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RNAi as Random Degradative PCR: siRNA Primers Convert mRNA into dsRNAs that Are Degraded to Generate New siRNAs

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Summary

In posttranscriptional gene silencing (PTGS), "quelling," and RNA interference (RNAi), 21–25 nucleotide RNA fragments are produced from the initiating dsRNA. These short interfering RNAs (siRNAs) mediate RNAi by an unknown mechanism. Here, we show that GFP and *Pp*-Luc siRNAs, isolated from a protein complex in *Drosophila* embryo extract, target mRNA degradation in vitro. Most importantly, these siRNAs, as well as a synthetic 21-nucleotide duplex GFP siRNA, serve as primers to transform the target mRNA into dsRNA. The nascent dsRNA is degraded to eliminate the incorporated target mRNA while generating new siRNAs in a cycle of dsRNA synthesis and degradation. Evidence is presented that mRNA-dependent siRNA incorporation to form dsRNA is carried out by an RNA-dependent RNA polymerase activity (RdRP).

Introduction

Fire and associates first reported on RNA interference (RNAi) when they demonstrated potent and specific genetic interference upon the injection of dsRNA into *C. elegans* (Fire et al., 1998). However, the underlying phenomenon, known as posttranscriptional gene silencing (PTGS), was initially described in studies on transgenic plants (Napoli et al., 1990). PTGS was observed when the introduction of all or a portion of a transgene occasionally resulted in the loss of expression from the corresponding endogenous gene with no disruption in gene transcription. Mature mRNA transcripts simply did not accumulate in the cytoplasm of the affected plants (Kooter et al., 1999; Plasterk and Ketting, 2000). The viral stocks used to make the transgene constructs were also known to generate dsRNA during the replication cycle and the phenomenon of PTGS was linked to replication-competent virus (Baulcombe, 1999a; Burton et al., 2000; Ruiz et al., 1998). Similar findings were also described in *Neurospora* where the phenomenon was termed "quelling" (Cogoni et al., 1996).

A remarkable aspect of this process is that the local induction of gene silencing can spread throughout the organism and is often heritable to the next generation. This is particularly true in plants (Palauqui and Vaucheret, 1998; Sonoda and Nishiguchi, 2000; Voinnet et al., 1998) but it has also been demonstrated in *C. elegans* (Grishok et al., 2000). Furthermore, only a few molecules of dsRNA per cell or embryo completely silence the corresponding gene, a result that led to the speculation the process might be catalytic in nature (Fire et al., 1998; Kennerdell and Carthew, 1998). Genetic studies in *Neurospora*, *Arabidopsis*, and *C. elegans* identified several genes involved in quelling, PTGS, and RNAi, and include members of the helicase family (Cogoni and Macino, 1999b; Dalmay et al., 2001), RNase III-related nucleases (Ketting et al., 1999), members of the Argonaute family (Catalanotto et al., 2000; Fagard et al., 2000; Hammond et al., 2001a; Tabara et al., 1999), and RNA-dependent RNA polymerases (RdRP) (Cogoni and Macino, 1999a; Dalmay et al., 2000; Mourrain et al., 2000; Smardon et al., 2000).

Molecular studies in plants (Hamilton and Baulcombe, 1999), *C. elegans* (Parrish et al., 2000), and *Drosophila* (Zamore et al., 2000) have also shown that a common feature shared among these various targeted RNA degradation processes is the production of short 21–25 nucleotide RNA fragments of both sense and antisense orientation derived from the input dsRNA. Recent studies using Schneider cell extracts prepared from dsRNA treated cells have identified Dicer, an ATP-dependent ribonuclease related to the RNase III family of nucleases that specifically cleaves double stranded RNA into 21-25 nucleotide pieces (Bernstein et al., 2001). The resulting small dsRNA fragments have been proposed to act as "guide RNAs" that target an associated nuclease complex, called RISC (RNA-induced silencing complex), (Hammond et al., 2000) to the corresponding mRNA through strand complementarity (Bass, 2000). However, this has not been demonstrated experimentally and the exact mechanism of action involving the 21-25 nucleotide RNAs remains to be elucidated.

With the recent development of *Drosophila* embryo (Tuschl et al., 1999) and Schneider cell extracts (Hammond et al., 2000) capable of carrying out the dsRNA-dependent targeted degradation of mRNA in vitro, we sought to further clarify the role of the 21–25 nucleotide RNAs in mRNA silencing. Tuschl and coworkers have shown that synthetic short dsRNAs, called siRNAs for short interfering RNAs, can mediate RNAi in *Drosophila* extracts by directing a cleavage event in the target RNA (Elbashir et al., 2001b). Here, we show that the siRNAs produced upon the addition of GFP and *Pp*-Luc dsRNA to *Drosophila* embryo extract can be enriched in a micrococcal nuclease-resistant fraction. Once these short RNAs are treated with calf-intestinal phosphatase, they mediate efficient RNAi in vitro. These siRNAs, as well as a synthetic 21-nucleotide GFP duplex siRNA, are shown to act as mRNA-specific primers that are incorporated during the subsequent conversion of the target mRNA into dsRNA. Nascent dsRNA is then cleaved by RNase III-related enzymes to degrade the mRNA while generating new siRNAs in the process. RdRP activity is shown to mediate the incorporation of a synthetic siRNA into nascent dsRNA. We propose that repeated cycles of dsRNA synthesis and concomitant siRNA/primer production result in targeted mRNA degradation, and that this process can account for the underlying mechanism responsible for PTGS, quelling, and RNAi.

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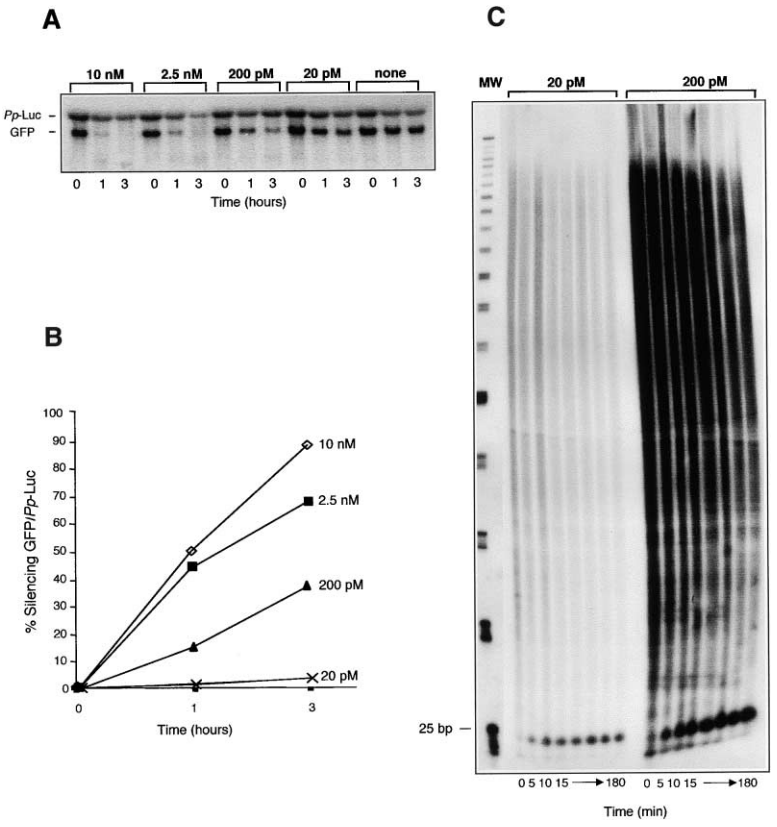

Figure 1. siRNAs Are Produced in the Absence of RNAi

(A) The rate of GFP mRNA (746 bp) degradation is proportional to the input concentration of GFP dsRNA (716 bp). Degradation does not occur below a threshold concentration of 20 pM dsRNA. *Pp*-Luc mRNA (1682 bp) is the nontargeted control mRNA. GFP and *Pp*-Luc indicate the positions of the full-length target and control RNAs, respectively.

