be useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or only in specific cellular compartments or tissues. The functional equivalent

of conditional mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the target genome.

5 The present invention may be useful in allowing the inhibition of genes that have been difficult to inhibit using other methods due to gene redundancy. Since the present methods may be used to deliver more than one dsRNA to a cell or organism, dsRNA identical or similar to more than one gene, wherein the genes have a redundant function during normal development, may be delivered.

10 If alternative splicing produced a family of transcripts that were distinguished by usage of characteristic exons, the present invention can target inhibition through the appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a protein factor that contained an alternatively spliced transmembrane domain may be expressed in both membrane bound and secreted forms. 15 Instead of isolating a nonsense mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone. That is, the subject method can be used for selected ablation of splicing variants.

20 The present invention may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the *in vitro* or *in vitro* introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

25 Alternatively, an organism may be engineered to produce dsRNA which produces commercially or medically beneficial results, for example, resistance to a pathogen or its pathogenic effects, improved growth, or novel developmental patterns.

Another aspect of the invention provides a method for attenuating expression of a target gene in mammalian cells, comprising introducing into the mammalian cells a single-30 stranded hairpin ribonucleic acid (shRNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA: (i) is a substrate for cleavage by a RNaseIII enzyme to produce a double-stranded RNA product, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) reduces

expression of said target gene in a manner dependent on the sequence of said complementary regions. In a preferred embodiment, the shRNA comprises a 3' overhang of about 1-4 nucleotides.

In a related aspect, the invention provides a method for attenuating expression of a target gene in mammalian cells, comprising introducing into the mammalian cells a single-stranded hairpin ribonucleic acid (shRNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA: (i) is cleaved in the mammalian cells to produce an RNA guide sequence that enters an Argonaut-containing complex, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions. In a preferred emodiment, the shRNA comprises a 3' overhang of about 1-4 nucleotides.

In yet another embodiment, the invention provides a method for attenuating expression of one or more target genes in mammalian cells, comprising introducing into the mammalian cells a variegated library of single-stranded hairpin ribonucleic acid (shRNA) species, each shRNA species comprising self complementary sequences of 19 to 100 nucleotides that form duplex regions and which hybridize under intracellular conditions to a target gene, wherein each of said hairpin RNA species: (i) is a substrate for cleavage by a RNaseIII enzyme to produce a double-stranded RNA product, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) if complementary to a target sequence, reduces expression of said target gene in a manner dependent on the sequence of said complementary regions. In a preferred embodiment, the shRNA comprises a 3' overhang of about 1-4 nucleotides.

In certain embodiments, the variegated library of shRNA species are arrayed a solid substrate.

In another embodiment, the method includes the further step of identifying shRNA species of said variegated library which produce a detected phenotype in the mammalian cells.

Yet another aspect of the invention provide a method of enhancing the potency / activity of an RNAi therapeutic for a mammalian patient, the RNAi therapeutic comprising an siRNA of 19-22 paired polynucleotides, the method comprising replacing the siRNA with a single-stranded hairpin RNA (shRNA) of the subject invention, wherein said duplex region comprises the same 19-22 paired polynucleotides of the siRNA. This aspect of the invention

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is partly based on the surprising discovery that shRNA constructs designed as Dicer substrates perform at least as well as, and in most cases much better / potent than the corresponding siRNA form of dsRNA (e.g., with the same eventual target guide sequence of about 22 nucleotides).

5 In certain embodiments, the half-maximum inhibition by the RNAi therapeutic is achieved by a concentration of the shRNA at least about 20%, or about 30%, 40%, 50%, 60%, 70%, 80%, 90% lower than that of the corresponding siRNA.

In another embodiment, the end-point inhibition by the shRNA is at least about 40%, or about 50%, 75%, 100%, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, or 10-fold higher than that of
10 the siRNA.

Another aspect of the invention provides a method of designing a short hairpin RNA (shRNA) construct for RNAi, the shRNA comprising a 3' overhang of about 1-4 nucleotides, the method comprising selecting the nucleotide about 21 bases 5' to the most 3'-end nucleotide as the first paired nucleotide in a cognate doubled-stranded siRNA with the same
15 3' overhang. Such shRNA can be used, for example, for RNAi in mammalian cells.

In one embodiment, the shRNA comprises about 15-45, preferably about 25-29 paired polynucleotides.

In one embodiment, the 3' overhang has 2 nucleotides.

In one embodiment, the shRNA, when cut by a Dicer enzyme (e.g., a human Dicer
20 enzyme), produces a product siRNA that is either identical to, or differ by a single basepair immediately 5' to the 3' overhang from the cognate siRNA.

In one embodiment, the shRNA construct has substantially the sameprofiles of off-target gene inhibition effects as compared to the cognate siRNA construct with substantially identical target sequences.

25

IV. Exemplification

The invention, now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention and are not intended to limit the
30 invention.

Example 1: An RNA-Directed Nuclease Mediates RNAi Gene Silencing

In a diverse group of organisms that includes *Caenorhabditis elegans*, *Drosophila*, planaria, hydra, trypanosomes, fungi and plants, the introduction of double-stranded RNAs inhibits gene expression in a sequence-specific manner (Sharp, *Genes and Development* **13**: 139-141, 1999; Sanchez-Alvarado and Newmark, *PNAS* **96**: 5049-5054, 1999; Lohman *et al.*,
5 *Developmental Biology* **214**: 211-214, 1999; Cogoni and Macino, *Nature* **399**: 166-169, 1999; Waterhouse *et al.*, *PNAS* **95**: 13959-13964, 1998; Montgomery and Fire, *Trends Genet.* **14**: 225-228, 1998; Ngo *et al.*, *PNAS* **95**: 14687-14692, 1998). These responses, called RNA interference or post-transcriptional gene silencing, may provide anti-viral defense, modulate transposition or regulate gene expression (Sharp, *Genes and Development* **13**: 139-141, 1999;
10 Montgomery and Fire, *Trends Genet.* **14**: 225-228, 1998; Tabara *et al.*, *Cell* **99**: 123-132, 1999; Ketting *et al.*, *Cell* **99**: 133-141, 1999; Ratcliff *et al.*, *Science* **276**: 1558-1560, 1997). We have taken a biochemical approach towards elucidating the mechanisms underlying this genetic phenomenon. Here we show that 'loss-of-function' phenotypes can be created in cultured *Drosophila* cells by transfection with specific double-stranded RNAs. This coincides
15 with a marked reduction in the level of cognate cellular messenger RNAs. Extracts of transfected cells contain a nuclease activity that specifically degrades exogenous transcripts homologous to transfected double-stranded RNA. This enzyme contains an essential RNA component. After partial purification, the sequence-specific nuclease co-fractionates with a discrete, ~25-nucleotide RNA species which may confer specificity to the enzyme through
20 homology to the substrate mRNAs.

Although double-stranded RNAs (dsRNAs) can provoke gene silencing in numerous biological contexts including *Drosophila* (Kennerdell *et al.*, *Cell* **95**: 1017-1026, 1998; Misquitta and Paterson, *PNAS* **96**: 1451-1456, 1999), the mechanisms underlying this phenomenon have remained mostly unknown. We therefore wanted to establish a
25 biochemically tractable model in which such mechanisms could be investigated.

Transient transfection of cultured, *Drosophila* S2 cells with a *lacZ* expression vector resulted in β-galactosidase activity that was easily detectable by an *in situ* assay (Fig. 1a). This activity was greatly reduced by co-transfection with a dsRNA corresponding to the first 300 nucleotides of the *lacZ* sequence, whereas co-transfection with a control dsRNA (*CD8*)
30 (Fig. 1a) or with single-stranded RNAs of either sense or antisense orientation (data not shown) had little or no effect. This indicated that dsRNAs could interfere, in a sequence-specific fashion, with gene expression in cultured cells.

To determine whether RNA interference (RNAi) could be used to target endogenous

genes, we transfected S2 cells with a dsRNA corresponding to the first 540 nucleotides of *Drosophila cyclin E*, a gene that is essential for progression into S phase of the cell cycle. During log-phase growth, untreated S2 cells reside primarily in G2/M (Fig. 1b). Transfection with *lacZ* dsRNA had no effect on cell-cycle distribution, but transfection with the *cyclin E*
5 dsRNA caused a G1-phase cell-cycle arrest (Fig. 1b). The ability of *cyclin E* dsRNA to provoke this response was length-dependent. Double-stranded RNAs of 540 and 400 nucleotides were quite effective, whereas dsRNAs of 200 and 300 nucleotides were less potent. Double-stranded *cyclin E* RNAs of 50 or 100 nucleotides were inert in our assay, and transfection with a single-stranded, antisense *cyclin E* RNA had virtually no effect.

10 One hallmark of RNAi is a reduction in the level of mRNAs that are homologous to the dsRNA. Cells transfected with the *cyclin E* dsRNA (bulk population) showed diminished endogenous *cyclin E* mRNA as compared with control cells (Fig. 1c). Similarly, transfection of cells with dsRNAs homologous to *fizzy*, a component of the anaphase-promoting complex (APC) or *cyclin A*, a cyclin that acts in S, G2 and M, also caused reduction of their cognate
15 mRNAs (Fig.1c). The modest reduction in *fizzy* mRNA levels in cells transfected with *cyclin A* dsRNA probably resulted from arrest at a point in the division cycle at which *fizzy* transcription is low (Wolf and Jackson, *Current Biology* **8**: R637-R639, 1998; Kramer *et al.*, *Current Biology* **8**: 1207-1210, 1998). These results indicate that RNAi may be a generally applicable method for probing gene function in cultured *Drosophila* cells.

20 The decrease in mRNA levels observed upon transfection of specific dsRNAs into *Drosophila* cells could be explained by effects at transcriptional or post-transcriptional levels. Data from other systems have indicated that some elements of the dsRNA response may affect mRNA directly (reviewed in Sharp, *Genes and Development* **13**: 139-141, 1999; Montgomery and Fire, *Trends Genet.* **14**: 225-228, 1998). We therefore sought to develop a
25 cell-free assay that reflected, at least in part, RNAi.

S2 cells were transfected with dsRNAs corresponding to either *cyclin E* or *lacZ*. Cellular extracts were incubated with synthetic mRNAs of *lacZ* or *cyclin E*. Extracts prepared from cells transfected with the 540-nucleotide *cyclin E* dsRNA efficiently degraded the *cyclin E* transcript; however, the *lacZ* transcript was stable in these lysates (Fig. 2a). Conversely,
30 lysates from cells transfected with the *lacZ* dsRNA degraded the *lacZ* transcript but left the *cyclin E* mRNA intact. These results indicate that RNAi ablates target mRNAs through the generation of a sequence-specific nuclease activity. We have termed this enzyme RISC (RNA-induced silencing complex). Although we occasionally observed possible intermediates in the degradation process (see Fig. 2), the absence of stable cleavage end-

products indicates an exonuclease (perhaps coupled to an endonuclease). However, it is possible that the RNAi nuclease makes an initial endonucleolytic cut and that non-specific exonucleases in the extract complete the degradation process (Shuttleworth and Colman, *EMBO J.* 7: 427-434, 1988). In addition, our ability to create an extract that targets *lacZ in*
5 *vitro* indicates that the presence of an endogenous gene is not required for the RNAi response.

To examine the substrate requirements for the dsRNA-induced, sequence-specific nuclease activity, we incubated a variety of *cyclin-E*-derived transcripts with an extract derived from cells that had been transfected with the 540-nucleotide *cyclin E* dsRNA (Fig.
10 2b, c). Just as a length requirement was observed for the transfected dsRNA, the RNAi nuclease activity showed a dependence on the size of the RNA substrate. Both a 600-nucleotide transcript that extends slightly beyond the targeted region (Fig. 2b) and an ~1-kilobase (kb) transcript that contains the entire coding sequence (data not shown) were completely destroyed by the extract. Surprisingly, shorter substrates were not degraded as
15 efficiently. Reduced activity was observed against either a 300- or a 220-nucleotide transcript, and a 100-nucleotide transcript was resistant to nuclease in our assay. This was not due solely to position effects because ~100-nucleotide transcripts derived from other portions of the transfected dsRNA behaved similarly (data not shown). As expected, the nuclease activity (or activities) present in the extract could also recognize the antisense strand of the
20 *cyclin E* mRNA. Again, substrates that contained a substantial portion of the targeted region were degraded efficiently whereas those that contained a shorter stretch of homologous sequence (~130 nucleotides) were recognized inefficiently (Fig. 2c, as600). For both the sense and antisense strands, transcripts that had no homology with the transfected dsRNA (Fig. 2b, Eout; Fig. 2c, as300) were not degraded. Although we cannot exclude the possibility
25 that nuclease specificity could have migrated beyond the targeted region, the resistance of transcripts that do not contain homology to the dsRNA is consistent with data from *C. elegans*. Double-stranded RNAs homologous to an upstream cistron have little or no effect on a linked downstream cistron, despite the fact that unprocessed, polycistronic mRNAs can be readily detected (Tabara *et al.*, *Science* **282**: 430-432, 1998; Bosher *et al.*, *Genetics* **153**:
30 1245-1256, 1999). Furthermore, the nuclease was inactive against a dsRNA identical to that used to provoke the RNAi response *in vitro* (Fig. 2b). In the *in vitro* system, neither a 5' cap nor a poly(A) tail was required, as such transcripts were degraded as efficiently as uncapped and non-polyadenylated RNAs.

Gene silencing provoked by dsRNA is sequence specific. A plausible mechanism for

determining specificity would be incorporation of nucleic-acid guide sequences into the complexes that accomplish silencing (Hamilton and Baulcombe, *Science* **286**: 950-952, 1999). In accord with this idea, pre-treatment of extracts with a Ca^{2+}-dependent nuclease (micrococcal nuclease) abolished the ability of these extracts to degrade cognate mRNAs
5 (Fig. 3). Activity could not be rescued by addition of non-specific RNAs such as yeast transfer RNA. Although micrococcal nuclease can degrade both DNA and RNA, treatment of the extract with DNAse I had no effect (Fig. 3). Sequence-specific nuclease activity, however, did require protein (data not shown). Together, our results support the possibility that the RNAi nuclease is a ribonucleoprotein, requiring both RNA and protein components.
10 Biochemical fractionation (see below) is consistent with these components being associated in extract rather than being assembled on the target mRNA after its addition.

In plants, the phenomenon of co-suppression has been associated with the existence of small (~25-nucleotide) RNAs that correspond to the gene that is being silenced (Hamilton and Baulcombe, *Science* **286**: 950-952, 1999). To address the possibility that a similar RNA
15 might exist in *Drosophila* and guide the sequence-specific nuclease in the choice of substrate, we partially purified our activity through several fractionation steps. Crude extracts contained both sequence-specific nuclease activity and abundant, heterogeneous RNAs homologous to the transfected dsRNA (Figs 2 and 4a). The RNAi nuclease fractionated with ribosomes in a high-speed centrifugation step. Activity could be extracted by treatment with high salt, and
20 ribosomes could be removed by an additional centrifugation step. Chromatography of soluble nuclease over an anion-exchange column resulted in a discrete peak of activity (Fig. 4b, *cyclin E*). This retained specificity as it was inactive against a heterologous mRNA (Fig. 4b, *lacZ*). Active fractions also contained an RNA species of 25 nucleotides that is homologous to the *cyclin E* target (Fig. 4b, northern). The band observed on northern blots may represent
25 a family of discrete RNAs because it could be detected with probes specific for both the sense and antisense *cyclin E* sequences and with probes derived from distinct segments of the dsRNA (data not shown). At present, we cannot determine whether the 25-nucleotide RNA is present in the nuclease complex in a double-stranded or single-stranded form.

RNA interference allows an adaptive defense against both exogenous and endogenous
30 dsRNAs, providing something akin to a dsRNA immune response. Our data, and that of others (Hamilton and Baulcombe, *Science* **286**: 950-952, 1999), is consistent with a model in which dsRNAs present in a cell are converted, either through processing or replication, into small specificity determinants of discrete size in a manner analogous to antigen processing. Our results suggest that the post-transcriptional component of dsRNA-dependent gene

silencing is accomplished by a sequence-specific nuclease that incorporates these small RNAs as guides that target specific messages based upon sequence recognition. The identical size of putative specificity determinants in plants (Hamilton and Baulcombe, *supra*) and animals predicts a conservation of both the mechanisms and the components of dsRNA-induced, post-transcriptional gene silencing in diverse organisms. In plants, dsRNAs provoke not only post-transcriptional gene silencing but also chromatin remodeling and transcriptional repression (Jones *et al.*, *EMBO J.* **17**: 6385-6393, 1998; Jones *et al.*, *Plant Cell* **11**: 2291-2301, 1999). It is now critical to determine whether conservation of gene-silencing mechanisms also exists at the transcriptional level and whether chromatin remodeling can be directed in a sequence-specific fashion by these same dsRNA-derived guide sequences.

Methods:

Cell culture and RNA methods S2 cells (Schneider, *J. Embryol Exp Morpho* **27**: 353-365, 1972) were cultured at 27°C in 90% Schneider's insect media (Sigma), 10% heat inactivated fetal bovine serum (FBS). Cells were transfected with dsRNA and plasmid DNA by calcium phosphate co-precipitation (DiNocera and Dawid, *PNAS* **80**: 7095-7098, 1983). Identical results were observed when cells were transfected using lipid reagents (for example, Superfect, Qiagen). For FACS analysis, cells were additionally transfected with a vector that directs expression of a green fluorescent protein (GFP)–US9 fusion protein (Kalejta *et al.*, *Exp Cell Res.* **248**: 322-328, 1999). These cells were fixed in 90% ice-cold ethanol and stained with propidium iodide at 25 μg/ml. FACS was performed on an Elite flow cytometer (Coulter). For northern blotting, equal loading was ensured by over-probing blots with a control complementary DNA (RP49). For the production of dsRNA, transcription templates were generated by polymerase chain reaction such that they contained T7 promoter sequences on each end of the template. RNA was prepared using the RiboMax kit (Promega). Confirmation that RNAs were double stranded came from their complete sensitivity to RNAse III. Target mRNA transcripts were synthesized using the Riboprobe kit (Promega) and were gel purified before use.

Extract preparation Log-phase S2 cells were plated on 15-cm tissue culture dishes and transfected with 30 μg dsRNA and 30 μg carrier plasmid DNA. Seventy-two hours after transfection, cells were harvested in PBS containing 5 mM EGTA, washed twice in PBS and once in hypotonic buffer (10 mM HEPES pH 7.3, 6 mM β-mercaptoethanol). Cells were suspended in 0.7 packed-cell volumes of hypotonic buffer containing *Complete* protease

inhibitors (Boehringer) and 0.5 units/ml of RNasin (Promega). Cells were disrupted in a dounce homogenizer with a type B pestle, and lysates were centrifuged at 30,000*g* for 20 min. Supernatants were used in an *in vitro* assay containing 20 mM HEPES pH 7.3, 110 mM KOAc, 1 mM $Mg(OAc)_2$, 3 mM EGTA, 2 mM $CaCl_2$, 1 mM DTT. Typically, 5 µl extract was used in a 10 µl assay that contained also 10,000 c.p.m. synthetic mRNA substrate.

Extract fractionation Extracts were centrifuged at 200,000*g* for 3 h and the resulting pellet (containing ribosomes) was extracted in hypotonic buffer containing also 1 mM $MgCl_2$ and 300 mM KOAc. The extracted material was spun at 100,000g for 1 h and the resulting supernatant was fractionated on Source 15Q column (Pharmacia) using a KCl gradient in buffer A (20 mM HEPES pH 7.0, 1 mM dithiothreitol, 1 mM $MgCl_2$). Fractions were assayed for nuclease activity as described above. For northern blotting, fractions were proteinase K/SDS treated, phenol extracted, and resolved on 15% acrylamide 8M urea gels. RNA was electroblotted onto Hybond N+ and probed with strand-specific riboprobes derived from cyclin E mRNA. Hybridization was carried out in 500 mM $NaPO_4$ pH 7.0, 15% formamide, 7% SDS, 1% BSA. Blots were washed in 1 × SSC at 37–45°C.

Example 2: Role for a Bidentate Ribonuclease in the Initiation Step of RNA Interference

Genetic approaches in worms, fungi and plants have identified a group of proteins that are essential for double-stranded RNA-induced gene silencing. Among these are ARGONAUTE family members (*e.g.* RDE1, QDE2) (Tabara *et al.*, *Cell* **99**: 123-132, 1999; Catalanotto *et al.*, *Nature* **404**: 245, 2000; Fagard *et al.*, *PNAS* **97**: 11650-11654, 2000), recQ-family helicases (MUT-7, QDE3) (Ketting *et al.*, *Cell* **99**: 133-141, 1999; Cogoni and Macino, *Science* **286**: 2342-2344, 1999), and RNA-dependent RNA polymerases (*e.g.*, EGO-1, QDE1, SGS2/SDE1) (Cogoni and Macino, *Nature* **399**: 166-169, 1999; Smardon *et al.*, *Current Biology* **10**: 169-178, 2000; Mourrain *et al.*, *Cell* **101**: 533-542, 2000; Dalmay *et al.*, *Cell* **101**: 543-553, 2000). While potential roles have been proposed, none of these genes has been assigned a definitive function in the silencing process. Biochemical studies have suggested that PTGS is accomplished by a multicomponent nuclease that targets mRNAs for degradation (Hammond *et al.*, *Nature* **404**: 293-296, 2000; Zamore *et al.*, *Cell* **101**: 25-33, 2000; Tuschl *et al.*, *Genes and Development* **13**: 3191-3197, 1999). We have shown that the specificity of this complex may derive from the incorporation of a small guide sequence that is homologous to the mRNA substrate (Hammond *et al.*, *Nature* **404**: 293-296, 2000).

Originally identified in plants that were actively silencing transgenes (Hamilton and Baulcombe, *Science* **286**: 950-952, 1999), these ~22 nt. RNAs have been produced during RNAi *in vitro* using an extract prepared from *Drosophila* embryos (Zamore *et al.*, *Cell* **101**: 25-33, 2000). Putative guide RNAs can also be produced in extracts from *Drosophila* S2 cells
5 (Fig. 5a). With the goal of understanding the mechanism of post-transcriptional gene silencing, we have undertaken both biochemical fractionation and candidate gene approaches to identify the enzymes that execute each step of RNAi.

Our previous studies resulted in the partial purification of a nuclease, RISC, that is an effector of RNA interference. See Example 1. This enzyme was isolated from *Drosophila* S2
10 cells in which RNAi had been initiated *in vitro* by transfection with dsRNA. We first sought to determine whether the RISC enzyme and the enzyme that initiates RNAi via processing of dsRNA into 22mers are distinct activities. RISC activity could be largely cleared from extracts by high-speed centrifugation (100,000 × g for 60 min.) while the activity that produces 22mers remained in the supernatant (Fig. 5b,c). This simple fractionation indicated
15 that RISC and the 22mer-generating activity are separable and thus distinct enzymes. However, it seems likely that they might interact at some point during the silencing process.

RNAse III family members are among the few nucleases that show specificity for double-stranded RNA (Nicholson, *FEMS Microbiol Rev* **23**: 371-390, 1999). Analysis of the *Drosophila* and *C. elegans* genomes reveals several types of RNAse III enzymes. First is the
20 canonical RNAse III which contains a single RNAse III signature motif and a double-stranded RNA binding domain (dsRBD; *e.g.* RNC_CAEEL). Second is a class represented by Drosha (Filippov *et al.*, *Gene* **245**: 213-221, 2000), a *Drosophila* enzyme that contains two RNAse III motifs and a dsRBD (CeDrosha in *C. elegans*). A third class contains two RNAse III signatures and an amino terminal helicase domain (*e.g. Drosophila* CG4792, CG6493, *C.*
25 *elegans* K12H4.8), and these had previously been proposed by Bass as candidate RNAi nucleases (Bass, *Cell* **101**: 235-238, 2000). Representatives of all three classes were tested for the ability to produce discrete, ~22 nt. RNAs from dsRNA substrates.

Partial digestion of a 500 nt. cyclin E dsRNA with purified, bacterial RNAse III produced a smear of products while nearly complete digestion produced a heterogeneous
30 group of ~11-17 nucleotide RNAs (not shown). In order to test the dual-RNAse III enzymes, we prepared T7 epitope-tagged versions of Drosha and CG4792. These were expressed in transfected S2 cells and isolated by immunoprecipitation using antibody-agarose conjugates. Treatment of the dsRNA with the CG4792 immunoprecipitate yielded ~22 nt. fragments

similar to those produced in either S2 or embryo extracts (Fig. 6a). Neither activity in extract nor activity in immunoprecipitates depended on the sequence of the RNA substrate since dsRNAs derived from several genes were processed equivalently (see Supplement 1). Negative results were obtained with Drosha and with immunoprecipitates of a DExH box 5 helicase (Homeless (Gillespie *et al.*, *Genes and Development* 9: 2495-2508, 1995); see Fig 6a,b). Western blotting confirmed that each of the tagged proteins was expressed and immunoprecipitated similarly (see Supplement 2). Thus, we conclude that CG4792 may carry out the initiation step of RNA interference by producing ~22 nt. guide sequences from dsRNAs. Because of its ability to digest dsRNA into uniformly sized, small RNAs, we have 10 named this enzyme Dicer (*Dcr*). *Dicer* mRNA is expressed in embryos, in S2 cells, and in adult flies, consistent with the presence of functional RNAi machinery in all of these contexts (see Supplement 3).

The possibility that Dicer might be the nuclease responsible for the production of guide RNAs from dsRNAs prompted us to raise an antiserum directed against the carboxy-15 terminus of the Dicer protein (Dicer-1, CG4792). This antiserum could immunoprecipitate a nuclease activity from either *Drosophila* embryo extracts or from S2 cell lysates that produced ~22 nt. RNAs from dsRNA substrates (Fig. 6C). The putative guide RNAs that are produced by the Dicer-1 enzyme precisely co-migrate with 22mers that are produced in extract and with 22mers that are associated with the RISC enzyme (Fig. 6 D,F). It had 20 previously been shown that the enzyme that produced guide RNAs in *Drosophila* embryo extracts was ATP-dependent (Zamore *et al.*, *Cell* **101**: 25-33, 2000). Depletion of this cofactor resulted in an ~6-fold lower rate of dsRNA cleavage and in the production of RNAs with a slightly lower mobility. Of interest was the fact that both Dicer-1 immunoprecipitates and extracts from S2 cells require ATP for the production of ~22mers (Fig. 6D). We do not 25 observe the accumulation of lower mobility products in these cases, although we do routinely observe these in ATP-depleted embryo extracts. The requirement of this nuclease for ATP is a quite unusual property. We hypothesize that this requirement could indicate that the enzyme may act processively on the dsRNA, with the helicase domain harnessing the energy of ATP hydrolysis both for unwinding guide RNAs and for translocation along the substrate.

30 Efficient induction of RNA interference in *C. elegans* and in *Drosophila* has several requirements. For example, the initiating RNA must be double-stranded, and it must be several hundred nucleotides in length. To determine whether these requirements are dictated by Dicer, we characterized the ability of extracts and of immunoprecipitated enzyme to digest various RNA substrates. Dicer was inactive against single stranded RNAs regardless of

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length (see Supplement 4). The enzyme could digest both 200 and 500 nucleotide dsRNAs but was significantly less active with shorter substrates (see Supplement 4). Double-stranded RNAs as short as 35 nucleotides could be cut by the enzyme, albeit very inefficiently (data not shown). In contrast, *E. coli* RNAse III could digest to completion dsRNAs of 35 or 22
5 nucleotides (not shown). This suggests that the substrate preferences of the Dicer enzyme may contribute to but not wholly determine the size dependence of RNAi.

To determine whether the Dicer enzyme indeed played a role in RNAi *in vitro*, we sought to deplete Dicer activity from S2 cells and test the effect on dsRNA-induced gene silencing. Transfection of S2 cells with a mixture of dsRNAs homologous to the two
10 *Drosophila* Dicer genes (CG4792 and CG6493) resulted in an ~6-7 fold reduction of Dicer activity either in whole cell lysates or in Dicer-1 immunoprecipitates (Fig. 7A,B). Transfection with a control dsRNA (murine caspase 9) had no effect. Qualitatively similar results were seen if Dicer was examined by Northern blotting (not shown). Depletion of Dicer in this manner substantially compromised the ability of cells to silence subsequently an
15 exogenous, GFP transgene by RNAi (Fig. 7C). These results indicate that Dicer is involved in RNAi *in vitro*. The lack of complete inhibition of silencing could result from an incomplete suppression of Dicer (which is itself required for RNAi) or could indicate that *in vitro*, guide RNAs can be produced by more than one mechanism (*e.g.* through the action of RNA-dependent RNA polymerases).

20 Our results indicate that the process of RNA interference can be divided into at least two distinct steps. According to this model, initiation of PTGS would occur upon processing of a double-stranded RNA by Dicer into ~22 nucleotide guide sequences, although we cannot formally exclude the possibility that another, Dicer-associated nuclease may participate in this process. These guide RNAs would be incorporated into a distinct nuclease complex
25 (RISC) that targets single-stranded mRNAs for degradation. An implication of this model is that guide sequences are themselves derived directly from the dsRNA that triggers the response. In accord with this model, we have demonstrated that ^{32}P-labeled, exogenous dsRNAs that have been introduced into S2 cells by transfection are incorporated into the RISC enzyme as 22 mers (Fig. 7E). However, we cannot exclude the possibility that RNA-
30 dependent RNA polymerases might amplify 22mers once they have been generated or provide an alternative method for producing guide RNAs.

The structure of the Dicer enzyme provokes speculation on the mechanism by which the enzyme might produce discretely sized fragments irrespective of the sequence of the dsRNA (see Supplement 1, Fig. 8a). It has been established that bacterial RNAse III acts on

its substrate as a dimer (Nicholson, *FEMS Microbiol Rev* **23**: 371-390, 1999; Robertson *et al.*, *J Biol Chem* **243**: 82-91, 1968; Dunn, *J Biol Chem* **251**: 3807-3814, 1976). Similarly, a dimer of Dicer enzymes may be required for cleavage of dsRNAs into ~22 nt. pieces. According to one model, the cleavage interval would be determined by the physical arrangement of the two 5 RNAse III domains within Dicer enzyme (Fig. 8a). A plausible alternative model would dictate that cleavage was directed at a single position by the two RIII domains in a single Dicer protein. The 22 nucleotide interval could be dictated by interaction of neighboring Dicer enzymes or by translocation along the mRNA substrate. The presence of an integral helicase domain suggests that the products of Dicer cleavage might be single-stranded 22 10 mers that are incorporated into the RISC enzyme as such.

A notable feature of the Dicer family is its evolutionary conservation. Homologs are found in *C. elegans* (K12H4.8), *Arabidopsis* (*e.g.*, CARPEL FACTORY (Jacobson *et al.*, *Development* **126**: 5231-5243, 1999), T25K16.4, AC012328_1), mammals (Helicase-MOI (Matsuda *et al.*, *Biochim Biophys Acta* **1490**: 163-169, 2000) and *S. pombe* (YC9A_SCHPO) 15 (Fig 8b, see Supplements 6,7 for sequence comparisons). In fact, the human Dicer family member is capable of generating ~22 nt. RNAs from dsRNA substrates (Supplement 5) suggesting that these structurally similar proteins may all share similar biochemical functions. It has been demonstrated that exogenous dsRNAs can affect gene function in early mouse embryos (Wianny *et al.*, *Nature Cell Biology* **2**: 70-75, 2000), and our results suggest that this 20 regulation may be accomplished by an evolutionarily conserved RNAi machinery.

In addition to RNAseIII and helicase motifs, searches of the PFAM database indicate that each Dicer family member also contains a ZAP domain (Fig 8c) (Sonnhammer *et al.*, *Proteins* **28**: 405-420, 1997). This sequence was defined based solely upon its conservation in the Zwille / ARGONAUTE / Piwi family that has been implicated in RNAi by mutations *in* 25 *C. elegans* (Rde-1) and *Neurospora* (Qde-2) (Tabara *et al.*, *Cell* **99**: 123-132, 1999; Catalanotto *et al.*, *Nature* **404**: 245, 2000). Although the function of this domain is unknown, it is intriguing that this region of homology is restricted to two gene families that participate in dsRNA-dependent silencing. Both the ARGONAUTE and Dicer families have also been implicated in common biological processes, namely the determination of stem-cell fates. A 30 hypomorphic allele of *carpel factory*, a member of the Dicer family in *Arabidopsis*, is characterized by increased proliferation in floral meristems (Jacobsen *et al.*, *Development* **126**: 5231-5243, 1999). This phenotype and a number of other characteristic features are also shared by *Arabidopsis ARGONAUTE* (*ago1-1*) mutants (Bohmert *et al.*, *EMBO J* **17**: 170-180, 1998; C. Kidner and R. Martiennsen, pers. comm.). These genetic analyses begin to

provide evidence that RNAi may be more than a defensive response to unusual RNAs but may also play important roles in the regulation of endogenous genes.

With the identification of Dicer as a catalyst of the initiation step of RNAi, we have begun to unravel the biochemical basis of this unusual mechanism of gene regulation. It will 5 be of critical importance to determine whether the conserved family members from other organisms, particularly mammals, also play a role in dsRNA-mediated gene regulation.

Methods:

Plasmid constructs. A full-length cDNA encoding Drosha was obtained by PCR 10 from an EST sequenced by the Berkeley *Drosophila* genome project. The *Homeless* clone was a gift from Gillespie and Berg (Univ. Washington). The T7 epitope-tag was added to the amino terminus of each by PCR, and the tagged cDNAs were cloned into pRIP, a retroviral vector designed specifically for expression in insect cells (E. Bernstein, unpublished). In this vector, expression is driven by the *Orgyia pseudotsugata* IE2 promoter (Invitrogen). Since no 15 cDNA was available for CG4792/Dicer, a genomic clone was amplified from a bacmid (BACR23F10; obtained from the BACPAC Resource Center in the Dept. of Human Genetics at the Roswell Park Cancer Institute). Again, during amplification, a T7 epitope tag was added at the amino terminus of the coding sequence. The human Dicer gene was isolated from a cDNA library prepared from HaCaT cells (GJH, unpublished). A T7-tagged version of 20 the complete coding sequence was cloned into pCDNA3 (Invitrogen) for expression in human cells (LinX-A).

Cell culture and extract preparation. *S2 and embryo culture.* S2 cells were cultured at 27°C in 5% CO_2 in Schneider's insect media supplemented with 10% heat inactivated fetal bovine serum (Gemini) and 1% antibiotic-antimycotic solution (GIBCO BRL). Cells were 25 harvested for extract preparation at 10×10^6 cells/ml. The cells were washed 1X in PBS and were resuspended in a hypotonic buffer (10 mM HEPES pH 7.0, 2 mM $MgCl_2$, 6 mM βME) and dounced. Cell lysates were spun 20,000 × g for 20 minutes. Extracts were stored at −80°C. *Drosophila* embryos were reared in fly cages by standard methodologies and were collected every 12 hours. The embryos were dechorionated in 50% chlorox bleach and 30 washed thoroughly with distilled water. Lysis buffer (10 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM DTT, 0.2 mM PMSF) was added to the embryos, and extracts were prepared by homogenization in a tissue grinder. Lysates were spun for two hours at 200,000 × g and were frozen at −80°C. LinX-A cells, a highly-

transfectable derivative of human 293 cells, (Lin Xie and GJH, unpublished) were maintained in DMEM/10% FCS.

Transfections and immunoprecipitations. S2 cells were transfected using a calcium phosphate procedure essentially as previously described (Hammond *et al.*, *Nature* **404**: 293-296, 2000). Transfection rates were ~90% as monitored in controls using an *in situ* β-galactosidase assay. LinX-A cells were also transfected by calcium phosphate co-precipitation. For immunoprecipitations, cells (~ $5x10^6$ per IP) were transfected with various clones and lysed three days later in IP buffer (125 mM KOAc, 1 mM MgOAc, 1 mM $CaCl_2$, 5 mM EGTA, 20 mM Hepes pH 7.0, 1 mM DTT, 1% NP-40 plus Complete protease inhibitors, Roche). Lysates were spun for 10 minutes at 14,000 × g and supernatants were added to T7 antibody-agarose beads (Novagen). Antibody binding proceeded for 4 hours at 4°C. Beads were centrifuged and washed in lysis buffer three times, and once in reaction buffer. The Dicer antiserum was raised in rabbits using a KLH-conjugated peptide corresponding to the C-terminal 8 amino acids of *Drosophila* Dicer-1 (CG4792).

Cleavage reactions. *RNA preparation.* Templates to be transcribed into dsRNA were generated by PCR with forward and reverse primers, each containing a T7 promoter sequence. RNAs were produced using Riboprobe (Promega) kits and were uniformly labeling during the transcription reaction with ^{32}P-UTP. Single-stranded RNAs were purified from 1% agarose gels. *dsRNA cleavage.* Five microliters of embryo or S2 extracts were incubated for one hour at 30°C with dsRNA in a reaction containing 20 mM Hepes pH 7.0, 2 mM MgOAc, 2 mM DTT, 1 mM ATP and 5% Superasin (Ambion). Immunoprecipitates were treated similarly except that a minimal volume of reaction buffer (including ATP and Superasin) and dsRNA were added to beads that had been washed in reaction buffer (see above). For ATP depletion, *Drosophila* embryo extracts were incubated for 20 minutes at 30°C with 2mM glucose and 0.375 U of hexokinase (Roche) prior to the addition of dsRNA.

Northern and Western analysis. Total RNA was prepared from *Drosophila* embryos (0-12 hour), from adult flies, and from S2 cells using Trizol (Lifetech). Messenger RNA was isolated by affinity selection using magnetic oligo-dT beads (Dynal). RNAs were electrophoresed on denaturing formaldehyde/agarose gels, blotted and probed with randomly primed DNAs corresponding to Dicer. For Western analysis, T7-tagged proteins were immunoprecipitated from whole cell lysates in IP buffer using anti-T7-antibody-agarose conjugates. Proteins were released from the beads by boiling in Laemmli buffer and were separated by electrophoresis on 8% SDS PAGE. Following transfer to nitrocellulose, proteins

were visualized using an HRP-conjugated anti-T7 antibody (Novagen) and chemiluminescent detection (Supersignal, Pierce).

RNAi of Dicer. *Drosophila* S2 cells were transfected either with a dsRNA corresponding to mouse caspase 9 or with a mixture of two dsRNAs corresponding to
5 *Drosophila* Dicer-1 and Dicer-2 (CG4792 and CG6493). Two days after the initial transfection, cells were again transfected with a mixture containing a GFP expression plasmid and either luciferase dsRNA or GFP dsRNA as previously described (Hammond *et al.*, *Nature* **404**: 293-296, 2000). Cells were assayed for Dicer activity or fluorescence three days after the second transfection. Quantification of fluorescent cells was done on a Coulter EPICS
10 cell sorter after fixation. Control transfections indicated that Dicer activity was not affected by the introduction of caspase 9 dsRNA.

Example 3: A Simplified Method for the Creation of Hairpin Constructs for RNA Interference.

15 In numerous model organisms, double stranded RNAs have been shown to cause effective and specific suppression of gene function (Bosher and Labouesse, *Nature Cell Biology* **2**: E31-E36, 2000). This response, termed RNA interference or post-transcriptional gene silencing, has evolved into a highly effective reverse genetic tool in *C. elegans*, *Drosophila*, plants and numerous other systems. In these cases, double-stranded RNAs can be
20 introduced by injection, transfection or feeding; however, in all cases, the response is both transient and systemic. Recently, stable interference with gene expression has been achieved by expression of RNAs that form snap-back or hairpin structures (Fortier and Belote, *Genesis* **26**: 240-244, 2000; Kennerdell and Carthew, *Nature Biotechnology* **18**: 896-898, 2000; Lam and Thummel, *Current Biology* **10**: 957-963, 2000; Shi *et al.*, *RNA* **6**: 1069-1076, 2000;
25 Smith *et al.*, *Nature* **407**: 319-320, 2000; Tavernarakis *et al.*, *Nature Genetics* **24**: 180-183, 2000). This has the potential not only to allow stable silencing of gene expression but also inducible silencing as has been observed in trypanosomes and adult *Drosophila* (Fortier and Belote, *Genesis* **26**: 240-244, 2000; Lam and Thummel, *Current Biology* **10**: 957-963, 2000; Shi *et al.*, *RNA* **6**: 1069-1076, 2000). The utility of this approach is somewhat hampered by
30 the difficulties that arise in the construction of bacterial plasmids containing the long inverted repeats that are necessary to provoke silencing. In a recent report, it was stated that more than 1,000 putative clones were screened to identify the desired construct (Tavernarakis *et al.*, *Nature Genetics* **24**: 180-183, 2000).

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The presence of hairpin structures often induces plasmid rearrangement, in part due to the *E. coli* sbc proteins that recognize and cleave cruciform DNA structures (Connelly *et al.*, *Genes Cell* 1: 285-291, 1996). We have developed a method for the construction of hairpins that does not require cloning of inverted repeats, per se. Instead, the fragment of the gene that 5 is to be silenced is cloned as a direct repeat, and the inversion is accomplished by treatment with a site-specific recombinase, either *in vitro* (or potentially *in vitro)* (see Fig 27). Following recombination, the inverted repeat structure is stable in a bacterial strain that lacks an intact SBC system (DL759). We have successfully used this strategy to construct numerous hairpin expression constructs that have been successfully used to provoke gene 10 silencing in *Drosophila* cells.

In the following examples, we use this method to express long dsRNAs in a variety of mammalian cell types. We show that such long dsRNAs mediate RNAi in a variety of cell types. Additionally, since the vector described in Figure 27 contains a selectable marker, dsRNAs produced in this manner can be stably expressed in cells. Accordingly, this method 15 allows not only the examination of transient effects of RNA suppression in a cell, but also the effects of stable and prolonged RNA suppression.

Methods:

Plasmids expressing hairpin RNAs were constructed by cloning the first 500 bps of 20 the GFP coding region into the FLIP cassette of pRIP-FLIP as a direct repeat. The FLIP cassette contains two directional cloning sites, the second of which is flanked by LoxP sites. The Zeocin gene, present between the cloning sites, maintains selection and stability. To create an inverted repeat for hairpin production, the direct repeat clones were exposed to Cre recombinase (Stratagene) *in vitro* and, afterwards, transformed into DL759 *E. coli*. These 25 bacteria permit the replication of DNA containing cruciform structures, which tend to form inverted repeats.

Example 4: Long dsRNAs Suppress Gene Expression in Mammalian Cells

Previous experiments have demonstrated that dsRNA, produced using a variety of 30 methods including via the construction of hairpins, can suppress gene expression in *Drosophila* cells. We now demonstrate that dsRNA can also suppress gene expression in mammalian cells in culture. Additionally, the power of RNAi as a genetic tool would be greatly enhanced by the ability to engineer stable silencing of gene expression. We therefore

undertook an effort to identify mammalian cells in which long dsRNAs could be used as RNAi triggers in the hope that these same cell lines would provide a platform upon which to develop stable silencing strategies. We demonstrate that RNA suppression can be mediated by stably expressing a long hairpin in a mammalian cell line. The ability to engineer stable
5 silencing of gene expression in cultured mammalian cells, in addition to the ability to transiently silence gene expression, has many important applications.

A. RNAi in Pluripotent Murine P19 Cells.

We first sought to determine whether long dsRNA triggers could induce sequence-specific silencing in cultured murine cells, both to develop this approach as a tool for probing
10 gene function and to allow mechanistic studies of dsRNA-induced silencing to be propagated to mammalian systems. We, therefore, attempted to extend previous studies in mouse embryos (Wianny *et al.*, *Nat. Cell Biol.* **2**: 70-75, 2000; Svoboda *et al.*, *Development* **127**: 4147-4156, 2000) by searching for RNAi-like mechanisms in pluripotent, embryonic cell types. We surveyed a number of cell lines of embryonic origin for the degree to which
15 generalized suppression of gene expression occurred upon introduction of dsRNA. As an assay, we tested the effects of dsRNA on the expression of GFP as measured *in situ* by counting fluorescent cells. As expected, in both human embryonic kidney cells (293) and mouse embryo fibroblasts, GFP expression was virtually eliminated irrespective of the sequence of the cotransfected dsRNA. In some pluripotent teratocarcinoma and teratoma cell
20 lines (*e.g.*, N-Tera1, F9), the PKR response was attenuated but still evident; however, in contrast, transfection of nonhomologous dsRNAs had no effect on the expression of reporter genes (*e.g.*, GFP or luciferase) either in mouse embryonic stem cells or in p19 embryonal carcinoma cells (Fig. 28).

Transfection of P19 embryonal carcinoma cells with GFP in the presence of cognate
25 dsRNA corresponding to the first ≈500 nts of the GFP coding sequence had a strikingly different effect. GFP expression was eliminated in the vast majority of cotransfected cells (Fig. 28), suggesting that these cultured murine cells might respond to dsRNA in a manner similar to that which we had previously demonstrated in cultured, *Drosophila* S2 cells (Hammond *et al.*, *Nature* **404**: 293-296, 2000).

30 To quantify the extent to which dsRNA could induce sequence-specific gene silencing, we used a dual luciferase reporter assay similar to that which had first been used to demonstrate RNAi in *Drosophila* embryo extracts (Tuschl *et al.*, *Genes Dev.* **13**: 3191-3197, 1999). P19 EC cells were transfected with a mixture of two plasmids that individually direct

the expression of firefly luciferase and *Renilla* luciferase. These were cotransfected with no dsRNA, with dsRNA that corresponds to the first ≈500 nts of the firefly luciferase, or with dsRNA corresponding to the first ≈500 nts of GFP as a control. Cotransfection with GFP dsRNA gave luciferase activities that were similar to the no-dsRNA control, both in the firefly/*Renilla* activity ratio and in the absolute values of both activities. In contrast, in cells that received the firefly luciferase dsRNA, the ratio of firefly to *Renilla* luciferase activity was reduced by up to 30-fold (250 ng, Fig. 29B). For comparison, we carried out an identical set of experiments in *Drosophila* S2 cells. Although qualitatively similar results were obtained, the silencing response was more potent. At equivalent levels of dsRNA, S2 cells suppressed firefly luciferase activity to virtually background levels.

The complementary experiment, in which dsRNA was homologous to *Renilla* luciferase, was also performed. Again, in this case, suppression of the expression of the *Renilla* enzyme was ≈10-fold (Fig. 29D). Thus, the dsRNA response in P19 cells was flexible, and the silencing machinery was able to adapt to dsRNAs directed against any of the reporters that were tested.

We took two approaches to test whether this response was specific for dsRNA. Pretreatment of the trigger with purified RNase III, a dsRNA-specific ribonuclease, before transfection greatly reduced its ability to provoke silencing. Furthermore, transfection of cells with single-stranded antisense RNAs directed against either firefly or *Renilla* luciferase had little or no effect on expression of the reporters (Fig. 29C and 29D). Considered together, these results provided a strong indication that double-stranded RNAs provoke a potent and specific silencing response in P19 embryonal carcinoma cells. Efficient silencing could be provoked with relatively low concentrations of dsRNA (25 ng/ml culture media; see Fig. 29A). The response was concentration-dependent, with maximal suppression of ≈20-fold being achieved at a dose of 1.5 µg/ml culture media. Silencing was established rapidly and was evident by 9 h post-transfection (the earliest time point examined). Furthermore, the response persisted without significant changes in the degree of suppression for up to 72 h following a single dose of dsRNA.

Figure 30 further shows wild-type P19 cells which have been co-transfected with either RFP or GFP (right panel). Note the robust expression of RFP or GFR respectively approximately 42 hours post-transfection. We isolated P19 clones which stably express a 500 nt. GFP hairpin. Such clones were then transfected with either RFP or GFP, and expression of RFP or GFP was assessed by visual inspection of the cells. The left panel demonstrates that a 500 nt GFP hairpin specifically suppresses expression of GFP in P19 cells.

B. RNAi in Embryonic Stem Cells.

To assess whether the presence of a sequence-specific response to dsRNA was a peculiarity of P19 cells or whether it also extended to normal murine embryonic cells, we performed similar silencing assays in mouse embryonic stem cells. Cotransfection of embryonic stem cells with noncognate dsRNAs (*e.g.*, GFP), again, had no dramatic effect on either the absolute values or the ratios of *Renilla* and firefly luciferase activity (Fig. 31). However, transfection with either firefly or *Renilla* luciferase dsRNA dramatically and specifically reduced the activity of the targeted enzyme (Fig. 31).

This result suggests that RNAi can operate in multiple murine cell types of embryonic origin, including normal embryonic stem cells. The ability to provoke silencing in a cell type that is normally used for the generation of genetic, mosaic animals suggests the possibility of eventually testing the biological effects of silencing both in culture and in reconstituted animal models. Our ability to successfully manipulate ES cell via RNAi allows the use of RNAi in the generation of transgenic and knock-out mice.

C. RNAi in Murine Somatic Cells.

RNAi effector pathways are likely to be present in mammalian somatic cells, based on the ability of siRNAs to induce transient silencing (Elbashir *et al.*, *Nature* **411**: 494-498, 2001). Furthermore, we have shown that RNAi initiator and effector pathways clearly exist in embryonic cells that can enforce silencing in response to long dsRNA triggers. We therefore sought to test whether the RNAi machinery might exist intact in some somatic cell lines.

Transfection of HeLa cells with luciferase reporters in combination with long dsRNA triggers caused a nearly complete suppression of activity, irrespective of the RNA sequence. In a murine myoblast cell line, C2C12, we noted a mixture of two responses. dsRNAs homologous to firefly luciferase provoked a sequence-specific effect, producing a degree of suppression that was slightly more potent than was observed upon transfection with cognate ≈21-nt siRNA (Elbashir *et al.*, *Nature* **411**: 494-498, 2001) (see Fig. 32A). However, with long dsRNA triggers, the specific effect was superimposed upon a generalized suppression of reporter gene expression that was presumably because of PKR activation (Fig. 32B).

Numerous mammalian viruses have evolved the ability to block PKR as an aid to efficient infection. For example, adenoviruses express VA RNAs, which mimic dsRNA with respect to binding but not to activation of PKR (Clarke *et al.*, *RNA* **1**: 7-20, 1995). Vaccinia virus uses two strategies to evade PKR. The first is expression of E3L, which binds and

masks dsRNAs (Kawagishi-Kobayashi *et al.*, *Virology* **276**: 424-434, 2000). The second is expression of K3L, which binds and inhibits PKR via its ability to mimic the natural substrate of this enzyme, eIF2α (Kawagishi-Kobayashi *et al.* 2000, *supra*).

Transfection of C2C12 cells with a vector that directs K3L expression attenuates the
5 generalized repression of reporter genes in response to dsRNA. However, this protein had no effect on the magnitude of specific inhibition by RNAi (Fig. 32C).

Figure 33 further shows the results of a transient co-transfection assay performed in Hela cells, CHO cells, and P19 cells. The cell lines were each transfected with plasmids expressing *Photinus pyralis* (firefly) and *Renila reniformis* (sea pansy) luciferases. The cells
10 lines were additionally transfected with 400 ng of 500nt dsRNAs corresponding to either firefly luciferase (dsLUC) or dsGFP. The results demonstrate that dsRNA can specifically mediate suppression in a multiple mammalian cells types in culture.

These results raise the possibility that, at least in some cell lines and/or cell types, blocking nonspecific responses to dsRNA will enable the use of long dsRNAs for the study of
15 gene function. This might be accomplished through the use of viral inhibitors, as described here, or through the use of cells isolated from animals that are genetically modified to lack undesirable responses.

D. Stable Suppression of Gene Expression Using RNAi.

To date, dsRNAs have been used to induce sequence-specific gene silencing in either
20 cultured mammalian cells or in embryos only in a transient fashion. However, the most powerful applications of genetic manipulation are realized only with the creation of stable mutants. The ability to induce silencing by using long dsRNAs offers the opportunity to translate into mammalian cells work from model systems such as *Drosophila*, plants, and *C. elegans* wherein stable silencing has been achieved by enforced expression of hairpin RNAs
25 (Kennerdell *et al.*, *Nat. Biotechnol.* **18**: 896-898, 2000; Smith *et al.*, *Nature* **407**: 319-320, 2000; Tavernarakis *et al.*, *Nat. Genet.* **24**: 180-183, 2000).

P19 EC cells were transfected with a control vector or with an expression vector that directs expression of a ≈500-nt GFP hairpin RNA from an RNA polymerase II promoter (cytomegalovirus). Colonies arising from cells that had stably integrated either construct were
30 selected and expanded into clonal cell lines. Each cell line was assayed for persistent RNAi by transient co-transfection with a mixture of two reporter genes, dsRED to mark transfected cells and GFP to test for stable silencing.

Transfection of clonal P19 EC cells that had stably integrated the control vector

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produced equal numbers of red and green cells, as would be expected in the absence of any specific silencing response (Fig. 34B), whereas cells that express the GFP hairpin RNA gave a very different result. These cells expressed the dsRED protein with an efficiency comparable to that observed in cells containing the control vector. However, the cells failed 5 to express the cotransfected GFP reporter (Fig. 34B). These data provide a strong indication that continuous expression of a hairpin dsRNA can provoke stable, sequence-specific silencing of a target gene.

In *Drosophila* S2 cells and *C. elegans*, RNAi is initiated by the Dicer enzyme, which processes dsRNA into ≈22-nt siRNAs (Bernstein *et al.*, *Nature* **409**: 363-366, 2001; Grishok 10 *et al.*, *Cell* **106**: 23-34, 2001; Hutvagner *et al.*, *Science* **293**: 834-838, 2001; Ketting *et al.*, *Genes Dev.* **15**: 2654-2659, 2001; Knight *et al.*, *Science* **293**: 2269-2271, 2001). In both, S2 cells and *C. elegans* experiments by using dsRNA to target Dicer suppress the RNAi response. Whether Dicer plays a central role in hairpin-induced gene silencing in P19 cells was tested by transfecting P19 cells stably transfected with GFP hairpin constructs with 15 mouse *Dicer* dsRNA. Treatment with *Dicer* dsRNA, but not control dsRNA, resulted in derepression of GFP (Fig. 34C).

E. dsRNA Induces Posttranscriptional Silencing.

A key feature of RNAi is that it exerts its effect at the posttranscriptional level by destruction of targeted mRNAs (Hammond *et al.*, *Nat. Rev. Genet.* **2**: 110-119, 2001). To test 20 whether dsRNAs induced silencing in mouse cells via posttranscriptional mechanisms, we used an assay identical to that, used initially to characterize RNAi responses in *Drosophila* embryo extracts (Tuschl *et al.*, *Genes Dev.* **13**: 3191-3197, 1999). We prepared lysates from P19 EC cells that were competent for *in vitro* translation of capped mRNAs corresponding to *Renilla* and firefly luciferase. Addition of nonspecific dsRNAs to these extracts had no 25 substantial effect on either the absolute amount of luciferase expression or on the ratio of firefly to *Renilla* luciferase (Fig. 35). In contrast, addition of dsRNA homologous to the firefly luciferase induced a dramatic and dose-dependent suppression of activity. Addition of RNA corresponding to only the antisense strand of the dsRNA had little effect, comparable to a nonspecific dsRNA control, and pretreatment of the dsRNA silencing trigger with RNase III 30 greatly reduced its potential to induce silencing *in vitro*. A second hallmark of RNAi is the production of small, ≈22-nt siRNAs, which determine the specificity of silencing. We found that such RNA species were generated from dsRNA in P19 cell extracts (Fig. 34D, *in vitro*), indicative of the presence of a mouse Dicer activity. These species were also produced in cells

that stably express GFP hairpin RNAs (Fig. 34D, *in vitro*). Considered together, the posttranscriptional nature of dsRNA-induced silencing, the association of silencing with the production of ≈22-nt siRNAs, and the dependence of this response on Dicer, a key player in the RNAi pathway, strongly suggests that dsRNA suppresses gene expression in murine cells
5 via a conventional RNAi mechanism.

F. RNAi-Mediated Gene Silencing Is Specific and Requires dsRNAs.

We carried out experiments to verify that the suppressive effects observed in the *in vitro* system were specific to double stranded RNA. Briefly, experiments were performed in accordance with the methods outlined above. Either dsRNA (ds), single-stranded RNA (ss),
10 or antisense-RNA (as) corresponding to firefly (FF) or *Renilla* (Ren) luciferase was added to the translation reaction. Following reactions performed at 30 °C for 1 hour, dual luciferase assays were performed using an Analytical Scientific Instruments model 3010 Luminometer.

Figure 36 summarizes the results of these experiments which demonstrate that the
15 suppression of gene expression observed in this *in vitro* assay is specific for dsRNA. These results further support the conclusion that dsRNA suppresses gene expression in this mammalian *in vitro* system in a manner consistent with post-transcriptional silencing.

G. Mammalian Cells Soaked with dsRNAs Results in Gene Silencing.

Studies of post-transcriptional silencing in invertebrates have demonstrated that
20 transfection or injection of the dsRNA is not necessary to achieve the suppressive affects. For example, dsRNA suppression in *C. elegans* can be observed by either soaking the worms in dsRNA, or by feeding the worms bacteria expressing the dsRNA of interest. We addressed whether dsRNA suppression in mammalian cells could be observed without transfection of the dsRNA. Such a result would present additional potential for easily using dsRNA
25 suppression in mammalian cells, and would also allow the use of dsRNA to suppress gene expression in cell types which have been difficult to transfect (*i.e.*, cell types with a low transfection efficiency, or cell types which have proven difficult to transfect at all).

P19 cells were grown in 6-well tissue culture plates to approximately 60% confluency in growth media (αMEM/10% FBS). Varying concentrations of firefly dsRNA were added to
30 the cultures, and cells were cultured for 12 hours in growth media + dsRNA. Cells were then transfected with plasmids expressing firefly or sea pansy luciferase, as described in detail above. Dual luciferase assays were carried out 12 hours post-transfection using an Analytical

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Scientific Instruments model 3010 Luminometer.

Figure 37 summarizes these results which demonstrate that dsRNA can suppress gene expression in mammalian cells without transfection. Culturing cells in the presence of dsRNA resulted in a dose dependent suppression of firefly luciferase gene expression.

5

Methods:

Cell Culture. P19 mouse embryonic carcinoma cells (American Type Culture Collection, CRL-1825) were cultured in α-MEM (GIBCO/BRL) supplemented with 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution (GIBCO/BRL). Mouse embryo 10 stem cells (J1, provided by S. Kim, Cold Spring Harbor Laboratory) were cultured in DMEM containing ESgro (Chemicon) according to the manufacturer's instructions. C2C12 murine myoblast cells (gift of N. Tonks, Cold Spring Harbor Laboratory) were cultured in DMEM (GIBCO/BRL) supplemented with 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution (GIBCO/BRL).

15 **RNA Preparation.** For the production of dsRNA, transcription templates were generated by PCR; they contained T7 promoter sequences on each end of the template (see Hammond *et al.* 2000, Nature 404: 293-296). dsRNAs were prepared by using the RiboMax kit (Ambion, Austin, TX). Firefly and *Renilla* luciferase mRNA transcripts were synthesized by using the Riboprobe kit (Promega) and were gel purified before use.

20 **Transfection and Gene Silencing Assays.** Cells were transfected with indicated amounts of dsRNA and plasmid DNA by using FuGENE6 (Roche Biochemicals) according to the manufacturer's instructions. Cells were transfected at 50-70% confluence in 12-well plates containing either 1 or 2 ml of medium per well. Dual luciferase assays (Promega) were carried out by co-transfecting cells with plasmids contain firefly luciferase under the control 25 of SV40 promoter (pGL3-Control, Promega) and *Renilla* luciferase under the control of the SV40 early enhancer/promoter region (pSV40, Promega). These plasmids were cotransfected by using a 1:1 or 10:1 ratio of pGL3-control (250 ng/well) to pRL-SV40. Both ratios yielded similar results. For some experiments, cells were transfected with vectors that direct expression of enhanced green fluorescent protein (EGFP)-US9 fusion protein (Kalejta *et al.*, 30 *Exp. Cell Res.* **248**: 322-328, 1999) or red fluorescent protein (RFP) (pDsRed N1, CLONTECH). RNAi in S2 cells was performed as described (Hammond *et al.*, *Nature* **404**: 293-296, 2000).

Plasmids expressing hairpin RNAs (RNAs with a self-complimentary stem loop) were constructed by cloning the first 500 bp of the EGFP coding region (CLONTECH) into the

FLIP cassette of pRIP-FLIP as a direct repeat. The FLIP cassette contains two directional cloning sites, the second of which sports flanking LoxP sites (see Fig. 35A). The Zeocin gene (Stratagene), present between the cloning sites, maintains selection and, thus, stability of the FLIP cassette. The FLIP cassette containing EGFP direct repeats was subcloned into pcDNA3 (Invitrogen). To create an inverted repeat for hairpin production, EGFP direct repeat clones were exposed to Cre recombinase (Stratagene) *in vitro* and, afterward, transformed into DL759 *Escherichia coli* (Connelly *et al.*, *Genes Cells* **1**: 285-291, 1996). These bacteria permit the replication of DNA containing cruciform structures, which tend to form from inverted repeats. DL759 transformants were screened for plasmids containing inverted repeats (≈50%).

Silencing of Dicer was accomplished by using a dsRNA comprising exon 25 of the mouse Dicer gene and corresponding to nucleotides 5284-5552 of the human Dicer cDNA.

***In vitro* Translation and *in vitro* Dicer Assays.** Logarithmically growing cells were harvested in PBS containing 5 mM EGTA washed twice in PBS and once in hypotonic buffer (10 mM Hepes, pH 7.3/6 mM β-mercaptoethanol). Cells were suspended in 0.7 packed-cell volumes of hypotonic buffer containing *Complete* protease inhibitors (Roche Molecular Biochemicals) and 0.5 units/ml of RNasin (Promega). Cells were disrupted in a Dounce homogenizer with a type B pestle, and lysates were centrifuged at 30,000 × *g* for 20 min. Supernatants were used in an *in vitro* translation assay containing capped m7G(5')pppG firefly and *Renilla* luciferase mRNA or in *in vitro* Dicer assays containing ^{32}P-labeled dsRNA. For *in vitro* translation assays, 5 μl of extract were mixed with 100 ng of firefly and *Renilla* mRNA along with 1 μg of dsRNA (or buffer)/10 mM DTT/0.5 mM spermidine/200 mM Hepes, 3.3 mM MgOAc/800 mM KOAc/1 mM ATP/1 mM GTP/4 units of Rnasin/215 μg of creatine phosphate/1 μg of creatine phosphate kinase/1 mM amino acids (Promega). Reactions were carried out for 1 h at 30 °C and quenched by adding 1× passive lysis buffer (Promega). Extracts were then assayed for luciferase activity. *In vitro* assays for Dicer activity were performed as described (Bernstein *et al.*, *Nature* **409**: 363-366, 2001).

Construction of Stable Silencing Lines. Ten-centimeter plates of P19 cells were transfected with 5 μg of GFP hairpin expression plasmid and selected for stable integrants by using G-418 (300 ng/ml) for 14 days. Clones were selected and screened for silencing of GFP.

Example 5: Compositions and Methods for Synthesizing siRNAs

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Previous results have indicated that short synthetic RNAs (siRNAs) can efficiently induce RNA suppression. Since short RNAs do not activate the non-specific PKR response, they offer a means for efficiently silencing gene expression in a range of cell types. However, the current state of the art with respect to siRNAs has several limitations. Firstly, siRNAs are
5 currently chemically synthesized at great cost (approx. $400/siRNA). Such high costs make siRNAs impractical for either small laboratories or for use in large scale screening efforts. Accordingly, there is a need in the art for methods for generating siRNAs at reduced cost.

We provide compositions and methods for synthesizing siRNAs by T7 polymerase. This approach allows for the efficient synthesis of siRNAs at a cost consistent with standard
10 RNA transcription reactions (approx. $16/siRNA). This greatly reduced cost makes the use of siRNA a reasonable approach for small laboratories, and also will facilitate their use in large-scale screening projects.

Figure 38 shows the method for producing siRNAs using T7 polymerase. Briefly, T7 polymerase is used to transcribe both a sense and antisense transcript. The transcripts are then
15 annealed to provide an siRNA. One of skill in the art will recognize that any one of the available RNA polymerases can be readily substituted for T7 to practice the invention (*i.e.*, T3, Sp6, etc.).

This approach is amenable to the generation of a single siRNA species, as well as to the generation of a library of siRNAs. Such a library of siRNAs can be used in any number of
20 high-throughput screens including cell based phenotypic screens and gene array based screens.

Example 6: Generation of Short Hairpin dsRNA and Suppression of Gene Expression Using Such Short Hairpins

25 Since the realization that small, endogenously encoded hairpin RNAs could regulate gene expression via elements of the RNAi machinery, we have sought to exploit this biological mechanism for the regulation of desired target genes. Here we show that short hairpin RNAs (shRNAs) can induce sequence-specific gene silencing in mammalian cells. As is normally done with siRNAs, silencing can be provoked by transfecting exogenously
30 synthesized hairpins into cells. However, silencing can also be triggered by endogenous expression of shRNAs. This observation opens the door to the production of continuous cells lines in which RNAi is used to stably suppress gene expression in mammalian cells. Furthermore, similar approaches should prove efficacious in the creation of transgenic

animals and potentially in therapeutic strategies in which long-term suppression of gene function is essential to produce a desired effect.

Several groups (Grishok *et al.*, *Cell* **106**: 23-34, 2001; Ketting *et al.*, *Genes & Dev.* **15**: 2654-2659, 2001; Knight *et al.*, *Science* **293**: 2269-2271, 2001; Hutvagner *et al.*, *Science* **293**: 834-838, 2001) have shown that endogenous triggers of gene silencing, specifically small temporal RNAs (stRNAs) *let-7* and *lin-4*, function at least in part through RNAi pathways. Specifically, these small RNAs are encoded by hairpin precursors that are processed by Dicer into mature, ~21-nt forms. Moreover, genetic studies in *C. elegans* have shown a requirement for Argonaute-family proteins in stRNA function. Specifically, alg-1 and alg-2, members of the EIF2c subfamily, are implicated both in stRNA processing and in their downstream effector functions (Grishok *et al.*, 2001, *supra*). We have recently shown that a component of RISC, the effector nuclease of RNAi, is a member of the Argonaute family, prompting a model in which stRNAs may function through RISC-like complexes, which regulate mRNA translation rather than mRNA stability (Hammond *et al.*, *Science* **293**: 1146-1150, 2001).

A. Short Hairpin RNAs Triggeedr Gene Silencing in Drosophila Cells.

We wished to test the possibility that we might retarget these small, endogenously encoded hairpin RNAs to regulate genes of choice with the ultimate goal of subverting this regulatory system for manipulating gene expression stably in mammalian cell lines and in transgenic animals. Whether triggered by long dsRNAs or by siRNAs, RNAi is generally more potent in the suppression of gene expression in *Drosophila* S2 cells than in mammalian cells. We therefore chose this model system in which to test the efficacy of short hairpin RNAs (shRNAs) as inducers of gene silencing.

Neither stRNAs nor the broader group of miRNAs that has recently been discovered form perfect hairpin structures. Indeed, each of these RNAs is predicted to contain several bulged nucleotides within their rather short (~30-nt) stem structures. Because the position and character of these bulged nucleotides have been conserved throughout evolution and among at least a subset of miRNAs, we sought to design retargeted miRNA mimics to conserve these predicted structural features. Only the *let-7* and *lin-4* miRNAs have known mRNA targets (Wightman *et al.*, *Cell* **75**: 855-862, 1993; Slack *et al.*, *Mol. Cell* **5**: 659-669, 2000). In both cases, pairing to binding sites within the regulated transcripts is imperfect, and in the case of *lin-4*, the presence of a bulged nucleotide is critical to suppression (Ha *et al.*, *Genes & Dev.* **10**: 3041-3050, 1996). We therefore also designed shRNAs that paired imperfectly with their

target substrates. A subset of these shRNAs is depicted in Figure 39A.

To permit rapid testing of large numbers of shRNA variants and quantitative comparison of the efficacy of suppression, we chose to use a dual-luciferase reporter system, as previously described for assays of RNAi in both *Drosophila* extracts (Tuschl *et al.*, *Genes & Dev.* **13**: 3191-3197, 1999) and mammalian cells (Caplen *et al.*, *Proc. Natl. Acad. Sci.* **98**: 9742-9747, 2001; Elbashir *et al.*, *Nature* **411**: 494-498, 2001). Cotransfection of firefly and *Renilla* luciferase reporter plasmids with either long dsRNAs or with siRNAs homologous to the firefly luciferase gene yielded an ~95% suppression of firefly luciferase without effect on *Renilla* luciferase (Fig. 39B; data not shown). Firefly luciferase could also be specifically silenced by co-transfection with homologous shRNAs. The most potent inhibitors were those composed of simple hairpin structures with complete homology to the substrate. Introduction of G-U basepairs either within the stem or within the substrate recognition sequence had little or no effect (Fig. 39A and 39B; data not shown).

These results show that short hairpin RNAs can induce gene silencing in *Drosophila* S2 cells with potency similar to that of siRNAs (Fig. 39B). However, in our initial observation of RNA interference in *Drosophila* S2 cells, we noted a profound dependence of the efficiency of silencing on the length of the dsRNA trigger (Hammond *et al.*, *Nature* **404**: 293-296, 2000). Indeed, dsRNAs of fewer than ~200 nt triggered silencing very inefficiently. Silencing is initiated by an RNase III family nuclease, Dicer, that processes long dsRNAs into ~22-nt siRNAs. In accord with their varying potency as initiators of silencing, long dsRNAs are processed much more readily than short RNAs by the Dicer enzyme (Bernstein *et al.*, *Nature* **409**: 363-366, 2001). We therefore tested whether shRNAs were substrates for the Dicer enzyme.

We had noted previously that *let-7* (Ketting *et al.*, *Genes & Dev.* **15**: 2654-2659, 2001) and other miRNAs (E. Bernstein, unpublished data) are processed by Dicer with an unexpectedly high efficiency as compared with short, nonhairpin dsRNAs. Similarly, Dicer efficiently processed shRNAs that targeted firefly luciferase, irrespective of whether they were designed to mimic a natural Dicer substrate (*let-7*) or whether they were simple hairpin structures (Fig. 39C). These data suggest that recombinant shRNAs can be processed by Dicer into siRNAs and are consistent with the idea that these short hairpins trigger gene silencing via an RNAi pathway.

B. Short Hairpin RNAs Activated Gene Silencing in Mammalian Cells.

Mammalian cells contain several endogenous systems that were predicted to hamper

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the application of RNAi. Chief among these is a dsRNA-activated protein kinase, PKR, which effects a general suppression of translation via phosphorylation of EIF-2α (Williams, *Biochem. Soc. Trans.* **25**: 509-513, 1997; Gil *et al.*, *Apoptosis* **5**: 107-114, 2000). Activation of these, and other dsRNA-responsive pathways, generally requires duplexes exceeding 30 bp
5 in length, possibly to permit dimerization of the enzyme on its allosteric activator (*e.g.*, Clarke *et al.*, *RNA* **1**: 7-20, 1995). Small RNAs that mimic Dicer products, siRNAs, presumably escape this limit and trigger specific silencing, in part because of their size. However, short duplex RNAs that lack signature features of siRNAs can efficiently induce silencing in *Drosophila* S2 cells but not in mammalian cells (A.A. Caudy, unpublished data).
10 Endogenously encoded miRNAs may also escape PKR surveillance because of their size but perhaps also because of the discontinuity of their duplex structure. Given that shRNAs of <30 bp were effective inducers of RNAi in *Drosophila* S2 cells, we tested whether these RNAs could also induce sequence-specific silencing in mammalian cells.

Human embryonic kidney (HEK293T) cells were cotransfected with chemically
15 synthesized shRNAs and with a mixture of firefly and *Renilla* luciferase reporter plasmids. As had been observed in S2 cells, shRNAs were effective inducers of gene silencing. Once again, hairpins designed to mimic *let-7* were consistently less effective than were simple hairpin RNAs, and the introduction of mismatches between the antisense strand of the shRNA and the mRNA target abolished silencing (Fig. 40A; data not shown). Overall, shRNAs were
20 somewhat less potent silencing triggers than were siRNAs. Whereas siRNAs homologous to firefly luciferase routinely yielded ~90%-95% suppression of gene expression, suppression levels achieved with shRNAs ranged from 80%-90% on average. As we also observe with siRNAs, the most important determinant of the potency of the silencing trigger is its sequence. We find that roughly 50% of both siRNAs and shRNAs are competent for
25 suppressing gene expression. However, neither analysis of the predicted structures of the target mRNA nor analysis of alternative structures in siRNA duplexes or shRNA hairpins has proved of predictive value for choosing effective inhibitors of gene expression.

We have adopted as a standard, shRNA duplexes containing 29 bp. However, the size of the helix can be reduced to ~25 nt without significant loss of potency. Duplexes as short as
30 22 bp can still provoke detectable silencing, but do so less efficiently than do longer duplexes. In no case did we observe a reduction in the internal control reporter (*Renilla* luciferase) that would be consistent with an induction of nonspecific dsRNA responses.

The ability of shRNAs to induce gene silencing was not confined to 293T cells. Similar results were also obtained in a variety of other mammalian cell lines, including

human cancer cells (HeLa), transformed monkey epithelial cells (COS-1), murine fibroblasts (NIH 3T3), and diploid human fibroblasts (IMR90; Fig. 40; data not shown).

