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On the Role of RNA Amplification in dsRNA-Triggered Gene Silencing

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Summary

We have investigated the role of trigger RNA amplification during RNA interference (RNAi) in *Caenorhabditis elegans*. Analysis of small interfering RNAs (siRNAs) produced during RNAi in *C. elegans* revealed a substantial fraction that cannot derive directly from input dsRNA. Instead, a population of siRNAs (termed secondary siRNAs) appeared to derive from the action of a cellular RNA-directed RNA polymerase (RdRP) on mRNAs that are being targeted by the RNAi mechanism. The distribution of secondary siRNAs exhibited a distinct polarity (5′→3′ on the antisense strand), suggesting a cyclic amplification process in which RdRP is primed by existing siRNAs. This amplification mechanism substantially augments the potency of RNAi-based surveillance, while ensuring that the RNAi machinery will focus on expressed mRNAs.

Introduction

RNA-mediated interference (RNAi) is a conserved gene silencing mechanism that recognizes double-stranded RNA (dsRNA) as a signal to trigger the sequence-specific degradation of homologous mRNA (see Sharp, 2001 for a recent review). Analyses of RNAi and related processes in diverse systems have uncovered several surprising properties, including the double-stranded character of the trigger RNA and a catalytic aspect of the interference reaction. Indeed, a few molecules of dsRNA are sufficient in *C. elegans* or *Drosophila* cells to trigger the decay of a much larger population of target mRNAs (Fire et al., 1998; Kennerdell and Carthew, 1998).

Several features of the RNAi mechanism have been proposed to contribute to the remarkable potency of the reaction. Some degree of amplification is likely to derive from cleavage of the dsRNA trigger into short pieces of 21–25 nt (called siRNAs) by the RNaseIII-like nuclease DICER (e.g., Zamore et al., 2000; Bernstein et al., 2001). For the most commonly used dsRNA triggers (500–1000 bp), this would result in a 20- to 40-fold increase in the molar ratio of trigger to target. A simple (single-use) utilization of the siRNAs would be sufficient to explain the molar efficiency of RNAi in extracts of *Drosophila*, but would be insufficient to account for in vivo potency in *C. elegans*. A multiround mechanism (use of a single siRNA for hundreds or thousands of rounds of target degradation) would be much more efficient.

An additional contribution to the potency of RNA-triggered gene silencing has been proposed to involve physical amplification of an aberrant RNA population through an RNA-directed RNA polymerase (RdRP) activity (Dougherty and Parks, 1995). By producing a large number of copies of a triggering RNA, an RdRP activity might dramatically increase the effectiveness of RNAi. The possibility of RdRP involvement in posttranscriptional gene silencing has been supported by the isolation of an endogenous RdRP activity from tomato (Schiebel et al., 1993a, 1993b, 1998), followed by subsequent demonstrations that factors with protein sequence homology to this RdRP were required for efficient silencing in fungal, nematode, and plant systems (Cogoni and Macino, 1999; Smardon et al., 2000; Dalmay et al., 2000; Mourrain et al., 2000).

A number of apparent constraints on the roles of RdRP activity in RNAi are suggested by experimental observations. Embryonic extracts from *Drosophila* with no measurable RdRP activity can carry out a complete RNAi reaction (Zamore et al., 2000; P. Zamore, personal communication). This, combined with the absence in available *Drosophila* or mammalian genomic sequences of a clear homolog of the RdRP-like genes implicated in other systems, argues that an RNAi reaction can proceed without RdRP. It should be noted, however, that formation of unstable (transient) copy RNAs during the in vitro reaction might be difficult to detect, and that additional enzymes (such as RNA polymerase II and retroviral type reverse transcriptases) are capable of polymerizing RNA in response to certain RNA templates (e.g., Diener, 1991; Filipovska and Konarska, 2000; Modahl et al., 2000). A more limited constraint on possible roles for RdRP in RNAi comes from experiments in which the two trigger strands have been modified differentially prior to injection into *C. elegans* or *Drosophila* (Parrish et al., 2000; Yang et al., 2000). These experiments showed a more stringent requirement for structure and sequence of the antisense strand of the original trigger, as compared to the sense strand. These “strand-preference” experiments do not rule out a role for RdRP in the interference reaction, but do severely limit models in which the RdRP carries out a multiround replication of a double-stranded trigger (e.g., Waterhouse et al., 1998) to produce exponential amplification: this type of exponential amplification would result in loss of memory of the difference between the original two strands and

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[4] These authors made equal contributions to this work.