(B) Data from (A) have been analyzed on the Fuji Phosphoimager and normalized to *Pp*-Luc mRNA over the concentration range of GFP dsRNA indicated.

(C) GFP siRNAs reach maximal levels in the processing reaction within 15 min in response to dsRNA concentrations that do (200 pM) or do not (20 pM) trigger RNAi. The specific activity of ^{32}P-UTP-labeled dsRNA added to the processing extract was approximately 1×10^9 cpm/μg. MW: 25 bp DNA ladder with the 25 nucleotide position indicated.

Results

Production of siRNAs Can Occur in the Absence of RNAi

Drosophila embryo extracts proficient in RNAi were prepared (Tuschl et al., 1999) to study the dsRNA-dependent degradation of mRNA. ^{32}P-labeled GFP mRNA (746 bp, capped and adenylated with 30 A residues) was selected as the target with ^{32}P-labeled *Photinus pyralis* luciferase (*Pp*-Luc) mRNA (1682 bp, capped and adenylated with 30 A residues) serving as the nontargeted control. Full-length unlabeled GFP dsRNA (716 bp) was used to activate targeted GFP mRNA degradation. Similar to the findings reported for dsRNA injected into *C. elegans* (Fire et al., 1998), the rate of GFP mRNA degradation in *Drosophila* embryo extracts was dependent upon the concentration of GFP dsRNA added to the reaction. Using the same conditions described previously (Tuschl et al., 1999) with 10–50 pM mRNAs and varying concentrations of dsRNA from 20 pM to 10 nM, maximal degradation rates for GFP mRNA were observed with GFP dsRNA concentrations above 10 nM, whereas no targeting was detected at or below 20 pM GFP dsRNA (Figures 1A and 1B). ^{32}P-labeled full-length GFP dsRNA was also used to look at the concentration-dependent appearance of the siRNAs produced under silencing and nonsilencing conditions. Although 20 pM GFP dsRNA did not support detectable targeted GFP mRNA degradation, the time course of appearance for the siRNAs was the same for both 20 pM and 200 pM GFP dsRNA and reached a plateau value within 15 min of incubation (Figure 1C). A similar time course for the appearance of siRNAs was also noted previously for *Drosophila* embryos injected with ^{32}P-labeled dsRNA (Yang et al., 2000). The amount of GFP siRNAs produced depended upon the input concentration of GFP dsRNA and this, in turn, was correlated with the level of RNAi in the reaction (Figure 1). Below a threshold concentration for the siRNAs, targeted mRNA degradation did not occur.

siRNAs Are Double Stranded and Protected in a Micrococcal Nuclease-Resistant Protein Complex

Previous studies have suggested that the processed short RNAs are double stranded and contained in a ribonucleoprotein complex (Elbashir et al., 2001b). We used micrococcal nuclease (Pelham and Jackson, 1976) to test whether or not the small RNAs could be isolated in a nuclease-resistant protein complex. In the first instance, extract was treated with micrococcal nuclease and blocked with EGTA prior to the addition of any RNAs. In agreement with previous observations (Hammond et al., 2000) micrococcal nuclease-treated extract was inactive in RNAi (Figure 2A, lane 2). This was not due to a nonspecific interaction between RNA and the nuclease (Wang and Gegenheimer, 1990) since the simultaneous addition of micrococcal nuclease, EGTA, and calcium to extracts was previously shown not to inhibit or affect RNAi (Hammond et al., 2000). The production of siRNAs was analyzed in the same reaction at 30 and 60 min time points using 10 nM full-length ^{32}P-labeled GFP dsRNA as a cleavage substrate. In micrococcal nuclease-treated extract, the rate of appearance

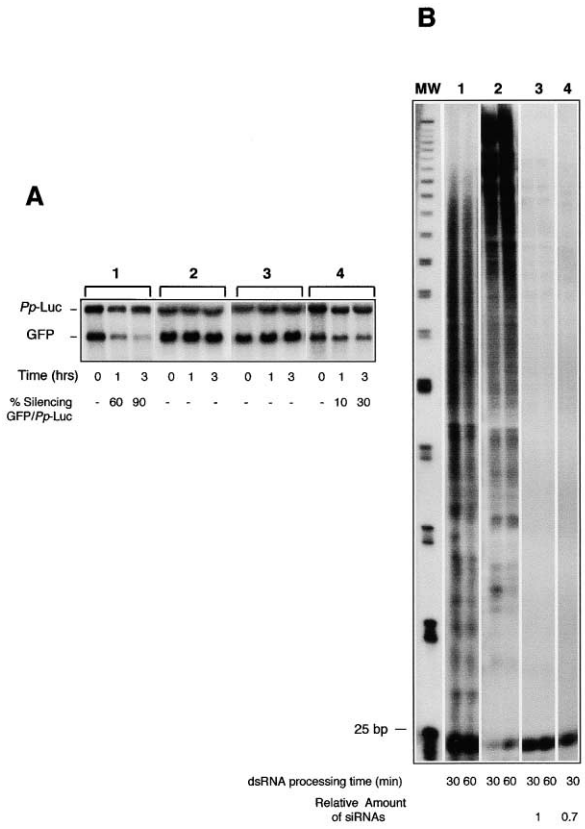

Figure 2. siRNAs Are Double Stranded and Associate in a Dynamic Ribonucleprotein Complex Protected from Micrococcal Nuclease Digestion

(A) Treatment of *Drosophila* embryo extract with micrococcal nuclease before or after the addition of GFP dsRNA inhibits RNAi: Lane 1, the standard reaction; Lane 2, addition of micrococcal nuclease prior to dsRNA; Lane 3, addition of dsRNA prior to micrococcal nuclease; and Lane 4, addition of dsRNA to the reaction in Lane 3 restores modest levels of RNAi. Percent silencing shown as GFP/*Pp*-Luc ratios is indicated below each lane.

(B) Addition of ^{32}P-UTP-labeled GFP dsRNA (200 pM, $>10^9$ cpm/μg) to extract prior to micrococcal nuclease treatment produces inactive siRNAs. Lane 1, control; Lane 2, addition of micrococcal nuclease prior to dsRNA; Lane 3, addition of dsRNA prior to micrococcal nuclease; and Lane 4, addition of 10 nM unlabeled GFP dsRNA to the reaction in Lane 3 chases the level of siRNAs. The initial input of labeled dsRNA was the same in all lanes shown, 2.5×10^5 cpm.

(C) The GFP siRNAs after micrococcal nuclease treatment, called Mn-siRNAs (Figure 2B, lane 3), are double stranded and resistant to Ribonuclease One digestion. Lane 1, siRNAs with no nuclease; Lane 2, siRNAs with RNase One; and Lane 3, denatured siRNAs with RNase One.

as well as the plateau value for the small RNAs was greatly reduced (Figure 2B, lane 2).

Extract incubated with unlabeled GFP dsRNA prior to micrococcal nuclease treatment also was unable to direct the degradation of GFP mRNA added subsequently to the reaction (Figure 2A, lane 3). However, an examination of the GFP siRNAs produced under these conditions revealed that the small RNAs remained resistant to nuclease digestion and were produced in amounts comparable to that seen in control reactions (Figure 2B, compare lanes 1 and 3). Furthermore, with the exception of the GFP siRNAs, most of the labeled GFP dsRNA was degraded during nuclease treatment (Figure 2B, lane 3).

