RNA interference is mediated by 21- and 22-nucleotide RNAs

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Double-stranded RNA (dsRNA) induces sequence-specific posttranscriptional gene silencing in many organisms by a process known as RNA interference (RNAi). Using a *Drosophila* in vitro system, we demonstrate that 21- and 22-nt RNA fragments are the sequence-specific mediators of RNAi. The short interfering RNAs (siRNAs) are generated by an RNase III–like processing reaction from long dsRNA. Chemically synthesized siRNA duplexes with overhanging 3′ ends mediate efficient target RNA cleavage in the lysate, and the cleavage site is located near the center of the region spanned by the guiding siRNA. Furthermore, we provide evidence that the direction of dsRNA processing determines whether sense or antisense target RNA can be cleaved by the siRNA–protein complex.

[*Key Words*: RNAi; posttranscriptional gene silencing; dsRNA; siRNA]

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The term RNA interference (RNAi) was coined after the discovery that injection of dsRNA into the nematode *Caenorhabditis elegans* leads to specific silencing of genes highly homologous in sequence to the delivered dsRNA (Fire et al. 1998). RNAi was also observed subsequently in insects (Kennerdell and Carthew 1998), frog (Oelgeschlager et al. 2000), and other animals including mice (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000) and is likely to also exist in human. RNAi is closely linked to the posttranscriptional gene-silencing (PTGS) mechanism of cosuppression in plants and quelling in fungi (Cogoni and Macino 1999; Catalanotto et al. 2000; Dalmay et al. 2000; Ketting and Plasterk 2000; Mourrain et al. 2000; Smardon et al. 2000), and some components of the RNAi machinery are also necessary for posttranscriptional silencing by cosuppression (Catalanotto et al. 2000; Dernburg et al. 2000; Ketting and Plasterk 2000). The topic has been reviewed recently (Fire 1999; Sharp 1999; Bass 2000; Bosher and Labouesse 2000; Plasterk and Ketting 2000; Sijen and Kooter 2000; see also the entire issue of *Plant Molecular Biology*, Vol. 43, issue 2/3, 2000).

The natural function of RNAi and cosuppression appears to be protection of the genome against invasion by mobile genetic elements such as transposons and viruses, which produce aberrant RNA or dsRNA in the host cell when they become active (Jensen et al. 1999; Ketting et al. 1999; Ratcliff et al. 1999; Tabara et al. 1999; Malinsky et al. 2000). Specific mRNA degradation prevents transposon and virus replication, although some viruses are able to overcome or prevent this process by expressing proteins that suppress PTGS (Anandalakshmi et al. 2000; Lucy et al. 2000; Voinnet et al. 2000).

DsRNA triggers the specific degradation of homologous RNAs only within the region of identity with the dsRNA (Zamore et al. 2000). The dsRNA is processed to 21–23-nt RNA fragments (Zamore et al. 2000). These short fragments were also detected in extracts prepared from *Drosophila melanogaster* Schneider 2 cells that were transfected with dsRNA before cell lysis (Hammond et al. 2000) or after injection of radiolabeled dsRNA into *D. melanogaster* embryos (Yang et al. 2000) or *C. elegans* adults (Parrish et al. 2000). RNA molecules of similar size also accumulate in plant tissue that exhibits PTGS (Hamilton and Baulcombe 1999). It has been suggested that the 21–23-nt fragments are the guide RNAs for target recognition (Hamilton and Baulcombe 1999; Hammond et al. 2000), which is supported by the finding that the target mRNA is cleaved in 21–23-nt intervals (Zamore et al. 2000).

Here, we use the established *Drosophila* in vitro system (Tuschl et al. 1999; Zamore et al. 2000) to explore further the mechanism of RNAi. It is demonstrated that synthetic 21- and 22-nt RNAs, when base paired with 3′ overhanging ends, act as the guide RNAs for sequence-specific mRNA degradation. Short 30-bp dsRNAs are inefficiently processed to 21- and 22-nt RNAs, which may explain why they are ineffective in mediating RNAi. Furthermore, we define the target RNA cleavage sites relative to the 21- and 22-nt short interfering RNAs (siRNAs) and provide evidence that the d

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target RNA can be cleaved by the siRNP endonuclease complex.