C. Synthesis of Effective Inhibitors of Gene Expression Using T7 RNA Polymerse.

The use of siRNAs to provoke gene silencing is developing into a standard
5 methodology for investigating gene function in mammalian cells. To date, siRNAs have been produced exclusively by chemical synthesis (*e.g.*, Caplen *et al.*, *Proc. Natl. Acad. Sci.* **98**: 9742-9747, 2001; Elbashir *et al.*, *Nature* **411**: 494-498, 2001). However, the costs associated with this approach are significant, limiting its potential utility as a tool for investigating in parallel the functions of large numbers of genes. Short hairpin RNAs are presumably
10 processed into active siRNAs *in vitro* by Dicer. Thus, these may be more tolerant of terminal structures, both with respect to nucleotide overhangs and with respect to phosphate termini. We therefore tested whether shRNAs could be prepared by *in vitro* transcription with T7 RNA polymerase.

Transcription templates that were predicted to generate siRNAs and shRNAs similar
15 to those prepared by chemical RNA synthesis were prepared by DNA synthesis (Fig. 41A,C). These were tested for efficacy both in S2 cells (data not shown) and in human 293 cells (Fig. 41B,D). Overall, the performance of the T7-synthesized hairpin or siRNAs closely matched the performance of either produced by chemical synthesis, both with respect to the magnitude of inhibition and with respect to the relative efficiency of differing sequences. Because T7
20 polymerase prefers to initiate at twin guanosine residues, however, it was critical to consider initiation context when designing *in vitro* transcribed siRNAs (Fig. 41B). In contrast, shRNAs, which are processed by Dicer (see Fig. 39C), tolerate the addition of these bases at the 5' end of the transcript.

Studies in *Drosophila* embryo extracts indicate that siRNAs possess 5'
25 phosphorylated termini, consistent with their production by an RNase III family nuclease. *In vitro*, this terminus is critical to the induction of RNAi by synthetic RNA oligonucleotides (Elbashir *et al.*, *EMBO J.* **20**: 6877-6888, 2001; Nykanen *et al.*, *Cell* **107**: 309-321, 2001). Chemically synthesized siRNAs are nonphosphorylated, and enzymatic addition of a 5' phosphate group *in vitro* prior to transfection does not increase the potency of the silencing
30 effect (A.A. Caudy, unpublished data). This suggests either that the requirement for phosphorylated termini is less stringent in mammalian cells or that a kinase efficiently phosphorylates siRNAs *in vitro*. RNAs synthesized with T7 RNA polymerase, however, possess 5' triphosphate termini. We therefore explored the possibility of synthesizing siRNAs

with T7 polymerase followed by treatment *in vitro* with pyrophosphatase to modify the termini to resemble those of siRNAs. Surprisingly, monophosphorylated siRNAs (data not shown) were as potent in inducing gene silencing as transcription products bearing triphosphate termini (Fig. 41B). This may suggest either that the requirement for 5 monophosphorylated termini is less stringent in mammalian cells or that siRNAs are modified *in vitro* to achieve an appropriate terminal structure.

Considered together, our data suggest that both shRNAs and siRNA duplexes can be prepared by synthesis with T7 RNA polymerase *in vitro*. This significantly reduces the cost of RNAi in mammalian cells and paves the way for application of RNAi on a whole-genome 10 scale.

D. Transcription of Small Hairpin RNAs In vitro by RNA Polymerase III.

Although siRNAs are an undeniably effective tool for probing gene function in mammalian cells, their suppressive effects are by definition of limited duration. Delivery of siRNAs can be accomplished by any of a number of transient transfection methodologies, and 15 both the timing of peak suppression and the recovery of protein levels as silencing decays can vary with both the cell type and the target gene. Therefore, one limitation on siRNAs is the development of continuous cell lines in which the expression of a desired target is stably silenced.

Hairpin RNAs, consisting of long duplex structures, have been proved as effective 20 triggers of stable gene silencing in plants, in *C. elegans*, and in *Drosophila* (Kennerdell *et al.*, *Nat. Biotechnol.* **18**: 896-898, 2000; Smith *et al.*, *Nature* **407**: 319-320, 2000; Tavernarakis *et al.*, *Nat. Genet.* **24**: 180-183, 2000). We have recently shown stable suppression of gene expression in cultured mammalian cells by continuous expression of a long hairpin RNA (Paddison *et al.*, *Proc. Natl. Acad. Sci.* **99**: 1443-1448, 2002). However, the scope of this 25 approach was limited by the necessity of expressing such hairpins only in cells that lack a detectable PKR response. In principle, shRNAs could bypass such limitations and provide a tool for evoking stable suppression by RNA in mammalian somatic cells.

To test this possibility, we initially cloned sequences encoding a firefly luciferase shRNA into a CMV-based expression plasmid. This was predicted to generate a capped, 30 polyadenylated RNA polymerase II transcript in which the hairpin was extended on both the 5' and 3' ends by vector sequences and poly(A). This construct was completely inert in silencing assays in 293T cells.

During our studies on chemically and T7-synthesized shRNAs, we noted that the

presence of significant single-stranded extensions (either 5' or 3' of the duplex) reduced the efficacy of shRNAs. We therefore explored the use of alternative promoter strategies in an effort to produce more defined hairpin RNAs. In particular, RNA polymerase III promoters have well-defined initiation and termination sites and naturally produce a variety of small, 5 stable RNA species. Although many Pol III promoters contain essential elements within the transcribed region, limiting their utility for our purposes; class III promoters use exclusively nontranscribed promoter sequences. Of these, the U6 snRNA promoter and the H1 RNA promoter have been well studied (Lobo *et al.*, *Nucleic Acids Res.* **18**: 2891-2899, 1990; Hannon *et al.*, *J. Biol. Chem.* **266**: 22796-22799, 1991; Chong *et al.*, *J. Biol. Chem.* **276**: 10 20727-20734, 2001).

By placing a convenient cloning site immediately behind the U6 snRNA promoter, we have constructed pShh-1, an expression vector in which short hairpins are harnessed for gene silencing. Into this vector either of two shRNA sequences derived from firefly luciferase were cloned from synthetic oligonucleotides. These were cotransfected with firefly and *Renilla* 15 luciferase expression plasmids into 293T cells. One of the two encoded shRNAs provoked effective silencing of firefly luciferase without altering the expression of the internal control (Fig. 42C). The second encoded shRNA also produced detectable, albeit weak, repression. In both cases, silencing was dependent on insertion of the shRNA in the correct orientation with respect to the promoter (Fig. 42C; data not shown). Although the shRNA itself is bilaterally 20 symmetric, insertion in the incorrect orientation would affect Pol III termination and is predicted to produce a hairpin with both 5' and 3' single-stranded extensions. Similar results were also obtained in a number of other mammalian cell lines including HeLa, COS-1, NIH 3T3, and IMR90 (Fig. 42; data not shown). pShh1-Ff1 was, however, incapable of effecting suppression of the luciferase reporter in *Drosophila* cells, in which the human U6 promoter is 25 inactive.

E. Dicer Is Required for shRNA-Mediated Gene Silencing.

As a definitive test of whether the plasmid-encoded shRNAs brought about gene silencing via the mammalian RNAi pathway, we assessed the dependence of suppression on an essential component of the RNAi pathway. We transfected pShh1-Ff1 along with an 30 siRNA homologous to human *Dicer*. Figure 43 shows that treatment of cells with *Dicer* siRNAs is able to completely depress the silencing induced by pShh1-Ff1. Addition of an unrelated siRNA had no effect on the magnitude of suppression by pShh1-Ff1. Importantly, *Dicer* siRNAs had no effect on siRNA-induced silencing of firefly luciferase. These results

are consistent with shRNAs operating via an RNAi pathway similar to those provoked by stRNAs and long dsRNAs. Furthermore, it suggests that siRNA-mediated silencing is less sensitive to depletion of the Dicer enzyme.

F. Stable shRNA-Mediated Gene Silencing of An Endogenous Gene.

5 The ultimate utility of encoded short hairpins will be in the creation of stable mutants that permit the study of the resulting phenotypes. We therefore tested whether we could create a cellular phenotype through stable suppression. Expression of activated alleles of the *ras* oncogene in primary mouse embryo fibroblasts (MEFs) induces a stable growth arrest that resembles, as a terminal phenotype, replicative senescence (Serrano *et al.*, *Cell* **88**: 593-602, 10 1997). Cells cease dividing and assume a typical large, flattened morphology. Senescence can be countered by mutations that inactivate the p53 tumor suppressor pathway (Serrano *et al.* 1997, *supra*). As a test of the ability of vector-encoded shRNAs to stably suppress an endogenous cellular gene, we generated a hairpin that was targeted to the mouse *p53* gene. As shown in Figure 44, MEFs transfected with pBabe-RasV12 fail to proliferate and show a 15 senescent morphology when cotransfected with an empty control vector. As noted previously by Serrano *et al.*, the terminally arrested state is achieved in 100% of drug-selected cells in culture by 8 d post-transfection. However, upon cotransfection of an activated *ras* expression construct with the pShh-p53, cells emerged from drug selection that not only fail to adopt a senescent morphology but also maintain the ability to proliferate for a minimum of several 20 weeks in culture (Fig. 44). These data strongly suggest that shRNA expression constructs can be used for the creation of continuous mammalian cell lines in which selected target genes are stably suppressed.

G. Simultaneous Introduction of Multiple Hairpin RNAs Does Not Produce Synergy.

In an attempt to further understand the mechanisms by which short hairpins suppress 25 gene expression, we examined the effects of transfecting cells with a mixture of two different short hairpins corresponding to firefly luciferase. Figure 45 summarizes the results of experiments which suggest that there is no synergistic affects on suppression of firefly luciferase gene expression obtained when cells are exposed to a mixture of such short hairpins.

30

Methods:

Cell culture. HEK 293T, HeLa, COS-1, MEF, and IMR90 cells were cultured in

DMEM (GIBCO BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (GIBCO BRL). NIH 3T3 cells were cultured in DMEM supplemented with 10% heat-inactivated calf serum and 1% antibiotic/antimycotic solution.

5 **RNA preparation.** Both shRNAs and siRNAs were produced *in vitro* using chemically synthesized DNA oligonucleotide templates (Sigma) and the T7 Megashortscript kit (Ambion). Transcription templates were designed such that they contained *T7* promoter sequences at the 5' end. shRNA transcripts subjected to *in vitro* Dicer processing were synthesized using a Riboprobe kit (Promega). Chemically synthesized RNAs were obtained 10 from Dharmacon, Inc.

Transfection and gene silencing assays. Cells were transfected with indicated amounts of siRNA, shRNA, and plasmid DNA using standard calcium phosphate procedures at 50%-70% confluence in 6-well plates. Dual luciferase assays (Promega) were carried out by cotransfecting cells with plasmids containing firefly luciferase under the control of the 15 SV40 promoter (pGL3-Control, Promega) and *Renilla* luciferase under the control of the SV40 early enhancer/promoter region (pSV40, Promega). Plasmids were cotransfected using a 1:1 ratio of pGL3-Control (250 ng/well) to pRL-SV40. RNAi in S2 cells was performed as previously described (Hammond *et al.*, *Nature* **404**: 293-296, 2000). For stable silencing, primary MEFs (a gift from S. Lowe, Cold Spring Harbor Laboratory, NY) were cotransfected 20 using Fugene 6 with pBabe-Ha-rasV12 and pShh-p53 (no resistance marker), according to the manufacturer's recommendations. Selection was for the presence of the activated *Ha-rasV12* plasmid, which carries a puromycin-resistance marker. The pShh-p53 plasmid was present in excess, as is standard in a cotransfection experiment. We have now generated a version of the U6 promoter vector (pSHAG-1) that is compatible with the GATEWAY system (Invitrogen), 25 and this can be used to transport the shRNA expression cassette into a variety of recipient vectors that carry *cis*-linked selectable markers. Furthermore, we have validated delivery of shRNAs using retroviral vectors. Updated plasmid information can be obtained at:

http://www.cshl.org/public/science/hannon.html.

Plasmids expressing hairpin RNAs. The U6 promoter region from -265 to +1 was 30 amplified by PCR, adding 5' *Kpn*I and 3' *Eco*RV sites for cloning into pBSSK$^+$. A linker/terminator oligonucleotide set bearing the U6 terminator sequence and linker ends of 5' *Eco*RV and 3' *Not*I was cloned into the promoter construct, resulting in a U6 cassette with an *Eco*RV site for insertion of new sequences. This vector has been named pShh1. Blunt-ended, double-stranded DNA oligonucleotides encoding shRNAs with between 19 and 29 bases of

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homology to the targeted gene were ligated into the *Eco*RV site to produce expression constructs. The oligonucleotide sequence used to construct Ffl was: TCCAATTCAGCGGGAGCCACCTGATGAAGCTTGATCGGGTGGCTCTCGCTGAGTT GGAATCCATTTTTTTT (SEQ ID NO: 38). This sequence is preceded by the sequence
5 GGAT, which is supplied by the vector, and contains a tract of more than five Ts as a Pol III terminator.

***In vitro* Dicer assays.** *In vitro* assays for Dicer activity were performed as described (Bernstein *et al.*, *Nature* **409**: 363-366, 2001).

10 **Example 7: Encoded Short Hairpins Function *In vitro***

An object of the present invention is to improve methods for generating siRNAs and short hairpins for use in specifically suppressing gene expression. Example 6 demonstrates that siRNAs and short hairpins are highly effective in specifically suppressing gene expression. Accordingly, it would be advantageous to combine the efficient suppression of
15 gene expression attainable using short hairpins and siRNAs with a method to encode such RNA on a plasmid and express it either transiently or stably.

Figure 46 demonstrates that short hairpins encoded on a plasmid are effective in suppressing gene expression. DNA oligonucleotides encoding 29 nucleotide hairpins corresponding to firefly luciferase were inserted into a vector containing the U6 promoter.
20 Three independent constructs were examined for their ability to specifically suppress firefly luciferase gene expression in 293T cells. siOligo1-2, siOligo1-6, and siOligo1-19 (construct in the correct orientation) each suppressed gene expression as effectively as siRNA. In contrast, siOligo1-10 (construct in the incorrect orientation) did not suppress gene expression. Additionally, an independent construct targeted to a different portion of the firefly luciferase
25 gene did not effectively suppress gene expression in either orientation (siOligo2-23, siOligo2-36).

The results summarized in Figure 46 demonstrate that transient expression of siRNAs and short hairpins encoded on a plasmid can efficiently suppress gene expression. One of skill can choose from amongst a range of vectors to either transiently or stably express an
30 siRNA or short hairpin. Non-limiting examples of vectors and strategies to stably express short dsRNAs are presented in Figures 47-49.

Example 8: dsRNA Suppression in the Absence of a PKR Response

One potential impediment to the use of RNAi to suppress gene expression in some cell types, is the non-specific PKR response that can be triggered by long dsRNAs. Numerous mammalian viruses have evolved the ability to block PKR in order to aid in the infection of potential host cells. For example, adenoviruses express RNAs which mimic dsRNA but do
5 not activate the PKR response. Vaccinia virus uses two strategies to evade PKR: the expression of E3L which binds and masks dsRNA; the expression of K3L to mimic the natural PKR substrate eIF2α.

Our understanding of the mechanisms by which viruses avoid the PKR response allows us to design approaches to circumvent the PKR response in cell types in which in
10 might be advantageous to suppression gene expression with long dsRNAs. Possible approaches include treating cells with an agent that inhibits protein kinase RNA-activated (PKR) apoptosis, such as by treatment with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKR. Accordingly, RNAi suppression of gene expression in such cell types could involve first inhibiting the PKR response, and then
15 delivering a dsRNA identical or similar to a target gene.

A. In a murine myoblast cell line, C2C12, we noted that the cells responded to long dsRNAs with a mixture of specific and non-specific (presumably PKR) responses. In order to attenuate the non-specific PKR response while maintaining the robust and specific suppression due to the long dsRNA, C2C12 cells were transfected with a vector that directs
20 K3L expression. This additional step successfully attenuated the PKR response, however expression of K3L protein had no effect on the magnitude of specific inhibition.

B. However, since the efficacy of such a two step approach had not been previously demonstrated, it was formerly possible that dsRNA suppression would not be possible in cells with a PKR response. Figure 50 summarizes results which demonstrate that
25 such a two step approach is possible, and that robust and specific dsRNA mediated suppression is possible in cells which had formerly possessed a robust PKR response.

Briefly, dual luciferase assay were carried out as described in detail above. The experiments were carried out using PKR$^{-/-}$ MEFs harvested from E13.5 PKR$^{-/-}$ mouse embryos. MEFs typically have a robust PKR response, and thus treatment with long dsRNAs
30 typically results in non-specific suppression of gene expression and apoptosis. However, in PKR$^{-/-}$ cells examined 12, 42, and 82 hours after transfection, expression of ds*Renilla* luciferase RNA specifically suppresses expression *Renilla reniformis* (sea pansy) luciferase. This suppression is stable over time.

These results demonstrate that the non-specific PKR response can be blocked without affecting specific suppression of gene expression mediated by dsRNA. This allows the use of long dsRNAs to suppress gene expression in a diverse range of cell types, including those that would be previously intractable due to the confounding influences of the non-specific 5 PKR response to long dsRNA.

Example 9: Suppression of Gene Expression using dsRNA which Corresponds to Non-Coding Sequence

Current models for the mechanisms which drive RNAi have suggested that the 10 dsRNA construct must contain coding sequence corresponding to the gene of interest. Although evidence has demonstrated that such coding sequence need not be a perfect match to the endogenous coding sequence (*i.e.*, it may be similar), it has been widely held that the dsRNA construct must correspond to coding sequence. We present evidence that contradicts the teachings of the prior art, and demonstrate that dsRNA corresponding to non-coding 15 regions of a gene can suppress gene function *in vitro*. These results are significant not only because they demonstrate that dsRNA identical or similar to non-coding sequences (*i.e.*, promoter sequences, enhancer sequences, or intronic sequences) can mediate suppression, but also because we demonstrate the *in vitro* suppression of gene expression using dsRNA technology in a mouse model.

20 We generated doubled stranded RNA corresponding to four segments of the mouse tyrosinase gene promoter. Three of these segments correspond to the proximal promoter and one corresponds to an enhancer (Fig. 51). The tyrosinase gene encodes the rate limiting enzyme involved in the melanin biosynthetic pathway (Bilodeau *et al.*, *Pigment Cell Research* **14**: 328-336, 2001). Accordingly, suppression of the tyrosinase gene is expected to 25 inhibit pigmentation.

Double stranded RNA corresponding to each of the above promoter segments was injected into the pronuclei of fertilized eggs. Pups were born after 19 days. In total 42/136 (31%) of the embryos were carried to term. This number is within the expected range for transgenesis (30-40%). Two pups out of 42 (5%) appear totally unpigmented at birth, 30 consistent with suppression of tyrosinase function.

Methods:

dsRNA from non-coding promoter region of tyrosinase gene. Four segments of the

mouse tyrosinase gene promoter were amplified by PCR using primers which incorporated T7 RNA polymerase promoters into the PCR products (shown in bold – Fig. 51). Sequences of the mouse tyrosinase gene 5' flanking regions were obtained from GenBank (accession number D00439 and X51743). The sequence of the tyrosinase enhancer, located approximately 12 kb upstream of the transcriptional start site, was also obtained from GenBank (accession number X76647).

The sequences of the primers used were as follows: note the sequence of the T7 RNA polymerase promoter is shown in bold:

(a) Tyrosinase enhancer (~12 kb upstream):

5' **TAATACGACTCACTATAGGG**CAAGGTCATAGTTCCTGCCAGCTG 3'

(SEQ ID NO: 39)

5' **TAATACGACTCACTATAGGG**CAGATATTTTCTTACCACCCACCC 3'

(SEQ ID NO: 40)

(b) -1404 to -1007:

5' **TAATACGACTCACTATAGGG**TTAAGTTTAACAGGAGAAGCTGGA 3'

(SEQ ID NO: 41)

5' **TAATACGACTCACTATAGGG**AAATCATTGCTTTCCTGATAATGC 3'

(SEQ ID NO: 42)

(c) -1003 to -506:

5' **TAATACGACTCACTATAGGG**TAGATTTCCGCAGCCCCAGTGTTC 3'

(SEQ ID NO: 43)

5' **TAATACGACTCACTATAGGG**GTTGCCTCTCATTTTTCCTTGATT 3'

(SEQ ID NO: 44)

(d) -505 to -85:

5' **TAATACGACTCACTATAGGG**TATTTTAGACTGATTACTTTTATAA 3'

(SEQ ID NO: 45)

5' **TAATACGACTCACTATAGGG**TCACATGTTTTGGCTAAGACCTAT 3'

(SEQ ID NO: 46)

PCR products were gel purified from 1% TAE agarose gels using QiaExII Gel Extraction Kit (Qiagen). Double stranded RNA was produced from these templates using T7-Megashortscript Kit (Ambion). Enzymes and unincorporated nucleotides were removed using Qiaquick MinElute PCR Purification Kit. RNA was phenol/chloroform extracted twice, and ethanol precipitated. Pellets were resuspended in injection buffer ((10 mM Tris (pH 7.5), 0.15 nM EDTA (pH 8.0)) at a concentration of 20 ng/ul and run on a 1% TAE agarose gel to confirm integrity.

Generation of mice: An equal mixture of double stranded RNA from each of the above primer sets was injected into the pronuclei of fertilized eggs from C57BL6J mice. A total of 136 injections was performed, and 34 embryos were implanted into each of 4 pseudopregnant CD-1 females. Pups were born after 19 days. In total, 42/136 (31%) of the embryos were carried to term. 2/42 pups (5%) appear totally unpigmented at birth.

It is not clear whether the RNAi mediated by dsRNA identical or similar to non-coding sequence works via the same mechanism as PTGS observed in the presence of dsRNA identical or similar to coding sequence. However, whether these results ultimately reveal similar or differing mechanisms does not diminish the tremendous utility of the compositions and methods of the present invention to suppress expression of one or more genes *in vitro* or *in vitro*.

The present invention demonstrates that dsRNA ranging in length from 20-500 nt can readily suppress expression of target genes both *in vitro* and *in vitro*. Furthermore, the present invention demonstrates that the dsRNAs can be generated using a variety of methods including the formation of hairpins, and that these dsRNAs can be expressed either stably or transiently. Finally, the present invention demonstrates that dsRNA identical or similar to non-coding sequences can suppress target gene expression.

Example 10: RNA interference in adult mice

RNA interference is an evolutionarily conserved surveillance mechanism that responds to double-stranded RNA by sequence-specific silencing of homologous genes. Here we show that transgene expression can be suppressed in adult mice by synthetic small interfering RNAs and by small-hairpin RNAs transcribed *in vitro* from DNA templates. We also show the therapeutic potential of this technique by demonstrating effective targeting of a

sequence from hepatitis C virus by RNA interference *in vitro*.

Small interfering RNAs (siRNAs) mimic intermediates in the RNA-interference (RNAi) pathway and can silence genes in somatic cells without activating non-specific suppression by double-stranded RNA-dependent protein kinase (Elbashir *et al.*, *Nature* **411**:
5 494-498, 2001). To investigate whether siRNAs also inhibit gene expression *in vitro*, we used a modification of hydrodynamic transfection methods (Zhang *et al.*, *Hum. Gene Therapy* **10**: 1735-1737, 1999; Liu *et al.*, *Gene Therapy* **6**: 1258-1266, 1999; Chang *et al.*, *J. Virol.* **75**: 3469-3473, 2001) to deliver naked siRNAs to the livers of adult mice. Either an siRNA derived from firefly luciferase or an unrelated siRNA was co-injected with a luciferase-
10 expression plasmid (for construct description and sequences, see Figure 52). We monitored luciferase expression in living animals using quantitative whole-body imaging (Contag, *et al.*, *Photochem. Photobiol.* **66**: 523-531, 1997) (see Fig. 53a, 54a), and found that it was dependent on reporter-plasmid dose.

In each experiment, serum measurements of a co-injected human α-1 antitrypsin
15 (hAAT) plasmid (Yant *et al.*, *Nature Genet.* **25**: 35-41, 2000) served to normalize transfection efficiency and to monitor non-specific translational inhibition. Average serum concentrations of hAAT after 74 h were similar in all groups.

Our results indicate that there was specific, siRNA-mediated inhibition of luciferase expression in adult mice ($P < 0.0115$) and that unrelated siRNAs had no effect ($P < 0.864$;
20 Fig. 53a, 53b). In 11 independent experiments, luciferase siRNAs reduced luciferase expression (as judged by emitted light) by an average of 81% (± 2.2%). These findings indicate that RNAi can downregulate gene expression in adult mice.

As RNAi degrades respiratory syncitial virus RNAs in culture (Bitko *et al.* 2001, BMC Microbiol. 1: 34), we investigated whether RNAi could be directed against a human
25 pathogenic RNA expressed in a mouse, namely that of hepatitis C virus (HCV). Infection by HCV (an RNA virus that infects 1 in 40 people worldwide) is the most common reason for liver transplantation in the United States and Europe. We fused the NS5B region (non-structural protein 5B, viral-polymerase-encoding region) of this virus with luciferase RNA and monitored RNAi by co-transfection *in vitro*. An siRNA targeting the NS5B region
30 reduced luciferase expression from the chimaeric HCV NS5B protein–luciferase fusion by 75% (± 6.8%; 6 animals per group). This result suggests that it may be feasible to use RNAi as a therapy against other important human pathogens.

Although our results show that siRNAs are functional in mice, delivery remains a major obstacle. Unlike siRNAs, functional small-hairpin RNAs (shRNAs) can be expressed

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in vitro from DNA templates using RNA polymerase III promoters (Paddison *et al.*, *Genes Dev.* **16**: 948-958, 2002; Tuschl, *Nature Biotechnol.* **20**: 446-448, 2002); they are as effective as siRNAs in inducing gene suppression. Expression of a cognate shRNA (pShh1-Ff1) inhibited luciferase expression by up to 98% (± 0.6%), with an average suppression of 92.8% (± 3.39%) in three independent experiments (see Fig. 54a, 54b). An empty shRNA-expression vector had no effect; reversing the orientation of the shRNA (pShh1-Ff1rev) insert prevents gene silencing because it alters the termination by RNA polymerase III and generates an improperly structured shRNA. These findings indicate that plasmid-encoded shRNAs can induce a potent and specific RNAi response in adult mice.

RNAi may find application in functional genomics or in identifying targets for designer drugs. It is a more promising system than gene-knockout mice because groups of genes can be simultaneously rendered ineffective without the need for time-consuming crosses. Gene therapy currently depends on the ectopic expression of exogenous proteins; however, RNAi may eventually complement this gain-of-function approach by silencing disease-related genes with DNA constructs that direct the expression of shRNAs. Our method of RNAi delivery could also be tailored to take advantage of developing viral and non-viral gene-transfer vectors in a clinical context.

Example 11: Germ-line transmission of RNAi in mice

MicroRNA molecules (miRNAs) are small, noncoding RNA molecules that have been found in a diverse array of eukaryotes, including mammals. miRNA precursors share a characteristic secondary structure, forming short 'hairpin' RNAs. Genetic and biochemical studies have indicated that miRNAs are processed to their mature forms by Dicer, an RNAse III family nuclease, and function through RNA-mediated interference (RNAi) and related pathways to regulate the expression of target genes (Hannon, *Nature* **418**: 244-251, 2002; Pasquinelli *et al.*, *Annu. Rev. Cell. Dev. Biol.* **18**: 495-513, 2002). Recently, we and others have remodeled miRNAs to permit experimental manipulation of gene expression in mammalian cells and have dubbed these synthetic silencing triggers 'short hairpin RNAs' (shRNAs) (Paddison *et al.*, *Cancer Cell* **2**: 17-23, 2002). Silencing by shRNAs requires the RNAi machinery and correlates with the production of small interfering RNAs (siRNAs), which are a signature of RNAi.

Expression of shRNAs can elicit either transient or stable silencing, depending upon whether the expression cassette is integrated into the genome of the recipient cultured cell

(Paddison *et al.*, *Cancer Cell* **2**: 17-23, 2002). shRNA expression vectors also induce gene silencing in adult mice following transient delivery (Lewis *et al.*, *Nat. Genet.* **32**: 107-108, 2002; McCaffrey *et al.*, *Nature* **418**: 38-39, 2002). However, for shRNAs to be a viable genetic tool in mice, stable manipulation of gene expression is essential. Hemann and 5 colleagues have demonstrated long-term suppression of gene expression *in vitro* following retroviral delivery of shRNA-expression cassettes to hematopoietic stem cells (Hemann *et al.*, *Nat. Genet.* in the press, 2003). Here we sought to test whether shRNA-expression cassettes that were passed through the mouse germ-line could enforce heritable gene silencing.

We began by taking standard transgenesis approaches (Gordon *et al.*, *Methods* 10 *Enzymol.* **225**: 747-771, 1993) using shRNAs directed against a variety of targets with expected phenotypes, including the genes encoding tyrosinase (albino), myosin VIIa (shaker), Bmp-5 (crinkled ears), Hox a-10 (limb defects), homogentisate 1,2,-dioxygenase (urine turns black upon exposure to air), Hairless (hair loss) and melanocortin 1 receptor (yellow). Three constructs per gene were linearized and injected into pronuclei to produce transgenic founder 15 animals. Although we noted the presence of the transgene in some animals, virtually none showed a distinct or reproducible phenotype that was expected for a hypomorphic allele of the targeted gene.

Therefore, we decided to take another approach: verifying the presence of the shRNA and its activity toward a target gene in cultured embryonic stem (ES) cells and then asking 20 whether those cells retained suppression in a chimeric animal *in vitro*. We also planned to test whether such cells could pass a functional RNAi-inducing construct through the mouse germ-line. For these studies, we chose to examine a novel gene, *Neil1*, which is proposed to have a role in DNA repair. Oxidative damage accounts for 10,000 DNA lesions per cell per day in humans and is thought to contribute to carcinogenesis, aging and tissue damage following 25 ischemia (Ames *et al.*, *Proc. Natl. Acad. Sci. USA* **90**: 7915-7922, 1993; Jackson *et al.*, *Mutat. Res.* **477**: 7-21, 2001). Oxidative DNA damage includes abasic sites, strand breaks and at least 20 oxidized bases, many of which are cytotoxic or pro-mutagenic (Dizdaroglu *et al.*, *Free Radic. Biol. Med.* **32**: 1102-1115, 2002). DNA *N*-glycosylases initiate the base excision repair pathway by recognizing specific bases in DNA and cleaving the sugar base bond to 30 release the damaged base (David *et al.*, *Chem. Rev.* **98**: 1221-1262, 1998).

The *Neil* genes are a newly discovered family of mammalian DNA *N*-glycosylases related to the Fpg/Nei family of proteins from *Escherichia coli* (Hazra *et al.*, *Proc. Natl. Acad. Sci. USA* **99**: 3523-3528, 2002; Bandaru *et al.*, *DNA Repair* **1**: 517-529, 2002). Neil1 recognizes and removes a wide spectrum of oxidized pyrimidines and ring-opened purines

from DNA, including thymine glycol (Tg), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 4,6-diamino-5-formidopyrimidine (FapyA). Tg, FapyG and FapyA are among the most prevalent oxidized bases produced by ionizing radiation (Dizdaroglu *et al. Free Radic. Biol. Med.* **32**: 1102-1115, 2002) and can block replicative DNA polymerases, which 5 can, in turn, cause cell death (Asagoshi *et al. J. Biol. Chem.* **277**: 14589-14597, 2002; Clark *et al., Biochemistry* **28**: 775-779, 1989).

The Nth1 and Ogg1 glycosylases each remove subsets of oxidized DNA bases that overlap with substrates of Neil1 (Nishimura, *Free Radic. Biol. Med.* **32**: 813-821, 2002; Asagoshi *et al., Biochemistry* **39**: 11389-11398, 2000; Dizdaroglu *et al., Biochemistry* **38**: 10 243-246, 1999). However, mice with null mutations in either *Nth1* (Ocampo *et al., Mol. Cell. Biol.* **22**: 6111-6121, 2002; Takao *et al., EMBO J.* **21**: 3486-3493, 2002) or *Ogg1* (Klungland *et al., Proc. Natl. Acad. Sci. USA* **96**: 13300-13305, 1999; Minowa *et al., Proc. Natl. Acad. Sci. USA* **97**: 4156-4161, 2000) are viable, raising the possibility that Neil1 activity tempers the loss of Nth1 or Ogg1. Recently, a residual Tg-DNA glycosylase activity in $Nth1^{-/-}$ mice 15 has been identified as Neil1 (Takao *et al., J. Biol. Chem.* **277**: 42205-42213, 2002).

We constructed a single shRNA expression vector targeting a sequence near the 5' end of the *Neil1* coding region. This vector was introduced into mouse embryonic stem cells by electroporation, and individual stable integrants were tested for expression of the Neil1 protein (see the weblink: http://www.cshl.edu/public/SCIENCE/hannon.html for detailed 20 procedures). The majority of cell lines showed an ~80% reduction in Neil1 protein, which correlated with a similar change in levels of *Neil1* mRNA. These cells showed an approximately two-fold increase in their sensitivity to ionizing radiation, consistent with a role for Neil1 in DNA repair. Two independent ES cell lines were injected into BL/6 blastocysts, and several high-percentage chimeras were obtained. These chimeras were out-25 crossed, and germ-line transmission of the shRNA-expression construct was noted in numerous F_1 progeny (13/27 for one line and 12/26 for the other).

To determine whether the silencing of *Neil1* that had been observed in ES cells was transmitted faithfully, we examined *Neil1* mRNA and protein levels. Both were reduced by approximately the same extent that had been observed in the engineered ES cells (Figs. 55, 30 56). Consistent with this having occurred through the RNAi pathway, we detected the presence of siRNAs corresponding to the shRNA sequence in F_1 animals that carry the shRNA expression vector but not in those that lack the vector (Fig. 56b).

The aforementioned data demonstrate that shRNAs can be used to create germ-line transgenic mice in which RNAi has silenced a target gene. These observations open the door

to using of RNAi as a complement to standard knock-out methodologies and provide a means to rapidly assess the consequences of suppressing a gene of interest in a living animal. Coupled with activator-dependent U6 promoters, the use of shRNAs will ultimately provide methods for tissue-specific, inducible and reversible suppression of gene expression in mice.

5

Example 12: Dicer cleaves a single siRNA from the end of each shRNA

We performed the following experiments in order to understand how Dicer processes shRNAs, and in order to permit comparison of the efficiency of different silencing triggers.

We began by producing ~70 chemically synthesized shRNAs, targeting various 10 endogenous genes and reporters. We initially focused on a detailed analysis of one set of four shRNAs that target firefly luciferase (Fig. 57a). The individual species differed in two distinct ways. First, the stems of the shRNAs were either 19 or 29 nucleotides in length. Second, each shRNA either contained or lacked a 2 nucleotide 3' overhang, identical to that produced by processing of pri-miRNAs by Drosha. Each species was end-labeled by 15 enzymatic phosphorylation and incubated with recombinant human Dicer. The 29 nt. shRNA bearing the 3' overhang was converted almost quantitatively into a 22 nt product by Dicer (Fig. 57b). In contrast, the 29 nt shRNA that lacked the overhang generated very little 22 nt labeled product, although there was a substantial depletion of the starting material. Neither 19 nt shRNA was cleaved to a significant extent by the Dicer enzyme. This result was not due to 20 the lack of dsRNA in the 19 nt shRNAs as all shRNA substrates were efficiently cleaved by bacterial RNAseIII (Fig. 57c). Parallel analysis of identical shRNA substrates that were produced by *in vitro* transcription with T7 polymerase and uniformly labeled clarified the results obtained with end–labeled substrates (not shown). Specifically, 19 nt shRNAs were not cleaved. However, both the overhung and the blunt 29 nucleotide shRNAs gave rise to 22 25 nt products, albeit at reduced levels in the latter case. These results suggest that Dicer requires a minimum stem length for productive cleavage. Furthermore, they are consistent with a hypothesis that the presence of a correct 3' overhang enhances the efficiency and specificity of cleavage, directing Dicer to cut ~22 nucleotides from the end of the substrate.

A number of previous studies have suggested that Dicer might function as an end-30 recognizing endonuclease, without positing a role for the 3' overhang. Processive Dicer cleavage was first implied by *in vitro* analysis of RISC cleavage (Zamore *et al.*, *Cell* **101**: 25-33, 2000). In *Drosophila* embryo extracts programmed for RISC assembly using a long dsRNA, phased cleavage sites occurred at approximately 22 nucleotide intervals along an

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mRNA substrate. Similarly, analysis of *C. elegans* Dicer in whole cell extracts (Ketting *et al.*, *Genes Dev* **15**: 2654-9, 2001) or purified human Dicer *in vitro* (Zhang *et al.*, *EMBO J* **21**: 5875-85, 2002) showed accumulation of discretely sized cleavage intermediates. Blocking of the ends of dsRNAs using either fold-back structures or chimeric RNA-DNA hybrids
5 attenuated, but did not abolish, the ability of human Dicer to generate siRNAs (Zhang *et al.*, *EMBO J* **21**: 5875-85, 2002). Finally, Lund and colleagues suggested that Dicer cleaved ~22 nt from the blunt end of an extended pre-miRNA, designed in part to mimic a pri-miRNA (see Lund *et al.*, *Science* **303**: 95-8, 2004).

Our results suggest that while the overhang is not obligate for Dicer processing of its
10 substrates (see Zhang *et al.*, *EMBO J* **21**: 5875-85, 2002, and Fig. 57b), this structure does aid in determining the specificity of cleavage. Furthermore, time courses of processing of blunt and overhung 29nt shRNAs do show a more rapid processing of the overhung substrate if reactions are performed in the linear range for the enzyme (not shown).

To map more precisely the position of Dicer cleavage in the shRNA, we used primer
15 extension analysis. The shRNAs described in Fig. 57a were reacted with recombinant human Dicer as shown in Fig. 57b. Total RNA was recovered from the processing reactions and used in primer extension assays. Consistent with direct analysis of the RNA, shRNAs with 19 nt stems failed to yield discrete extension products. The extension products that would be predicted from the unreacted substrate are not seen due to secondary structure of the
20 uncleaved precursor (Fig. 58a). Both of the 29 nt shRNAs give rise to extension products with the overhung precursor giving a relatively discrete product of 20 nucleotides, as predicted for a cleavage precisely 22 nt from the 3' end of the substrate (Fig. 58b). The blunt-ended precursor gave a distribution of products, as was predicted from the analysis of uniformly and end-labeled RNAs.

25 In *Drosophila*, Dicer2 acts in a complex with a double-stranded RNA binding protein, R2D2 (Liu *et al.*, *Science* **301**: 1921-5, 2003). Similarly, biochemical evidence from *C. elegans* suggests that its Dicer binds RDE-1, RDE-4 and DRH-1 (Tabara *et al.*, *Cell* **109**: 861-71, 2002). These results suggest that the human enzyme might also function as part of a larger complex, which could show altered cleavage specificities. Therefore, we also mapped
30 the cleavage of our shRNAs *in vitro*. Precursors were transfected into cells, and the processed form of each was isolated by virtue of its co-immunoprecipitation with human Argonaute proteins, Ago1 and Ago2. Primer extension suggested identical cleavage specificities upon exposure of shRNAs to Dicer *in vitro* and in living cells (Fig. 58c).

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Example 13: shRNAs are generally more effective than siRNAs

Since each shRNA gave rise to a single, predictable 22 nt sequence in RISC, we compared the efficacy of shRNAs and siRNAs. Toward this goal, we selected 43 sequences targeting a total of 6 genes (3-9 sequences per gene). For each sequence, we synthesized a 21 nt siRNA (19 base stem) and 19 and 29 nt shRNAs that were predicted to give Dicer products that were either identical to the siRNAs or that differed by the addition of one 3' nucleotide (Fig. 59a). Each RNA species was transfected into HeLa cells at a relatively high concentration (100 nM). The level of suppression was determined by semi-quantitative RT-PCR and the performance of each shRNA compared to the performance of the corresponding siRNA (Fig. 59b). Comparison of 19 nt shRNAs with siRNAs revealed that there was little difference in endpoint inhibition with these species (left panel). A comparison of siRNAs with 29 nt shRNAs gave a different result. Clustering of the comparison data points above the diagonal indicated consistently better endpoint inhibition with the 29 nt shRNAs (right panel).

The generally better endpoint inhibition observed with 29 nt shRNAs led us to investigate in more detail the performance of these silencing triggers as compared to siRNAs. Seventeen complete sets comprising an siRNA, a 19 nt shRNA and a 29 nt shRNA were examined for suppression in titration experiments. In all cases, the 19 nt shRNAs performed as well as or worse than the corresponding siRNAs. In contrast, 29 nt shRNAs exceeded the performance of siRNAs in the majority of cases. Four representative examples, targeting MAPK-14 are shown in Fig. 59c. Several 29 nt shRNAs (*e.g.*, see MAPK14-1) showed both significantly greater endpoint inhibition and efficacy at lower concentrations than the corresponding siRNA. In other cases(*e.g.*, see MAPK14-2 and MAPK-14-4), the maximal level of suppression for the 29 nt. shRNA was approximately two-fold greater than the maximal level of suppression for the corresponding siRNA. Finally, in a minority of cases, exemplified by MAPK14-3, the performance of the three types of silencing triggers was similar. Importantly, in only one case out of 17 did we note that the 29 nt shRNA with a 2 nt. 3' overhang performed less effectively than the corresponding siRNA (data not shown).

Example 14: siRNAs and shRNAs give similar profiles of off-target effects at saturation

Sequence specificity is a critical parameter in RNAi experiments. Microarray analysis has revealed down-regulation of many non-targeted transcripts following transfection of siRNAs into HeLa cells (Jackson *et al.*, *Nat Biotechnol* **21**: 635-7, 2003). Notably, these gene

expression signatures differed between different siRNAs targeting the same gene. Many of the "off target" transcripts contained sites of partial identity to the individual siRNA, possibly explaining the source of the effects. To examine potential off-target effects of synthetic shRNAs, we compared shRNA signatures with those of siRNAs derived from the same target
5 sequence. Using microarray gene expression profiling, we obtained a genome-wide view of transcript suppression in response to siRNA and shRNA transfection. Fig. 60 (a and b) shows heat maps of signatures produced in HeLa cells 24 hours after transfection of 19 nt and 29 nt shRNAs compared with those generated by corresponding siRNAs. 19 nt shRNAs produced signatures that resembled, but were not identical to, those of corresponding siRNAs. In
10 contrast, the signatures of the 29 nt shRNAs (Fig. 60a) were nearly identical to those of the siRNAs.

These results indicate that off target effects may be inherent to the use of synthetic RNAs for eliciting RNAi and cannot be ameliorated by intracellular processing of an upstream precursor in the RNAi pathway. Furthermore, the agreement between the signatures
15 of 29 nt shRNAs and siRNAs is consistent with precise intracellular processing of the shRNA to generate a single siRNA rather than a random sampling of the hairpin stem by Dicer. The basis of the divergence between the signature of the 19 nt shRNA and the corresponding siRNA is presently unclear.

Considered together, our results indicate that chemically synthesized, 29 nt shRNAs
20 are often substantially more effective triggers of RNAi than are siRNAs. While not wishing to be bound by any particular theory, a possible mechanistic explanation for this finding may lie in the fact that 29 nt shRNAs are substrates for Dicer processing both *in vitro* and *in vitro*. We originally suggested that siRNAs might be passed from Dicer to RISC in a solid state reaction on the basis of an interaction between Dicer and Argonaute2 in *Drosophila* S2 cell
25 extracts (Hammond *et al.*, *Science* **293**: 1146-50, 2001). More recently, results from several laboratories have strongly suggested a model for assembly of the RNAi effector complex in which a multi-protein assembly containing Dicer and accessory proteins interacts with an Argonaute protein and actively loads one strand of the siRNA or miRNA into RISC (Lee *et al.*, *Cell* **117**: 69-81, 2004; Pham *et al.*, *Cell* **117**: 83-94, 2004; Tomari *et al.*, *Cell* **116**: 831-
30 41, 2004). Our result is consistent with a model where Dicer substrates, derived from nuclear processing of pri-miRNAs or cytoplasmic delivery of pre-miRNA mimetics, are loaded into RISC more effectively than siRNAs. Our data support such a model, since it is not the hairpin structure of the synthetic RNA that determines its increased efficacy but the fact that the shRNA is a Dicer substrate that correlates with enhanced potency. Again, not wishing to be

bound by any particular theory, it is possible that even siRNAs enter RISC via a Dicer-mediated assembly pathway. Our data may also reflect an increased affinity of Dicer for longer duplexes substrates. Alternatively, hairpin RNAs, such as miRNA precursors, might interact with specific cellular proteins that facilitate delivery of these substrates to Dicer, whereas siRNAs might not benefit from such chaperones.

Overall, our results provide an improved method for triggering RNAi in mammalian cells that uses higher potency RNAi triggers. Mapping the single 22 nt sequence that appears in RISC from each of these shRNAs now permits the combination of this more effective triggering method with rules for effective siRNA design.

Methods

RNA sequence design

Each set of RNAs began with the choice of a single 19-mer sequence. These 19mers were used directly to create siRNAs. To create shRNAs with 19-mer stems, we appended a 4-base loop (either CCAA or UUGG) to the end of the 19-mer sense strand target sequence followed by the 19-mer complementary sequence and a UU overhang. To create 29-mer stems, we increased the length of the 19-mer target sequence by adding 1 base upstream and 9 bases downstream from the target region and used the same loop sequence and UU overhang. All synthetic RNA molecules used in this study were purchased from Dharmacon.

Dicer processing

RNA hairpins corresponding to luciferase were end -labeled with [γ-^{32}P] ATP and T4 Polynucleotide kinase. 0.1 pmoles of RNA were then processed with 2 units of Dicer (Stratagene) at 37°C for 2 hours. Reaction products were trizol extracted, isopropanol precipitated, run on an 18% polyacrylamide, 8M urea denaturing gel. For RNaseIII digestion, 0.1 pmoles were digested with 1 unit of *E. coli* RNase III (NEB) for 30 minutes at 37°C and analyzed as described above. For primer extension analysis, hairpins were processed with Dicer at 37°C for 2 hours, followed by heat inactivation of the enzyme. DNA primers were 5' labeled with PNK and annealed to 0.05 pmole of RNA as follows : 95°C for one minute, 10 minutes at 50°C and then 1 min on ice. Extensions were carried out at 42°C for 1 hour using MoMLV reverse transcriptase. Products were analyzed by electrophoresis on a 8M Urea/20% polyacrylamide gel. For analysis of *in vitro* processing, LinxA cells were transfected in 10 cm plates using Mirus TKO (10 μg hairpin RNA) or Mirus LT4 reagent for DNA transfection (12

μg of tagged Agol/Ago 2 DNA; J. Liu, unpublished). Cells were lysed and immunoprecipitated after 48 hours using with myc Antibody (9E14) Antibody. Immunoprecipitations were washed 3 × in lysis buffer and treated with DNase for 15 minutes. Immunoprecipitates were then primer extended as described above.

5 **siRNA and shRNA Transfections and mRNA Quantitation**

HeLa cells were transfected in 96-well plates by use of Oligofectamine (Invitrogen) with the final nanomolar concentrations of each synthetic RNA indicated in the graphs. RNA quantitation was performed by Real-time PCR, using appropriate Applied Biosystems TaqMan™ primer probe sets. The primer probe set used for MAPK14 was Hs00176247_m1.
10 RNA values were normalized to RNA for HGUS (probe 4310888E).

Microarray Gene Expression Profiling

HeLa cells were transfected in 6-well plates by use of Oligofectamine. RNA from transfected cells was hybridized competitively with RNA from mock-transfected cells (treated with transfection reagent in the absence of synthetic RNA). Total RNA was purified
15 by Qiagen RNeasy kit, and processed as described previously (Hughes *et al.*, *Nat Biotechnol* **19**: 342-7, 2001) for hybridization to microarrays containing oligonucleotides corresponding to approximately 21,000 human genes. Ratio hybridizations were performed with fluorescent label reversal to e liminate dye bias. Microarrays were purchased from Agilent Technologies. Error models have been described previously (Hughes *et al.*, *Nat Biotechnol* **19**: 342-7,
20 2001). Data were analyzed using Rosetta Resolver™ software.

Supplementary Table 1. Sequences of the siRNAs used in this study

Gene	Accession number	Target sequence ID	Target sequence
IGF1R	NM_000875	IGF1R-1	GGAUGCACCAUCUUCAAGG (SEQ ID NO: 47)
IGF1R	NM_000875	IGF1R-2	GACAAAAUCCCCAUCAGGA (SEQ ID NO: 48)
IGF1R	NM_000875	IGF1R-3	ACCGCAAAGUCUUUGAGAA (SEQ ID NO: 49)
IGF1R	NM_000875	IGF1R-4	GUCCUGACAUGCUGUUUGA (SEQ ID NO: 50)
IGF1R	NM_000875	IGF1R-5	GACCACCAUCAACAAUGAG (SEQ ID NO: 51)
IGF1R	NM_000875	IGF1R-6	CAAAUUAUGUGUUUCCGAA (SEQ ID NO: 52)
IGF1R	NM_000875	IGF1R-7	CGCAUGUGCUGGCAGUAUA (SEQ ID NO: 53)
IGF1R	NM_000875	IGF1R-8	CCGAAGAUUUCACAGUCAA (SEQ ID NO: 54)
IGF1R	NM_000875	IGF1R-9	ACCAUUGAUUCUGUUACUU (SEQ ID NO: 55)
KIF11	NM_004523	KIF11-1	CUGACAAGAGCUCAAGGAA (SEQ ID NO: 56)

KIF11	NM_004523	KIF11-2	CGUUCUGGAGCUGUUGAUA (SEQ ID NO: 57)
KIF11	NM_004523	KIF11-3	GAGCCCAGAUCAACCUUUA (SEQ ID NO: 58)
KIF11	NM_004523	KIF11-4	GGCAUUAACACACUGGAGA (SEQ ID NO: 59)
KIF11	NM_004523	KIF11-5	GAUGGCAGCUCAAAGCAAA (SEQ ID NO: 60)
KIF11	NM_004523	KIF11-6	CAGCAGAAAUCUAAGGAUA (SEQ ID NO: 61)
KIF14	NM_014875	KIF14-1	CAGGGAUGCUGUUUGGAUA (SEQ ID NO: 62)
KIF14	NM_014875	KIF14-2	ACUGACAACAAAGUGCAGC (SEQ ID NO: 63)
KIF14	NM_014875	KIF14-3	AAACUGGGAGGCUACUUAC (SEQ ID NO: 64)
KIF14	NM_014875	KIF14-4	CACUGAAUGUGGGAGGUGA (SEQ ID NO: 65)
KIF14	NM_014875	KIF14-5	GUCUGGGUGGAAAUUCAAA (SEQ ID NO: 66)
KIF14	NM_014875	KIF14-6	CAUCUUUGCUGAAUCGAAA (SEQ ID NO: 67)
KIF14	NM_014875	KIF14-7	GGGAUUGACGGCAGUAAGA (SEQ ID NO: 68)
KIF14	NM_014875	KIF14-8	CAGGUAAAGUCAGAGACAU (SEQ ID NO: 69)
KIF14	NM_014875	KIF14-9	CUCACAUUGUCCACCAGGA (SEQ ID NO: 70)
KNSL1	NM_004523	KNSL1-1	GACCUGUGCCUUUUAGAGA (SEQ ID NO: 71)
KNSL1	NM_004523	KNSL1-2	AAAGGACAACUGCAGCUAC (SEQ ID NO: 72)
KNSL1	NM_004523	KNSL1-3	GACUUCAUUGACAGUGGCC (SEQ ID NO: 73)
MAPK14	NM_139012	MAPK14-1	AAUAUCCUCAGGGGUGGAG (SEQ ID NO: 74)
MAPK14	NM_139012	MAPK14-2	GUGCCUCUUGUUGCAGAGA (SEQ ID NO: 75)
MAPK14	NM_139012	MAPK14-3	GAAGCUCUCCAGACCAUUU (SEQ ID NO: 76)
MAPK14	NM_001315	MAPK14-4	CUCCUGAGAUCAUGCUGAA (SEQ ID NO: 77)
MAPK14	NM_001315	MAPK14-5	GCUGUUGACUGGAAGAACA (SEQ ID NO: 78)
MAPK14	NM_001315	MAPK14-6	GGAAUUCAAUGAUGUGUAU (SEQ ID NO: 79)
MAPK14	NM_001315	MAPK14-7	CCAUUUCAGUCCAUCAUUC (SEQ ID NO: 80)
PLK	NM_005030	PLK-1	CCCUGUGUGGGACUCCUAA (SEQ ID NO: 81)
PLK	NM_005030	PLK-2	CCGAGUUAUUCAUCGAGAC (SEQ ID NO: 82)
PLK	NM_005030	PLK-3	GUUCUUUACUUCUGGCUAU (SEQ ID NO: 83)
PLK	NM_005030	PLK-4	CGCCUCAUCCUCUACAAUG (SEQ ID NO: 84)
PLK	NM_005030	PLK-5	AAGAGACCUACCUCCGGAU (SEQ ID NO: 85)
PLK	NM_005030	PLK-6	GGUGUUCGCGGGCAAGAUU (SEQ ID NO: 86)
PLK	NM_005030	PLK-7	CUCCUUAAAUAUUUCCGCA (SEQ ID NO: 87)
PLK	NM_005030	PLK-8	AAGAAGAACCAGUGGUUCG (SEQ ID NO: 88)
PLK	NM_005030	PLK-9	CUGAGCCUGAGGCCCGAUA (SEQ ID NO: 89)

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25

V. Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.
30 All of the above-cited references and publications are hereby incorporated by reference.

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We Claim:

1. A method for attenuating expression of a target gene in mammalian cells, comprising introducing into the mammalian cells a single-stranded hairpin ribonucleic acid (shRNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA:

   (i) is a substrate for cleavage by a RNaseIII enzyme to produce a double-stranded RNA product,

   (ii) does not produce a general sequence-independent killing of the mammalian cells, and

   (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions, and,

   wherein said shRNA comprises a 3' overhang of about 1-4 nucleotides.

2. A method for attenuating expression of a target gene in mammalian cells, comprising introducing into the mammalian cells a single-stranded hairpin ribonucleic acid (shRNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA:

   (i) is cleaved in the mammalian cells to produce an RNA guide sequence that enters an Argonaut-containing complex,

   (ii) does not produce a general sequence-independent killing of the mammalian cells, and

   (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions, and,

   wherein said shRNA comprises a 3' overhang of about 1-4 nucleotides.

3. A method for attenuating expression of one or more target genes in mammalian cells, comprising introducing into the mammalian cells a variegated library of single-stranded hairpin ribonucleic acid (shRNA) species, each shRNA species comprising self complementary sequences of 19 to 100 nucleotides that form duplex regions and which hybridize under intracellular conditions to a target gene, wherein each of said hairpin RNA species:

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(i) is a substrate for cleavage by a RNaseIII enzyme to produce a double-stranded RNA product,

(ii) does not produce a general sequence-independent killing of the mammalian cells, and

5 (iii) if complementary to a target sequence, reduces expression of said target gene in a manner dependent on the sequence of said complementary regions, and,

wherein said shRNA comprises a 3' overhang of about 1-4 nucleotides.

4. The method of claim 1, 2, or 3, wherein the shRNA comprises a 3' overhang of 2 nucleotides.

10 5. The method of claim 1, 2, or 3, wherein the shRNA comprises self-complementary sequences of 25 to 29 nucleotides that form duplex regions.

6. The method of claim 1, 2, or 3, wherein the self-complementary sequences are 29 nucleotides in length.

7. The method of claim 1, 2, or 3, wherein the shRNA is transfected or microinjected
15 into said mammalian cells.

8. The method of claim 1, 2, or 3, wherein the shRNA is a transcriptional product that is transcribed from an expression construct introduced into said mammalian cells, which expression construct comprises a coding sequence for transcribing said shRNA, operably linked to one or more transcriptional regulatory sequences.

20 9. The method of claim 8, wherein said transcriptional regulatory sequences include a promoter for an RNA polymerase.

10. The method of claim 9, wherein said RNA polymerase is a cellular RNA polymerase.

11. The method of claim 9, wherein said promoter is a U6 promoter, a T7 promoter, a T3 promoter, or an SP6 promoter.

25 12. The method of claim 8, wherein said transcriptional regulatory sequences includes an inducible promoter.

13. The method of claim 8, wherein said mammalian cells are stably transfected with said expression construct.

14. The method of claim 1, 2 or 3, wherein the mammalian cells are primate cells.

30 15. The method of claim 14, wherein the primate cells are human cells.

16. The method of claim 1 or 2, wherein the shRNA is introduced into the mammalian cells in cell culture or in an animal.

17. The method of claim 1 or 2, wherein expression of the target is attenuated by at least 33 percent relative expression in cells not treated said hairpin RNA.

5 18. The method of claim 1 or 2, wherein the target gene is an endogenous gene or a heterologous gene relative to the genome of the mammalian cell.

19. The method of claim 1 or 2, wherein the self complementary sequences hybridize under intracellular conditions to a non-coding sequence of the target gene selected from a promoter sequence, an enhancer sequence, or an intronic sequence.

10 20. The method of claim 1 or 2, wherein the shRNA includes one or more modifications to phosphate-sugar backbone or nucleosides residues.

21. The method of claim 3, wherein said variegated library of shRNA species are arrayed a solid substrate.

22. The method of claim 3, including the further step of identifying shRNA species of
15 said variegated library which produce a detected phenotype in said mammalian cells.

23. The method of claim 1, 2, or 3, wherein the shRNA is a chemically synthesized product or an *in vitro* transcription product.

24. A method of enhancing the potency / activity of an RNAi therapeutic for a mammalian patient, said RNAi therapeutic comprising an siRNA of 19-22 paired
20 polynucleotides, the method comprising replacing said siRNA with a single-stranded hairpin RNA (shRNA) of claim 1 or 2, wherein said duplex region comprises the same 19-22 paired polynucleotides of said siRNA.

25. The method of claim 24, wherein said shRNA comprises a 3' overhang of 2 nucleotides.

25 26. The method of claim 24, wherein the half-maximum inhibition by said RNAi therapeutic is achieved by a concentration of said shRNA at least about 20% lower than that of said siRNA.

27. The method of claim 26, wherein the half-maximum inhibition by said RNAi therapeutic is achieved by a concentration of said shRNA at least about 100% lower
30 than that of said siRNA.

28. The method of claim 24, wherein the end-point inhibition by said shRNA is at least about 40% higher than that of said siRNA.

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29. The method of claim 24, wherein the end-point inhibition by said shRNA is at least about 2-6 fold higher than that of said siRNA.

30. A method of designing a short hairpin RNA (shRNA) construct for RNAi, said shRNA comprising a 3' overhang of about 1-4 nucleotides, the method comprising
5 selecting the nucleotide about 21 bases 5' to the most 3'-end nucleotide as the first paired nucleotide in a cognate doubled-stranded siRNA with the same 3' overhang.

31. The method of claim 30, wherein said shRNA comprises 25-29 paired polynucleotides.

32. The method of claim 31, wherein said shRNA, when cut by a Dicer enzyme, produces
10 a product siRNA that is either identical to, or differ by a single basepair immediately 5' to the 3' overhang from, said cognate siRNA.

33. The method of claim 32, wherein said Dicer enzyme is a human Dicer.

34. The method of claim 30, wherein said 3' overhang has 2 nucleotides.

35. The method of claim 30, wherein said shRNA is for RNAi in mammalian cells.

Abstract

The present invention provides methods for attenuating gene expression in a cell, especially in a mammalian cell, using gene-targeted double stranded RNA (dsRNA), such as a hairpin RNA. The dsRNA contains a nucleotide sequence that hybridizes under physiologic
5 conditions of the cell to the nucleotide sequence of at least a portion of the gene to be inhibited (the "target" gene).

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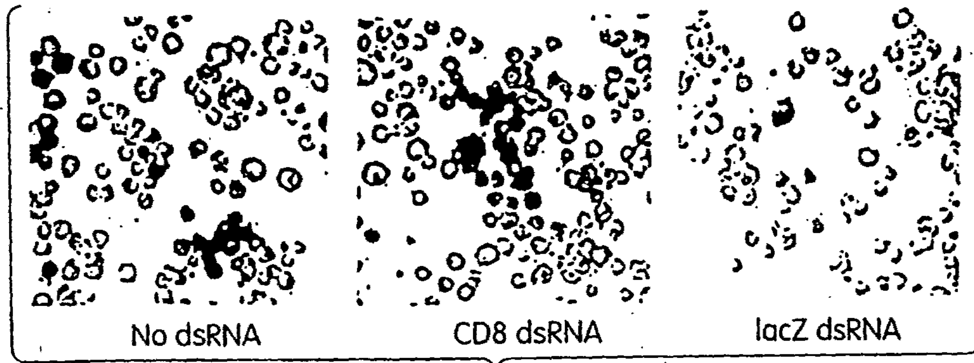


