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Evidence that siRNAs Function as Guides, Not Primers, in the *Drosophila* and Human RNAi Pathways

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Summary

In *Drosophila*, two features of small interfering RNA (siRNA) structure—5′ phosphates and 3′ hydroxyls—are reported to be essential for RNA interference (RNAi). Here, we show that as in *Drosophila*, a 5′ phosphate is required for siRNA function in human HeLa cells. In contrast, we find no evidence in flies or humans for a role in RNAi for the siRNA 3′ hydroxyl group. Our in vitro data suggest that in both flies and mammals, each siRNA guides endonucleolytic cleavage of the target RNA at a single site. We conclude that the underlying mechanism of RNAi is conserved between flies and mammals and that RNA-dependent RNA polymerases are not required for RNAi in these organisms.

Introduction

In diverse eukaryotes, double-stranded RNA (dsRNA) triggers the destruction of mRNA sharing sequence with the double strand (Hutvágner and Zamore, 2002b; Hannon, 2002). In animals and basal eukaryotes, this process is called RNA interference (RNAi) (Fire et al., 1998). There is now wide agreement that RNAi is initiated by the conversion of dsRNA into 21–23 nt fragments by the multidomain RNase III enzyme, Dicer (Billy et al., 2001; Bernstein et al., 2001; Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001; Martens et al., 2002). These short RNAs are known as small interfering RNAs (siRNAs), and they direct the degradation of target RNAs complementary to the siRNA sequence (Zamore et al., 2000; Elbashir et al., 2001c, 2001b, 2001a; Nykänen et al., 2001; Elbashir et al., 2002). In addition to its role in initiating RNAi, Dicer also cleaves ~70 nt precursor RNA stem-loop structures into single-stranded 21–23 nt RNAs known as microRNAs (miRNAs; Hutvágner et al., 2001; Grishok et al., 2001; Ketting et al., 2001; Reinhart et al., 2002). Like siRNAs, miRNAs bear 5′ monophosphate and 3′ hydroxyl groups, the signatures of RNase III cleavage products (Hutvágner et al., 2001; Elbashir et al., 2001b). miRNAs are hypothesized to function in animals as translational repressors (Lee et al., 1993; Wightman et al., 1993; Ha et al., 1996; Moss et al., 1997; Olsen and Ambros, 1999; Reinhart et al., 2000; Zeng et al., 2002; Seggerson et al., 2002). The conversion of dsRNA into siRNAs requires additional protein cofactors that may recruit the dsRNA to Dicer or stabilize the siRNA products (Tabara et al., 1999; Hammond et al., 2001; Grishok et al., 2001; Tabara et al., 2002). How siRNAs direct target cleavage and whether a single mechanism explains the function of siRNAs in posttranscriptional gene silencing in plants, quelling in fungi, and RNAi in animals remain unknown. Furthermore, how siRNAs are permitted to enter the RNAi pathway while other 21–23 nt RNAs seem to be excluded cannot yet be fully explained.

Three models have been proposed for RNAi in *Drosophila*. Each model seeks to explain the mechanism by which siRNAs direct target RNA destruction. In one model (Figure 1), target destruction requires an RNA-dependent RNA polymerase (RdRP) to convert the target mRNA into dsRNA (Lipardi et al., 2001). The RdRP is hypothesized to use single-stranded siRNAs as primers for the target RNA-templated synthesis of complementary RNA (cRNA). The resulting cRNA/target RNA hybrid is proposed to then be cleaved by Dicer, destroying the mRNA and generating new siRNAs in the process. Key features of this model are that the ATP-dependent, dsRNA-specific endonuclease Dicer acts twice in the RNAi pathway, that target destruction should require nucleotide triphosphates to support the production of cRNA, and that a 3′ hydroxyl group is essential for siRNA function, since siRNAs are proposed to serve as primers for new RNA synthesis.

A second model proposes that single-stranded siRNAs do not act as primers for an RdRP, but instead assemble along the length of the target RNA and are then ligated together by an RNA ligase to generate cRNA (Lipardi et al., 2001; Nishikura, 2001). The cRNA/target RNA hybrid would then be destroyed by Dicer. This model predicts that target recognition and destruction should require ATP (or perhaps an NAD-derived high-energy cofactor) to catalyze ligation, as well as to support Dicer cleavage. Like the first model, the ligation hypothesis predicts that an siRNA 3′ hydroxyl group should be required for RNAi. Furthermore, a 5′ phosphate should be required for siRNA ligation, but ribonucleotide triphosphates other than ATP should not be required for target destruction.

A third model (Figure 1) hypothesizes that two distinct enzymes or enzyme complexes act in the RNAi pathway (Hammond et al., 2000; Zamore et al., 2000; Nykänen et al., 2001). As in the first model, Dicer is proposed to generate siRNAs from dsRNA. These siRNAs are then incorporated into a second enzyme complex, the RNA-induced silencing complex (RISC), in an ATP-dependent step or series of steps during which the siRNA duplex is unwound into single strands. The resulting single-stranded siRNA is proposed to guide the RISC to recognize and cleave the target RNA in a step or series of steps requiring no nucleotide cofactors whatsoever. The absence of a nucleotide triphosphate requirement for target recognition and cleavage is a key feature of this model.

We have previously demonstrated by two different experimental protocols that both recognition and endonucleolytic cleavage of a target RNA proceeds efficiently in the presence of less than 50 nM ATP, a concentration

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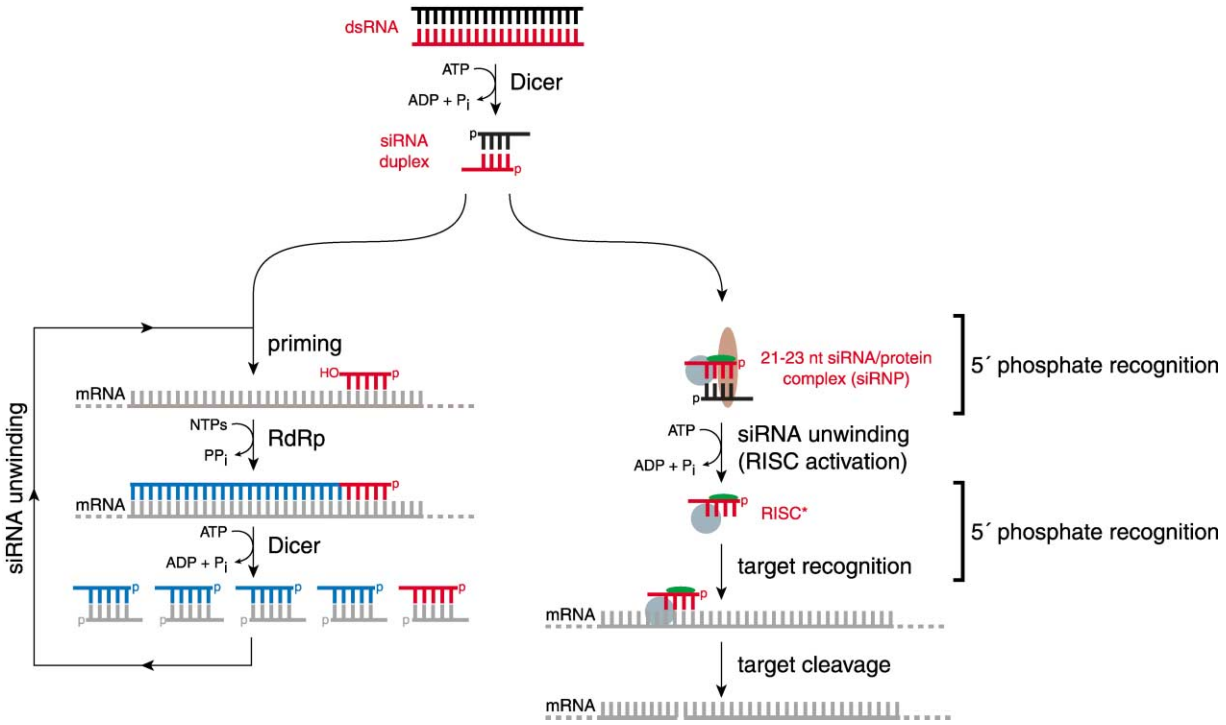