When additional unlabeled GFP dsRNA was added to the reaction shown in Figure 2A, lane 3, modest RNAi was observed—about 30% of control values (Figure 2A, lane 4). Similarly, unlabeled GFP dsRNA added to extract preincubated with ^{32}P-labeled GFP dsRNA also resulted in roughly a 30% decrease in the amount of ^{32}P-labeled GFP siRNAs (Figure 2B, lane 4). Taken together, these results indicate that the production of the siRNAs is a dynamic process involving cleavage and displacement by the dsRNA substrate.

The resistance of the siRNAs to micrococcal nuclease digestion suggested the small RNAs were in a protein complex. This was confirmed upon treatment of the protected siRNAs with proteinase K, as described by Tuschl (Tuschl et al., 1999), which now rendered the siRNAs digestible by micrococcal nuclease (data not shown, but similar to Figure 2C). The same short RNAs were also resistant (Figure 2C) to digestion with Ribonuclease One (RNase One), a single-strand-specific ribonuclease that can cut gaps and single base-pair mismatches (Meador et al., 1990).

siRNAs Require a 3′-Hydroxyl Group to Direct RNAi In Vitro

Using micrococcal nuclease and 500 ng of unlabeled GFP dsRNA, we prepared an RNA fraction enriched for the GFP-specific siRNAs for further testing in RNAi reactions. Addition of this RNA fraction to a silencing reaction did not trigger the degradation of GFP mRNA (Figures 3A and 3B, control versus Mn-siRNAs). The products of RNA digestion with micrococcal nuclease are mononucleotides and oligonucleotides with terminal 3′-phosphate groups (Krupp and Gross, 1979). Tuschl and coworkers have recently shown that the siRNAs derived from dsRNA incubated in *Drosophila* extracts have 5′-monophosphate and 3′-hydroxyl groups (Elbashir et al., 2001b). We speculated that a phosphate group on the 3′-hydroxyl position might inhibit the function of the GFP siRNAs in some aspect of the silencing process. In order to check this, the enriched GFP Mn-siRNAs were treated with calf-intestinal alkaline phosphatase (CIP) to remove any phosphate groups prior to retesting for RNAi (Mossner et al., 1980). Phosphatase treatment restored mRNA target degradation to a level comparable to that observed with 500 ng of full-sized GFP dsRNA (Figures 3A and 3B,control versus CIP-Mn-siRNAs). The slight trimming of the siRNAs during nuclease treatment did not impair their silencing activity and they remained within the 21–25 nucleotide size range, as noted in Figures 2B and 2C. This was a direct

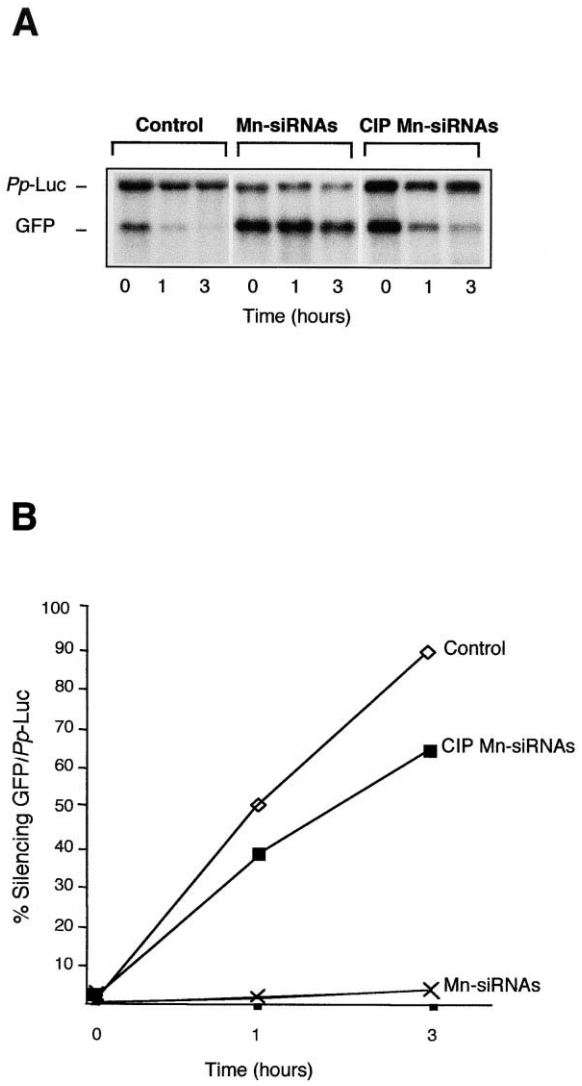

Figure 3. siRNAs Require a 3′-Hydroxyl Group to Function in RNAi

(A) GFP siRNAs produced from 500 ng of full-length GFP dsRNA (716 bp) and treated with micrococcal nuclease trigger efficient RNAi if they are treated with calf-intestinal alkaline phosphatase (CIP). Control, 500 ng GFP dsRNA under standard reaction conditions; Mn-siRNAs, siRNAs derived from 500 ng of GFP dsRNA treated with micrococcal nuclease; CIP Mn-siRNAs, Mn-siRNAs treated with CIP. GFP (746 bp) and *Pp*-Luc (1682 bp) mRNA positions are indicated. (B) Quantification of the GFP/*Pp*-Luc band intensity ratios shown in (A) were measured with the Fuji Phosphoimager.

demonstration that the GFP siRNAs produced in a silencing reaction efficiently target degradation of the corresponding mRNA and that this activity required a 3′ hydroxyl group on the siRNAs.

siRNAs Behave as Primers and Are Incorporated into dsRNA

The requirement for a 3′-hydroxyl group on the GFP siRNAs to degrade the target mRNA indicated that they might serve as primers for an RdRP activity in RNAi. If this was correct, labeled siRNAs would be incorporated into RNA product dependent specifically upon the use of the complementary template RNA. ^{32}P-labeled siRNAs (2.5×10^5 cpm), corresponding to 250 pg of dsRNA, were prepared from full-size GFP and *Pp*-Luc ^{32}P-labeled dsRNAs using the micrococcal nuclease digestion procedure and assayed for template RNA-dependent incorporation into larger RNAs. Full-size GFP (716 bp) and *Pp*-Luc (1652 bp) single strand, double strand, and capped/adenylated RNAs were used as templates. The reaction products were analyzed on denaturing 1.5% agarose formaldehyde gels. Consistent with their role as primers for RdRP activity, the labeled GFP siRNAs were incorporated specifically into full-size RNA for all the templates (Figure 4A). Heterologous RNA/siRNA combinations did not result in any detectable siRNA incorporation (Figure 4A) and neither single- nor double-stranded DNA substrates served as templates for siRNA uptake (data not shown).

siRNAs Are Incorporated into dsRNA that Is Subsequently Degraded

We wanted to determine if the full-length RNAs labeled with siRNAs were double stranded. The reaction products were digested with RNase one and found to be resistant to nuclease digestion under conditions that completely degraded single-stranded GFP RNA transcribed in vitro with T7 RNA polymerase (Figure 4B). The slight sensitivity of the dsRNAs derived from the different RNA templates suggested that some of them might have contained gaps or mismatches susceptible to RNase one digestion. RNase T1, which does not degrade dsRNA but cleaves single stranded RNA 3′ to G residues, also did not degrade the siRNA-labeled RNAs, as shown for GFP mRNA derived dsRNA (Figure 4B). Therefore, the siRNA-abeled RNAs are double stranded.