Length requirements for processing of dsRNA to 21- and 22-nt RNA fragments

Lysate prepared from *D. melanogaster* syncytial embryos recapitulates RNAi in vitro, providing a tool for biochemical analysis of the mechanism of RNAi (Tuschl et al. 1999; Zamore et al. 2000). In vitro and in vivo analysis of the length requirements of dsRNA for RNAi has revealed that short dsRNA (<150 bp) are less effective than longer dsRNAs in degrading target mRNA (Ngo et al. 1998; Tuschl et al. 1999; Caplen et al. 2000; Hammond et al. 2000). The reasons for reduction in mRNA degrading efficiency are not understood. We therefore examined the precise length requirement of dsRNA for target RNA degradation under optimized conditions in the *Drosophila* lysate. Three series of dsRNAs were synthesized and directed against firefly luciferase (*Pp*-luc) reporter RNA. The dual luciferase assay was used to monitor specific suppression of target RNA expression (Tuschl et al. 1999; (Fig. 1A,B). Specific 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. The effect was independent of the target position and the degree of inhibition of *Pp*-luc mRNA expression correlated with the length of the dsRNA; that is, long dsRNAs were more effective than short dsRNAs.

It has been suggested that the 21–23-nt RNA fragments generated by processing of dsRNAs are the mediators of RNA interference and cosuppression (Hamilton and Baulcombe 1999; Hammond et al. 2000; Zamore et al. 2000). We therefore analyzed the rate of 21–23-nt fragment formation for a subset of dsRNAs ranging in size from 501 to 29 bp. Formation 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. 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. The 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 RNAs.

Mapping of the cleavage sites on sense and antisense target RNAs

Addition of dsRNA and 5′-capped target RNA to the *Drosophila* lysate results in sequence-specific degradation of the target RNA (Tuschl et al. 1999). The target mRNA is only cleaved within the region of identity with the dsRNA, and many of the target cleavage sites are separated by 21–23 nt (Zamore et al. 2000). Thus, the number of cleavage sites for a given dsRNA was expected to roughly correspond to the length of the dsRNA divided by 21. We mapped the target cleavage sites on a sense and an antisense target RNA that was 5′ radiolabeled at the cap (Zamore et al. 2000; Fig. 3A,B). Stable 5′ cleavage products were separated on a sequencing gel, and the position of cleavage was determined by comparison with a partial RNase T1 and an alkaline hydrolysis ladder from the target RNA.

Consistent with the previous observation (Zamore et al. 2000), all target RNA cleavage sites were located within the region of identity to the dsRNA. The 39-bp dsRNA produced a strong and a weak (often hardly de-