Fig. 1A

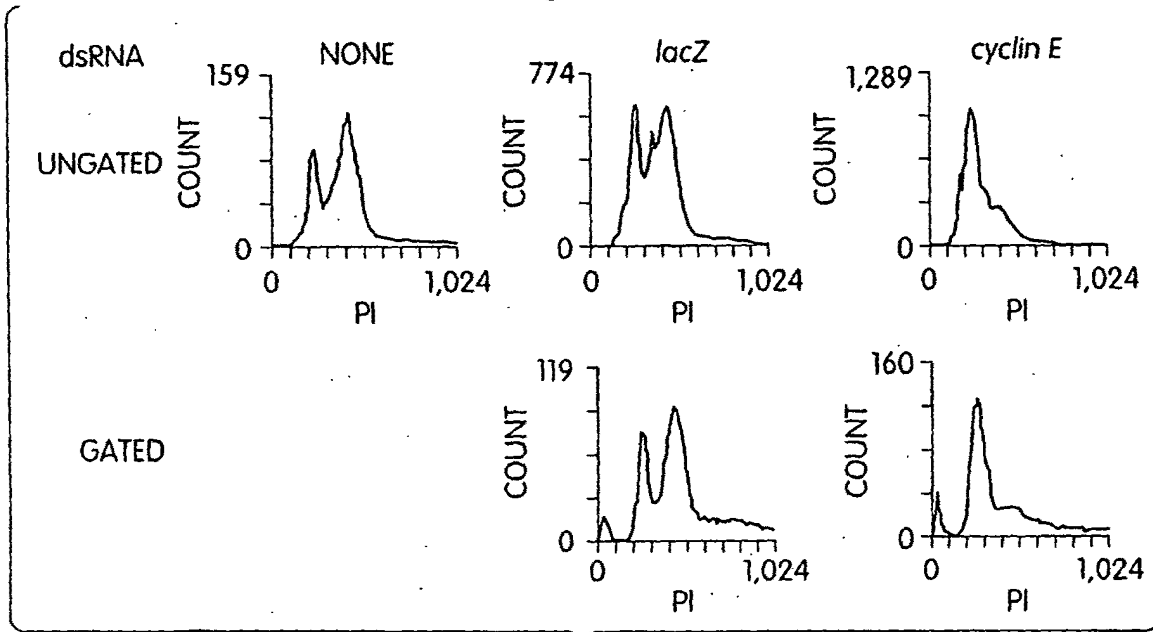


Fig. 1B

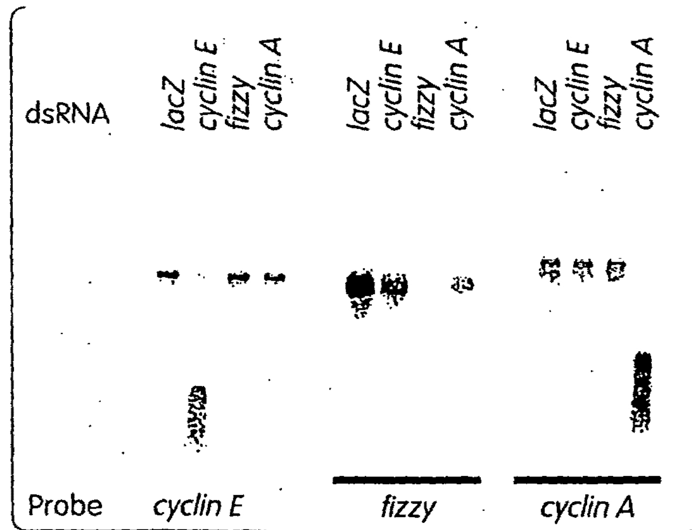


Fig. 1C

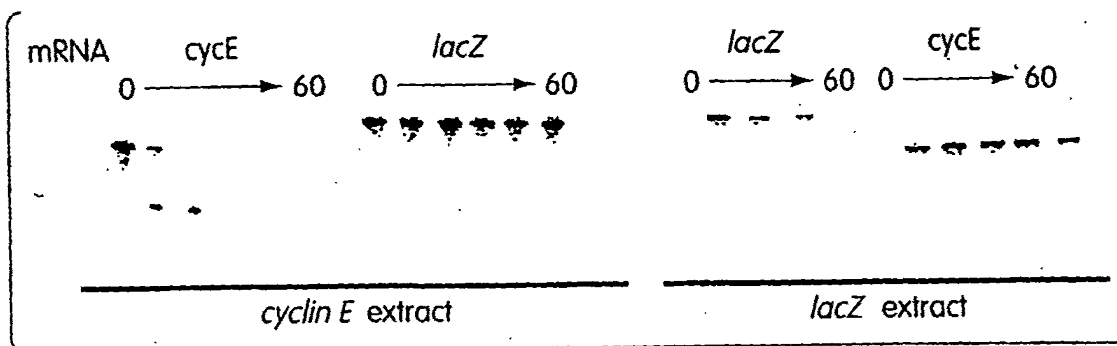


Fig. 2A

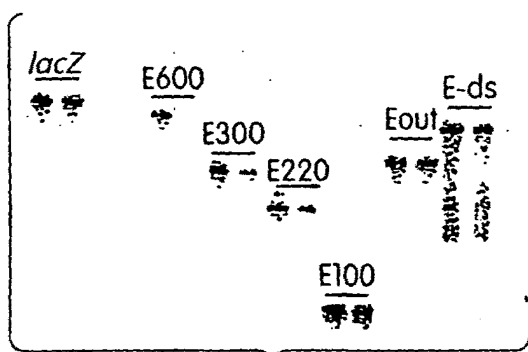


Fig. 2B

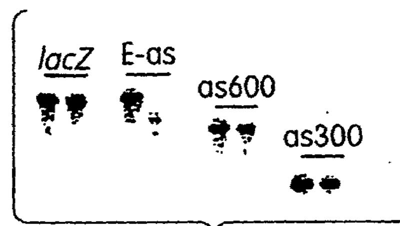


Fig. 2C

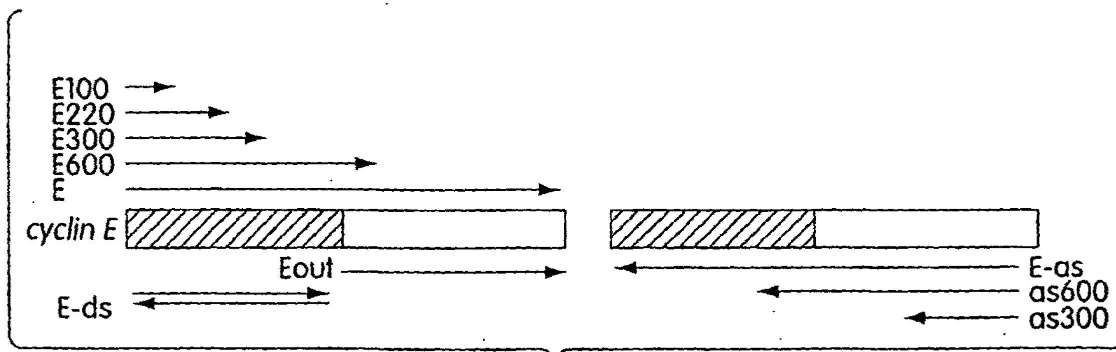


Fig. 2D

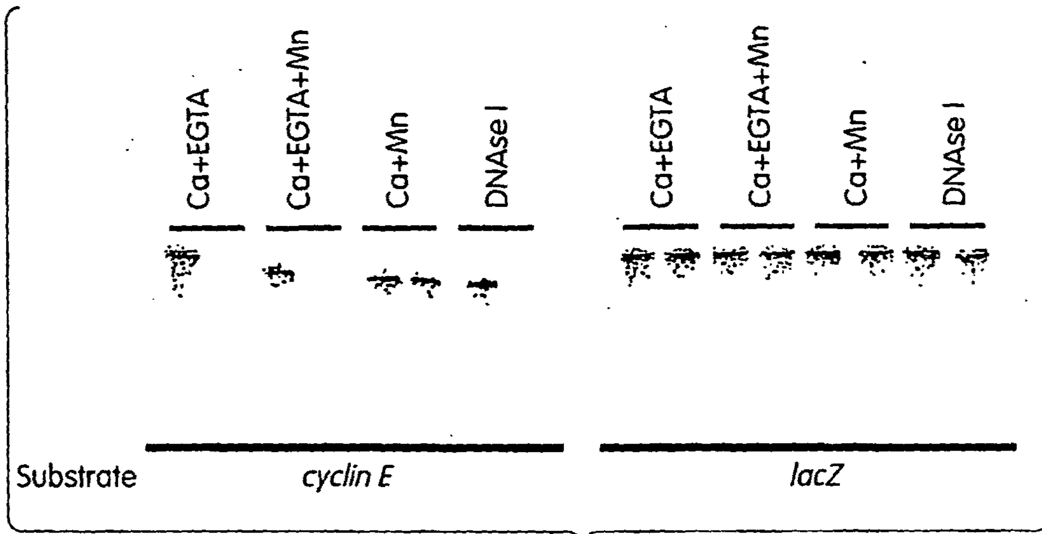


Fig. 3

Fig. 4A

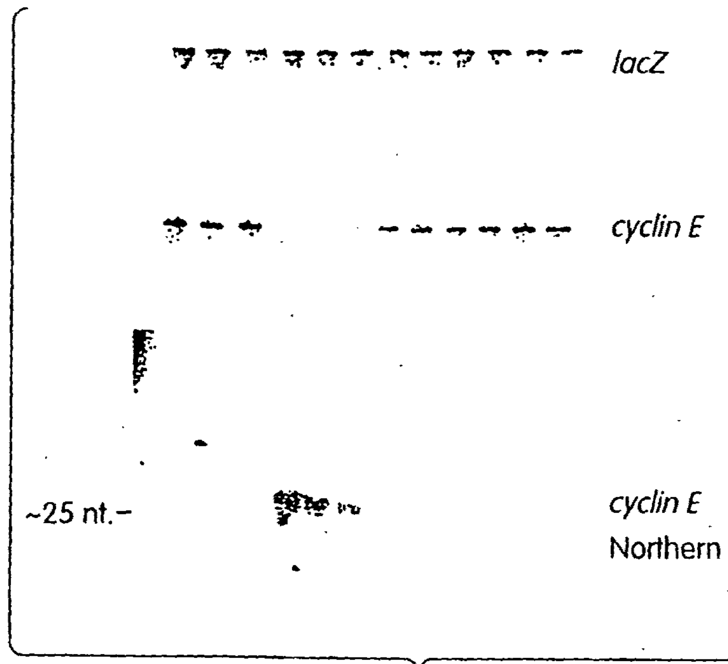


Fig. 4B

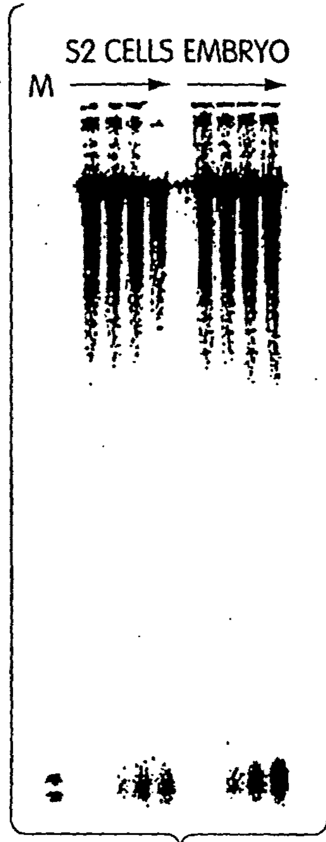


Fig. 5A

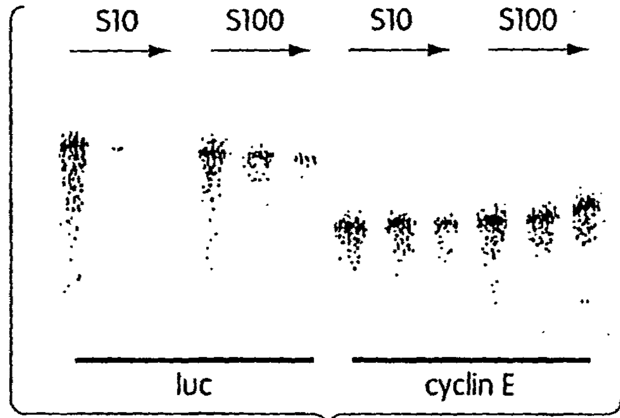


Fig. 5B

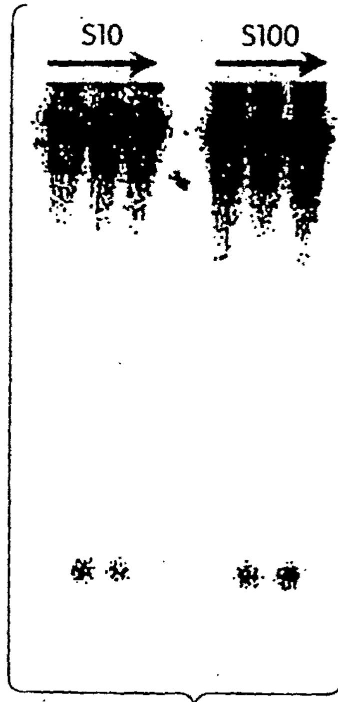


Fig. 5C

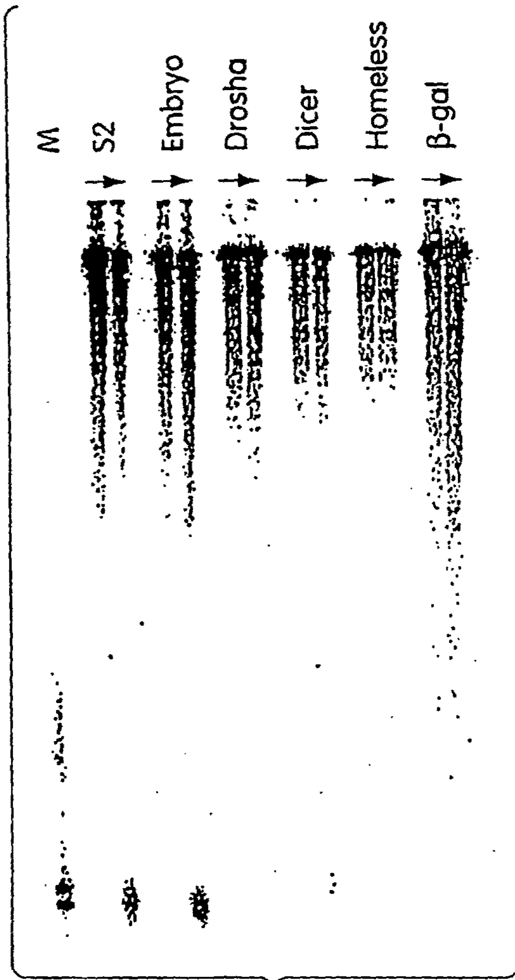


Fig. 6A

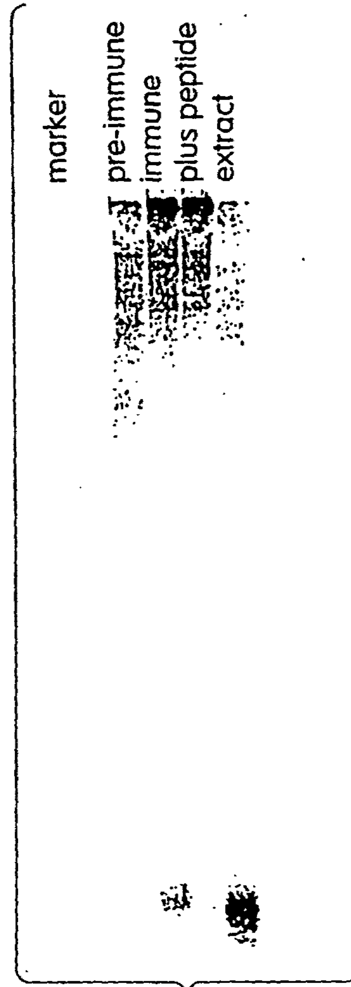


Fig. 6C

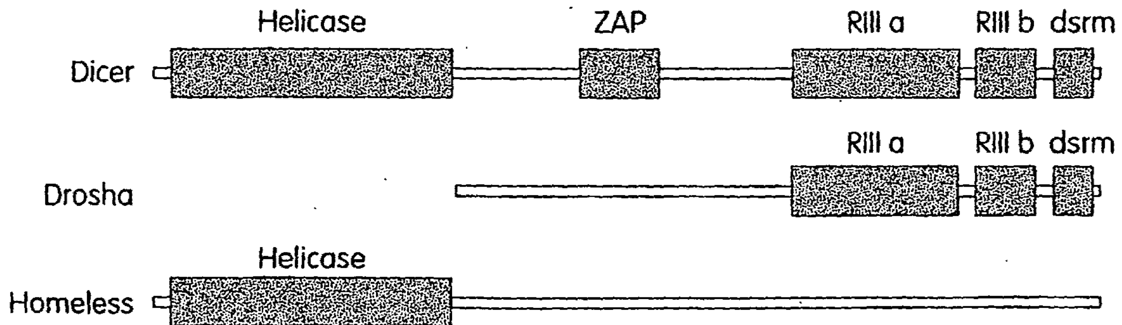


Fig. 6B

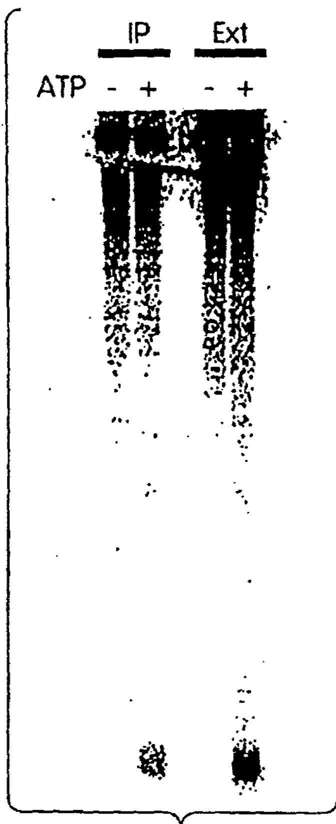


Fig. 6D

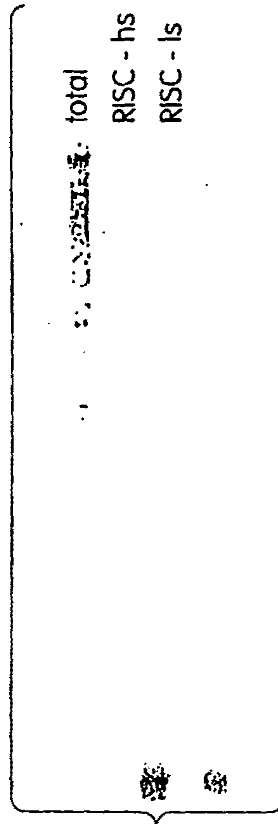


Fig. 6E

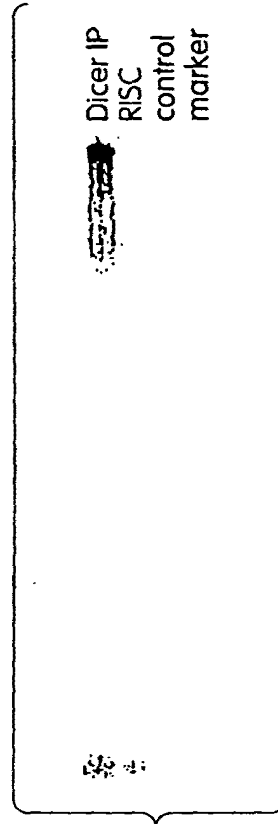


Fig. 6F

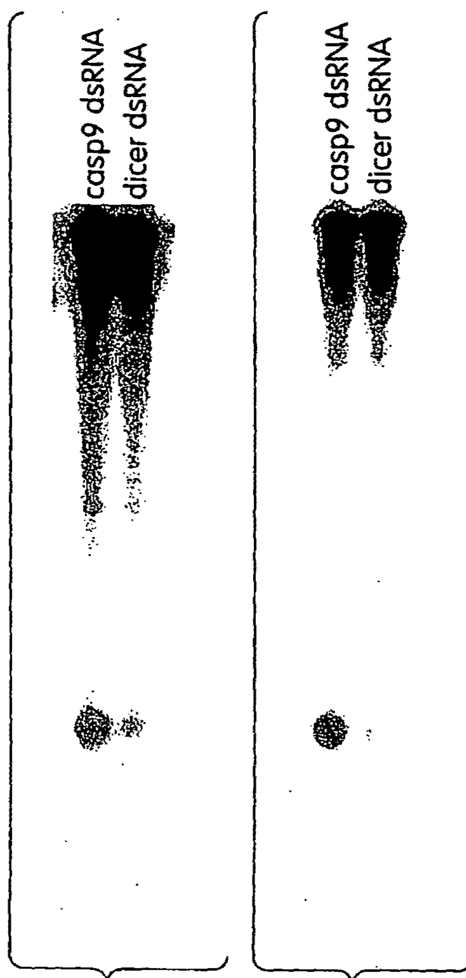


Fig. 7A Fig. 7B

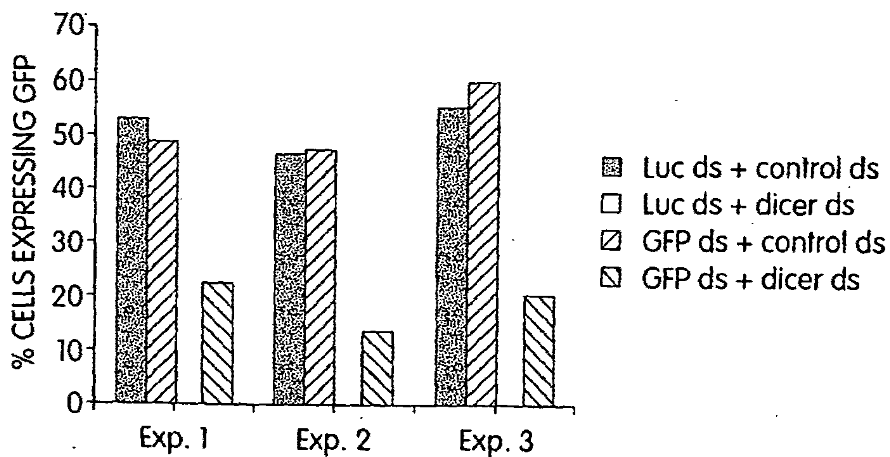


Fig. 7C

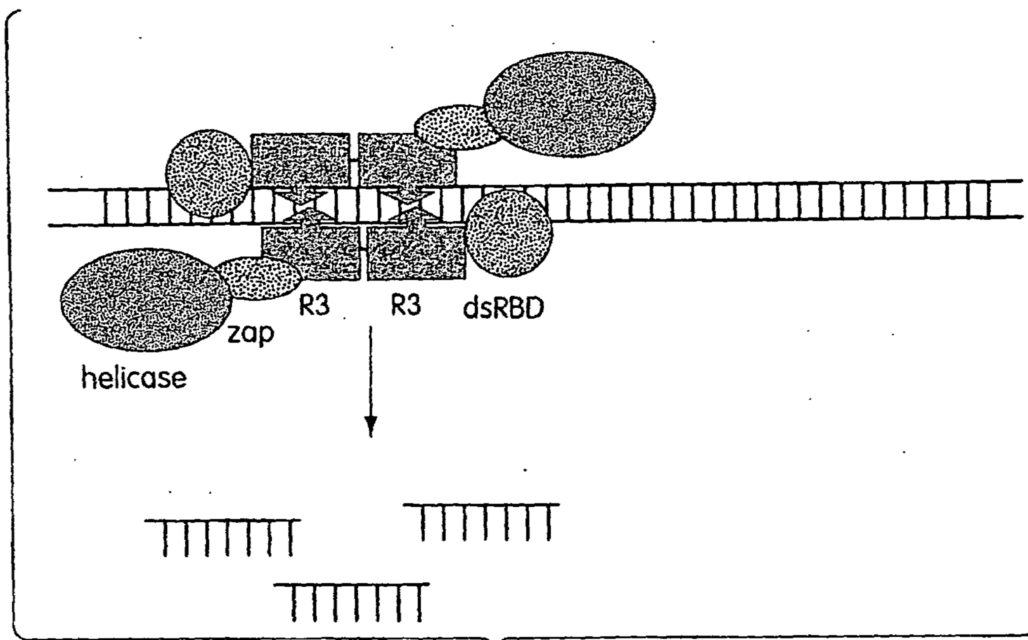


Fig. 8A

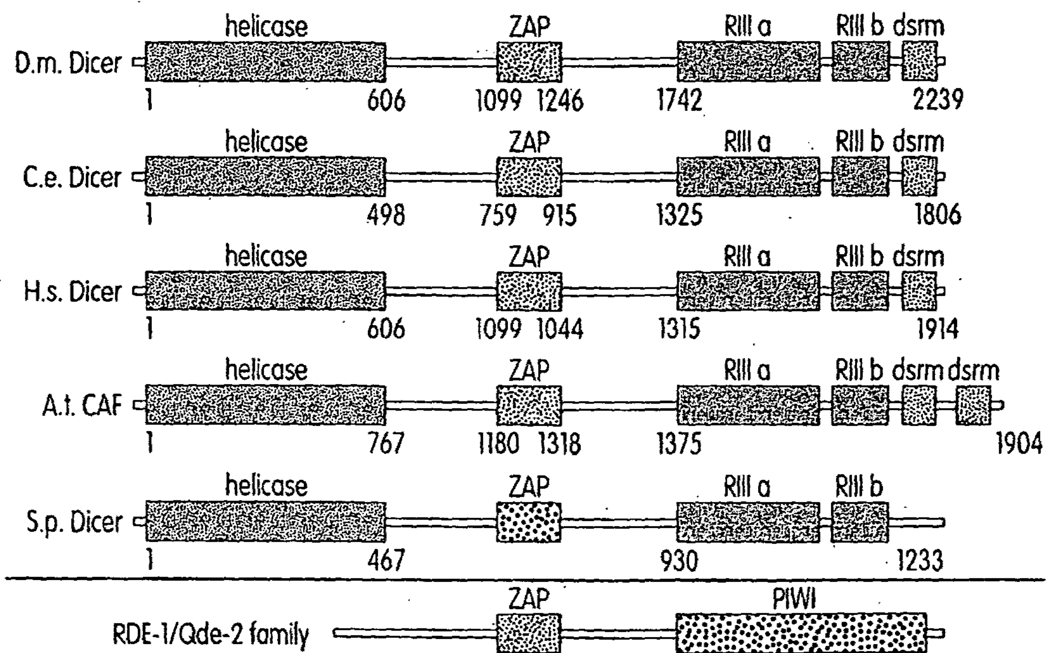


Fig. 8B

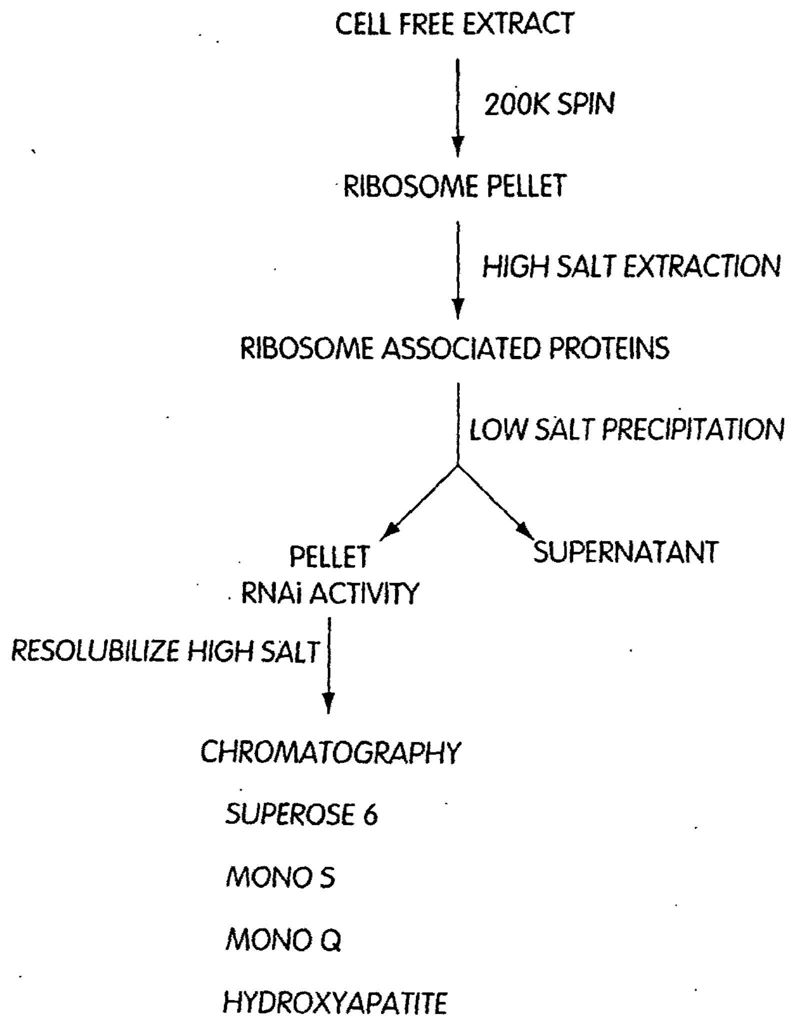


Fig. 9

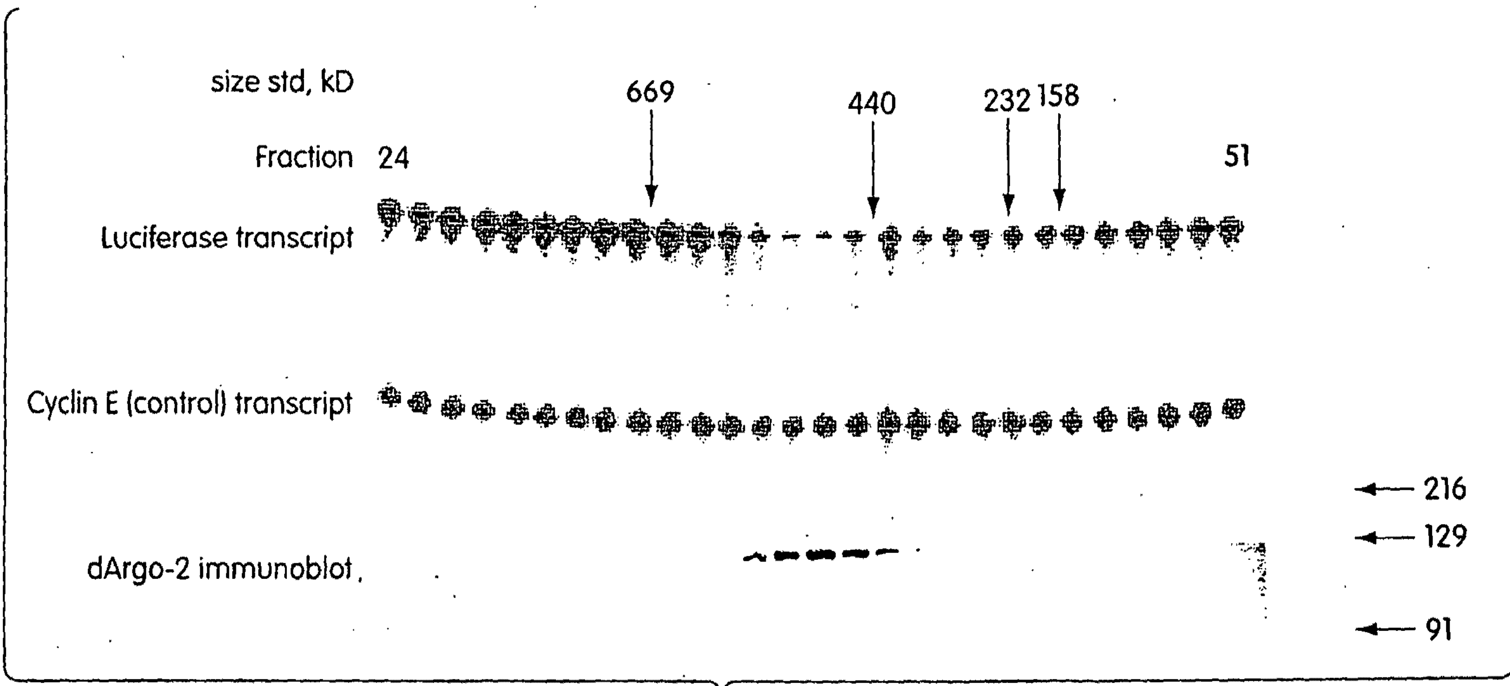


Fig. 10

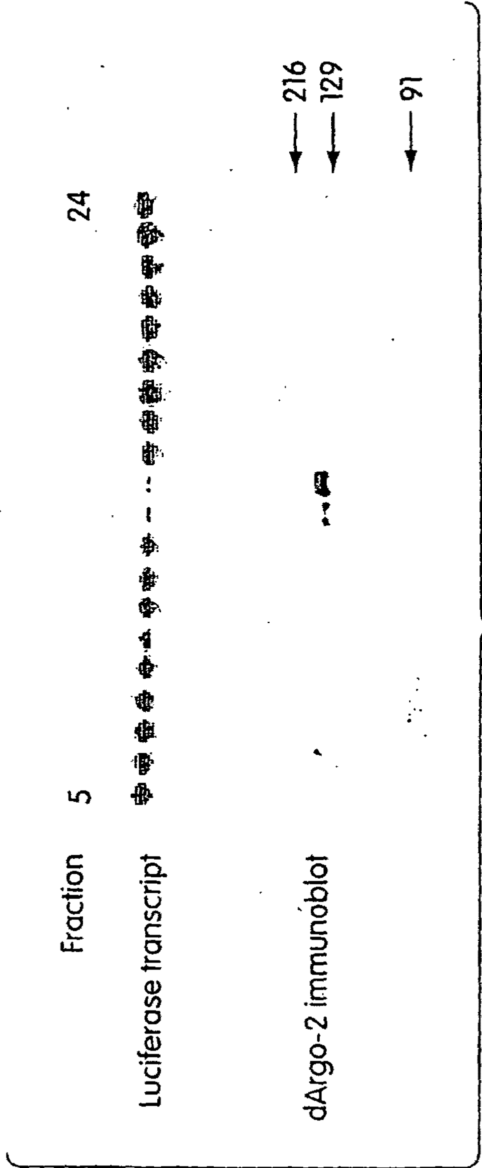


Fig. 11

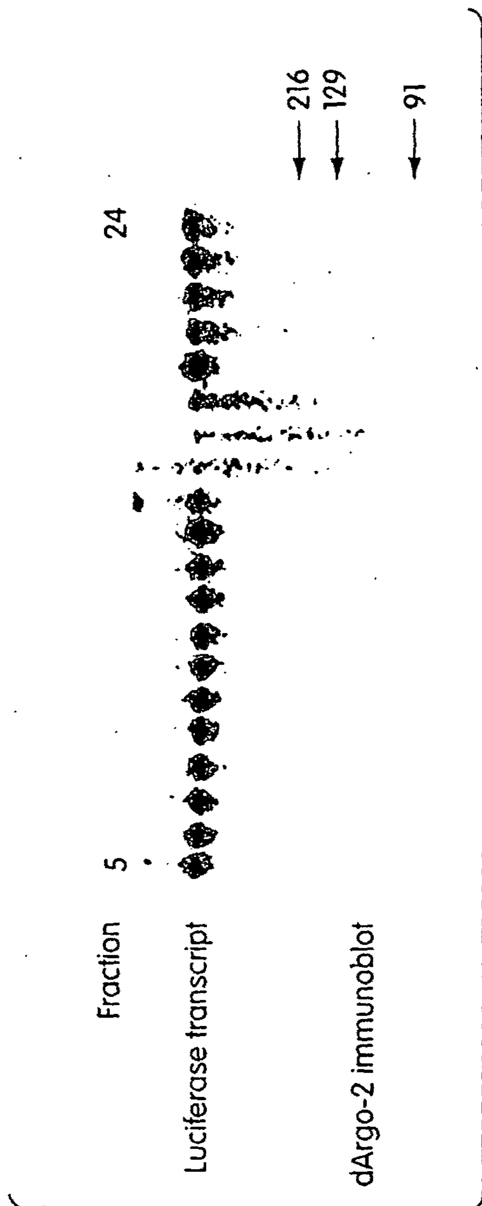


Fig. 12

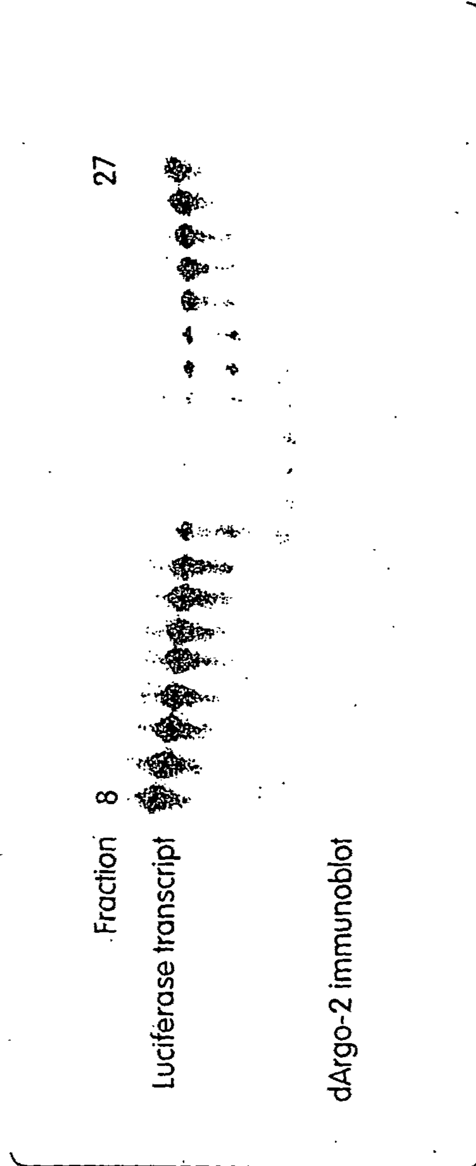


Fig. 13

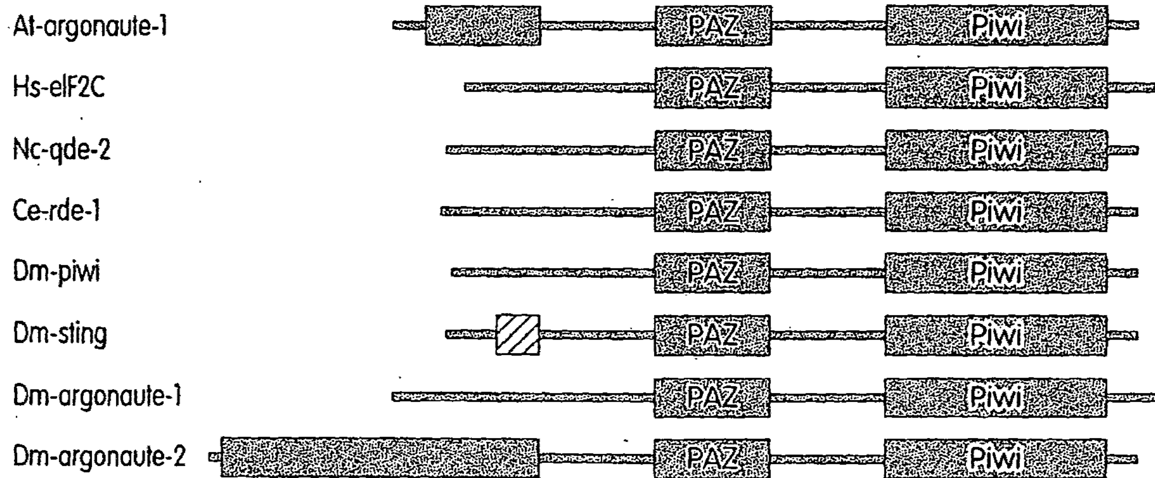


Fig. 14

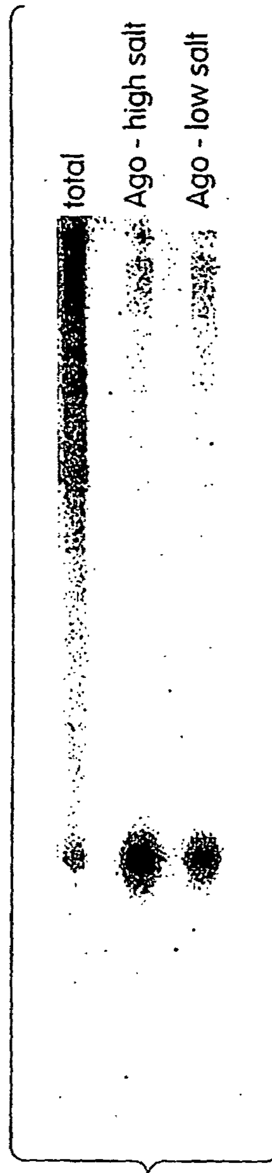


Fig. 15

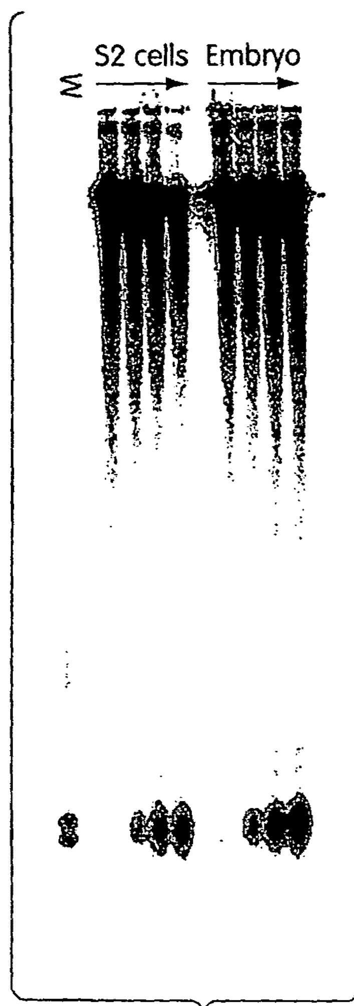


Fig. 16

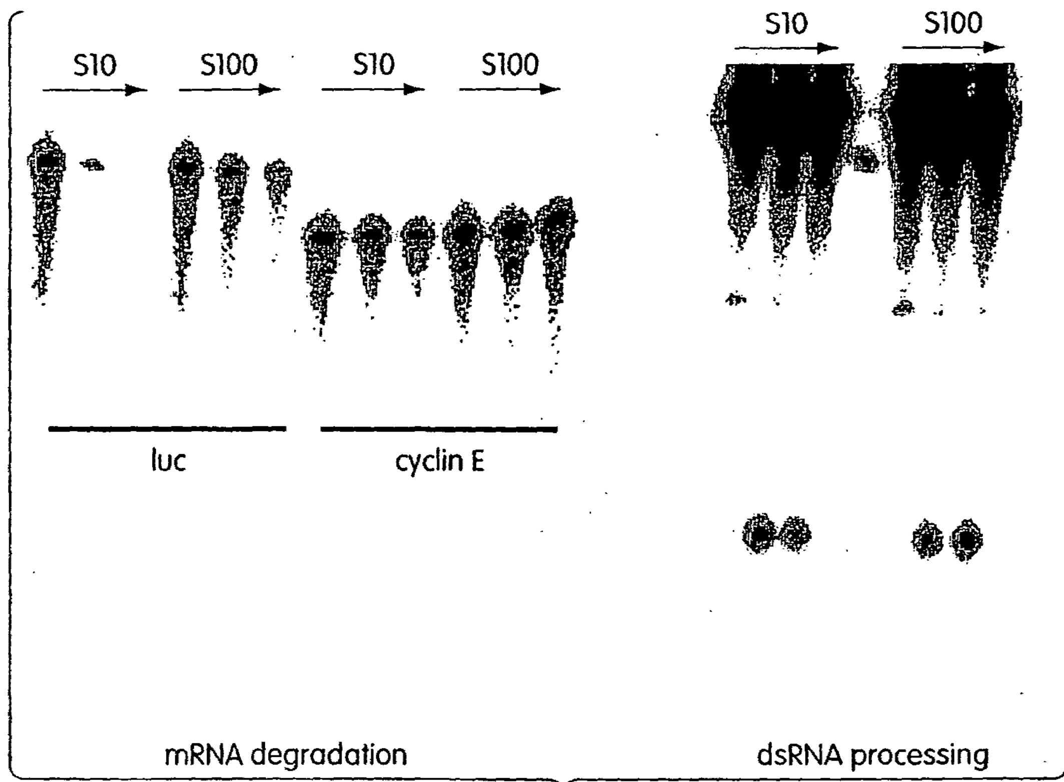


Fig. 17

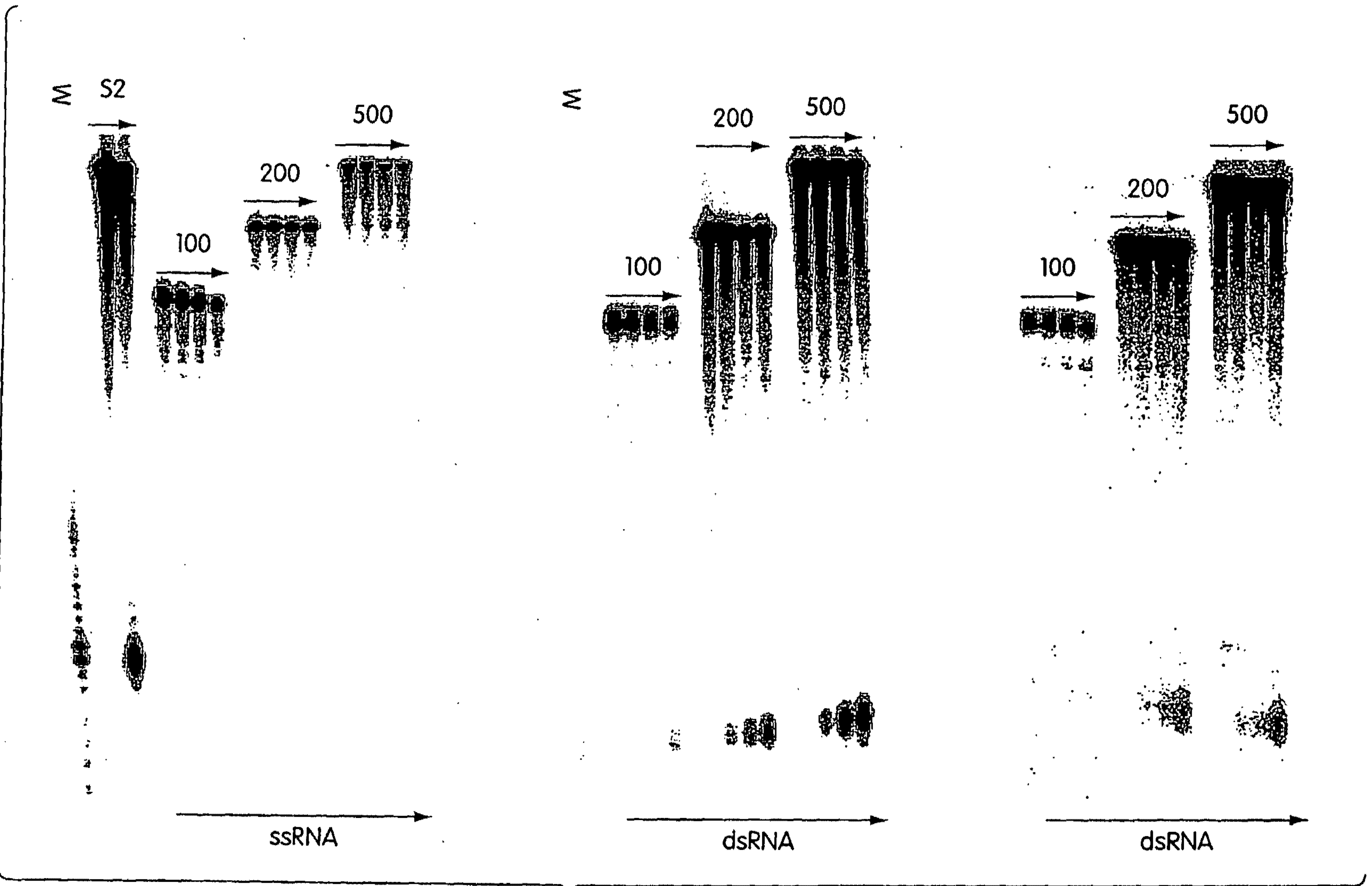
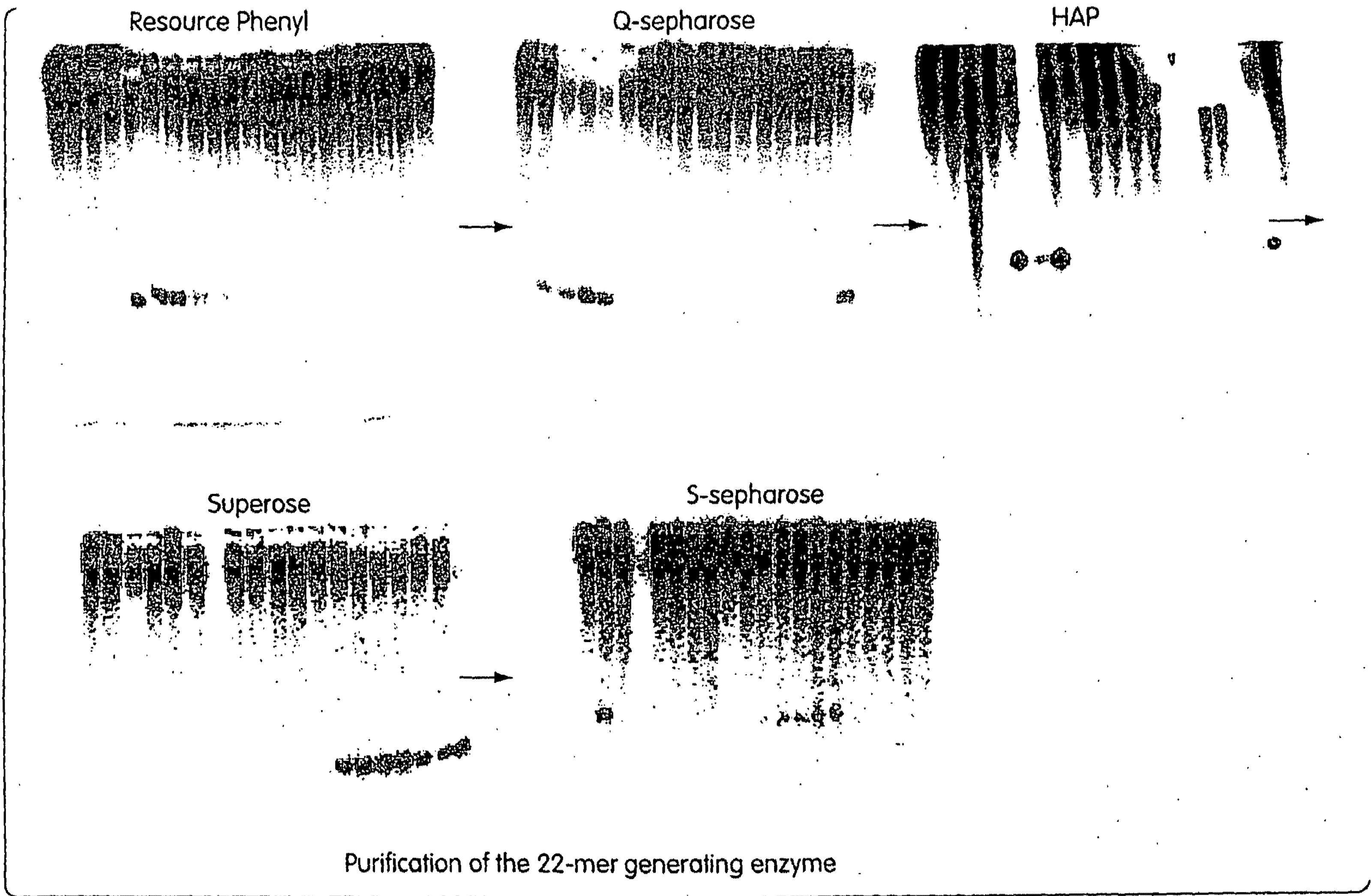


Fig. 18



Purification of the 22-mer generating enzyme

Fig. 19

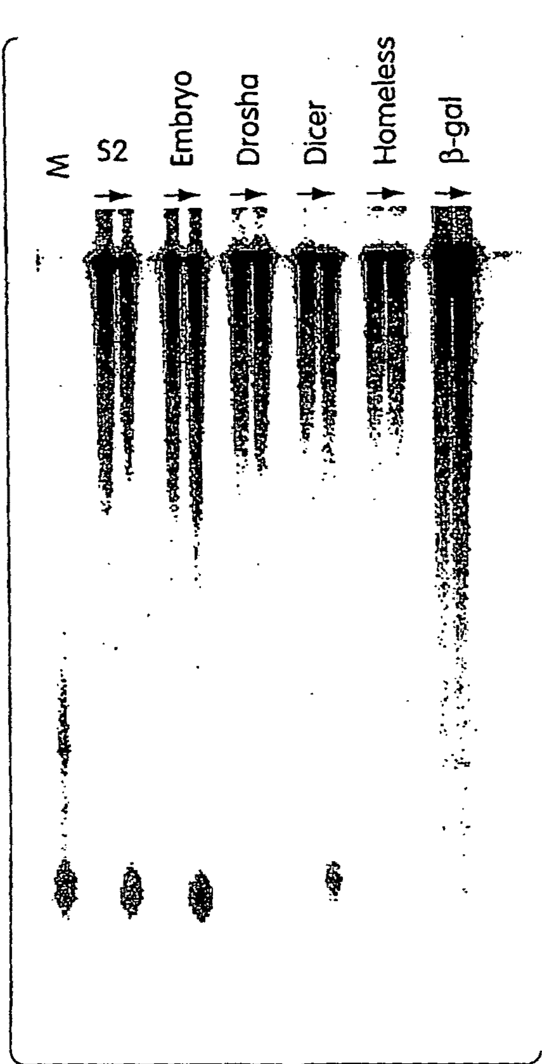


Fig. 20A

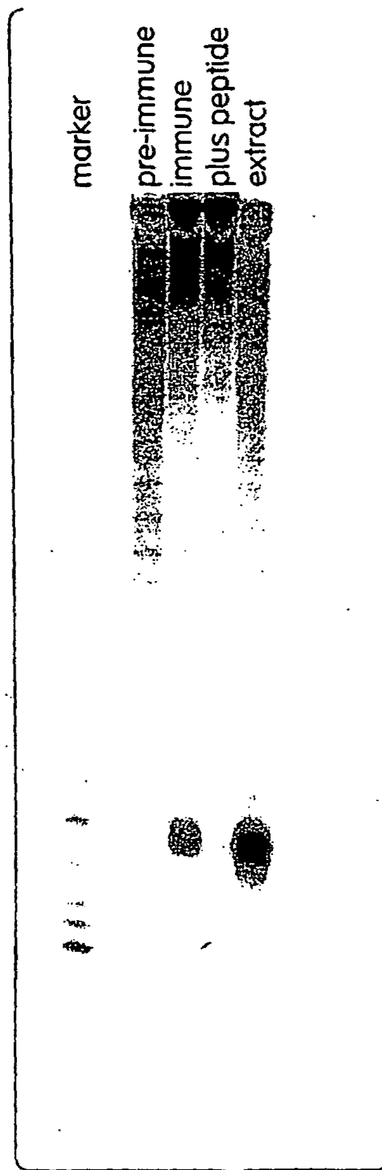


Fig. 20C

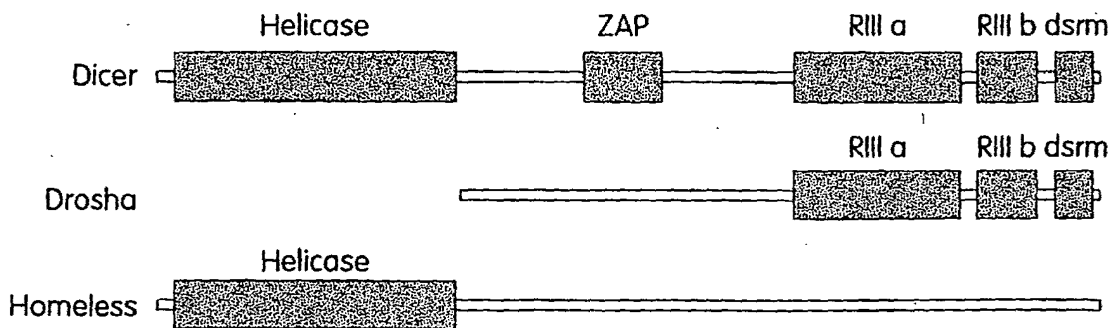


Fig. 20B

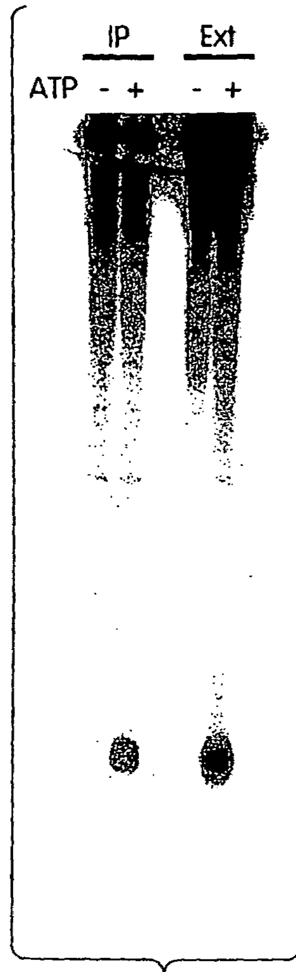


Fig. 21

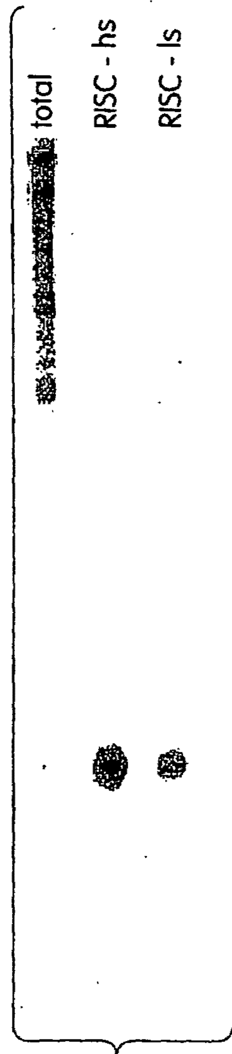


Fig. 22A

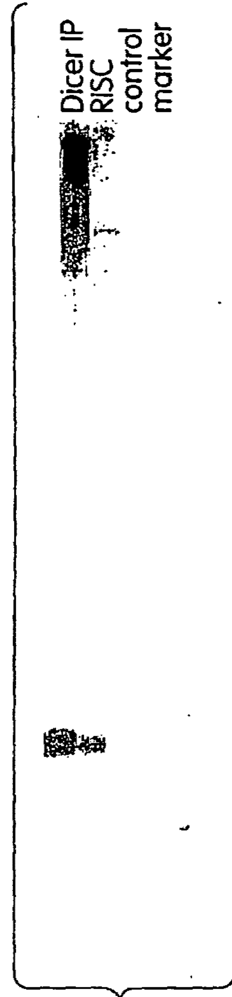


Fig. 22B

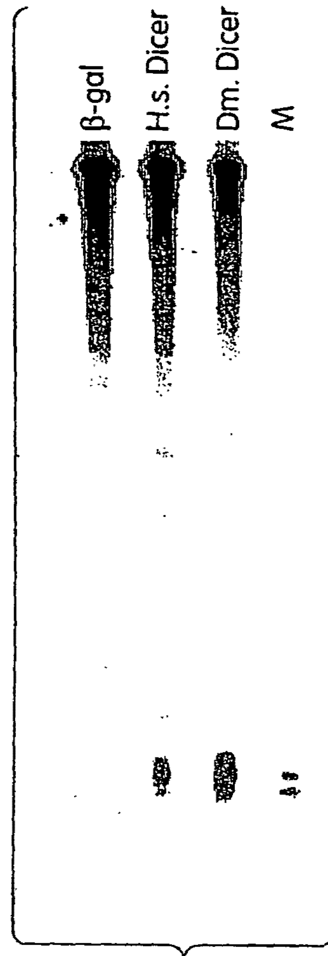


Fig. 23

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QCVEVVLASPCHNKAIRVGR**SFFK**MSDPNNRHELDDGYEALVGLYQAFMLGDRPFLNV
DISHKSFPISMPMIEYLERFSLKAK**INNTTNLDYSR**RFLEPFLRGINVVYTPPQSFQS
APRVYRVNGLSR**APASSETFEHDGK**KVTIASYFHSRNYPLKFPQLHCLNVGSSIKSIL
LPIELCSIEEGQALNRKDGATQVANMIKYAATSTNVRKRKIMNLLQYFQHNLDPTISR
FGIRIANDFIVVSTRVLSPPQVEYHSKRFTMVKNGSWRMDGMK**FLEPKPK**AHKCAVLY
CDPRSGRKMNYTQLNDFGNLIISQGKAVNISLDSDVTYRPFTDDERSLDTIFADLKRS
QHDLAIVIIPQFRISYDTIKQKAELQHGILTQCIKQFTVERKCNNQTIGNILLKINSK
LNGINHKIKDDPRLPMMKNTMYIGADVTHPSPDQREIPSVVGVAASHDPYGASYNMQY
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Fig. 24

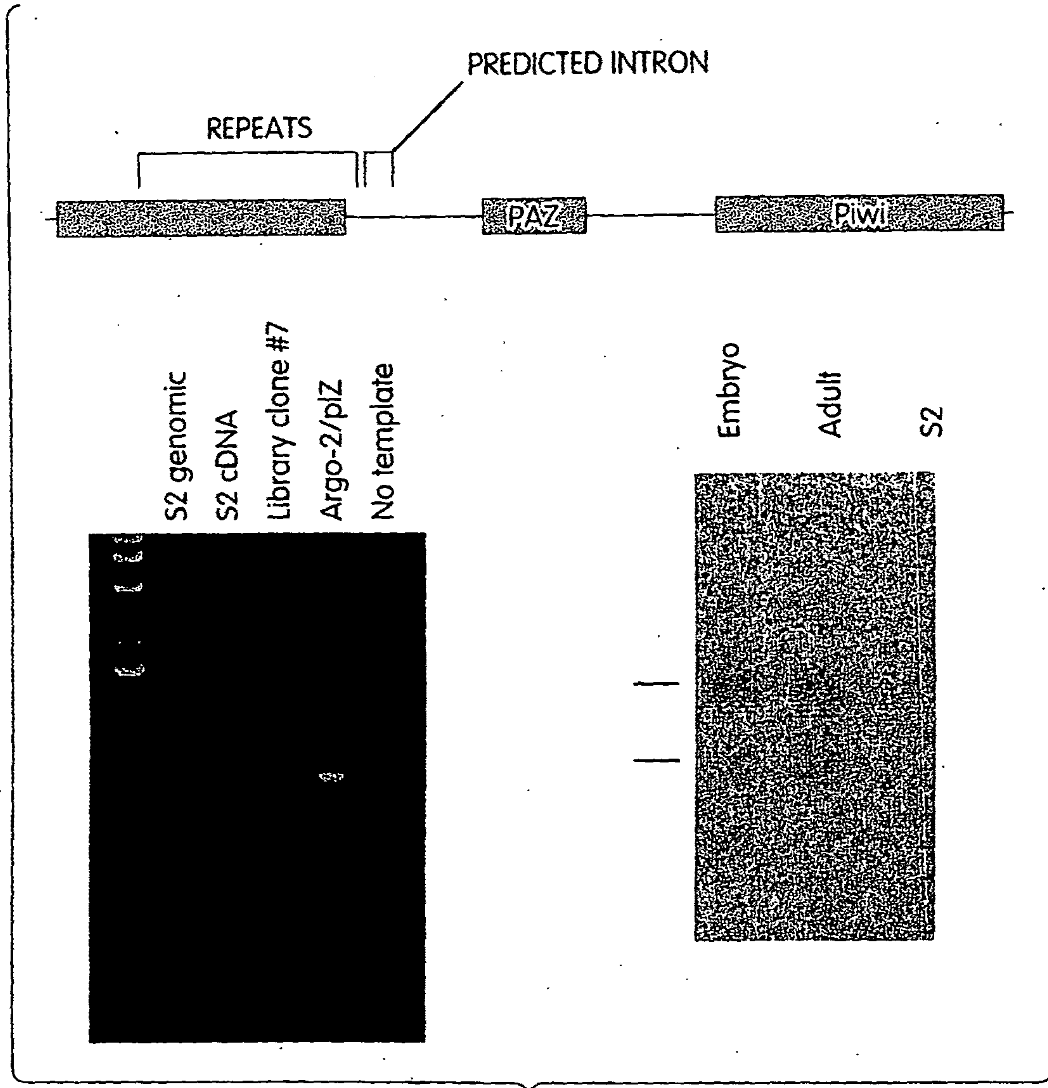


Fig. 25

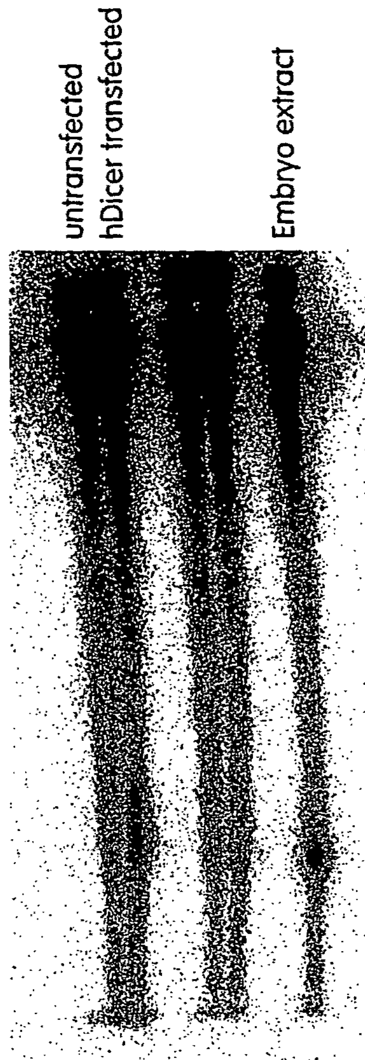


Fig. 26

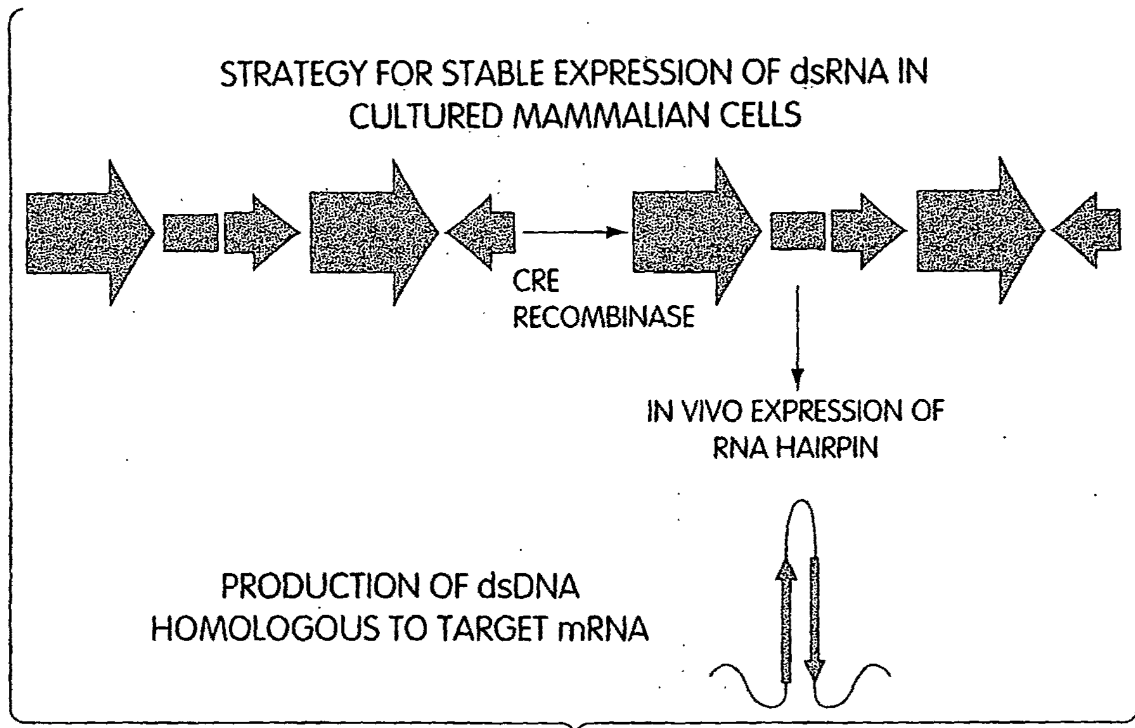


Fig. 27

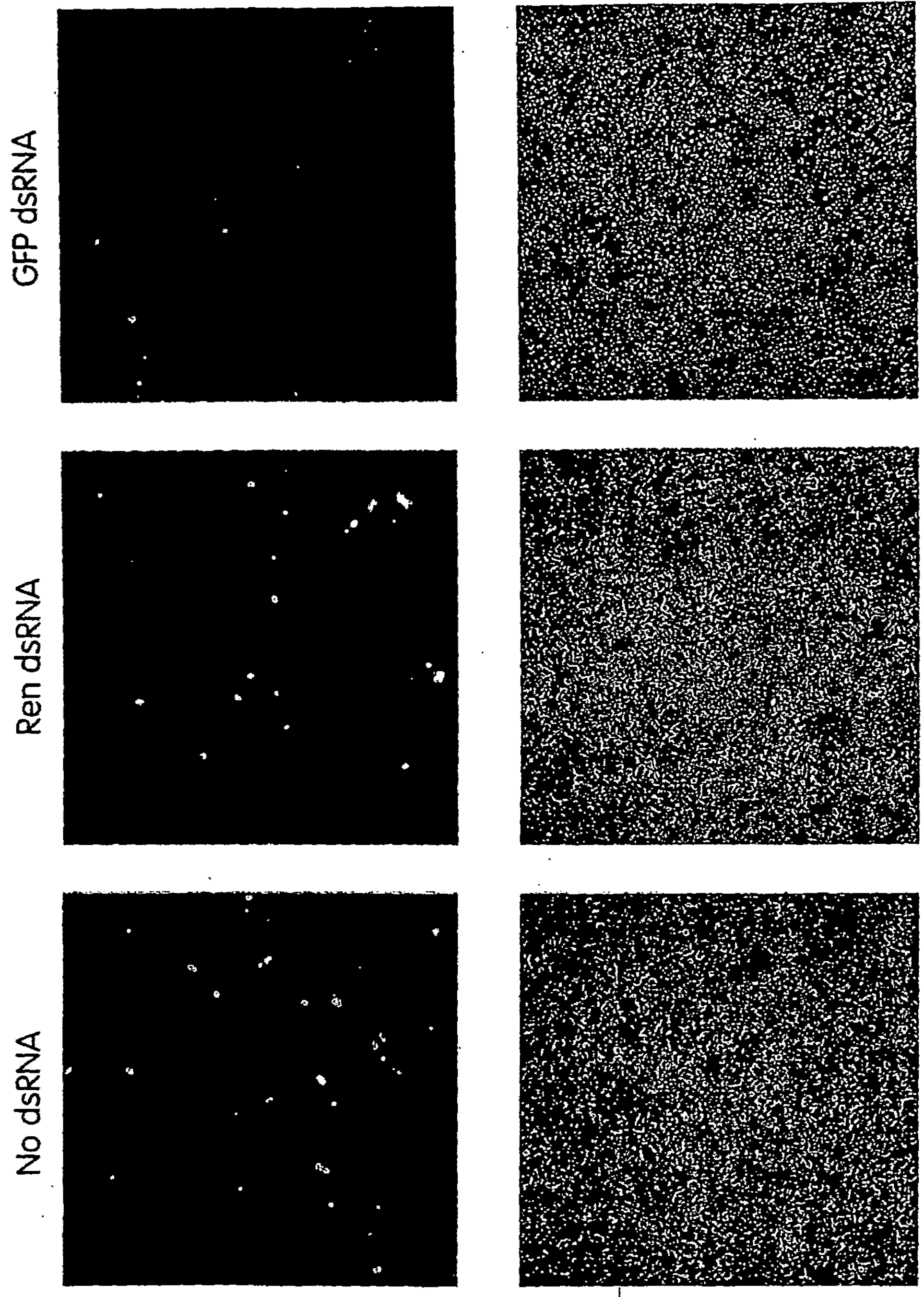


Fig. 28

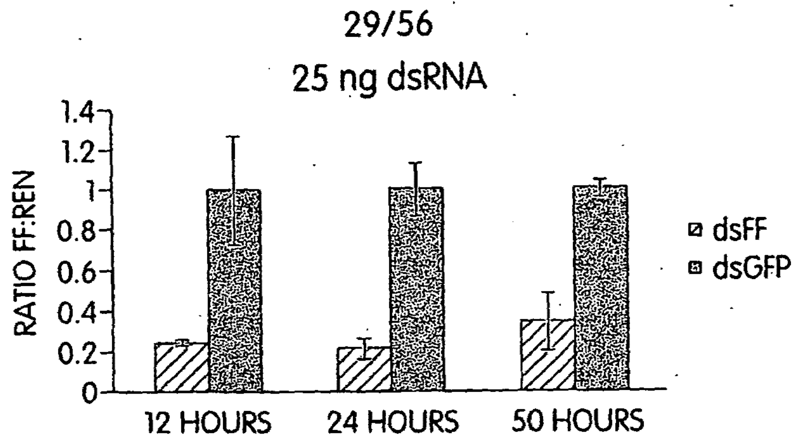


Fig. 29A

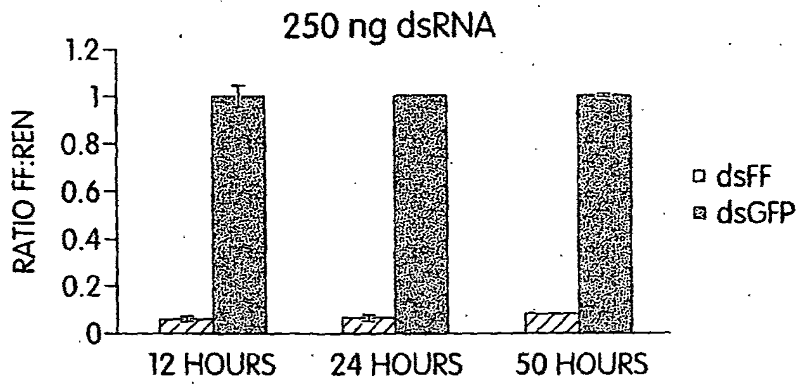


Fig. 29B

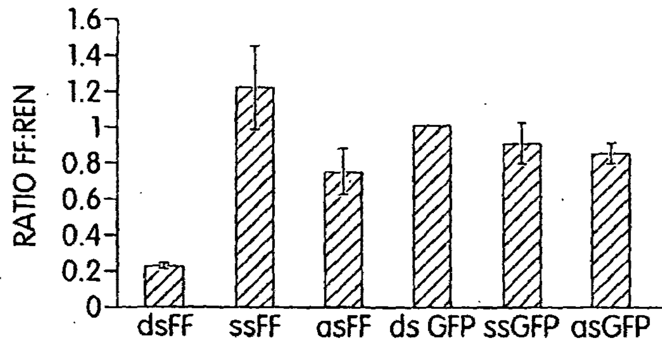


Fig. 29C

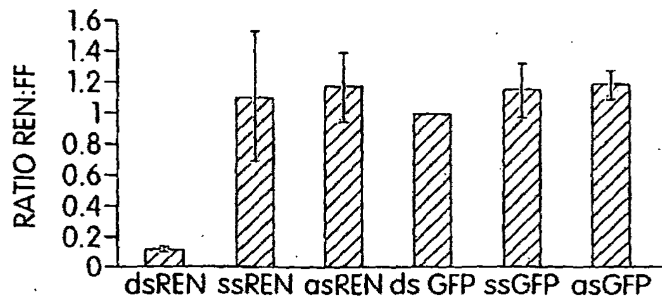
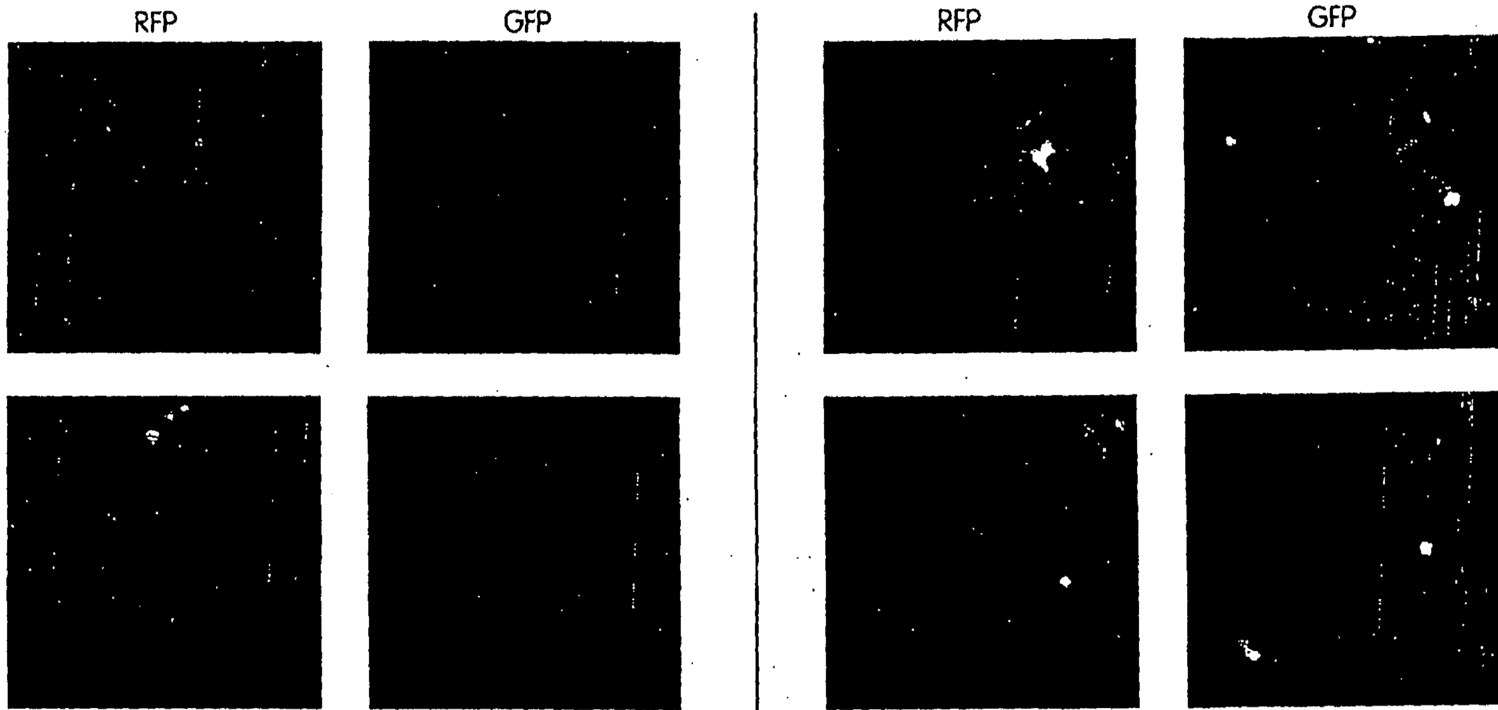