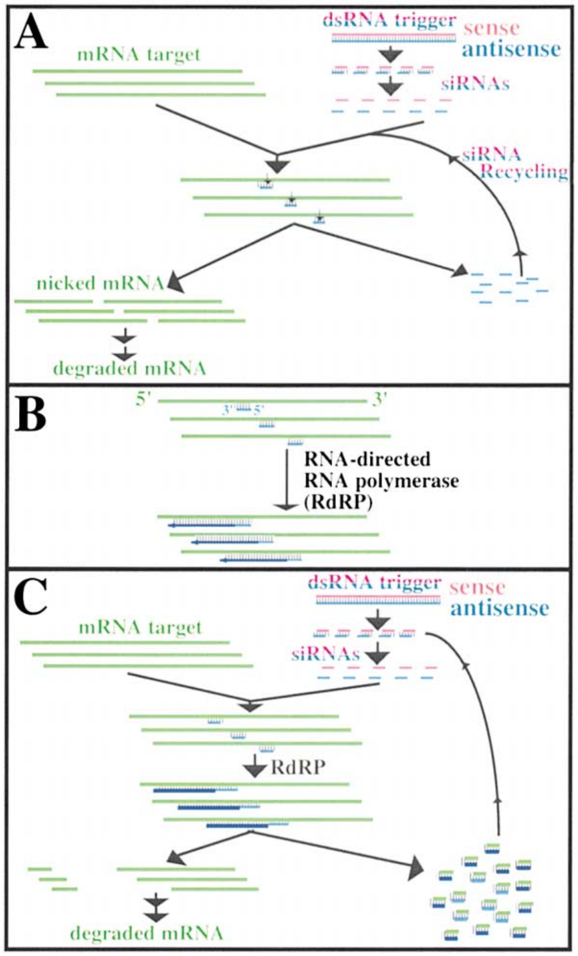


Figure 1. Could siRNA-Primed Copying of Target RNAs by an RNA-Directed RNA Polymerase Contribute to RNAi?

(A) A current model of the nucleic acid alterations during RNA interference based primarily on in vitro studies of RNAi in *Drosophila* extracts (e.g., Zamore et al., 2000; Hammond et al., 2001; Bernstein et al., 2001; Elbashir et al., 2001). After cleavage of the dsRNA trigger into short siRNA segments, the individual antisense siRNAs pair with complementary mRNAs, with degradation of mRNA and (eventual) recycling of siRNAs.
(B) shows that at the heart of the working model is an intermediate with the antisense strand of an siRNA hybridized to an mRNA target. Since the siRNAs possess a 3′-terminal hydroxyl group, the resulting intermediate might function as a template for elongation by an RdRP activity.
(C) shows a possible consequence of the reactions proposed in (A) and (B), with the sequential activity of RdRP and a dsRNA-specific nuclease (e.g., DICER) leading to a target-dependent amplification of the siRNA population.

Of the numerous roles proposed for RdRP during gene silencing, we were most intrigued by the possibility (Figure 1) that antisense siRNAs that have annealed to a ssRNA target might be elongated by RdRP to produce longer stretches of dsRNA (Sijen and Kooter, 2000). This model is particularly attractive in that (1) siRNAs are known to have a 3′ hydroxyl group (Elbashir et al., 2001), which would be poised for elongation by an RNA poly-

amplification of the initial population of siRNAs at the expense of target transcripts, and (3) this mode of amplification utilizes the two input strands of the RNA trigger differentially; thus, there is no inconsistency with earlier results which had shown more stringent chemical requirements for the antisense strand of the initial trigger RNA (Parrish et al., 2000; Yang et al., 2000).

