A Dissertation Presented

By

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Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 26, 2005

Biochemistry and Molecular Pharmacology

BIOCHEMICAL MECHANISM OF RNA INTERFERENCE IN HIGHER
ORGANISMS

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August 26, 2005

ABSTRACT

RNA interference (RNAi) is an evolutionarily conserved, sequence-specific gene silencing pathway found in eukaryotes, in which 21-nucleotide, small interfering RNAs (siRNAs) guide destruction of a corresponding target mRNA. RNAi is a natural mechanism for both genome surveillance and gene regulation. Moreover, siRNAs can be transfected into cultured mammalian cells, causing the sequence-specific 'knock down' of an mRNA. My work in the Zamore lab has centered around the *Drosophila* in vitro system and cultured mammalian cells to study the RNA interference (RNAi) pathway. small interfering RNAs (siRNAs) are incorporated into the RNA-induced silencing complex (RISC), which culminates in the cleavage of a complementary target mRNA. Previous work proved that certain structural features of siRNAs are essential for RNAi in flies, including the requirement for 5′ phosphates and 3′ hydroxyl groups. In cultured mammalian cells, the requirement for a 5′ phosphate also holds true, but we found no evidence to support the necessity for 3′ hydroxyls in either system. In addition, siRNAs can act as single strands entering the pathway downstream of double-stranded siRNAs, both of which are competent in directing the cleavage of its cognate mRNA at a single site.

While these key features are a requirement for functional siRNAs, alone they do not determine the efficiency to which an siRNA can enter the RISC. In fact, both strands of an siRNA can enter RISC to a different degree as determined by the stabilities of the 5′ ends of the siRNA strand, a phenomenon termed 'functional asymmetry'. This

characteristic is also reflected in another class of small RNAs involved in gene silencing known as microRNAs (miRNAs), which are processed from long hairpin RNA structures into mature, single-stranded non-coding RNAs. The asymmetric loading of siRNAs suggests that miRNAs are initially generated from siRNA-like duplexes cleaved from the stem of the hairpins. The strand whose 5′ end is less tightly paired will be processed into the mature miRNA, while the other strand is destroyed. By applying the rules of siRNA asymmetry it is possible to predict which side of the stem will be processed into the mature miRNA, a finding verified experimentally by our lab and others. This discovery also has additional implications in designing highly effective siRNAs and in reducing siRNA off-target effects.

We used these results to design siRNAs that target the single nucleotide polymorphism in superoxide dismutase that causes the familial form of amyotrophic lateral sclerosis (ALS), but leave the wild-type mRNA intact and functional. Our experiments have helped define the 'rules' for creating SNP-specific siRNAs. In particular, we found that only siRNAs with a purine:purine mismatch to the allele not intended for destruction show good discrimination. The placement of the mismatch in a tiled set of siRNAs shows that mismatches located in the 5′ region of the siRNA, a region shown to be responsible for siRNA binding, can not discriminate between alleles. In contrast, mismatches in the 3′ region of the siRNA, the region contributing to catalysis, discriminate between wild-type and mutant alleles. This work is an important step in creating allele-specific siRNAs as therapeutics for dominant negative genetic diseases.

But how does RISC cleave its target? By isolating both the 5′ and 3′ cleavage products produced by RISC in the *Drosophila* in vitro system, we discovered that RISC acts as a Mg^{2+}-dependent endonuclease that cleaves a single phosphodiester bond in the mRNA target, leaving 5′ phosphate and 3′ hydroxyl groups. These findings were a critical step in the demonstration that Argonaute, a protein known to be a component of RISC, is the RNAi endonuclease.

ACKNOWLEDGEMENTS

scientific community. For all of their support and help, I would like to thank Karen Welch, Barbara Bucciaglia, and Tiffanie Covello. My graduate school days and our day to day lab life would not run so smoothly without Tiffanie. To all of my committee members (Mary Munson, Zuoshang Xu, Craig Peterson, Bob Matthews, Steve Miller, and Greg Hannon) for their insightful comments and suggestions. Also, to our collaborators at UMASS – Neil Aronin and his lab (particularly Lori Kennington), and Zuoshang Xu (particularly Hongliu Ding) who have been such a great team to work with in tackling the questions of RNAi and disease – for all of their comments and discussions I am truly grateful. For one of my greatest friends here at UMASS, I would not have made it through grad school, or remained as sane as possible, if it weren't for Jared Auclair and our coffee/ice cream runs and talks. To Melissa and Pam for all of the fun that we have had, all of the laughs, and for being such great friends – I will miss you greatly. For some of my greatest science buddies and Red Sox fans, Julie Claycomb and Alex Ensminger – for being such great friends, for all of the fun that we have had, and for many more memories to come.