The siRNA-labeled RNAs could also serve as substrate for cleavage by RNase III-related enzymes. To test this, the full-length GFP dsRNA (shown in Figure 4A) was gel purified and incubated in extract. As shown in Figure 4C, lane 3, the siRNA-labeled RNA was processed to produce new GFP siRNAs.

Given this result, we speculated that longer incubation of the siRNA incorporation reaction would show degradation of the dsRNA and accumulation of the corresponding siRNAs. This was observed when ^{32}P-labeled GFP and *Pp*-Luc siRNAs and the cognate RNA templates were used to produce dsRNA (Figures 5A and 5B). The products were analyzed over a 3 hr period. The full-length dsRNAs appeared rapidly within 2–5 min for both the *Pp*-Luc and GFP templates (Figure 5A, panels 1 and 3) and reached a maximum level within 5–10 min of incubation before the onset of degradation was observed. A longer exposure revealed that the siRNAs were incorporated into shorter products that were degraded during the time course of the reaction (Figure 5A, panels 2 and 4). By the end of the incubation period, more than 90% of the siRNA-labeled dsRNA was degraded while nascent siRNAs continually increased over the time course of the reaction (Figures 5A and 5B). SiRNA levels did not plateau within 15 min, as observed with the addition of labeled dsRNA to the extract, but continued to accumulate (Figure 5B). This result confirmed that the siRNA-labeled RNA was double stranded since single-stranded RNA is not a substrate for RNase III-related

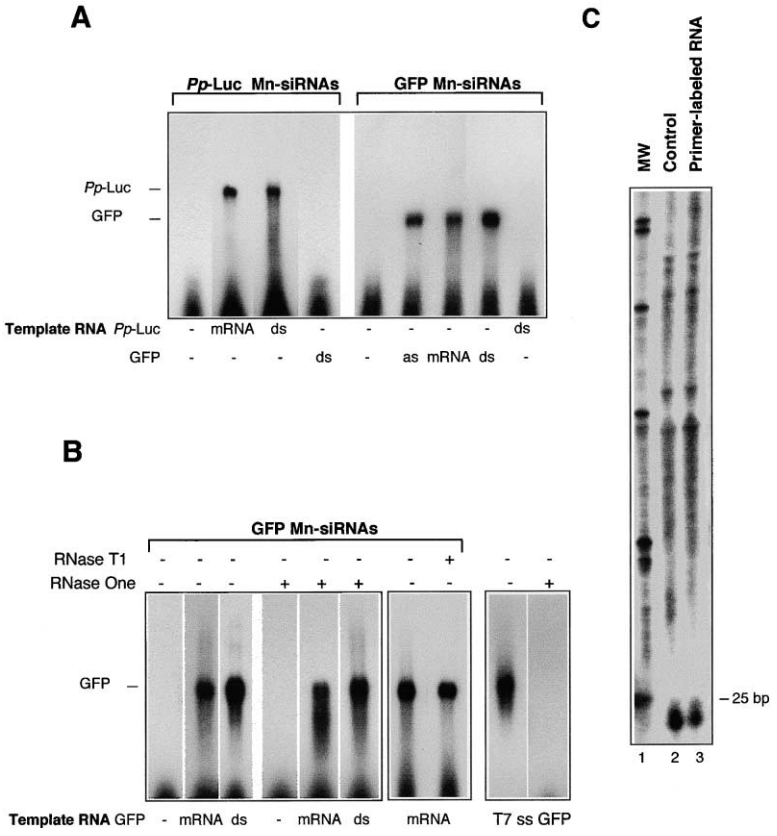

Figure 4. siRNAs Are mRNA-Specific Primers for dsRNA Synthesis

(A) ^{32}P-UTP labeled GFP or *Pp*-Luc Mn-siRNAs, prepared from the full-length dsRNAs by the micrococcal nuclease/CIP method (see Experimental Procedures), were incubated in extract with the cognate and heterologous RNA templates and assayed for incorporation into larger RNAs on 1.5% agarose formaldehyde gels. *Pp*-Luc and GFP siRNAs were incorporated up to the corresponding full-size RNAs only with the homologous single- or double-stranded RNA templates. Incubation of siRNAs in the absence of template or with the heterologous template resulted in no product. Single-stranded antisense RNA (as), double-stranded RNA (ds), and capped-polyA RNA (mRNA) were all templates for primer incorporation. GFP (746 bp) and *Pp*-Luc (1682 bp) mark the positions of the corresponding full-length RNAs.

(B) siRNAs are incorporated into RNA that is double-stranded and resistant to digestion by Ribonuclease One and Ribonuclease T1. siRNAs-labeled GFP RNA produced from either single- or double-stranded templates is resistant to two different nucleases that do not digest dsRNA. T7 ss GFP, single strand control RNA transcribed in vitro with T7 RNA polymerase. The same notation as in (A) is used here.

(C) Full-length siRNA-labeled GFP dsRNA is processed into new siRNAs. Full-length GFP dsRNA, as shown in (A), was gel purified as described in Experimental Procedures and incubated in extract for processing. Lane 1, 25 bp ladder; Lane 2, marked control, processed GFP dsRNA (90,000 cpm input); Lane 3, new GFP siRNAs produced from the full-length GFP RNA labeled by siRNA incorporation (90,000 cpm input).

enzymes and does not produce siRNAs. When GFP dsRNA was used as template for siRNA incorporation, the production of new siRNAs was significantly slower and reached a lower level compared to that seen with mRNA template (Figure 5B, compare dsGFP to ssGFP). This can be explained by the fact that cold dsRNA template competes with the newly labeled dsRNA for the cleavage reaction, as shown in the previous results (Figure 2B, compare lanes 3 and 4). SiRNAs incubated in the absence of template remained at the same position and intensity and did not shift into new products at all time points (Figure 5B, no template).

Therefore, siRNAs are used to convert the target mRNA into dsRNA, which is then cleaved by RNase III activity in the extract to eliminate the target RNA while producing a new set of siRNAs to repeat the process.

A Synthetic 21 nt GFP Duplex siRNA Is Incorporated into Full-Length GFP dsRNA

It has been recently shown that synthetic 21 nt duplex siRNAs can mediate RNAi in both *Drosophila* embryo extracts and in Schneider cells (Elbashir et al., 2001b). We prepared a ^{32}P-labeled 21 nt duplex siRNA corresponding to nucleotides 26–44 in the GFP coding region with two additional uridine residues on the 3′ end of each strand (Figure 6A). This siRNA was tested for template-specific incorporation into full-length GFP dsRNA using sense and antisense GFP and *Pp*-Luc RNAs. Specific incorporation would provide evidence for RdRP activity in the extract. This synthetic duplex siRNA primed the synthesis of essentially full-length GFP dsRNA only when the antisense RNA strand was used as template (Figure 6B). The sense strand template primed the synthesis of the predicted 44 nucleotide fragment corresponding to the number of base pairs from the 3′ end of the siRNA to the GFP AUG start codon at the 5′ end of the template (Figure 6B). *Pp*-Luc templates did not produce any incorporation of the GFP siRNA (Figure 6B). Therefore, both strands of the siRNA were used as primers on the appropriate template strand and were incorporated into dsRNA as a single primer event.