The model in Figure 1C leads to a number of testable predictions; in particular, we would expect to observe a population of secondary siRNAs after RdRP-mediated synthesis of duplex RNAs followed by cleavage by RNaseIII/DICER activity. These secondary triggers would be derived primarily from sequences upstream of the initial trigger region on the target mRNA and would be expected to induce a secondary RNA interference reaction directed to any homologous target RNA.

In this paper, we demonstrate the production and biological activity of RdRP-dependent secondary triggers during RNA interference in *C. elegans*.

Results

Biochemical Evidence for Secondary siRNAs

We first sought to demonstrate the existence of secondary siRNAs through direct analysis of RNA populations. Although the appearance of short RNAs in the 21–25 nt range has universally been observed in studies of RNA-triggered gene silencing, the abundance of such RNAs varies considerably between systems. In particular, siRNAs observed during RNAi are apparently much less abundant in *C. elegans* than in plants and *Drosophila* (e.g., Hamilton and Baulcombe, 1999; Parrish et al., 2000; Yang et al., 2000). In order to characterize populations of siRNA from *C. elegans* in detail, we used RNase protection assays. ^{32}P labeled ssRNA molecules (used as probes) were hybridized to denatured cellular RNA, and the resulting material treated with ssRNA-specific ribonucleases to degrade any unhybridized probe. We used single-stranded probes from the sense strand in order to detect the siRNA signal while avoiding a background due to breakdown products of the cellular mRNA target. To generate a large mass of *C. elegans* actively performing RNAi, we used a procedure in which animals are grown on bacteria engineered to express high levels of a specific dsRNA (Timmons and Fire, 1998; Fraser et al., 2000).

Each RNase-protection experiment involves two segments: a dsRNA trigger produced in bacteria and a probe RNA used to detect siRNA molecules. Figure 2 shows results for two target genes: the muscle-specific gene *unc-22* and the germline-specific gene *pos-1*. In each case, the strongest siRNA signals were obtained when the trigger and probe sequences corresponded. This population of siRNAs would be expected from models in which a dsRNA-specific nuclease cleaves the original dsRNA trigger to produce siRNA segments. In addition to the trigger-coincident siRNAs, we also detected populations of small antisense RNAs that correspond to regions of the target gene outside the original trigger. We tentatively refer to these as secondary siRNAs. The secondary siRNAs were generally detected at levels