I also could not have made the decision to study Biology if it were not for several key people before I even came to graduate school. For my high school Biology teacher, Joan Imhoff, for instilling the love of Biology in me, and for Denise McKeon and Caro-Beth Stewart in college for making me realize how exciting research could be – if it was not for Dr. Stewart I would not have been able to realize my ability and love for science. She

never doubted me and always encouraged my ideas and thinking, and for that I am extremely grateful.

To my family – for always being supportive of me no matter what, and for always being there as a shoulder to lean on or an ear to listen. I am truly blessed with such a wonderful family who has always believed in me, has taught me the meaning of hard work and "stick-to-itiveness", respect, and being a good person. For a life filled with so many wonderful memories and fun times, I am always appreciative. To my parents, especially, for being (what I think) the best parents in the entire world for giving me a lifetime of wonderful memories, and for making home a place that I always look forward to visiting. For always being there, just a phone call away, when I needed anything – through tough decisions, times of self-doubt, and for never once questioning my capabilities, but most importantly teaching me how to be a good person and lead a life that I would be proud of – if I could be even half of what they are, I would know that I had done well. And, last but not least, to my husband Nelson, who is my favorite lab partner, for always believing in me when I didn't have the sense to believe in myself, for helping me through tough decisions and transitions in my life, for being my best friend, for sticking by me no matter what, and for reminding me not to take myself too seriously. For all of your support, your advice, the countless laughs that we have shared, for being my source of entertainment even when you don't mean to be, I know that even when things in life seem bad, they could never be bad with you by my side. I look forward to a bright and exciting

future with you. Thank you for everything – I appreciate it more than you will ever know.

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The RNA-Induced Silencing Complex is a Mg^{2+}-Dependent Endonuclease

Chapter IV has been published previously as:
D.S. Schwarz, Y. Tomari, and P.D. Zamore. "The RNA-Induced Silencing Complex Is a Mg^{2+}-Dependent Endonuclease." Curr Biol 14: 787 – 791. (2004) © Elsevier Ltd.

Selective Silencing by RNAi of a Dominant Allele that
Causes Amyotrophic Lateral Sclerosis

Chapter V has been published previously as:
H. Ding*, D.S. Schwarz, A. Keene, E.B. Affar, L. Fenton,
X. Xia, Y. Shi, P.D. Zamore, and Z. Xu. "Selective
Silencing by RNAi of a Dominant Allele that Causes
Amyotrophic Lateral Sclerosis." Aging Cell 2: 209 – 217.
(2003) ©Blackwell Publishing Ltd.

Application of Functionally Asymmetric siRNA Design
to Single Nucleotide Discrimination

Chapter VI is a manuscript in preparation as:
D.S. Schwarz, H. Ding, L. Kennington, J.T. Moore, N.
Aronin, ,J.M. Schelter, P.S. Linsley, P.D. Zamore, and Z. Xu.

Why do miRNAs Live in the miRNP?

The Appendix has been published previously as:
D.S. Schwarz, and P.D. Zamore. "Why do miRNAs
Live in the miRNP?" Genes & Dev 16: 1025 – 1031.
(2002) © Cold Spring Harbor Laboratory Press.

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CHAPTER I: INTRODUCTION

RNA Interference: A Historical Perspective

Disruption of gene expression is the first step in identifying the putative function of a gene and its gene product in a particular pathway or developmental process. In the early 1980's, Izant and Weintraub decided to use antisense RNA as a molecular tool to decipher gene function in cultured cell lines as an alternative approach to classical genetics. By introducing a construct containing the sequence of a gene of interest in the reverse orientation (or complimentary strand), gene expression and activity were reduced (Izant and Weintraub, 1984). Several groups went on to confirm this result in cultured cells (Holt et al., 1986; Hunts et al., 1986; Izant and Weintraub, 1985; McGarry and Lindquist, 1986), Xenopus oocytes (Harland and Weintraub, 1985; Wormington, 1986), and Drosophila embryos (Rosenberg et al., 1985).

In 1991, Moerman and colleagues extended this antisense inhibition technique to the organismal level by injecting the construct into worms (Fire et al., 1991). When plasmids targeting unc-22 and unc-54, myofilament proteins found in muscle, were injected into oocytes of *Caenorhabditis elegans*, endogenous protein levels dropped and phenotypes similar to mutants were observed (Fire et al., 1991). Four years later Guo and Kemphues turned to the antisense inhibition technique when attempts to obtain clones for germline transformation rescue failed. Instead of using plasmids, they injected *in vitro*

synthesized RNA corresponding to their gene of interest, par-1, directly into the gonads of the worm (Guo and Kemphues, 1995). While the mock and unrelated RNA injections showed no change in phenotype, both sense and antisense RNA displayed *par-1* mutant phenotypes (Guo and Kemphues, 1995).