Figure 1. Two Models Proposed for the RNAi Pathway in *Drosophila*

Both models postulate that dsRNA is converted to siRNA by the ATP-dependent endoribonuclease Dicer, but the models differ as to the subsequent function of siRNAs. In the "random degradative PCR" model (at left), siRNAs are postulated to function as primers for the target RNA-templated synthesis of cRNA by an RdRP. The resulting dsRNA is then proposed to be cleaved by Dicer into a new crop of siRNAs, which can prime the conversion of additional target RNAs into dsRNA. In the endonucleolytic cleavage model for RNAi (at right), siRNAs are proposed to be incorporated into an endonuclease complex distinct from Dicer, the RISC. Assembly of the RISC is proposed to be ATP dependent, whereas endonucleolytic cleavage of the target RNA is postulated to require no high-energy cofactors.

likely to be insufficient to support either the synthesis of new RNA or the ligation of multiple siRNAs into cRNA (Nykänen et al., 2001). However, our data also revealed an absolute requirement for a 5′ phosphate for siRNAs to direct target RNA cleavage in *Drosophila* embryo lysates, a finding we interpreted as reflecting an authentication step in the assembly of the RNAi-enzyme complex, the RISC. We envisioned that the 5′ phosphate was involved in obligatory noncovalent interactions with one or more protein components of the RNAi pathway. Nonetheless, the 5′ phosphate requirement might formally reflect a requirement for the phosphate group in covalent interactions, such as the ligation of multiple siRNAs to generate cRNA (Nishikura, 2001).

Here, we more fully define the mechanism of RNAi in flies and mammals by examining the requirement for a 5′ phosphate and a 3′ hydroxyl group on the antisense strand of the siRNA duplex. First, we analyze the role of these functional groups in siRNA function in vitro, using both *Drosophila* and human cell-free systems that recapitulate siRNA-directed target RNA destruction. Then, we validate our findings in vivo in human HeLa cells. Our data support a model for the RNAi pathway in which siRNAs function as guides for an endonuclease complex that mediates target RNA destruction. We find that the requirement for a 5′ phosphate is conserved between *Drosophila* and human cells and that an siRNA 3′ hydroxyl is dispensable in both systems. Our data argue against an obligatory role for an RdRP in *Drosophila* or human RNAi, despite the clear requirement for such enzymes in PTGS in plants, quelling in *Neurospora crassa*, and RNAi in *C. elegans* and *Dictyostelium discoideum* (Cogoni and Macino, 1999; Smardon et al., 2000; Dalmay et al., 2000; Mourrain et al., 2000; Sijen et al., 2001; Martens et al., 2002). In this respect, the mechanism of RNAi in flies and mammals appears to be distinct from that of PTGS, quelling, and RNAi in worms and *Dictyostelium*, suggesting that the pathway in flies and mammals may be more restricted in the range of triggers that can elicit an RNAi response.

Results and Discussion

Requirement for the siRNA 5′ Phosphate in Human RNAi

Synthetic siRNAs bearing a 5′ hydroxyl can efficiently mediate RNAi both in vitro in *Drosophila* embryo lysates and in vivo in cultured human cells (Elbashir et al., 2001b, 2001a; Nykänen et al., 2001). However, in the *Drosophila* in vitro system, an endogenous kinase rapidly converts the 5′ hydroxyl group to a phosphate (Nykänen et al., 2001). Blocking siRNA phosphorylation by substituting the 5′ hydroxyl with a methoxy moiety completely blocks RNAi in *Drosophila* embryo lysates (Nykänen et al., 2001). Furthermore, 5′ phosphorylated siRNAs more efficiently trigger RNAi in vivo in *Drosophila* embryos than

do 5′ hydroxyl-containing siRNAs (Boutla et al., 2001). 5′ hydroxyl-containing, synthetic siRNAs that trigger RNAi in cultured mammalian cells (Elbashir et al., 2001a, 2002), in mice (McCaffrey et al., 2002; Lewis et al., 2002), and perhaps even in plants (Klahre et al., 2002) may likewise be phosphorylated by a cellular kinase prior to entering the RNAi pathway.

To determine if a 5′ phosphate is required for RNAi in mammals, we first analyzed mammalian RNAi in vitro, using HeLa cell S100 extract. These reactions accurately recapitulate the known features of siRNA-directed RNAi in mammalian cell culture: exquisite sequence specificity (Elbashir et al., 2001a) and target RNA cleavage (Holen et al., 2002). RNAi reactions were performed in HeLa S100 extracts using siRNA duplexes in which the antisense strand, which we refer to as the guide strand, contained either a 5′ hydroxyl or a 5′ methoxy group (Figure 2A) and a chimeric target RNA in which nucleotides 62 to 81 were complementary to the siRNA (Figure 2B). When the guide strand of the siRNA duplex contained a 5′ hydroxyl group, and could, therefore, be phosphorylated, it directed cleavage of the target RNA within the sequence complementary to the siRNA (Figure 3). Target cleavage directed by this siRNA occurred at the same site in the HeLa S100 as in *Drosophila* embryo lysate. These data suggest that endonucleolytic cleavage of the target RNA is a common feature of RNAi in flies and mammals. siRNAs with a 5′ methoxy group cannot be phosphorylated by nucleic acid kinases and cannot direct RNAi in lysates of *Drosophila* embryos (Nykänen et al., 2001). Such siRNAs were likewise unable to direct cleavage of the target RNA in the HeLa S100 reaction (Figure 3A). Although the exogenous, methoxy-blocked siRNA did not trigger sequence-specific target cleavage, an endogenous HeLa RISC complex that contains the miRNA *let-7* (Hutvágner and Zamore, 2002a) cleaved the chimeric target RNA within the *let-7* complementary sequence near its 3′ end (Figure 2B) in all of the human in vitro RNAi reactions. This diagnostic 5′ cleavage product (indicated by an asterisk) serves as an internal control for these and subsequent in vitro HeLa S100 reactions. Our data suggest that mammalian RNAi, like RNAi in *Drosophila* (Nykänen et al., 2001; Boutla et al., 2001), requires the siRNA 5′ phosphate for target cleavage and suggest that 5′ hydroxyl-containing siRNA duplexes must be phosphorylated by a cellular kinase before they become competent to mediate RNAi in human cells. Consistent with this idea, 5′ hydroxyl-containing siRNAs are rapidly 5′ phosphorylated after only 5 min incubation in the HeLa S100 (Figure 3B). Thus, like *Drosophila*, human cells contain a nucleic acid kinase that can add a 5′ phosphate to a synthetic siRNA.