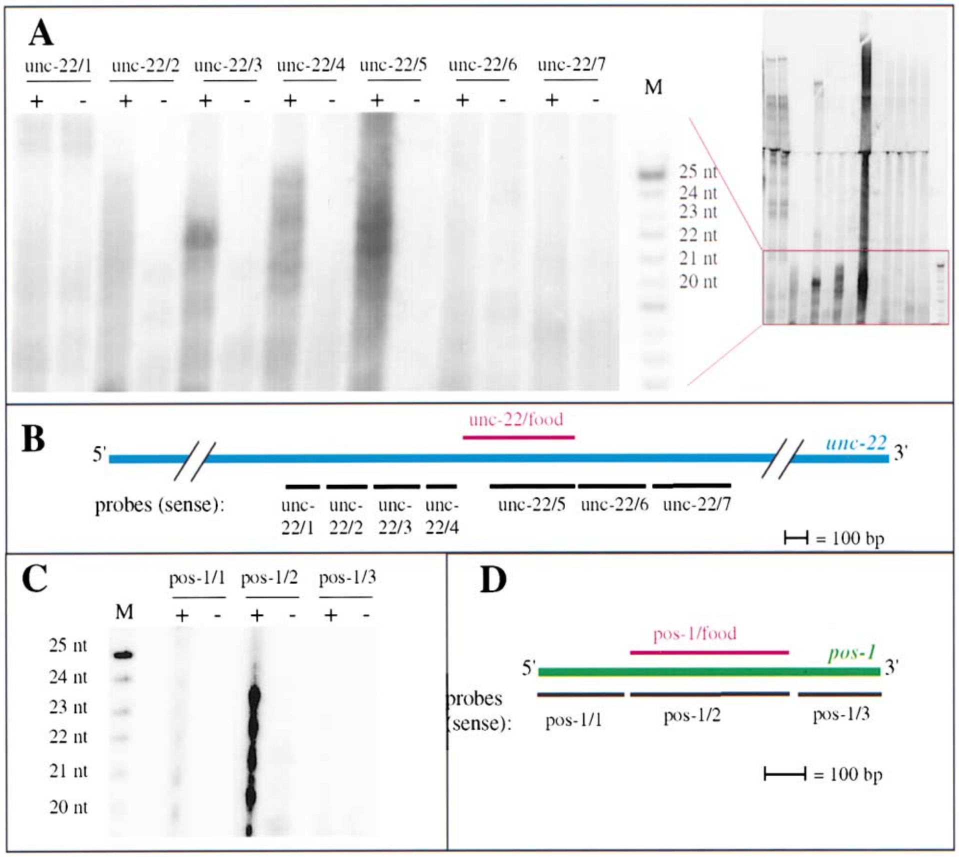


Figure 2. Biochemical Detection of Secondary siRNAs

Analysis of small RNAs from wild-type animals grown on *E. coli* expressing dsRNA segments of *unc-22* or *pos-1*. Total RNA was isolated and RNase protection assays were performed using various *unc-22* or *pos-1* specific probes (all of sense polarity).

(A) Products of RNase protection assay (right: protected fragments of probe resolved on polyacrylamide-urea gel; left: detail of 16–30 nt portion of gel). Feeding on *unc-22* dsRNA yielded siRNAs from the dsRNA segment comprising the food, but also produced siRNAs mapping upstream of this region. Lanes designated "+": RNA from animals fed *unc-22* dsRNA. To determine levels of probe-derived background, negative controls ("−") were carried out by performing RNase protections with yeast tRNA as input RNA. A similar background in the siRNA size range was observed in RNase protection assays on RNA from animals grown on induced bacteria containing the feeding vector L4440 with no insert (data not shown). RNase protection assays have also been carried out using RNA from IPTG-induced *E. coli* producing *unc-22* dsRNA; these showed some level of probe protection but no protected fragments in the siRNA size range (data not shown). Labels above the lanes indicate probes. "M": ^{32}P-labeled 25 nt RNA oligonucleotide marker.

(B) Map of *unc-22* mRNA with positions of probes and bacterially produced dsRNA.

(C) Secondary siRNAs are also produced upon feeding with *E. coli* producing *pos-1* dsRNA. Since *pos-1* is a germline-specific gene, RNA was isolated from egg preparations. "+": *C. elegans* populations fed with *E. coli* producing *pos-1* dsRNA; "−": equivalent RNA preparations from animals grown on *E. coli* containing the empty L4440 vector.

(D) Map of *pos-1* mRNA with positions of probes and bacterially produced dsRNA.

different combinations of trigger and probe sequences. Although the detection limits of the system preclude a definitive measurement of siRNA levels for each trigger/probe combination, two points emerge rather clearly from the analysis. First, occurrence of a detectable secondary antisense population was limited to cases in which the probe sequence was upstream (closer to the 5′ end of the target mRNA) as compared with the trigger sequence. Second, the abundance of secondary siRNA molecules appeared to decrease as a function of distance from the primary trigger.

Transitive RNAi

sequences for degradation. To test this hypothesis, it is necessary to distinguish between targeting by the initial dsRNA trigger and by the secondary siRNAs. This is most conveniently carried out by means of a "transitive RNAi" assay. Essentially, such an assay entails a cell with two populations of target RNA: the first population (primary target) has a segment which matches the dsRNA trigger; the second population has no homology to the initial dsRNA trigger, but has a segment which is identical to the primary target.