The dsRNA produced with the synthetic GFP duplex siRNA was also synthesized and degraded with kinetics similar to the GFP dsRNA produced with micrococcal nuclease-generated GFP siRNAs (Figure 6D). This result suggests that a single primer in the natural siRNA population would be capable of priming the entire GFP dsRNA. Using the synthetic GFP siRNA, we never saw the intermediate products noted with the natural siRNAs (Figure 6C, panel 2 versus Figure 5A, panels 2 and 4). These intermediate products may represent numerous

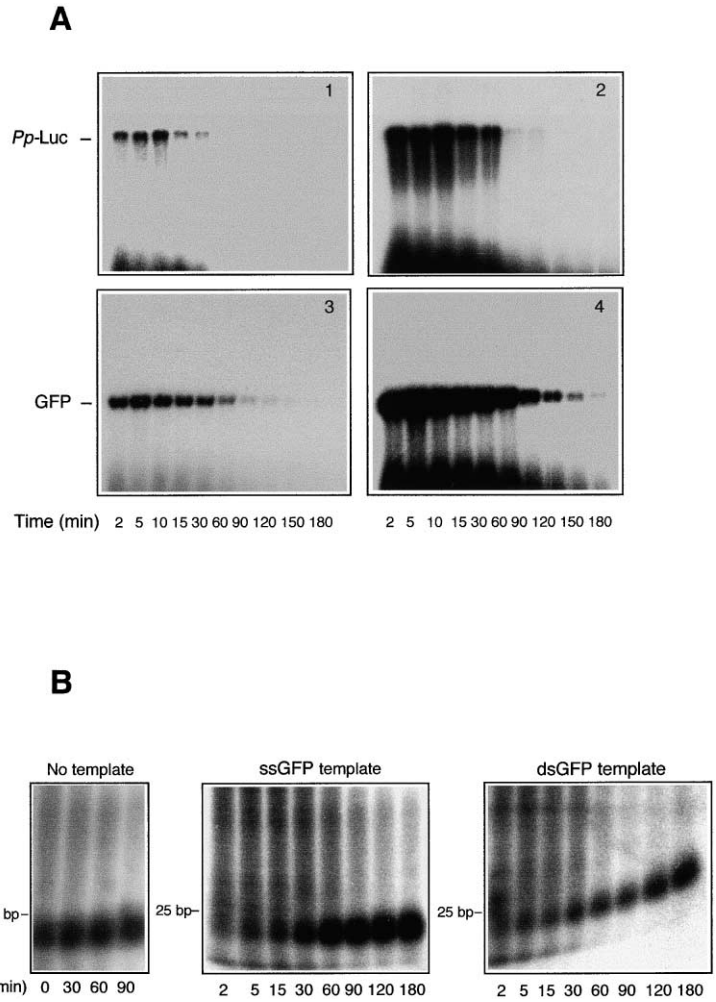

Figure 5. siRNAs Are Incorporated into dsRNA Prior to Degradation and the Formation of New siRNAs

(A) Nascent full-length GFP and *Pp*-Luc dsRNAs labeled by siRNA incorporation are synthesized then degraded upon prolonged incubation in extract. Labeled siRNAs are rapidly incorporated into dsRNAs up to the full-length of the template RNAs. Panels 1 and 3, short exposure (20 min) of *Pp*-Luc and GFP siRNA incorporation into dsRNA showing a maximal labeling rate at 5 to 10 min. Panels 2 and 4, long exposure (2 hr) of panels 1 and 3 to reveal shorter labeled products. The full-length products are essentially absent by 180 min of incubation.

(B) New siRNAs are produced during the degradation of the full-length GFP and *Pp*-Luc dsRNAs labeled by siRNA incorporation. No template, the siRNAs are not incorporated (see Figure 4A, minus template) and remain at a constant level at each time point; ssGFP template, using the reaction shown above in (A) panel 3, new siRNAs are derived from the labeled GFP dsRNA and continue to accumulate during the incubation period; dsGFP, GFP dsRNA was used as a template for siRNA incorporation. New siRNAs accumulate more slowly during the reaction period due to the competition for cleavage by cold GFP dsRNA. Images are from the Fuji Phosphoimager.

single primer extensions from various primer positions along the target RNA.

Efforts to use unlabeled siRNAs and ^{32}P-UTP to follow dsRNA synthesis were unsuccessful due to the high levels of nonspecific UTP incorporation in the extract that were unaffected by α-amanitin and actinomycin D. Nevertheless, the incorporation of the synthetic 21 nucleotide GFP duplex siRNA into dsRNA is consistent with the presence of RdRP in the extract and its role in the siRNA-dependent generation of dsRNA from the mRNA target.

Discussion

Model for RNAi

Our findings complement and clarify the previously proposed models concerning the mechanism of PTGS, quelling, and RNAi (Bass, 2000; Baulcombe, 1999b, 2000; Carthew, 2001; Cogoni and Macino, 2000; Gura, 2000; Hammond et al., 2001b; Hunter, 2000; Kooter et al., 1999; Maine, 2000; Marx, 2000; Meins, 2000; Plasterk and Ketting, 2000; Sharp, 2001; Sijen et al., 2001). Most importantly, we demonstrate the template-specific incorporation of the 21–25 nucleotide RNAs, or siRNAs, to generate dsRNA that is subsequently cleaved by RNase III activity into new siRNAs. In this way, mRNA is degraded through a cycle of "degradative-PCR" (Figure 7). We present substantial evidence for RdRP activity in *Drosophila* extracts and suggest siRNA incorporation into dsRNA involves RdRP, the crucial step in the amplification of the target RNA for rapid degradation by RNase III-type activity. Although we cannot exclude that siRNAs may be incorporated into dsRNA by a direct "guide" mechanism not involving RdRP, such a process would not give the sufficient amplification of the double-stranded RNA target. This would be needed to trigger efficient RNAi with substoichiometric levels of the initiating double-stranded trigger RNA. Consistent with the genetic screens in other lower eukaryotes, our results suggest a role for RdRP in *Drosophila* RNAi as well.

Double-Stranded RNA as the Target for Degradation and the Source for New siRNAs

The requirement for a dsRNA trigger as the effector for silencing can be partially explained by the nature of the dsRNA cleavage step required for siRNA production. As previously noted by Fire and associates (Parrish et al., 2000) and reiterated by Sharp (Sharp, 2001), any factor that significantly alters the double-stranded nature of the dsRNA trigger, such as sequence divergence or