Role of the siRNA 3′ Hydroxyl Group in Flies and Mammals

Both siRNAs produced by enzymatic cleavage of dsRNA and those prepared by chemical synthesis contain 3′ hydroxyl termini (Elbashir et al., 2001b). Experiments using nuclease-treated siRNAs suggested that a 3′ phosphate blocks RNAi in *Drosophila* embryo lysates (Lipardi et al., 2001), a finding consistent with authentication of siRNA 3′ structure by the RNAi machinery, with siRNAs acting as primers for cRNA synthesis, or with RNA-templated ligation of multiple siRNAs into cRNA. To determine if the siRNA 3′ hydroxyl group plays an essential role in RNAi, we synthesized two siRNAs in which the 3′ hydroxyl group of the guide strand was blocked (Figure 2). In one siRNA, the 3′ hydroxyl was replaced by a 2′,3′ dideoxy terminus. In the other, the 3′ position contained 3-amino-propyl phosphoester (3′ "amino modifier"). Each of the blocked siRNA guide strands was analyzed by electrospray mass spectrometry to confirm its identity and purity. The two modified siRNA guide strands, as well as a 3′ hydroxyl-containing control strand, were annealed to a standard 21 nt siRNA sense strand. The three resulting siRNA duplexes were tested for their ability to direct cleavage of a complementary target RNA in an in vitro RNAi reaction containing *Drosophila* embryo lysate. Figure 4A shows that the two 3′-blocked siRNAs produced the same degree of target cleavage as the 3′ hydroxyl-containing siRNA control.

Next, we repeated the experiment in HeLa S100 extract to determine if an siRNA 3′ hydroxyl group is required for RNAi in mammalian cells. 3′ modification of an siRNA has been reported to be permitted for RNAi in mammalian cells (Holen et al., 2002), but it was not shown in those experiments that all of the siRNA was 3′ modified. In contrast to the 5′ methoxy modification, which completely blocked target RNA cleavage in the HeLa S100 reaction, 3′ modification had no effect on the efficiency or specificity of RNAi (Figure 4B). The identity and purity of these siRNAs was confirmed by electrospray mass spectrometry. However, we could envision that a fraction of the siRNA guide strand was cleaved within the single-stranded, two nucleotide, 3′ overhang by a nuclease in the HeLa S100, regenerating the 3′ hydroxyl. If this occurred, the cleaved siRNAs could then act as primers. To exclude this possibility, we performed RNAi reactions using progressively shorter guide siRNAs blocked at the 3′ end by either a 2′,3′ dideoxy or a 3′ amino modifier group. The 20 or 19 nt guide strands were annealed to the same 21 nt sense siRNA strand. Figure 4B shows that target RNA cleavage occurred in all cases, although the efficiency of cleavage decreased as the siRNA guide strand was shortened, even when it contained a 3′ hydroxyl terminus. If the 3′ blocked 21 nt siRNA was active because it had been shortened to a 20-mer, it could not have attained the activity of the 3′ hydroxyl 21 nt siRNA. Similarly, if nucleolytic removal of the 3′ block accounted for the activity of the 20 nt guide siRNA, it should have only been as active as the 19 nt, 3′ hydroxyl-containing siRNA. These results suggest that the 3′ hydroxyl group of the siRNA guide strand does not play an obligatory role in siRNA-directed RNAi in flies or mammals.

Single-Stranded siRNAs

All current models for RNAi—including those that propose siRNA to function as guides for an endonuclease and models that propose siRNAs to act as primers for target-RNA templated RNA synthesis—predict that siRNAs ultimately function as single strands. In fact, in *Drosophila* embryos, single-stranded antisense siRNAs corresponding to the *Notch* mRNA elicited *Notch* phenotypes in 12% of injected embryos, although the expres-

A

guide strand length (nt)	guide strand 5′ end	guide strand 3′ end	siRNA sequence
21	OH	OH	5′-UCGAAGUAUUCCGCGUACGUG-3′ 3′-UUAGCUUCAUAAGGCGCAUGC-5′
21	CH_3O	OH	5′-CH_3O-dTCGAAGUAUUCCGCGUACGUG-3′ 3′-UUAGCUUCAUAAGGCGCAUGC-5′
21	OH	2′,3′ ddC	5′-UCGAAGUAUUCCGCGUACGUddC-3′ 3′-UUAGCUUCAUAAGGCGCAUGC-5′
21	OH	AM	5′-UCGAAGUAUUCCGCGUACGUG-AM-3′ 3′-UUAGCUUCAUAAGGCGCAUGC-5′
20	OH	OH	5′-UCGAAGUAUUCCGCGUACGU-3′ 3′-UUAGCUUCAUAAGGCGCAUGC-5′
20	OH	2′,3′ ddC	5′-UCGAAGUAUUCCGCGUACGddC-3′ 3′-UUAGCUUCAUAAGGCGCAUGC-5′
20	OH	AM	5′-UCGAAGUAUUCCGCGUACGU-AM-3′ 3′-UUAGCUUCAUAAGGCGCAUGC-5′
20	OH	OH	5′-UCGAAGUAUUCCGCGUACGC-3′ 3′-UUAGCUUCAUAAGGCGCAUGC-5′
19	OH	OH	5′-UCGAAGUAUUCCGCGUACG-3′ 3′-UUAGCUUCAUAAGGCGCAUGC-5′
19	OH	AM	5′-UCGAAGUAUUCCGCGUACG-AM-3′ 3′-UUAGCUUCAUAAGGCGCAUGC-5′
21	OH	OH	5′-UGAGGUAGUAGGUUGUAUAGU-3′ 3′-UUACUCCAUCAUCCAACAUAU-5′
21	OH, 2′dT	AM	5′-dTGAGGUAGUAGGUUGUAUAGU-AM-3′ 3′-UUACUCCAUCAUCCAACAUAU-5′

B

Figure 2. RNAs Used in This Study

(A) Photinus pyralis (firefly) luciferase (blue) and *let-7* (red) siRNAs used in this study. The guide strand (antisense strand) is shown 5′-to-3′ as the upper strand of each siRNA. Single-stranded siRNAs used in Figures 4, 5, and 6 correspond to the indicated guide strands. ddC, dideoxy Cytosine; AM, amino modifier. siRNAs corresponding to firefly luciferase sequence are blue; those corresponding to *let-7* sequence are red.

(B) A schematic representation of the chimeric target RNA, indicating the relative positions of firefly luciferase sequences and sequences complementary to the *let-7* miRNA found naturally in HeLa cells.

sivity was quite low (Boutla et al., 2001). Furthermore, single-stranded RNAs of various lengths trigger RNAi in *C. elegans*, but only when they contain a 3′ hydroxyl group, suggesting that single-stranded siRNA functions in that organism as a primer for an RdRP (Tijsterman et al., 2002). Consistent with single-stranded siRNAs acting in nematodes as primers that direct the production of new dsRNA, they fail to trigger RNAi in the absence of Dicer (Dcr-1) (Tijsterman et al., 2002).