Guo and Kemphues' control experiment posed the puzzle as to why the sense RNA would elicit a mutant phenotype. Three years later, Fire and Mello decided to test the potency of sense or anti-sense RNA preparations alone, as well as a preparation of double-stranded RNA (dsRNA). Their hypothesis was that the original transcripts from Guo and Kemphues, which were transcribed using bacteriophage RNA polymerase, were contaminated with aberrant RNA products resulting in a small population of RNA with double-stranded character that may be acting as the silencing trigger for mRNA destruction. Fire and Mello made a breakthrough when they observed dsRNA injected into the worm was at least 10 times more effective at silencing than either single-stranded RNA alone, and that in situ hybridizations indicated that levels of targeted mRNAs were reduced, albeit to varying degrees (Fire et al., 1998). They named this gene silencing phenomenon RNA Interference, or RNAi.

In worms, RNAi was not only potent; it was specific, and yet the silencing effect could cross cellular boundaries as well as be inherited in the progeny. DsRNA corresponding to intron and promoter sequences did not elicit a decrease in gene expression (Fire et al., 1998), dsRNA targeting exon sequences did not alter levels of pre-mRNAs (Ngo et al., 1998), and cistrons within a polycistronic message could be targeted separately (Montgomery et al., 1998), which suggests that the silencing mechanism acts

post-transcriptionally. Interfering RNAs could exhibit silencing activity in a variety of worm tissues, whether the RNA was injected just in the worm gonads, or if the worms were soaked in RNA solution, and even when the worms ingested dsRNA expressed by bacteria (Tabara et al., 1998; Timmons et al., 2001; Timmons and Fire, 1998). These observations suggest a possible mechanism for transporting the effector molecules between cells, or a mechanism for amplifying the active molecules. Such mechanisms might even explain the interesting observation that silencing by RNAi could be passed on to progeny two generations removed from the initial parent subjected to the RNA treatment (Grishok et al., 2000).

Subsequently, the use of dsRNA to mediate gene silencing was utilized in various organisms including *Drosophila* (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999), mouse oocytes (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000), planaria (Sanchez Alvarado and Newmark, 1999), hydra (Lohmann et al., 1999), zebrafish (Wargelius et al., 1999) and trypanosomes (Ngo et al., 1998). Additionally, long dsRNA recapitulated silencing in vitro in *Drosophila* embryo lysates (Tuschl et al., 1999; Zamore et al., 2000) and in cultured *Drosophila* S2 cells (Caplen et al., 2000; Clemens et al., 2000; Hammond et al., 2000). While the use of dsRNA was effective in deciphering gene function, the exact mechanism in which silencing occurred remained unclear.

In order to dissect the steps of the silencing pathway and address mechanistic questions including which protein cofactors are involved, what energy requirements may be necessary, and what steps and intermediates exist, an in vitro system that recapitulates the gene silencing activity was needed. The first two such systems were a *Drosophila*

embryo lysate system (Tuschl et al., 1999) and extracts prepared from *Drosophila* S2 cells (Hammond et al., 2000). First it was shown that in vitro transcribed and annealed renilla or firefly luciferase long dsRNAs specifically targeted and destroyed only the corresponding luciferase mRNAs as determined by luciferase activity (Tuschl et al., 1999). Additionally, preincubation of the long dsRNA in lysate increased the efficiency of gene silencing, possibly allowing for processing of the long dsRNA into an active form (Tuschl et al., 1999). In addition, a minimum length of dsRNA was required for robust interference, and an unrelated competitor dsRNA was able to reduce the efficiency of related dsRNA by potentially titrating out necessary proteins or cofactors for the conversion of the long dsRNA into an active form capable of mediating specific interference (Tuschl et al., 1999).

Using a similar approach, Hannon and colleagues utilized a lacZ expression vector to look at specific gene silencing in *Drosophila* S2 cells co-transfected with unrelated or specific long dsRNA using β-galactosidase staining as a means for assaying gene knock-down (Hammond et al., 2000). In addition, cyclin E, cyclin A, and fizzy could be targeted by this method as well, the first example of RNAi against an endogenous gene in vitro (Hammond et al., 2000). Extracts made from the transiently transfected cells confirmed that the silencing was specific to homologous mRNAs, and that the sequence-specific target degradation must be dependent on a nuclease activity, termed the RNA-Induced Silencing Complex (RISC) (Hammond et al., 2000). The group postulated that the nuclease complex contained an RNA component based on co-fractionation experiments revealing the appearance of small RNA species approximately