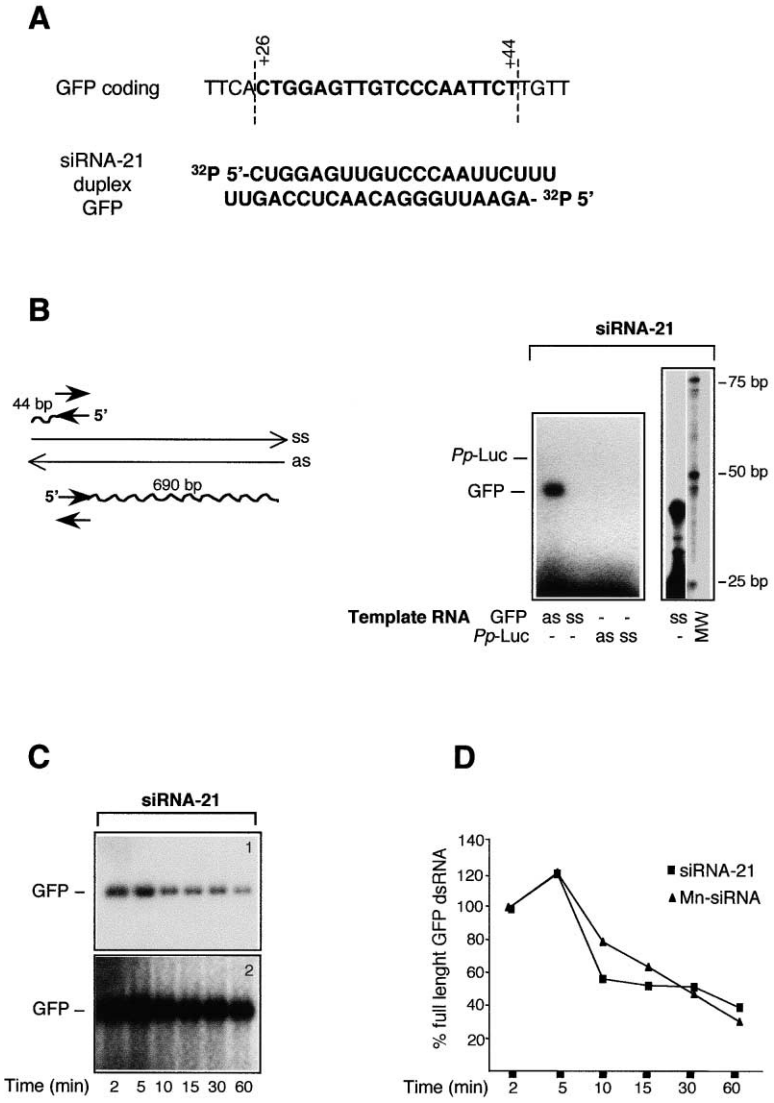

Figure 6. Incorporation of a Synthetic 21 Nucleotide Duplex GFP siRNA into dsRNA

(A) The GFP coding region and the sequence of the synthetic 21 nucleotide duplex GFP siRNA are indicated. The siRNA corresponds to nucleotides 26–44 in the GFP coding region with two additional uridine residues on the 3′ end. The 5′ end of each strand of the siRNA was labeled with [γ-^{32}P]ATP (see Experimental Procedures).

(B) Left, the expected product lengths for incorporation of each strand of the synthetic 21 nucleotide GFP siRNA using full-length GFP sense and antisense RNA templates are shown: 690 bp with the antisense strand template and 44 bp with the sense strand template. Right, the ^{32}P-labeled siRNA incorporation into full-length GFP dsRNA occurs only with the GFP antisense RNA template and gives the expected 44 bp product with the sense strand template. *Pp*-Luc templates give no product. GFP and *Pp*-Luc mark the positions of the full-length RNAs and MW denotes the 25 bp ladder.

(C) GFP dsRNA produced with the synthetic 21 nucleotide duplex GFP siRNA is rapidly synthesized and then degraded upon prolonged incubation in the reaction (Panel 1). Note with longer exposure the absence of intermediate products for the early time points for GFP dsRNA formation (Panel 2). GFP marks the position of the full-length RNA (690bp). Panels 1 and 2 were exposed for 30 min and 2 hr, respectively.

(D) GFP dsRNA produced either with micrococcal nuclease generated GFP siRNAs (Mn-siRNA, data from Figure 5A, GFP) or the synthetic 21 nucleotide duplex GFP siRNA (siRNA-21) is synthesized and degraded with the same kinetics in the incorporation reaction. Note that maximal levels of GFP dsRNA occur at 5 min for both synthetic and natural siRNAs. Data were quantified on the Fuji Phosphoimager.

chemical modification, affects silencing substantially. In the model proposed here, any changes in strand complementarity could presumably reduce the susceptibility of the triggering dsRNA to RNase III-type cleavage. The nature of the sense and antisense strands in the triggering dsRNA would also play a role in the efficacy of silencing since the RdRP amplification step would depend upon the production and functionality of the siRNAs. We show that both strands of a synthetic 21 nucleotide GFP duplex siRNA function as primers to give the expected RNA products when the appropriate GFP template strand is used (Figure 6).

The length of the siRNAs may be an important aspect of their function. Previous reports indicated that 29–36 nucleotide dsRNAs transcribed in vitro do not direct RNAi efficiently in *Drosophila* extracts (Elbashir et al., 2001b), and that a 26 nucleotide dsRNA, also transcribed in vitro, when injected into worms, triggered lower than expected levels of RNA interference at 25°C and none at 16°C (Parrish et al., 2000). However, chemically synthesized 21 and 22 nucleotide siRNAs can mediate targeted RNA cleavage in *Drosophila* embryo extracts and in Schneider cells (Elbashir et al., 2001a, 2001b). The siRNAs produced in *Drosophila* embryo extract by micrococcal nuclease and CIP treatment are essentially as efficient on a weight basis in RNAi as the full-length dsRNA from which they were derived, suggesting there is some optimal length for siRNAs in RNAi. The conservation in the size range for the small RNAs associated with silencing in all the species examined proposes that it may be closely correlated with primer function. This could be due to some unique property of primer activity in a protein complex that has yet to be identified.

We demonstrate that siRNAs require a 3′ hydroxyl group for function in RNAi. The authentic siRNAs, produced in *Drosophila* extracts by RNase III-related enzymes such as Dicer (Bernstein et al., 2001), have been chemically characterized and shown to have a 5′ phosphate and a 3′ hydroxyl group (Elbashir et al., 2001b). The micrococcal nuclease generated siRNAs described here are functional in RNAi and can be incorporated into dsRNA after phosphatase treatment (Figure 3) to remove the 3′ phosphate group produced by the nuclease diges-