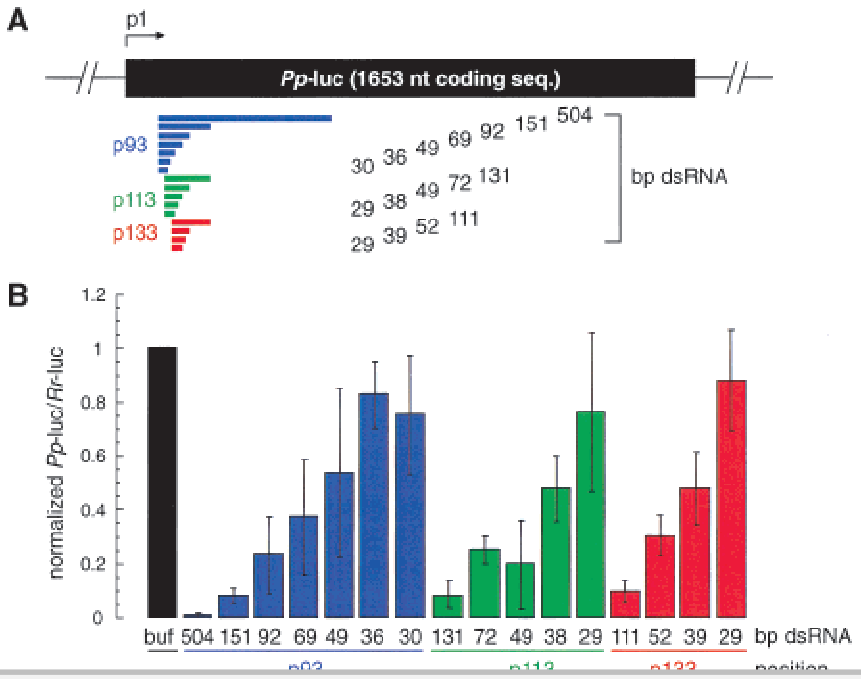

Figure 1. Double-stranded RNA as short as 38 bp can mediate RNAi. (*A*) Graphic representation of dsRNAs used for targeting *Pp*-luc mRNA. Three series of blunt-ended dsRNAs covering a range of 29–504 bp were prepared. The position of the first nucleotide of the sense strand of the dsRNA is indicated relative to the start codon of *Pp*-luc mRNA (p1). (*B*) RNA interference assay (Tuschl et al. 1999). Ratios of target *Pp*-luc to control *Rr*-luc activity were normalized to a buffer control (black bar). DsRNAs (5 nM) were preincubated in *Drosophila* lysate at 25°C for 15 min before the addition of 7-methyl-guanosine-capped *Pp*-luc and *Rr*-luc mRNAs (~50 pM). The incubation was continued for another hour and then analyzed by the dual luciferase assay (Promega). The data are the average from at

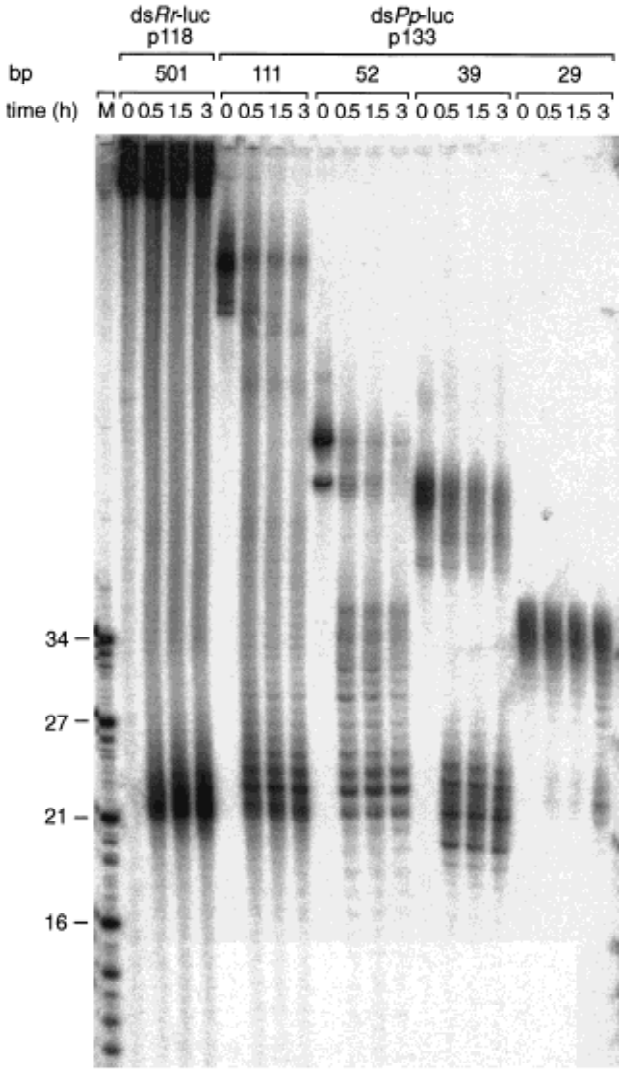

Figure 2. A 29-bp dsRNA is only slowly processed to 21–23-nt fragments. Time course of 21–23-mer formation from processing of internally ^{32}P-labeled dsRNAs (5 nM) in the *Drosophila* lysate. The length and source of the dsRNA are indicated. An RNA size marker (M) has been loaded in the left lane, and the fragment sizes are indicated. Double bands at time zero are caused by incompletely denatured dsRNA.