Fig. 29D



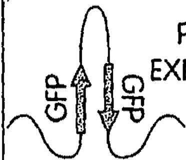


Fig. 30

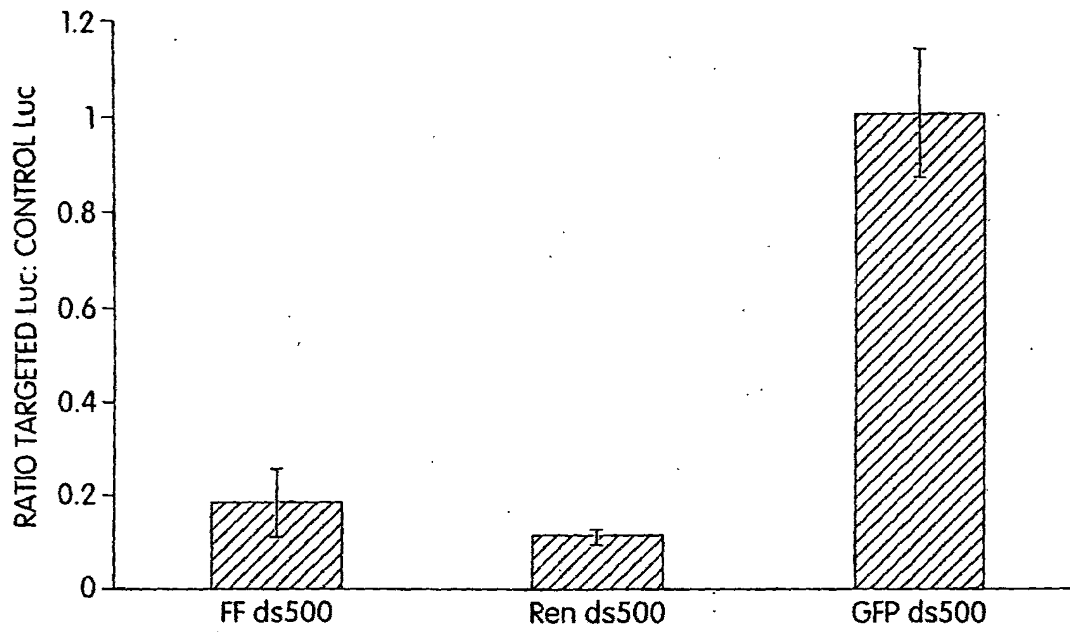


Fig. 31

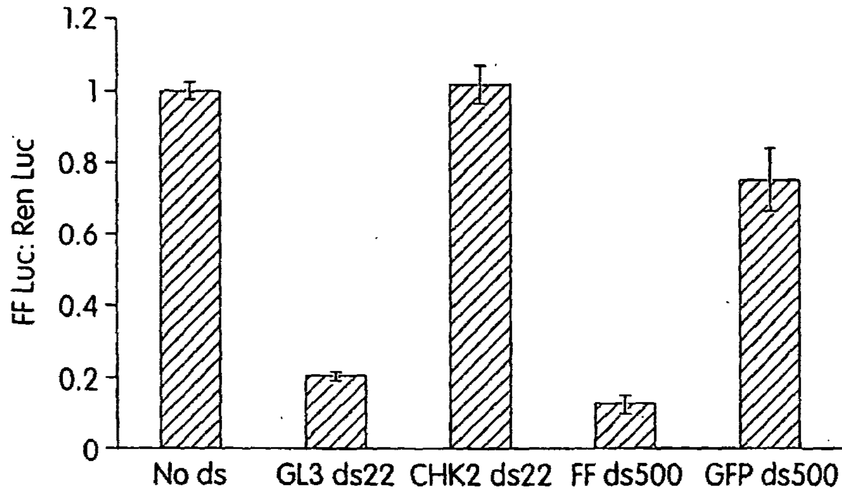


Fig. 32A

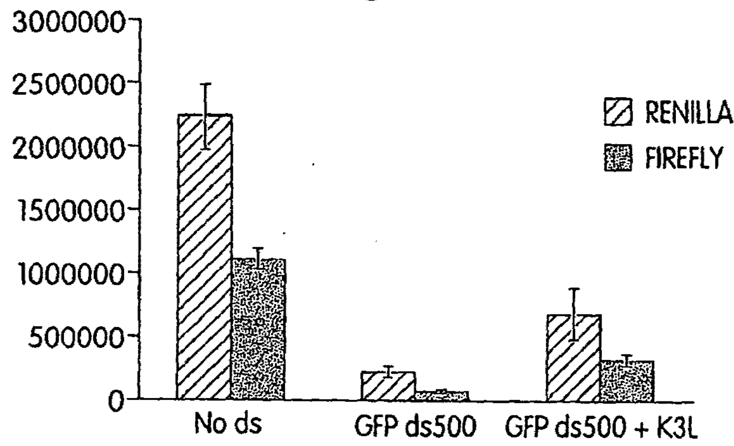


Fig. 32B

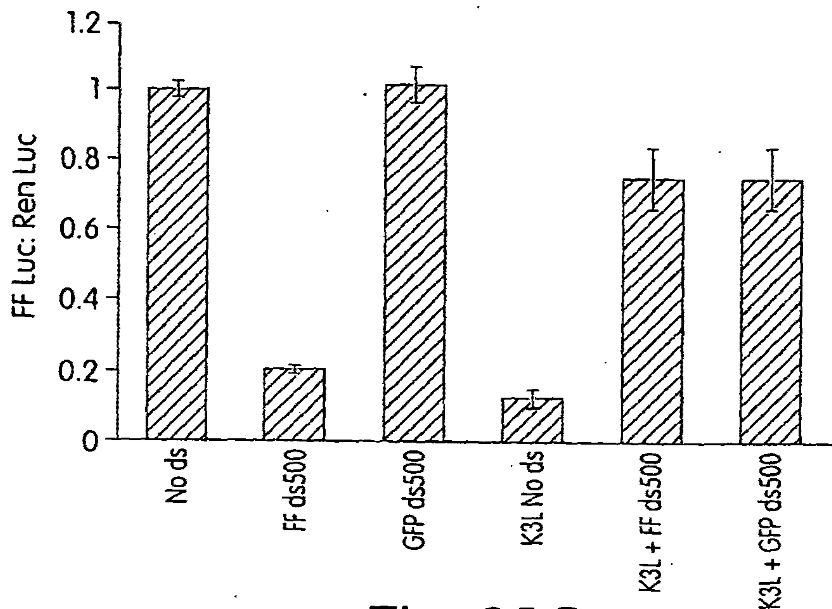


Fig. 32C

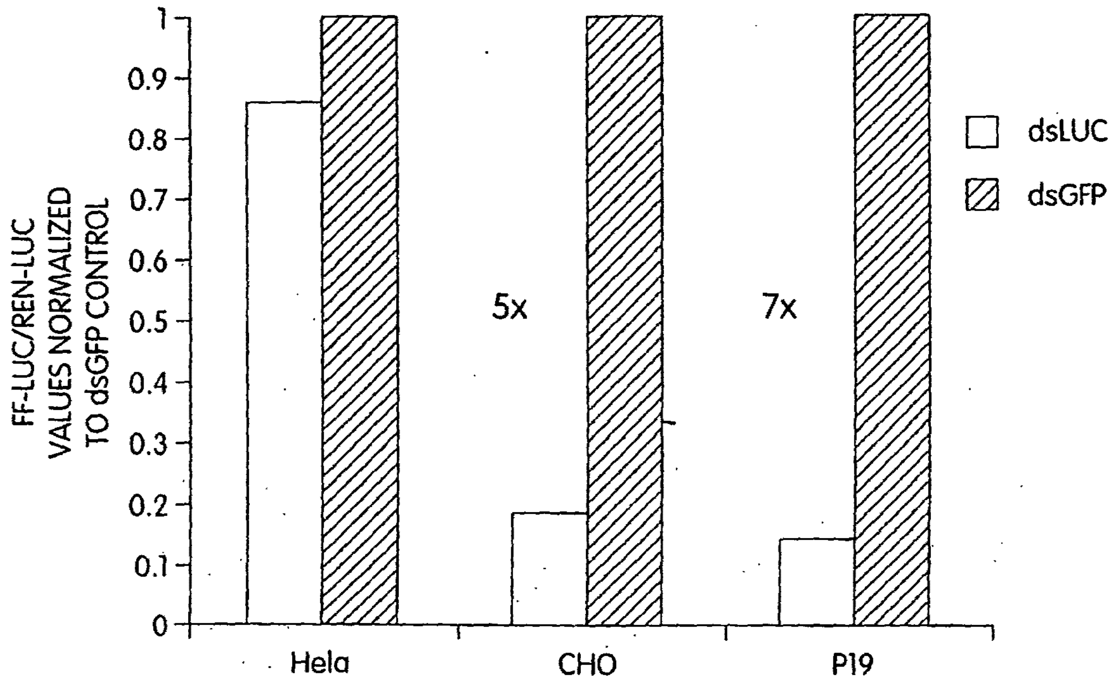


Fig. 33

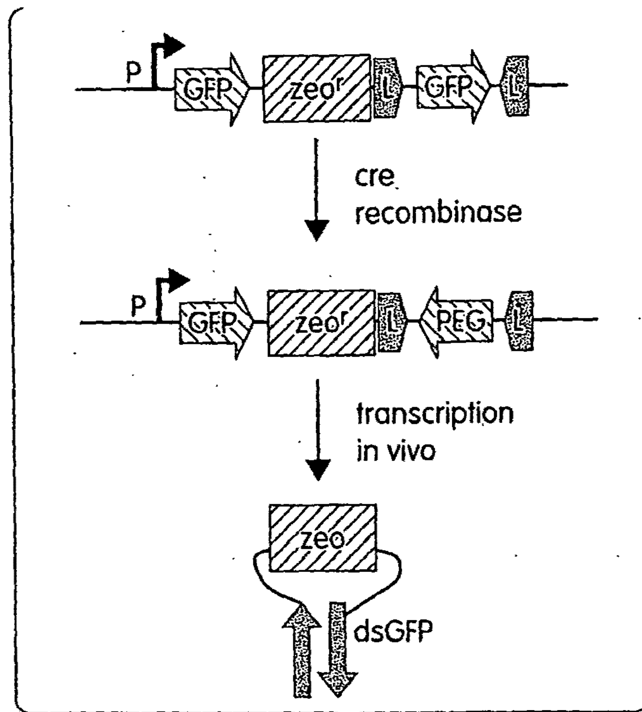


Fig. 34A

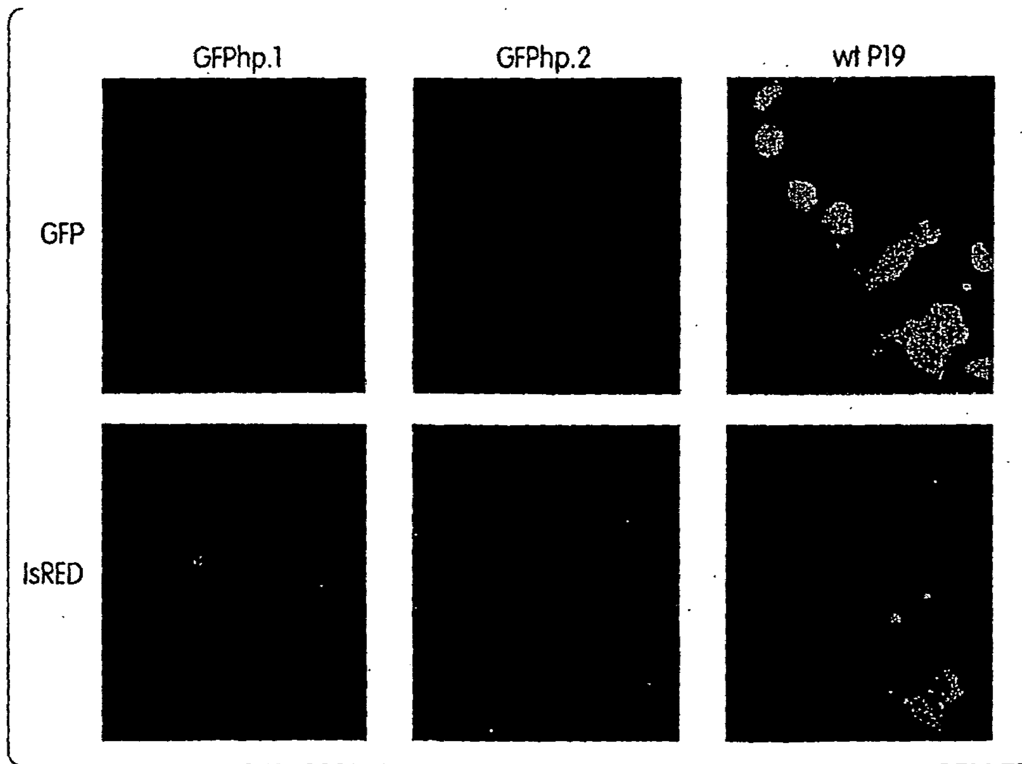
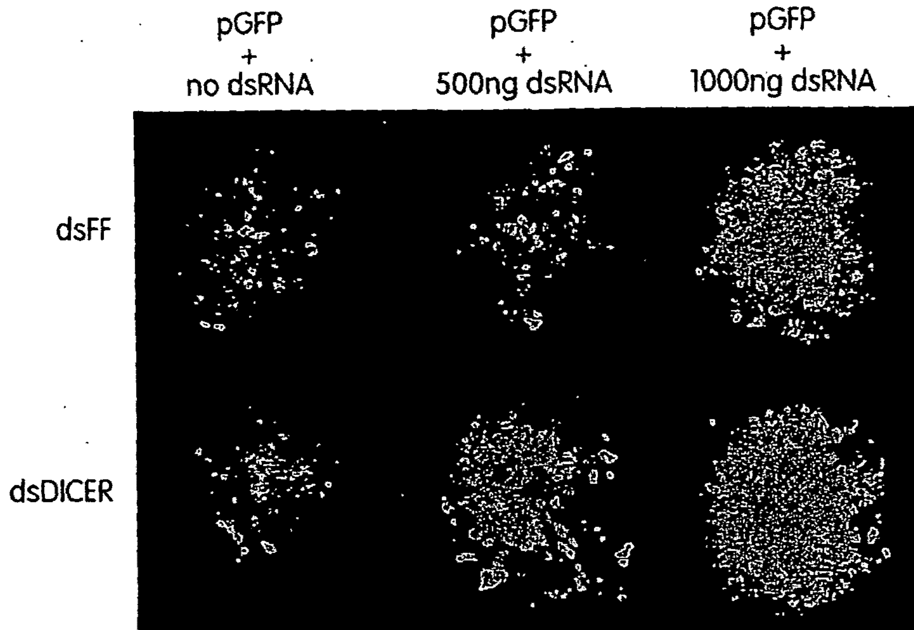


Fig. 34B



P19 GFP HAIRPIN CLONE NUMBER #10
48 HRS POST-TRANSFECTION
FLUORESCENT MICROSCOPY SUPERIMPOSED WITH BRIGHT FIELD

Fig. 34C

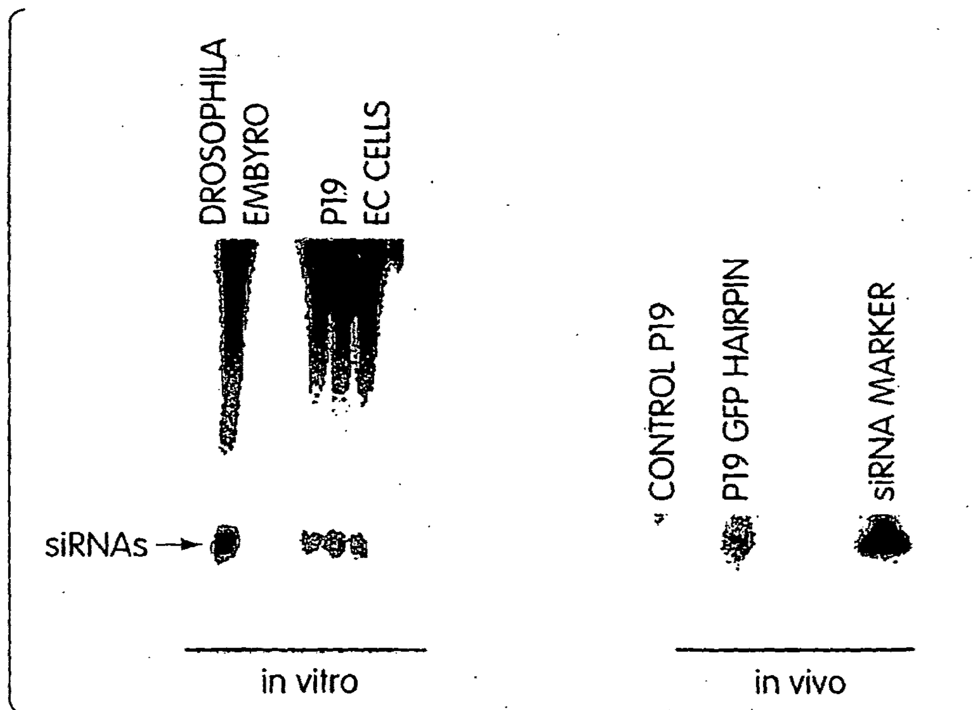


Fig. 34D

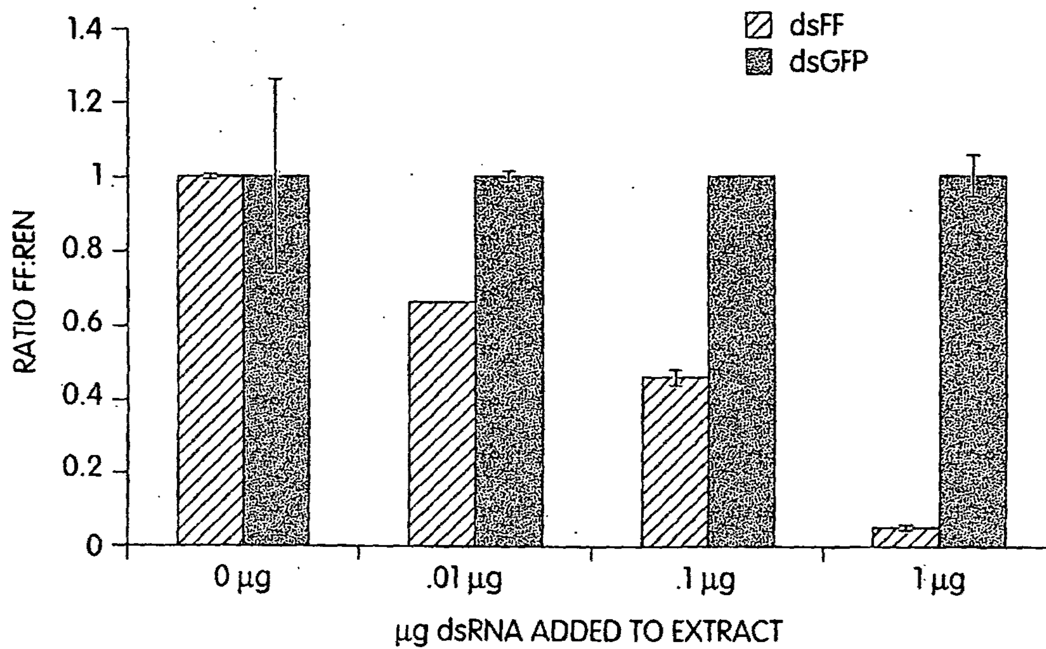


Fig. 35

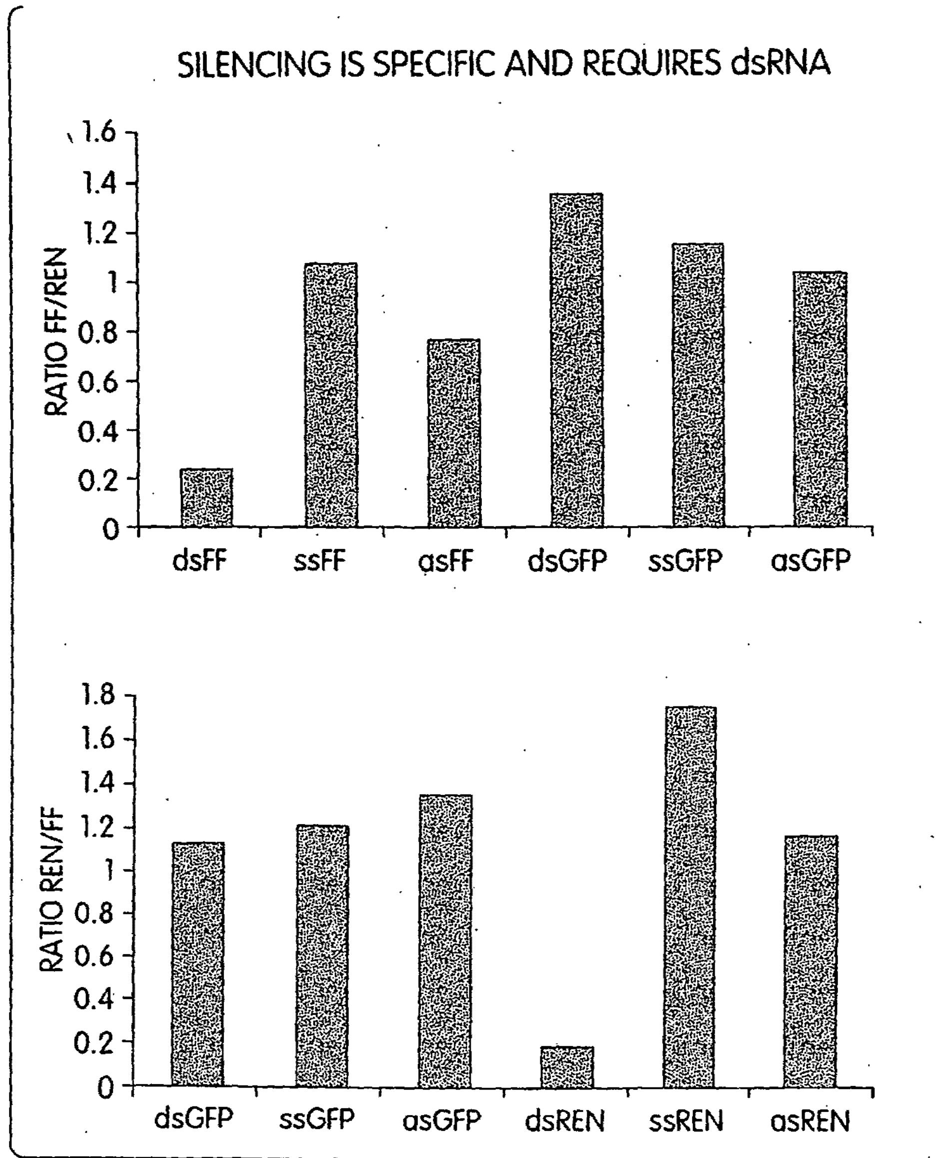


Fig. 36

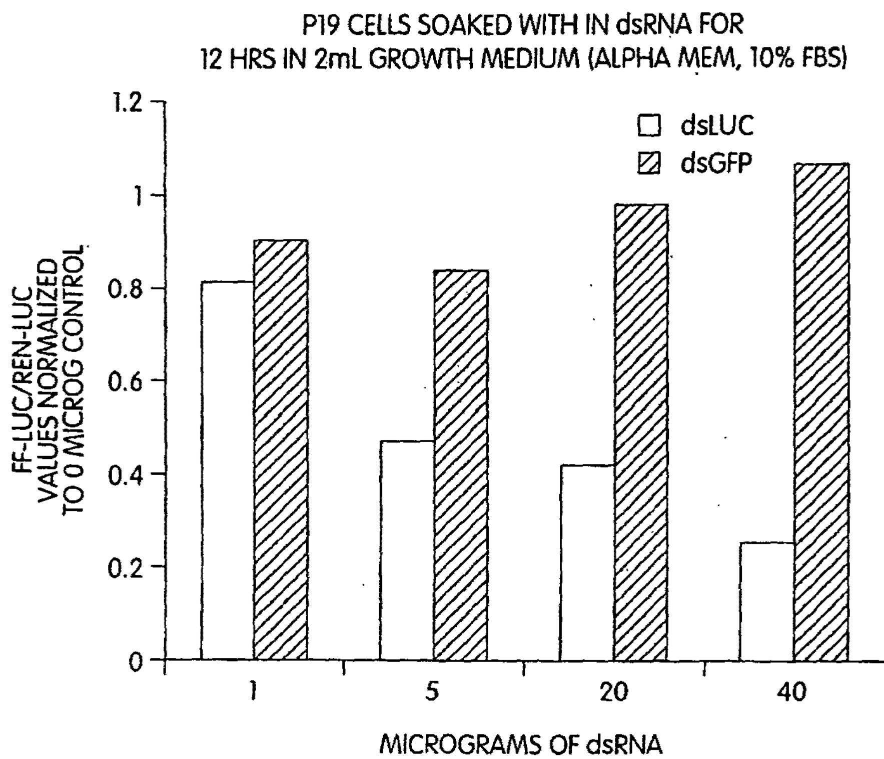


Fig. 37

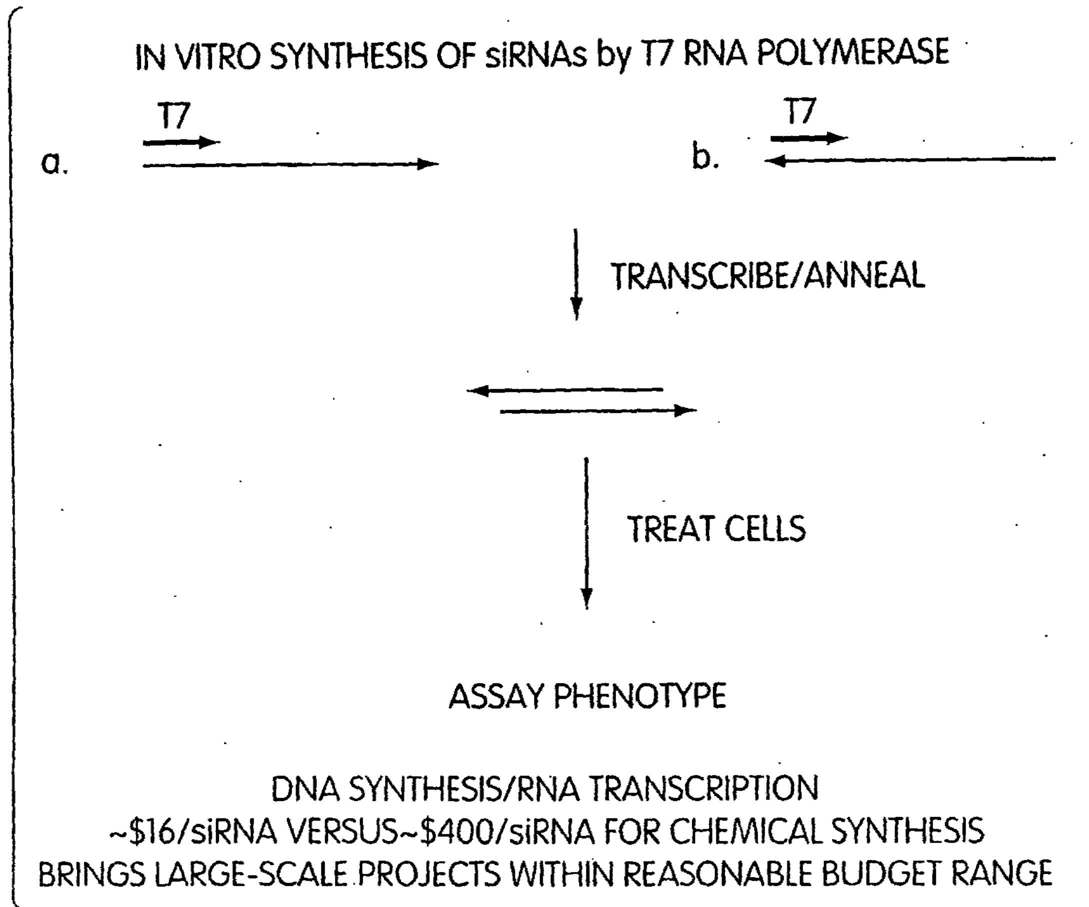


Fig. 38

siRNA

```
  UCGAAGUACUCAGCGUAAGUG
AAAGCUUCAUGAGUCGCAUUC
```

cshFf

```
                                 U
CAUCGACUGAAAUCCCUGGUAAUCCGUUG U
GUAGCUGACUUUAGGGACCAUUAGGCAAC A
                                 A
```

cshFf-L7

```
                              ----------      U
CAUCGACUGAAAUCCCUGGUAAUCCGUUU          GGGGC \
GUAGCUGAUUUUAGGGACUAUUAGGUAAA          UCCCG C
                              UAGGGUAUCG      U
```

cshFf-L7m

```
                GCC           ----------      U
CAUCGACUGAAAUCCC   GUAAUCCGUUU          GGGGC \
GUAGCUGAUUUUAGGG   UAUUAGGUAAA          UCCCG C
                AC-           UAGGGUAUCG      U
```

Fig. 39A

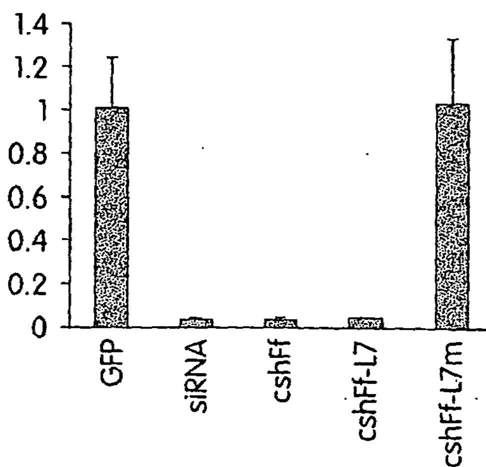


Fig. 39B

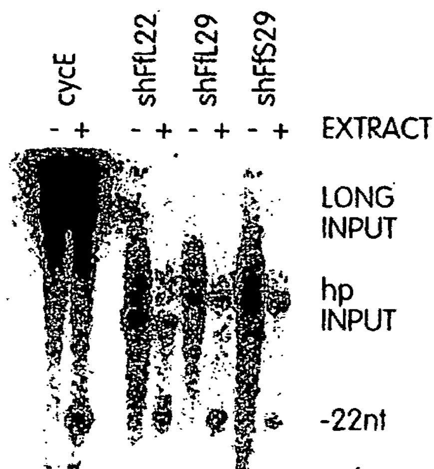


Fig. 39C

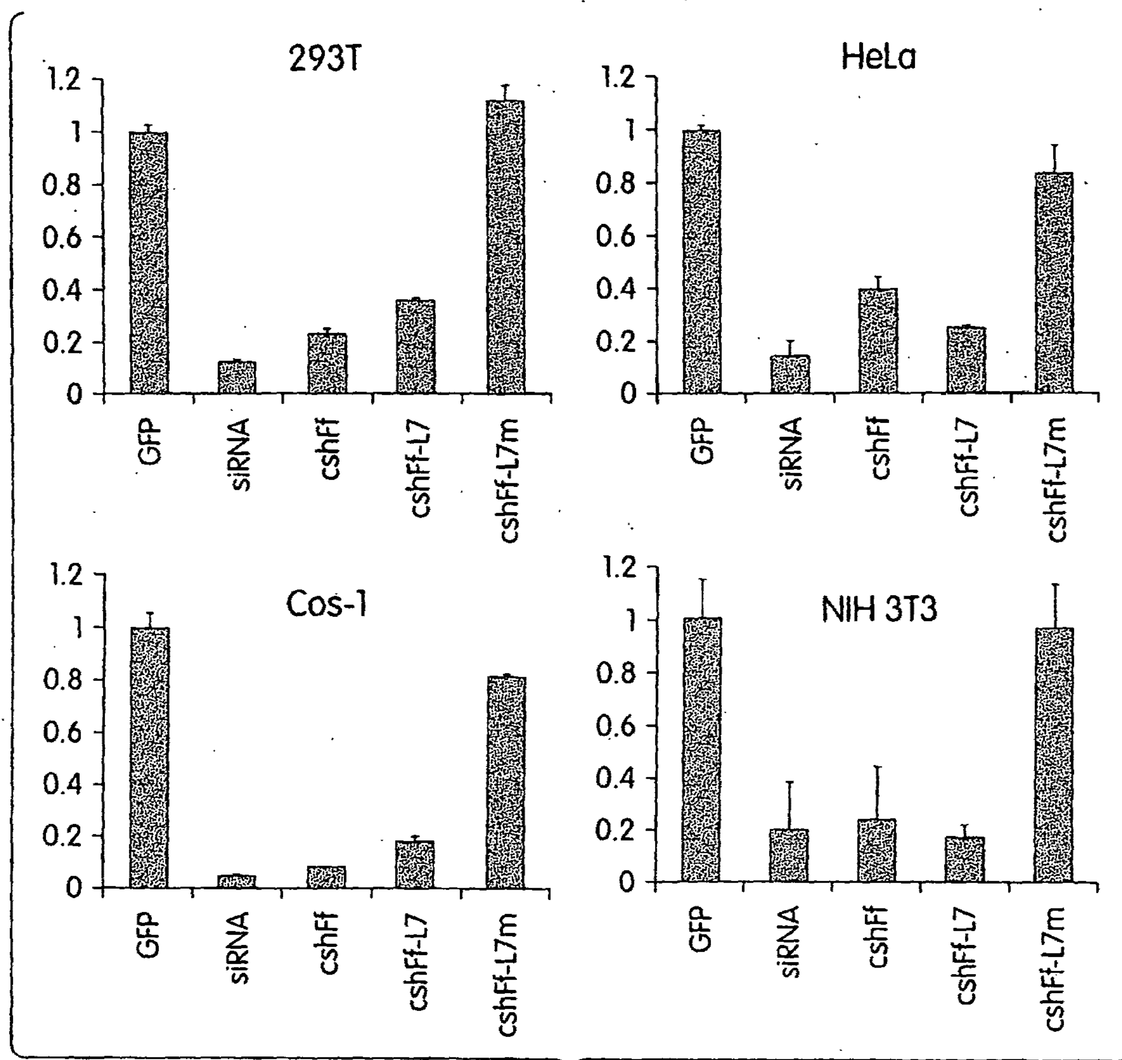


Fig. 40

siRNA

```
  UCGAAGUACUCAGCGUAAGUG
AAAGCUUCAUGAGUCGCAUUC
```

T7siRNA

```
GGUCGAAGUACUCAGCGUAAGAA
AAAGCUUCATGAGUCGCAUUCGG
```

T7siFf-2

```
  GGUUGUGGAUCUGGAUACCGG
UUCCAACACCUAGACCUAUGG
```

T7siFf-3

```
  GGUGCCAACCCUAUUCUCCUU
GACCACGGUUGGGAUAAGAGG
```

T7siFf-8

```
  GGCUAUGAAGAGAGUACGCCCU
UUCCGAUACUUCUCUCAUGCGG
```

Fig. 41A

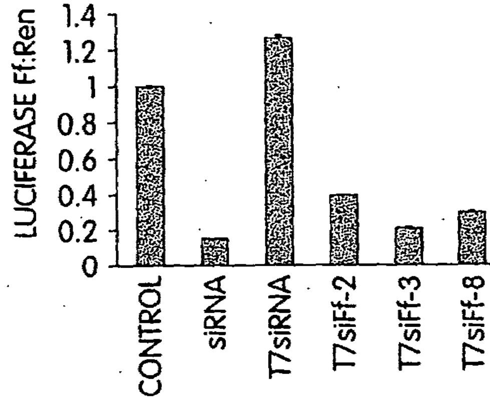


Fig. 41B

T7shFf29

```
GGU|                             U
   CGAAGUACUCAGCGUAAGUGAUGUCCAC U
   GUUUUGUGGGUUGUGUUUGUUGUGGGUG A
  G^                             A
```

T7shFf27

```
GGU|                           U
   CGAAGUACUCAGCGUAAGUGAUGUCC U
   GUUUUGUGGGUUGUGUUUGUUGUGGG A
  G^                           A
```

T7shFf25

```
GGU|                         U
   CGAAGUACUCAGCGUAAGUGAUGU U
   GUUUUGUGGGUUGUGUUUGUUGUG A
  G^                         A
```

T7shFf22

```
GGU|                      U
   CGAAGUACUCAGCGUAAGUGA U
   GUUUUGUGGGUUGUGUUUGUU A
  G^                      A
```

T7shFf29-5'T

```
GGCUCGAGU|                             U
         CGAAGUACUCAGCGUAAGUGAUGUCCAC U
         GUUUUGUGGCUUGUGUUUGUUGUGGGUG A
G--------^                             A
```

T7shFf29-3'T

```
-----G|                               U
      GUCGAAGUACUCAGCGUAAGUGAUGUCCAC U
      CGGUUUUGUGGGUUGUGUUUGUUGUGGGUG A
 GAGCU^                               A
```

Fig. 41C

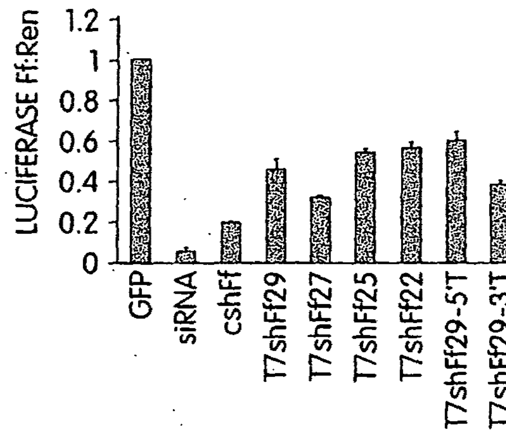


Fig. 41D

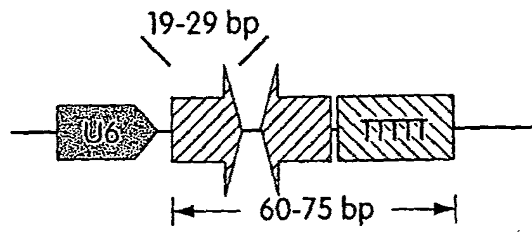


Fig. 42A

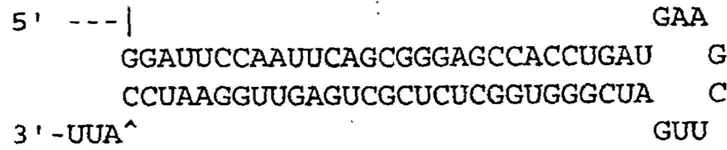


Fig. 42B

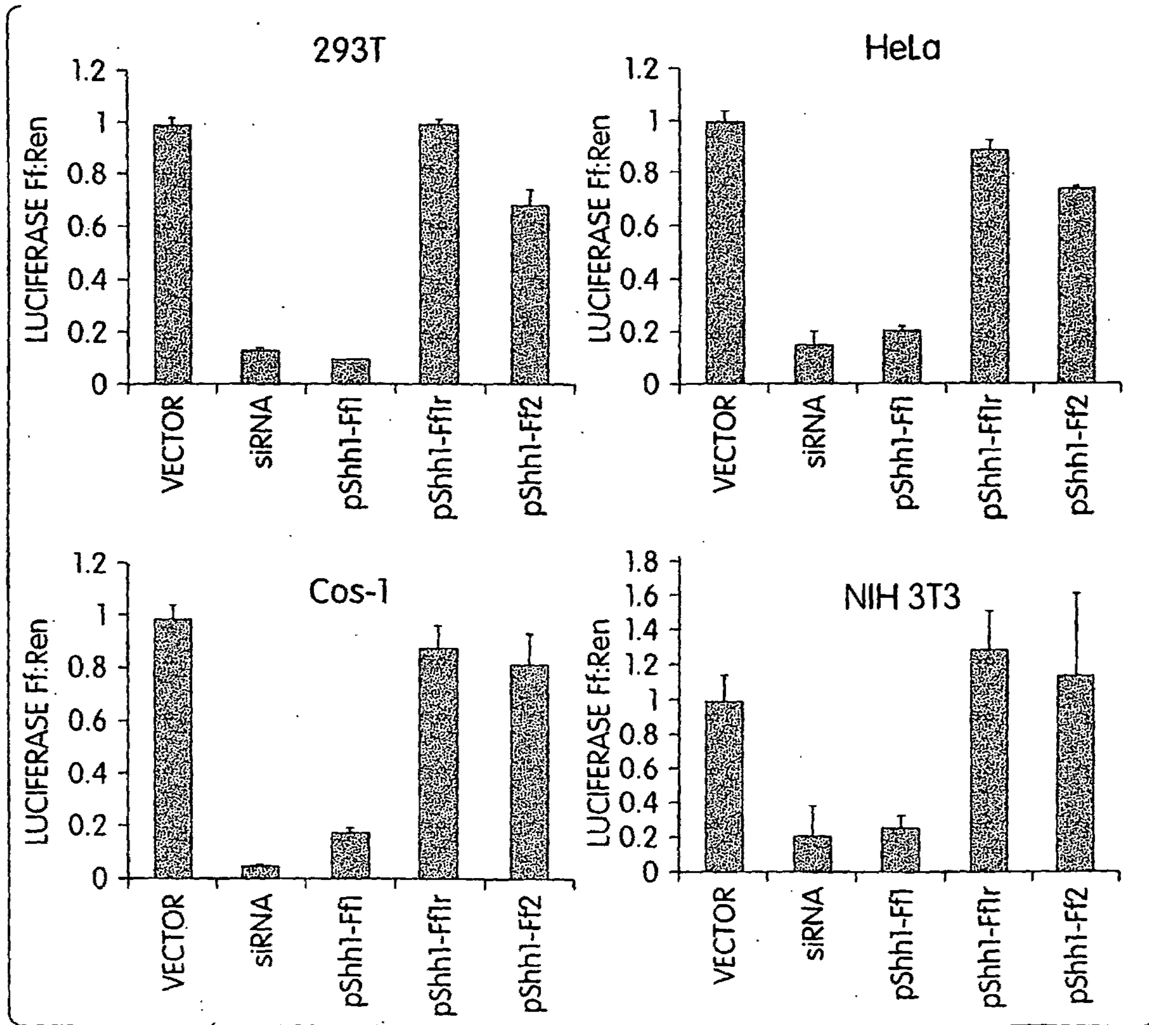


Fig. 42C

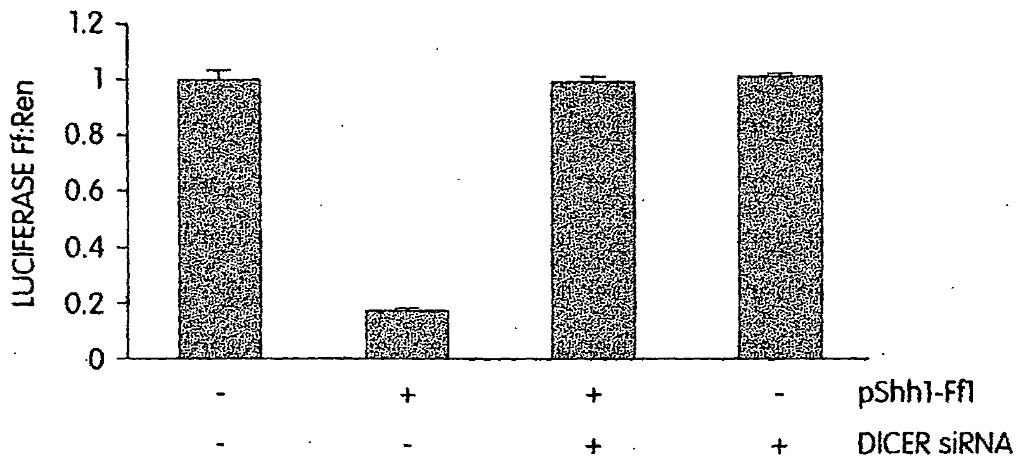


Fig. 43

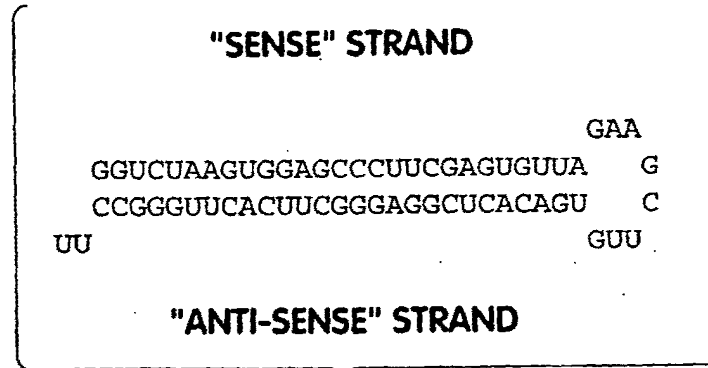


Fig. 44A

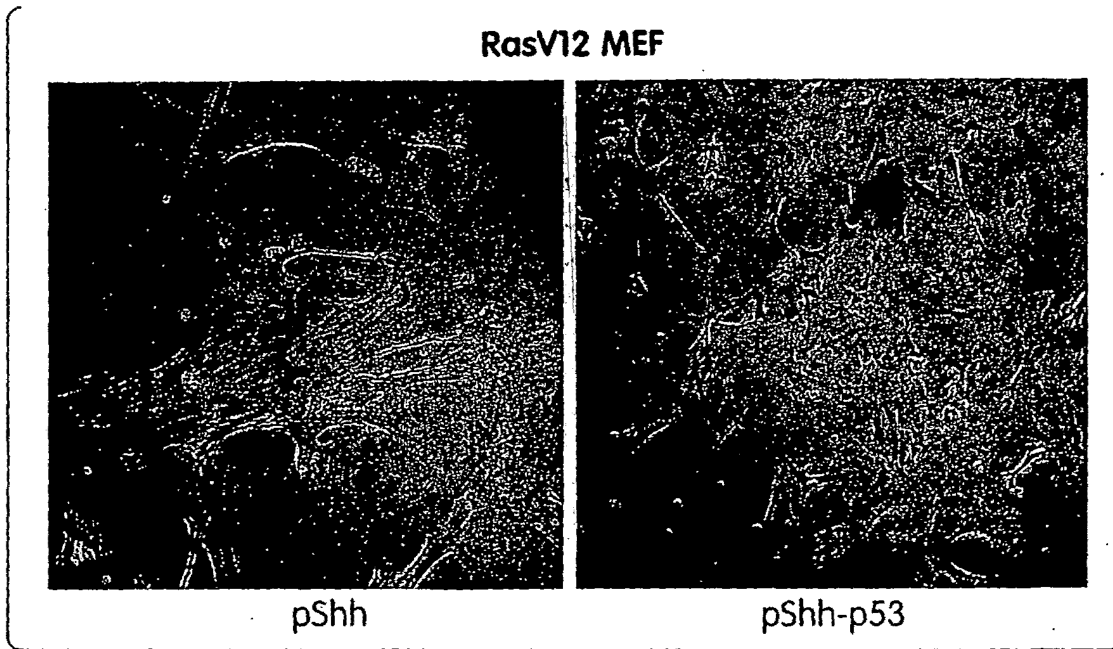


Fig. 44B

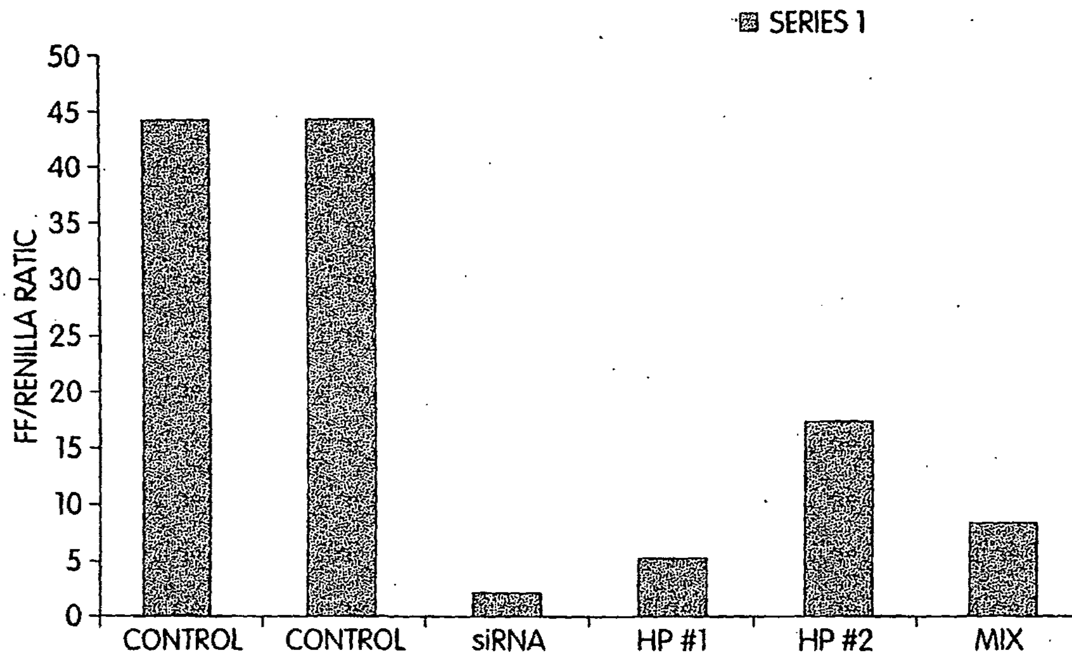


Fig. 45

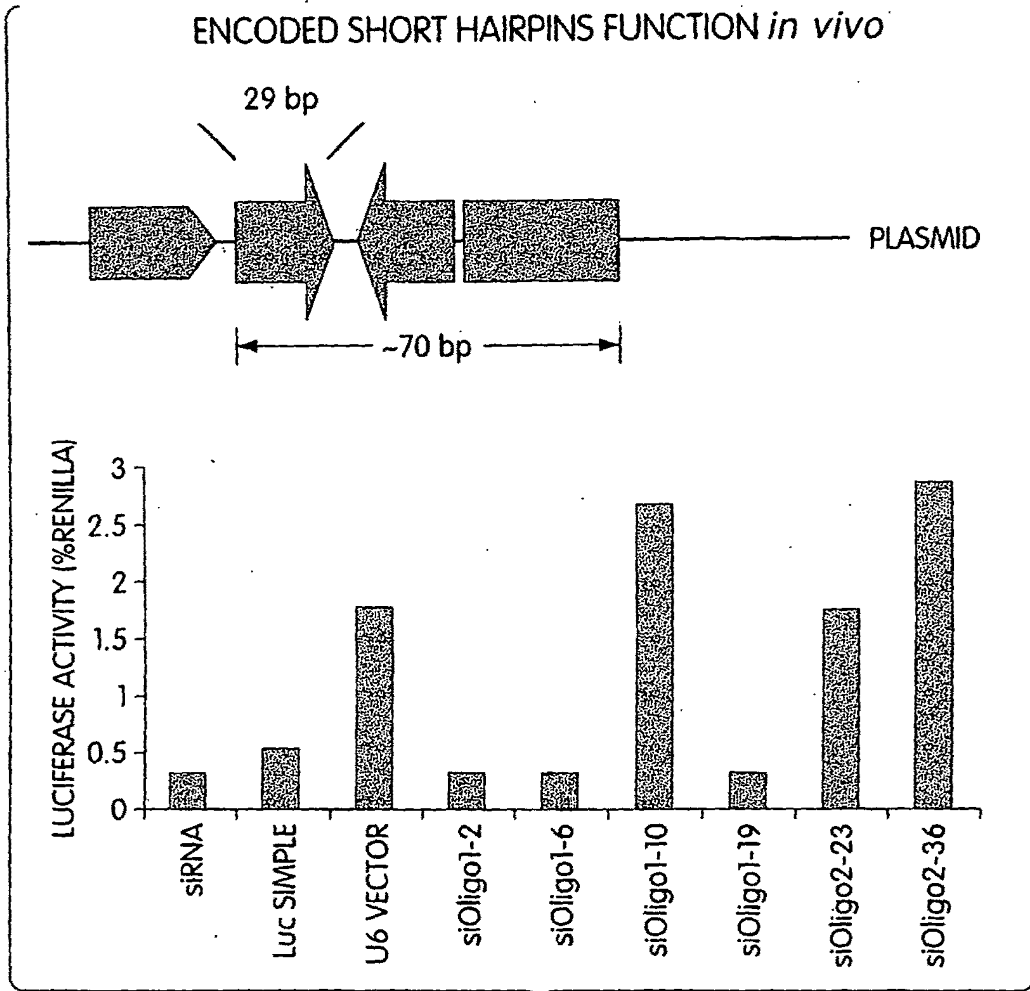
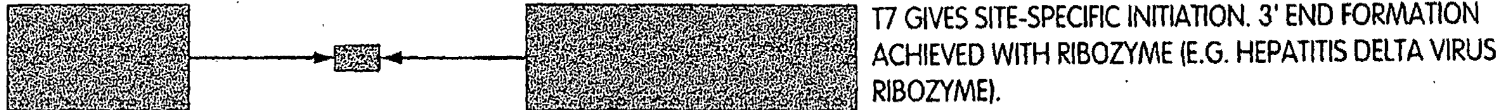
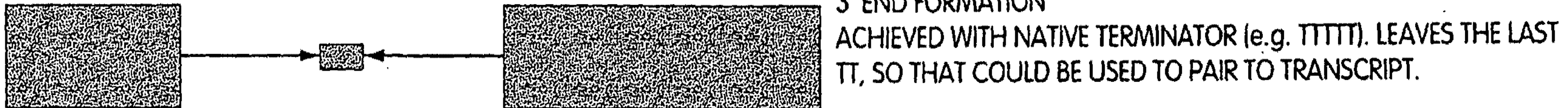


Fig. 46

STABLE SUPPRESSION BY SHORT dsRNAs - STABLE EXPRESSION STRATEGIES





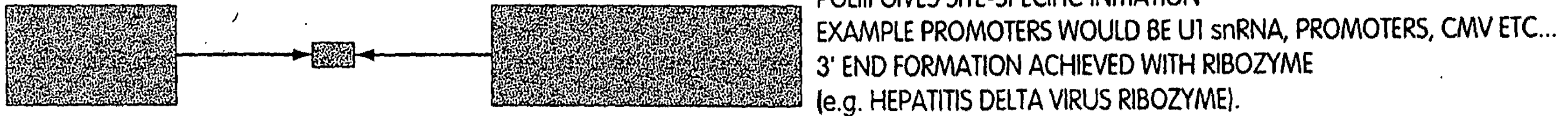


Fig. 47

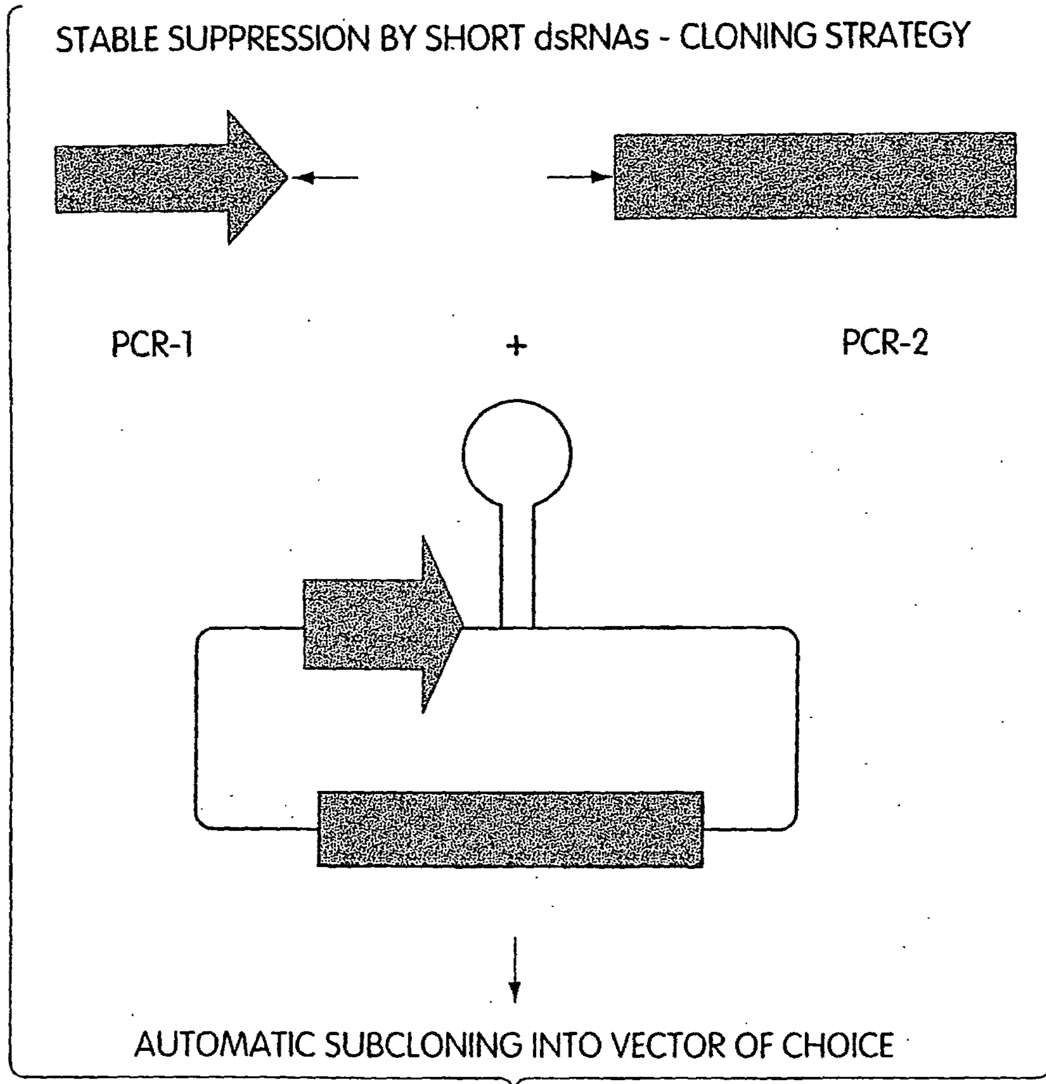


Fig. 48

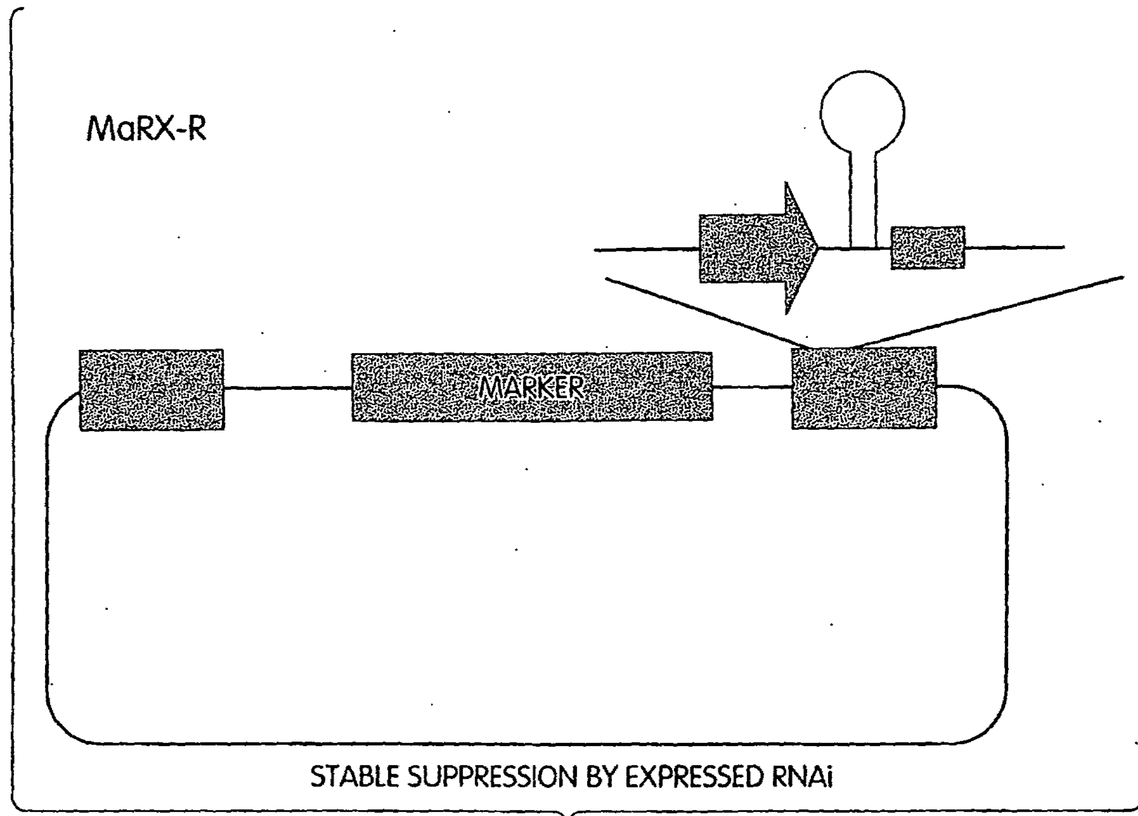


Fig. 49

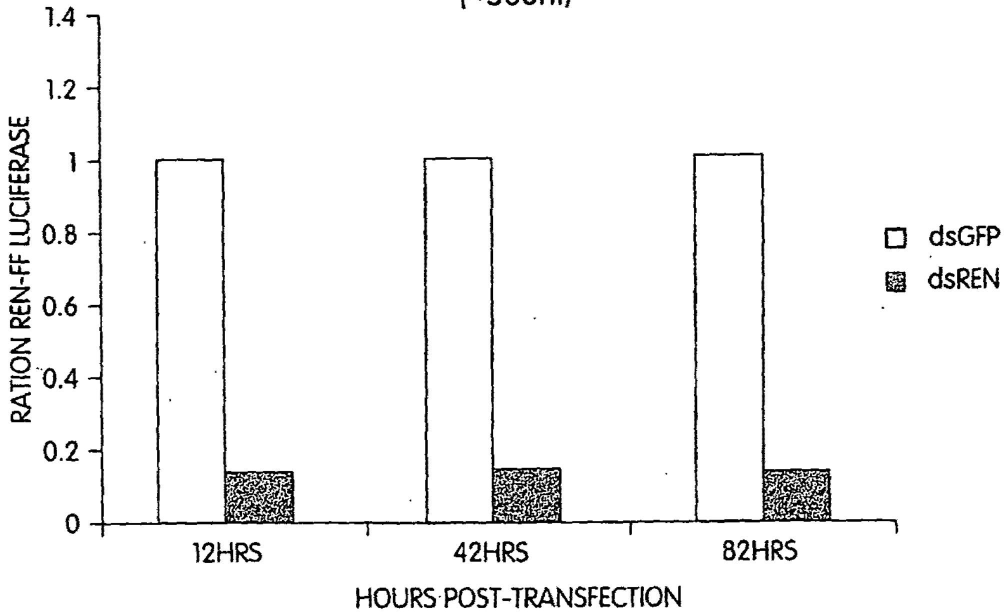


Fig. 50

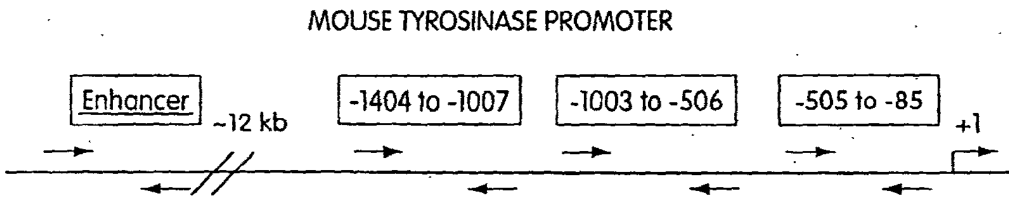


Fig. 51

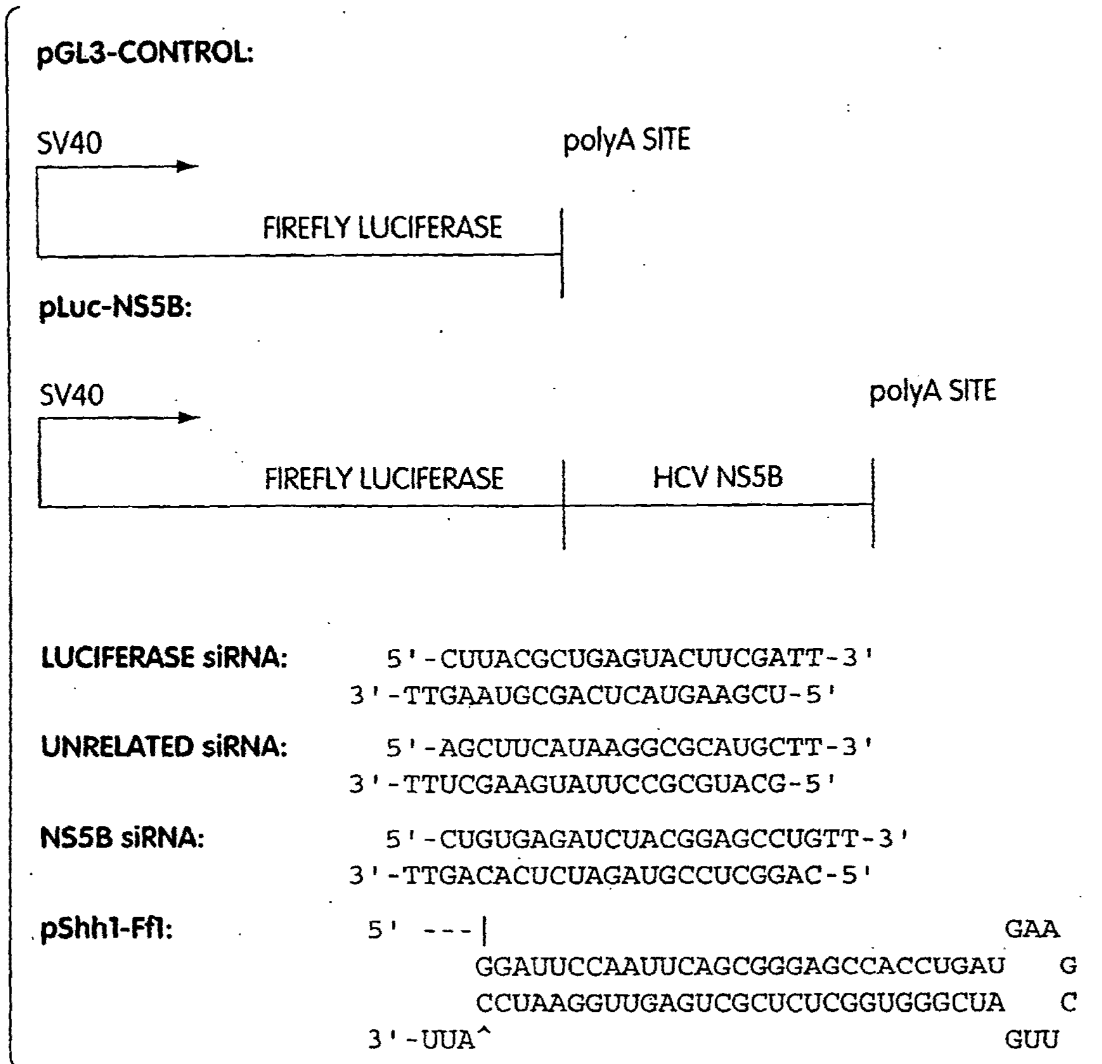


Fig. 52

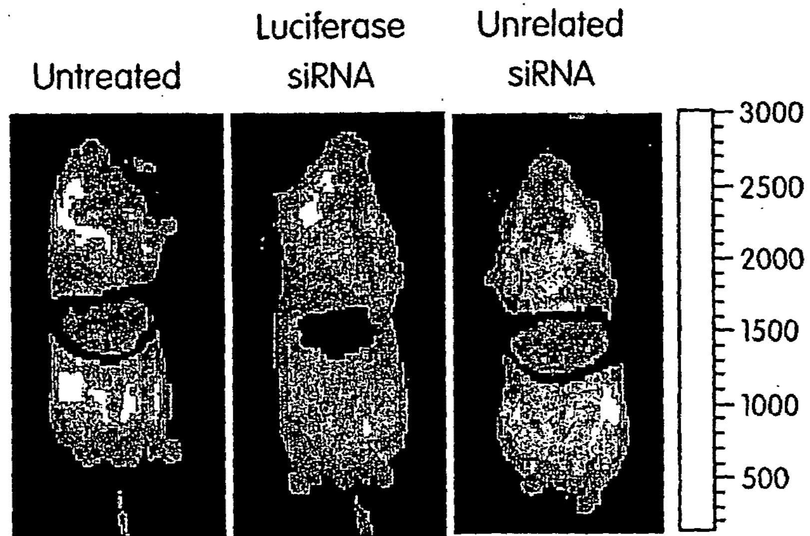


Fig. 53A

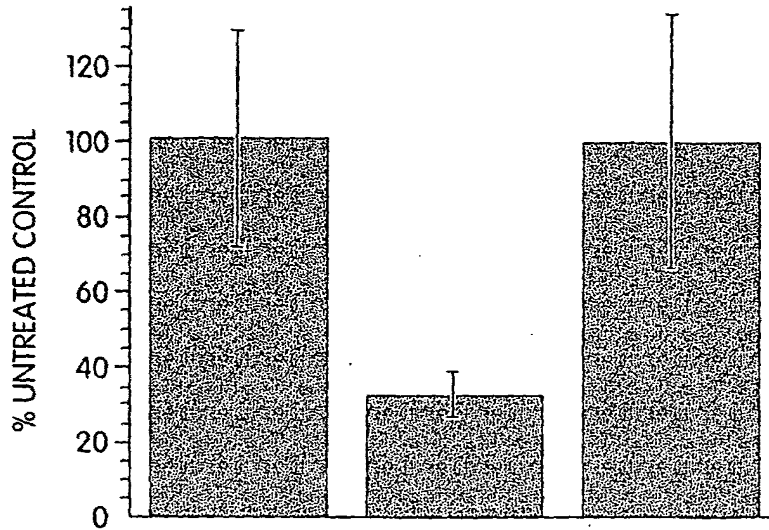


Fig. 53B

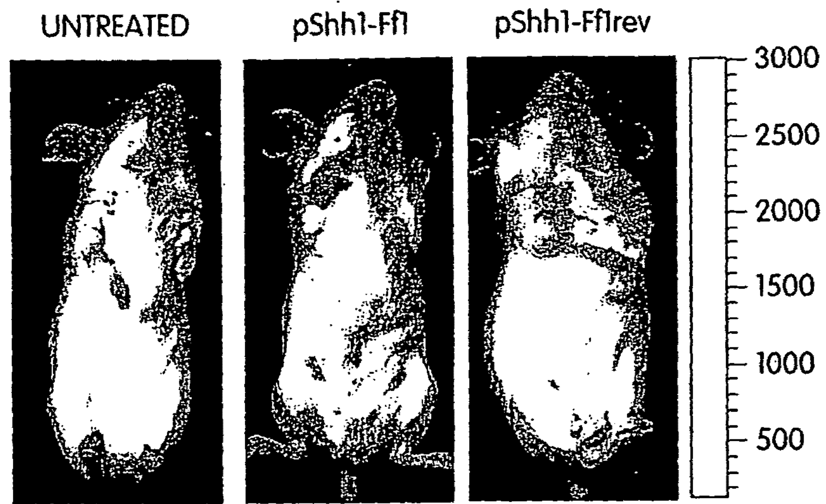


Fig. 54A

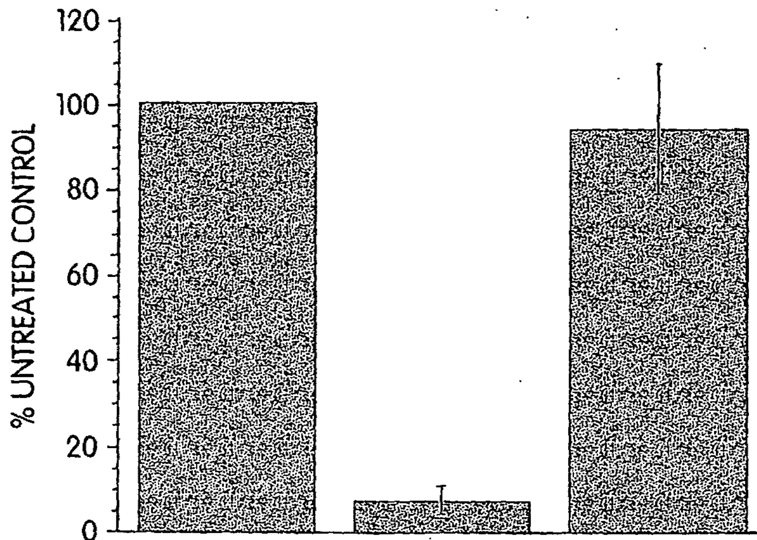


Fig. 54B

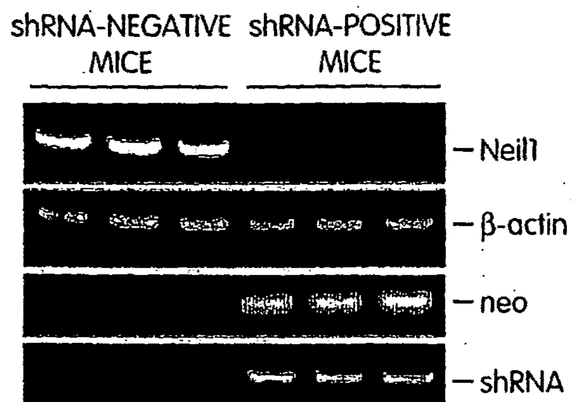


Fig. 55A

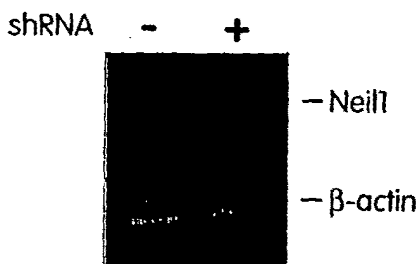


Fig. 55B

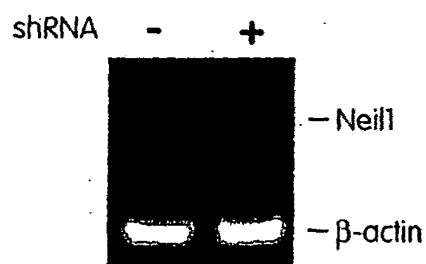


Fig. 55C

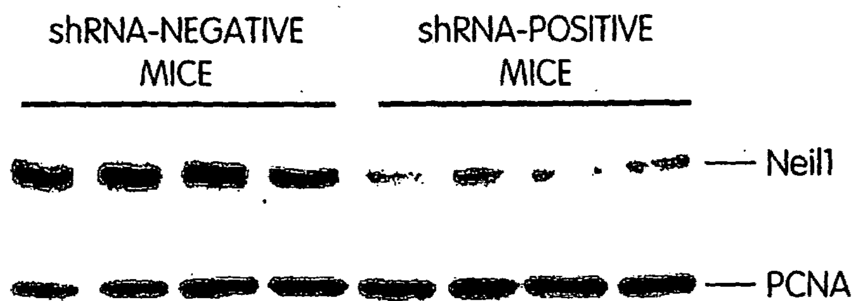


Fig. 56A

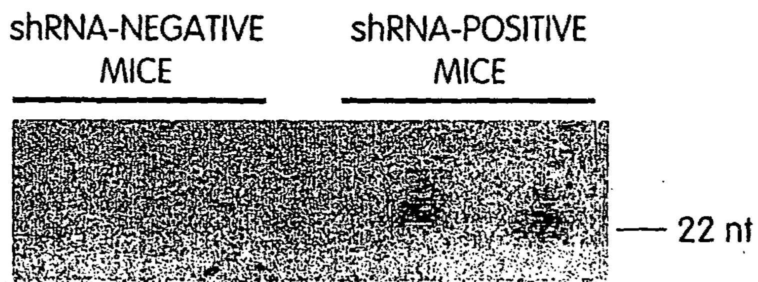


Fig. 56B

Fig. 57 A

29 nt. shRNA with overhang

```
NNNNNNNNNNNNNNNNNNNNNNNNNNNNN C
                               C
UUNNNNNNNNNNNNNNNNNNNNNNNNNNNNN A
                               A
```

29mer shRNA no overhang

```
NNNNNNNNNNNNNNNNNNNNNNNNNNNNN C
                              C
NNNNNNNNNNNNNNNNNNNNNNNNNNNNN A
                              A
```

19 nt. shRNA with overhang

```
NNNNNNNNNNNNNNNNNNN C
                     C
UUNNNNNNNNNNNNNNNNNNN A
                     A
```

19mer shRNA

```
NNNNNNNNNNNNNNNNNNN C
                     C
NNNNNNNNNNNNNNNNNNN A
                     A
```

Luciferase 29mer

AGUUGCGCCCGCGAAUGAUAUUUAUAAUG

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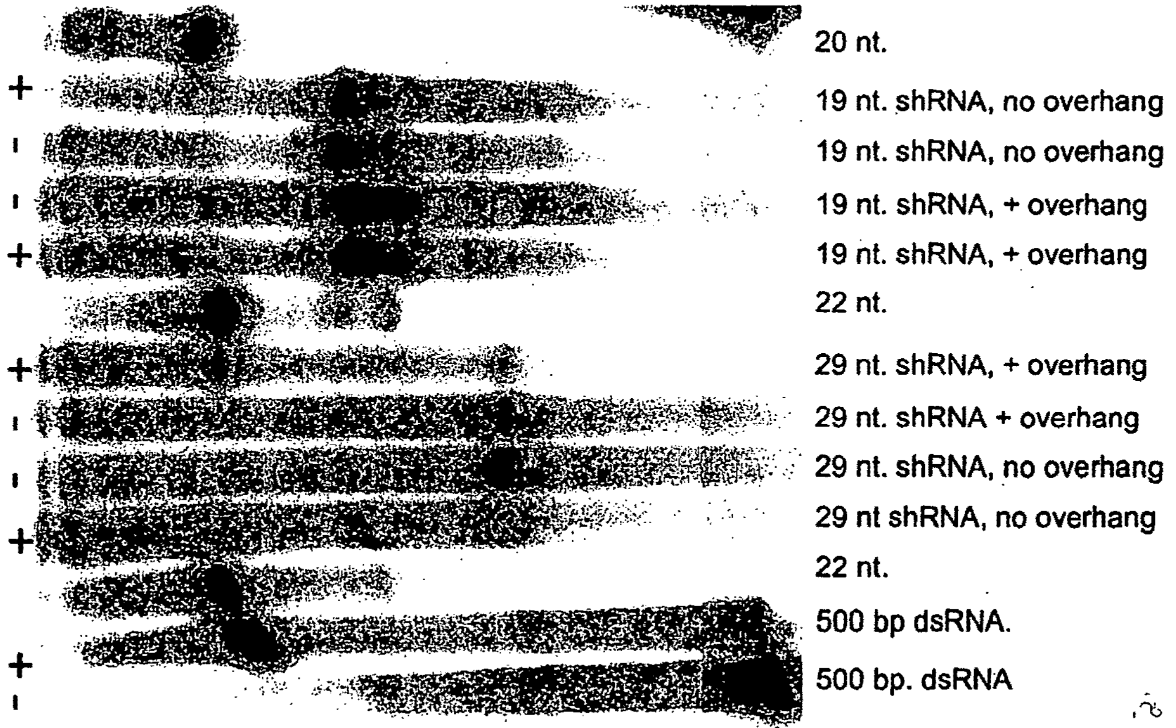