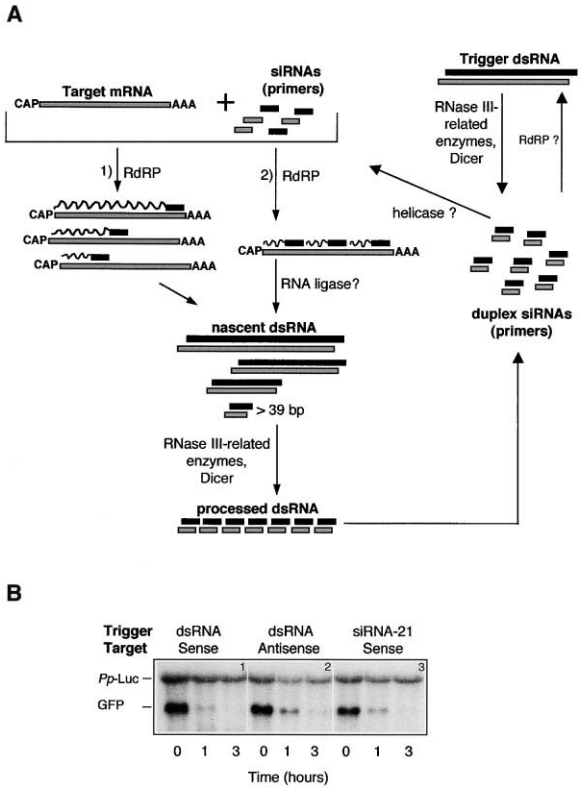

Figure 7. A Model for RNAi

(A) RNAi is initiated by a threshold concentration of the trigger dsRNA, which is immediately cleaved by RNase III-related enzymes, such as Dicer, to generate duplex siRNAs. The siRNAs are proposed to interact with the target mRNA as primers to form dsRNA in the following ways: (1) siRNA primers align randomly along the template strand, one per template, and are extended by RdRP to form dsRNA; and (2) multiple siRNA primers align along the template strand and are extended by RdRP then ligated by a putative RNA ligase. If the siRNAs were "guide" RNAs, they would only be ligated along the template strand by RNA ligase to form larger dsRNA. However, a "guide" mechanism for primer incorporation could not amplify the target dsRNA sufficiently when substiochiometric levels of dsRNA are used to initiate RNAi, so this mechanism is not considered likely. Importantly, unlike RdRP, there is no genetic evidence for the involvement of RNA ligase in RNAi. Even though dsRNA can direct siRNA incorporation in vitro, this may play a minor role in the overall RNAi process and the potential amplification of the trigger dsRNA since dsRNA is rapidly degraded. Genetic screens also suggest a role for helicase, which may be involved in unwinding the siRNAs for dsRNA synthesis. Ultimately, the nascent dsRNA greater than 39 bp in length is then cleaved by RNase III-related enzymes and this step generates new siRNAs to amplify mRNA degradation. It is not clear what fraction of the dsRNA generates siRNAs. This cycle is repeated with the new siRNAs until the target mRNA is degraded. (B) The model predicts that the GFP dsRNA trigger RNA should target both sense and antisense GFP transcripts, and that the GFP siRNA primer used in Figure 6 should direct GFP mRNA degradation since it generates dsRNA greater than 39 bp. This is what is observed using either 1 μg of GFP dsRNA or 1 μg GFP siRNA-21 to target GFP sense or antisense mRNA: Panel 1, GFP dsRNA with GFP mRNA; Panel 2, GFP dsRNA with GFP antisense mRNA; and Panel 3, GFP siRNA-21 with GFP mRNA.

tion. The 21 nucleotide synthetic siRNA primer (Figure 6) also has a 3′ hydroxyl group that would be required for incorporation into dsRNA by RdRP activity. Whether or not the 3′ hydroxyl group is also used in a primer ligation step remains to be determined.

In agreement with the results reported using the synthetic siRNAs hybridized to RNA (Elbashir et al., 2001b), we show that cleavage of the GFP target RNA occurs after the synthetic GFP siRNA is incorporated into dsRNA. If cleavage occurred in the template RNA immediately upon binding to the synthetic GFP siRNA, no full-length GFP dsRNA would have been observed. Therefore, cleavage occurs in the nascent dsRNA in regions inside and outside the zone represented by the initial siRNA since the primers are extended to make dsRNA. The fact that the synthetic GFP siRNA is extended to the 5′ end of the sense strand template (Figure 6B) would also restrict cleavage, in this instance, to the region upstream of the 3′ terminus of the siRNA. Any region of the target RNA converted into duplex by a given siRNA would be subject to digestion by RNase III activity.

A Role for RdRP in *Drosophila* RNAi

It has been proposed previously that there is no amplification of the trigger dsRNA in RNAi in *C. elegans* (Sharp, 2001), based upon the effects of asymmetric strand substitutions in the input dsRNA (Parrish et al., 2000). We provide evidence that both single- and double-stranded RNAs can serve as templates for siRNA incorporation into dsRNA in *Drosophila* extract. However, the rapid degradation of dsRNA suggests that amplification of the trigger dsRNA is of limited value. In the substitution experiments described by Fire et al. (Parrish et al., 2000), modifications on the sense strand had no effect, but the same substitutions on the antisense strand rendered the modified dsRNA inactive in RNAi when injected into worms. Since any amplification of the modified dsRNA would dilute out the substitution effects, it was concluded that the target dsRNA was not amplified (Sharp, 2001). Our results also indicate that the antisense siRNA strand would be the most important for the synthesis of new dsRNA from the mRNA template (Figure 6).

Genetic studies have identified several mutants in *C. elegans*, *Neurospora crassa*, and *Arabidopsis thaliana* that resist RNA interference. These include mutants that affect the initiation of silencing activity (rde-*1* and *rde-4* in *C. elegans* [Tabara et al., 1999], *qde-2* in *Neurospora* [Catalanotto et al., 2000], and *AGO-1* in *Arabidopsis* [Fagard et al., 2000]), mutants in the effectors of silencing (*rde-2* and *mut-7* in *C. elegans* [Ketting et al., 1999], the later related to RNase D), mutants in helicase (*qde-3* in *Neurospora* [Cogoni and Macino, 1999b], and *SDE-3* in *Arabidopsis* [Dalmay et al., 2001]), and mutants in RNA-dependent RNA polymerase (*ego-1* in *C. elegans* [Smardon et al., 2000], *qde-1* in *Neurospora* [Cogoni and Macino, 1999a], and SGS-2/*SDE-1* in *Arabidopsis* [Dalmay et al., 2000; Mourrain et al., 2000]). Although by sequence comparison an RdRP homolog in *Drosophila* has not yet been identified, our results suggest the presence of an RdRP gene.

RdRP-dependent as well as -independent mechanisms may be involved in the generation of dsRNA up to the full-length of the target RNA, according to one of the following schemes: (1) a single siRNA primer would be extended from various positions along individual template strands by RdRP to generate dsRNAs; and (2) different siRNAs would associate along a single template RNA and be extended by RdRP to the adjacent

siRNA primer. The extension products would be ligated to generate dsRNAs. This model would require RdRP activity as well as an RNA ligase step; and (3) dsRNAs would be formed by a primer "guide" mechanism where they would align along the template for subsequent ligation. All these mechanisms could generate dsRNA of sufficient length to be cleaved by RNase III-type activity since this requires a minimum of 39 base pairs (Elbashir et al., 2001b). Our results favor the first and second models since RdRP activity would be required to amplify the target dsRNA sufficiently when substiochiometric amounts of the trigger dsRNA are involved in initiating RNAi (Figure 7). A primer "guide" mechanism would require the involvement of an RNA ligase in order to generate larger dsRNAs, and genetic screens have not identified related genes as candidates essential for RNAi. As previously noted, RdRP genes have been shown to be involved in posttranscriptional gene silencing in three different lower eukaryotes. In addition, the "guide" primer ligation model is not supported by observations using synthetic siRNAs. siRNAs generated from dsRNAs greater than 111 nucleotides in length are not well defined and are derived from several overlapping regions of different lengths (18–24 nucleotides) to make the siRNA population heterogeneous in composition (Elbashir et al., 2001b). It is unlikely that a "guide" mechanism could sort out the precise siRNAs for ligation along the target RNA to rapidly generate the full-length dsRNA. The most convincing evidence for the involvement of RdRP activity in *Drosophila* RNAi comes from our results using the synthetic 21 nucleotide GFP duplex siRNA where full-length GFP dsRNA was produced from a single primer (Figure 6) with the same time course of synthesis and degradation as dsRNA produced using the micrococcal-nuclease-generated GFP siRNAs (Figure 6D). The extension of both strands of the synthetic siRNA in a template-dependent manner to yield the expected dsRNA products (Figure 6B) would specifically require an RdRP. The role for helicase activity in RNAi, as shown for *qde-3* in *Neurospora* (Cogoni and Macino, 1999b) and *SDE-3* in *Arabidopsis* (Dalmay et al., 2001) in the genetic screens, may be to unwind the primers or the dsRNA trigger, but this remains to be demonstrated.

Predictions from the Degradative PCR Model

Double-stranded RNA is processed into siRNA primers that convert the target mRNA into dsRNA for subsequent degradation and the formation of new siRNAs. Since the siRNA primers are double stranded, they should direct the degradation of either sense or antisense cognate target RNAs. This is exactly what is observed when either sense or antisense GFP mRNA is incubated in extract with GFP dsRNA (Figure 7B). Therefore, dsRNA representing transcripts derived from opposite strands of a complementary template would be targeted simultaneously to effect silencing of more than a single gene in some instances. The siRNA primer model also suggests a single siRNA should target transcript degradation as long as the primer extended product is of sufficient length to be cleaved by RNase III activity, roughly 39 nucleotides. When the siRNA-21 shown in Figure 6 is used in a silencing assay, GFP mRNA is selectively degraded, as predicted, since the extended primer produces dsRNA 44 bp long (Figure 7B). Therefore, a single primer is sufficient to target mRNA silencing. This result also predicts that a primer producing the longest dsRNA product would be the most efficient since the second generation of siRNAs would represent more of the target RNA.