We examined if the guide siRNA strand alone could trigger target cleavage in an in vitro RNAi reaction containing either *Drosophila* embryo lysate or human HeLa cell S100. We first examined if single-stranded siRNA could direct target RNA cleavage in *Drosophila* embryo lysates (Figure 5A). For this experiment, we used siRNA with the sequence of the miRNA *let-7* (Figure 2A). Cleavage of the target RNA (Figure 2B) by a *let-7*-containing siRNA duplex produces a diagnostic 522 nt 5′ product (Hutvágner and Zamore, 2002a). When the synthetic siRNA was used as a single strand, the target RNA was not cleaved (Figure 5A). Similarly, a single-stranded siRNA of the same sequence but bearing a 2′ deoxy thymidine (dT) instead of uracil as its first nucleotide, was also a poor trigger of target cleavage. However,

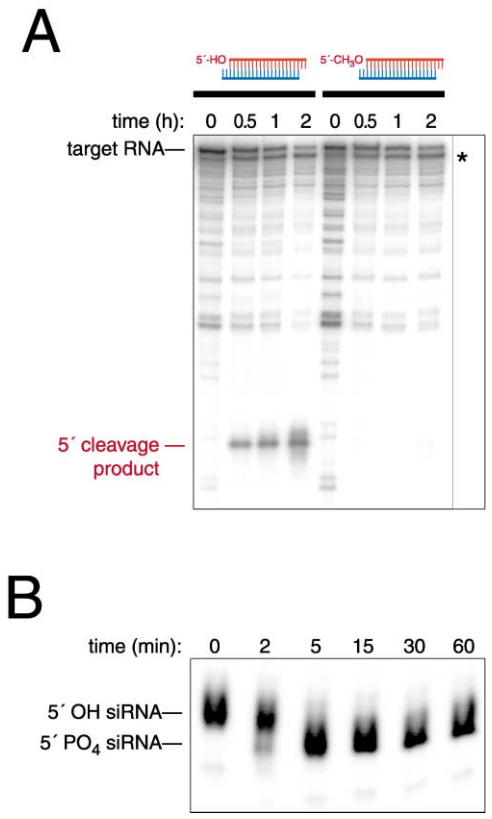


Figure 3. The siRNA 5′ Phosphate Group Is Required for siRNA-Directed Target Cleavage in HeLa S100 Extracts

(A) RNAi in vitro in human HeLa cell S100 extract. At left, a time course of in vitro RNAi for a standard siRNA; at right, for an siRNA duplex bearing a 5′ methoxy guide strand. The asterisk indicates the position of a 5′ cleavage product catalyzed by an endogenous, human *let-7*-programmed RISC complex, which cleaves this target RNA within a *let-7* complementary sequence located near the 3′ end of the RNA (Hutvágner and Zamore, 2002a). This cleavage product serves as an internal control.

(B) Phosphorylation status of the guide strand of an siRNA duplex upon incubation in HeLa S100. An siRNA duplex containing a guide strand 3′-end-labeled with α-^{32}P cordycepin (3′ deoxyadenosine triphosphate) was incubated in a standard HeLa S100 RNAi reaction, then analyzed on a 15% sequencing gel. Phosphorylation accelerates the gel mobility of the labeled siRNA strand, because it adds two additional negative charges. The radiolabeled RNA is 3′ deoxy; therefore, we infer that the added phosphate is on the 5′ end.

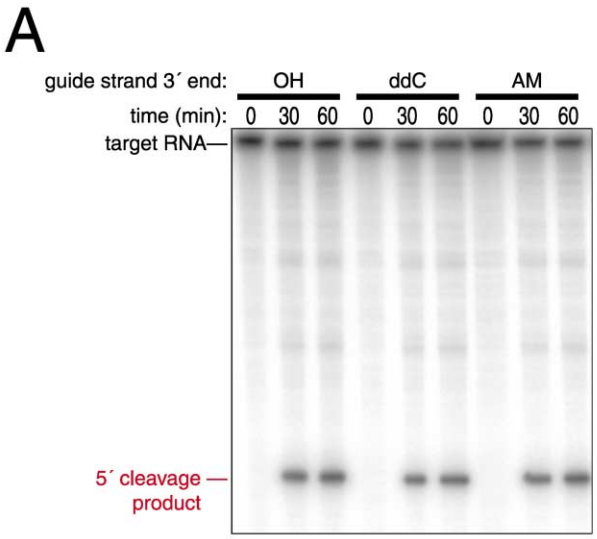
both these siRNAs contain a 5′ hydroxyl, and a 5′ phosphate is required for siRNA duplexes to trigger target RNA cleavage in *Drosophila* embryo lysates (Nykänen et al., 2001). Therefore, we considered that the defect with the single-stranded siRNAs might be that they lacked a 5′ phosphate and cannot obtain one because they are not substrates for the *Drosophila* kinase. In support of this hypothesis, when the single-stranded siRNA starting with dT was prephosphorylated with polynucleotide kinase, it directed target cleavage.

To confirm these findings, we examined the activity of a second single-stranded siRNA, complementary to the luciferase portion of the target RNA. When prephosphorylated, this single-stranded siRNA again directed target cleavage in *Drosophila* embryo lysate, albeit less efficiently than the same molar concentration of an siRNA duplex (Figure 5B). Cleavage occurred at precisely the same site in the target RNA for both single-stranded and double-stranded siRNAs, suggesting that the single-stranded siRNA entered the RNAi pathway, rather than triggered RNA destruction by a different route. The same single-stranded siRNA sequence bearing a 5′ methoxy group did not direct target RNA cleavage (Figure 5B). Together, the experiments in Figure 5 demonstrate that single-stranded siRNAs—like the guide strands of siRNA duplexes—do not function in the RNAi pathway unless they bear a 5′ phosphate.

To determine if single-stranded siRNAs trigger target destruction in *Drosophila* embryo lysates by acting as primers, we modified the 3′ end of the siRNA to 2′,3′ dideoxy. As with double-stranded siRNAs, blocking the 3′ end of the single-stranded siRNA had no effect on the efficiency or specificity with which the target was cleaved (Figure 5B). We note that the efficiency of target cleavage by single-stranded siRNAs is significantly less than that of siRNA duplexes. The lower efficiency might simply reflect the remarkably short lifespan of single-stranded siRNA in the *Drosophila* embryo lysate: the vast majority is destroyed within the first 2 min of incubation (Figure 5C). One explanation for the requirement for a 5′ phosphate might be that, without it, the single-stranded siRNA is destroyed even faster. This explanation is unlikely, because the rate of single-stranded RNA destruction is only 1.4-fold faster for 5′ hydroxy siRNAs (Figure 5C). More likely is that the 5′ phosphate of the single-stranded siRNA is required for its entry into the RISC and that because a small fraction of 5′ phosphorylated, single-stranded siRNA enters the RISC, it is protected from degradation, enhancing its stability in the lysate.

Next, we examined if single-stranded siRNAs could function to trigger RNAi in HeLa S100 extracts. Again, single-stranded siRNAs directed target cleavage at the same site as the corresponding siRNA duplex (Figure 6A). Prephosphorylation of single-stranded siRNA was not required for it to function in target cleavage in HeLa S100, but blocking the 5′ end with a methoxy group completely eliminated RNAi (Figure 6B). These results suggest that a 5′ phosphate is required for mammalian RNAi, but that the nucleic acid kinase(s) responsible for phosphorylating siRNAs in HeLa S100 acts on single-stranded siRNA, unlike its *Drosophila* counterpart. Blocking the 3′ end of the single-stranded siRNA had no effect on the ability of the single-stranded siRNA to cleave the target RNA in HeLa S100 (Figure 6A). Thus, the structural requirements for single-stranded siRNA function in target cleavage are conserved between flies and mammals: a 5′ phosphate is required, but a 3′ hydroxyl is not.