Figure 3 shows an example of transitive RNAi in which both primary and secondary target RNAs are transgene-derived transcripts carrying *gfp*. The primary target in

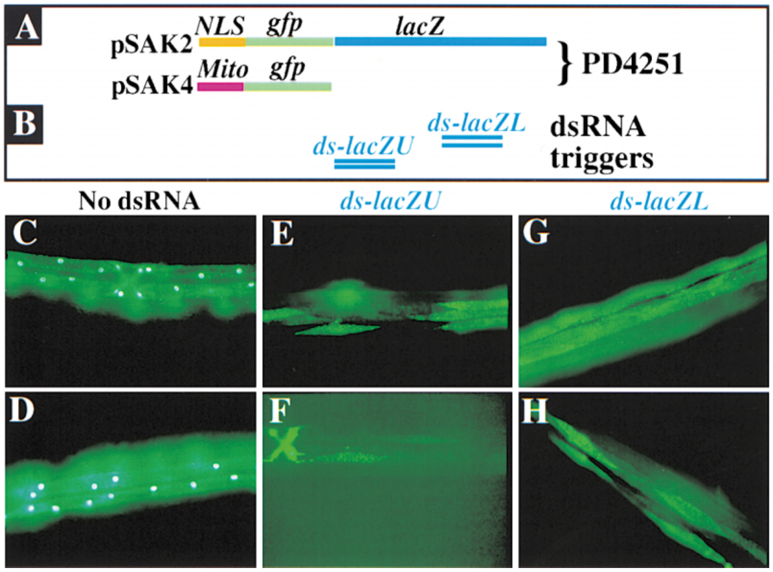


Figure 3. Assays for Transitive RNAi Using Distinct *gfp* Transgenes

The transgenic line used for this assay (PD4251) carries two different *gfp* reporter constructs (A). pSAK2 produces nuclear-localized GFP fused at the C terminus to additional sequences encoding *E. coli* β-galactosidase (*lacZ*). pSAK4 produces mitochondrially localized GFP with no additional sequences at the C terminus. PD4251 animals express both nuclear and mitochondrial GFP forms in all cells of the body musculature (Fire et al., 1998). Young adult progeny of adult animals injected with specific dsRNA segments (B) were examined to determine the level of interference with nuclear- and mitochondrial-targeted *gfps*.

(C and D) Mock injected control animals with both GFP isoforms expressed in each muscle cell.

(E and F) Progeny of animals injected with ds-*lacZU.* This injection produced a strong transitive RNAi effect, interfering in a majority of cells not only with the nuclear targeted *gfp*::*lacZ* transgene, but also with the mitochondrial-targeted *gfp*. (A bright "X" shape in [F] shows vulval muscles fortuitously included in the photo; these cells are generally nonresponsive to parentally injected dsRNA; Fire et al., 1998)

(G and H) Progeny of animals injected with ds-*lacZL*. This segment had only a modest effect on the expression of mitochondrially targeted *gfp*, so that the majority of cells continue to produce GFP in mitochondria but not nuclei. (F) and (H) are representative of the strongest transitive RNAi response in each population, while (E) and (G) are representative of the weakest effect. As negative controls, PD4251 animals injected with a variety of unrelated dsRNA segments (*unc-22*A, *unc-22*B, *lin-26*IVS3) showed no evident decrease in either nuclear or mitochondrial GFP. Animals injected with *gfp* dsRNA show near-complete (98%) loss of both nuclear and mitochondrial GFP (Fire et al., 1998).