Experimental Procedures

Drosophila Embryo Extracts and RNAi Assay

Drosophila embryo extracts were prepared and subfractionated using the procedures previously described by Tuschl et al. (1999) and Hammond et al. (2000). Aliquots of all extract preparations were stored at −80°C. In vitro RNAi reactions were performed as described by Tuschl with 10–50 pM (final concentration) ^{32}P-UTP-labeled capped and adenylated target and control mRNAs and 10 nM unlabeled dsRNA as the trigger RNA unless otherwise noted. Reaction products were analyzed on 1.5% formaldehyde gels and processed as previously described (Tuschl et al., 1999).

RNA Synthesis

Unlabeled and labeled RNAs were prepared using one of the following Ambion kits, as recommended by the company. After synthesis, the reactions were phenol extracted and precipitated with isopropanol as recommended. For unlabeled dsRNA, the MegaScript T3 and T7 kits were used. For labeled dsRNA, the T3 and T7 MaxiScript kits were used with [^{32}P]UTP (800 Ci/mmole) as the only source of UTP. For capped and adenylated labeled mRNA, the SP6 Message Machine Kit was used with 40 μCi UTP (Redivue 3000 Ci/mmole) and the appropriate GFP (716 bp) and *Pp*-Luc (1652 bp) plasmids. Where indicated, the RNAs were annealed, as previously described by (Misquitta and Paterson (1999) and Tuschl et al. (1999) and digested with RNase one to eliminate single strand contamination.

Analysis of dsRNA Processing

High specific activity GFP dsRNA ($>1 \times 10^9$ cpm/μg) was incubated for various times, up to 3 hr, at the indicated concentrations in *Drosophila* extract, as described for RNAi assays. After treatment, samples were prepared for analysis on 6% sequencing gels, as previously noted (Zamore et al., 2000). A 25 bp DNA ladder was used as a molecular weight marker and was labeled with [α-^{32}P]dCTP according to the manufacturer (Life Sciences). In order to process siRNA-labeled GFP dsRNA, the RNA was fractionated on a 4% mini DNA-sequencing gel, the band of full-length dsRNA was excised, and the material electro-eluted. The eluted material was phenol extracted and precipitated with 20 μg of glycogen carrier, dissolved in TEN (10 mM Tris [pH 7.5], 0.1 mM EDTA, and 20 mM NaCl), annealed, and processed for siRNA production in a 30 min incubation as described above.

Preparation of siRNAs

For preparative amounts of siRNAs, 500 ng to 10 μg of unlabeled dsRNA was added to 100 μl of S100 extract, prepared from embryo lysate according to Hammond et al. (2000), and adjusted to a final concentration of 100 mM potassium acetate, 30 mM HEPES (pH 7.4), 2 mM Mg acetate, 5 mM DDT, 10 mM creatine phosphate, 1μg/ml creatine kinase, 500 μM ATP, and 500 μM α-amanatin and incubated 30 min at 25°C. After processing, micrococcal nuclease (Roche) was added to a final concentration of 60 U/50 μl of reaction in the presence of 2 mM $CaCl_2$ and incubated for 30 min at 37°C. This step was repeated and the reaction was stopped by the addition of EGTA to a final concentration of 10 mM. The siRNA preparation was digested with 100 μg/ml proteinase K for 15 min at 65°C in 100 mM Tris-HCl (pH 7.5), 12.5 mM EDTA, 150 mM NaCl, and 1% SDS, phenol extracted and precipitated with ethanol using 40 μg of glycogen as carrier (Tuschl et al., 1999). The precipitated material was dissolved in 50 μl of water. Ten microliters of the siRNAs were treated with 20 U of calf-intestinal phosphatase in New England Biolabs buffer 3 in a final volume of 50 μl for 1 hr at 37°C. The sample was phenol extracted and precipitated with ethanol. The material was dissolved in 10 μl of water for further use. For the preparation of very high specific activity siRNAs, RNA was transcribed in vitro (see below) using [^{32}P]UTP (800 Ci/mmole) as the only source of UTP to give dsRNAs with specific activities $>10^9$

cpm /μg. Typically, 1 μg of labeled dsRNA was processed as above to generate highly labeled siRNAs.

Two synthetic 21 nucleotide RNAs were synthesized by the Yale Keck Labs (trityl-off) representing nucleotides 26–44 in the GFP coding region and containing two additional U residues on the 3′ end: RNA-1, CUGGAGUUGUCCCAAUUCUUU and RNA-2, UUGAC CUCAACAGGGUUAAGA. To label the 21 nucleotide RNAs, 0.1 μg of each RNA was labeled in a 10 μl reaction using T4 polynucleotide kinase (NEB) with 3 μl of [γ-^{32}P]ATP (crude, ICN 6000 Ci/mmol) and passed over a G-25 spin column in 50 μl final volume (preadjusted with TE) to yield a specific activity of $>10^9$ cpm/μg for each 21 nucleotide RNA. Kinase was inactivated by incubation at 70°C for 10 min after the addtion of 1 μl of 0.5 M EDTA, as recommended by the manufacturer. Twenty microliters of each labeled synthetic RNA was annealed in a 50 μl reaction to form the siRNA as described (Elbashir et al., 2001b). Unlabeled siRNAs were prepared by annealing 7.5 μg of each RNA in 50 μl as previously noted.

SiRNA Incorporation Assay

S100 fraction, prepared from embryo extract, was used instead of crude extract under standard RNAi reaction conditions with 500 μM α-amanitin, 2.5×10^5 cpm of ^{32}P-UTP labeled siRNAs, and 3 μg of RNA template (either double- or single-stranded RNA), incubated at 25°C for 60 min or the indicated times for the time course reactions. The primer incorporation reaction was stopped with proteinase K/SDS buffer and extracted as before (Tuschl et al., 1999). Labeled RNA products were analyzed on 1.5% agarose formaldehyde gels or 6% DNA sequencing gels. For incorporation reactions using the 21 nucleotide synthetic duplex siRNA, 1 μg of unlabeled siRNA was mixed with 3×10^6 cpm of labeled siRNA along with 3 μg of template and incubated in extract as described above.

Ribonuclease One and Ribonuclease T1 Digestions

Digestions of single- and double-stranded RNAs as well as the products of the siRNA incorporation reactions were carried out using Ribonuclease One (Promega, 10 μU/3 μg of cold template) for up to 5 min at 37°C in buffer supplied by the manufacturer. Ribonuclease One digests RNA to nucleotide monophosphates so gel purification of dsRNA was not required. Reactions were then phenol extracted as before and analyzed on 1.5% agarose formaldehyde gels. For Ribonuclease T1 (Roche) digestion, identical RNA samples were dissolved in 50 μl of TE (10 mM Tris [pH 7.5] and 0.1 mM EDTA) and digested with 100–500 units of enzyme for 30 min at 37°C. The reactions were phenol extracted and precipitated with 20 μg of glycogen carrier prior to gel analysis.

Image Analysis

Radioactivity was detected by exposing the dried agarose gel (dried under vacuum) onto an Immobilon-NY+ membrane or sequence gel directly to Kodak AR-2 film at −80°C with a Dupont screen or a Fuji Image plate, the latter quantified with a Fujix Bas 2000 using Image Gauge 3.0 software.

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