Together, these data support the view that siRNAs do not direct target RNA destruction by priming the synthesis of new RNA, nor are siRNAs ligated together to generate cRNA. Both processes should require a 3′ hydroxyl group, which is dispensable for target cleavage in either *Drosophila* or human cell extracts. Instead, our data suggest that siRNAs act as guides to direct a protein endoribonuclease to cleave the target RNA. The finding that single-stranded siRNAs can function as guides in the RNAi pathway suggests that each individual RISC contains only one siRNA strand. Consistent



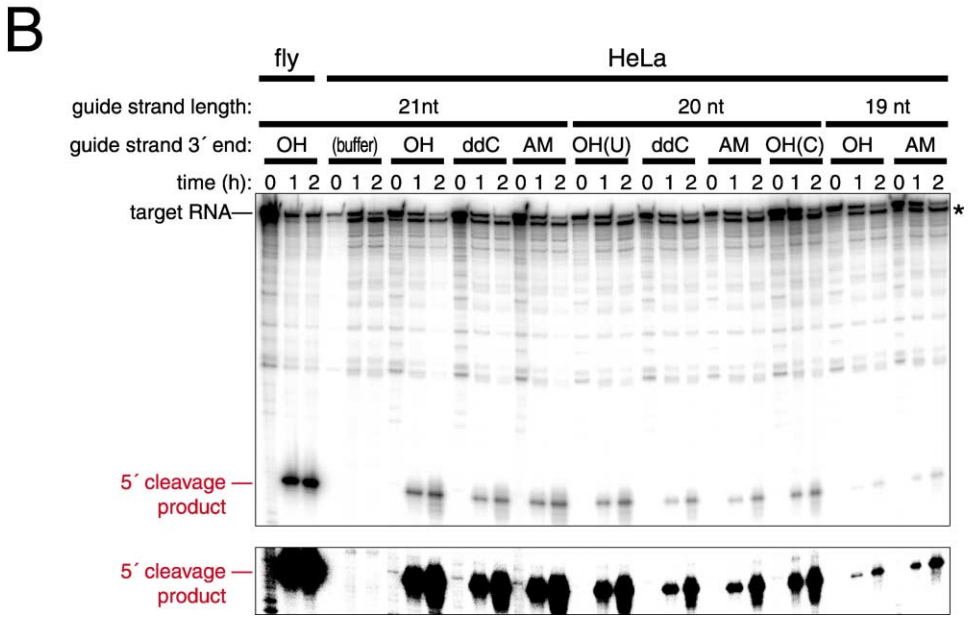


Figure 4. The siRNA 3′ Hydroxyl Is Dispensable for siRNA-Directed Target Cleavage in *Drosophila* and Human Cell Extracts

(A) 3′-blocked siRNAs trigger RNAi in *Drosophila* embryo lysates with the same efficiency as 3′-hydroxyl-containing siRNAs. ddC, 2′,3′ dideoxy C; AM, amino modifier.

(B) 3′-blocked siRNAs trigger RNAi in HeLa S100 extracts with the same efficiency as standard, 3′-hydroxyl-containing siRNAs. An overexposure of the region of the gel containing the 5′ cleavage product is shown in the lower panel. The asterisk marks the internal control 5′ cleavage product described in Figure 3.

with this view, in HeLa cell S100 extracts, the single stranded miRNA, *let-7*, is in an endogenous RISC that catalyzes multiple rounds of cleavage of a perfectly complementary target RNA (Hutvágner and Zamore, 2002a).

Previously, it was proposed that the siRNA 5′ phosphate was recognized twice during the assembly of the siRNA-containing endoribonuclease complex (Nykänen et al., 2001) (Figure 1). That study placed one 5′ phosphate recognition event before siRNA duplex unwinding but could not distinguish whether the 5′ phosphate is required subsequently at the unwinding step itself or after unwinding is complete. The absence of target cleavage by single-stranded siRNAs lacking a 5′ phosphate suggests that the second phosphate recognition step occurs after the siRNA duplex is unwound. In both *Drosophila* embryo lysates and human HeLa S100, cleavage directed by single-stranded siRNA was less efficient than RNAi triggered by siRNA duplexes. This inefficiency correlated with the general instability of short RNA in the in vitro extracts, as determined by