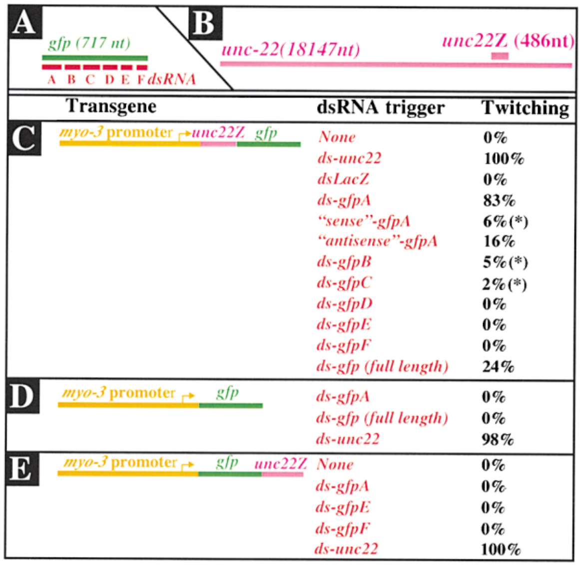
target encodes a mitochondrially targeted GFP (MtGFP) which has no sequences from *lacZ* (both transgene mRNAs are driven by the *myo-3* promoter). As a control, animals carrying only one of the two transgene constructs show the expected effects: both GFPs are dramatically reduced in progeny of animals injected with dsRNA corresponding to GFP, while only the NLS-GFP-LACZ construct is affected by dsRNAs corresponding to *lacZ* (data not shown). A line carrying both transgene constructs produces both nuclear LACZ-GFP and mitochondrial GFP (PD4251; Figures 3C and 3D). Injection of dsRNA segments from *lacZ* into the line carrying both transgenes produces a transitive effect: reduction of both nuclear GFP-*lacZ* and mitochondrial GFP. Of two different *lacZ* segments tested, a trigger that was located just 3′ to the *gfp*::*lacZ* junction (*ds-lacZU*) was most potent in the transitive RNAi assay, producing reduction of mitochondrial GFP to background in 60% of targeted cells, while a dsRNA trigger located further downstream (*ds-lacZL*) produced a more modest effect (reduction of GFP in 28% of cells) (Figure 3 and data not shown).

A second example of transitive RNAi is presented in Figure 4. In this case, the primary target is an *unc-22*::*gfp* fusion transgene (Figure 4C), while the secondary target is an endogenous gene (*unc-22*; Brenner, 1974; Moerman et al., 1988). Injection of dsRNA corresponding to *gfp* into wild-type animals (no transgene) produced no phenotype; injection of ds*gfp* RNA into animals carrying a transgene expressing GFP alone produced a decrease in GFP but no *unc-22* phenotype. Injection of ds*gfp* RNA into animals expressing the *unc-22*::*gfp* transgene

To test whether transitive RNAi could proceed with endogenous genes as targets, we carried out the two experiments shown in Figure 5. In-frame deletion alleles of *unc-22* and *unc-52* provide a useful genetic tool: these alleles each produce proteins that lose a fraction of the coding region (658 amino acids for *unc-22*(*st528*); 150 amino acids for *unc-52*(*ra511*)) but retain full wild-type function (Kiff et al., 1988; Fire et al., 1991; Rogalski et al., 1993; Mullen et al., 1999). As expected, dsRNAs corresponding to the deleted regions produced strong gene-specific RNAi effects in wild-type animals, but no effect in animals homozygous for the corresponding deletion alleles. The test for transitive RNAi in each case consists of introducing these trigger RNAs into heterozygous animals carrying both wild-type and mutant alleles. In each case, we found a strong transitive RNAi effect: heterozygotes exhibited interference with both deletion and wild-type alleles. These experiments demonstrate that transitive RNAi is not limited to transgene targets, but can also target physiological expression of cellular genes.