tectable) cleavage site in the sense target RNA separated by 19 nt. The antisense target was only cleaved once, by the 39-bp dsRNA. The predominant cleavage site of the sense strand and the cleavage site of the antisense strand are located 10 nt from the 5′ end of the region covered by the dsRNA (Fig. 3B). The 52-bp dsRNA, which shares the same 5′ end as the 39-bp dsRNA, produces the same strong cleavage site on the sense target, located 10 nt from the 5′ end of the region of identity with the dsRNA in addition to two weaker cleavage sites 23 and 24 nt downstream of the first site. The antisense target was only cleaved once, again 10 nt from the 5′ end of the region covered by its respective dsRNA. Mapping of the cleavage sites for the 38–49-bp dsRNAs shown in Figure 1 was always located 7–10 nt downstream from the 5′ boundary of the region covered by the dsRNA (data not shown). This suggests that the point-of-target RNA cleavage can be determined by the end of the dsRNA and could imply that processing to 21–23mers starts from the ends of the duplex.

Cleavage sites on sense and antisense targets for the longer 111-bp dsRNA were much more frequent than anticipated, and most of them appear in clusters separated by 20–23 nt (Fig. 3A,B). As for the shorter dsRNAs, the first cleavage site on the sense target is 10 nt from the 5′ end of the region spanned by the dsRNA, and the first cleavage site on the antisense target is located 9 nt from the 5′ end of region covered by the dsRNA. It is unclear what causes this disordered cleavage, but one possibility could be that longer dsRNAs may not only get processed from the ends but also internally, or there are some specificity determinants for dsRNA processing that we do not yet understand. Some irregularities to the 21–23 nt spacing were also noted previously (Zamore et al. 2000).

dsRNA is processed to 21- and 22-nt RNAs by an RNase III–like mechanism

To understand better the molecular basis of dsRNA processing and target RNA recognition, we decided to analyze the sequences of the 21–23-nt fragments generated by processing of 39-, 52-, and 111-bp dsRNAs in the *Drosophila* lysate. We first examined the 5′ and 3′ termini of the RNA fragments. Periodate oxidation of gel-purified 21–23-nt RNAs followed by β-elimination indicated the presence of a terminal 2′ and 3′ hydroxyl (data not shown). The 21–23mers were also responsive to alkaline phosphatase treatment, implying the presence of a 5′ terminal phosphate (data not shown). The presence of 5′ phosphate and 3′ hydroxyl termini suggests that the dsRNA could be processed by an enzymatic activity similar to *Escherichia coli* RNase III (for reviews, see Dunn 1982; Nicholson 1999; Robertson 1982, 1990).

To directionally clone the 21–23-nt RNA fragments, 3′ and 5′ adapter oligonucleotides were ligated to the purified 21–23 mers using T4 RNA ligase. The ligation products were reverse transcribed, PCR-amplified, concatamerized, cloned, and sequenced. Over 220 short RNAs were sequenced from dsRNA processing reactions of the 39-, 52-, and 111-bp dsRNAs (Fig. 4A). We found the following length distribution: 1% 18 nt, 5% 19 nt, 12% 20 nt, 45% 21 nt, 28% 22 nt, 6% 23 nt, and 2% 24 nt. Sequence analysis of the 5′ terminal nucleotide of the processed fragments indicated that oligonucleotides with a 5′ guanosine were underrepresented. This bias was most likely introduced by T4 RNA ligase, which discriminates against 5′ phosphorylated guanosine as donor oligonucleotide (Romaniuk et al. 1982); no significant sequence bias was seen at the 3′ end. Many of the ~21-nt fragments originating from the 3′ ends of the sense or antisense strand of the duplexes include 3′ nucleotides that are derived from untemplated addition