Fig. 57 B

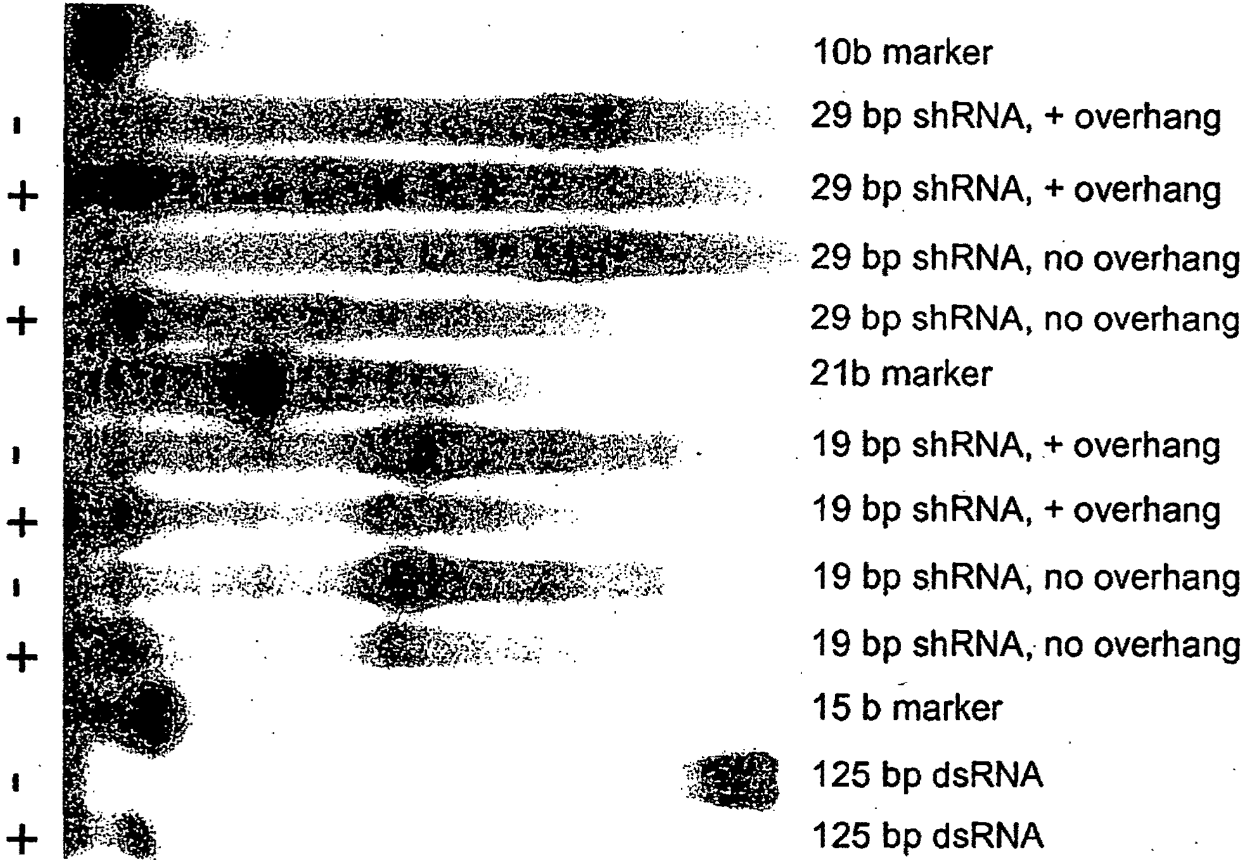

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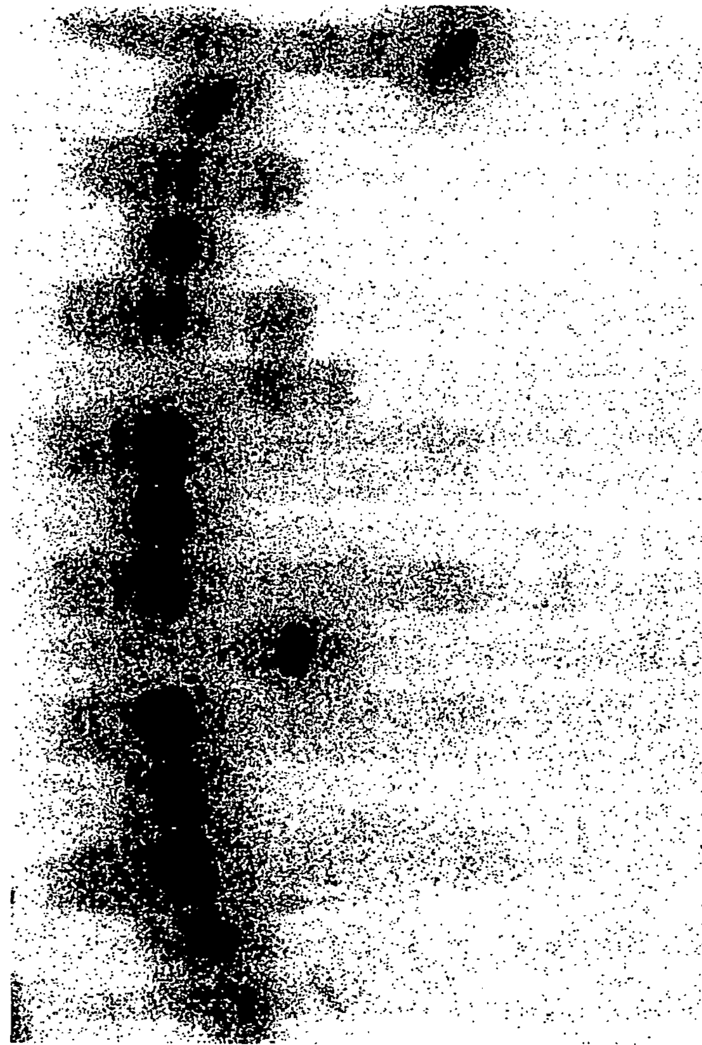
Fig. 57 C



RT	Dicer	
		25 b marker
-	-	21 nt. siRNA
+	-	21 nt siRNA
-	-	22 nt. siRNA
+	-	22 nt. siRNA
		20 b marker
+	-	19 bp shRNA, + overhang
-	+	19 bp shRNA, + overhang
+	+	19 bp shRNA, + overhang
		22 b marker
+	-	19 bp shRNA, no overhang
-	+	19 bp shRNA, no overhang
+	+	19 bp shRNA, no overhang
+	-	primer only
		15 b marker

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Fig. 58 A

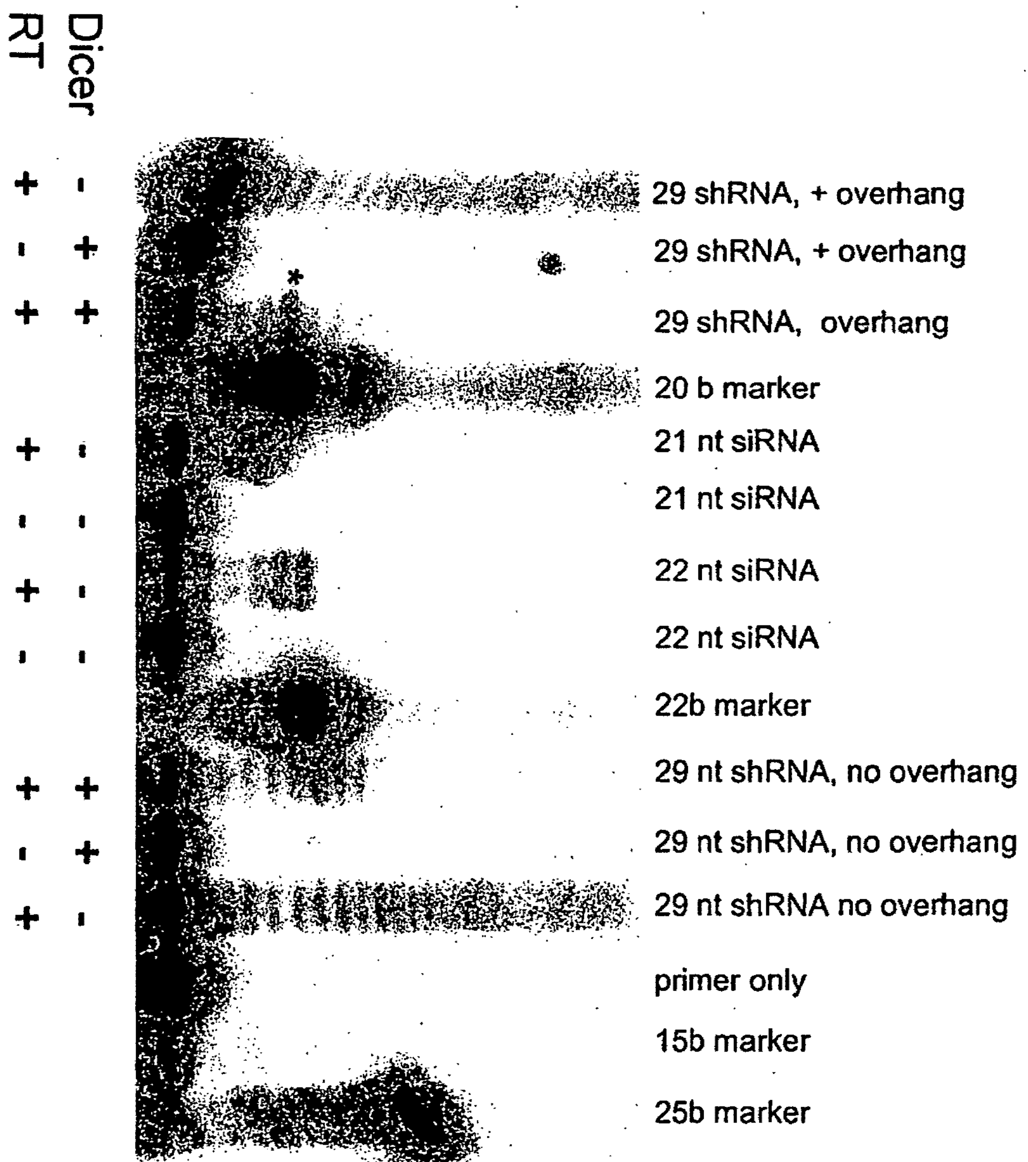

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Fig. 58 B

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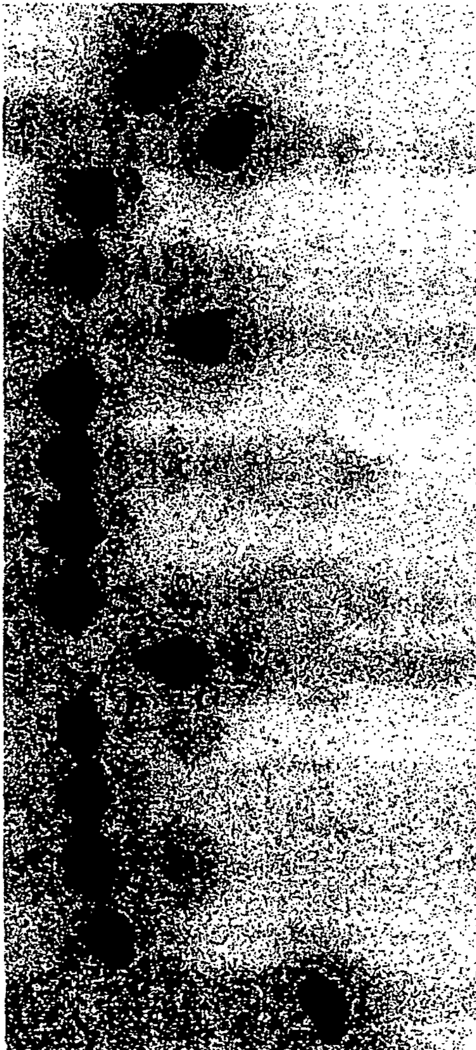
RT		
+		Primer only
		21 b marker
-		in vitro 29 nt shRNA
+		in vitro 29 nt shRNA
		22 b marker
-		in vivo 29 nt shRNA, Ago2 IP
+		in vivo 29 nt shRNA, Ago2 IP
-		in vivo 29 nt shRNA, Ago1 IP
+		in vivo 29 nt shRNA, Ago1 IP
		20 b marker
+		22 nt siRNA
-		22 nt siRNA
+		21 nt siRNA
-		21 nt siRNA
		25 b marker

Fig. 58 C

Fig. 59 A

siRNAs

19mer

NNNNNNNNNNNNNNNNNNNdTdT

dTdTNNNNNNNNNNNNNNNNNNN

Synthetic 19mer shRNAs

19mer of siRNA

NNNNNNNNNNNNNNNNNNN U U

UUNNNNNNNNNNNNNNNNNNN G G

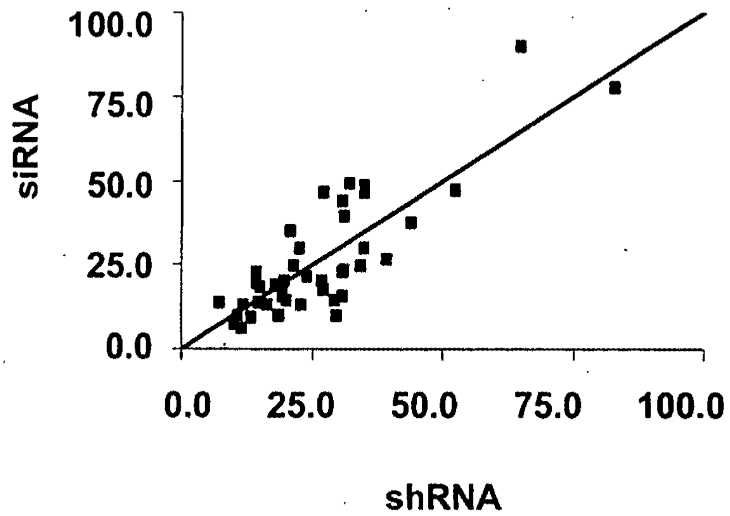
Synthetic 29mer shRNAs

19mer of siRNA

XNNNNNNNNNNNNNNNNNNNXXXXXXXXX U U

UUXNNNNNNNNNNNNNNNNNNNXXXXXXXXX G G

Fig. 59 B



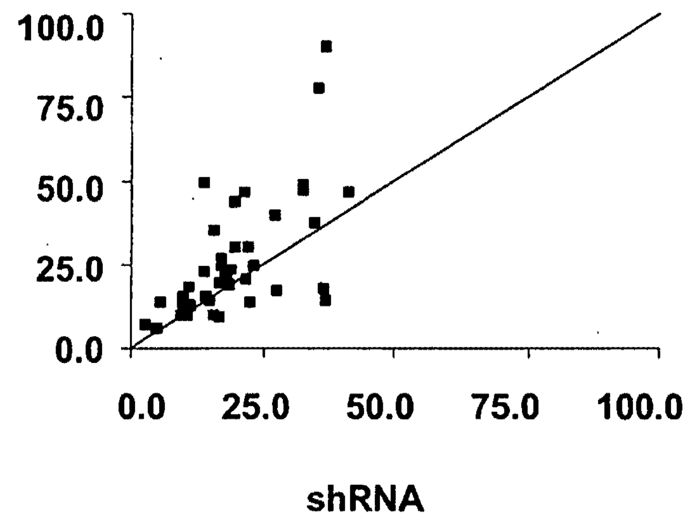


Fig. 59 C

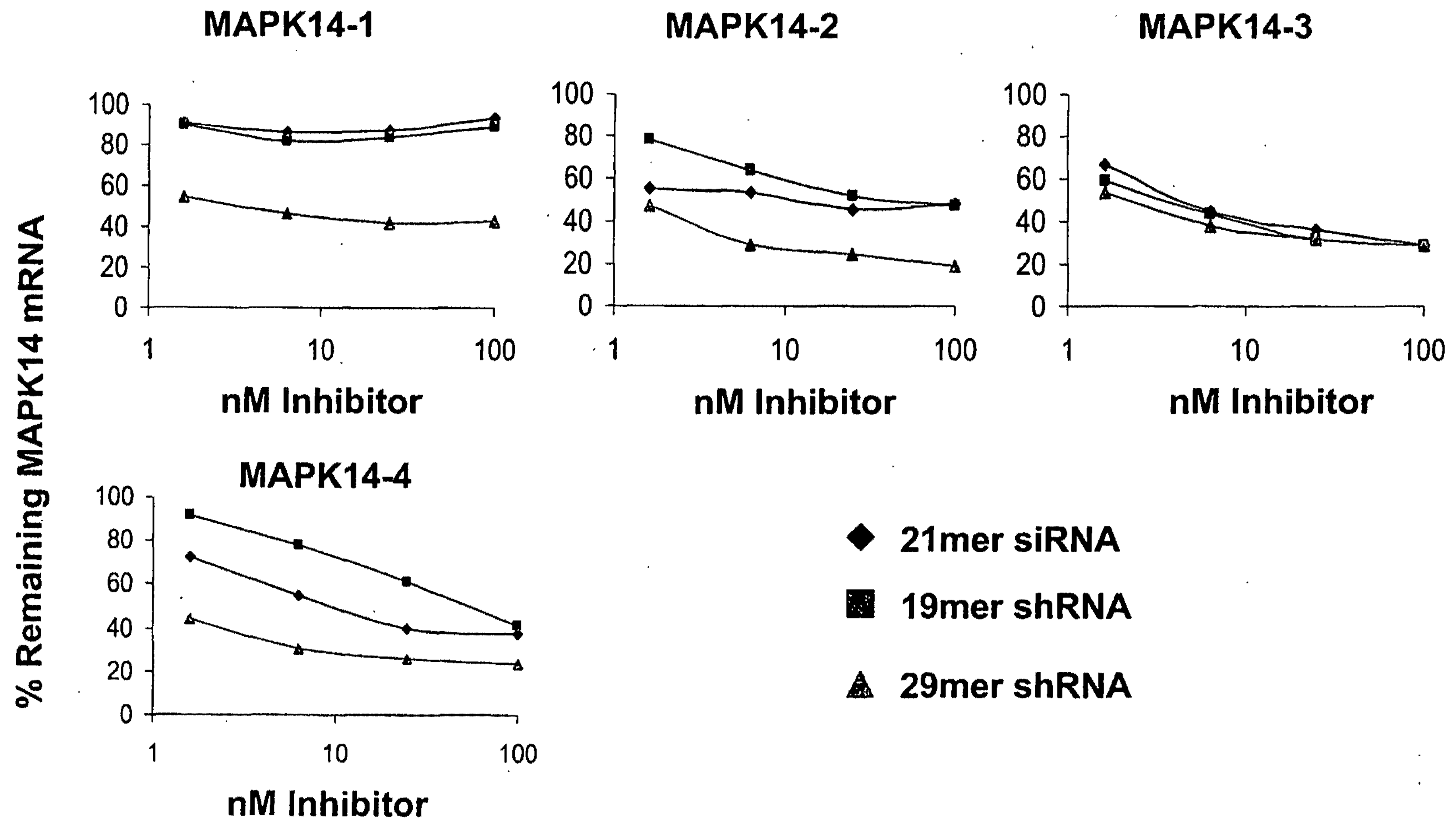


Fig. 60 A

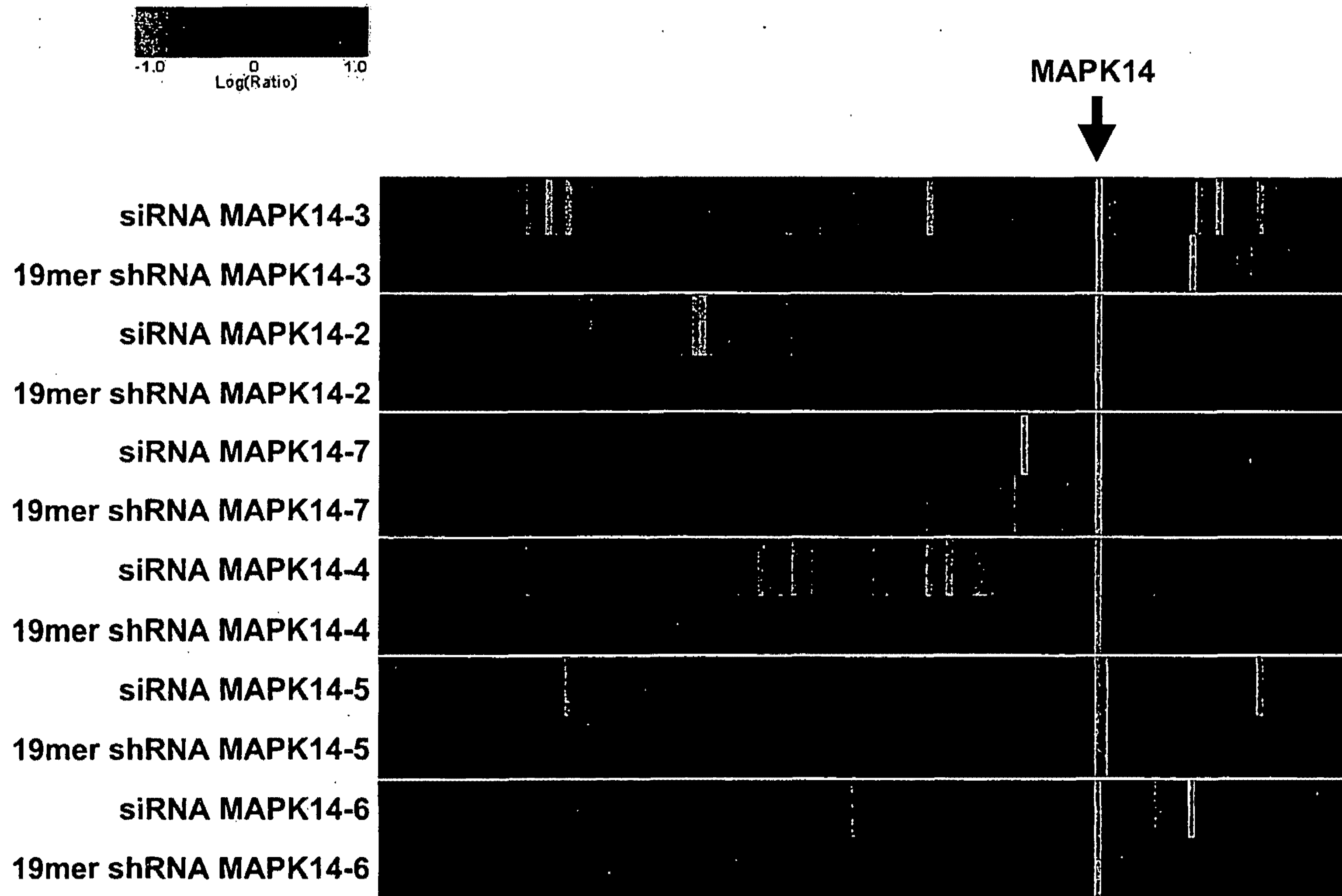


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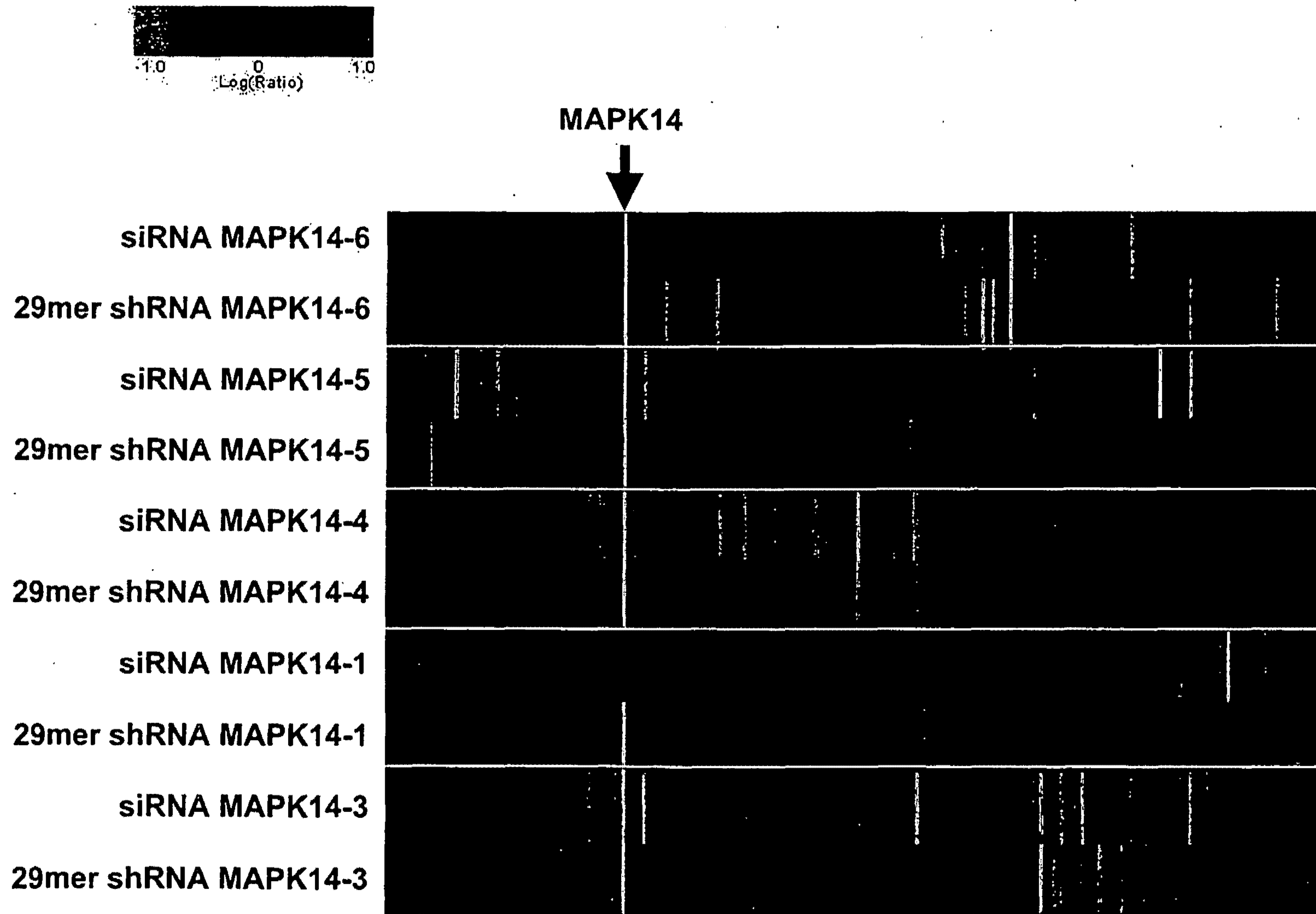
Fig. 60 B



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I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EV543610569US, on the date shown below and in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: August 20, 2007 Signature:
(Scott Whittemore)

Docket No.: CSHL-P08-010
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Hannon *et al.*		Confirmation No.:	Not Yet Assigned
Application No.:	Not Yet Assigned		
		Art Unit:	Not Yet Assigned
Filed:	August 20, 2007		
For: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		Examiner:	Not Yet Assigned

PRELIMINARY AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

Prior to examination on the merits, please amend the above-identified U.S. patent application as follows:

Amendments to the specification begin on page 2 of this paper.

Amendments to the claims begin on page 3 of this paper.

Remarks begin on page 5 of this paper.

AMENDMENTS TO THE CLAIMS

- Please insert the following new paragraph immediately after the title and immediately before the section "Government Support":

Related Applications:

This application is a continuation application of U.S. Serial No. 11/791,554, filed on May 23, 2007, which is a national stage filing under 35 U.S.C. § 371 of International Application No. PCT/US2005/042488, filed on November 23, 2005, which is a continuation application of U.S. Serial No. 10/997,086, filed on November 23, 2004, which is a continuation-in-part of U.S. Ser. No. 10/350,798, filed on January 24, 2003, which is a continuation-in-part of U.S. Ser. No. 10/055,797, filed on January 22, 2002, which is a continuation-in-part of International Application No. PCT/US01/08435, filed on March 16, 2001, which claims the benefit of the filing date from U.S. Provisional Application Nos. 60/189,739, filed on March 16, 2000, and 60/243,097, filed on October 24, 2000. U.S. Ser. No. 10/350,798 is also a continuation-in-part of U.S. Ser. No. 09/866,557, filed on May 24, 2001, which is also a continuation-in-part of International Application No. PCT/US01/08435, filed on March 16, 2001. U.S. Ser. No. 10/350,798 is also a continuation-in-part of U.S. Ser. No. 09/858,862, filed on May 16, 2001, which is also a continuation-in-part of International Application No. PCT/US01/08435, filed on March 16, 2001. The specifications of such applications are incorporated by reference herein. International Application PCT/US01/08435 and International Application PCT/US2005/042488 were both published under PCT Article 21(2) in English.

- Please delete the paragraph (under the title "Related Applications") between the sections titled "Government Support" and "Background of the Invention."

AMENDMENTS TO THE CLAIMS

1-35. **(Canceled)**

36. **(New)** A method for producing a double-stranded RNA (dsRNA) product capable of reducing expression of a target gene in a mammalian cell, said method comprising:
    (1) providing a cell-free mixture comprising:
        (a) an RNaseIII enzyme, and,
        (b) an RNA substrate comprising a duplex region that is a substrate for the RNaseIII,
    (2) allowing said substrate to be cleaved by the RNaseIII enzyme to generate the dsRNA product of about 22 bp in length.

37. **(New)** The method of claim 35, wherein the substrate is a single strand RNA comprising self-complementary sequences that form a duplex region.

38. **(New)** The method of claim 35, wherein the substrate comprises two complementary strands that form a duplex region.

39. **(New)** The method of claim 35, wherein the substrate is at least 25, 50, 100, 200, 300, 400, or 400-800 bp.

40. **(New)** The method of claim 35, wherein the dsRNA product is identical to a potion of the target gene.

41. **(New)** The method of claim 35, wherein the dsRNA product hybridizes with a potion of the target gene under wash conditions of 0.2 × SSC at 65°C.

42. **(New)** The method of claim 35, wherein the RNaseIII enzyme is a Dicer.

43. **(New)** The method of claim 35, wherein the RNaseIII enzyme is at least 75% identical to SEQ ID NO: 2 or 4.

44. **(New)** The method of claim 35, wherein the RNaseIII enzyme is encoded by a polynucleotide that hybridizes under wash conditions of 0.2 × SSC at 65°C to SEQ ID NO: 1 or 3.

45. **(New)** The method of claim 35, wherein the RNaseIII enzyme is recombinantly expressed or purified.

46. **(New)** The method of claim 45, wherein the RNaseIII enzyme is recombinantly expressed from an expression vector comprising at least one transcriptional regulatory sequence selected from a promoter, an enhancer, or other expression control elements.

47. **(New)** The method of claim 35, further comprising purifying the dsRNA product from the mixture.

48. **(New)** The method of claim 35, further comprising storing the purified dsRNA product dry or in an aqueous solution.

49. **(New)** A cell-free mixture for generating a double-stranded RNA product capable of reducing expression of a target gene in a mammalian cell, said mixture comprising:

(1) an RNaseIII enzyme, and,

(2) a RNA substrate comprising a duplex region that is a substrate for the RNaseIII, wherein said substrate is capable of being cleaved by the RNaseIII enzyme to generate the dsRNA product of about 22 bp in length.

REMARKS

Upon entry of this amendment, new Claims 36-49 are pending. Claims 1-35 are canceled without prejudice. Applicants reserve the right to prosecute claims of identical or similar scope in one or more future continuation or divisional applications.

New Claims 36-49 are added to clarify the subject matter being claimed. Support can be found throughout the specification.

For example, the paragraph bridging pages 32-33 (corresponding to page 16, 4th full paragraph in the parent application U.S.S.N. 09/858,862, filed on May 16, 2001) describes a method of using a cell-free mixture and an RNAi enzyme, such as RNaseIII (*e.g.*, a recombinantly expressed Dicer), to potentiate RNAi.

Page 41, 2nd full paragraph (corresponding to page 24, 1st full paragraph in the parent application U.S.S.N. 09/858,862) describes various lengths of the dsRNA substrates, and the 22-mer product of Dicer-cleavage.

Page 9, 3rd full paragraph (corresponding to page 5, 2nd full paragraph in the parent application U.S.S.N. 09/858,862) describes that the substrate may be a single self-complementary RNA strand or two complementary RNA strands, be identical to the target gene or be able to hybridize to the target gene.

Page 33, 1st paragraph (corresponding to page 16, last full paragraph in the parent application U.S.S.N. 09/858,862x) describes that the RNaseIII enzyme may be a Dicer, one at least 75% identical to SEQ ID NO: 2 or 4, or one that is encoded by a polynucleotide that hybridizes under wash conditions of 0.2 × SSC at 65°C to SEQ ID NO: 1 or 3.

Page 43, 1st paragraph (corresponding to page 25, 1st paragraph in the parent application U.S.S.N. 09/858,862) describes purifying the enzymatically synthesized dsRNA before using, and/or storage of the dsRNA.

Therefore, all claims are fully supported by the specification, and is at least entitled to the benefit of the filing date of the parent application U.S.S.N. 09/858,862 – May 16, 2001, possibly even earlier.

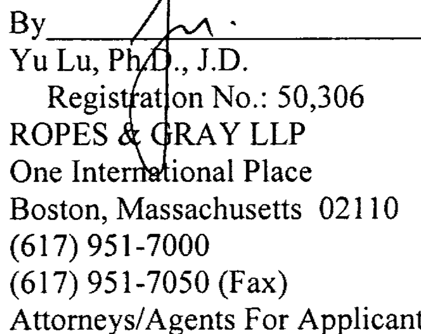
CONCLUSION

In view of the above amendment, Applicants believe the pending application is in condition for allowance.

Applicants believe no fee is due in connection with the filing of this amendment. If, however, any fee associated with the filing of this preliminary amendment (except for the filing fee, examination fee, and search fee, which are NOT being paid at this time) is due, please charge the fees due or credit any overpayments to Deposit Account No. **18-1945**, from which the undersigned is authorized to draw under Order No. **CSHL-P08-010**.

Dated: August 20, 2007

Respectfully submitted,



By________________________

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(617) 951-7050 (Fax)
Attorneys/Agents For Applicant

EV543610569US

Application Data Sheet

Application Information

Application number::	Not Yet Assigned
Filing Date::	8/20/2007
Application Type::	Regular
Subject Matter::	Utility
Suggested Group Art Unit::	1635
CD-ROM or CD-R?::	None
Sequence submission?::	None
Computer Readable Form (CRF)?::	No
Title::	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE
Attorney Docket Number::	CSHL-P08-010
Request for Early Publication?::	No
Request for Non-Publication?::	No
Total Drawing Sheets::	67
Small Entity?::	Yes
Petition included?::	No
Secrecy Order in Parent Appl.?::	No

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State or Province of Residence::	NY
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State or Province of mailing address::	NY
Postal or Zip Code of mailing address::	11952

Correspondence Information

Correspondence Customer Number::	28120

Representative Information

Representative Customer Number:: 28120

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	Continuation of	11/791,554	05/23/07
11/791,554	National Stage of	PCT/US2005/042488	11/23/05
PCT/US2005/042488	Continuation of	10/997,086	11/23/04
10/997,086	Continuation-in-part of	10/350,798	01/24/03
10/350,798	Continuation-in-part of	09/858,862	05/16/01
09/858,862	Continuation-in-part of	US01/08435	03/16/01
10/350,798	Continuation-in-part of	09/866,557	05/24/01
09/866,557	Continuation-in-part of	US01/08435	03/16/01
10/350,798	Continuation-in-part of	10/055,797	01/22/02
10/055,797	Continuation-in-part of	US01/08435	03/16/01
US01/08435	An application claiming the benefit under 35 USC 119(e)	60/189,739	03/16/00
US01/08435	An application claiming the benefit under 35 USC 119(e)	60/243,097	10/24/00

Foreign Priority Information

Assignee Information

08/20/2007

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Approved for use through 7/31/2006. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD
Substitute for Form PTO-875

Application or Docket Number
11/894,676

APPLICATION AS FILED – PART I

FOR	NUMBER FILED (Column 1)	NUMBER EXTRA (Column 2)	SMALL ENTITY RATE ($)	SMALL ENTITY FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
BASIC FEE (37 CFR 1:16(a), (b), or (c))	N/A	N/A	N/A	155		N/A	
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	255		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	105		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	14	*	X 25=		OR	X 50=	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2	*	X 105=			X 200=	
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR			260			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))			N/A			N/A	
			TOTAL	775		TOTAL	

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED – PART II

AMENDMENT A

	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X =		OR	X =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X =		OR	X =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					N/A		OR	N/A	
					TOTAL ADD'T FEE		OR	TOTAL ADD'T FEE	

AMENDMENT B

	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X =		OR	X =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X =		OR	X =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					N/A		OR	N/A	
					TOTAL ADD'T FEE		OR	TOTAL ADD'T FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD
Substitute for Form PTO-875

Application or Docket Number: 11/894,676
Filing Date: 08/20/2007
☐ To be Mailed

APPLICATION AS FILED – PART I

SMALL ENTITY ☒ OR OTHER THAN SMALL ENTITY

FOR	NUMBER FILED (Column 1)	NUMBER EXTRA (Column 2)	SMALL ENTITY RATE ($)	SMALL ENTITY FEE ($)		OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☒ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	150		N/A	
☒ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	250		N/A	
☒ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	100		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	14 minus 20 =	* 0	X $25 =	0	OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 minus 3 =	* 0	X $100 =	0		X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
			TOTAL	500		TOTAL	

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED – PART II

SMALL ENTITY OR OTHER THAN SMALL ENTITY

AMENDMENT	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/LAWRENCE BRITT JR/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/894,676	08/20/2007	Gregory J. Hannon	CSHL-P08-010

CONFIRMATION NO. 8161

28120
ROPES & GRAY LLP
PATENT DOCKETING 39/41
ONE INTERNATIONAL PLACE
BOSTON, MA 02110-2624

FORMALITIES LETTER

OC000000026587580

Date Mailed: 11/05/2007

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The statutory basic filing fee is missing.
  *Applicant must submit $***155** *to complete the basic filing fee for a small entity.*
- The oath or declaration is missing.
  A properly signed oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.
  Note: If a petition under 37 CFR 1.47 is being filed, an oath or declaration in compliance with 37 CFR 1.63 signed by all available joint inventors, or if no inventor is available by a party with sufficient proprietary interest, is required.
- This application clearly fails to comply with the requirements of 37 CFR. 1.821-1.825. Applicant's attention is directed to the final rulemaking notice published at 55 FR 18230 (May 1, 1990), and 1114 OG 29 (May 15, 1990). If the effective filing date is on or after July 1, 1998, see the final rulemaking notice published at 63 FR 29620 (June 1, 1998) and 1211 OG 82 (June 23, 1998). If the effective filing date is on or after September 8, 2000, see the final rulemaking notice published in the Federal Register at 65 FR 54604 (September 8, 2000) and 1238 OG 145 (September 19, 2000). Applicant must provide an initial computer readable form (CRF) copy of the "Sequence Listing", an initial paper or compact disc copy of the "Sequence Listing", **as well as an amendment specifically directing its entry into the application**. Applicant must also provide a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b), or 1.825(d). If applicant desires the sequence listing in the instant application to be identical with that of another application on file in the U.S. Patent and Trademark Office, such request in accordance with 37 CFR 1.821(e) may be submitted in lieu of a new CRF.

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

To Download Patentin Software, visit http://www.uspto.gov/web/patents/software.htm
For questions regarding compliance to these requirements, please contact:

- **For Rules Interpretation, call (571) 272-0951**
- **For Patentin Software Program Help, call Patent EBC at 1-866-217-9197 or directly at 703-305-3028 / 703-308-6845 between the hours of 6 a.m. and 12 midnight, Monday through Friday, EST.**
- **Send e-mail correspondence for Patentin Software Program Help @ ebc@uspto.gov**

The applicant needs to satisfy supplemental fees problems indicated below.

The required item(s) identified below must be timely submitted to avoid abandonment:

- To avoid abandonment, a surcharge (for late submission of filing fee, search fee, examination fee or oath or declaration) as set forth in 37 CFR 1.16(f) of $**65** for a small entity in compliance with 37 CFR 1.27, must be submitted with the missing items identified in this notice.

SUMMARY OF FEES DUE:

Total additional fee(s) required for this application is $**840** for a small entity

- $**155** Statutory basic filing fee.
- $**65** Surcharge.
- The application search fee has not been paid. Applicant must submit $**255** to complete the search fee.
- The application examination fee has not been paid. Applicant must submit $**105** to complete the examination fee for a small entity in compliance with 37 CFR 1.27.
- The specification and drawings contain more than 100 pages. Applicant owes $**260** for **66** pages in excess of **100** pages for a small entity in compliance with 37 CFR 1.27.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

/rrobel/

Office of Initial Patent Examination (571) 272-4000 or 1-800-PTO-9199

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
11/894,676	08/20/2007	1635	0.00	CSHL-P08-010	14	2

CONFIRMATION NO. 8161

28120
ROPES & GRAY LLP
PATENT DOCKETING 39/41
ONE INTERNATIONAL PLACE
BOSTON, MA 02110-2624

FILING RECEIPT

OC000000026587579

Date Mailed: 11/05/2007

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please write to the Office of Initial Patent Examination's Filing Receipt Corrections. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

Gregory J. Hannon, Huntington, NY;
Patrick J. Paddison, Oyster Bay, NY;
Despina C. Siolas, Mattituck, NY;

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 11/791,554
which is a 371 of PCT/US2005/042488 11/23/2005
which is a CON of 10/997,086 11/23/2004
which is a CIP of 10/350,798 01/24/2003
which is a CIP of 10/055,797 01/22/2002
which is a CIP of PCT/US01/08435 03/16/2001
which claims benefit of 60/189,739 03/16/2000
and claims benefit of 60/243,097 10/24/2000
and said 10/350,798 01/24/2003
is a CIP of 09/866,557 05/24/2001
which is a CIP of PCT/US01/08435 03/16/2001
and said 10/350,798 01/24/2003
is a CIP of 09/858,862 05/16/2001
which is a CIP of PCT/US01/08435 03/16/2001

Foreign Applications

If Required, Foreign Filing License Granted: 11/02/2007

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 11/894,676**

Projected Publication Date: To Be Determined - pending completion of Missing Parts

Non-Publication Request: No

Early Publication Request: No
**** SMALL ENTITY ****
Title

Methods and compositions for RNA interference

Preliminary Class

514

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

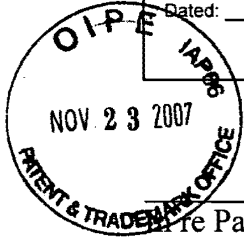
NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

IFW

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service as First Class Mail, on the date shown below and in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: November 20, 2007 Signature: [signature] (Scott Whittemore)



Docket No.: CSHL-P08-010
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Hannon *et al.*		Confirmation No.:	8161
Application No.:	11/894,676		
		Art Unit:	1635
Filed:	August 20, 2007		
For: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		Examiner:	Not Yet Assigned

SECOND PRELIMINARY AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Prior to examination on the merits, please amend the above-identified U.S. patent application as follows:

Amendments to the specification begin on page 2 of this paper.

Remarks begin on page 3 of this paper.

AMENDMENTS TO THE CLAIMS

- Please replace the paragraph first inserted in the Preliminary Amendment filed on August 20, 2007, with the following re-written paragraph:

Related Applications:

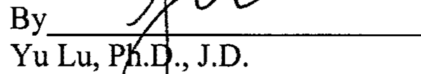
This application is a continuation application of ~~U.S. Serial No. 11/791,554, filed on May 23, 2007, which is a national stage filing under 35 U.S.C. § 371 of International Application No. PCT/US2005/042488, filed on November 23, 2005, which is a continuation application of~~ U.S. Serial No. 10/997,086, filed on November 23, 2004, which is a continuation-in-part of U.S. Ser. No. 10/350,798, filed on January 24, 2003, which is a continuation-in-part of U.S. Ser. No. 10/055,797, filed on January 22, 2002, which is a continuation-in-part of International Application No. PCT/US01/08435, filed on March 16, 2001, which claims the benefit of the filing date from U.S. Provisional Application Nos. 60/189,739, filed on March 16, 2000, and 60/243,097, filed on October 24, 2000. U.S. Ser. No. 10/350,798 is also a continuation-in-part of U.S. Ser. No. 09/866,557, filed on May 24, 2001, which is also a continuation-in-part of International Application No. PCT/US01/08435, filed on March 16, 2001. U.S. Ser. No. 10/350,798 is also a continuation-in-part of U.S. Ser. No. 09/858,862, filed on May 16, 2001, which is also a continuation-in-part of International Application No. PCT/US01/08435, filed on March 16, 2001. The specifications of such applications are incorporated by reference herein. International Application PCT/US01/08435 ~~and International Application PCT/US2005/042488 were both~~ was published under PCT Article 21(2) in English.

REMARKS

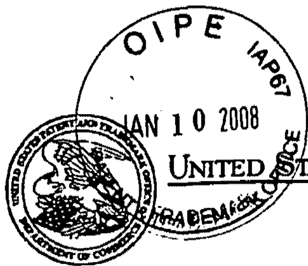
Applicants believe no fee is due in connection with the filing of this amendment. If, however, any fee associated with the filing of this preliminary amendment is due, please charge the fees due or credit any overpayments to Deposit Account No. **18-1945**, from which the undersigned is authorized to draw under Order No. **CSHL-P08-010**.

Dated: November 20, 2007

Respectfully submitted,

By 

Yu Lu, Ph.D., J.D.
Registration No.: 50,306
ROPES & GRAY LLP
One International Place
Boston, Massachusetts 02110
(617) 951-7000
(617) 951-7050 (Fax)
Attorneys/Agents For Applicant



1FW/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/894,676	08/20/2007	Gregory J. Hannon	CSHL-P08-010

CONFIRMATION NO. 8161

28120
ROPES & GRAY LLP
PATENT DOCKETING 39/41
ONE INTERNATIONAL PLACE
BOSTON, MA 02110-2624

01/10/2008 WASFAW1 00000075 181945 11894676
01 FC:2011 155.00 DA
02 FC:2111 255.00 DA
03 FC:2311 105.00 DA
04 FC:2051 65.00 DA
~~05 FC:2085 390.00 DA~~

FORMALITIES LETTER

OC000000026587580

Date Mailed: 11/05/2007

01/11/2008 WASFAW1 00000046 181945 11894676
01 FC:2081 390.00 DA

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The statutory basic filing fee is missing.
  Applicant must submit $155 to complete the basic filing fee for a small entity.
- The oath or declaration is missing.
  A properly signed oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.
  Note: If a petition under 37 CFR 1.47 is being filed, an oath or declaration in compliance with 37 CFR 1.63 signed by all available joint inventors, or if no inventor is available by a party with sufficient proprietary interest, is required.
- This application clearly fails to comply with the requirements of 37 CFR. 1.821-1.825. Applicant's attention is directed to the final rulemaking notice published at 55 FR 18230 (May 1, 1990), and 1114 OG 29 (May 15, 1990). If the effective filing date is on or after July 1, 1998, see the final rulemaking notice published at 63 FR 29620 (June 1, 1998) and 1211 OG 82 (June 23, 1998). If the effective filing date is on or after September 8, 2000, see the final rulemaking notice published in the Federal Register at 65 FR 54604 (September 8, 2000) and 1238 OG 145 (September 19, 2000). Applicant must provide an initial computer readable form (CRF) copy of the "Sequence Listing", an initial paper or compact disc copy of the "Sequence Listing", **as well as an amendment specifically directing its entry into the application**. Applicant must also provide a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b), or 1.825(d). If applicant desires the sequence listing in the instant application to be identical with that of another application on file in the U.S. Patent and Trademark Office, such request in accordance with 37 CFR 1.821(e) may be submitted in lieu of a new CRF.

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

To Download Patentin Software, visit http://www.uspto.gov/web/patents/software.htm
For questions regarding compliance to these requirements, please contact:

- **For Rules Interpretation, call (571) 272-0951**
- **For Patentin Software Program Help, call Patent EBC at 1-866-217-9197 or directly at 703-305-3028 / 703-308-6845 between the hours of 6 a.m. and 12 midnight, Monday through Friday, EST.**
- **Send e-mail correspondence for Patentin Software Program Help @ ebc@uspto.gov**

The applicant needs to satisfy supplemental fees problems indicated below.

The required item(s) identified below must be timely submitted to avoid abandonment:

- To avoid abandonment, a surcharge (for late submission of filing fee, search fee, examination fee or oath or declaration) as set forth in 37 CFR 1.16(f) of **$65** for a small entity in compliance with 37 CFR 1.27, must be submitted with the missing items identified in this notice.

SUMMARY OF FEES DUE:

Total additional fee(s) required for this application is **$840** for a small entity

- **$155** Statutory basic filing fee.
- **$65** Surcharge.
- The application search fee has not been paid. Applicant must submit **$255** to complete the search fee.
- The application examination fee has not been paid. Applicant must submit **$105** to complete the examination fee for a small entity in compliance with 37 CFR 1.27.
- The specification and drawings contain more than 100 pages. Applicant owes **$260** for **66** pages in excess of **100** pages for a small entity in compliance with 37 CFR 1.27.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

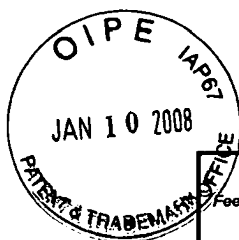
Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

/rrobel/

Office of Initial Patent Examination (571) 272-4000 or 1-800-PTO-9199



PTO/SB/17 (10-07)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no person are required to respond to a collection of information unless it displays a valid OMB control number.

Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2008

[x] Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT	($) 970.00

Complete if Known	
Application Number	11/894,676
Filing Date	August 20, 2007
First Named Inventor	Gregory J. Hannon
Examiner Name	Not Yet Assigned
Art Unit	1635
Attorney Docket No.	CSHL-P08-010

METHOD OF PAYMENT (check all that apply)

[ ] Check [ ] Credit Card [ ] Money Order [ ] None [ ] Other (please identify): ____

[x] Deposit Account Deposit Account Number: 18-1945 Deposit Account Name: Ropes & Gray LLP

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

[x] Charge fee(s) indicated below
[ ] Charge fee(s) indicated below, **except for the filing fee**
[x] Charge any additional fee(s) or underpayments of fee(s) under 37 CFR 1.16 and 1.17
[x] Credit any overpayments

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

Application Type	FILING FEES Fee ($)	FILING FEES Small Entity Fee ($)	SEARCH FEES Fee ($)	SEARCH FEES Small Entity Fee ($)	EXAMINATION FEES Fee ($)	EXAMINATION FEES Small Entity Fee ($)	Fees Paid ($)
Utility	310	155	510	255	210	105	515.00
Design	210	105	100	50	130	65	
Plant	210	105	310	155	160	80	
Reissue	310	155	510	255	620	310	
Provisional	210	105	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	210	105
Multiple dependent claims	370	185

Total Claims		Extra Claims		Fee ($)		Fee Paid ($)
14	- 20 =		x		=	

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		Extra Claims		Fee ($)		Fee Paid ($)
2	- 3 =		x		=	

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims

Fee ($)	Fee Paid ($)

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $260 ($130 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		Extra Sheets		Number of each additional 50 or fraction thereof		Fee ($)		Fee Paid ($)
215	- 100 =	115	/50 =	3	(round up to a whole number) x	130.00	=	390.00

4. OTHER FEE(S) **Fees Paid ($)**

Non-English Specification, $130 fee (no small entity discount)
Other (e.g., late filing surcharge): 2051 Surcharge-Late oath or declaration 65.00

SUBMITTED BY					
Signature	[signature]	Registration No. (Attorney/Agent)	50,306	Telephone	(617) 951-7268
Name (Print/Type)	Yu Lu, Ph.D., J.D.			Date	January 7, 2008

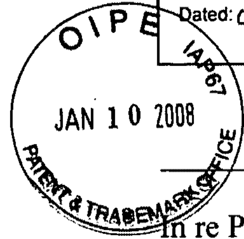
I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: MS Missing Parts, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: 01/07/08 Signature: [signature] (Scott Whittemore)

10858046_1.DOC

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: MS Missing Parts, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: 01/07/08 Signature:
(Scott Whittemore)



Docket No.: CSHL-P08-010
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Hannon et al.

Confirmation No.: 8161

Application No.: 11/894,676

Art Unit: 1635

Filed: August 20, 2007

Examiner: Not Yet Assigned

For: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

RESPONSE TO NOTICE TO FILE MISSING PARTS OF APPLICATION

MS Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Notice to File Missing Parts of Application – Filing Date Granted mailed November 5, 2007, Applicants respectfully submit a Declaration, the Filing Fee for the Application (as shown on accompanying Fee Transmittal), a paper copy of the Sequence Listing under 37 CFR 1.821(c), a Request Under 37 CFR 1.821(e), and a Statement Pursuant to 37 CFR 1.821(f), an Information Disclosure Statement, and Part 2 Copy of Notice.

Applicants request the entry of the paper copy of the Sequence Listing submitted herewith as part of the specification.

Please charge our Deposit Account No. **18-1945** in the amount of **$970.00** covering the fees due in the above-captioned case. The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter

filed in this application by this firm) to our Deposit Account No. **18-1945**, under Order No. **CSHL-P08-010**. A duplicate copy of this paper is enclosed.

Dated: January 7, 2008

Respectfully submitted,

By_______________

Yu Lu, Ph.D., J.D.
Registration No.: 50,306
ROPES & GRAY LLP
One International Place
Boston, Massachusetts 02110
(617) 951-7000
(617) 951-7050 (Fax)
Attorneys/Agents For Applicant

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: MS Missing Parts, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: 01/07/08 Signature: [signature]
(Scott Whittemore)

OIPE IAP67
JAN 10 2008
PATENT & TRADEMARK OFFICE

Docket No.: CSHL-P08-010
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Hannon et al.

Application No.: 11/894,676 Confirmation No.: 8161

Filed: August 20, 2007 Art Unit: 1635

For: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE Examiner: Not Yet Assigned

REQUEST UNDER 37 C.F.R. § 1.821(e) AND STATEMENT PURSUANT TO 37 C.F.R. § 1.821(f)

MS Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

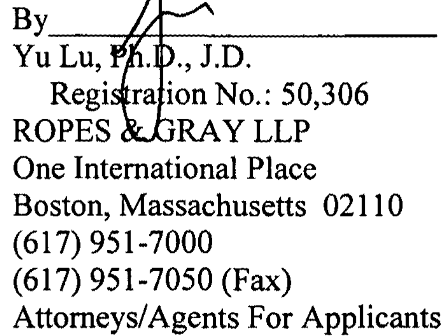
Pursuant to 37 C.F.R. § 1.821(e), it is hereby requested that the computer readable form (CRF) of the sequence listing filed in U.S. Serial No. 10/997,086 (filed on November 23, 2004), serve as the CRF for the subject application.

Applicants' Attorney hereby states that the Sequence Listing for the above-captioned application is identical to that which was filed in U.S. Serial No. 10/997,086, filed on November 23, 2004; Applicants' Attorney further states that the contents of the "Sequence Listing" in paper form submitted herewith as required by 37 C.F.R. § 1.821(c) and the CRF incorporated hereinabove as required by 37 C.F.R. § 1.821(e) are the same as required by 37 C.F.R. § 1.821(f).

Applicants believe no fee other than those listed in the accompanying Fee Transmittal is due with this response. However, if any other fee is due, please charge our Deposit Account No. **18-1945**, from which the undersigned is authorized to draw under Order No. **CSHL-P08-010**.

Dated: January 7, 2008

Respectfully submitted,



By____________________

Yu Lu, Ph.D., J.D.
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One International Place
Boston, Massachusetts 02110
(617) 951-7000
(617) 951-7050 (Fax)
Attorneys/Agents For Applicants

SEQUENCE LISTING

<110> Hannon, Gregory J.
Paddison, Patrick J.
Siolas, Despina C.

<120> METHODS AND COMPOSITIONS FOR RNA
INTERFERENCE

<130> CSHL-P05-010

<140> US 10/997,086
<141> 2004-11-23

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 1               5                  10                  15

atg acc cct gct tcc tca cca atg ggt cct ttc ttt gga ctg cca tgg   96
Met Thr Pro Ala Ser Ser Pro Met Gly Pro Phe Phe Gly Leu Pro Trp
            20                  25                  30

caa caa gaa gca att cat gat aac att tat acg cca aga aaa tat cag  144
Gln Gln Glu Ala Ile His Asp Asn Ile Tyr Thr Pro Arg Lys Tyr Gln
        35                  40                  45
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Asn Thr Gly Ser Gly Lys Thr Phe Ile Ala Ser Thr Thr Leu Leu Lys
 65                  70                  75                  80

agc tgt ctc tat cta gat cta ggg gag act tca gct aga aat gga aaa    288
Ser Cys Leu Tyr Leu Asp Leu Gly Glu Thr Ser Ala Arg Asn Gly Lys
                 85                  90                  95

agg acg gtg ttc ttg gtc aac tct gca aac cag gtt gct caa caa gtg    336
Arg Thr Val Phe Leu Val Asn Ser Ala Asn Gln Val Ala Gln Gln Val
            100                 105                 110

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Ser Ala Val Arg Thr His Ser Asp Leu Lys Val Gly Glu Tyr Ser Asn
        115                 120                 125

cta gaa gta aat gca tct tgg aca aaa gag aga tgg aac caa gag ttt    432
Leu Glu Val Asn Ala Ser Trp Thr Lys Glu Arg Trp Asn Gln Glu Phe
    130                 135                 140

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145                 150                 155                 160

ttg aaa aat ggt tac tta tca ctg tca gac att aac ctt ttg gtg ttt    528
Leu Lys Asn Gly Tyr Leu Ser Leu Ser Asp Ile Asn Leu Leu Val Phe
                165                 170                 175

gat gag tgt cat ctt gca atc cta gac cac ccc tat cga gaa ttt atg    576
Asp Glu Cys His Leu Ala Ile Leu Asp His Pro Tyr Arg Glu Phe Met
            180                 185                 190

aag ctc tgt gaa att tgt cca tca tgt cct cgc att ttg gga cta act    624
Lys Leu Cys Glu Ile Cys Pro Ser Cys Pro Arg Ile Leu Gly Leu Thr
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225                 230                 235                 240

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            260                 265                 270

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Ser Val His Ser Lys Glu Arg Asp Ser Thr Leu Ile Ser Lys Gln Ile
    290                 295                 300

cta tca gac tgt cgt gcc gta ttg gta gtt ctg gga ccc tgg tgt gca    960
Leu Ser Asp Cys Arg Ala Val Leu Val Val Leu Gly Pro Trp Cys Ala
305                 310                 315                 320

gat aaa gta gct gga atg atg gta aga gaa cta cag aaa tac atc aaa    1008
Asp Lys Val Ala Gly Met Met Val Arg Glu Leu Gln Lys Tyr Ile Lys
                325                 330                 335

cat gag caa gag gag ctg cac agg aaa ttt tta ttg ttt aca gac act    1056
His Glu Gln Glu Glu Leu His Arg Lys Phe Leu Leu Phe Thr Asp Thr
            340                 345                 350

ttc cta agg aaa ata cat gca cta tgt gaa gag cac ttc tca cct gcc    1104
Phe Leu Arg Lys Ile His Ala Leu Cys Glu Glu His Phe Ser Pro Ala
        355                 360                 365

tca ctt gac ctg aaa ttt gta act cct aaa gta atc aaa ctg ctc gaa    1152
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    370                 375                 380

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Ile Leu Arg Lys Tyr Lys Pro Tyr Glu Arg His Ser Phe Glu Ser Val
385                 390                 395                 400

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Glu Trp Tyr Asn Asn Arg Asn Gln Asp Asn Tyr Val Ser Trp Ser Asp
                405                 410                 415

tct gag gat gat gat gag gat gaa gaa att gaa gaa aaa gag aag cca    1296
Ser Glu Asp Asp Asp Glu Asp Glu Glu Ile Glu Glu Lys Glu Lys Pro
            420                 425                 430

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Glu Thr Asn Phe Pro Ser Pro Phe Thr Asn Ile Leu Cys Gly Ile Ile
        435                 440                 445

ttt gtg gaa aga aga tac aca gca gtt gtc tta aac aga ttg ata aag    1392
Phe Val Glu Arg Arg Tyr Thr Ala Val Val Leu Asn Arg Leu Ile Lys
    450                 455                 460

gaa gct ggc aaa caa gat cca gag ctg gct tat atc agt agc aat ttc    1440
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465                 470                 475                 480

ata act gga cat ggc att ggg aag aat cag cct cgc aac aac acg atg    1488
Ile Thr Gly His Gly Ile Gly Lys Asn Gln Pro Arg Asn Asn Thr Met
                485                 490                 495

gaa gca gaa ttc aga aaa cag gaa gag gta ctt agg aaa ttt cga gca    1536
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        515                 520                 525

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    530                 535                 540

tat cga tcc tat gtt caa tct aaa gga aga gca agg gca ccc atc tct    1680
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545                 550                 555                 560

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Ser Lys Ser Val Asp Thr Gly Glu Thr Asp Ile Asp Pro Val Met Asp
        595                 600                 605

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Asp Asp His Val Phe Pro Pro Tyr Val Leu Arg Pro Asp Asp Gly Gly
    610                 615                 620

cca cga gtc aca atc aac acg gcc att gga cac atc aat aga tac tgt    1920
Pro Arg Val Thr Ile Asn Thr Ala Ile Gly His Ile Asn Arg Tyr Cys
625                 630                 635                 640

gct aga tta cca agt gat ccg ttt act cat cta gct cct aaa tgc aga    1968
Ala Arg Leu Pro Ser Asp Pro Phe Thr His Leu Ala Pro Lys Cys Arg
                645                 650                 655

acc cga gag ttg cct gat ggt aca ttt tat tca act ctt tat ctg cca    2016
Thr Arg Glu Leu Pro Asp Gly Thr Phe Tyr Ser Thr Leu Tyr Leu Pro
            660                 665                 670

att aac tca cct ctt cga gcc tcc att gtt ggt cca cca atg agc tgt    2064
Ile Asn Ser Pro Leu Arg Ala Ser Ile Val Gly Pro Pro Met Ser Cys
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Val Arg Leu Ala Glu Arg Val Val Ala Leu Ile Cys Cys Glu Lys Leu
    690                 695                 700

cac aaa att ggc gaa ctg gat gac cat ttg atg cca gtt ggg aaa gag    2160
His Lys Ile Gly Glu Leu Asp Asp His Leu Met Pro Val Gly Lys Glu
705                 710                 715                 720

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Thr Val Lys Tyr Glu Glu Glu Leu Asp Leu His Asp Glu Glu Glu Thr
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agt gtt cca gga aga cca ggt tcc acg aaa cga agg cag tgc tac cca    2256
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785                 790                 795                 800

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His Phe Pro Val Tyr Thr Arg Ser Gly Glu Val Thr Ile Ser Ile Glu
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Ser Thr Lys Tyr Thr Lys Glu Thr Pro Phe Val Phe Lys Leu Glu Asp
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Tyr Gln Asp Ala Val Ile Ile Pro Arg Tyr Arg Asn Phe Asp Gln Pro
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His Arg Phe Tyr Val Ala Asp Val Tyr Thr Asp Leu Thr Pro Leu Ser
945                 950                 955                 960