Structural Requirements for Triggering of Transitive RNAi

Certain features of transitive RNAi are illuminated by the requirements for structure and dose of the primary trigger. A prediction of the model in Figure 1C is that the effect should exhibit a defined polarity, with interference depending on the order of the two segments in the primary target mRNA. This was the case, as shown by the lack of sensitivity to transitive RNA when the order



	Transgene	dsRNA trigger	Twitching
C	myo-3 promoter unc22Z gfp	None	0%
		ds-unc22	100%
		dsLacZ	0%
		ds-gfpA	83%
		"sense"-gfpA	6%(*)
		"antisense"-gfpA	16%
		ds-gfpB	5%(*)
		ds-gfpC	2%(*)
		ds-gfpD	0%
		ds-gfpE	0%
		ds-gfpF	0%
		ds-gfp (full length)	24%
D	myo-3 promoter gfp	ds-gfpA	0%
		ds-gfp (full length)	0%
		ds-unc22	98%
E	myo-3 promoter gfp unc22Z	None	0%
		ds-gfpA	0%
		ds-gfpE	0%
		ds-gfpF	0%
		ds-unc22	100%

Figure 4. Assays for Transitive RNAi Using a Chimeric *unc-22*::*gfp* Transgene

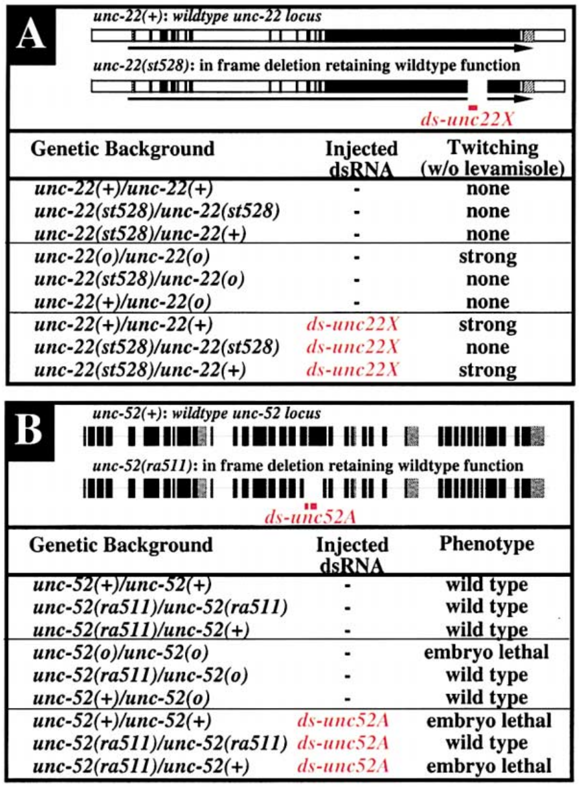
Transgenic lines used for these assays carry the *C. elegans myo-3* promoter driving the indicated combinations of the *gfp* coding region (717 nt) and a segment within the *unc-22* gene (*unc-22z*; 486 nt). Following propagation of clonal transgenic lines for several generations, transitive RNAi was assayed by injecting adults with a variety of dsRNAs. After ∼3.5 days, injected animals and postinjection progeny (>50 animals derived from 5–20 injected parents) were scored for twitching in levamisole. Assays marked with an "*" showed twitching predominantly in the injected adults; the remaining positive assays showed twitching in both injected adults and progeny, while negative assays showed twitching in neither injected adults nor progeny.

(A and B) Segments used in this analysis. mRNA structures are shown; the *gfp* coding region is interrupted in each DNA construct by three 51 nt introns. The *gfp*-derived dsRNAs (Parrish et al., 2000) were each functional in primary RNAi, as assayed by reduction of GFP in injected adults and progeny.

(C) A twitching phenotype was observed when the injected dsRNA corresponded to sequences from *gfp* downstream of the *unc-22*::*gfp* junction. Note that *ds-gfpA* produced the most effective twitching response, presumably by producing the highest molar concentration of siRNAs immediately downstream of the *unc-22*::*gfp* junction.

(D and E) Transitive RNA was specific to the structure and arrangement of the initial dsRNA trigger and transgene.