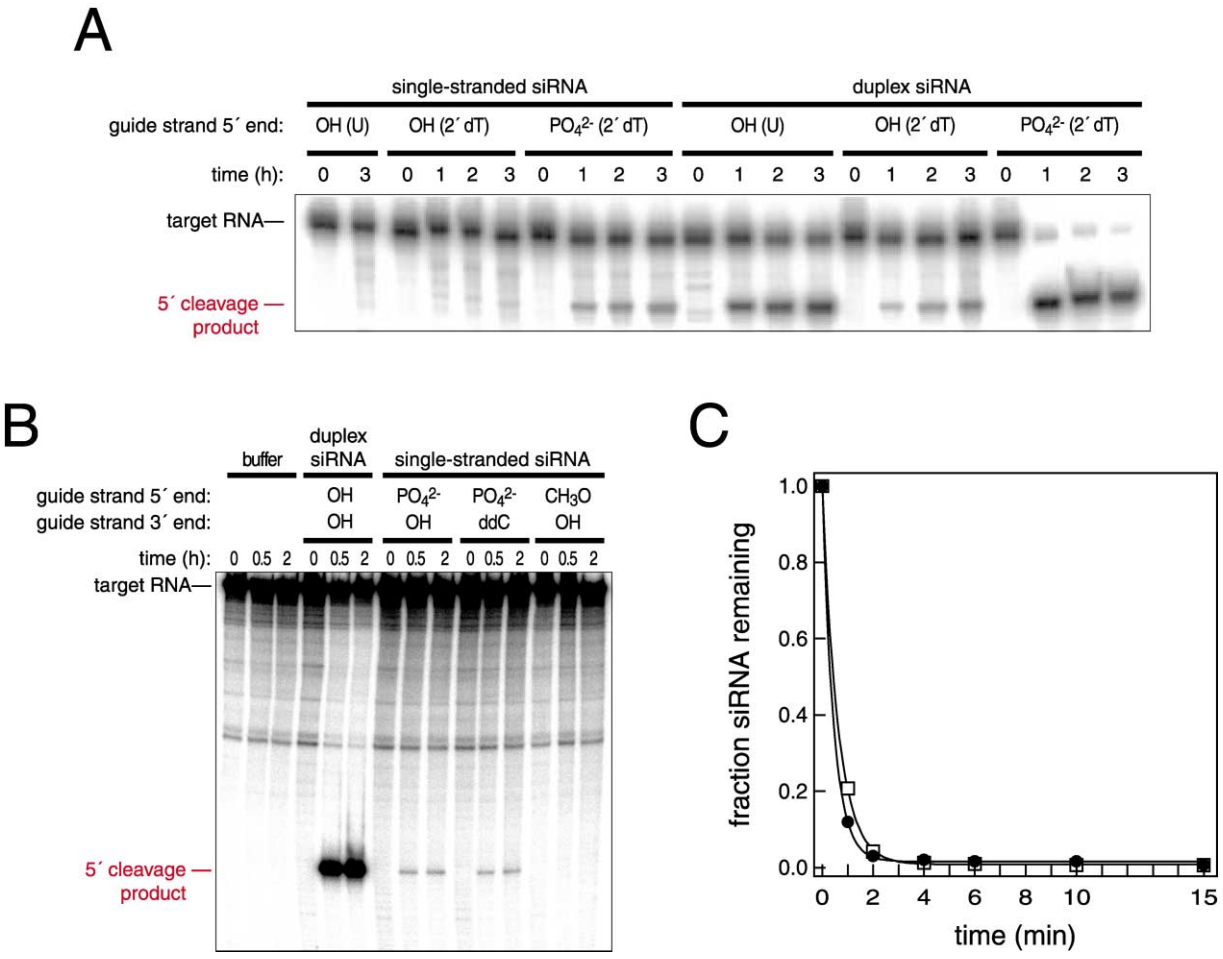


Figure 5. Single-Stranded siRNA Guides Target Cleavage in *Drosophila* Embryo Lysates

(A) Single-stranded siRNAs with the sequence of the miRNA *let-7* triggered target cleavage in *Drosophila* embryo lysate, but only if the 5′ end was prephosphorylated.

(B) Single-stranded siRNAs complementary to firefly luciferase sequence triggered target cleavage in *Drosophila* embryo lysate, even if the 3′ end was blocked (2′,3′ddC). No target cleavage was observed using an siRNA with a 5′ methoxy group.

(C) Rate of degradation of single-stranded siRNA in the *Drosophila* embryo lysate. siRNA single strands were 3′ end-labeled with α-^{32}P cordycepin and their stability measured with (filled circles) or without (open squares) a 5′ phosphate. The curves represent the best-fit to a single exponential, consistent with pseudo first-order kinetics for single-stranded siRNA decay. The difference in rates is 1.4-fold (with versus without a 5′ phosphate).

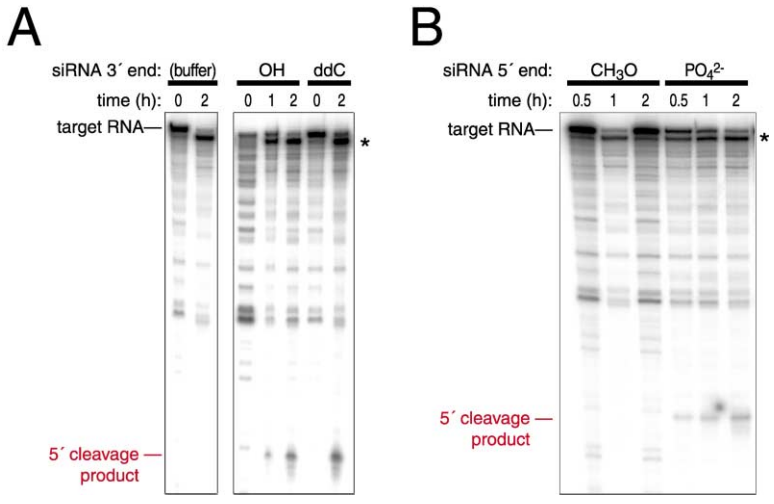
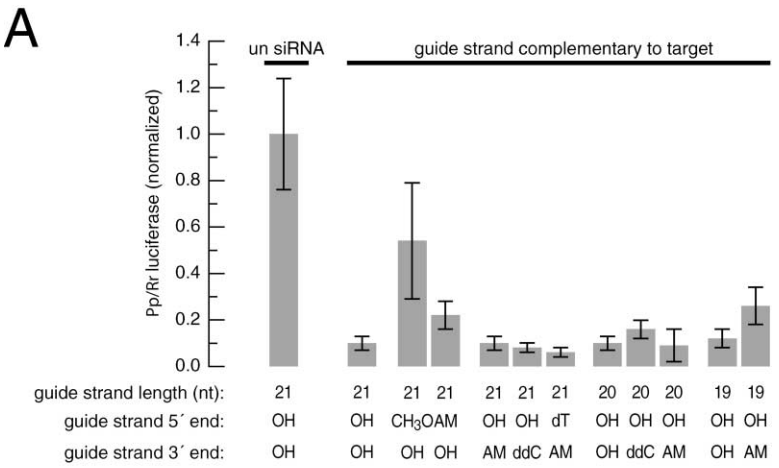


Figure 6. A 5′ Phosphate but Not a 3′ Hydroxyl Is Required for Single-Stranded Antisense siRNAs to Trigger RNAi in HeLa S100 Extract

(A) Single-stranded siRNA triggered target cleavage in HeLa S100, even if the 3′ end of the siRNA was blocked (2′,3′ dideoxy).

(B) Blocking the 5′ end of the siRNA with a methoxy group eliminated the ability of the single-stranded RNA to trigger RNAi. The asterisk marks the control 5′ cleavage product described in Figure 3.



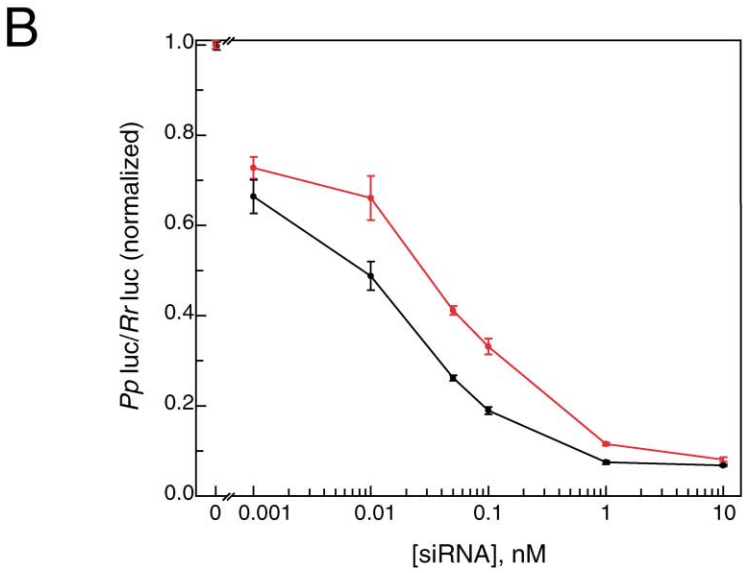


Figure 7. A 5′ Phosphate but Not a 3′ Hydroxyl Is Required for siRNA Duplexes to Trigger RNAi In Vivo in Cultured Human HeLa Cells

(A) siRNA duplexes were examined for their ability to silence the *Photinus pyralis* (Pp; firefly) luciferase target reporter, relative to the *Renilla reniformis* (Rr) luciferase control reporter. ddC, 2′,3′ dideoxy C; AM, amino modifier.