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Lys Phe Pro Ser Pro Glu Tyr Glu Thr Phe Ala Glu Tyr Tyr Lys Thr
                965                 970                 975

aag tac aac ctt gac cta acc aat ctc aac cag cca ctg ctg gat gtg    2976
Lys Tyr Asn Leu Asp Leu Thr Asn Leu Asn Gln Pro Leu Leu Asp Val
            980                 985                 990
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gac cac aca tct tca aga ctt aat ctt ttg aca cct cga cat ttg aat    3024
Asp His Thr Ser Ser Arg Leu Asn Leu Leu Thr Pro Arg His Leu Asn
        995                 1000                1005

cag aag ggg aaa gcg ctt cct tta agc agt gct gag aag agg aaa gcc    3072
Gln Lys Gly Lys Ala Leu Pro Leu Ser Ser Ala Glu Lys Arg Lys Ala
    1010                1015                1020

aaa tgg gaa agt ctg cag aat aaa cag ata ctg gtt cca gaa ctc tgt    3120
Lys Trp Glu Ser Leu Gln Asn Lys Gln Ile Leu Val Pro Glu Leu Cys
1025                1030                1035                1040

gct ata cat cca att cca gca tca ctg tgg aga aaa gct gtt tgt ctc    3168
Ala Ile His Pro Ile Pro Ala Ser Leu Trp Arg Lys Ala Val Cys Leu
                1045                1050                1055

ccc agc ata ctt tat cgc ctt cac tgc ctt ttg act gca gag gag cta    3216
Pro Ser Ile Leu Tyr Arg Leu His Cys Leu Leu Thr Ala Glu Glu Leu
            1060                1065                1070

aga gcc cag act gcc agc gat gct ggc gtg gga gtc aga tca ctt cct    3264
Arg Ala Gln Thr Ala Ser Asp Ala Gly Val Gly Val Arg Ser Leu Pro
        1075                1080                1085

gcg gat ttt aga tac cct aac tta gac ttc ggg tgg aaa aaa tct att    3312
Ala Asp Phe Arg Tyr Pro Asn Leu Asp Phe Gly Trp Lys Lys Ser Ile
    1090                1095                1100

gac agc aaa tct ttc atc tca att tct aac tcc tct tca gct gaa aat    3360
Asp Ser Lys Ser Phe Ile Ser Ile Ser Asn Ser Ser Ser Ala Glu Asn
1105                1110                1115                1120

gat aat tac tgt aag cac agc aca att gtc cct gaa aat gct gca cat    3408
Asp Asn Tyr Cys Lys His Ser Thr Ile Val Pro Glu Asn Ala Ala His
                1125                1130                1135

caa ggt gct aat aga acc tcc tct cta gaa aat cat gac caa atg tct    3456
Gln Gly Ala Asn Arg Thr Ser Ser Leu Glu Asn His Asp Gln Met Ser
            1140                1145                1150

gtg aac tgc aga acg ttg ctc agc gag tcc cct ggt aag ctc cac gtt    3504
Val Asn Cys Arg Thr Leu Leu Ser Glu Ser Pro Gly Lys Leu His Val
        1155                1160                1165

gaa gtt tca gca gat ctt aca gca att aat ggt ctt tct tac aat caa    3552
Glu Val Ser Ala Asp Leu Thr Ala Ile Asn Gly Leu Ser Tyr Asn Gln
    1170                1175                1180

aat ctc gcc aat ggc agt tat gat tta gct aac aga gac ttt tgc caa    3600
Asn Leu Ala Asn Gly Ser Tyr Asp Leu Ala Asn Arg Asp Phe Cys Gln
1185                1190                1195                1200

gga aat cag cta aat tac tac aag cag gaa ata ccc gtg caa cca act    3648
Gly Asn Gln Leu Asn Tyr Tyr Lys Gln Glu Ile Pro Val Gln Pro Thr
                1205                1210                1215

acc tca tat tcc att cag aat tta tac agt tac gag aac cag ccc cag    3696
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Thr Ser Tyr Ser Ile Gln Asn Leu Tyr Ser Tyr Glu Asn Gln Pro Gln
            1220                1225                1230

ccc agc gat gaa tgt act ctc ctg agt aat aaa tac ctt gat gga aat    3744
Pro Ser Asp Glu Cys Thr Leu Leu Ser Asn Lys Tyr Leu Asp Gly Asn
        1235                1240                1245

gct aac aaa tct acc tca gat gga agt cct gtg atg gcc gta atg cct    3792
Ala Asn Lys Ser Thr Ser Asp Gly Ser Pro Val Met Ala Val Met Pro
    1250                1255                1260

ggt acg aca gac act att caa gtg ctc aag ggc agg atg gat tct gag    3840
Gly Thr Thr Asp Thr Ile Gln Val Leu Lys Gly Arg Met Asp Ser Glu
1265                1270                1275                1280

cag agc cct tct att ggg tac tcc tca agg act ctt ggc ccc aat cct    3888
Gln Ser Pro Ser Ile Gly Tyr Ser Ser Arg Thr Leu Gly Pro Asn Pro
                1285                1290                1295

gga ctt att ctt cag gct ttg act ctg tca aac gct agt gat gga ttt    3936
Gly Leu Ile Leu Gln Ala Leu Thr Leu Ser Asn Ala Ser Asp Gly Phe
            1300                1305                1310

aac ctg gag cgg ctt gaa atg ctt ggc gac tcc ttt tta aag cat gcc    3984
Asn Leu Glu Arg Leu Glu Met Leu Gly Asp Ser Phe Leu Lys His Ala
        1315                1320                1325

atc acc aca tat cta ttt tgc act tac cct gat gcg cat gag ggc cgc    4032
Ile Thr Thr Tyr Leu Phe Cys Thr Tyr Pro Asp Ala His Glu Gly Arg
    1330                1335                1340

ctt tca tat atg aga agc aaa aag gtc agc aac tgt aat ctg tat cgc    4080
Leu Ser Tyr Met Arg Ser Lys Lys Val Ser Asn Cys Asn Leu Tyr Arg
1345                1350                1355                1360

ctt gga aaa aag aag gga cta ccc agc cgc atg gtg gtg tca ata ttt    4128
Leu Gly Lys Lys Lys Gly Leu Pro Ser Arg Met Val Val Ser Ile Phe
                1365                1370                1375

gat ccc cct gtg aat tgg ctt cct cct ggt tat gta gta aat caa gac    4176
Asp Pro Pro Val Asn Trp Leu Pro Pro Gly Tyr Val Val Asn Gln Asp
            1380                1385                1390

aaa agc aac aca gat aaa tgg gaa aaa gat gaa atg aca aaa gac tgc    4224
Lys Ser Asn Thr Asp Lys Trp Glu Lys Asp Glu Met Thr Lys Asp Cys
        1395                1400                1405

atg ctg gcg aat ggc aaa ctg gat gag gat tac gag gag gag gat gag    4272
Met Leu Ala Asn Gly Lys Leu Asp Glu Asp Tyr Glu Glu Glu Asp Glu
    1410                1415                1420

gag gag gag agc ctg atg tgg agg gct ccg aag gaa gag gct gac tat    4320
Glu Glu Glu Ser Leu Met Trp Arg Ala Pro Lys Glu Glu Ala Asp Tyr
1425                1430                1435                1440

gaa gat gat ttc ctg gag tat gat cag gaa cat atc aga ttt ata gat    4368
Glu Asp Asp Phe Leu Glu Tyr Asp Gln Glu His Ile Arg Phe Ile Asp
                1445                1450                1455
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aat atg tta atg ggg tca gga gct ttt gta aag aaa atc tct ctt tct   4416
Asn Met Leu Met Gly Ser Gly Ala Phe Val Lys Lys Ile Ser Leu Ser
            1460                1465                1470

cct ttt tca acc act gat tct gca tat gaa tgg aaa atg ccc aaa aaa   4464
Pro Phe Ser Thr Thr Asp Ser Ala Tyr Glu Trp Lys Met Pro Lys Lys
        1475                1480                1485

tcc tcc tta ggt agt atg cca ttt tca tca gat ttt gag gat ttt gac   4512
Ser Ser Leu Gly Ser Met Pro Phe Ser Ser Asp Phe Glu Asp Phe Asp
    1490                1495                1500

tac agc tct tgg gat gca atg tgc tat ctg gat cct agc aaa gct gtt   4560
Tyr Ser Ser Trp Asp Ala Met Cys Tyr Leu Asp Pro Ser Lys Ala Val
1505                1510                1515                1520

gaa gaa gat gac ttt gtg gtg ggg ttc tgg aat cca tca gaa gaa aac   4608
Glu Glu Asp Asp Phe Val Val Gly Phe Trp Asn Pro Ser Glu Glu Asn
                1525                1530                1535

tgt ggt gtt gac acg gga aag cag tcc att tct tac gac ttg cac act   4656
Cys Gly Val Asp Thr Gly Lys Gln Ser Ile Ser Tyr Asp Leu His Thr
            1540                1545                1550

gag cag tgt att gct gac aaa agc ata gcg gac tgt gtg gaa gcc ctg   4704
Glu Gln Cys Ile Ala Asp Lys Ser Ile Ala Asp Cys Val Glu Ala Leu
        1555                1560                1565

ctg ggc tgc tat tta acc agc tgt ggg gag agg gct gct cag ctt ttc   4752
Leu Gly Cys Tyr Leu Thr Ser Cys Gly Glu Arg Ala Ala Gln Leu Phe
    1570                1575                1580

ctc tgt tca ctg ggg ctg aag gtg ctc ccg gta att aaa agg act gat   4800
Leu Cys Ser Leu Gly Leu Lys Val Leu Pro Val Ile Lys Arg Thr Asp
1585                1590                1595                1600

cgg gaa aag gcc ctg tgc cct act cgg gag aat ttc aac agc caa caa   4848
Arg Glu Lys Ala Leu Cys Pro Thr Arg Glu Asn Phe Asn Ser Gln Gln
                1605                1610                1615

aag aac ctt tca gtg agc tgt gct gct gct tct gtg gcc agt tca cgc   4896
Lys Asn Leu Ser Val Ser Cys Ala Ala Ala Ser Val Ala Ser Ser Arg
            1620                1625                1630

tct tct gta ttg aaa gac tcg gaa tat ggt tgt ttg aag att cca cca   4944
Ser Ser Val Leu Lys Asp Ser Glu Tyr Gly Cys Leu Lys Ile Pro Pro
        1635                1640                1645

aga tgt atg ttt gat cat cca gat gca gat aaa aca ctg aat cac ctt   4992
Arg Cys Met Phe Asp His Pro Asp Ala Asp Lys Thr Leu Asn His Leu
    1650                1655                1660

ata tcg ggg ttt gaa aat ttt gaa aag aaa atc aac tac aga ttc aag   5040
Ile Ser Gly Phe Glu Asn Phe Glu Lys Lys Ile Asn Tyr Arg Phe Lys
1665                1670                1675                1680

aat aag gct tac ctt ctc cag gct ttt aca cat gcc tcc tac cac tac   5088
Asn Lys Ala Tyr Leu Leu Gln Ala Phe Thr His Ala Ser Tyr His Tyr
                1685                1690                1695
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aat act atc act gat tgt tac cag cgc tta gaa ttc ctg gga gat gcg   5136
Asn Thr Ile Thr Asp Cys Tyr Gln Arg Leu Glu Phe Leu Gly Asp Ala
            1700                1705                1710

att ttg gac tac ctc ata acc aag cac ctt tat gaa gac ccg cgg cag   5184
Ile Leu Asp Tyr Leu Ile Thr Lys His Leu Tyr Glu Asp Pro Arg Gln
        1715                1720                1725

cac tcc ccg ggg gtc ctg aca gac ctg cgg tct gcc ctg gtc aac aac   5232
His Ser Pro Gly Val Leu Thr Asp Leu Arg Ser Ala Leu Val Asn Asn
    1730                1735                1740

acc atc ttt gca tcg ctg gct gta aag tac gac tac cac aag tac ttc   5280
Thr Ile Phe Ala Ser Leu Ala Val Lys Tyr Asp Tyr His Lys Tyr Phe
1745                1750                1755                1760

aaa gct gtc tct cct gag ctc ttc cat gtc att gat gac ttt gtg cag   5328
Lys Ala Val Ser Pro Glu Leu Phe His Val Ile Asp Asp Phe Val Gln
                1765                1770                1775

ttt cag ctt gag aag aat gaa atg caa gga atg gat tct gag ctt agg   5376
Phe Gln Leu Glu Lys Asn Glu Met Gln Gly Met Asp Ser Glu Leu Arg
            1780                1785                1790

aga tct gag gag gat gaa gag aaa gaa gag gat att gaa gtt cca aag   5424
Arg Ser Glu Glu Asp Glu Glu Lys Glu Glu Asp Ile Glu Val Pro Lys
        1795                1800                1805

gcc atg ggg gat att ttt gag tcg ctt gct ggt gcc att tac atg gat   5472
Ala Met Gly Asp Ile Phe Glu Ser Leu Ala Gly Ala Ile Tyr Met Asp
    1810                1815                1820

agt ggg atg tca ctg gag aca gtc tgg cag gtg tac tat ccc atg atg   5520
Ser Gly Met Ser Leu Glu Thr Val Trp Gln Val Tyr Tyr Pro Met Met
1825                1830                1835                1840

cgg cca cta ata gaa aag ttt tct gca aat gta ccc cgt tcc cct gtg   5568
Arg Pro Leu Ile Glu Lys Phe Ser Ala Asn Val Pro Arg Ser Pro Val
                1845                1850                1855

cga gaa ttg ctt gaa atg gaa cca gaa act gcc aaa ttt agc ccg gct   5616
Arg Glu Leu Leu Glu Met Glu Pro Glu Thr Ala Lys Phe Ser Pro Ala
            1860                1865                1870

gag aga act tac gac ggg aag gtc aga gtc act gtg gaa gta gta gga   5664
Glu Arg Thr Tyr Asp Gly Lys Val Arg Val Thr Val Glu Val Val Gly
        1875                1880                1885

aag ggg aaa ttt aaa ggt gtt ggt cga agt tac agg att gcc aaa tct   5712
Lys Gly Lys Phe Lys Gly Val Gly Arg Ser Tyr Arg Ile Ala Lys Ser
    1890                1895                1900

gca gca gca aga aga gcc ctc cga agc ctc aaa gct aat caa cct cag   5760
Ala Ala Ala Arg Arg Ala Leu Arg Ser Leu Lys Ala Asn Gln Pro Gln
1905                1910                1915                1920

gtt ccc aat agc tga                                               5775
Val Pro Asn Ser  *
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Gln Gln Glu Ala Ile His Asp Asn Ile Tyr Thr Pro Arg Lys Tyr Gln
        35                  40                  45
Val Glu Leu Leu Glu Ala Ala Leu Asp His Asn Thr Ile Val Cys Leu
    50                  55                  60
Asn Thr Gly Ser Gly Lys Thr Phe Ile Ala Ser Thr Thr Leu Leu Lys
65                  70                  75                  80
Ser Cys Leu Tyr Leu Asp Leu Gly Glu Thr Ser Ala Arg Asn Gly Lys
                85                  90                  95
Arg Thr Val Phe Leu Val Asn Ser Ala Asn Gln Val Ala Gln Gln Val
            100                 105                 110
Ser Ala Val Arg Thr His Ser Asp Leu Lys Val Gly Glu Tyr Ser Asn
        115                 120                 125
Leu Glu Val Asn Ala Ser Trp Thr Lys Glu Arg Trp Asn Gln Glu Phe
    130                 135                 140
Thr Lys His Gln Val Leu Ile Met Thr Cys Tyr Val Ala Leu Asn Val
145                 150                 155                 160
Leu Lys Asn Gly Tyr Leu Ser Leu Ser Asp Ile Asn Leu Leu Val Phe
                165                 170                 175
Asp Glu Cys His Leu Ala Ile Leu Asp His Pro Tyr Arg Glu Phe Met
            180                 185                 190
Lys Leu Cys Glu Ile Cys Pro Ser Cys Pro Arg Ile Leu Gly Leu Thr
        195                 200                 205
Ala Ser Ile Leu Asn Gly Lys Trp Asp Pro Glu Asp Leu Glu Glu Lys
    210                 215                 220
Phe Gln Lys Leu Glu Lys Ile Leu Lys Ser Asn Ala Glu Thr Ala Thr
225                 230                 235                 240
Asp Leu Val Val Leu Asp Arg Tyr Thr Ser Gln Pro Cys Glu Ile Val
                245                 250                 255
Val Asp Cys Gly Pro Phe Thr Asp Arg Ser Gly Leu Tyr Glu Arg Leu
            260                 265                 270
Leu Met Glu Leu Glu Glu Ala Leu Asn Phe Ile Asn Asp Cys Asn Ile
        275                 280                 285
Ser Val His Ser Lys Glu Arg Asp Ser Thr Leu Ile Ser Lys Gln Ile
    290                 295                 300
Leu Ser Asp Cys Arg Ala Val Leu Val Val Leu Gly Pro Trp Cys Ala
305                 310                 315                 320
Asp Lys Val Ala Gly Met Met Val Arg Glu Leu Gln Lys Tyr Ile Lys
                325                 330                 335
His Glu Gln Glu Glu Leu His Arg Lys Phe Leu Leu Phe Thr Asp Thr
            340                 345                 350
Phe Leu Arg Lys Ile His Ala Leu Cys Glu Glu His Phe Ser Pro Ala
        355                 360                 365
Ser Leu Asp Leu Lys Phe Val Thr Pro Lys Val Ile Lys Leu Leu Glu
    370                 375                 380
Ile Leu Arg Lys Tyr Lys Pro Tyr Glu Arg His Ser Phe Glu Ser Val
385                 390                 395                 400
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Glu Trp Tyr Asn Asn Arg Asn Gln Asp Asn Tyr Val Ser Trp Ser Asp
                405                 410                 415
Ser Glu Asp Asp Asp Glu Asp Glu Glu Ile Glu Glu Lys Glu Lys Pro
            420                 425                 430
Glu Thr Asn Phe Pro Ser Pro Phe Thr Asn Ile Leu Cys Gly Ile Ile
        435                 440                 445
Phe Val Glu Arg Arg Tyr Thr Ala Val Val Leu Asn Arg Leu Ile Lys
    450                 455                 460
Glu Ala Gly Lys Gln Asp Pro Glu Leu Ala Tyr Ile Ser Ser Asn Phe
465                 470                 475                 480
Ile Thr Gly His Gly Ile Gly Lys Asn Gln Pro Arg Asn Asn Thr Met
                485                 490                 495
Glu Ala Glu Phe Arg Lys Gln Glu Glu Val Leu Arg Lys Phe Arg Ala
            500                 505                 510
His Glu Thr Asn Leu Leu Ile Ala Thr Ser Ile Val Glu Glu Gly Val
        515                 520                 525
Asp Ile Pro Lys Cys Asn Leu Val Val Arg Phe Asp Leu Pro Thr Glu
    530                 535                 540
Tyr Arg Ser Tyr Val Gln Ser Lys Gly Arg Ala Arg Ala Pro Ile Ser
545                 550                 555                 560
Asn Tyr Ile Met Leu Ala Asp Thr Asp Lys Ile Lys Ser Phe Glu Glu
                565                 570                 575
Asp Leu Lys Thr Tyr Lys Ala Ile Glu Lys Ile Leu Arg Asn Lys Cys
            580                 585                 590
Ser Lys Ser Val Asp Thr Gly Glu Thr Asp Ile Asp Pro Val Met Asp
        595                 600                 605
Asp Asp His Val Phe Pro Pro Tyr Val Leu Arg Pro Asp Asp Gly Gly
    610                 615                 620
Pro Arg Val Thr Ile Asn Thr Ala Ile Gly His Ile Asn Arg Tyr Cys
625                 630                 635                 640
Ala Arg Leu Pro Ser Asp Pro Phe Thr His Leu Ala Pro Lys Cys Arg
                645                 650                 655
Thr Arg Glu Leu Pro Asp Gly Thr Phe Tyr Ser Thr Leu Tyr Leu Pro
            660                 665                 670
Ile Asn Ser Pro Leu Arg Ala Ser Ile Val Gly Pro Pro Met Ser Cys
        675                 680                 685
Val Arg Leu Ala Glu Arg Val Val Ala Leu Ile Cys Cys Glu Lys Leu
    690                 695                 700
His Lys Ile Gly Glu Leu Asp Asp His Leu Met Pro Val Gly Lys Glu
705                 710                 715                 720
Thr Val Lys Tyr Glu Glu Glu Leu Asp Leu His Asp Glu Glu Glu Thr
                725                 730                 735
Ser Val Pro Gly Arg Pro Gly Ser Thr Lys Arg Arg Gln Cys Tyr Pro
            740                 745                 750
Lys Ala Ile Pro Glu Cys Leu Arg Asp Ser Tyr Pro Arg Pro Asp Gln
        755                 760                 765
Pro Cys Tyr Leu Tyr Val Ile Gly Met Val Leu Thr Thr Pro Leu Pro
    770                 775                 780
Asp Glu Leu Asn Phe Arg Arg Arg Lys Leu Tyr Pro Pro Glu Asp Thr
785                 790                 795                 800
Thr Arg Cys Phe Gly Ile Leu Thr Ala Lys Pro Ile Pro Gln Ile Pro
                805                 810                 815
His Phe Pro Val Tyr Thr Arg Ser Gly Glu Val Thr Ile Ser Ile Glu
            820                 825                 830
Leu Lys Lys Ser Gly Phe Met Leu Ser Leu Gln Met Leu Glu Leu Ile
        835                 840                 845
Thr Arg Leu His Gln Tyr Ile Phe Ser His Ile Leu Arg Leu Glu Lys
    850                 855                 860
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Pro Ala Leu Glu Phe Lys Pro Thr Asp Ala Asp Ser Ala Tyr Cys Val
865                 870                 875                 880
Leu Pro Leu Asn Val Val Asn Asp Ser Ser Thr Leu Asp Ile Asp Phe
                885                 890                 895
Lys Phe Met Glu Asp Ile Glu Lys Ser Glu Ala Arg Ile Gly Ile Pro
            900                 905                 910
Ser Thr Lys Tyr Thr Lys Glu Thr Pro Phe Val Phe Lys Leu Glu Asp
        915                 920                 925
Tyr Gln Asp Ala Val Ile Ile Pro Arg Tyr Arg Asn Phe Asp Gln Pro
    930                 935                 940
His Arg Phe Tyr Val Ala Asp Val Tyr Thr Asp Leu Thr Pro Leu Ser
945                 950                 955                 960
Lys Phe Pro Ser Pro Glu Tyr Glu Thr Phe Ala Glu Tyr Tyr Lys Thr
                965                 970                 975
Lys Tyr Asn Leu Asp Leu Thr Asn Leu Asn Gln Pro Leu Leu Asp Val
            980                 985                 990
Asp His Thr Ser Ser Arg Leu Asn Leu Leu Thr Pro Arg His Leu Asn
        995                 1000                1005
Gln Lys Gly Lys Ala Leu Pro Leu Ser Ser Ala Glu Lys Arg Lys Ala
    1010                1015                1020
Lys Trp Glu Ser Leu Gln Asn Lys Gln Ile Leu Val Pro Glu Leu Cys
1025                1030                1035                1040
Ala Ile His Pro Ile Pro Ala Ser Leu Trp Arg Lys Ala Val Cys Leu
                1045                1050                1055
Pro Ser Ile Leu Tyr Arg Leu His Cys Leu Leu Thr Ala Glu Glu Leu
            1060                1065                1070
Arg Ala Gln Thr Ala Ser Asp Ala Gly Val Gly Val Arg Ser Leu Pro
        1075                1080                1085
Ala Asp Phe Arg Tyr Pro Asn Leu Asp Phe Gly Trp Lys Lys Ser Ile
    1090                1095                1100
Asp Ser Lys Ser Phe Ile Ser Ile Ser Asn Ser Ser Ser Ala Glu Asn
1105                1110                1115                1120
Asp Asn Tyr Cys Lys His Ser Thr Ile Val Pro Glu Asn Ala Ala His
                1125                1130                1135
Gln Gly Ala Asn Arg Thr Ser Ser Leu Glu Asn His Asp Gln Met Ser
            1140                1145                1150
Val Asn Cys Arg Thr Leu Leu Ser Glu Ser Pro Gly Lys Leu His Val
        1155                1160                1165
Glu Val Ser Ala Asp Leu Thr Ala Ile Asn Gly Leu Ser Tyr Asn Gln
    1170                1175                1180
Asn Leu Ala Asn Gly Ser Tyr Asp Leu Ala Asn Arg Asp Phe Cys Gln
1185                1190                1195                1200
Gly Asn Gln Leu Asn Tyr Tyr Lys Gln Glu Ile Pro Val Gln Pro Thr
                1205                1210                1215
Thr Ser Tyr Ser Ile Gln Asn Leu Tyr Ser Tyr Glu Asn Gln Pro Gln
            1220                1225                1230
Pro Ser Asp Glu Cys Thr Leu Leu Ser Asn Lys Tyr Leu Asp Gly Asn
        1235                1240                1245
Ala Asn Lys Ser Thr Ser Asp Gly Ser Pro Val Met Ala Val Met Pro
    1250                1255                1260
Gly Thr Thr Asp Thr Ile Gln Val Leu Lys Gly Arg Met Asp Ser Glu
1265                1270                1275                1280
Gln Ser Pro Ser Ile Gly Tyr Ser Ser Arg Thr Leu Gly Pro Asn Pro
                1285                1290                1295
Gly Leu Ile Leu Gln Ala Leu Thr Leu Ser Asn Ala Ser Asp Gly Phe
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Asn Leu Glu Arg Leu Glu Met Leu Gly Asp Ser Phe Leu Lys His Ala
        1315                1320                1325
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Ile Thr Thr Tyr Leu Phe Cys Thr Tyr Pro Asp Ala His Glu Gly Arg
    1330                1335                1340
Leu Ser Tyr Met Arg Ser Lys Lys Val Ser Asn Cys Asn Leu Tyr Arg
1345                1350                1355                1360
Leu Gly Lys Lys Lys Gly Leu Pro Ser Arg Met Val Val Ser Ile Phe
                1365                1370                1375
Asp Pro Pro Val Asn Trp Leu Pro Pro Gly Tyr Val Val Asn Gln Asp
            1380                1385                1390
Lys Ser Asn Thr Asp Lys Trp Glu Lys Asp Glu Met Thr Lys Asp Cys
        1395                1400                1405
Met Leu Ala Asn Gly Lys Leu Asp Glu Asp Tyr Glu Glu Glu Asp Glu
    1410                1415                1420
Glu Glu Glu Ser Leu Met Trp Arg Ala Pro Lys Glu Glu Ala Asp Tyr
1425                1430                1435                1440
Glu Asp Asp Phe Leu Glu Tyr Asp Gln Glu His Ile Arg Phe Ile Asp
                1445                1450                1455
Asn Met Leu Met Gly Ser Gly Ala Phe Val Lys Lys Ile Ser Leu Ser
            1460                1465                1470
Pro Phe Ser Thr Thr Asp Ser Ala Tyr Glu Trp Lys Met Pro Lys Lys
        1475                1480                1485
Ser Ser Leu Gly Ser Met Pro Phe Ser Ser Asp Phe Glu Asp Phe Asp
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Tyr Ser Ser Trp Asp Ala Met Cys Tyr Leu Asp Pro Ser Lys Ala Val
1505                1510                1515                1520
Glu Glu Asp Asp Phe Val Val Gly Phe Trp Asn Pro Ser Glu Glu Asn
                1525                1530                1535
Cys Gly Val Asp Thr Gly Lys Gln Ser Ile Ser Tyr Asp Leu His Thr
            1540                1545                1550
Glu Gln Cys Ile Ala Asp Lys Ser Ile Ala Asp Cys Val Glu Ala Leu
        1555                1560                1565
Leu Gly Cys Tyr Leu Thr Ser Cys Gly Glu Arg Ala Ala Gln Leu Phe
    1570                1575                1580
Leu Cys Ser Leu Gly Leu Lys Val Leu Pro Val Ile Lys Arg Thr Asp
1585                1590                1595                1600
Arg Glu Lys Ala Leu Cys Pro Thr Arg Glu Asn Phe Asn Ser Gln Gln
                1605                1610                1615
Lys Asn Leu Ser Val Ser Cys Ala Ala Ala Ser Val Ala Ser Ser Arg
            1620                1625                1630
Ser Ser Val Leu Lys Asp Ser Glu Tyr Gly Cys Leu Lys Ile Pro Pro
        1635                1640                1645
Arg Cys Met Phe Asp His Pro Asp Ala Asp Lys Thr Leu Asn His Leu
    1650                1655                1660
Ile Ser Gly Phe Glu Asn Phe Glu Lys Lys Ile Asn Tyr Arg Phe Lys
1665                1670                1675                1680
Asn Lys Ala Tyr Leu Leu Gln Ala Phe Thr His Ala Ser Tyr His Tyr
                1685                1690                1695
Asn Thr Ile Thr Asp Cys Tyr Gln Arg Leu Glu Phe Leu Gly Asp Ala
            1700                1705                1710
Ile Leu Asp Tyr Leu Ile Thr Lys His Leu Tyr Glu Asp Pro Arg Gln
        1715                1720                1725
His Ser Pro Gly Val Leu Thr Asp Leu Arg Ser Ala Leu Val Asn Asn
    1730                1735                1740
Thr Ile Phe Ala Ser Leu Ala Val Lys Tyr Asp Tyr His Lys Tyr Phe
1745                1750                1755                1760
Lys Ala Val Ser Pro Glu Leu Phe His Val Ile Asp Asp Phe Val Gln
                1765                1770                1775
Phe Gln Leu Glu Lys Asn Glu Met Gln Gly Met Asp Ser Glu Leu Arg
            1780                1785                1790
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Arg Ser Glu Glu Asp Glu Glu Lys Glu Glu Asp Ile Glu Val Pro Lys
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Ala Met Gly Asp Ile Phe Glu Ser Leu Ala Gly Ala Ile Tyr Met Asp
    1810                1815                1820
Ser Gly Met Ser Leu Glu Thr Val Trp Gln Val Tyr Tyr Pro Met Met
1825                1830                1835                1840
Arg Pro Leu Ile Glu Lys Phe Ser Ala Asn Val Pro Arg Ser Pro Val
                1845                1850                1855
Arg Glu Leu Leu Glu Met Glu Pro Glu Thr Ala Lys Phe Ser Pro Ala
            1860                1865                1870
Glu Arg Thr Tyr Asp Gly Lys Val Arg Val Thr Val Glu Val Val Gly
        1875                1880                1885
Lys Gly Lys Phe Lys Gly Val Gly Arg Ser Tyr Arg Ile Ala Lys Ser
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1905                1910                1915                1920
Val Pro Asn Ser
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Met Ala Phe His Trp Cys Asp Asn Asn Leu His Thr Thr Val Phe Thr
 1               5                  10                  15

ccg cgc gac ttt cag gtg gag cta ctg gcc acc gcc tac gag cgg aac    96
Pro Arg Asp Phe Gln Val Glu Leu Leu Ala Thr Ala Tyr Glu Arg Asn
            20                  25                  30

acg att att tgc ctg ggc cat cga agt tcc aag gag ttt ata gcc ctc    144
Thr Ile Ile Cys Leu Gly His Arg Ser Ser Lys Glu Phe Ile Ala Leu
        35                  40                  45

aag ctg ctc cag gag ctg tcg cgt cga gca cgc cga cat ggt cgt gtc    192
Lys Leu Leu Gln Glu Leu Ser Arg Arg Ala Arg Arg His Gly Arg Val
    50                  55                  60

agt gtc tat ctc agt tgc gag gtt ggc acc agc acg gaa cca tgc tcc    240
Ser Val Tyr Leu Ser Cys Glu Val Gly Thr Ser Thr Glu Pro Cys Ser
65                  70                  75                  80

atc tac acg atg ctc acc cac ttg act gac ctg cgg gtg tgg cag gag    288
Ile Tyr Thr Met Leu Thr His Leu Thr Asp Leu Arg Val Trp Gln Glu
                85                  90                  95

cag ccg gat atg caa att ccc ttt gat cat tgc tgg acg gac tat cac    336
Gln Pro Asp Met Gln Ile Pro Phe Asp His Cys Trp Thr Asp Tyr His
            100                 105                 110

gtt tcc atc cta cgg cca gag gga ttt ctt tat ctg ctc gaa act cgc    384
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Val Ser Ile Leu Arg Pro Glu Gly Phe Leu Tyr Leu Leu Glu Thr Arg
        115                 120                 125

gag ctg ctg ctg agc agc gtc gaa ctg atc gtg ctg gaa gat tgt cat    432
Glu Leu Leu Leu Ser Ser Val Glu Leu Ile Val Leu Glu Asp Cys His
    130                 135                 140

gac agc gcc gtt tat cag agg ata agg cct ctg ttc gag aat cac att    480
Asp Ser Ala Val Tyr Gln Arg Ile Arg Pro Leu Phe Glu Asn His Ile
145                 150                 155                 160

atg cca gcg cca ccg gcg gac agg cca cgg att ctc gga ctc gct gga    528
Met Pro Ala Pro Pro Ala Asp Arg Pro Arg Ile Leu Gly Leu Ala Gly
                165                 170                 175

ccg ctg cac agc gcc gga tgt gag ctg cag caa ctg agc gcc atg ctg    576
Pro Leu His Ser Ala Gly Cys Glu Leu Gln Gln Leu Ser Ala Met Leu
            180                 185                 190

gcc acc ctg gag cag agt gtg ctt tgc cag atc gag acg gcc agt gat    624
Ala Thr Leu Glu Gln Ser Val Leu Cys Gln Ile Glu Thr Ala Ser Asp
        195                 200                 205

att gtc acc gtg ttg cgt tac tgt tcc cga ccg cac gaa tac atc gta    672
Ile Val Thr Val Leu Arg Tyr Cys Ser Arg Pro His Glu Tyr Ile Val
    210                 215                 220

cag tgc gcc ccc ttc gag atg gac gaa ctg tcc ctg gtg ctt gcc gat    720
Gln Cys Ala Pro Phe Glu Met Asp Glu Leu Ser Leu Val Leu Ala Asp
225                 230                 235                 240

gtg ctc aac aca cac aag tcc ttt tta ttg gac cac cgc tac gat ccc    768
Val Leu Asn Thr His Lys Ser Phe Leu Leu Asp His Arg Tyr Asp Pro
                245                 250                 255

tac gaa atc tac ggc aca gac cag ttt atg gac gaa ctg aaa gac ata    816
Tyr Glu Ile Tyr Gly Thr Asp Gln Phe Met Asp Glu Leu Lys Asp Ile
            260                 265                 270

ccc gat ccc aag gtg gac ccc ctg aac gtc atc aac tca cta ctg gtc    864
Pro Asp Pro Lys Val Asp Pro Leu Asn Val Ile Asn Ser Leu Leu Val
        275                 280                 285

gtg ctg cac gag atg ggt cct tgg tgc acg cag cgg gct gca cat cac    912
Val Leu His Glu Met Gly Pro Trp Cys Thr Gln Arg Ala Ala His His
    290                 295                 300

ttt tac caa tgc aat gag aag tta aag gtg aag acg ccg cac gaa cgt    960
Phe Tyr Gln Cys Asn Glu Lys Leu Lys Val Lys Thr Pro His Glu Arg
305                 310                 315                 320

cac tac ttg ctg tac tgc cta gtg agc acg gcc ctt atc caa ctg tac    1008
His Tyr Leu Leu Tyr Cys Leu Val Ser Thr Ala Leu Ile Gln Leu Tyr
                325                 330                 335

tcc ctc tgc gaa cac gca ttc cat cga cat tta gga agt ggc agc gat    1056
Ser Leu Cys Glu His Ala Phe His Arg His Leu Gly Ser Gly Ser Asp
            340                 345                 350
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tca cgc cag acc atc gaa cgc tat tcc agc ccc aag gtg cga cgt ctg    1104
Ser Arg Gln Thr Ile Glu Arg Tyr Ser Ser Pro Lys Val Arg Arg Leu
        355                 360                 365

ttg cag aca ctg agg tgc ttc aag ccg gaa gag gtg cac acc caa gcg    1152
Leu Gln Thr Leu Arg Cys Phe Lys Pro Glu Glu Val His Thr Gln Ala
    370                 375                 380

gac gga ctg cgc aga atg cgg cat cag gtg gat cag gcg gac ttc aat    1200
Asp Gly Leu Arg Arg Met Arg His Gln Val Asp Gln Ala Asp Phe Asn
385                 390                 395                 400

cgg tta tct cat acg ctg gaa agc aag tgc cga atg gtg gat caa atg    1248
Arg Leu Ser His Thr Leu Glu Ser Lys Cys Arg Met Val Asp Gln Met
                405                 410                 415

gac caa ccg ccg acg gag aca cga gcc ctg gtg gcc act ctt gag cag    1296
Asp Gln Pro Pro Thr Glu Thr Arg Ala Leu Val Ala Thr Leu Glu Gln
            420                 425                 430

att ctg cac acg aca gag gac agg cag acg aac aga agc gcc gct cgg    1344
Ile Leu His Thr Thr Glu Asp Arg Gln Thr Asn Arg Ser Ala Ala Arg
        435                 440                 445

gtg act cct act cct act ccc gct cat gcg aag ccg aaa cct agc tct    1392
Val Thr Pro Thr Pro Thr Pro Ala His Ala Lys Pro Lys Pro Ser Ser
    450                 455                 460

ggt gcc aac act gca caa cca cga act cgt aga cgt gtg tac acc agg    1440
Gly Ala Asn Thr Ala Gln Pro Arg Thr Arg Arg Arg Val Tyr Thr Arg
465                 470                 475                 480

cgc cac cac cgg gat cac aat gat ggc agc gac acg ctc tgc gca ctg    1488
Arg His His Arg Asp His Asn Asp Gly Ser Asp Thr Leu Cys Ala Leu
                485                 490                 495

att tac tgc aac cag aac cac acg gct cgc gtg ctc ttt gag ctt cta    1536
Ile Tyr Cys Asn Gln Asn His Thr Ala Arg Val Leu Phe Glu Leu Leu
            500                 505                 510

gcg gag att agc aga cgt gat ccc gat ctc aag ttc cta cgc tgc cag    1584
Ala Glu Ile Ser Arg Arg Asp Pro Asp Leu Lys Phe Leu Arg Cys Gln
        515                 520                 525

tac acc acg gac cgg gtg gca gat ccc acc acg gag ccc aaa gag gct    1632
Tyr Thr Thr Asp Arg Val Ala Asp Pro Thr Thr Glu Pro Lys Glu Ala
    530                 535                 540

gag ttg gag cac cgg cgg cag gaa gag gtg cta aag cgc ttc cgc atg    1680
Glu Leu Glu His Arg Arg Gln Glu Glu Val Leu Lys Arg Phe Arg Met
545                 550                 555                 560

cat gac tgc aat gtc ctg atc ggt act tcg gtg ctg gaa gag ggc atc    1728
His Asp Cys Asn Val Leu Ile Gly Thr Ser Val Leu Glu Glu Gly Ile
                565                 570                 575

gat gtg ccc aag tgc aat ttg gtt gtg cgc tgg gat ccg cca acc aca    1776
Asp Val Pro Lys Cys Asn Leu Val Val Arg Trp Asp Pro Pro Thr Thr
            580                 585                 590
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tat cgc agt tac gtt cag tgc aaa ggt cga gcc cgt gct gct cca gcc    1824
Tyr Arg Ser Tyr Val Gln Cys Lys Gly Arg Ala Arg Ala Ala Pro Ala
        595                 600                 605

tat cat gtc att ctc gtc gct ccg agt tat aaa agc cca act gtg ggg    1872
Tyr His Val Ile Leu Val Ala Pro Ser Tyr Lys Ser Pro Thr Val Gly
    610                 615                 620

tca gtg cag ctg acc gat cgg agt cat cgg tat att tgc gcg act ggt    1920
Ser Val Gln Leu Thr Asp Arg Ser His Arg Tyr Ile Cys Ala Thr Gly
625                 630                 635                 640

gat act aca gag gcg gac agc gac tct gat gat tca gcg atg cca aac    1968
Asp Thr Thr Glu Ala Asp Ser Asp Ser Asp Asp Ser Ala Met Pro Asn
                645                 650                 655

tcg tcc ggc tcg gat ccc tat act ttt ggc acg gca cgc gga acc gtg    2016
Ser Ser Gly Ser Asp Pro Tyr Thr Phe Gly Thr Ala Arg Gly Thr Val
            660                 665                 670

aag atc ctc aac ccc gaa gtg ttc agt aaa caa cca ccg aca gcg tgc    2064
Lys Ile Leu Asn Pro Glu Val Phe Ser Lys Gln Pro Pro Thr Ala Cys
        675                 680                 685

gac att aag ctg cag gag atc cag gac gaa ttg cca gcc gca gcg cag    2112
Asp Ile Lys Leu Gln Glu Ile Gln Asp Glu Leu Pro Ala Ala Ala Gln
    690                 695                 700

ctg gat acg agc aac tcc agc gac gaa gcc gtc agc atg agt aac acg    2160
Leu Asp Thr Ser Asn Ser Ser Asp Glu Ala Val Ser Met Ser Asn Thr
705                 710                 715                 720

tct cca agc gag agc agt aca gaa caa aaa tcc aga cgc ttc cag tgc    2208
Ser Pro Ser Glu Ser Ser Thr Glu Gln Lys Ser Arg Arg Phe Gln Cys
                725                 730                 735

gag ctg agc tct tta acg gag cca gaa gac aca agt gat act aca gcc    2256
Glu Leu Ser Ser Leu Thr Glu Pro Glu Asp Thr Ser Asp Thr Thr Ala
            740                 745                 750

gaa atc gat act gct cat agt tta gcc agc acc acg aag gac ttg gtg    2304
Glu Ile Asp Thr Ala His Ser Leu Ala Ser Thr Thr Lys Asp Leu Val
        755                 760                 765

cat caa atg gca cag tat cgc gaa atc gag cag atg ctg cta tcc aag    2352
His Gln Met Ala Gln Tyr Arg Glu Ile Glu Gln Met Leu Leu Ser Lys
    770                 775                 780

tgc gcc aac aca gag ccg ccg gag cag gag cag agt gag gcg gaa cgt    2400
Cys Ala Asn Thr Glu Pro Pro Glu Gln Glu Gln Ser Glu Ala Glu Arg
785                 790                 795                 800

ttt agt gcc tgc ctg gcc gca tac cga ccc aag ccg cac ctg cta aca    2448
Phe Ser Ala Cys Leu Ala Ala Tyr Arg Pro Lys Pro His Leu Leu Thr
                805                 810                 815

ggc gcc tcc gtg gat ctg ggt tct gct ata gct ttg gtc aac aag tac    2496
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Gly Ala Ser Val Asp Leu Gly Ser Ala Ile Ala Leu Val Asn Lys Tyr
            820                 825                 830

tgc gcc cga ctg cca agc gac acg ttc acc aag ttg acg gcg ttg tgg    2544
Cys Ala Arg Leu Pro Ser Asp Thr Phe Thr Lys Leu Thr Ala Leu Trp
        835                 840                 845

cgc tgc acc cga aac gaa agg gct gga gtg acc ctg ttt cag tac aca    2592
Arg Cys Thr Arg Asn Glu Arg Ala Gly Val Thr Leu Phe Gln Tyr Thr
    850                 855                 860

ctc cgt ctg ccc atc aac tcg cca ttg aag cat gac att gtg ggt ctt    2640
Leu Arg Leu Pro Ile Asn Ser Pro Leu Lys His Asp Ile Val Gly Leu
865                 870                 875                 880

ccg atg cca act caa aca ttg gcc cgc cga ctg gct gcc ttg cag gct    2688
Pro Met Pro Thr Gln Thr Leu Ala Arg Arg Leu Ala Ala Leu Gln Ala
                885                 890                 895

tgc gtg gaa ctg cac agg atc ggt gag tta gac gat cag ttg cag cct    2736
Cys Val Glu Leu His Arg Ile Gly Glu Leu Asp Asp Gln Leu Gln Pro
            900                 905                 910

atc ggc aag gag gga ttt cgt gcc ctg gag ccg gac tgg gag tgc ttt    2784
Ile Gly Lys Glu Gly Phe Arg Ala Leu Glu Pro Asp Trp Glu Cys Phe
        915                 920                 925

gaa ctg gag cca gag gac gaa cag att gtg cag cta agc gat gaa cca    2832
Glu Leu Glu Pro Glu Asp Glu Gln Ile Val Gln Leu Ser Asp Glu Pro
    930                 935                 940

cgt ccg gga aca acg aag cgt cgt cag tac tat tac aaa cgc att gca    2880
Arg Pro Gly Thr Thr Lys Arg Arg Gln Tyr Tyr Tyr Lys Arg Ile Ala
945                 950                 955                 960

tcc gaa ttt tgc gat tgc cgt ccc gtt gcc gga gcg cca tgc tat ttg    2928
Ser Glu Phe Cys Asp Cys Arg Pro Val Ala Gly Ala Pro Cys Tyr Leu
                965                 970                 975

tac ttt atc caa ctg acg ctc caa tgt ccg att ccc gaa gag caa aac    2976
Tyr Phe Ile Gln Leu Thr Leu Gln Cys Pro Ile Pro Glu Glu Gln Asn
            980                 985                 990

acg cgg gga cgc aag att tat ccg ccc gaa gat gcg cag cag gga ttc    3024
Thr Arg Gly Arg Lys Ile Tyr Pro Pro Glu Asp Ala Gln Gln Gly Phe
        995                 1000                1005

ggc att cta acc acc aaa cgg ata ccc aag ctg agt gct ttc tcg ata    3072
Gly Ile Leu Thr Thr Lys Arg Ile Pro Lys Leu Ser Ala Phe Ser Ile
    1010                1015                1020

ttc acg cgt tcc ggt gag gtg aag gtt tcc ctg gag tta gct aag gaa    3120
Phe Thr Arg Ser Gly Glu Val Lys Val Ser Leu Glu Leu Ala Lys Glu
1025                1030                1035                1040

cgc gtg att cta act agc gaa caa ata gtc tgc atc aac gga ttt tta    3168
Arg Val Ile Leu Thr Ser Glu Gln Ile Val Cys Ile Asn Gly Phe Leu
                1045                1050                1055
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aac tac acg ttc acc aat gta ctg cgt ttg caa aag ttt ctg atg ctc    3216
Asn Tyr Thr Phe Thr Asn Val Leu Arg Leu Gln Lys Phe Leu Met Leu
            1060                1065                1070

ttc gat ccg gac tcc acg gaa aat tgt gta ttc att gtg ccc acc gtg    3264
Phe Asp Pro Asp Ser Thr Glu Asn Cys Val Phe Ile Val Pro Thr Val
        1075                1080                1085

aag gca cca gct ggc ggc aag cac atc gac tgg cag ttt ctg gag ctg    3312
Lys Ala Pro Ala Gly Gly Lys His Ile Asp Trp Gln Phe Leu Glu Leu
    1090                1095                1100

atc caa gcg aat gga aat aca atg cca cgg gca gtg ccc gat gag gag    3360
Ile Gln Ala Asn Gly Asn Thr Met Pro Arg Ala Val Pro Asp Glu Glu
1105                1110                1115                1120

cgc cag gcg cag ccg ttt gat ccg caa cgc ttc cag gat gcc gtc gtt    3408
Arg Gln Ala Gln Pro Phe Asp Pro Gln Arg Phe Gln Asp Ala Val Val
                1125                1130                1135

atg ccg tgg tat cgc aac cag gat caa ccg cag tat ttc tat gtg gcg    3456
Met Pro Trp Tyr Arg Asn Gln Asp Gln Pro Gln Tyr Phe Tyr Val Ala
            1140                1145                1150

gag ata tgt cca cat cta tcc cca ctc agc tgc ttc cct ggt gac aac    3504
Glu Ile Cys Pro His Leu Ser Pro Leu Ser Cys Phe Pro Gly Asp Asn
        1155                1160                1165

tac cgc acg ttc aag cac tac tac ctc gtc aag tat ggt ctg acc ata    3552
Tyr Arg Thr Phe Lys His Tyr Tyr Leu Val Lys Tyr Gly Leu Thr Ile
    1170                1175                1180

cag aat acc tcg cag ccg cta ttg gac gtg gat cac acc agt gcg cgg    3600
Gln Asn Thr Ser Gln Pro Leu Leu Asp Val Asp His Thr Ser Ala Arg
1185                1190                1195                1200

tta aac ttc ctc acg cca cga tac gtt aat cgc aag ggc gtt gct ctg    3648
Leu Asn Phe Leu Thr Pro Arg Tyr Val Asn Arg Lys Gly Val Ala Leu
                1205                1210                1215

ccc act agt tcg gag gag aca aag cgg gca aag cgc gag aat ctc gaa    3696
Pro Thr Ser Ser Glu Glu Thr Lys Arg Ala Lys Arg Glu Asn Leu Glu
            1220                1225                1230

cag aag cag atc ctt gtg cca gag ctc tgc act gtg cat cca ttc ccc    3744
Gln Lys Gln Ile Leu Val Pro Glu Leu Cys Thr Val His Pro Phe Pro
        1235                1240                1245

gcc tcc ttg tgg cga act gcc gtg tgc ctg ccc tgc atc ctg tac cgc    3792
Ala Ser Leu Trp Arg Thr Ala Val Cys Leu Pro Cys Ile Leu Tyr Arg
    1250                1255                1260

ata aat ggt ctt cta ttg gcc gac gat att cgg aaa cag gtt tct gcg    3840
Ile Asn Gly Leu Leu Leu Ala Asp Asp Ile Arg Lys Gln Val Ser Ala
1265                1270                1275                1280

gat ctg ggg ctg gga agg caa cag atc gaa gat gag gat ttc gag tgg    3888
Asp Leu Gly Leu Gly Arg Gln Gln Ile Glu Asp Glu Asp Phe Glu Trp
                1285                1290                1295
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ccc atg ctg gac ttt ggg tgg agt cta tcg gag gtg ctc aag aaa tcg    3936
Pro Met Leu Asp Phe Gly Trp Ser Leu Ser Glu Val Leu Lys Lys Ser
            1300                1305                1310

cgg gag tcc aaa caa aag gag tcc ctt aag gat gat act att aat ggc    3984
Arg Glu Ser Lys Gln Lys Glu Ser Leu Lys Asp Asp Thr Ile Asn Gly
        1315                1320                1325

aaa gac tta gct gat gtt gaa aag aaa ccg act agc gag gag acc caa    4032
Lys Asp Leu Ala Asp Val Glu Lys Lys Pro Thr Ser Glu Glu Thr Gln
    1330                1335                1340

cta gat aag gat tca aaa gac gat aag gtt gag aaa agt gct att gaa    4080
Leu Asp Lys Asp Ser Lys Asp Asp Lys Val Glu Lys Ser Ala Ile Glu
1345                1350                1355                1360

cta atc att gag gga gag gag aag ctg caa gag gct gat gac ttc att    4128
Leu Ile Ile Glu Gly Glu Glu Lys Leu Gln Glu Ala Asp Asp Phe Ile
                1365                1370                1375

gag ata ggc act tgg tca aac gat atg gcc gac gat ata gct agt ttt    4176
Glu Ile Gly Thr Trp Ser Asn Asp Met Ala Asp Asp Ile Ala Ser Phe
            1380                1385                1390

aac caa gaa gac gac gac gag gat gac gcc ttc cat ctc cca gtt tta    4224
Asn Gln Glu Asp Asp Asp Glu Asp Asp Ala Phe His Leu Pro Val Leu
        1395                1400                1405

ccg gca aac gtt aag ttc tgt gat cag caa acg cgc tac ggt tcg ccc    4272
Pro Ala Asn Val Lys Phe Cys Asp Gln Gln Thr Arg Tyr Gly Ser Pro
    1410                1415                1420

aca ttt tgg gat gtg agc aat ggc gaa agc ggc ttc aag ggt cca aag    4320
Thr Phe Trp Asp Val Ser Asn Gly Glu Ser Gly Phe Lys Gly Pro Lys
1425                1430                1435                1440

agc agt cag aat aag cag ggt ggc aag ggc aaa gca aag ggt ccg gca    4368
Ser Ser Gln Asn Lys Gln Gly Gly Lys Gly Lys Ala Lys Gly Pro Ala
                1445                1450                1455

aag ccc aca ttt aac tat tat gac tcg gac aat tcg ctg ggt tcc agc    4416
Lys Pro Thr Phe Asn Tyr Tyr Asp Ser Asp Asn Ser Leu Gly Ser Ser
            1460                1465                1470

tac gat gac gac gat aac gca ggt ccg ctc aat tac atg cat cac aac    4464
Tyr Asp Asp Asp Asp Asn Ala Gly Pro Leu Asn Tyr Met His His Asn
        1475                1480                1485

tac agt tcg gat gac gac gat gtg gca gat gat atc gat gcg gga cgc    4512
Tyr Ser Ser Asp Asp Asp Asp Val Ala Asp Asp Ile Asp Ala Gly Arg
    1490                1495                1500

att gcg ttc acc tcc aag aat gaa gcg gag act att gaa acc gca cag    4560
Ile Ala Phe Thr Ser Lys Asn Glu Ala Glu Thr Ile Glu Thr Ala Gln
1505                1510                1515                1520

gaa gtg gaa aag cgc cag aag cag ctg tcc atc atc cag gcg acc aat    4608
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Glu Val Glu Lys Arg Gln Lys Gln Leu Ser Ile Ile Gln Ala Thr Asn
                1525                1530                1535

gct aac gag cgg cag tat cag cag aca aag aac ctg ctc att gga ttc    4656
Ala Asn Glu Arg Gln Tyr Gln Gln Thr Lys Asn Leu Leu Ile Gly Phe
            1540                1545                1550

aat ttt aag cat gag gac cag aag gaa cct gcc act ata aga tat gaa    4704
Asn Phe Lys His Glu Asp Gln Lys Glu Pro Ala Thr Ile Arg Tyr Glu
        1555                1560                1565

gaa tcc ata gct aag ctc aaa acg gaa ata gaa tcc ggc ggc atg ttg    4752
Glu Ser Ile Ala Lys Leu Lys Thr Glu Ile Glu Ser Gly Gly Met Leu
    1570                1575                1580

gtg ccg cac gac cag cag ttg gtt cta aaa aga agt gat gcc gct gag    4800
Val Pro His Asp Gln Gln Leu Val Leu Lys Arg Ser Asp Ala Ala Glu
1585                1590                1595                1600

gct cag gtt gca aag gta tcg atg atg gag cta ttg aag cag ctg ctg    4848
Ala Gln Val Ala Lys Val Ser Met Met Glu Leu Leu Lys Gln Leu Leu
                1605                1610                1615

ccg tat gta aat gaa gat gtg ctg gcc aaa aag ctg ggt gat agg cgc    4896
Pro Tyr Val Asn Glu Asp Val Leu Ala Lys Lys Leu Gly Asp Arg Arg
            1620                1625                1630

gag ctt ctg ctg tcg gat ttg gta gag cta aat gca gat tgg gta gcg    4944
Glu Leu Leu Leu Ser Asp Leu Val Glu Leu Asn Ala Asp Trp Val Ala
        1635                1640                1645

cga cat gag cag gag acc tac aat gta atg gga tgc gga gat agt ttt    4992
Arg His Glu Gln Glu Thr Tyr Asn Val Met Gly Cys Gly Asp Ser Phe
    1650                1655                1660

gac aac tat aac gat cat cat cgg ctg aac ttg gat gaa aag caa ctg    5040
Asp Asn Tyr Asn Asp His His Arg Leu Asn Leu Asp Glu Lys Gln Leu
1665                1670                1675                1680

aaa ctg caa tac gaa cga att gaa att gag cca cct act tcc acg aag    5088
Lys Leu Gln Tyr Glu Arg Ile Glu Ile Glu Pro Pro Thr Ser Thr Lys
                1685                1690                1695

gcc ata acc tca gcc ata tta cca gct ggc ttc agt ttc gat cga caa    5136
Ala Ile Thr Ser Ala Ile Leu Pro Ala Gly Phe Ser Phe Asp Arg Gln
            1700                1705                1710

ccg gat cta gtg ggc cat cca gga ccc agt ccc agc atc att ttg caa    5184
Pro Asp Leu Val Gly His Pro Gly Pro Ser Pro Ser Ile Ile Leu Gln
        1715                1720                1725

gcc ctc aca atg tcc aat gct aac gat ggc atc aat ctg gag cga ctg    5232
Ala Leu Thr Met Ser Asn Ala Asn Asp Gly Ile Asn Leu Glu Arg Leu
    1730                1735                1740

gag aca att gga gat tcc ttt cta aag tat gcc att acc acc tac ttg    5280
Glu Thr Ile Gly Asp Ser Phe Leu Lys Tyr Ala Ile Thr Thr Tyr Leu
1745                1750                1755                1760
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tac atc acc tac gag aat gtg cac gag gga aaa cta agt cac ctg cgc    5328
Tyr Ile Thr Tyr Glu Asn Val His Glu Gly Lys Leu Ser His Leu Arg
                1765                1770                1775

tcc aag cag gtt gcc aat ctc aat ctc tat cgt ctg ggc aga cgt aag    5376
Ser Lys Gln Val Ala Asn Leu Asn Leu Tyr Arg Leu Gly Arg Arg Lys
            1780                1785                1790

aga ctg ggt gaa tat atg ata gcc act aaa ttc gag ccg cac gac aat    5424
Arg Leu Gly Glu Tyr Met Ile Ala Thr Lys Phe Glu Pro His Asp Asn
        1795                1800                1805

tgg ctg cca ccc tgc tac tac gtg cca aag gag cta gag aag gcg ctc    5472
Trp Leu Pro Pro Cys Tyr Tyr Val Pro Lys Glu Leu Glu Lys Ala Leu
    1810                1815                1820

atc gag gcg aag atc ccc act cac cat tgg aag ctg gcc gat ctg cta    5520
Ile Glu Ala Lys Ile Pro Thr His His Trp Lys Leu Ala Asp Leu Leu
1825                1830                1835                1840

gac att aag aac cta agc agt gtg caa atc tgc gag atg gtt cgc gaa    5568
Asp Ile Lys Asn Leu Ser Ser Val Gln Ile Cys Glu Met Val Arg Glu
                1845                1850                1855

aaa gcc gat gcc ctg ggc ttg gag cag aat ggg ggt gcc caa aat gga    5616
Lys Ala Asp Ala Leu Gly Leu Glu Gln Asn Gly Gly Ala Gln Asn Gly
            1860                1865                1870

caa ctt gac gac tcc aat gat agc tgc aat gat ttt agc tgt ttt att    5664
Gln Leu Asp Asp Ser Asn Asp Ser Cys Asn Asp Phe Ser Cys Phe Ile
        1875                1880                1885

ccc tac aac ctt gtt tcg caa cac agc att ccg gat aag tct att gcc    5712
Pro Tyr Asn Leu Val Ser Gln His Ser Ile Pro Asp Lys Ser Ile Ala
    1890                1895                1900

gat tgc gtc gaa gcc ctc att gga gcc tat ctc att gag tgc gga ccc    5760
Asp Cys Val Glu Ala Leu Ile Gly Ala Tyr Leu Ile Glu Cys Gly Pro
1905                1910                1915                1920

cga ggg gct tta ctc ttt atg gcc tgg ctg ggc gtg aga gtg ctc cct    5808
Arg Gly Ala Leu Leu Phe Met Ala Trp Leu Gly Val Arg Val Leu Pro
                1925                1930                1935

atc aca agg cag ttg gac ggg ggt aac cag gag caa cga ata ccc ggt    5856
Ile Thr Arg Gln Leu Asp Gly Gly Asn Gln Glu Gln Arg Ile Pro Gly
            1940                1945                1950

agc aca aaa ccg aat gcc gaa aat gtg gtc acc gtt tac ggt gca tgg    5904
Ser Thr Lys Pro Asn Ala Glu Asn Val Val Thr Val Tyr Gly Ala Trp
        1955                1960                1965

ccc acg ccg cgt agt cca ctg ctg cac ttt gct cca aat gct acg gag    5952
Pro Thr Pro Arg Ser Pro Leu Leu His Phe Ala Pro Asn Ala Thr Glu
    1970                1975                1980

gag ctg gac cag tta cta agc ggc ttt gag gag ttt gag gag agt ttg    6000
Glu Leu Asp Gln Leu Leu Ser Gly Phe Glu Glu Phe Glu Glu Ser Leu
1985                1990                1995                2000
```

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gga tac aag ttc cgg gat cgg tcg tac ctg ttg caa gcc atg aca cat   6048
Gly Tyr Lys Phe Arg Asp Arg Ser Tyr Leu Leu Gln Ala Met Thr His
                2005                2010                2015

gcc agt tac acg ccc aat cga ttg acg gat tgc tat cag cgt ctg gag   6096
Ala Ser Tyr Thr Pro Asn Arg Leu Thr Asp Cys Tyr Gln Arg Leu Glu
            2020                2025                2030

ttc ctg ggc gat gct gtt cta gat tac ctc att acg cgg cat tta tac   6144
Phe Leu Gly Asp Ala Val Leu Asp Tyr Leu Ile Thr Arg His Leu Tyr
        2035                2040                2045

gaa gat ccc cgc cag cat tct cca ggc gca tta acg gat ttg cgg tca   6192
Glu Asp Pro Arg Gln His Ser Pro Gly Ala Leu Thr Asp Leu Arg Ser
    2050                2055                2060

gca ctg gtg aat aat aca ata ttc gcc tcc ctg gct gtt cgc cat ggc   6240
Ala Leu Val Asn Asn Thr Ile Phe Ala Ser Leu Ala Val Arg His Gly
2065                2070                2075                2080

ttc cac aag ttc ttc cgg cac ctc tcg ccg ggc ctt aac gat gtg att   6288
Phe His Lys Phe Phe Arg His Leu Ser Pro Gly Leu Asn Asp Val Ile
                2085                2090                2095

gac cgt ttt gtg cgg atc cag cag gag aat gga cac tgc atc agt gag   6336
Asp Arg Phe Val Arg Ile Gln Gln Glu Asn Gly His Cys Ile Ser Glu
            2100                2105                2110

gag tac tac tta ttg tcc gag gag gag tgc gat gac gcc gag gac gtt   6384
Glu Tyr Tyr Leu Leu Ser Glu Glu Glu Cys Asp Asp Ala Glu Asp Val
        2115                2120                2125

gag gtg ccc aag gca ttg ggc gac gtt ttc gag tcg atc gca ggt gcc   6432
Glu Val Pro Lys Ala Leu Gly Asp Val Phe Glu Ser Ile Ala Gly Ala
    2130                2135                2140

att ttt ctc gac tca aac atg tcg ctg gac gtg gtt tgg cac gta tat   6480
Ile Phe Leu Asp Ser Asn Met Ser Leu Asp Val Val Trp His Val Tyr
2145                2150                2155                2160

agc aac atg atg agc ccg gag atc gag cag ttc agc aac tca gtg cca   6528
Ser Asn Met Met Ser Pro Glu Ile Glu Gln Phe Ser Asn Ser Val Pro
                2165                2170                2175

aaa tcg ccc att cgg gag ctc ctc gag ctg gag ccg gaa acc gcc aag   6576
Lys Ser Pro Ile Arg Glu Leu Leu Glu Leu Glu Pro Glu Thr Ala Lys
            2180                2185                2190

ttc ggc aag ccc gag aag ctg gcg gat ggg cga cgg gtg cgc gtt acc   6624
Phe Gly Lys Pro Glu Lys Leu Ala Asp Gly Arg Arg Val Arg Val Thr
        2195                2200                2205

gtg gat gtc ttc tgc aaa gga acc ttc cgt ggc atc gga cgc aac tat   6672
Val Asp Val Phe Cys Lys Gly Thr Phe Arg Gly Ile Gly Arg Asn Tyr
    2210                2215                2220

cgc att gcc aag tgc acg gcg gcc aaa tgc gca ttg cgc caa ctc aaa   6720
```

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Arg Ile Ala Lys Cys Thr Ala Ala Lys Cys Ala Leu Arg Gln Leu Lys
2225                2230                2235                2240

aag cag ggc ttg ata gcc aaa aaa gac taa                                6750
Lys Gln Gly Leu Ile Ala Lys Lys Asp  *
                2245


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<213> Drosophila melanogaster

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Pro Arg Asp Phe Gln Val Glu Leu Leu Ala Thr Ala Tyr Glu Arg Asn
            20                  25                  30
Thr Ile Ile Cys Leu Gly His Arg Ser Ser Lys Glu Phe Ile Ala Leu
        35                  40                  45
Lys Leu Leu Gln Glu Leu Ser Arg Arg Ala Arg Arg His Gly Arg Val
    50                  55                  60
Ser Val Tyr Leu Ser Cys Glu Val Gly Thr Ser Thr Glu Pro Cys Ser
65                  70                  75                  80
Ile Tyr Thr Met Leu Thr His Leu Thr Asp Leu Arg Val Trp Gln Glu
                85                  90                  95
Gln Pro Asp Met Gln Ile Pro Phe Asp His Cys Trp Thr Asp Tyr His
            100                 105                 110
Val Ser Ile Leu Arg Pro Glu Gly Phe Leu Tyr Leu Leu Glu Thr Arg
        115                 120                 125
Glu Leu Leu Leu Ser Ser Val Glu Leu Ile Val Leu Glu Asp Cys His
    130                 135                 140
Asp Ser Ala Val Tyr Gln Arg Ile Arg Pro Leu Phe Glu Asn His Ile
145                 150                 155                 160
Met Pro Ala Pro Pro Ala Asp Arg Pro Arg Ile Leu Gly Leu Ala Gly
                165                 170                 175
Pro Leu His Ser Ala Gly Cys Glu Leu Gln Gln Leu Ser Ala Met Leu
            180                 185                 190
Ala Thr Leu Glu Gln Ser Val Leu Cys Gln Ile Glu Thr Ala Ser Asp
        195                 200                 205
Ile Val Thr Val Leu Arg Tyr Cys Ser Arg Pro His Glu Tyr Ile Val
    210                 215                 220
Gln Cys Ala Pro Phe Glu Met Asp Glu Leu Ser Leu Val Leu Ala Asp
225                 230                 235                 240
Val Leu Asn Thr His Lys Ser Phe Leu Leu Asp His Arg Tyr Asp Pro
                245                 250                 255
Tyr Glu Ile Tyr Gly Thr Asp Gln Phe Met Asp Glu Leu Lys Asp Ile
            260                 265                 270
Pro Asp Pro Lys Val Asp Pro Leu Asn Val Ile Asn Ser Leu Leu Val
        275                 280                 285
Val Leu His Glu Met Gly Pro Trp Cys Thr Gln Arg Ala Ala His His
    290                 295                 300
Phe Tyr Gln Cys Asn Glu Lys Leu Lys Val Lys Thr Pro His Glu Arg
305                 310                 315                 320
His Tyr Leu Leu Tyr Cys Leu Val Ser Thr Ala Leu Ile Gln Leu Tyr
                325                 330                 335
Ser Leu Cys Glu His Ala Phe His Arg His Leu Gly Ser Gly Ser Asp
            340                 345                 350
```

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Ser Arg Gln Thr Ile Glu Arg Tyr Ser Ser Pro Lys Val Arg Arg Leu
        355                 360                 365
Leu Gln Thr Leu Arg Cys Phe Lys Pro Glu Glu Val His Thr Gln Ala
    370                 375                 380
Asp Gly Leu Arg Arg Met Arg His Gln Val Asp Gln Ala Asp Phe Asn
385                 390                 395                 400
Arg Leu Ser His Thr Leu Glu Ser Lys Cys Arg Met Val Asp Gln Met
                405                 410                 415
Asp Gln Pro Pro Thr Glu Thr Arg Ala Leu Val Ala Thr Leu Glu Gln
            420                 425                 430
Ile Leu His Thr Thr Glu Asp Arg Gln Thr Asn Arg Ser Ala Ala Arg
        435                 440                 445
Val Thr Pro Thr Pro Thr Pro Ala His Ala Lys Pro Lys Pro Ser Ser
    450                 455                 460
Gly Ala Asn Thr Ala Gln Pro Arg Thr Arg Arg Arg Val Tyr Thr Arg
465                 470                 475                 480
Arg His His Arg Asp His Asn Asp Gly Ser Asp Thr Leu Cys Ala Leu
                485                 490                 495
Ile Tyr Cys Asn Gln Asn His Thr Ala Arg Val Leu Phe Glu Leu Leu
            500                 505                 510
Ala Glu Ile Ser Arg Arg Asp Pro Asp Leu Lys Phe Leu Arg Cys Gln
        515                 520                 525
Tyr Thr Thr Asp Arg Val Ala Asp Pro Thr Thr Glu Pro Lys Glu Ala
    530                 535                 540
Glu Leu Glu His Arg Arg Gln Glu Glu Val Leu Lys Arg Phe Arg Met
545                 550                 555                 560
His Asp Cys Asn Val Leu Ile Gly Thr Ser Val Leu Glu Glu Gly Ile
                565                 570                 575
Asp Val Pro Lys Cys Asn Leu Val Val Arg Trp Asp Pro Pro Thr Thr
            580                 585                 590
Tyr Arg Ser Tyr Val Gln Cys Lys Gly Arg Ala Arg Ala Ala Pro Ala
        595                 600                 605
Tyr His Val Ile Leu Val Ala Pro Ser Tyr Lys Ser Pro Thr Val Gly
    610                 615                 620
Ser Val Gln Leu Thr Asp Arg Ser His Arg Tyr Ile Cys Ala Thr Gly
625                 630                 635                 640
Asp Thr Thr Glu Ala Asp Ser Asp Ser Asp Asp Ser Ala Met Pro Asn
                645                 650                 655
Ser Ser Gly Ser Asp Pro Tyr Thr Phe Gly Thr Ala Arg Gly Thr Val
            660                 665                 670
Lys Ile Leu Asn Pro Glu Val Phe Ser Lys Gln Pro Pro Thr Ala Cys
        675                 680                 685
Asp Ile Lys Leu Gln Glu Ile Gln Asp Glu Leu Pro Ala Ala Ala Gln
    690                 695                 700
Leu Asp Thr Ser Asn Ser Ser Asp Glu Ala Val Ser Met Ser Asn Thr
705                 710                 715                 720
Ser Pro Ser Glu Ser Ser Thr Glu Gln Lys Ser Arg Arg Phe Gln Cys
                725                 730                 735
Glu Leu Ser Ser Leu Thr Glu Pro Glu Asp Thr Ser Asp Thr Thr Ala
            740                 745                 750
Glu Ile Asp Thr Ala His Ser Leu Ala Ser Thr Thr Lys Asp Leu Val
        755                 760                 765
His Gln Met Ala Gln Tyr Arg Glu Ile Glu Gln Met Leu Leu Ser Lys
    770                 775                 780
Cys Ala Asn Thr Glu Pro Pro Glu Gln Glu Gln Ser Glu Ala Glu Arg
785                 790                 795                 800
Phe Ser Ala Cys Leu Ala Ala Tyr Arg Pro Lys Pro His Leu Leu Thr
                805                 810                 815
```

```
Gly Ala Ser Val Asp Leu Gly Ser Ala Ile Ala Leu Val Asn Lys Tyr
            820                     825                     830
Cys Ala Arg Leu Pro Ser Asp Thr Phe Thr Lys Leu Thr Ala Leu Trp
        835                     840                     845
Arg Cys Thr Arg Asn Glu Arg Ala Gly Val Thr Leu Phe Gln Tyr Thr
    850                     855                     860
Leu Arg Leu Pro Ile Asn Ser Pro Leu Lys His Asp Ile Val Gly Leu
865                     870                     875                     880
Pro Met Pro Thr Gln Thr Leu Ala Arg Arg Leu Ala Ala Leu Gln Ala
                885                     890                     895
Cys Val Glu Leu His Arg Ile Gly Glu Leu Asp Asp Gln Leu Gln Pro
            900                     905                     910
Ile Gly Lys Glu Gly Phe Arg Ala Leu Glu Pro Asp Trp Glu Cys Phe
        915                     920                     925
Glu Leu Glu Pro Glu Asp Glu Gln Ile Val Gln Leu Ser Asp Glu Pro
    930                     935                     940
Arg Pro Gly Thr Thr Lys Arg Arg Gln Tyr Tyr Tyr Lys Arg Ile Ala
945                     950                     955                     960
Ser Glu Phe Cys Asp Cys Arg Pro Val Ala Gly Ala Pro Cys Tyr Leu
                965                     970                     975
Tyr Phe Ile Gln Leu Thr Leu Gln Cys Pro Ile Pro Glu Glu Gln Asn
            980                     985                     990
Thr Arg Gly Arg Lys Ile Tyr Pro Pro Glu Asp Ala Gln Gln Gly Phe
        995                     1000                    1005
Gly Ile Leu Thr Thr Lys Arg Ile Pro Lys Leu Ser Ala Phe Ser Ile
    1010                    1015                    1020
Phe Thr Arg Ser Gly Glu Val Lys Val Ser Leu Glu Leu Ala Lys Glu
1025                    1030                    1035                    1040
Arg Val Ile Leu Thr Ser Glu Gln Ile Val Cys Ile Asn Gly Phe Leu
                1045                    1050                    1055
Asn Tyr Thr Phe Thr Asn Val Leu Arg Leu Gln Lys Phe Leu Met Leu
            1060                    1065                    1070
Phe Asp Pro Asp Ser Thr Glu Asn Cys Val Phe Ile Val Pro Thr Val
        1075                    1080                    1085
Lys Ala Pro Ala Gly Gly Lys His Ile Asp Trp Gln Phe Leu Glu Leu
    1090                    1095                    1100
Ile Gln Ala Asn Gly Asn Thr Met Pro Arg Ala Val Pro Asp Glu Glu
1105                    1110                    1115                    1120
Arg Gln Ala Gln Pro Phe Asp Pro Gln Arg Phe Gln Asp Ala Val Val
                1125                    1130                    1135
Met Pro Trp Tyr Arg Asn Gln Asp Gln Pro Gln Tyr Phe Tyr Val Ala
            1140                    1145                    1150
Glu Ile Cys Pro His Leu Ser Pro Leu Ser Cys Phe Pro Gly Asp Asn
        1155                    1160                    1165
Tyr Arg Thr Phe Lys His Tyr Tyr Leu Val Lys Tyr Gly Leu Thr Ile
    1170                    1175                    1180
Gln Asn Thr Ser Gln Pro Leu Leu Asp Val Asp His Thr Ser Ala Arg
1185                    1190                    1195                    1200
Leu Asn Phe Leu Thr Pro Arg Tyr Val Asn Arg Lys Gly Val Ala Leu
                1205                    1210                    1215
Pro Thr Ser Ser Glu Glu Thr Lys Arg Ala Lys Arg Glu Asn Leu Glu
            1220                    1225                    1230
Gln Lys Gln Ile Leu Val Pro Glu Leu Cys Thr Val His Pro Phe Pro
        1235                    1240                    1245
Ala Ser Leu Trp Arg Thr Ala Val Cys Leu Pro Cys Ile Leu Tyr Arg
    1250                    1255                    1260
Ile Asn Gly Leu Leu Leu Ala Asp Asp Ile Arg Lys Gln Val Ser Ala
1265                    1270                    1275                    1280
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Asp Leu Gly Leu Gly Arg Gln Gln Ile Glu Asp Glu Asp Phe Glu Trp
                1285                1290                1295
Pro Met Leu Asp Phe Gly Trp Ser Leu Ser Glu Val Leu Lys Lys Ser
            1300                1305                1310
Arg Glu Ser Lys Gln Lys Glu Ser Leu Lys Asp Asp Thr Ile Asn Gly
        1315                1320                1325
Lys Asp Leu Ala Asp Val Glu Lys Lys Pro Thr Ser Glu Glu Thr Gln
    1330                1335                1340
Leu Asp Lys Asp Ser Lys Asp Asp Lys Val Glu Lys Ser Ala Ile Glu
1345                1350                1355                1360
Leu Ile Ile Glu Gly Glu Glu Lys Leu Gln Glu Ala Asp Asp Phe Ile
                1365                1370                1375
Glu Ile Gly Thr Trp Ser Asn Asp Met Ala Asp Asp Ile Ala Ser Phe
            1380                1385                1390
Asn Gln Glu Asp Asp Asp Glu Asp Asp Ala Phe His Leu Pro Val Leu
        1395                1400                1405
Pro Ala Asn Val Lys Phe Cys Asp Gln Gln Thr Arg Tyr Gly Ser Pro
    1410                1415                1420
Thr Phe Trp Asp Val Ser Asn Gly Glu Ser Gly Phe Lys Gly Pro Lys
1425                1430                1435                1440
Ser Ser Gln Asn Lys Gln Gly Gly Lys Gly Lys Ala Lys Gly Pro Ala
                1445                1450                1455
Lys Pro Thr Phe Asn Tyr Tyr Asp Ser Asp Asn Ser Leu Gly Ser Ser
            1460                1465                1470
Tyr Asp Asp Asp Asp Asn Ala Gly Pro Leu Asn Tyr Met His His Asn
        1475                1480                1485
Tyr Ser Ser Asp Asp Asp Asp Val Ala Asp Asp Ile Asp Ala Gly Arg
    1490                1495                1500
Ile Ala Phe Thr Ser Lys Asn Glu Ala Glu Thr Ile Glu Thr Ala Gln
1505                1510                1515                1520
Glu Val Glu Lys Arg Gln Lys Gln Leu Ser Ile Ile Gln Ala Thr Asn
                1525                1530                1535
Ala Asn Glu Arg Gln Tyr Gln Gln Thr Lys Asn Leu Leu Ile Gly Phe
            1540                1545                1550
Asn Phe Lys His Glu Asp Gln Lys Glu Pro Ala Thr Ile Arg Tyr Glu
        1555                1560                1565
Glu Ser Ile Ala Lys Leu Lys Thr Glu Ile Glu Ser Gly Gly Met Leu
    1570                1575                1580
Val Pro His Asp Gln Gln Leu Val Leu Lys Arg Ser Asp Ala Ala Glu
1585                1590                1595                1600
Ala Gln Val Ala Lys Val Ser Met Met Glu Leu Leu Lys Gln Leu Leu
                1605                1610                1615
Pro Tyr Val Asn Glu Asp Val Leu Ala Lys Lys Leu Gly Asp Arg Arg
            1620                1625                1630
Glu Leu Leu Leu Ser Asp Leu Val Glu Leu Asn Ala Asp Trp Val Ala
        1635                1640                1645
Arg His Glu Gln Glu Thr Tyr Asn Val Met Gly Cys Gly Asp Ser Phe
    1650                1655                1660
Asp Asn Tyr Asn Asp His His Arg Leu Asn Leu Asp Glu Lys Gln Leu
1665                1670                1675                1680
Lys Leu Gln Tyr Glu Arg Ile Glu Ile Glu Pro Pro Thr Ser Thr Lys
                1685                1690                1695
Ala Ile Thr Ser Ala Ile Leu Pro Ala Gly Phe Ser Phe Asp Arg Gln
            1700                1705                1710
Pro Asp Leu Val Gly His Pro Gly Pro Ser Pro Ser Ile Ile Leu Gln
        1715                1720                1725
Ala Leu Thr Met Ser Asn Ala Asn Asp Gly Ile Asn Leu Glu Arg Leu
    1730                1735                1740
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Glu Thr Ile Gly Asp Ser Phe Leu Lys Tyr Ala Ile Thr Thr Tyr Leu
1745                1750                1755                1760
Tyr Ile Thr Tyr Glu Asn Val His Glu Gly Lys Leu Ser His Leu Arg
                1765                1770                1775
Ser Lys Gln Val Ala Asn Leu Asn Leu Tyr Arg Leu Gly Arg Arg Lys
            1780                1785                1790
Arg Leu Gly Glu Tyr Met Ile Ala Thr Lys Phe Glu Pro His Asp Asn
        1795                1800                1805
Trp Leu Pro Pro Cys Tyr Tyr Val Pro Lys Glu Leu Glu Lys Ala Leu
    1810                1815                1820
Ile Glu Ala Lys Ile Pro Thr His His Trp Lys Leu Ala Asp Leu Leu
1825                1830                1835                1840
Asp Ile Lys Asn Leu Ser Ser Val Gln Ile Cys Glu Met Val Arg Glu
                1845                1850                1855
Lys Ala Asp Ala Leu Gly Leu Glu Gln Asn Gly Gly Ala Gln Asn Gly
            1860                1865                1870
Gln Leu Asp Asp Ser Asn Asp Ser Cys Asn Asp Phe Ser Cys Phe Ile
        1875                1880                1885
Pro Tyr Asn Leu Val Ser Gln His Ser Ile Pro Asp Lys Ser Ile Ala
    1890                1895                1900
Asp Cys Val Glu Ala Leu Ile Gly Ala Tyr Leu Ile Glu Cys Gly Pro
1905                1910                1915                1920
Arg Gly Ala Leu Leu Phe Met Ala Trp Leu Gly Val Arg Val Leu Pro
                1925                1930                1935
Ile Thr Arg Gln Leu Asp Gly Gly Asn Gln Glu Gln Arg Ile Pro Gly
            1940                1945                1950
Ser Thr Lys Pro Asn Ala Glu Asn Val Val Thr Val Tyr Gly Ala Trp
        1955                1960                1965
Pro Thr Pro Arg Ser Pro Leu Leu His Phe Ala Pro Asn Ala Thr Glu
    1970                1975                1980
Glu Leu Asp Gln Leu Leu Ser Gly Phe Glu Glu Phe Glu Glu Ser Leu
1985                1990                1995                2000
Gly Tyr Lys Phe Arg Asp Arg Ser Tyr Leu Leu Gln Ala Met Thr His
                2005                2010                2015
Ala Ser Tyr Thr Pro Asn Arg Leu Thr Asp Cys Tyr Gln Arg Leu Glu
            2020                2025                2030
Phe Leu Gly Asp Ala Val Leu Asp Tyr Leu Ile Thr Arg His Leu Tyr
        2035                2040                2045
Glu Asp Pro Arg Gln His Ser Pro Gly Ala Leu Thr Asp Leu Arg Ser
    2050                2055                2060
Ala Leu Val Asn Asn Thr Ile Phe Ala Ser Leu Ala Val Arg His Gly
2065                2070                2075                2080
Phe His Lys Phe Phe Arg His Leu Ser Pro Gly Leu Asn Asp Val Ile
                2085                2090                2095
Asp Arg Phe Val Arg Ile Gln Gln Glu Asn Gly His Cys Ile Ser Glu
            2100                2105                2110
Glu Tyr Tyr Leu Leu Ser Glu Glu Glu Cys Asp Asp Ala Glu Asp Val
        2115                2120                2125
Glu Val Pro Lys Ala Leu Gly Asp Val Phe Glu Ser Ile Ala Gly Ala
    2130                2135                2140
Ile Phe Leu Asp Ser Asn Met Ser Leu Asp Val Val Trp His Val Tyr
2145                2150                2155                2160
Ser Asn Met Met Ser Pro Glu Ile Glu Gln Phe Ser Asn Ser Val Pro
                2165                2170                2175
Lys Ser Pro Ile Arg Glu Leu Leu Glu Leu Glu Pro Glu Thr Ala Lys
            2180                2185                2190
Phe Gly Lys Pro Glu Lys Leu Ala Asp Gly Arg Arg Val Arg Val Thr
        2195                2200                2205
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Val Asp Val Phe Cys Lys Gly Thr Phe Arg Gly Ile Gly Arg Asn Tyr
2210 2215 2220
Arg Ile Ala Lys Cys Thr Ala Ala Lys Cys Ala Leu Arg Gln Leu Lys
2225 2230 2235 2240
Lys Gln Gly Leu Ile Ala Lys Lys Asp
2245

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Gln Pro Gln Gln Leu Gln Gln Pro Gln Gln Leu Gln Gln Pro Gln Gln
35 40 45
Leu Gln Gln Pro Gln Gln Gln Gln Gln Gln Gln Pro His Gln Gln Gln
50 55 60
Gln Gln Ser Ser Arg Gln Gln Pro Ser Thr Ser Ser Gly Gly Ser Arg
65 70 75 80
Ala Ser Gly Phe Gln Gln Gly Gly Gln Gln Gln Lys Ser Gln Asp Ala
85 90 95
Glu Gly Trp Thr Ala Gln Lys Lys Gln Gly Lys Gln Gln Val Gln Gly
100 105 110
Trp Thr Lys Gln Gly Gln Gln Gly Gly His Gln Gln Gly Arg Gln Gly
115 120 125
Gln Asp Gly Gly Tyr Gln Gln Arg Pro Pro Gly Gln Gln Gln Gly Gly
130 135 140
His Gln Gln Gly Arg Gln Gly Gln Glu Gly Gly Tyr Gln Gln Arg Pro
145 150 155 160
Pro Gly Gln Gln Gln Gly Gly His Gln Gln Gly Arg Gln Gly Gln Glu
165 170 175
Gly Gly Tyr Gln Gln Arg Pro Ser Gly Gln Gln Gln Gly Gly His Gln
180 185 190
Gln Gly Arg Gln Gly Gln Glu Gly Gly Tyr Gln Gln Arg Pro Pro Gly
195 200 205
Gln Gln Gln Gly Gly His Gln Gln Gly Arg Gln Gly Gln Glu Gly Gly
210 215 220
Tyr Gln Gln Arg Pro Ser Gly Gln Gln Gln Gly Gly His Gln Gln Gly
225 230 235 240
Arg Gln Gly Gln Glu Gly Gly Tyr Gln Gln Arg Pro Ser Gly Gln Gln
245 250 255
Gln Gly Gly His Gln Gln Gly Arg Gln Gly Gln Glu Gly Gly Tyr Gln
260 265 270
Gln Arg Pro Ser Gly Gln Gln Gln Gly Gly His Gln Gln Gly Arg Gln
275 280 285
Gly Gln Glu Gly Gly Tyr Gln Gln Arg Pro Pro Gly Gln Gln Pro Asn
290 295 300
Gln Thr Gln Ser Gln Gly Gln Tyr Gln Ser Arg Gly Pro Pro Gln Gln
305 310 315 320
Gln Gln Ala Ala Pro Leu Pro Leu Pro Pro Gln Pro Ala Gly Ser Ile
325 330 335
Lys Arg Gly Thr Ile Gly Lys Pro Gly Gln Val Gly Ile Asn Tyr Leu
340 345 350

```
Asp Leu Asp Leu Ser Lys Met Pro Ser Val Ala Tyr His Tyr Asp Val
        355                 360                 365
Lys Ile Met Pro Glu Arg Pro Lys Lys Phe Tyr Arg Gln Ala Phe Glu
    370                 375                 380
Gln Phe Arg Val Asp Gln Leu Gly Gly Ala Val Leu Ala Tyr Asp Gly
385                 390                 395                 400
Lys Ala Ser Cys Tyr Ser Val Asp Lys Leu Pro Leu Asn Ser Gln Asn
                405                 410                 415
Pro Glu Val Thr Val Thr Asp Arg Asn Gly Arg Thr Leu Arg Tyr Thr
            420                 425                 430
Ile Glu Ile Lys Glu Thr Gly Asp Ser Thr Ile Asp Leu Lys Ser Leu
        435                 440                 445
Thr Thr Tyr Met Asn Asp Arg Ile Phe Asp Lys Pro Met Arg Ala Met
    450                 455                 460
Gln Cys Val Glu Val Val Leu Ala Ser Pro Cys His Asn Lys Ala Ile
465                 470                 475                 480
Arg Val Gly Arg Ser Phe Phe Lys Met Ser Asp Pro Asn Asn Arg His
                485                 490                 495
Glu Leu Asp Asp Gly Tyr Glu Ala Leu Val Gly Leu Tyr Gln Ala Phe
            500                 505                 510
Met Leu Gly Asp Arg Pro Phe Leu Asn Val Asp Ile Ser His Lys Ser
        515                 520                 525
Phe Pro Ile Ser Met Pro Met Ile Glu Tyr Leu Glu Arg Phe Ser Leu
    530                 535                 540
Lys Ala Lys Ile Asn Asn Thr Thr Asn Leu Asp Tyr Ser Arg Arg Phe
545                 550                 555                 560
Leu Glu Pro Phe Leu Arg Gly Ile Asn Val Val Tyr Thr Pro Pro Gln
                565                 570                 575
Ser Phe Gln Ser Ala Pro Arg Val Tyr Arg Val Asn Gly Leu Ser Arg
            580                 585                 590
Ala Pro Ala Ser Ser Glu Thr Phe Glu His Asp Gly Lys Lys Val Thr
        595                 600                 605
Ile Ala Ser Tyr Phe His Ser Arg Asn Tyr Pro Leu Lys Phe Pro Gln
    610                 615                 620
Leu His Cys Leu Asn Val Gly Ser Ser Ile Lys Ser Ile Leu Leu Pro
625                 630                 635                 640
Ile Glu Leu Cys Ser Ile Glu Glu Gly Gln Ala Leu Asn Arg Lys Asp
                645                 650                 655
Gly Ala Thr Gln Val Ala Asn Met Ile Lys Tyr Ala Ala Thr Ser Thr
            660                 665                 670
Asn Val Arg Lys Arg Lys Ile Met Asn Leu Leu Gln Tyr Phe Gln His
        675                 680                 685
Asn Leu Asp Pro Thr Ile Ser Arg Phe Gly Ile Arg Ile Ala Asn Asp
    690                 695                 700
Phe Ile Val Val Ser Thr Arg Val Leu Ser Pro Pro Gln Val Glu Tyr
705                 710                 715                 720
His Ser Lys Arg Phe Thr Met Val Lys Asn Gly Ser Trp Arg Met Asp
                725                 730                 735
Gly Met Lys Phe Leu Glu Pro Lys Pro Lys Ala His Lys Cys Ala Val
            740                 745                 750
Leu Tyr Cys Asp Pro Arg Ser Gly Arg Lys Met Asn Tyr Thr Gln Leu
        755                 760                 765
Asn Asp Phe Gly Asn Leu Ile Ile Ser Gln Gly Lys Ala Val Asn Ile
    770                 775                 780
Ser Leu Asp Ser Asp Val Thr Tyr Arg Pro Phe Thr Asp Asp Glu Arg
785                 790                 795                 800
Ser Leu Asp Thr Ile Phe Ala Asp Leu Lys Arg Ser Gln His Asp Leu
                805                 810                 815
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Ala Ile Val Ile Ile Pro Gln Phe Arg Ile Ser Tyr Asp Thr Ile Lys
            820                 825                 830
Gln Lys Ala Glu Leu Gln His Gly Ile Leu Thr Gln Cys Ile Lys Gln
        835                 840                 845
Phe Thr Val Glu Arg Lys Cys Asn Asn Gln Thr Ile Gly Asn Ile Leu
    850                 855                 860
Leu Lys Ile Asn Ser Lys Leu Asn Gly Ile Asn His Lys Ile Lys Asp
865                 870                 875                 880
Asp Pro Arg Leu Pro Met Met Lys Asn Thr Met Tyr Ile Gly Ala Asp
                885                 890                 895
Val Thr His Pro Ser Pro Asp Gln Arg Glu Ile Pro Ser Val Val Gly
            900                 905                 910
Val Ala Ala Ser His Asp Pro Tyr Gly Ala Ser Tyr Asn Met Gln Tyr
        915                 920                 925
Arg Leu Gln Arg Gly Ala Leu Glu Glu Ile Glu Asp Met Phe Ser Ile
    930                 935                 940
Thr Leu Glu His Leu Arg Val Tyr Lys Glu Tyr Arg Asn Ala Tyr Pro
945                 950                 955                 960
Asp His Ile Ile Tyr Tyr Arg Asp Gly Val Ser Asp Gly Gln Phe Pro
                965                 970                 975
Lys Ile Lys Asn Glu Glu Leu Arg Cys Ile Lys Gln Ala Cys Asp Lys
            980                 985                 990
Val Gly Cys Lys Pro Lys Ile Cys Cys Val Ile Val Val Lys Arg His
        995                 1000                1005
His Thr Arg Phe Phe Pro Ser Gly Asp Val Thr Thr Ser Asn Lys Phe
    1010                1015                1020
Asn Asn Val Asp Pro Gly Thr Val Val Asp Arg Thr Ile Val His Pro
1025                1030                1035                1040
Asn Glu Met Gln Phe Phe Met Val Ser Gly Gln Ala Ile Gln Gly Thr
                1045                1050                1055
Ala Lys Pro Thr Arg Tyr Asn Val Ile Glu Asn Thr Gly Asn Leu Asp
            1060                1065                1070
Ile Asp Leu Leu Gln Gln Leu Thr Tyr Asn Leu Cys His Met Phe Pro
        1075                1080                1085
Arg Cys Asn Arg Ser Val Ser Tyr Pro Ala Pro Ala Tyr Leu Ala His
    1090                1095                1100
Leu Val Ala Ala Arg Gly Arg Val Tyr Leu Thr Gly Thr Asn Arg Phe
1105                1110                1115                1120
Leu Asp Leu Lys Lys Glu Tyr Ala Lys Arg Thr Ile Val Pro Glu Phe
                1125                1130                1135
Met Lys Lys Asn Pro Met Tyr Phe Val
            1140                1145
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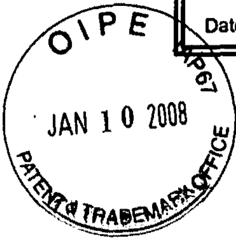
nnuu 64

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail, in an envelope addressed to: MS Missing Parts, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Dated: 01/07/08 Signature: Scott Whittemore (Scott Whittemore)



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

DECLARATION FOR PATENT APPLICATION

COPY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

the specification of which was filed on November 23, 2004 as Application No. 10/997,086.