Interference showed a dose response to the concentration of primary trigger, with a modest interference response observed at doses as low as 1.5 × 10^6 molecules per injected parent (data not shown). Given the expression levels of *unc-22* (Fire et al., 1991), and assuming equal dispersion of trigger RNA among the cells of the affected progeny, this corresponds to a stoichiometry on the order of ∼100 molecules of trigger RNA for ∼5000 molecules of target mRNA in each muscle cell of the affected animals. Triggering also appeared to be structure-specific: although some interference was observed with sense or antisense RNA preparations alone, there was a dramatic stimulation upon mixing the two preparations. As with previous studies (e.g., Fire et al., 1998), it was not straightforward to distinguish whether residual activity of our ssRNA preparations was



Genetic Background	Injected dsRNA	Twitching (w/o levamisole)
unc-22(+)/unc-22(+)	-	none
unc-22(st528)/unc-22(st528)	-	none
unc-22(st528)/unc-22(+)	-	none
unc-22(o)/unc-22(o)	-	strong
unc-22(st528)/unc-22(o)	-	none
unc-22(+)/unc-22(o)	-	none
unc-22(+)/unc-22(+)	ds-unc22X	strong
unc-22(st528)/unc-22(st528)	ds-unc22X	none
unc-22(st528)/unc-22(+)	ds-unc22X	strong

Genetic Background	Injected dsRNA	Phenotype
unc-52(+)/unc-52(+)	-	wild type
unc-52(ra511)/unc-52(ra511)	-	wild type
unc-52(ra511)/unc-52(+)	-	wild type
unc-52(o)/unc-52(o)	-	embryo lethal
unc-52(ra511)/unc-52(o)	-	wild type
unc-52(+)/unc-52(o)	-	wild type
unc-52(+)/unc-52(+)	ds-unc52A	embryo lethal
unc-52(ra511)/unc-52(ra511)	ds-unc52A	wild type
unc-52(ra511)/unc-52(+)	ds-unc52A	embryo lethal

Figure 5. Transitive RNAi Can Operate on Native Chromosomal Genes

(A) Maps of wild-type *unc-22* and an in-frame deletion (*st528*) that retains wild-type function (Moerman et al., 1988; Benian et al., 1993; Kiff et al., 1988; black, exons; white, introns). *unc-22* null mutants exhibit a strong twitching behavior in the absence of levamisole (we used *unc-22*(*e66*) as a canonical null for this analysis; Brenner, 1974). The strong twitching phenotype is not seen with animals that have a single functional dose of the wild-type or *st528* allele. Following injection of *ds-unc22X* RNA, twitching without levamisole was observed in 100% of *unc-22*(+) animals, 0% of *unc-22*(*st528*)/*unc-22*(*st528*) animals, and 60% of *unc-22*(*st528*)/+ animals.

(B) Maps of wild-type *unc-52* and a deletion allele that removes nonessential sequences (*unc-52*(*ra511*); Mullen et al., 1999; black, exons; white, introns; hatched, alternatively spliced exons). The null phenotype for *unc-52* is a zygotic-effect embryonic lethality with paralysis (Williams and Waterston, 1994; Rogalski et al., 1993). A chromosomal deficiency (mnT11; Herman et al., 1982) was used to definitively determine *unc-52*(+)/*unc-52*(o) and *unc-52*(*ra511*)/*unc-52*(o) phenotypes. Animals that have a single functional dose of the wild-type or *ra511* allele show no lethal or visible phenotype. Following injection of *ds-unc52A* RNA, embryonic lethality with paralysis was observed in 100% of *unc-52*(+) animals, 0% of *unc-52*(*ra511*)/*unc-52*(*ra511*) animals, and 100% of *unc-52*(*ra511*)/+ animals.

initial triggering reaction is either fully dependent on, or greatly stimulated by, delivery of a trigger RNA with double-stranded character.

Not all potential trigger RNAs were capable of producing transitive interference. For each target RNA, we observed a graded effect as a function of distance between primary and secondary target sequences. The precise relationship between distance and effectiveness ap-

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