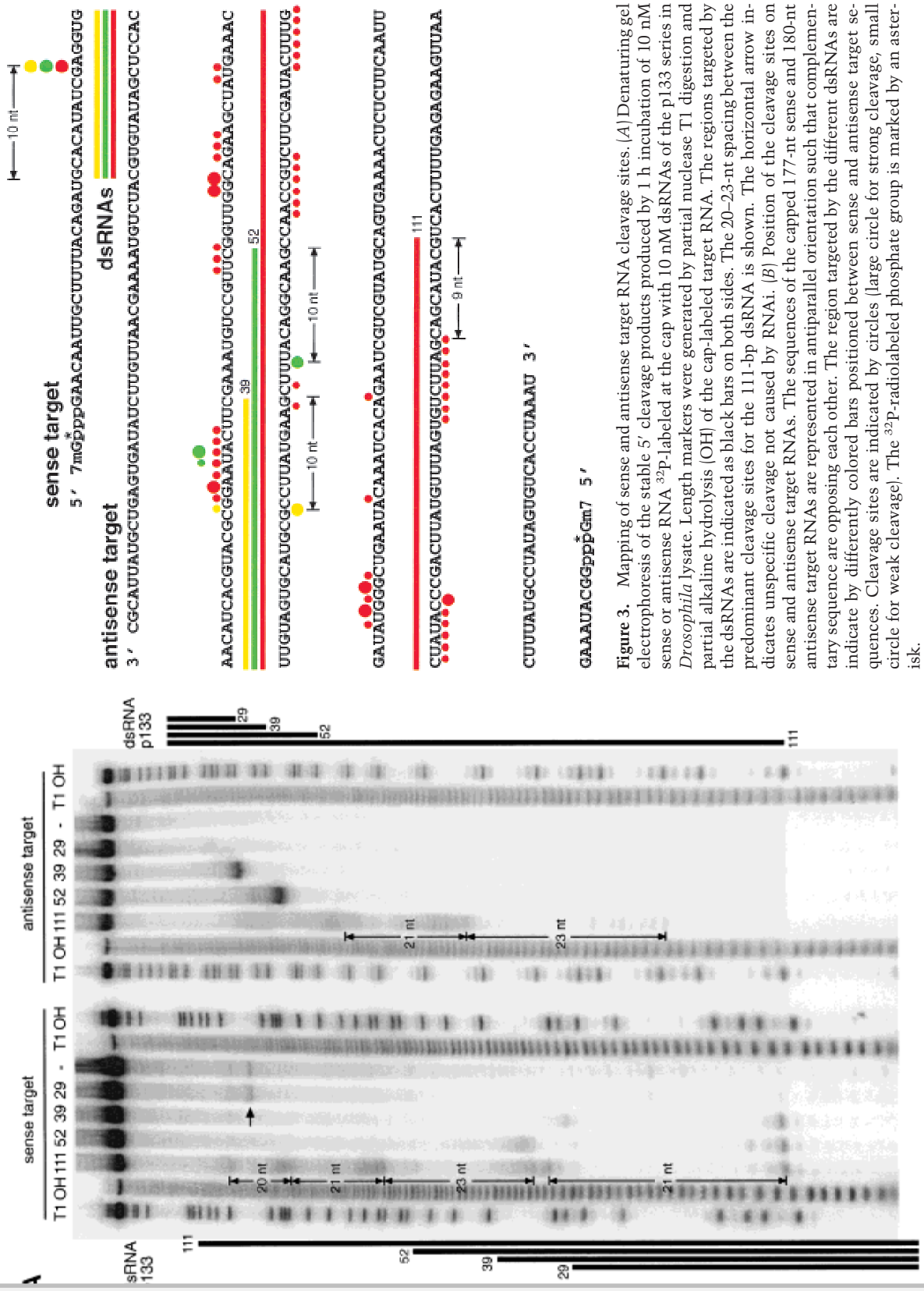

Figure 3. Mapping of sense and antisense target RNA cleavage sites. (*A*) Denaturing gel electrophoresis of the stable 5′ cleavage products produced by 1 h incubation of 10 nM sense or antisense RNA ^{32}P-labeled at the cap with 10 nM dsRNAs of the p133 series in *Drosophila* lysate. Length markers were generated by partial nuclease T1 digestion and partial alkaline hydrolysis (OH) of the cap-labeled target RNA. The regions targeted by the dsRNAs are indicated as black bars on both sides. The 20–23-nt spacing between the predominant cleavage sites for the 111-bp dsRNA is shown. The horizontal arrow indicates unspecific cleavage not caused by RNAi. (*B*) Position of the cleavage sites on sense and antisense target RNAs. The sequences of the capped 177-nt sense and 180-nt antisense target RNAs are represented in antiparallel orientation such that complementary sequence are opposing each other. The region targeted by the different dsRNAs are indicate by differently colored bars positioned between sense and antisense target sequences. Cleavage sites are indicated by circles (large circle for strong cleavage, small circle for weak cleavage). The ^{32}P-radiolabeled phosphate group is marked by an asterisk.

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