(B) Relative efficacy at limiting siRNA concentrations for siRNA duplexes with guide strands bearing either hydroxy (black symbols) or ddC (red symbols) 3′ termini. Data are the average ± standard deviation for three trials.

measuring single-stranded siRNA half-life using 3′ radiolabeled siRNAs (Figure 5C) and by Northern hybridization (data not shown).

siRNAs Need Not Function as Primers to Trigger RNAi in HeLa Cells

To assess if our in vitro results accurately predict the RNAi mechanism in vivo, cultured human cells were used to assess the structural requirements for siRNA function. Synthetic siRNAs were cotransfected into HeLa cells with plasmids expressing target (*Photinus pyralis, Pp*) and control (*Renilla reniformis, Rr*) luciferase mRNAs. Luciferase expression was measured, and target (firefly) luciferase levels were normalized to the *Renilla* control. The results of these experiments are shown in Figure 7.

First, the requirement for a 5′ phosphate observed in *Drosophila* and HeLa extracts was conserved in vivo (Figure 7A). A 5′ hydroxyl-containing siRNA duplex triggered efficient gene silencing in vivo, reducing expression of the target luciferase >90%. In contrast, a 5′ methoxy-modified siRNA reduced firefly luciferase levels by only 2-fold. This small reduction may reflect inhibition of translation, perhaps by an antisense mechanism. Alternatively, some of the methoxy-blocked siRNA may inefficiently enter the RNAi pathway in vivo. An siRNA in which the guide strand contained a 5′ amino modifier group—6-amino-hexyl phosphoester—was significantly more effective in suppressing target mRNA expression than the siRNA with the 5′ methoxy group (Figure 7A). This finding is consistent with the idea that a 5′ phosphate group is required for siRNA function, but that the 5′ phosphate participates in noncovalent interactions only, since the modified 5′ phosphate should be less able to act as an electron acceptor. The in vivo studies agree with the in vitro results: a 5′ phosphate is essential for efficient siRNA function in flies and mammals. However, in flies only duplex siRNAs can be 5′ phosphorylated by cellular kinases, whereas in mammals, both single-stranded and double-stranded siRNAs are phosphorylated.

Consistent with the view that the core function of siRNA in human cells is as guides, not primers, blocking the 3′ end of the siRNA guide strand had no effect on

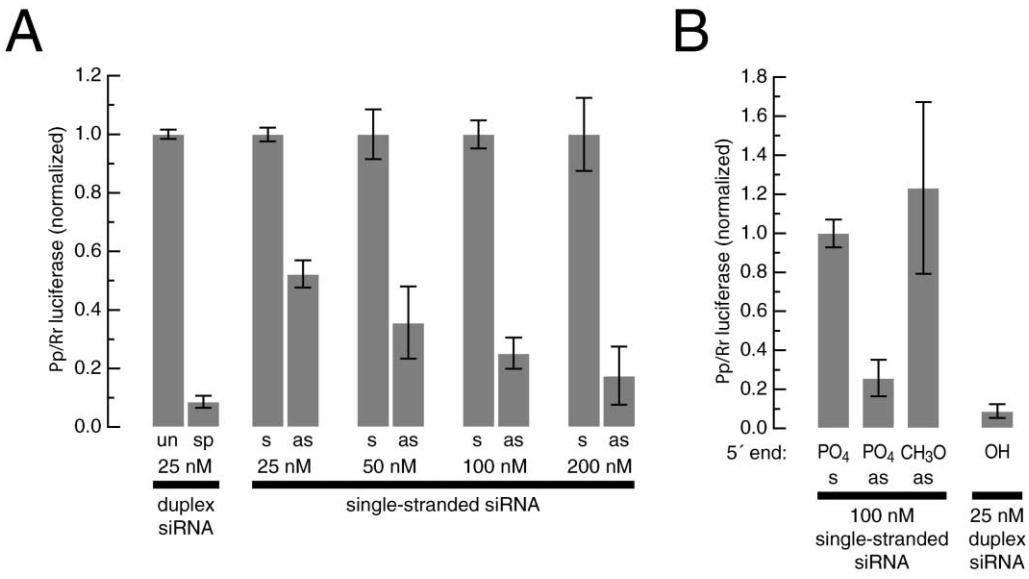


Figure 8. Single-Stranded siRNA Triggers Gene Silencing in HeLa Cells

(A) Single-stranded siRNA silencing as a function of siRNA concentration.

(B) Blocking the 5′ end of single-stranded siRNAs prevented their triggering target gene silencing. Gray bars indicate the average ± standard deviation for three trials. Un, siRNA unrelated in sequence to the target RNA; sp, specific siRNA corresponding to the target RNA; s, sense strand; as, antisense strand.

RNAi in vivo. siRNA duplexes in which the guide strand contained a 3′ hydroxyl, a 2′,3′ dideoxy, or a 3′ amino modifier were all equally effective in triggering RNAi in vivo (Figure 7A). The silencing activity in vivo of a 21 nt, 3′-blocked siRNA guide strand was greater than that of a 20 nt, 3′ hydroxy siRNA guide strand, indicating that the 3′ block was not removed in vivo. We believe that these data exclude an obligatory role for the siRNA 3′ hydroxyl group in RNAi in mammalian cells and argue that siRNAs do not normally trigger target destruction in human cells by functioning as primers.

These experiments were conducted at siRNA concentrations where the siRNA is not limiting for RNA silencing. An siRNA function in priming the synthesis of dsRNA might be used when siRNAs are limiting. We tested the relative ability of siRNA duplexes in which the guide strand either contained a 3′ hydroxyl or a 2′,3′ dideoxy group at low siRNA concentrations (Figure 7B). We find that the efficacy of the two types of siRNAs did differ when siRNA was rate limiting for target mRNA silencing, but never by more than 1.8-fold. The observed difference in efficacy between the two types of siRNAs does not seem sufficiently great to support the view that the 3′ hydroxyl group of the siRNA is used to prime the synthesis of dsRNA from the target mRNA. If the siRNA were used to prime dsRNA synthesis, the production of new dsRNA by an RdRP using the siRNA as a primer should have amplified the silencing activity of the 3′ hydroxy but not the 2′,3′ dideoxy siRNA at limiting concentrations. For example, if the 3′ hydroxy guide strand had primed synthesis of one molecule of dsRNA (~130 bp long based on the site of siRNA/target complementarity) for each target mRNA molecule, and this new dsRNA was then Diced into just two of the possible six new siRNAs, at least a 2-fold difference between the two siRNAs should have been observed. This analysis fails to take into account the new crop of siRNAs acting in a subsequent cycle of priming, which would further amplify the difference between 3′ deoxy and 3′ hydroxy siRNA at limiting concentrations. The simplest interpretation of our finding that 3′ hydroxy siRNAs trigger no significant amplification of RNA silencing relative to 3′ blocked siRNAs is that an siRNA-primed, RdRP-dependent cycle of siRNA amplification plays no productive role in RNAi in cultured HeLa cells, even at low siRNA concentrations. The small difference in efficacy between 3′ OH and 2′,3′ dideoxy siRNAs likely indicates that the blocked siRNAs have a subtle defect such as a lower affinity for components of the RNAi machinery, slightly reduced intracellular half-life, or a minor reduction in phosphorylation rate. This defect may result from the 2′ deoxy modification of the terminal nucleotide, rather than the 3′ block, since siRNAs with 2′ deoxythymidine tails have been reported to be less efficient than those containing uracil in HeLa cells (Hohjoh, 2002).