In the event that the filing date and/or Application No. are not entered above at the time I execute this document, and if such information is deemed necessary, I hereby authorize and request my attorneys/agent(s) at **Ropes & Gray LLP**, One International Place, Boston, 02110-2624, to insert above the filing date and/or Application No. of said application.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by an amendment, if any, specifically referred to herein.

I acknowledge the duty to disclose all information known to me that is material to patentability in accordance with Title 37, Code of Federal Regulations, § 1.56.

FOREIGN PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

[x] no such foreign applications have been filed

[ ] such foreign application have been filed as follows:

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Application Number	Country	Date of Filing	Priority Claimed Under 35 USC 119
			___ Yes No ___
			___ Yes No ___
			___ Yes No ___

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Application Number	Country	Date of Filing

CLAIM FOR BENEFIT OF EARLIER U.S. PROVISIONAL APPLICATIONS

I hereby claim priority benefits under Title 35, United States Code §119(e), of any United States provisional patent application(s) listed below:

[ ] no such U.S. provisional applications have been filed.

[x] such U.S. provisional application have been filed as follows:

Application Number	Date of Filing	Priority Claimed Under 35 USC 119
60/189739	March 16, 2000	x Yes No ___
60/243097	October 24, 2000	x Yes No ___
		___ Yes No ___

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)

I hereby claim the benefit under Title 35, United States Code, §120 of the United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information that is material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56 which became available to me between the filing date of the prior application and the national or PCT international filing date of this application:

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☐ no such U.S./PCT applications have been filed.

☒ such U.S./PCT application have been filed as follows:

Application Number	Relationship	Parent Application	Date of Filing
This Application	Continuation-in-part	10/350798	January 24, 2003
10/350798	Continuation-in-part	09/858862	May 16, 2001
09/858862	Continuation-in-part	US01/08435	March 16, 2001
10/350798	Continuation-in-part	09/866557	May 24, 2001
09/866557	Continuation-in-part	US01/08435	March 16, 2001
10/350798	Continuation-in-part	10/055797	January 22, 2002
10/055797	Continuation-in-part	US01/08435	March 16, 2001

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint:

All practitioners at Customer Number 28120

all of **Ropes & Gray LLP**, One International Place, Boston, 02110-2624, jointly, and each of them severally, my attorneys at law/patent agent(s), with full power of substitution, delegation and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, and to transact all business in the U. S. Patent and Trademark Office connected therewith.

Please mail all correspondence to Matthew P. Vincent, whose address is:

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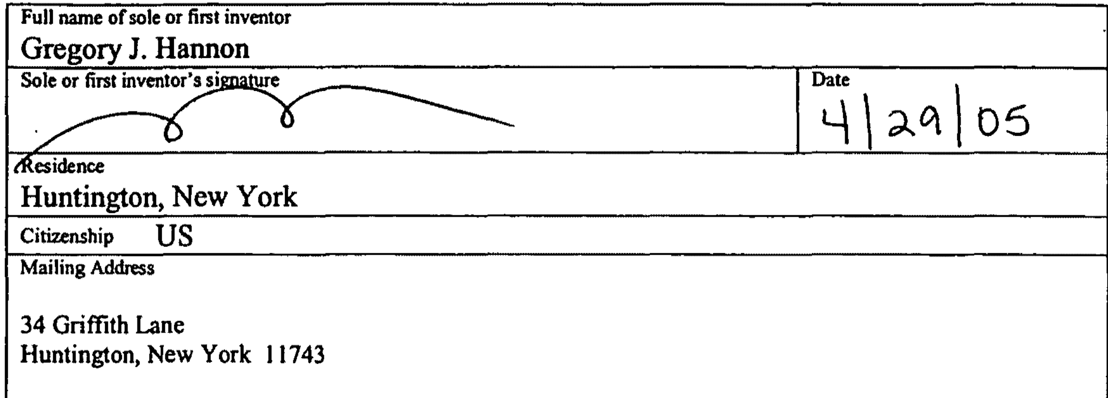
Boston, 02110-2624

Please direct telephone calls to: Matthew P. Vincent at (617) 951-7739.

Please direct facsimiles to: (617) 951-7050

Benitec - Exhibit 1002 - page 393



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Sole or first inventor's signature	Date
	4/29/05

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Citizenship US

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Second inventor's signature	Date

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Third inventor's signature	Date

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Citizenship US	
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Third inventor's signature Despina C Siolas	Date 5/2/05.
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Full name of sole or first inventor Gregory J. Hannon	
Sole or first inventor's signature	Date
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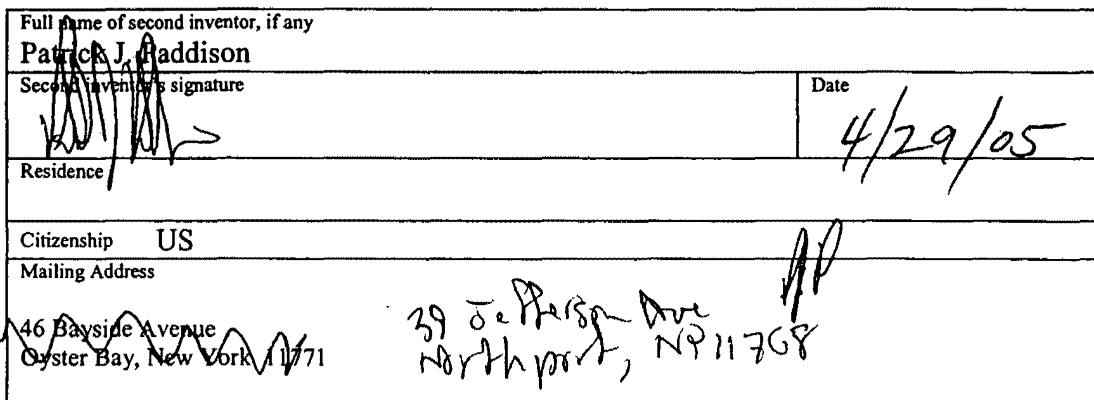
Citizenship US	
Mailing Address 34 Griffith Lane Huntington, New York 11743	



Full name of second inventor, if any Patrick J. Paddison	
Second inventor's signature	Date 4/29/05
Residence	
Citizenship US	
Mailing Address ~~46 Bayside Avenue~~ ~~Oyster Bay, New York 11771~~ 39 Jefferson Ave Northport, NY 11768	

Full name of third inventor, if any Despina C. Siolas	
Third inventor's signature	Date
Residence Mattituck, New York	
Citizenship US	
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OIPE JAN 10 2008 PATENT & TRADEMARK OFFICE

Docket No.: CSHL-P08-010
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Hannon et al.

Application No.:	11/894,676	Confirmation No.:	8161
Filed:	August 20, 2007	Art Unit:	1635
For:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE	Examiner:	Not Yet Assigned

INFORMATION DISCLOSURE STATEMENT (IDS)

MS Missing Parts
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P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 CFR 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the references listed on the attached PTO/SB/08. It is respectfully requested that the information be expressly considered during the prosecution of this application, and that the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

This Information Disclosure Statement is filed before the mailing date of a first Office Action on the merits as far as is known to the undersigned (37 CFR 1.97(b)(3)).

The documents listed on the attached form PTO/SB/08 are not supplied because they were previously cited by or submitted to the Office in prior application number 10/997,086 filed November 23, 2004 and relied upon in this application for an earlier filing date under 35 U.S.C. 120.

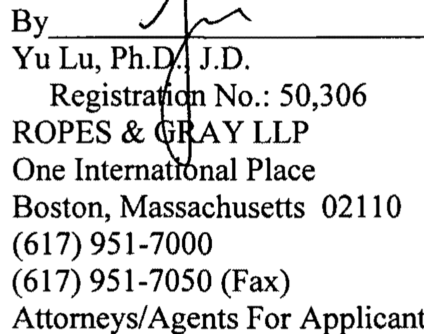
In accordance with 37 CFR 1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR 1.56(a) exists. In accordance with 37 CFR 1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

It is submitted that the Information Disclosure Statement is in compliance with 37 CFR 1.98 and the Examiner is respectfully requested to consider the listed references.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. **18-1945**, under Order No. **CSHL-P08-010**.

Dated: January 7, 2008

Respectfully submitted,



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Substitute for form 1449/PTO				*Complete if Known*	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	11/894,676
				Filing Date	August 20, 2007
				First Named Inventor	Gregory J. Hannon
				Art Unit	1635
(Use as many sheets as necessary)				Examiner Name	Not Yet Assigned
Sheet	1	of	7	Attorney Docket Number	CSHL-P08-010

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Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AA	US-20020086356-A1	07-04-2002	Tuschl et al.	
	AB	US-20020114784-A1	08-22-2002	Li et al.	
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Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	BA	WO-00/01846	01-13-2000	Devgen Nv et al.		
	BB	WO-00/44895	08-03-2000	Kreutzer Roland et al.		
	BC	WO-00/63364	10-26-2000	American Home Prod et al.		
	BD	WO-01/49844	07-12-2001	Univ Rutgers et al.		
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	BG	WO-94/01550	01-20-1994	Hybridon Inc et al.		
	BH	WO-99/49029	09-30-1999	Gene Australia Limited Ag et al.		
	BI	WO-00/44914	08-03-2000	Medical College Of Georgia Res et al.		
	BJ	WO-01/29058	04-26-2001	Univ Massachusetts et al.		
	BK	WO-01/36646	05-25-2001	Cancer Res Campaign Tech et al.		
	BL	WO-01/48183	07-05-2001	Devgen Nv et al.		

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	BM	WO-01/75164	10-11-2001	Whitehead Institute for Biomedical Research; Max-Planck-Gesellschaft Zur Forderung Der Wissenschaften E.V.; Massachusetts Institute of Technology; University of Masachusetts Medical Center		
	BN	WO-02/059300	08-01-2002	J & J Res Pty Ltd et al.		
	BO	WO-02/068635	09-06-2002	Novartis Forschungsstiftung Zw et al.		
	BP	WO-99/32619	07-01-1999	Carnegie Inst Of Washington et al.		

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. * CITE NO.: Those application(s) which are marked with an single asterisk (*) next to the Cite No. are not supplied (under 37 CFR 1.98(a)(2)(iii)) because that application was filed after June 30, 2003 or is available in the IFW. ** CITE NO.: Those document(s) which are marked with an double asterisk (**) next to the Cite No. are not supplied because they were previously cited by or submitted to the Office in a prior application relied upon in this application for an earlier filing date under 35 U.S.C. 120. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
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	CB	Ambros, "Dicing Up RNAs," Science 293: 811-813 (2001).	
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	CN1	Guo, "par-1, a Gene Required for Establishing Polarity in C. elegans Embryos, Encodes a Putative Ser/Thr Kinase that is Asymmetrically Distributed," Cell 81:611-620 (1995).	
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	CW1	Hasuwa, H., et al., "Small interfering RNA and gene silencing in transgenic mice and rats," FEBS Letters, 532:227-230 (2002).	
	CX1	He, et al., "A microRNA polycistron as a potential human oncogene," Nature, 435(7043):828-33 (2005).	
	CY1	He, et al., "MicroRNAs: small RNAs with a big role in gene regulation," Nat Rev Genet., 5(7):522-31 (2004).	
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	CM3	Paddison, et al., "Short hairpin activated gene silencing in mammalian cells," Methods Mol Biol., 265:85-100 (2004).	
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Examiner Signature		Date Considered	

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(Based on PTO 04-07 version)

Substitute for form 1449/PTO				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	11/894,676
(Use as many sheets as necessary)				Filing Date	August 20, 2007
				First Named Inventor	Gregory J. Hannon
				Art Unit	1635
				Examiner Name	Not Yet Assigned
Sheet	7	of	7	Attorney Docket Number	CSHL-P08-010

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	CL4	Smith, et al., "Total silencing by intron-spliced hairpin RNAs," Nature, 407:319-320 (2000).	
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	CN4	Song, et al., "The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes," Nat. Struct. Biol. 10(12):1026-32 (2003).	
	CO4	Svoboda, et al., "RNAi and expression of retrotransposons MuERV-L and IAP in preimplantation mouse embryos," Dev. Biol., 269(1):276-85 (2004).	
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	CQ4	Tabara, et al., "The dsRNA Binding Protein RDE-4 Interacts with RDE-1, DCR-1, and a DExH-Box Helicase to Direct RNAi in C. elegans," Cell, 109:861-871. (2002).	
	CR4	Tabara, et al., "The rde-1 Gene, RNA Interference, and Transposon Silencing in C. elegans," Cell, 99:123-132 (1999).	
	CS4	Tavernarakis, et al., "Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes," Nat. Genet., 24:180-183 (2000).	
	CT4	Timmons, et al., "Specific interference by ingested dsRNA," Nature, 395:854 (1998).	
	CU4	Tomari, et al., "RISC Assembly Defects in the Drosophila RNAi Mutant armitage", Cell 116:831-841 (2004).	
	CV4	Tuschl, et al. "Targeted mRNA degradation by double-stranded RNA in vitro," Genes Dev., 13:3191-3197 (1999).	
	CW4	Ui-Tei, et al., "Sensitive Assay of RNA Interference in Drosophila and Chinese Hamster Cultured Cells Using Firefly Luciferase Gene as Target," FEBS Letters, 479:79-82 (2000).	
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	CZ4	Wassenegger, "A model for RNA-mediated gene silencing in higher plants," Plant Mol. Biol. 37:349-362 (1998).	
	CA5	Waterhouse, et al., "Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA," PNAS 95:13959-13964 (1998).	
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	CD5	Zamore, et al., "RNAi: Double-Stranded RNA Directs the ATP-Dependent Cleavage of mRNA at 21 to 23 Nucleotide Intervals." Cell 101:25-33 (2000).	
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[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

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Met Thr Pro Ala Ser Ser Pro Met Gly Pro Phe Phe Gly Leu Pro Trp
            20                  25                  30

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Asn Thr Gly Ser Gly Lys Thr Phe Ile Ala Ser Thr Thr Leu Leu Lys
65                  70                  75                  80

agc tgt ctc tat cta gat cta ggg gag act tca gct aga aat gga aaa    288
Ser Cys Leu Tyr Leu Asp Leu Gly Glu Thr Ser Ala Arg Asn Gly Lys
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agg acg gtg ttc ttg gtc aac tct gca aac cag gtt gct caa caa gtg    336
Arg Thr Val Phe Leu Val Asn Ser Ala Asn Gln Val Ala Gln Gln Val
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Ser Ala Val Arg Thr His Ser Asp Leu Lys Val Gly Glu Tyr Ser Asn
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Leu Glu Val Asn Ala Ser Trp Thr Lys Glu Arg Trp Asn Gln Glu Phe
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act aag cac cag gtt ctc att atg act tgc tat gtc gcc ttg aat gtt    480
Thr Lys His Gln Val Leu Ile Met Thr Cys Tyr Val Ala Leu Asn Val
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ttg aaa aat ggt tac tta tca ctg tca gac att aac ctt ttg gtg ttt    528
Leu Lys Asn Gly Tyr Leu Ser Leu Ser Asp Ile Asn Leu Leu Val Phe
                165                 170                 175

gat gag tgt cat ctt gca atc cta gac cac ccc tat cga gaa ttt atg    576
Asp Glu Cys His Leu Ala Ile Leu Asp His Pro Tyr Arg Glu Phe Met
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aag ctc tgt gaa att tgt cca tca tgt cct cgc att ttg gga cta act    624
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gat aaa gta gct gga atg atg gta aga gaa cta cag aaa tac atc aaa    1008
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Ser Leu Asp Leu Lys Phe Val Thr Pro Lys Val Ile Lys Leu Leu Glu
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Ile Leu Arg Lys Tyr Lys Pro Tyr Glu Arg His Ser Phe Glu Ser Val
385                 390                 395                 400

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                405                 410                 415

tct gag gat gat gat gag gat gaa gaa att gaa gaa aaa gag aag cca    1296
Ser Glu Asp Asp Asp Glu Asp Glu Glu Ile Glu Glu Lys Glu Lys Pro
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gag aca aat ttt cct tct cct ttt acc aac att ttg tgc gga att att    1344
Glu Thr Asn Phe Pro Ser Pro Phe Thr Asn Ile Leu Cys Gly Ile Ile
        435                 440                 445

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Phe Val Glu Arg Arg Tyr Thr Ala Val Val Leu Asn Arg Leu Ile Lys
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gaa gct ggc aaa caa gat cca gag ctg gct tat atc agt agc aat ttc    1440
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465                 470                 475                 480

ata act gga cat ggc att ggg aag aat cag cct cgc aac aac acg atg    1488
Ile Thr Gly His Gly Ile Gly Lys Asn Gln Pro Arg Asn Asn Thr Met
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gaa gca gaa ttc aga aaa cag gaa gag gta ctt agg aaa ttt cga gca    1536
Glu Ala Glu Phe Arg Lys Gln Glu Glu Val Leu Arg Lys Phe Arg Ala
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Tyr Arg Ser Tyr Val Gln Ser Lys Gly Arg Ala Arg Ala Pro Ile Ser
545                 550                 555                 560

aat tat ata atg tta gcg gat aca gac aaa ata aaa agt ttt gaa gaa    1728
Asn Tyr Ile Met Leu Ala Asp Thr Asp Lys Ile Lys Ser Phe Glu Glu
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gac ctt aaa acc tac aaa gct att gaa aag atc ttg aga aac aag tgt    1776
Asp Leu Lys Thr Tyr Lys Ala Ile Glu Lys Ile Leu Arg Asn Lys Cys
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Asp Asp His Val Phe Pro Pro Tyr Val Leu Arg Pro Asp Asp Gly Gly
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Pro Arg Val Thr Ile Asn Thr Ala Ile Gly His Ile Asn Arg Tyr Cys
625                 630                 635                 640

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Ala Arg Leu Pro Ser Asp Pro Phe Thr His Leu Ala Pro Lys Cys Arg
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Val Arg Leu Ala Glu Arg Val Val Ala Leu Ile Cys Cys Glu Lys Leu
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705                 710                 715                 720

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Thr Val Lys Tyr Glu Glu Glu Leu Asp Leu His Asp Glu Glu Glu Thr
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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
11/894,676	08/20/2007	1635	970	CSHL-P08-010	14	2

CONFIRMATION NO. 8161

UPDATED FILING RECEIPT

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Applicant(s)

Gregory J. Hannon, Huntington, NY;
Patrick J. Paddison, Northport, NY;
Despina C. Siolas, Mattituck, NY;

Power of Attorney: The patent practitioners associated with Customer Number 28120

Domestic Priority data as claimed by applicant

This application is a CON of 10/997,086 11/23/2004
which is a CIP of 10/350,798 01/24/2003
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and claims benefit of 60/243,097 10/24/2000
and said 10/350,798 01/24/2003
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and said 10/350,798 01/24/2003
is a CIP of 09/858,862 05/16/2001
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Foreign Applications

If Required, Foreign Filing License Granted: 11/02/2007

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 11/894,676**

Projected Publication Date: 06/26/2008

Non-Publication Request: No

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**** SMALL ENTITY ****
Title

Methods and compositions for RNA interference

Preliminary Class

514

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CONFIRMATION NO. 8161

28120
ROPES & GRAY LLP
PATENT DOCKETING 39/41
ONE INTERNATIONAL PLACE
BOSTON, MA02110-2624

Date Mailed. 06/26/2008

NOTICE OF NEW OR REVISED PROJECTED PUBLICATION DATE

The above-identified application has a new or revised projected publication date. The current projected publication date for this application is 09/04/2008. If this is a new projected publication date (there was no previous projected publication date), the application has been cleared by Licensing & Review or a secrecy order has been rescinded and the application is now in the publication queue.

If this is a revised projected publication date (one that is different from a previously communicated projected publication date), the publication date has been revised due to processing delays in the USPTO or the abandonment and subsequent revival of an application. The application is anticipated to be published on a date that is more than six weeks different from the originally-projected publication date.

More detailed publication information is available through the private side of Patent Application Information Retrieval (PAIR) System. The direct link to access PAIR is currently http://pair.uspto.gov. Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Questions relating to this Notice should be directed to the Office of Patent Publication at 1-888-786-0101.

PART 1 - ATTORNEY/APPLICANT COPY

PTO/SB/82 (01-06)
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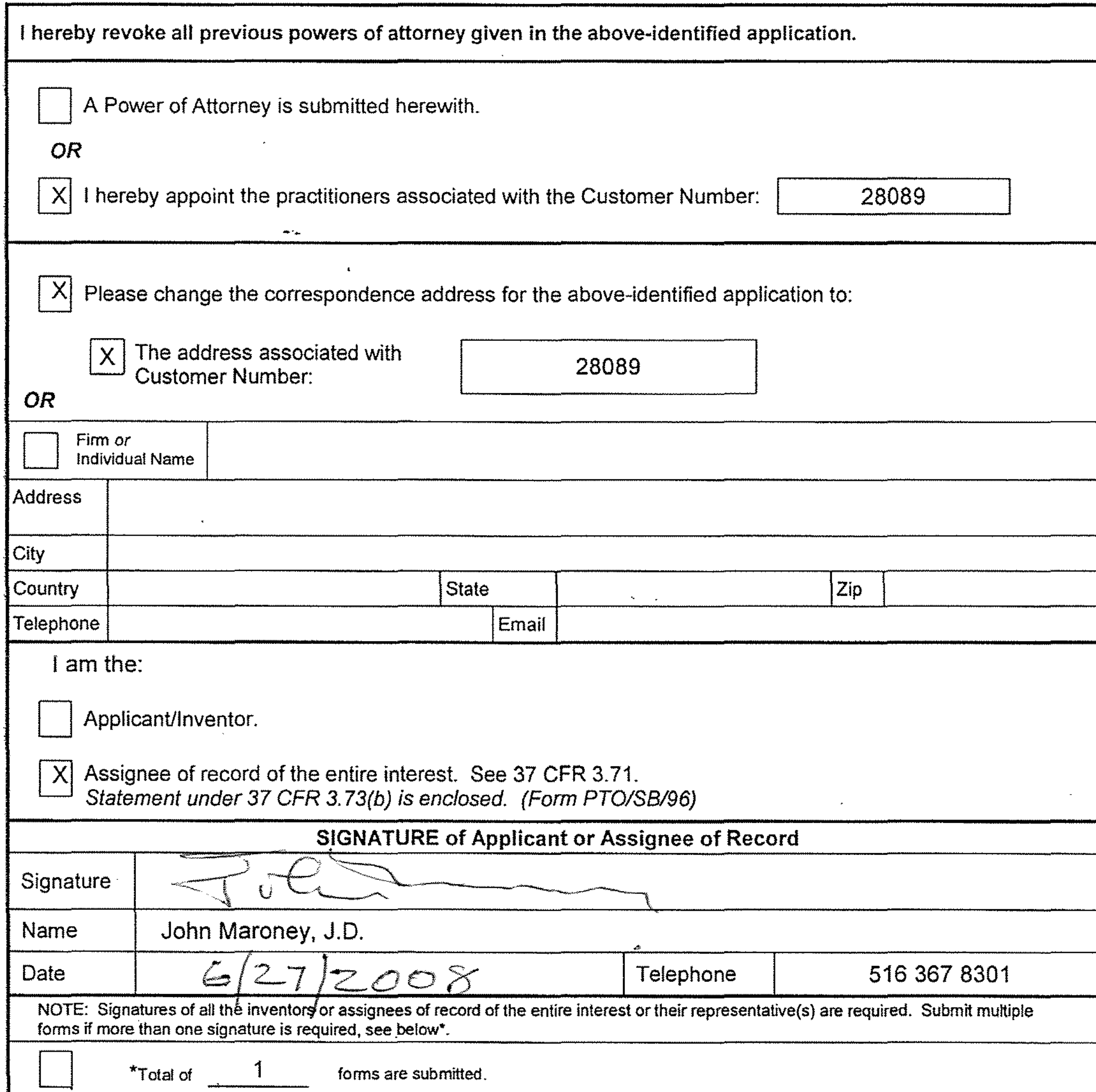
REVOCATION OF POWER OF ATTORNEY WITH NEW POWER OF ATTORNEY AND CHANGE OF CORRESPONDENCE ADDRESS		
	Application Number	11/894,676 – Conf #8161
	Filing Date	08/20/2007
	First Named Inventor	Gregory J. Hannon
	Art Unit	N/A
	Examiner Name	N/A
	Attorney Docket Number	287000.134US1



I hereby revoke all previous powers of attorney given in the above-identified application.

☐ A Power of Attorney is submitted herewith.

OR

☒ I hereby appoint the practitioners associated with the Customer Number: 28089

☒ Please change the correspondence address for the above-identified application to:

☒ The address associated with Customer Number: 28089

OR

☐ Firm *or* Individual Name					
Address					
City					
Country		State		Zip	
Telephone		Email			

I am the:

☐ Applicant/Inventor.

☒ Assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96)

SIGNATURE of Applicant or Assignee of Record			
Signature	[signature]		
Name	John Maroney, J.D.		
Date	6/27/2008	Telephone	516 367 8301

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☐ *Total of 1 forms are submitted.

US1DOCS 6728880V1

PTO/SB/96 (01-08)
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STATEMENT UNDER 37 CFR 3.73(b)

Applicant/Patent Owner: Gregory Hannon et al.

Application No./Patent No.: 11/894,676 Filed/Issue Date: 08/20/2007

Entitled: Methods and compositions for RNA interference

Cold Spring Harbor Laboratory (Name of Assignee), a Educational Corporation (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that it is:

1. [X] the assignee of the entire right, title, and interest; or

2. [ ] an assignee of less than the entire right, title and interest.
(The extent (by percentage) of its ownership interest is ______ %)

in the patent application/patent identified above by virtue of either:

A. [X] An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 020427, Frame 0756, or for which a copy thereof is attached.

OR

B. [ ] A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: ______ To: ______
The document was recorded in the United States Patent and Trademark Office at Reel ______, Frame ______, or for which a copy thereof is attached.

2. From: ______ To: ______
The document was recorded in the United States Patent and Trademark Office at Reel ______, Frame ______, or for which a copy thereof is attached.

3. From: ______ To: ______
The document was recorded in the United States Patent and Trademark Office at Reel ______, Frame ______, or for which a copy thereof is attached.

[ ] Additional documents in the chain of title are listed on a supplemental sheet.

[X] As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (*i.e.*, a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Signature

6/27/2008
Date

John Maroney, J.D.
Printed or Typed Name

516 367 8301
Telephone Number

Vice President, Office of Technology Transfer
Title

US1DOCS 6728887V1

Electronic Acknowledgement Receipt

EFS ID:	3627520
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	28120
Filer:	Jane Maureen Love/sophie murray
Filer Authorized By:	Jane Maureen Love
Attorney Docket Number:	CSHL-P08-010
Receipt Date:	16-JUL-2008
Filing Date:	20-AUG-2007
Time Stamp:	16:51:48
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes) /Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	287000_134US1_POA.pdf	69070 db4cb22f035f4dfc955e6d3a46be1423f625ffd5	no	1

Warnings:

Information:

2	Assignee showing of ownership per 37 CFR 3.73(b).	287000_134US1_Statement.pdf	74379 dca68181c459f4e5cbc5d7add829be8fa5116ca4	no	1
Warnings:					
Information:					
		Total Files Size (in bytes):	143449		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/894,676	08/20/2007	Gregory J. Hannon	287000.134US1

CONFIRMATION NO. 8161

28089
WILMERHALE/NEW YORK
399 PARK AVENUE
NEW YORK, NY 10022

POA ACCEPTANCE LETTER

OC000000031144221

Date Mailed: 07/25/2008

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 07/16/2008.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

/mnguyen/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/894,676	08/20/2007	Gregory J. Hannon	CSHL-P08-010

CONFIRMATION NO. 8161

POWER OF ATTORNEY NOTICE

28120
ROPES & GRAY LLP
PATENT DOCKETING 39/41
ONE INTERNATIONAL PLACE
BOSTON, MA 02110-2624

Date Mailed: 07/25/2008

NOTICE REGARDING CHANGE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 07/16/2008.

- The Power of Attorney to you in this application has been revoked by the assignee who has intervened as provided by 37 CFR 3.71. Future correspondence will be mailed to the new address of record(37 CFR 1.33).

/mnguyen/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

PTO/SB/08a (03-08)
Approved for use through 07/31/2008. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99)**		
	Application Number	11894676
	Filing Date	2007-08-20
	First Named Inventor	Gregory J. HANNON
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	0287000.00130US3

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							☐

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NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	11894676
	Filing Date	2007-08-20
	First Named Inventor	Gregory J. HANNON
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	0287000.00130US3

	1	European Search Report for European PAtent Application No 05857008.6, mailed May 8, 2008	☐

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)	Application Number		11894676
	Filing Date		2007-08-20
	First Named Inventor	Gregory J. HANNON	
	Art Unit		N/A
	Examiner Name	Not Yet Assigned	
	Attorney Docket Number		0287000.00130US3

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☒ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☒ See attached certification statement.

☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ None

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Jane M. Love, Ph.D./	Date (YYYY-MM-DD)	2008-08-08
Name/Print	Jane M. Love, Ph.D.	Registration Number	42812

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	3751482
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	28089
Filer:	Jane Maureen Love/sophie murray
Filer Authorized By:	Jane Maureen Love
Attorney Docket Number:	287000.134US1
Receipt Date:	08-AUG-2008
Filing Date:	20-AUG-2007
Time Stamp:	17:06:15
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes) /Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement Letter	287000_130US3_IDS.pdf	86446 c3613eeca7864ef8e10001b5df0656eca6733b25	no	2

Warnings:

Information:

2	Information Disclosure Statement (IDS) Filed (SB/08)	287000_130US3_SB08.pdf	754914 a3c928745bd5cb7f2ebafc60a0af1e642c46eef1	no	4
Warnings:					
Information:					
A U.S. Patent Number Citation or a U.S. Publication Number Citation is required in the Information Disclosure Statement (IDS) form for autoloading of data into USPTO systems. You may remove the form to add the required data in order to correct the Informational Message if you are citing U.S. References. If you chose not to include U.S. References, the image of the form will be processed and be made available within the Image File Wrapper (IFW) system. However, no data will be extracted from this form. Any additional data such as Foreign Patent Documents or Non Patent Literature will be manually reviewed and keyed into USPTO systems.					
3	NPL Documents	European_Search_Report.pdf	172165 0abeb82051ff727563c7913f123c4a82022e2fcb	no	3
Warnings:					
Information:					
		Total Files Size (in bytes):	1013525		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Docket No.: 0287000.00130US3
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gregory J. Hannon et al.	Confirmation No.:	8161
Application No.:	11/894,676	Art Unit:	N/A
Filed:	August 20, 2007	Examiner:	Not Yet Assigned
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT (IDS)

Dear Sir:

Applicants state that each item of information contained in the Information Disclosure Statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing date of this Information Disclosure Statement. No fee is required.

Enclosed please find a copy of the European Search Report corresponding to European Patent Application No 05857008.6, mailed on May 8, 2008. The documents cited in the European Search Report but not listed on the attached PTO form SB/08 are already of record in the subject application.

Applicants request that the Examiner initial and return a copy of the enclosed Form PTO SB-08 with the next communication.

6777447

Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 08-0219, under Order No. 0287000.00130US3 from which the undersigned is authorized to draw.

Respectfully submitted,

Dated: August 8, 2008

__/Jane M. Love, Ph.D./__________________
Jane M. Love, Ph.D.
Registration No.: 42,812
Attorney for Applicant(s)

Wilmer Cutler Pickering Hale and Dorr LLP
399 Park Avenue
New York, New York 10022
(212) 230-8800 (telephone)
(212) 230-8888 (facsimile)

PTO/SB/08a (03-08)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99)**		
	Application Number	11894676
	Filing Date	2007-08-20
	First Named Inventor	Gregory J. HANNON
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	0287000.00130US3

U.S.PATENTS

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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FOREIGN PATENT DOCUMENTS

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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	99/32619	WO		1999-07-01	Fire et al.		☐

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NON-PATENT LITERATURE DOCUMENTS

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99)**		
	Application Number	11894676
	Filing Date	2007-08-20
	First Named Inventor	Gregory J. HANNON
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	0287000.00130US3

	1	Bosher et al., "RNA interference can target pre-mRNA: consequences for gene expression in a Caenorhabditis elegans operon," Genetics, Vol 153, No 3, p. 1245-1256 (November 1999)	☐
	2	European Search report for European Patent application No 03732052.0, mailed May 23, 2008	☐
	3	Hasuwa et al., "Small interfering RNA and gene silencing in transgenic mice and rats," FEBS Letters, Elsevier, Amsterdam, NL, Vol 532, pp. 227-230 (December 2002)	☐
	4	Manche et al., "Interactions between double-stranded RNA regulators and the proteinkinase Dai," Molecular and cellular Biology, Amercian Society for Microbiology, Washington, US, Vol 12, pp. 5238-5248 (November 1992)	☐

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)	Application Number		11894676
	Filing Date		2007-08-20
	First Named Inventor	Gregory J. HANNON	
	Art Unit		N/A
	Examiner Name	Not Yet Assigned	
	Attorney Docket Number		0287000.00130US3

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☒ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☒ See attached certification statement.

☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ None

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Jane M. Love, Ph.D./	Date (YYYY-MM-DD)	2008-08-14
Name/Print	Jane M. Love, Ph.D.	Registration Number	42812

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Docket No.: 0287000.00130US3
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gregory J. Hannon et al.	Confirmation No.:	8161
Application No.:	11/894,676	Art Unit:	N/A
Filed:	August 20, 2007	Examiner:	Not Yet Assigned
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT (IDS)

Dear Sir:

Applicants state that each item of information contained in the Information Disclosure Statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing date of this Information Disclosure Statement. No fee is required.

Enclosed please find a copy of the European Search Report corresponding to European Patent application EP 03732052.0, mailed on May 23, 2008.

This Information Disclosure Statement is being filed before the mailing date of an Office Action on the merits.

Applicants request that the Examiner initial and return a copy of the enclosed Form PTO SB-08 with the next communication.

6782352

Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 08-0219, under Order No. 0287000.00130US3 from which the undersigned is authorized to draw.

Respectfully submitted,

Dated: August 14, 2008

_/Jane M. Love, Ph.D./___________________
Jane M. Love, Ph.D.
Registration No.: 42,812
Attorney for Applicant(s)

Wilmer Cutler Pickering Hale and Dorr LLP
399 Park Avenue
New York, New York 10022
(212) 230-8800 (telephone)
(212) 230-8888 (facsimile)

Electronic Acknowledgement Receipt

EFS ID:	3780893
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	28089
Filer:	Jane Maureen Love/sophie murray
Filer Authorized By:	Jane Maureen Love
Attorney Docket Number:	287000.134US1
Receipt Date:	14-AUG-2008
Filing Date:	20-AUG-2007
Time Stamp:	18:01:40
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes) /Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Filed (SB/08)	287000_130US3_Suppl_SB 08.pdf	773922 9fd56c6904bd9dfb5e0580650bbe9f8a9 dbb44db	no	4

Warnings:

Information:

A U.S. Patent Number Citation or a U.S. Publication Number Citation is required in the Information Disclosure Statement (IDS) form for autoloading of data into USPTO systems. You may remove the form to add the required data in order to correct the Informational Message if you are citing U.S. References. If you chose not to include U.S. References, the image of the form will be processed and be made available within the Image File Wrapper (IFW) system. However, no data will be extracted from this form. Any additional data such as Foreign Patent Documents or Non Patent Literature will be manually reviewed and keyed into USPTO systems.

2	Foreign Reference	WO09932619A1.pdf	2589603 98433601d9651c4193662d1d024d97c3c8373af6	no	54
Warnings:					
Information:					
3	NPL Documents	Bosher.pdf	1327739 c976d56bcf6de8459f05a39bd14dd6249f4692cf	no	12
Warnings:					
Information:					
4	NPL Documents	European_Search_Report_03732052.pdf	148429 f3899bbf9484ef3849ed19e9a310e6060c1a3106	no	2
Warnings:					
Information:					
5	NPL Documents	Hasuwa.pdf	496322 10794c3ebd6b2a7c99fa9de458e9f430d734657c	no	4
Warnings:					
Information:					
6	NPL Documents	Manche.pdf	1038293 7e5138e58171b852097c6803df1c85b0bed6b86c	no	11
Warnings:					
Information:					
7	Information Disclosure Statement Letter	287000_130US3_Suppl_IDS.pdf	87288 9687c6905a7a06947b8fc9b814a65bd2ede31dd4	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	6461596		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Hannon et al.	Confirmation No.:	4518
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Dear Sir:

In accordance with Applicants' duty of candor and good faith in dealing with the Office, as set forth in 37 CFR §1.56(a), and in accordance with 37 CFR §1.97 and §1.98, this Information Disclosure Statement, and the accompanying form PTO/SB/08, is being filed to bring to the attention of the Office certain facts pertaining to the prosecution of U.S. patent application 11/894,676 (hereinafter the "present application"), and also pertaining to U.S. patent applications 60/243,097, 09/858,862 , 09/866,557, 10/055,797, 10/350,798, 10/997,086, 11/330,043, 11/791,554, 12/152,655, and 12/152,837 and to international patent applications PCT/US01/08345, PCT/US03/01963, and PCT/US05/42488.

This Information Disclosure Statement (IDS) is being filed before the mailing of a first Office Action on the merits. Accordingly, no fees are believed to be due. However, in the event that any unforeseen fees are due, the Director is hereby authorized to charge any such fees, or credit any overpayment in fees, to Deposit Account No. 08-0219.

Applicants request that the Examiner initial and return a copy of the enclosed form PTO-SB-08 with the next communication.

The **Facts** disclosed are listed beginning on page 2 of this paper.

The **Exhibits** to this Information Disclosure Statement are listed on page 6 of this paper and on the accompanying form PTO/SB/08.

Additional **Remarks** are provided on page 7 of this paper.

FACTS

The following facts are brought to the attention of the Office:

1. International Patent Application PCT/US98/27233, which was published on July 1, 1999 with International Publication Number WO/99/32619, and which lists Andrew Fire as the first named inventor/applicant, was cited to the Office during the prosecution of the present application in the Information Disclosure Statement of January 7, 2008. International Patent Application PCT/US98/27233 is referred to hereinafter as the “Fire PCT.”

2. U.S. Patent No. 6,506,559, which issued on January 14, 2003, and which lists Andrew Fire as the first named inventor, was cited to the Office during the prosecution of the present application in the Information Disclosure Statement of January 7, 2008. U.S. Patent No. 6,506,559 is referred to hereinafter as the “Fire patent.”

3. The Fire PCT and the Fire patent are referred to collectively hereinafter as the “Fire specifications.”

4. The specification of the present application contains numerous sections of text that are the same as, or very similar to, text in the Fire specifications. A “marked-up” version of related application 09/866,557 is attached hereto as Exhibit A. In this marked-up version of the present application, text that is the same as text in the Fire specifications is highlighted.

5. U.S. patent application numbers 60/189,739, 60/243,097, 09/858,862, 09/866,557, 10/055,797, 10/350,798, 10/997,086, 11/330,043, 11/791,554, 12/152,655, and 12/152,837

(hereinafter the "related U.S. patent applications"), and international patent applications PCT/US01/08345, PCT/US03/01963, and PCT/US05/42488, are related to the present application. These related patent applications, together with the present application, are referred to herein as "the Hannon RNAi applications." With the exception of U.S. patent application number 60/189,739, all of the Hannon RNAi applications contain sections of text that are the same as, or very similar to, text in the specification the Fire specifications. Information Disclosure Statements disclosing this fact are being filed for each of the affected Hannon RNAi applications that are currently pending. The first filed of the Hannon RNAi applications to contain sections of text that are the same as, or very similar to, text in the specification the Fire specifications, was U.S. provisional patent application 60/243,097 ("the '097 application"), to which the present application claims priority. A "marked-up" version of the '097 application is attached hereto as Exhibit B. In this marked-up version of the '097 application, text that is the same as text in the Fire specifications is highlighted.

6. Cold Spring Harbor Laboratory (hereinafter the "CSHL") is the assignee of the entire right, title, and interest in the present application.

7. Dr. Vladimir Drozdoff, J.D. (hereinafter Dr. Drozdoff) is a Senior Licensing Associate and Patent Attorney for CSHL's Office of Technology Transfer. Dr. Drozdoff first began working at CSHL on February 11, 2008. As indicated in the Declaration of Dr. Drozdoff , attached hereto as Exhibit C, Dr. Drozdoff first became aware of the apparent "copying" of text from the Fire specifications into the Hannon RNAi applications upon reviewing the Office Action of 9/4/2007 in the file of the related U.S. patent application 09/866,557 (hereinafter the "Office Action") during the week of February 25, 2008. The Office Action states, on both page 8 and page 10, that "*the disclosure of cell[s]/organisms of the instant specification at pages 21-22 is essentially verbatim of the disclosure of Fire et al. at column 8*." As indicated in the attached Declaration of Dr. Drozdoff , after reviewing the Office Action, Dr. Drozdoff compared the text of the Fire specifications with the text of application 09/866,557 and with the text of the '097 provisional application and thereby discovered the full extent of the apparent "copying" of text from the Fire specifications. The textual similarities between the Fire specifications and application 09/866,557 can be seen in the "marked-

up" version of the present application attached hereto as Exhibit A. The textual similarities between the Fire specifications and the '097 application can be seen in the "marked-up" version of the '097 application attached hereto as Exhibit B.

8. Mr. John Maroney, J.D. (hereinafter Mr. Maroney) is the Vice President, Legal Counsel, and Director of CSHL's Office of Technology Transfer. As indicated in the Declaration of Mr. Maroney, attached hereto as Exhibit D, Mr. Maroney first became aware of the apparent "copying" of text from the Fire specifications into the Hannon RNAi applications upon being advised of the same by Dr. Drozdoff on March 3, 2008.

9. Professor Gregory Hannon (hereinafter "Professor Hannon") is a named inventor of the currently pending claims and is a Professor and Howard Hughes Medical Institute (HHMI) Investigator at CSHL. As indicated in the Declaration of Professor Hannon, attached hereto as Exhibit E, Professor Hannon first became aware of the apparent "copying" of text from the Fire specifications into the Hannon RNAi applications upon being advised of the same by Dr. Drozdoff and Mr. Maroney on March 18, 2008.

10. As indicated in the Declarations of Dr. Drozdoff, Mr. Maroney, and Professor Hannon, attached hereto as Exhibits C, D, and E, respectively, to the extent that any papers were filed with the Office, or any statements were made to the Office, during the prosecution of the present application, including any statements made to the Office about the Fire patent or the Fire PCT or any statements made about the present application that involved sections that are the same as sections of the Fire specifications, all such statements were made without any knowledge on the part of Dr. Drozdoff, Mr. Maroney, or Professor Hannon, or, to the best of their knowledge, on the part of CSHL, that the specification of the present application contains text that is the same as, or very similar to, text from the Fire specifications.

11. As indicated in the Declarations of Dr. Drozdoff and Mr. Maroney, attached hereto as Exhibits C and D respectively, after learning of the of the apparent "copying" of text from the Fire

specifications into the Hannon RNAi applications, Dr. Drozdoff and Mr. Maroney, acting on behalf of CSHL, diligently sought new counsel to advise CSHL in connection with the present application.

12. On May 8, 2008, CSHL engaged the undersigned as new counsel to advise CSHL in connection with the present application.

13. Between May 8, 2008 and June 13, 2008, Dr. Drozdoff and Mr. Maroney, acting on behalf of CSHL, had numerous meetings and teleconferences with Dr. Jane M. Love (the undersigned) and others of Wilmer Cutler Pickering Hale and Dorr, LLP (hereinafter WCPHD).

14. On June 13, 2008 CSHL revoked all previous powers of attorney given in the present application, and appointed the undersigned and those practitioners associated with Customer number 28089 as Attorneys of record for the present application, by executing a Revocation of Power of Attorney with New Power of Attorney and Change of Correspondence Address form (form PTO/SB/82).

15. On June 13, 2008, Dr. Love filed the above mentioned executed form PTO/SB/82 with the Office.

16. On June 13, 2008. Dr. Love telephoned Examiners McGarry, Vivlemore, and Chong, and SPE Schultz, to schedule an interview to discuss the facts described herein and in the attached Declarations.

17. On July 28, 2008. Dr. Love, together with Mr. Maroney, Dr. Drozdoff, and Professor Hannon, conducted an interview at the Patent Office with Examiners McGarry, Vivlemore, and Chong, and SPE Schultz, to discuss the facts described herein and in the attached Declarations.

EXHIBITS

The following Exhibits are submitted with this Information Disclosure Statement:

Exhibit A: Marked-up copy of related application 09/866,557 (filed 5/24/2001)
Exhibit B: Marked-up copy of provisional patent application 60/243,097 (filed 10/24/2000)
Exhibit C: Declaration of Dr. Vladimir Drozdoff (executed 8/5/2008)
Exhibit D: Declaration of Mr. John Maroney (executed 8/5/2008)
Exhibit E: Declaration of Professor Gregory Hannon (executed 8/5/2008)
Exhibit F: Letter of April 22, 2008 (referred to in Exhibit D)
Exhibit G: Letter of April 28, 2008 (referred to in Exhibit D)
Exhibit H: Letter of April 29, 2008 (referred to in Exhibit D)
Exhibit I: Letter of May 9, 2008 (referred to in Exhibit D)
Exhibit J: Letter of June 4, 2008 (referred to in Exhibit D)
Exhibit K: Letter of June 13, 2008 (referred to in Exhibit D)

Exhibits A and B provide marked-up copies of the present application and provisional patent application 60/243,097, respectively, in which text that is the same as text in the Fire specifications is highlighted. To the best of my knowledge, these marked-up documents are accurate.

In these marked-up applications, the numerals in the margins denote the page numbers of the corresponding sections of the Fire PCT.

In Exhibit A (application 09/866,557 as published), the following paragraphs contain copied text: [0037], [0120]-[0126], [0128]-[0131], [0133]-[0139], [0141]-[0144], and [0146-0151].

In Exhibit B (provisional patent application 60/243,097), the following pages contain copied text: page 3 (final two paragraphs), pages 4-13 (all paragraphs), and page 14 (first three paragraphs).

REMARKS

This paper is being filed to advise the Office of the above facts relating to the apparent "copying" of certain sections of the Fire specifications during the drafting of the present application, and to advise the Office of Mr. Maroney's, Dr. Drozdoff's, Professor Hannon's, the undersigned's, and, to the best of Mr. Maroney's, Dr. Drozdoff's and Professor Hannon's knowledge, CSHL's, lack of knowledge of, or complicity in, the same. This paper is also being filed to advise the Office that any previous statements made during the prosecution of the present application, and any previous declarations made by the inventors or others, were made without any knowledge, on the part of Mr. Maroney, Dr. Drozdoff, Professor Hannon, the undersigned, or, to the best of Mr. Maroney's, Dr. Drozdoff's and Professor Hannon's knowledge, anyone at CSHL, that the present application contains text that appears to have been copied from the Fire specifications.

If any further information is needed in relation to the above facts and remarks, the Examiner is invited to contact the undersigned at his convenience.

Dated: August 28, 2008

/Jane M. Love, Ph.D./
Jane M. Love, Ph.D.
Registration No.: 42,812
Attorney for Applicant(s)

Wilmer Cutler Pickering Hale and Dorr LLP
399 Park Avenue
New York, New York 10022
(212) 230-8800 (telephone)
(212) 230-8888 (facsimile)

PTO/SB/08a (01-08)
Approved for use through 07/31/2008. OMB 0651-0031
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Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet	1	of	3

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

PTO/SB/08b (01-08)
Approved for use through 07/31/2008. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449/PTO				*Complete if Known*	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				**Application Number**	**11/894,676-Conf.** #8161
				Filing Date	August 20, 2007
				First Named Inventor	Gregory J. HANNON
				Art Unit	1635
(Use as many sheets as necessary)				Examiner Name	K. Chong
Sheet	2	of	**3**	Attorney Docket Number	0287000.00130US3

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CA	Marked-up copy of application 09/866,557 (filed 5/24/2001)	
	CB	Marked-up copy of provisional patent application 60/243,097 (filed 10/24/2000)	
	CC	Declaration of Dr. Vladimir Drozdoff (executed 8/5/2008)	
	CD	Declaration of Mr. John Maroney (executed 8/5/2008)	
	CE	Declaration of Professor Gregory Hannon (executed 8/5/2008)	
	CF	Letter of April 22, 2008 from Douglass N. Ellis, Jr. of Ropes & Gray LLP to John Maroney, Esq. of Cold Spring Harbor Laboratory	
	CG	Letter of April 28, 2008 from John Maroney of Cold Spring Harbor Laboratory to Douglass N. Ellis, Jr. of Robes & Gray LLP	
	CH	Letter of April 29, 2008 from Douglass N. Ellis, Jr. from Robes & Gray LLP to John Maroney, Esq. of Cold Spring Harbor Laboratory	
	CI	Letter of May 9, 2008 to Eric R. Hubbard, Esq. of Robes & Gray LLP from John Maroney, Esq. of Cold Spring Harbor Laboratory	
	CJ	Letter of June 4, 2008 from Eric R. Hubbard of Robes & Gray LLP to John Maroney, Esq. of Cold Spring Harbor Laboratory	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

US1DOCS 6797646v1

PTO/SB/08b (01-08)
Approved for use through 07/31/2008. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Substitute for form 1449/PTO				*Complete if Known*	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				**Application Number**	**11/894,676-Conf.** #8161
				Filing Date	August 20, 2007
				First Named Inventor	Gregory J. HANNON
				Art Unit	1635
(Use as many sheets as necessary)				Examiner Name	K. Chong
Sheet	3	of	**3**	Attorney Docket Number	0287000.00130US3

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CK	Letter of June 13, 2008 from John Maroney, Esq. of Cold Spring Harbor Laboratory to James Haley, Esq. of Robes & Gray LLP	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

US1DOCS 6797646v1

Electronic Acknowledgement Receipt

EFS ID:	3852954
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	28089
Filer:	Jane Maureen Love/Carolyn DeCasseres
Filer Authorized By:	Jane Maureen Love
Attorney Docket Number:	287000.130US3
Receipt Date:	28-AUG-2008
Filing Date:	20-AUG-2007
Time Stamp:	15:28:53
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement Letter	287000_130US3_IDS_82808.pdf	126125 d921a7d5b9e145a5a454bc0477487736de331209	no	7

Warnings:

Information:

2	Information Disclosure Statement (IDS) Filed (SB/08)	287000_130US3_SB08_82808.pdf	147687 62564d38289891906b8075702cba9c19b043b009	no	3

Warnings:

Information:

This is not an USPTO supplied IDS fillable form

3	NPL Documents	287000_130US3_ExhibitA_82708.pdf	2176830 8cddf86ced9dd540c7039dece9ac418de7afc816	no	56

Warnings:

The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing

Information:

4	NPL Documents	287000_130US3_ExhibitB_82708.pdf	3036502 6a2d15d1b5f68e0d3ed273828b09435fce9326cc	no	54

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Information:

5	NPL Documents	287000_130US3_ExhibitC_82708.pdf	147114 378a90215fe4ca66a20823048e9363ead80c95f3	no	3

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Information:

6	NPL Documents	287000_130US3_ExhibitD_82708.pdf	364525 e98a29f1084d5cfc5129f03b56019cc382f97aa1	no	7

Warnings:

Information:

7	NPL Documents	287000_130US3_ExhibitE_82708.pdf	162016 37f64ac65ae97649390c8a16d982af2839e9b7db	no	3

Warnings:

Information:

8	NPL Documents	287000_130US3_ExhibitF_82708.pdf	95331 45af266dd4cd8057501f15997217a436f290b437	no	3

Warnings:

Information:

9	NPL Documents	287000_130US3_ExhibitG_82708.pdf	70075 d488e6510cbdf9babb607576eefde21ef2064863	no	2

Warnings:

Information:

10	NPL Documents	287000_130US3_ExhibitH_82708.pdf	33441 91e33de6c155fbfdec06ba9ee5f0e90ba3a7e416	no	1
Warnings:					
Information:					
11	NPL Documents	287000_130US3_ExhibitI_82708.pdf	148127 200880b5703f99a172f840c85d73e9f590202986	no	4
Warnings:					
Information:					
12	NPL Documents	287000_130US3_ExhibitJ_82708.pdf	929748 297a1d4da06c5e040bb2de7e76b62861c7febc33	no	3
Warnings:					
Information:					
13	NPL Documents	287000_130US3_ExhibitK_82708.pdf	179658 baf59f1261077790f176cbe098297322855fe622	no	6
Warnings:					
Information:					
Total Files Size (in bytes):			7617179		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3

CONFIRMATION NO. 8161

28089
WILMERHALE/NEW YORK
399 PARK AVENUE
NEW YORK, NY 10022

PUBLICATION NOTICE

Title: Methods and compositions for RNA interference

Publication No.US-2008-0213861-A1
Publication Date:09/04/2008

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Office of Public Records. The Office of Public Records can be reached by telephone at (703) 308-9726 or (800) 972-6382, by facsimile at (703) 305-8759, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently http://pair.uspto.gov/. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INTERVIEW SUMMARY

Dear Sir:

Further to the interview of July 28, 2008 held with Examiners Vivlemore, Chong, McGarry and Schultz (hereinafter "the Examiners"), and in response to the Interview Summary dated August 6, 2008 as issued by the Office for related application 09/858,862, Applicants hereby submit this record of the substance of the interview, as required by 37 C.F.R. §1.133(b). No fees are believed to be due for the filing of this paper. However, in the event that any unforeseen fees are due, the Director is hereby authorized to charge any such fee, or credit any overpayment of fees, to Deposit Account No. 08-0219.

During the interview of July 28, 2008, the undersigned, together with Applicants' representatives, Mr. John Maroney, Dr. Drozdoff, and Professor Hannon, disclosed that the present application, and certain related applications, contain disclosure that appears to have been copied from the specification of International Patent Application PCT/US98/27233 (WO/99/32619, hereinafter the "Fire PCT"). Applicants' representatives explained that the apparent and unauthorized copying had taken place, to the best of their information and belief, without the knowledge, consent or involvement of anyone at Cold Spring Harbor Laboratory. The undersigned advised the Examiners of Applicants' intention to file an Information Disclosure Statement and associated declarations by Mr. John Maroney, Dr. Drozdoff, and Professor Hannon providing

further details regarding Applicants' investigation of the facts and circumstances relating to the copying. The Information Disclosure Statement and associated Declarations were subsequently filed with the Patent Office on August 28, 2008.

There was no discussion of specific claims, claim amendments, or the merits of the application during the Interview. Furthermore, other than the discussion of the apparent copying from the Fire PCT, there was no discussion of prior art during the interview.

Applicants believe that the above provides an accurate record of the substance of the interview of July 28, 2008. If any further information is needed, the Examiner is invited to contact the undersigned at her convenience.

Dated: September 8, 2008

/Jane M. Love, Ph.D./
Jane M. Love, Ph.D.
Registration No.: 42,812
Attorney for Applicant(s)

Wilmer Cutler Pickering Hale and Dorr LLP
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(212) 230-8800 (telephone)
(212) 230-8888 (facsimile)

Electronic Acknowledgement Receipt

EFS ID:	3903778
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	28089
Filer:	Jane Maureen Love/Carolyn DeCasseres
Filer Authorized By:	Jane Maureen Love
Attorney Docket Number:	287000.130US3
Receipt Date:	08-SEP-2008
Filing Date:	20-AUG-2007
Time Stamp:	16:52:08
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant summary of interview with examiner	287000_130US3_InterviewSummary_9808.pdf	76349 8b22c12449e0107d0c08aa56809bd7d053612087	no	2

Warnings:

Information:

Total Files Size (in bytes):	76349

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New International Application Filed with the USPTO as a Receiving Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

28089 7590 10/09/2008
WILMERHALE/NEW YORK
399 PARK AVENUE
NEW YORK, NY 10022

EXAMINER	
CHONG, KIMBERLY	

ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
10/09/2008	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

michael.mathewson@wilmerhale.com
teresa.carvalho@wilmerhale.com
sharon.matthews@wilmerhale.com

PTOL-90A (Rev. 04/07)

Office Action Summary	Application No. 11/894,676	Applicant(s) HANNON ET AL.	
	Examiner KIMBERLY CHONG	Art Unit 1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *1* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *23 November 2007*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-35* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) *1-35* are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)

3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.

4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

5) ☐ Notice of Informal Patent Application

6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 1-2, 4-20 and 23, drawn to a method for attenuating expression of a target gene in mammalian cells comprising introducing into the mammalian cells a shRNA, classifiable in class 435, subclass 375.

II. Claims 3-15 and 21-23, drawn to a method for attenuating expression of a target gene in mammalian cells comprising introducing into the mammalian cells a variegated library of shRNA, classifiable in class 435, subclass 375.

III. Claims 24-29, drawn to a method of enhancing the potency/activity of an RNAi therapeutic for a mammalian patient, said RNAi therapeutic comprising a siRNA of 19-22 paired polynucleotide, the method comprising replacing said siRNA with an shRNA, classifiable in class 435, subclass 6.

IV. Claims 30-35, drawn to a method of designing a shRNA construct for RNAi, said shRNA comprising a 3' overhang of about 1-4 nucleotides, classifiable in class 435, subclass 6.

The inventions are distinct, each from the other because of the following reasons:

Inventions of groups I, II, III and IV are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have

different designs, modes of operation, and effects (MPEP § 802.01 and § 806.06). In the instant case, the different inventions are drawn to materially different methods with different modes of operation. For example, the method of group I involves attenuation of expression of a target gene by administering a single shRNA, whereas the method of group II involves attenuation of one or more target genes by administering a multitude of shRNAs. The method of group III involves enhancing the potency or activity of a RNAi therapeutic by replacing a siRNA with a shRNA, which is materially different than administering a shRNA or variegated library of shRNA to mammalian cells. The method of group IV involves designing a shRNA construct for RNAi which involves method steps not necessarily found in the methods of groups I, II or III. Furthermore restriction is proper because the subject matter is divergent and non-coextensive and a search for one would not necessarily reveal art against the other. It is therefore a burden to search these inventions in a single application.

Restriction for examination purposes as indicated is proper because all these inventions listed in this action are independent or distinct for the reasons given above and there would be a serious search and examination burden if restriction were not required because one or more of the following reasons apply:

(a) the inventions have acquired a separate status in the art in view of their different classification;

(b) the inventions have acquired a separate status in the art due to their recognized divergent subject matter;

(c) the inventions require a different field of search (for example, searching different classes/subclasses or electronic resources, or employing different search queries);

(d) the prior art applicable to one invention would not likely be applicable to another invention;

(e) the inventions are likely to raise different non-prior art issues under 35 U.S.C. 101 and/or 35 U.S.C. 112, first paragraph.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a invention to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected invention.**

The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144.

If claims are added after the election, applicant must indicate which of these claims are readable upon the elected invention.

Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is

the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

This application contains claims directed to the following patentably distinct species. Claim 11 is directed to patentably distinct promoters. The species are independent or distinct because claims to the different species recite the mutually exclusive characteristics of such species. In addition, these species are not obvious variants of each other based on the current record.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claim 1 is generic.

There is an examination and search burden for these patentably distinct species due to their mutually exclusive characteristics. The species require a different field of search (e.g., searching different classes/subclasses or electronic resources, or employing different search queries); and/or the prior art applicable to one species would

not likely be applicable to another species; and/or the species are likely to raise different non-prior art issues under 35 U.S.C. 101 and/or 35 U.S.C. 112, first paragraph.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected species**, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

The election of the species may be made with or without traverse. To preserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the election of species requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected species.

Should applicant traverse on the ground that the species are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the species unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other species.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which depend from or otherwise require all the limitations of an allowable generic claim as provided by 37 CFR 1.141.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Kimberly Chong whose telephone number is 571-272-3111. The examiner can normally be reached Monday thru Friday between 7-4 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, James Schultz can be reached at 571-272-0763. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file folder(s) as well as general patent information available to the public. For more information about the PAIR system, see http://pair-direct.uspto.gov.

For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

/Kimberly Chong/
Examiner
Art Unit 1635

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AND RESPONSE TO OFFICE ACTION

This paper is filed in response to the October 9, 2008 Office Action, setting a one-month period for reply. Applicants request a five-month extension of the time up to and including April 9, 2009. Accordingly, this paper is being timely filed. The Director is authorized to charge the required fee for the extension of time and any other fees occasioned by this paper, and/or to credit any overpayment in fees, to Deposit Account No. 08-0219.

Amendments to the Claims begin on page 2.
Remarks begin on page 4.

AMENDMENT

In The Claims

Please amend the claims as follows, without prejudice. This listing of the claims will replace all prior versions and listings of claims in the application:

1-49. (Cancelled)

50. (New) A method for attenuating expression of a target gene in a mammalian cell, the method comprising introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising:

(i) an RNA polymerase promoter, and

(ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region,

wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and

wherein the short hairpin RNA molecule is expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner.

51. (New) The method of claim 50, wherein the short hairpin RNA is stably expressed in the mammalian cell.

52. (New) The method of claim 50, wherein the expression construct further comprises LTR sequences located 5' and 3' of the sequence encoding the short hairpin RNA molecule.

53. (New) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region of at least 20 nucleotides.

54. (New) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region of at least 21 nucleotides.

55. (New) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region of at least 22 nucleotides.

56. (New) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region of at least 25 nucleotides.

57. (New) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region of 29 nucleotides.

58. (New) The method of claim 50, wherein the short hairpin RNA molecule has a total length of about 70 nucleotides.

59. (New) The method of claim 50, wherein the RNA polymerase promoter comprises a pol II promoter or a pol III promoter.

60. (New) The method of claim 59, wherein the pol III promoter comprises a U6, an H1, or an SRP promoter.

61. (New) The method of claim 59, wherein the pol II promoter comprises a U1 or a CMV promoter.

REMARKS

I. Status of the Claims & Priority Support

By this paper, all previously pending claims are canceled, without prejudice, and new claims 50-61 are added. The new claims adopt the claim language of pending parent application Serial No. 10/997,086, of which this application is a continuation. No new matter has been added.

The new claims are entitled to the benefit of priority of the filing date of U.S. application serial number 10/055,797. Applicants reserve the right to assert entitlement to an earlier priority date in future prosecution. Support for the claims is found in the specification of the present application and in the specification of the grandparent application U.S.S.N. 10/055,797. The below table illustrates some of the locations where such support can be found.