Our in vitro studies suggest that single-stranded siRNAs can enter the RNAi pathway, albeit inefficiently. To test if single-stranded siRNAs could trigger mRNA silencing in vivo, we substituted various concentrations of single-stranded, sense or antisense siRNA for siRNA duplexes in our HeLa cell cotransfections (Figure 8A). As the concentration of antisense single strand was increased, the expression of the firefly luciferase decreased relative to the Renilla internal control. Note that single-stranded siRNAs are less efficient than siRNA duplexes: it takes nearly eight times more single-stranded siRNA to approach the potency of the corresponding duplex. This inefficiency may simply reflect rapid degradation of the majority of the transfected single-stranded siRNA before it can enter the RISC com-

plex. Cells may possess a mechanism that stabilizes siRNA duplexes and shuttles them to the RISC as single strands without exposing them to degradatory enzymes. Thus, if endogenous siRNAs are double-stranded in vivo, they may be double-stranded so as to facilitate their entry into the RNAi pathway and to exclude them from a competing pathway that degrades small, single-stranded RNA. Alternatively, single-stranded siRNAs may bypass a key step in RISC assembly, making them less efficient than duplexes in triggering RNAi. The dramatic instability of single-stranded siRNAs in vitro may simply reflect their inefficiency in assembling into a RISC, which could protect them from degradation.

Gene silencing by single-stranded siRNA was sequence specific, and single-stranded sense siRNA did not alter the expression of the target RNA (Figure 8B). Thus, it is unlikely that siRNAs themselves are copied by an RdRP in mammalian cells, since copying the sense siRNA should generate the antisense siRNA strand. However, copying sense siRNA into a duplex would not generate the characteristic 3′ overhanging ends of siRNAs. Such 3′ overhangs might be required for siRNA unwinding and/or efficient RISC assembly. Prephosphorylation of single-stranded siRNA did not enhance its potency in HeLa cells, consistent with our observations in HeLa S100 extracts, but blocking phosphorylation with a 5′ methoxy group abolished silencing, pointing to the importance of 5′ phosphorylation for single-stranded siRNA function in vivo (Figure 8B). Our findings are not entirely unexpected, since endogenous, single-stranded miRNAs enter the RNAi pathway in HeLa cells (Hutvágner and Zamore, 2002a). Superficially, the finding that single-stranded siRNAs can elicit RNA silencing blurs the distinction between RNAi and antisense effects. We have presented here evidence that single-stranded siRNAs trigger the same pathway as siRNA duplexes: both guide endonucleolytic cleavage of target RNAs at the same site, and both require 5′ phosphates but not 3′ hydroxyl groups to function.

Our in vitro experiments with *Drosophila* embryo lysates and HeLa S100 extracts and our in vivo experiments in HeLa cells argue against siRNAs functioning as primers in the RNAi pathway. These findings are consistent with the absence of any genes encoding canonical RdRPs in the currently available release of either the *Drosophila* or human genome. A hallmark of the involvement of RdRPs in posttranscriptional silencing is the spread of silencing beyond the confines of an initial trigger dsRNA or siRNA into regions of the target RNA 5′ to the silencing trigger. In *C. elegans*, this spreading ("transitive RNAi") is manifest in the production of new siRNAs corresponding to target sequences not contained in the exogenous trigger dsRNA (Sijen et al., 2001). Furthermore, small RNAs as long as 40 nt can initiate silencing in worms, but only if they contain 3′ hydroxyls, suggesting that they act as primers for the synthesis of cRNA (Tijsterman et al., 2002). In contrast, 5′ spreading is not detected in *Drosophila*, either in vitro (Zamore et al., 2000), in cultured *Drosophila* S2 cells (Celotto and Graveley, 2002), or in vivo in flies (J.-Y. Roignant and C. Antoniewski, personal communication). Our data support the view that, in both flies and mammals, siRNAs trigger target RNA destruction not by acting as primers but rather by guiding a protein endoribonuclease to a site on the target RNA that is complementary to one strand of the siRNA. The observation that the target cleavage site is across from the center of the complementary siRNA (Elbashir et al., 2001c, 2001b) is consistent with an enzyme other than Dicer acting in target RNA destruction and not with models that propose that Dicer destroys target RNAs. Furthermore, mammalian extracts depleted of Dicer still catalyze siRNA-directed target cleavage (Martinez et al., 2002).

Will it be possible to design siRNAs to degrade just one of several mRNA isoforms that differ at only a single nucleotide? If siRNAs do not act as RdRP primers in flies and mammals, then there is no fear that the silencing signal will spread 5′ to a region of sequence common to the entire family of mRNAs. Despite earlier concerns that such siRNAs would not be possible (Nishikura, 2001), our data suggest that isoform- and polymorphism-specific siRNAs will be used in mammals in the future to dissect the function of individual gene isoforms and perhaps even to treat inherited autosomal dominant human diseases.

Experimental Procedures

General Methods

Drosophila embryo lysate preparation, in vitro RNAi reactions, and cap-labeling of target RNAs using Guanylyl transferase were carried out as previously described (Zamore et al., 2000). Human S100 extracts were prepared as described (Dignam et al., 1983). HeLa S100 was substituted for *Drosophila* embryo lysate in an otherwise standard RNAi reaction, except that incubation was at 37° instead of 25°C. Cleavage products of RNAi reactions were analyzed by electrophoresis on 8% denaturing acrylamide gels. 3′ end labeling with α-^{32}P cordycepin and determination of 5′ phosphorylation status were according to Nykänen et al. (2001). Gels were dried and exposed to image plates (Fuji), which were scanned with a Fuji FLA-5000 phosphorimager. Images were analyzed using Image Reader FLA-5000 version 1.0 (Fuji) and Image Gauge version 3.45 (Fuji).

siRNA Preparation

Synthetic RNAs (Dharmacon) were deprotected according to the manufacturer's protocol and processed as previously described (Nykänen et al., 2001). siRNA strands were annealed (Elbashir et al., 2001a) and used at 100 nM final concentration unless otherwise noted. siRNA single strands were phosphorylated with polynucleotide kinase (New England Biolabs) and 1 mM ATP according to the manufacturer's directions.

Tissue Culture

siRNA transfections were as described (Elbashir et al., 2001a). In brief, cultured HeLa cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies). Cells were trypsinized and seeded at 1×10^5 cells/ml in 24-well plates (5×10^4 cells/well) in DMEM supplemented with 10% FBS. Twenty-four hours after seeding, 1 μg pGL2 control firefly luciferase (*Pp*-luc GL2; Promega) and 0.1 μg pRL-TK Renilla luciferase (*Rr*-luc; Promega) plasmids and the luciferase siRNA (25 nM) were cotransfected with LipofectAMINE 2000 reagent (Invitrogen) in DMEM (Life Technologies) lacking serum and antibiotics according to manufacturer's instructions. Media was replaced 4 hr after transfection with DMEM containing 10% fetal bovine serum (Life Technologies); 1 day after transfection, the cells were lysed in 1x Passive Lysis Buffer (Promega) according to the manufacturer's instructions. Luciferase expression was determined by the Dual luciferase assay kit (Promega) using a Mediators PhL luminometer. Data analysis was performed using Excel (Microsoft) and IgorPro 5.0 (Wavemetrics). Experiments were performed in triplicate, and error was propagated through all calculations.

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