Claim Language	Support in 10/055,797 U.S. Pub. No. 2003/0084471	Support in 11/894,676 U.S. Pub. No. 2008/0213861
50. A method for attenuating expression of a target gene in a mammalian cell, the method comprising introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising: (i) an RNA polymerase promoter, and (ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region, wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and wherein the short hairpin RNA molecule is expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner.	[0019] "Yet another aspect of the present invention provides a method for attenuating expression of a target gene in cultured cells, comprising introducing an expression vector having a "coding sequence" which, when transcribed, produces double stranded RNA (dsRNA) in the cell in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene… In [some] embodiments, the vector includes a coding sequence which forms a hairpin." [0027] Still another aspect of the present invention provides an assay for identifying nucleic acid sequences, either coding or non-coding sequences, responsible for conferring a particular phenotype in a cell, comprising [0028] (i) constructing a variegated library of nucleic acid sequences from a cell in an orientation relative to a promoter to produce double stranded DNA; [0029] (ii) introducing the variegated dsRNA library into a culture of target cells;	See same text in paragraphs [0017], [0025], [0118], [220], and [345]. Note that FIG. 42 in the '797 application corresponds to FIG. 46 in the '676 application.

Claim Language	Support in 10/055,797 U.S. Pub. No. 2003/0084471	Support in 11/894,676 U.S. Pub. No. 2008/0213861
	[0030] (iii) identifying members of the library which confer a particular phenotype on the cell, and identifying the sequence from a cell which correspond, such as being identical or homologous, to the library member. [0093] FIG. 42: Encoded short hairpins specifically suppress gene expression in vivo. DNA oligonucleotides encoding 29 nucleotide hairpins corresponding to firefly luciferase were inserted into a vector containing the U6 promoter. Three independent constructs were examined for their ability to specifically suppress firefly luciferase gene expression in 293T cells. [0174] In an exemplary embodiment, the subject invention provides an arrayed library of RNAi constructs. [0252] "FIG. 42 demonstrates that short hairpins encoded on a plasmid are effective in suppressing gene expression. DNA oligonucleotides encoding 29 nucleotide hairpins corresponding to firefly luciferase were inserted into a vector containing the U6 promoter. Three independent constructs were examined for their ability to specifically suppress firefly luciferase gene expression in [mammalian] 293T cells."	
51. The method of claim 50, wherein the short hairpin RNA is stably expressed in the mammalian cell.	[0019] "In certain embodiments, the vectors are episomal, e.g., and transfection is transient. In other embodiments, the vectors are chromosomally integrated, e.g., to produce a stably transfected cell line." [0024] "The double-stranded RNA may be an siRNA or a hairpin, and may be expressed transiently or stably." [0253] "One of skill can choose from amongst a range of vectors to either transiently or stably express an siRNA or short hairpin. Non-limiting examples of vectors and strategies to stably express short dsRNAs are presented in FIGS. 43-45."	See same text in paragraphs [0017], [0022], and [0346]. Note that FIGS. 43-45 in the '797 application correspond to FIGS. 47-49 in the '676 application.
52. The method of claim 50, wherein the expression construct further comprises LTR sequences	[0253] "One of skill can choose from amongst a range of vectors to either transiently or stably express an siRNA or short hairpin.	See same text in paragraph [0346].

Claim Language	Support in 10/055,797 U.S. Pub. No. 2003/0084471	Support in 11/894,676 U.S. Pub. No. 2008/0213861
located 5' and 3' of the sequence that, when transcribed, forms the short hairpin RNA molecule.	Non-limiting examples of vectors and strategies to stably express short dsRNAs are presented in FIGS. 43-45." See Figure 45, which illustrates a vector for stable expression in which the sequence encoding the hairpin RNA is flanked by LTR sequences.	Note that FIG. 45 in the '797 application corresponds to FIG. 49 in the '676 application. See Figure 49, which illustrates a vector for stable expression in which the sequence encoding the hairpin RNA is flanked by LTR sequences.
53. The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region of at least 20 nucleotides.	[0017] "In certain preferred embodiments, the length of the dsRNA is at least 20, 21 or 22 nucleotides in length, e.g., corresponding in size to RNA products produced by Dicer-dependent cleavage."	See same text in paragraph [0015].
54. The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region of at least 21 nucleotides.	See paragraph 17, as excerpted above.	See same text in paragraph [0015].
55. The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region of at least 22 nucleotides.	See paragraph 17, as excerpted above.	See same text in paragraph [0015].
56. The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region of at least 25 nucleotides.	[0017] "In certain embodiments, the dsRNA construct is at least 25, 50, 100, 200, 300 or 400 bases."	See same text in paragraph [0015].
57. The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region of 29 nucleotides.	[0252] "FIG. 42 demonstrates that short hairpins encoded on a plasmid are effective in suppressing gene expression. DNA oligonucleotides encoding 29 nucleotide hairpins corresponding to firefly luciferase were inserted into a vector containing the U6 promoter."	See same text in paragraph [345].
58. The method of claim 50, wherein the short hairpin RNA molecule has a total length of about 70 nucleotides.	See FIG. 42, showing a plasmid containing a short hairpin construct having a total length of about 70 nucleotides.	See FIG. 46, which is the same as FIG. 42 in the '797 application.
59. The method of claim 50, wherein the RNA polymerase promoter comprises a pol II promoter or a pol III promoter.	See FIG. 43, which illustrates strategies for stable expression and shows pol II, and pol III promoters. See also paragraph [0252] "…29 nucleotide hairpins…were inserted into a vector containing the U6 promoter." The U6 promoter is a pol III promoter.	See FIG. 47, which is the same as FIG. 43 in the '797 application. See also paragraph [0345] which contains the same text as paragraph [0252] in the '797 application.

Claim Language	Support in 10/055,797 U.S. Pub. No. 2003/0084471	Support in 11/894,676 U.S. Pub. No. 2008/0213861
60. The method of claim 59, wherein the pol III promoter comprises a U6, an H1, or an SRP promoter.	See FIG. 42, which shows a vector with a U6 promoter, and FIG. 43, which lists U6 snRNA, H1 RNA, and SRP RNA promoters. See also paragraph [0252] "…29 nucleotide hairpins…were inserted into a vector containing the U6 promoter."	See FIG. 46, which is the same as FIG. 42 in the '797 application. See also paragraph [0345] which contains the same text as paragraph [0252] in the '797 application.
61. The method of claim 59, wherein the pol II promoter comprises a U1 or a CMV promoter.	See FIG. 43, which lists U1 snRNA and CMV promoters.	See FIG. 47, which is the same as FIG. 43 in the '797 application.

II. Response to Restriction Requirement

The Office Action of October 9, 2008 required election under 35 U.S.C. § 121 from among the following groups:

I. Claims 1-2, 4-20 and 23, drawn to a method for attenuating expression of a target gene in mammalian cells comprising introducing into the mammalian cells a shRNA, classifiable in class 435, subclass 375;

II. Claims 3-15 and 21-23, drawn to a method for attenuating expression of a target gene in mammalian cells comprising introducing into the mammalian cells a variegated library of shRNA, classifiable in class 435, subclass 375;

III. Claims 24-29, drawn to a method of enhancing the potency/activity of an RNAi therapeutic for a mammalian patient, said RNAi therapeutic comprising a siRNA of 19-22 paired polynucleotides, the method comprising replacing said siRNA with an shRNA, classifiable in class 435, subclass 6; and

IV. Claims 30-35, drawn to a method of designing a shRNA construct for RNAi, said shRNA comprising a 3' overhang of about 1-4 nucleotides, classifiable in class 435, subclass 6.

Initially, Applicants note that the above restriction requirement was drawn without regard to the Preliminary Amendment filed with this application on August 20, 2007, cancelling claims 1-35 and adding claims 36-49. Nonetheless, by this paper, Applicants cancel all pending claims and add

new claims 50-61. All of the new claims are drawn to Group II, which Applicants elect without traverse.

The Office Action also required an election of species, asserting that claim 11 was drawn to patentably distinct promoters. Applicants elect, with travserse, the U6 promoter. Claims 50-59 are generic and encompass the elected species. Claim 60 recites the elected species. Applicants submit that the combined search and examination of all recited promoters would not impose a serious burden on the Examiner, and that, therefore, the requirement for an election of species is not proper under MPEP § 808.01(a) and/or MPEP § 808.02. Applicants submit that searches for art pertinent to each species will necessarily be co-extensive, and that accordingly, there would be no serious burden associated with searching all species simultaneously.

CONCLUSION

Consideration of this paper, and early allowance of this application, are respectfully requested. If it would advance prosecution, the Examiner is invited to contact the undersigned to discuss the contents of this paper.

Dated: April 9, 2009

Respectfully submitted,

/Anne-Marie C. Yvon/

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Electronic Patent Application Fee Transmittal

Application Number:	11894676
Filing Date:	20-Aug-2007
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Filer:	Anne-Marie Yvon/Patricia Ierardi
Attorney Docket Number:	287000.130US3

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 5 months with $0 paid	2255	1	1175	1175

Benitec - Exhibit 1002 - page 473

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
		Total in USD ($)		**1175**

Electronic Acknowledgement Receipt

EFS ID:	5124824
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	28089
Filer:	Anne-Marie Yvon/Patricia Ierardi
Filer Authorized By:	Anne-Marie Yvon
Attorney Docket Number:	287000.130US3
Receipt Date:	09-APR-2009
Filing Date:	20-AUG-2007
Time Stamp:	14:55:05
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1175
RAM confirmation Number	982
Deposit Account	080219
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Extension of Time	287000_130US3_EOT_040909.pdf	93049 cd53bc9113e6b3c5adb4c843deed450852d1ca45	no	1

Warnings:

Information:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
2		287000_130US3_Amendment_040909.pdf	97775 653a8041465a2521ce85176d4481c754d3eca736	yes	8

Multipart Description/PDF files in .zip description

Document Description	Start	End
Response to Election / Restriction Filed	1	1
Claims	2	3
Applicant Arguments/Remarks Made in an Amendment	4	8

Warnings:

Information:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
3	Fee Worksheet (PTO-06)	fee-info.pdf	30256 1952258a05623d912667350e874b40e99ca89cd1	no	2

Warnings:

Information:

Total Files Size (in bytes):	221080

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/22 (10-08)
Approved for use through 10/31/2008. OMB 0651-0031
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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)
FY 2009
(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)

Docket Number (Optional)
0287000.00130US3

Application Number 11/894,676-Conf. #8161

Filed August 20, 2007

For METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

Art Unit 1635

Examiner K. Chong

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$130	$65	$ ______
☐	Two months (37 CFR 1.17(a)(2))	$490	$245	$ ______
☐	Three months (37 CFR 1.17(a)(3))	$1110	$555	$ ______
☐	Four months (37 CFR 1.17(a)(4))	$1730	$865	$ ______
X	Five months (37 CFR 1.17(a)(5))	$2350	$1175	$ 1,175.00

- [X] Applicant claims small entity status. See 37 CFR 1.27.
- [ ] A check in the amount of the fee is enclosed.
- [ ] Payment by credit card. Form PTO-2038 is attached.
- [X] The Director has already been authorized to charge fees in this application to a Deposit Account.
- [X] The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 08-0219 .

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

- [ ] applicant/inventor.
- [ ] assignee of record of the entire interest. See 37 CFR 3.71.
  Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).
- [X] attorney or agent of record. Registration Number 52,390
- [ ] attorney or agent under 37 CFR 1.34.
  Registration number if acting under 37 CFR 1.34 ______

/Anne-Marie C. Yvon/	April 9, 2009
Signature	Date
Anne-Marie C. Yvon	(212) 230-8800
Typed or printed name	Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

- [ ] Total of 1 forms are submitted.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD
Substitute for Form PTO-875

Application or Docket Number: 11/894,676
Filing Date: 08/20/2007
☐ To be Mailed

APPLICATION AS FILED – PART I

SMALL ENTITY ☒ OR OTHER THAN SMALL ENTITY

FOR	NUMBER FILED (Column 1)	NUMBER EXTRA (Column 2)	SMALL ENTITY RATE ($)	SMALL ENTITY FEE ($)		OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
			TOTAL			TOTAL	

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED – PART II

SMALL ENTITY OR OTHER THAN SMALL ENTITY

AMENDMENT

04/09/2009	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 12	Minus	** 20	= 0	X $26 =	0	OR	X $ =	
Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	X $110 =	0	OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT

	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/SANDRA L. TUCKER SMITH/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

28089 7590 05/04/2009
WILMERHALE/NEW YORK
399 PARK AVENUE
NEW YORK, NY 10022

EXAMINER	
CHONG, KIMBERLY	

ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
05/04/2009	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

michael.mathewson@wilmerhale.com
teresa.carvalho@wilmerhale.com
sharon.matthews@wilmerhale.com

PTOL-90A (Rev. 04/07)

Office Action Summary	Application No.	Applicant(s)	
	11/894,676	HANNON ET AL.	
	Examiner	Art Unit	
	KIMBERLY CHONG	1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *09 April 2009*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *50-61* is/are pending in the application.

4a) Of the above claim(s) *61* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *50-60* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *20 August 2007* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08)
Paper No(s)/Mail Date *1/10/08,8/8/08,8/14/05,8/28/08*.
4) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

Applicant's election with traverse of Group I and the U6 promoter, in the reply filed on 04/09/2009 is acknowledged. Applicant argues the combined search and examination of all the promoters would not impose a serious burden on the Examiner because searches for each species will be co-extensive. This argument is not persuasive because the species are not obvious variants of each other and a search for a RNA expression construct comprising a U6 promoter will not necessarily return art for a construct comprising a pol II promoter such as CMV, for example.

Therefore, the requirement is still deemed proper and is therefore made FINAL.

Status of the Application

Claims 50-61 are pending. Claims 50-60 are currently under examination. Claim 61 and non-elected subject matter are withdrawn as being drawn to a non-elected invention.

Information Disclosure Statement

The submission of the Information Disclosure Statements on 01/10/2008, 08/08/2008, 08/14/2008 and 08/28/2008 is in compliance with 37 CFR 19.7. The information disclosure statements have been considered by the examiner and signed copies have been placed in the file.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970);and, *In re Thorington,* 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 50-60 are provisionally rejected under the judicially created doctrine of double patenting over claims 1, 2, 7-20, 24 and 59-63 of copending Application No. 10/350,798. This is a provisional double patenting rejection since the conflicting claims have not yet been patented.

Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims are directed to a method of attenuating gene expression in a mammalian cell comprising introducing a library of RNA expression constructs wherein the construct comprises a promoter and a shRNA targeted to a gene. The claims of the ’798 application are directed to the same method using a dsRNA performed in vertebrate cells. The specification of the ’798 application discloses the dsRNA can be a short a hairpin RNA on page 5 and discloses using a library of RNAi constructs on page 38 and it would be obvious to use a shRNA and a library of RNAi constructs in the methods of the ’798 application.

Thus the instant claims and claims 1, 2, 7-20, 24 and 59-63 of copending Application No. 10/350,798 overlap in scope. This is a provisional obviousness-type double patenting rejection.

Claims 50-60 are provisionally rejected under the judicially created doctrine of double patenting over claims 1, 2, 6-7, 9-10 and 23-28 of copending Application No. 09/858,862. This is a provisional double patenting rejection since the conflicting claims have not yet been patented.

Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims are directed to a method of attenuating gene expression in a mammalian cell comprising introducing a library of RNA expression constructs wherein the construct comprises a promoter and a shRNA targeted to a gene. The claims of the ’862 application are directed to the same method using a dsRNA. The specification of the ’862 application discloses the dsRNA can be a short a hairpin RNA and discloses using a library of RNAi constructs on pages 3 and 23-24 and it would be obvious to use a shRNA and a library of RNAi constructs in the methods of the ’862 application.

Thus the instant claims and claims 1, 2, 6-7, 9-10 and 23-28 of copending Application No. 09/858,862 overlap in scope. This is a provisional obviousness-type double patenting rejection.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claim 52 is rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This is a new matter rejection.

Claim 52 recites the expression construct further comprises LTR sequences located 5' and 3' of the sequence encoding the short hairpin RNA molecule and points to Figure 49 for support.

Figure 49 does not explicitly disclose a construct with LTR sequences located at the 5’ and 3’ ends of a sequence encoding a shRNA. The Figure shown does not indicate which part of the supposed construct is a LTR sequence or indicate 5’ or 3’ ends of a sequence. Furthermore, the brief description of the figure in the instant specification does not disclose the claimed limitations.

If Applicant believes that such support is present in the specification and claimed priority documents, Applicant should point, with particularity, to where such support is to be found.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent.

The changes made to 35 U.S.C. 102(e) by the American Inventors Protection Act of 1999 (AIPA) and the Intellectual Property and High Technology Technical Amendments Act of 2002 do not apply when the reference is a U.S. patent resulting directly or indirectly from an international application filed before November 29, 2000. Therefore, the prior art date of the reference is determined under 35 U.S.C. 102(e) prior to the amendment by the AIPA (pre-AIPA 35 U.S.C. 102(e)).

Claims 50-51 and 53-58 are rejected under 35 U.S.C. 102(e) as being anticipated by Fire et al. (US Patent Number 6,506,559).

The claims are drawn to a method of attenuating expression of a target gene in a mammalian cells comprising introducing into mammalian cells a library of RNA expression constructs wherein each construct comprises a promoter and wherein the shRNA comprises at least 20, 21, 22 or 25 double-stranded region, or a 29 nucleotide double stranded region or is about 70 nucleotides in length.

Fire et al. disclose a method of attenuating expression of a target gene in mammalian cells (see column 8, lines 12-19) wherein the RNA can be formed by a

single self-complementary RNA i.e. a hairpin RNA (see column 7, lines 42-44) and wherein inhibition is sequence specific (see column 7, lines 49-52). Fire et al. discloses each strand of the dsRNA may be at least 25 nucleotides in length up to 400 nucleotides in length, which would meet the limitation of claims 53-58 because the dsRNA has at least 20, 21, 22 or 25 nucleotides, has 29 nucleotides and about 70 nucleotides. Fire et al. additionally teach the dsRNA is a transcriptional product of a RNA polymerase, a bacteriophage RNA promoter or a T7, T3 or a SP6 promoter (see column 7, lines 5-15 and Figure 5A) and disclose introducing into mammalian cells a library of RNA expression constructs (see columns 12-13). Fire et al. further discloses Fire et al. teach the shRNA be targeted to intracellular regions or untranscribed regions of a target gene (see column 9).

Thus, the instant claims are anticipated by Fire et al.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 50-59 are rejected under 35 U.S.C. 103(a) as being unpatentable over Fire et al. (US Patent Number 6,506,559 cited on Applicant's IDS filed 01/10/2008),

Good et al. (Gene Therapy 1997 cited on Applicant's IDS filed 01/10/2008) and Noonberg et al. (US Patent NO. 5,624,803).

The claims are drawn to a method of attenuating expression of a target gene in a mammalian cells comprising introducing into mammalian cells a library of RNA expression constructs wherein each construct comprises a promoter and a shRNA wherein the construct comprises LTR sequences and wherein the shRNA comprises at least 20, 21, 22 or 25 double-stranded region, or a 29 nucleotide double stranded region or is about 70 nucleotides in length and wherein the promoter is a pol III, U6 promoter.

Fire et al. disclose a method of attenuating expression of a target gene in mammalian cells (see column 8, lines 12-19) wherein the RNA can be formed by a single self-complementary RNA i.e. a hairpin RNA (see column 7, lines 42-44) and wherein inhibition is sequence specific (see column 7, lines 49-52). Fire et al. discloses each strand of the dsRNA may be at least 25 nucleotides in length up to 400 nucleotides in length, which would meet the limitation of claims 53-58 because the dsRNA has at least 20, 21, 22 or 25 nucleotides, has 29 nucleotides and about 70 nucleotides. Fire et al. additionally teach the dsRNA is a transcriptional product of a RNA polymerase, a bacteriophage RNA promoter or a T7, T3 or a SP6 promoter (see column 7, lines 5-15 and Figure 5A) and disclose introducing into mammalian cells a library of RNA expression constructs (see columns 12-13). Fire et al. further discloses Fire et al. teach the shRNA be targeted to intracellular regions or untranscribed regions of a target gene (see column 9). Fire et al. does not specifically disclose the expression construct comprises a pol III or specifically a U6 promoter.

Good et al. teach an expression construct comprising a U6 promoter and a coding sequence for a hairpin RNA wherein the expression construct is capable of efficiently expressing small hairpin RNA (see at least Figure 1).

Noonberg et al. teach an expression construct for generation of short-sequence specific oligonucleotides for the purpose of gene regulation wherein the construct comprises a U6 promoter (see columns 7-8). Noonberg et al. teach such constructs facilitate delivery of oligonucleotides to any target cell.

It would have been obvious to one of ordinary skill in the art to use a U6 promoter in the RNA expression construct to generate shRNA that are capable of attenuating expression of a target gene.

It was well known in the art that pol III promoters such as U6 promoters could be used to efficiently generate inhibitory oligonucleotides as taught by Noonberg et al. and given Good et al. teach a construct comprising U6 promoters were capable of expressing shRNA, one of ordinary skill in the art would have used a U6 promoter to generate the shRNA of Fire et al. One of ordinary skill in the art would have expected to be able to generate the shRNA of Fire et al. from a RNA construct that was capable of attenuating expression of a target gene because this was taught by Good et al.

Thus in the absence of evidence to the contrary, the invention as a whole would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made.

Claims 50-59 are rejected under 35 U.S.C. 103(a) as being unpatentable over Kreutzer et al. (US Application No. 20040102408), Lieber et al. (US Patent No.

6,130,092 cited on Applicant's IDS filed 01/10/2008), Good et al. (Gene Therapy 1997 cited on Applicant's IDS filed 01/10/2008) and Noonberg et al. (US Patent NO. 5,624,803).

The claims are drawn to a method of attenuating expression of a target gene in a mammalian cells comprising introducing into mammalian cells a library of RNA expression constructs wherein each construct comprises a promoter and a shRNA wherein the construct comprises LTR sequences and wherein the shRNA comprises at least 20, 21, 22 or 25 double-stranded region, or a 29 nucleotide double stranded region or is about 70 nucleotides in length and wherein the promoter is a pol III, U6 promoter.

Kreutzer et al. disclose a method of attenuating expression of a target gene in mammalian cells using a shRNA comprising10-1000 nucleotide base pairs, preferably 15-49base pairs (see page 2). The shRNA having 49 nucleotide base pairs would meet the limitation of "about 70 nucleotides" in claim 58. Kreutzer et al. teach expression of shRNA using an expression vector (see pages 2-3). Kreutzer et al. does not teach introduction of a library of RNA expression constructs and do not specifically teach the expression construct comprises a pol III or specifically a U6 promoter.

Lieber et al. teach a method of searching for a function gene comprising making randomized ribozyme libraries and introducing h ribozyme libraries into mammalian cells, selecting cells into which the library expression systems were introduced and analyzing the phenotypes of the cells (see Figure 2 and columns 3 and 8 and claims 1-8). Lieber et al. teach the ribozymes are is chemically synthesized by transcription using expression cassettes comprising pol II or pol III promoters (see column 3).

Good et al. teach an expression construct comprising a U6 promoter and a coding sequence for a hairpin RNA wherein the expression construct is capable of efficiently expressing small hairpin RNA and LTR sequences flanking the RNA sequences (see entire document and at least Figure 1).

Noonberg et al. teach an expression construct for generation of short-sequence specific oligonucleotides for the purpose of gene regulation wherein the construct comprises a U6 promoter (see columns 7-8). Noonberg et al. teach such constructs facilitate delivery of oligonucleotides to any target cell.

It would have been obvious to one of ordinary skill in the art to use a library of RNA expression constructs capable of expression shRNA and obvious to use a U6 promoter in the RNA expression construct to generate shRNA that are capable of attenuating expression of a target gene.

Lieber et al. teach identifying a gene responsible for a particular phenotype is crucial to important any biological mechanism and our understanding of disease and teach the use of a library expression system that can identify genes that are specifically involved in producing a particular phenotype by knocking down intracellular expression, one would have clearly been motivated to incorporate a shRNA in the library expression system to attenuate the expression of a target gene and identify the function os said gene.

It was well known in the art that pol III promoters such as U6 promoters could be used to efficiently generate inhibitory oligonucleotides as taught by Noonberg et al. and given Good et al. teach a construct comprising U6 promoters were capable of

expressing shRNA, one of ordinary skill in the art would have used a U6 promoter to generate the shRNA of Kreutzer et al. One would have a reasonable expectation of success at using a library of RNA constructs because Lieber et al. teach efficient identification of target genes using short inhibitory RNA molecules and would have expected to be able to use the shRNA of Kreutzer et al. One of ordinary skill in the art would have expected to be able to generate the shRNA of Kreutzer et al. from a RNA construct that was capable of attenuating expression of a target gene because this was taught by Good et al.

Thus in the absence of evidence to the contrary, the invention as a whole would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Kimberly Chong whose telephone number is 571-272-3111. The examiner can normally be reached Monday thru Friday between 7-4 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, James (Doug) Schultz can be reached at 571-272-0763. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

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/Kimberly Chong/
Primary Examiner
Art Unit 1635

Notice of References Cited	Application/Control No. 11/894,676	Applicant(s)/Patent Under Reexamination HANNON ET AL.	
	Examiner KIMBERLY CHONG	Art Unit 1635	Page 1 of 1

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*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-5,624,803	04-1997	Noonberg et al.	435/6
*	B	US-2004/0102408	05-2004	Kreutzer et al.	514/044
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
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	S					
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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
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*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No. 11/894,676	Applicant(s)/Patent under Reexamination HANNON ET AL.
	Examiner KIMBERLY CHONG	Art Unit 1635

SEARCHED

Class	Subclass	Date	Examiner

SEARCH NOTES (INCLUDING SEARCH STRATEGY)

	DATE	EXMR
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11/894,676	08/20/2007 **RULE**	514	1635	287000.130US3

APPLICANTS

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** **CONTINUING DATA** *************************

This application is a CON of 10/997,086 11/23/2004
which is a CIP of 10/350,798 01/24/2003 ABN
which is a CIP of 10/055,797 01/22/2002 ABN
which is a CIP of PCT/US01/08435 03/16/2001
which claims benefit of 60/189,739 03/16/2000
and claims benefit of 60/243,097 10/24/2000
and said 10/350,798 01/24/2003
is a CIP of 09/866,557 05/24/2001 ABN
which is a CIP of PCT/US01/08435 03/16/2001
and said 10/350,798 01/24/2003
is a CIP of 09/858,862 05/16/2001
which is a CIP of PCT/US01/08435 03/16/2001

** **FOREIGN APPLICATIONS** *************************

** **IF REQUIRED, FOREIGN FILING LICENSE GRANTED** ** ** SMALL ENTITY **
11/02/2007

Foreign Priority claimed ❑ Yes ☑ No 35 USC 119(a-d) conditions met ❑ Yes ☑ No Verified and Acknowledged /KIMBERLY CHONG/ Examiner's Signature	❑ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		NY	67	14	2

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TITLE

Methods and compositions for RNA interference

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BIB (Rev. 05/07).

EAST Search History

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	0	expression adj constrcut	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/04/26 20:53
L2	26169	expression adj construct	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/04/26 20:54
L3	65	l2 same U6	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/04/26 20:54
L4	56	l3 and sirna	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/04/26 20:54
L5	55	l3 and oligonucleotide	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/04/26 20:54
L6	8	l3 same oligonucleotide	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/04/26 20:54

4/26/2009 8:55:32 PM

Used in Lieu of PTO/SB/08A/B
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Substitute for form 1449/PTO				*Complete if Known*	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	11/894,676
(Use as many sheets as necessary)				Filing Date	August 20, 2007
				First Named Inventor	Gregory J. Hannon
				Art Unit	1635
				Examiner Name	Not Yet Assigned
Sheet	1	of	7	Attorney Docket Number	CSHL-P08-010

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Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AA	US-20020086356-A1	07-04-2002	Tuschl et al.	
	AB	US-20020114784-A1	08-22-2002	Li et al.	
	AC	US-20030051263-A1	03-13-2003	Fire et al.	
	AD	US-20030055020-A1	03-20-2003	Fire et al.	
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	AF	US-20030084471-A1	05-01-2003	Beach et al.	
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	AH	US-20040086884-A1	05-06-2004	Beach et al.	
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	AP	US-6,326,193	12-04-2001	Liu et al.	
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	AR	US-6,573,099-A1	06-03-2003	Graham et al.	
	AS	US-6,605,429	08-12-2003	Barber et al.	

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	BA	WO-00/01846	01-13-2000	Devgen Nv et al.		
	BB	WO-00/44895	08-03-2000	Kreutzer Roland et al.		
	BC	WO-00/63364	10-26-2000	American Home Prod et al.		
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	BL	WO-01/48183	07-05-2001	Devgen Nv et al.		

Examiner Signature		Date Considered	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.C./

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Substitute for form 1449/PTO				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	11/894,676
				Filing Date	August 20, 2007
				First Named Inventor	Gregory J. Hannon
(Use as many sheets as necessary)				Art Unit	1635
				Examiner Name	Not Yet Assigned
Sheet	2	of	7	Attorney Docket Number	CSHL-P08-010

	BM	WO-01/75164	10-11-2001	Whitehead Institute for Biomedical Research; Max-Planck-Gesellschaft Zur Forderung Der Wissenschaften E.V.; Massachusetts Institute of Technology; University of Masachusetts Medical Center		
	BN	WO-02/059300	08-01-2002	J & J Res Pty Ltd et al.		
	BO	WO-02/068635	09-06-2002	Novartis Forschungsstiftung Zw et al.		
	BP	WO-99/32619	07-01-1999	Carnegie Inst Of Washington et al.		

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. * CITE NO.: Those application(s) which are marked with an single asterisk (*) next to the Cite No. are not supplied (under 37 CFR 1.98(a)(2)(iii)) because that application was filed after June 30, 2003 or is available in the IFW. ** CITE NO.: Those document(s) which are marked with an double asterisk (**) next to the Cite No. are not supplied because they were previously cited by or submitted to the Office in a prior application relied upon in this application for an earlier filing date under 35 U.S.C. 120. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

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Examiner* Initials	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CA	Agrawal, et al., "Antisense therapeutics: is it as simple as complementary base recognition?," Molecular Medicine Today, 61:72-81 (2000).	
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	CN	Carmell et al., "The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis," Genes Dev., 16(21):2733-42 (2002).	

Examiner Signature		Date Considered	

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				First Named Inventor	Gregory J. Hannon
				Art Unit	1635
(Use as many sheets as necessary)				Examiner Name	Not Yet Assigned
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	2	European Search report for European Patent application No 03732052.0, mailed May 23, 2008	☐
	3	Hasuwa et al., "Small interfering RNA and gene silencing in transgenic mice and rats," FEBS Letters, Elsevier, Amsterdam, NL, Vol 532, pp. 227-230 (December 2002)	☐
	4	Manche et al., "Interactions between double-stranded RNA regulators and the proteinkinase Dai," Molecular and cellular Biology, Amercian Society for Microbiology, Washington, US, Vol 12, pp. 5238-5248 (November 1992)	☐

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EXAMINER SIGNATURE

Examiner Signature	/Kimberly Chong/	Date Considered	04/26/2009

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Substitute for form 1449A/PTO				*Complete if Known*	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	11/894,676-Conf. #8161
				Filing Date	August 20, 2007
				First Named Inventor	Gregory J. HANNON
				Art Unit	1635
(Use as many sheets as necessary)				Examiner Name	K. Chong
Sheet	1	of	3	Attorney Docket Number	0287000.00130US3

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

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				Filing Date	August 20, 2007
				First Named Inventor	Gregory J. HANNON
				Art Unit	1635
(Use as many sheets as necessary)				Examiner Name	K. Chong
Sheet	2	of	**3**	Attorney Docket Number	0287000.00130US3

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	CA	Marked-up copy of application 09/866,557 (filed 5/24/2001)	
	CB	Marked-up copy of provisional patent application 60/243,097 (filed 10/24/2000)	
	CC	Declaration of Dr. Vladimir Drozdoff (executed 8/5/2008)	
	CD	Declaration of Mr. John Maroney (executed 8/5/2008)	
	CE	Declaration of Professor Gregory Hannon (executed 8/5/2008)	
	CF	Letter of April 22, 2008 from Douglass N. Ellis, Jr. of Ropes & Gray LLP to John Maroney, Esq. of Cold Spring Harbor Laboratory	
	CG	Letter of April 28, 2008 from John Maroney of Cold Spring Harbor Laboratory to Douglass N. Ellis, Jr. of Robes & Gray LLP	
	CH	Letter of April 29, 2008 from Douglass N. Ellis, Jr. from Robes & Gray LLP to John Maroney, Esq. of Cold Spring Harbor Laboratory	
	CI	Letter of May 9, 2008 to Eric R. Hubbard, Esq. of Robes & Gray LLP from John Maroney, Esq. of Cold Spring Harbor Laboratory	
	CJ	Letter of June 4, 2008 from Eric R. Hubbard of Robes & Gray LLP to John Maroney, Esq. of Cold Spring Harbor Laboratory	

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Substitute for form 1449/PTO				*Complete if Known*	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				**Application Number**	**11/894,676-Conf.** #8161
				Filing Date	August 20, 2007
				First Named Inventor	Gregory J. HANNON
				Art Unit	1635
(Use as many sheets as necessary)				Examiner Name	K. Chong
Sheet	3	of	3	Attorney Docket Number	0287000.00130US3

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	CK	Letter of June 13, 2008 from John Maroney, Esq. of Cold Spring Harbor Laboratory to James Haley, Esq. of Robes & Gray LLP	

Examiner Signature	/Kimberly Chong/	Date Considered	04/26/2009

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Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
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Signature	/Anne-Marie C. Yvon/
Typed or Printed Name	Anne-Marie C. Yvon
Date	May 8, 2009
Telephone	(212) 230-8800

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EFS ID:	5300161
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	28089
Filer:	Anne-Marie Yvon/Patricia Ierardi
Filer Authorized By:	Anne-Marie Yvon
Attorney Docket Number:	287000.130US3
Receipt Date:	08-MAY-2009
Filing Date:	20-AUG-2007
Time Stamp:	14:02:45
Application Type:	Utility under 35 USC 111(a)

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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Change of Address	287000_130US3_ChangeofCorrespAddress_050809.pdf	68805 7a572df58c98bc7ce40ebe9bcf65d8882dbf35db	no	1

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AND RESPONSE TO OFFICE ACTION

This paper is filed in response to the May 4, 2009 Office Action. A reply was originally due on August 4, 2009. Applicants request a three-month extension of time, up to and including November 4, 2009. Accordingly, this paper is being timely filed. The Director is authorized to charge the required fee for the extension of time and any other fees occasioned by this paper, and/or to credit any overpayment in fees, to Deposit Account No. 08-0219.

Amendments to the Claims begin on page 2.
Amendments to the Drawings begin on page 4.
Remarks begin on page 5.

AMENDMENT

In the Claims

Please amend the claims as follows, without prejudice. This listing of the claims will replace all prior versions and listings of claims in the application:

1-49. (Cancelled)

50. (Currently amended) A method for attenuating expression of a target gene in a mammalian cell, the method comprising

introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising:

(i) an RNA polymerase promoter, and

(ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides, such that the short hairpin RNA does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells,

wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and

wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, whereby expression of the target gene is inhibited.

51. (Cancelled)

52. (Previously presented) The method of claim 50, wherein the expression construct further comprises LTR sequences located 5' and 3' of the sequence encoding the short hairpin RNA molecule.

53. (Cancelled)

54. (Currently amended) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 21 nucleotides.

55. (Currently amended) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 22 nucleotides.

56. (Currently amended) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 25 nucleotides.

57. (Currently amended) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of 29 nucleotides.

58. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule has a total length of about 70 nucleotides.

59. (Previously presented) The method of claim 50, wherein the RNA polymerase promoter comprises a pol II promoter or a pol III promoter.

60. (Previously presented) The method of claim 59, wherein the pol III promoter comprises a U6, an H1, or an SRP promoter.

61. (Withdrawn) The method of claim 59, wherein the pol II promoter comprises a U1 or a CMV promoter.

62. (New) The method of claim 50, wherein the short hairpin RNA molecule attenuates expression of the target gene in the mammalian cell by at least about 60%.

63. (New) The method of claim 50, wherein the short hairpin RNA molecule attenuates expression of the target gene in the mammalian cell by about 60% to about 90%.

In the Drawings

Please replace Figure 49 as filed with the attached version of Figure 49, labeled Replacement Sheet.

REMARKS

I. Status of the Claims & Priority Support

Claims 50, 52, and 54-63 are pending in this application. Claims 50 and 54-57 are amended; claims 62 and 63 are added. Claims 51 and 53 are cancelled without prejudice to pursue the subject of that claim in a future application. Figure 49 is replaced to include labeling of the LTRs, which was present in Figure 45 of priority application Serial No. 10/055,797. These amendments raise no issue of new matter. Support for these amendments can be found throughout the present specification and in the parent application, U.S.S.N. 10/055,797.

Examples of support for the remaining amendments to claim 50 and for new claims 62 and 63 can be found in the publication of the present application (U.S. Publication No. 2008/0213861) and from the publication of priority application 10/055,797 (U.S. Publication No. 2003/0084471; "the '797 application"), as illustrated in the table below.

Claim Language	Support
50. A method for attenuating expression of a target gene in a mammalian cell, the method comprising introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising: (i) an RNA polymerase promoter, and (ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides, such that the short hairpin RNA does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells, wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, whereby expression of the target gene is inhibited.	Support for "20 nucleotides" can be found in paragraph [0015] of the present application, and in paragraph [0017] of the '797 application. Support for "29 nucleotides" can be found in Figures 37 and 42 of the '797 application, which figures depict short hairpins having a 29-nucleotide double stranded region. See also corresponding figure legends at paragraphs[0088] and [0093] of the '797 application. Example 7 of the '797 application shows RNAi using a 29-nucleotide hairpin. Figures 39A, 41C, 42A, 42B, 44A, 46, 52, 57A and 59A of the present application depict hairpins where the double-stranded region of the hairpin ranges in length from 19 to 29 nucleotides. Support for "wherein the short hairpin RNA molecule expressed is of a length sufficient to not provoke a non-specific PKR or PKR-like response," can be found, for example, in paragraphs [0107] and [0249] of the '797 application, and in paragraph [0141] of the present application.
62. The method of claim 50, wherein the short hairpin RNA molecule attenuates expression of the target gene in the mammalian cell by at least about 60%.	Support for claim 62 can be found in paragraph [0213] of the present application. Support can also be found in the results shown in Figure 42 of the '797 application, where siOligo 1-2, siOligo 1-6, and siOligo 1-19 demonstrate highly

Claim Language	Support
	effective attenuation of target gene expression. See also paragraph [0252] of the '797 application.
63. The method of claim 50, wherein the short hairpin RNA molecule attenuates expression of the target gene in the mammalian cell by about 60% to about 90%.	Support for claim 63 can be found in paragraph [0213] of the present application. Support can also be found in the results shown in Figure 42 of the '797 application, where siOligo 1-2, siOligo 1-6, and siOligo 1-19 demonstrate highly effective attenuation of target gene expression. See also paragraph [0252] of the '797 application.

The presently pending claims are fully supported by the application. The reliance upon drawings for written description is permitted under the law. The claimed invention need not be described literally word-for word in the specification; the inventor can use "such descriptive means as words, structures, figures, diagrams, formulas, etc., that set forth the claimed invention." *Lockwood v. Am. Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d (BNA) 1961, 1966 (Fed. Cir. 1997); *see Ralston Purina Co. v. Far-Mar-Co, Inc.*, 772 F.2d 1570, 1576, 227 USPQ 177, 180 (Fed. Cir. 1985) (disclosure taken with the knowledge of those skilled in the art may be sufficient support for claims).

The present application and the '797 parent application both disclose short hairpin RNA molecules of lengths that are short enough to not provoke a PKR or a PKR-like response. Examples are disclosed in the application showing molecules comprising a double-stranded region which consists of at least 20 nucleotides but not more than 29 nucleotides. For example, the figures illustrate these claimed elements and, along with descriptive words in the specification, provide full support for the claims.

II. Double Patenting

Claims 50-60 were provisionally rejected under the judicially created doctrine of double patenting over claims 1, 2, 7-20, 24, and 59-63 of application Serial No. 10/350,798 ("the '798 application"). The '798 application is abandoned, as was indicated in a Notice of Abandonment dated May 21, 2008. Therefore, the rejection of the present application over the '798 application is improper and should be withdrawn.

Claims 50-60 were provisionally rejected under the judicially created doctrine of double patenting over claims 1, 2, 6, 7, 9, 10, and 23-28 of application Serial No. 09/858,862 ("the '862 application"), which has been allowed.

In response, applicants traverse the rejection. The claims of the present application are patentably distinct from those allowed in the '862 application. The claims of the '862 application involve the introduction of double stranded RNA into a mammalian cell in culture. In contrast, the claims of the present application involve the introduction of an expression construct encoding a short hairpin RNA into a mammalian cell. In the claims of the '862 application, RNA is introduced into the cell, whereas in the present claims, DNA is introduced into the cell. As is discussed below, and in the accompanying Declaration under 37 C.F.R. § 1.132, the introduction of an expression vector encoding short hairpin RNA is not obvious in view of a method whereby double-stranded RNA is introduced into a cell.

Moreover, the claims of the '862 application require that the cell into which the double-stranded RNA is introduced, be engineered with a recombinant gene encoding a Dicer activity, an Argonaut activity, or both. Such a requirement is absent from the present claims, providing another basis of patentable distinctness.

Applicants request reconsideration and withdrawal of the double patenting rejections.

III. Written Description

Claim 52 was rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking adequate written description. The Examiner asserts that the recitation of 5' and 3' LTR sequences is new matter.

Figure 49 has been amended to include the designation of the LTR regions in an exemplary vector of the invention. Figure 49 is the equivalent of Figure 45 in the parent '797 application, to which the present application claims priority. The figure depicts a promoter sequence and a hairpin sequence within an LTR, *i.e.*, with LTR sequences 5' and 3' to the hairpin sequence, as recited in claim 52. As discussed above, Figures can be used to support the written description of claim terms. Accordingly, Applicants request reconsideration and withdrawal of the new matter rejection.

IV. Anticipation

Claims 50, 51, and 53-58 were rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by Fire et al.

In response, Applicants traverse the rejection. Fire et al. do not disclose the claimed invention. The Examiner has used impermissible hindsight in the reading of the Fire et al. publication, as evidenced by the picking and choosing of disparate words, phrases, and numbers that are sprinkled throughout the Fire et al. publication to patchwork together this rejection. It is only with the knowledge of Applicants' disclosure that the Examiner improperly recasts bits and pieces of Fire et al. as allegedly describing the presently claimed invention.

The pending claims are directed to a method for attenuating expression of a target gene in a mammalian cell, the method comprising introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising: (i) an RNA polymerase promoter, and (ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides, such that the short hairpin RNA does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells, wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, whereby expression of the target gene is inhibited. The elements recited in this claim are not disclosed by Fire et al.

To anticipate under 35 U.S.C. § 102, a reference must explicitly or inherently disclose every element of the claimed invention. *See In re Gleave*, 560 F.3d 1331, 1334 (Fed. Cir. 2009). In the present case, the context of the passages in Fire et al. relied upon by the Examiner, and the Fire et al. disclosure as a whole, do not support an anticipation rejection.

As the Court of Appeals for the Federal Circuit held in its *Net MoneyIN, Inc. v. Verisign, Inc.* decision, elements in an anticipatory reference must be arranged as recited in the claim. "[I]t is not enough that the prior art reference . . . includes multiple, distinct teachings that the artisan might somehow combine to achieve the claimed invention." *Net MoneyIN*, 545 F.3d at 1371 (citing *In re Arkley*, 455 F.2d 586, 587 (C.C.P.A. 1972)). "Because the hallmark of anticipation is prior invention, the prior art reference—in order to anticipate under 35 U.S.C. § 102—must not only disclose all elements of the claim within the four corners of the document, but must also disclose those elements 'arranged as in the claim.'" *Net MoneyIN, Inc. v. Verisign, Inc.*, 545 F.3d 1359, 1369 (Fed. Circ. 2008) (quoting *Connell v. Sears, Roebuck & Co.*, 722 F.2d

1542, 1548 (Fed. Cir. 1983)). "[T]he 'arranged as in the claim' requirement applies to all claims and refers to the need for an anticipatory reference to show all of the limitations of the claims arranged or combined in the same way as recited in the claims, not merely in a particular order. The test is thus more accurately understood to mean 'arranged or combined in the same way as in the claim.'" *Net MoneyIN*, 545 F.3d at 1370. "We thus hold that unless a reference discloses within the four corners of the document not only all of the limitations claimed but also all of the limitations arranged or combined in the same way as recited in the claim, it cannot be said to prove prior invention of the thing claimed and, thus, cannot anticipate under 35 U.S.C. § 102." *Net MoneyIN*, 545 F.3d at 1371.

Here, the Fire et al. reference lacks any disclosure of a short hairpin RNA molecule comprising a double-stranded region consisting of at least 20 nucleotides but not more than 29 nucleotides. In particular, Fire et al. do not disclose use of short hairpin RNA molecules in mammalian cells, wherein the double-stranded region is limited in length such that the hairpin RNA molecules do not trigger a PKR response in the cell. As discussed below, such a finding is unsupported by any of the Examiner's arguments.

(1) Alleged anticipatory disclosure of hairpin RNAs

The Examiner states Fire et al. discloses "single self-complementary RNA, i.e. a hairpin RNA (see column 7, lines 42-44)." The sentence that the Examiner relies upon in Fire et al. reads: "The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands." Col. 7, ll. 42-44. There is no apparent antecedent basis for "the double-stranded structure" in Fire et al. The characteristics and the details of the structure of the "single self-complementary RNA" are not provided. The Examiner does not identify any disclosure in Fire et al. that defines "the double-stranded structure" or describes what that structure is in the context of a DNA expression construct.

(2) Alleged anticipatory disclosure of length of the double-stranded region

The Examiner states that "Fire et al. discloses each strand of the dsRNA may be at least 25, 50 or 100 nucleotides in length which would meet the limitation of claims 41-46 because the shRNA has at least 20, 21, 22 or 25 nucleotides, has 29 nucleotides and about 70 nucleotides." However, Fire et al. do not disclose, as the Examiner asserts, that each strand of dsRNA may be at least 25, 50 or 100 in length. Instead, Fire et al. states: "The length of the identical nucleotide sequences may be at least 25, 50, 100, 200, 300 or 400 bases." Col. 8, ll. 5-6 (emphasis added).

This length does not necessarily equate to the length of the double-stranded region, which may be longer. Importantly, the Fire et al. disclosure therefore provides no specific limitation on the length of the *double-stranded* region. Only with knowledge of the claimed invention has the Examiner attributed such meaning to this passage. Moreover, the open-ended range disclosed by Fire et al. provides no upper limit on the length of the double-stranded region. There is no description in this passage of "a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides," as presently claimed.

In fact, as the Examiner has previously admitted, "Fire et al. is silent as to the specific length of the dsRNAs." February 12, 2007 Office Action at p. 6. The only double-stranded RNAs disclosed by Fire et al. range in length from 299 to 1033 nucleotides. See Table 1, col. 22-24. The present claims recite a specific range of nucleotides that comprise the double-stranded region of the short hairpin RNA molecule; Fire et al. do not disclose this element. In other words, Fire et al. lack any disclosure of "a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region is at least 20 nucleotides but not more than 29 nucleotides," as recited in the presently pending claims.

In that Fire et al. provide no disclosure regarding the length of the double-stranded region, a mere broad disclosure with respect to overall RNA length cannot anticipate the present claims, which recite a specific, narrow range of length of the double-stranded region. *See Atofina v. Great Lakes Chem. Corp.*, 441 F.3d 991 (Fed. Cir. 2006). In *Atofina*, the court held that the prior art disclosure of a chemical synthesis method, performed within a broad range of temperatures using a broad range of ingredient ratios, did not anticipate claims reciting a narrower temperature range and overlapping ingredient ratios. The *Atofina* holding is particularly instructive, given that the ranges disclosed by Fire et al., apart from the mention of 1033 nucleotides, have no upward limit and therefore could not reasonably be interpreted to describe the presently claimed numerical range of the double-stranded region of a short hairpin RNA with any specificity.

(3) Alleged disclosure of use of expression constructs encoding shRNA in mammalian cells

The Examiner states that "Fire et al. additionally teach the shRNA can be transcribed via an expression construct comprising a RNA polymerase, a bacteriophage RNA promoter or a T7, T3 or a SP6 promoter (see columns 8 9 and Figure 5A)." Office Action at p. 8. Again, the

Examiner has read into the Fire et al. reference additional disclosure based on hindsight. This passage of Fire et al. merely discloses that RNA can be synthesized in cells, e.g., bacterial or nematode cells, or in phages. It provides no teaching as to choice of cell type or promoter, and especially to expression in mammalian cells. In contrast to the statement in the Office Action above, Fire et al. actually states "[t]he RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and production of an expression construct are known in the art (see also WO 97/32016, U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein)." Col. 9, ll. 11-16.

Moreover, Figure 5A of Fire et al., also cited by the Examiner as teaching that "the shRNA can be transcribed via an expression construct comprising a RNA polymerase, a bacteriophage RNA promoter or a T7, T3 or a SP6 promoter" shows only an expression plasmid in a bacterial cell with a T7 promoter, a "gene of interest," and an antibiotic resistance gene. Figure 5A does not show expression of a short hairpin RNA molecule in a mammalian cell, nor does it show stable expression.

Indeed, all of the patents cited by Fire et al. as providing guidance regarding the use and production of expression constructs, relate to expression in plants. There is no disclosure by Fire et al. specifically teaching expression of short hairpin RNA molecules in mammalian cells to inhibit target gene expression therein. Fire et al. provide no bare mention, let alone enabling teaching, of an expression vector for expressing a short hairpin RNA molecule of 20-29 nucleotides in length in a mammalian cell in order to inhibit gene expression.

The Examiner relies on col. 8, lines 8-12 in Fire et al., which states that the "cell with the target gene may be derived from or contained in any organism. The organism may be a plant, animal, protozoan, bacterium, virus, or fungus." There is no disclosure here of "mammalian cells" as asserted by the Examiner. This passage merely lists a broad spectrum of organisms ranging from fungi, to microbes, to plants, to animals, to mold. Indeed, in this passage there is no disclosure of "attenuating expression of a target gene in mammalian cells." Fire et al. demonstrate attenuation of target gene expression only in the invertebrate *C. elegans*.

The Examiner's reliance on at least six disparate passages in Fire et al. in an attempt to support an anticipation rejection is not within the bounds of the law. In *Lindemann Maschinenfabrik GMBH v. American Hoist and Derrick Co.*, the Court of Appeals for the

Federal Circuit reversed a district court finding of anticipation because "[t]he district court's analysis treated the claims as mere catalogs of separate parts, in disregard of the part-to-part relationships set forth in the claims and that give the claims their meaning." 730 F.2d 1452, 1459 (Fed. Cir. 1984). While the prior art reference in *Lindemann* contained each separate element of the claimed invention, it "disclose[d] an entirely different device, composed of parts distinct from those of the claimed invention, and operating in a different way to process different material differently." *Lindemann*, 730 F.2d at 1458. Here, the Fire et al. reference lacks both disclosure of each element of the presently claimed invention (e.g., short hairpin RNA molecules), and any specific embodiment or other disclosure directed to use of short hairpin RNA in mammalian cells.

Moreover, as Applicants have previously noted, the present Examiner's interpretation of Fire et al. conflicts with the entirely different interpretation of the Fire et al. disclosure the Patent Office has taken in examining U.S.S.N. 10/283,190 (a related Fire application having the identical disclosure to Fire et al.). Notably, there the Examiner stated "[t]he discovery that short RNAs can mediate RNA interference in mammalian cells without invoking the PKR response was made after filing of the instant application." That statement correctly reflects the state of the art at the time Fire et al. was filed. Indeed, it is illogical for the Examiner here to respond by asserting that the inconsistent position taken by the Patent Office in examining enablement of Fire et al., the identical disclosure, "ha[s] no relevance." That it was made by a different Examiner in an application unrelated to the present one does not detract from its accuracy.

In view of the amendments to the claims, the law, and the arguments above, Applicants request reconsideration and withdrawal of the anticipation rejection over Fire et al.

V. <u>Obviousness</u>

Claims 50-59 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Fire et al., Good et al., and Noonberg et al. Claims 50-59 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Kreutzer et al., Lieber et al., Good et al., and Noonberg et al. These rejections are traversed.

In reply, Applicants traverse the rejection. None of the cited references, alone or in any combination, disclose or suggest a method for attenuating target gene expression in a mammalian cell by introducing an expression vector encoding a short hairpin RNA. The claimed invention

provides a solution to the problem of inhibiting gene expression in mammalian cells without provoking PKR-mediated apoptosis, overcoming technical difficulties that are not encountered or addressed using the prior art methods in non-mammalian cells.

According to the state of the art at the time of the invention, the skilled artisan would have had no reasonable expectation of success that sequence specific target gene attenuation could be achieved by using an expression vector encoding a short hairpin RNA molecule (shRNA) having a double-stranded region consisting of between 20 and 29 nucleotides. Moreover, one would have understood the process of RNAi to be mediated by 21- and 22-nucleotide small interfering RNAs (siRNAs) generated from longer double-stranded RNAs (dsRNAs), as disclosed by Elbashir et al. (2001) *Nature* 411:494-98.

One of ordinary skill would have known the literature to indicate that the RNAi response was sharply length dependent. For example, both *in vitro* and *in vivo* analysis of the length requirements of dsRNA had revealed that dsRNAs of fewer than 150 bp in length appeared less effective than longer dsRNAs, and in some cases ineffective, in their ability to degrade target mRNA. *See* Elbashir et al. (2001) *Genes Dev.* 15:188-200; Bernstein et al. (2001) *Nature* 409:363-66. In view of the state of the art at the time, the skilled artisan would have had no motivation to employ RNA molecules comprising short dsRNA structures that must be processed within the cell to activate RNAi. There was no realization that the ineffectiveness of such molecules in mediating RNAi could have been overcome by expressing RNA molecules within the cell in the form of a hairpin structure, as taught and claimed in the present application. In particular, the skilled artisan would not have expected that an RNA hairpin having a double-stranded region of 20 to 29 nucleotides in length would undergo processing to an siRNA or would be effective in triggering sequence specific gene attenuation through RNAi.

As evidence of the non-obviousness of the claimed invention, Applicants submit the attached Declaration under 37 C.F.R. § 1.132 by Dr. Nouria Hernandez. As Dr. Hernandez states, it would not have been obvious to one of ordinary skill in the art at the time of the invention that one could attenuate target gene expression in a mammalian cell by introducing an expression construct encoding a short hairpin RNA molecule having a double-stranded region of 20-29 nucleotides. Indeed, according to Dr. Hernandez it was unexpected that the claimed method would result in effective target gene attenuation, and one of ordinary skill at the time of

the invention would have had no reasonable expectation that it would do so. Accordingly, Applicants request reconsideration and withdrawal of this ground of rejection.

CONCLUSION

Consideration of this paper, and early allowance of this application, are respectfully requested. If it would advance prosecution, the Examiner is invited to contact the undersigned to discuss the contents of this paper.

Dated: November 4, 2009

Respectfully submitted,

/Anne-Marie C. Yvon/

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Anne-Marie C. Yvon, Ph.D.
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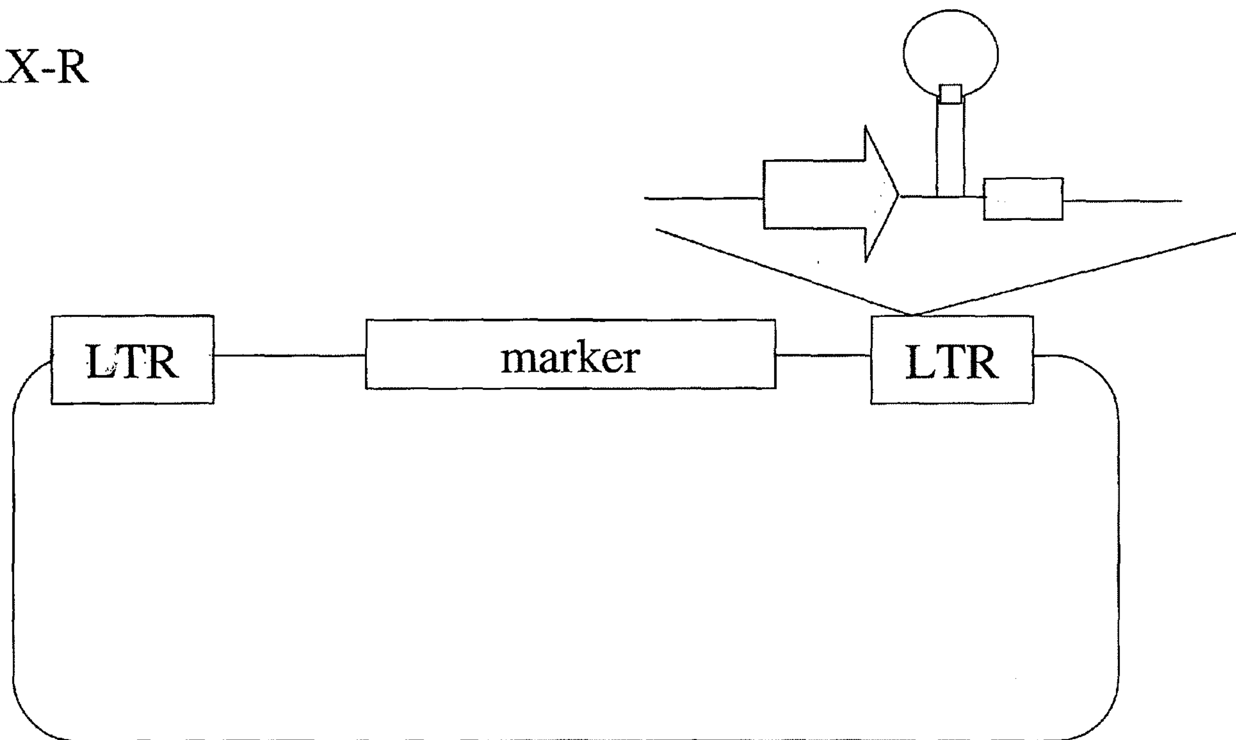

Stable suppression by expressed RNAi

Fig. 49

Replacement Sheet

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Docket No.: 287000-130-US3

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration of Professor Nouria Hernandez, Ph.D. Under 37 C.F.R §1.132

I, Nouria Hernandez, Ph.D., hereby declare and state that:

1. I am a Professor of Biology , and the Director of the Centre intégratif de génomique at the Université de Lausanne.

2. I am informed that the pending claims of this U.S. patent application, U.S. Serial No. 11/894,676, filed August 20, 2007 are directed to methods for attenuating expression of a target gene in a mammalian cell, the method comprising introducing into a mammalian cell a library of RNA expression constructs comprising: (i) an RNA polymerase promoter, and (ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region consisting of 20 to 29 base pairs, wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate

expression of the target gene in a sequence specific manner, whereby expression of the target gene is inhibited. A listing of the pending claims are attached at **Exhibit A**.

3. I am informed that the '676 application was filed based on a parent application, U.S. Serial No. 10/055,797, and is entitled to a priority date of January 22, 2002, which is the filing date of the '797 application.

4. At the time of the filing of the '797 application, i.e., around January 2002, I was an Investigator of the Howard Hughes Medical Institute and a Professor at Cold Spring Harbor Laboratory.

5. I am informed that the U.S. Patent Examiner has rejected the claims as obvious in over Fire et al. (USPN 6,506,559), Good et al. (Gene Therapy 1997) and Noonberg et al. (USPN 5,624,803). I am also informed that the Examiner rejected the pending claims as obvious over Kreutzer et al. (US Application No. 2004/0102408), Lieber et al. (USPN 6,130,092, Good et al. (Gene Therapy 1997) and Noonberg et al. (USPN 5,624,803).

6. I understand the claimed methods are all directed to using RNA interference (RNAi) to stably attenuate expression of the target gene in a sequence specific manner in a mammalian cell, without activating a non-sequence specific PK response. As discussed below, the claimed methods would not have been obvious to a person of ordinary skill in the art as of January 21, 2002.

7. In particular, a person of ordinary skill would have had no reasonable expectation that one could successfully carry out sequence specific gene silencing by using an expression vector encoding a short hairpin RNA molecule having a double-stranded

region consisting of 20 to 29 base pairs (bp). As discussed below, the leading literature in the field would have taught away from using an expressed short hairpin molecule, which to have gene silencing activity, must first be processed in the cell.

8. As of January 21, 2002, the ordinary skilled scientist would have recognized RNA interference as a process of sequence-specific post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Moreover, one would have understood the process of RNAi to be mediated by 21- and 22-nucleotide small interfering RNAs (siRNAs) generated from longer double-stranded RNAs (dsRNAs). See for example, Elbashir et al. (24 May 2001) *Nature*, 411:494-498 (Elbashir 2001(b)).

9. The literature, however, also indicated that the RNAi response was sharply length dependent. For example, both *in vitro* and *in vivo* analysis of the length requirements of dsRNA had revealed that dsRNAs less than 150 base pairs (bp) in length appeared less effective than longer dsRNAs (and in some cases ineffective) in their ability to degrade target mRNA. See, for example, Elbashir et al. (2001) *Genes Dev.* 15:188-200. (Elbashir 2001(a)), and literature cited therein. See Bernstein et al. at page 364 and also Supplemental Figure 4 (Bernstein et al. (2001) *Nature* 409:363-366).

10. Significantly, more extensive analysis of these length requirements demonstrated that dsRNA precursors comprising even shorter double-stranded regions, for example 30 bp or less, were not effectively processed to the 21- and 22-nt siRNAs that carried out gene silencing by mediating sequence-specific degradation of the target mNRA. See, for example, Elbashir 2001(a). In view of this literature, a person of ordinary skill would not

have expected that an RNA hairpin having a double-stranded region consisting of 20 to 29 bp in length would undergo processing to an siRNA or would be effective in triggering sequence specific gene attenuation through RNAi.

11. For example, Elbashir et al. (2001) *Genes Dev.* 15:188-200 discourages use of RNA precursors comprising double-stranded regions of shorter than 38 bp in length as a means of attenuating target genes through RNAi. Here, the authors used an established *Drosophila in vitro* system to explore the RNAi mechanism. As part of this study, Elbashir et al. examined "the precise length requirement of dsRNA for targeting RNA degradation under optimized conditions in the *Drosophila* lysate." See, Figure 1A and 1B of Elbashir et al. Notably, the authors observed that a minimal length requirement appeared to be an intrinsic feature of the RNAi mechanism; below a certain length, double-stranded RNA would not mediate an RNAi response. In particular, the authors conclude that "[s]pecific inhibition of target RNA expression was detected for dsRNAs as short as 38 bp, but dsRNAs of 29-36 bp were not effective in this process."

12. The authors suggest the lack of RNAi by such short dsRNAs could be explained by the inability of such short dsRNAs to be efficiently processed into the 21 to 22 nt guide RNAs that mediate RNA interference and cosuppression: "[s]hort 30-bp dsRNAs are inefficiently processed to 21- and 22-nt RNAs, which may explain why they are ineffective in mediating RNAi."

13. In particular, the authors "analyzed the rate of 21-23-nt fragment formation for a subset of dsRNAs ranging in size from 501 to 29 bp." The authors show the results in Figure 2, and state "[f]ormation of 21-23-nt fragments in Drosophila lysate (Fig. 2) was

readily detectable for 39-501 bp dsRNAs but was significantly delayed for the 29-bp dsRNA." The authors conclude that "this observation is consistent with a role of 21-23-nt fragments in guiding mRNA cleavage and provides an explanation for the lack of RNAi by 30 bp dsRNAs."

14. In discussing their findings, Elbashir et al. remark that "[t]he length dependence of 21-23 mer formation is likely to reflect a mechanism to prevent the undesired activation of RNAi by short intramolecular base-paired structures of cellular RNA." Further, "suppression of RNAi by single-stranded regions flanking short dsRNA and the reduced rate of siRNA formation from short 30-bp dsRNAs may explain why structured regions within mRNAs do not lead to activation of RNAi." (See page 197). As one of skill, I would have understood the reference here to "short intramolecular base-paired structures of cellular RNA" to include short hairpin RNA structures. In this regard, the ordinary skilled scientist would have understood Elbashir et al. to expressly teach away from using short hairpin RNAs having double-stranded regions of less than 30 bp, for example, 20-29 bp in length, to mediate RNAi.

15. Elabshir et al. (24 May 2001) *Nature*, 411:494-498 (Elbashir 2001(b)), demonstrated that as in Drosophila, 21- and 22- nucleotide siRNAs mimicking Dicer processed forms, could also effect transient attenuation of gene expression in mammalian cells.

16. However, in view of Elbashir et al. 2001(a), one of ordinary skill would have been taught away from using short hairpins, as presently claimed. In particular, Elbashir et al. 2001(a) disclosed negative results that would have caused one to expect that a short

hairpin RNA with a double-stranded region consisting of 20-29 bp in length (a) would not be processed to the 21- and 22-nt siRNA structures necessary to mediate RNAi and (b) would consequently be ineffective in mediating RNAi.

17. Nothing presented in Elbashir 2001(b) provides evidence to the contrary. Notably, none of the additional findings in mammalian cells reported in Elbashir 2001(b) questioned the conclusion of Elbashir 2001(a) that a minimal length requirement appeared to be an intrinsic feature of the RNAi processing mechanism. Absent from Elbashir 2001(b) is any data suggesting that, apart from 21- and 22-nt forms mimicking Dicer products requiring no further processing in the cell, RNA having double-stranded regions short enough to avoid a PK response (that is, 30 bp or less), would be processed and mediate an RNAi response.

18. A person of ordinary skill in the art as of January 21, 2002 would have, moreover, avoided using longer hairpins in mammalian cells because he/she would have been aware of the PK (or interferon) response exhibited by mammalian cells. For example, in Elbashir 2001(b), the authors state "[i]n the interferon response, dsRNA >30 bp binds and activates the protein kinase PKR and 2',5'-oliogoadenylate synthetase." The authors state "[t]hese responses are intrinsically sequence nonspecific to the inducing dsRNA." On page 496 of Elbashir 2001(b), the authors state "[n]onspecific reduction in reporter-gene expression by dsRNA > 30 bp was expected as part of the interferon response."

19. In sum, even in view of the references relied upon by the Examiner, the claimed invention would not have been obvious to a person of ordinary skill in the art at that time. In view of the available literature, and in particular, both Elbashir papers, there would

have been no expectation of success that one could use an RNA molecule comprising a double-stranded region consisting of 20-29 bp to mediate RNAi and avoid the PK response induced by RNA with longer double-stranded regions. Elbashir et al. 2001(a) disclosed negative results that would have caused one to expect that a short hairpin RNA with a double-stranded region of from 20-29 bp in length would be ineffective in degrading target mRNA.

I hereby declare that all statements believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 29 October 2009 By: Nouria Hernandez

Nouria Hernandez, Ph.D.

Exhibit A: Pending Claims – USSN 11/894,676

50. A method for attenuating expression of a target gene in a mammalian cell, the method comprising

introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising:

(i) an RNA polymerase promoter, and

(ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides, such that the short hairpin RNA does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells,

wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and

wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, whereby expression of the target gene is inhibited.

52. The method of claim 50, wherein the expression construct further comprises LTR sequences located 5' and 3' of the sequence encoding the short hairpin RNA molecule.

54. The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 21 nucleotides.

55. The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 22 nucleotides.

56. The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 25 nucleotides.

57. The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of 29 nucleotides.

58. The method of claim 50, wherein the short hairpin RNA molecule has a total length of about 70 nucleotides.

59. The method of claim 50, wherein the RNA polymerase promoter comprises a pol II promoter or a pol III promoter.

60. The method of claim 59, wherein the pol III promoter comprises a U6, an H1, or an SRP promoter.

Exhibit A: Pending Claims – USSN 11/894,676

61. The method of claim 59, wherein the pol II promoter comprises a U1 or a CMV promoter.

62. The method of claim 50, wherein the short hairpin RNA molecule attenuates expression of the target gene in the mammalian cell by at least about 60%.

63. The method of claim 50, wherein the short hairpin RNA molecule attenuates expression of the target gene in the mammalian cell by about 60% to about 90%.

Electronic Patent Application Fee Transmittal

Application Number:	11894676
Filing Date:	20-Aug-2007
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Filer:	Anne-Marie Yvon/Patricia Ierardi
Attorney Docket Number:	287000.130US3

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with $0 paid	2253	1	555	555

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
		Total in USD ($)		**555**

Electronic Acknowledgement Receipt

EFS ID:	6389479
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Anne-Marie Yvon/Patricia Ierardi
Filer Authorized By:	Anne-Marie Yvon
Attorney Docket Number:	287000.130US3
Receipt Date:	04-NOV-2009
Filing Date:	20-AUG-2007
Time Stamp:	15:51:46
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$555
RAM confirmation Number	11022
Deposit Account	080219
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
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Warnings:

Information:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
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Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Claims	2	3
Drawings-only black and white line drawings	4	4
Applicant Arguments/Remarks Made in an Amendment	5	14

Warnings:

Information:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
3	Drawings-only black and white line drawings	287000_130US3_ReplacementSheet_110409.pdf	19331 1600fdcaaa8416580caa4536c15563e8eee125cd	no	1

Warnings:

Information:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
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5	NPL Documents	287000_130US3_ElbashirNature_110409.pdf	197581 b0a9d8f0b8d9b908a37ec18cba642151bfaf45ee	no	5

Warnings:

Information:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
6	NPL Documents	287000_130US3_Elbashirshort_110409.pdf	2087585 8feb8dbe91add05e55fb48709f00468a05cb509b	no	13

Warnings:

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7	Rule 130, 131 or 132 Affidavits	287000_130US3_Rule132DeclandExA_110409.pdf	2481834 942254c234614da14630940d0241a80bd43ac75c	no	9
Warnings:					
Information:					
8	Fee Worksheet (PTO-875)	fee-info.pdf	30223 d9898e7adad801d88b80a8cdb4e11743e19c0b2c	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	5726292	

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)
FY 2009
(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)

Docket Number (Optional)
0287000.00130US3

Application Number 11/894,676-Conf. #8161 | Filed August 20, 2007

For METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

Art Unit 1635 | Examiner K. Chong

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$130	$65	$
☐	Two months (37 CFR 1.17(a)(2))	$490	$245	$
X	Three months (37 CFR 1.17(a)(3))	$1110	$555	$ 555.00
☐	Four months (37 CFR 1.17(a)(4))	$1730	$865	$
☐	Five months (37 CFR 1.17(a)(5))	$2350	$1175	$

[X] Applicant claims small entity status. See 37 CFR 1.27.

[ ] A check in the amount of the fee is enclosed.

[X] Payment by credit card. Form PTO-2038 is attached.

[X] The Director has already been authorized to charge fees in this application to a Deposit Account.

[X] The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 08-0219 .

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the
[ ] applicant/inventor.
[ ] assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).
[X] attorney or agent of record. Registration Number 52,390
[ ] attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 ______

/Anne-Marie C. Yvon/ — Signature
November 4, 2009 — Date

Anne-Marie C. Yvon — Typed or printed name
(212) 230-8800 — Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

[ ] Total of 1 forms are submitted.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	
11/894,676	08/20/2007	☐ To be Mailed

APPLICATION AS FILED – PART I

FOR	NUMBER FILED (Column 1)	NUMBER EXTRA (Column 2)	SMALL ENTITY ☒ RATE ($)	SMALL ENTITY FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
			TOTAL			TOTAL	

* If the difference in column 1 is less than zero, enter “0” in column 2.

APPLICATION AS AMENDED – PART II

AMENDMENT	11/04/2009	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 12	Minus	** 20	= 0	X $26 =	0	OR	X $ =	
	Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	X $110 =	0	OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write “0” in column 3.
** If the “Highest Number Previously Paid For” IN THIS SPACE is less than 20, enter “20”.
*** If the “Highest Number Previously Paid For” IN THIS SPACE is less than 3, enter “3”.
The “Highest Number Previously Paid For” (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/Kim Downing/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 12/29/2009
WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER	
CHONG, KIMBERLY	
ART UNIT	PAPER NUMBER
1635	
MAIL DATE	DELIVERY MODE
12/29/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

PTOL-90A (Rev. 04/07)

Interview Summary	Application No.	Applicant(s)	
	11/894,676	HANNON ET AL.	
	Examiner	**Art Unit**	
	KIMBERLY CHONG	1635	

All participants (applicant, applicant's representative, PTO personnel):

(1) Kimberly Chong, Tracy Vivlemore.

(2) Jane Love, .

(3) John Maroney.

(4) Anne-Marie Yvon.

Date of Interview: 17 December 2009.

Type: a)☒ Telephonic b)☐ Video Conference
c)☐ Personal [copy given to: 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☐ No.
If Yes, brief description: _____.

Claim(s) discussed: _____.

Identification of prior art discussed: _____.

Agreement with respect to the claims f)☐ was reached. g)☐ was not reached. h)☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: See Continuation Sheet.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

/Kimberly Chong/
Primary Examiner AU 1635

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:

- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:

1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: Discussed the rejection of record and specifically the prior art reference Fire et al. Discussed whether Fire et al. actually disclose a dsRNA with at least 25 nucleotide strands and whether Fire et al. discloses and dsRNA within the claimed reange of at leat 20 but no more that 29 nucleotides of which the Examiner believes the Fire et al. reference to disclose these specific limitation. Discussed whether Fire et al. discloses a hairpin RNA molecule that is 'stably expressed". Since this is a new limitation in the claim amendments, the Examiner stated the application would need to be further examined to see if in fact the prior art references would teach the new limitation. No agreements were made with respect to overcoming the rejection of record with the newly amended claims.

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