25 nucleotides in length (Hammond et al., 2000), similar in size to the small RNAs detected in plants undergoing silencing (Hamilton and Baulcombe, 1999). The production of shorter, approximately 21 – 23 nucleotide, double-stranded fragments from long dsRNA in a target-independent fashion was shown in *Drosophila* embryo lysates (Zamore et al., 2000), also detected by Northern analysis in S2 lysates (Hammond et al., 2000), injection of radiolabeled dsRNA in worms (Parrish et al., 2000), and injection of dsRNA in single *Drosophila* embryos (Yang et al., 2000). However, Zamore and colleagues demonstrated an ATP-dependence requirement for cleavage of the corresponding mRNA, and that target cleavage occurred at 21 – 23 nt intervals, the same length of the processed long dsRNA, indicating that these small RNA fragments were guiding the sequence-specific mRNA cleavage (Zamore et al., 2000).

What are the structural characteristics and mechanistic actions of these small interfering RNAs (siRNAs), and how are they produced? It had been postulated, based on the size of siRNAs, that an RNase III enzyme may be involved in the processing the long dsRNA (Bass, 2000). Chemical analysis of the 5′ and 3′ ends of the siRNAs revealed the presence of a terminal 2′, 3′ hydroxyl and a 5′ phosphate, similar to the termini observed following RNase III processing (Elbashir et al., 2001b). The enzyme was later identified as Dicer, which contains a helicase domain and two RNase III domains (Bernstein et al., 2001). siRNAs from long dsRNA processing reactions were directionally cloned and the sequences and size distribution were analyzed. The majority of the siRNAs cloned were 21 nucleotides long, but were as small as 18 nucleotides and as a long as 24 nucleotides (Elbashir et al., 2001b). Previously the size of siRNAs were

predicted to be approximately 21 – 23 nucleotides long (Zamore et al., 2000), as analyzed by the processing of radiolabeled dsRNA, but cloning of siRNAs revealed a small portion of total siRNAs fall outside of that range (Elbashir et al., 2001b).

To test if the short RNAs were the final determinants for guiding silencing activity to specific target RNAs, the Tuschl lab chemically synthesized short RNAs that resembled siRNAs in structure to further study the requirements for efficient target mRNA cleavage. siRNAs with 2 nucleotide overhangs on the 3′ end, a signature of RNase III enzyme cleavage, resulted in the most efficient RNAi and play a role in recognition or binding of the RNA or protein components of RISC (Elbashir et al., 2001b). The Zamore group found that 5′ phosphates on the siRNA enhanced entry into the RISC, which they conclude may be a licensing step in the pathway, allowing only true siRNAs into the silencing pathway (Nykanen et al., 2001). Subsequently, it was found that siRNAs cleave cognate mRNAs across from nucleotides 10 and 11 from the 5′ end of the siRNA, and single nucleotide mismatches between the siRNA and mRNA can abolish cleavage by the elusive "Slicer" (Elbashir et al., 2001c).

Experimental results from in vitro studies using fly embryo lysates, and results observed in vivo, lead to a revised view of how the RNAi pathway functions. From these first few key papers it became clear that long dsRNA was processed by the RNAse III enzyme Dicer into shorter dsRNAs, siRNAs, that acted as the sequence-specific guides for target mRNA destruction. These siRNAs were the key RNA component in the RISC, which guides the nuclease responsible for the cleavage that results in gene silencing. These dsRNA intermediates could be separated into their single-strand components that

could target cognate sequences, either sense or anti-sense sequences, and cleave the mRNA across from the middle of the siRNA. The important steps in RNAi were coming to light, but the key proteins were still not identified.

Genetic studies in worms and *Neurospora*, combined with biochemical data from in vitro fly systems, would give more clues and insight as to how long dsRNA mediates specific gene silencing. Although cloning of endogenous siRNAs with sequences corresponding to transposons reaffirmed a role for RNAi in transposon silencing (Elbashir et al., 2001b), the genetic evidence for this role was first shown in worms (Ketting et al., 1999; Tabara et al., 1999). The Mello group searched for mutants that were resistant to RNAi and found two genes, *rde-1* and *rde-4*, that when mutated showed no obvious developmental or growth defects yet prevented worms from initiating RNAi (Tabara et al., 1999). However, several other RNAi mutants including *rde-2*, *rde-3*, *mut-2*, and *mut-7* exhibited reduced fertility, chromosome loss, and displayed mobilization of endogenous transposons, giving a first glimpse as to the natural role of RNA interference in repression of transposon hopping or in silencing other potentially harmful and foreign molecules such as viruses (Tabara et al., 1999). Interestingly, the Plasterk group showed that when looking for mutants that displayed activation of Tc1 transposons in the germline, some of these mutants also showed resistance to RNAi (Ketting et al., 1999). One of the genes identified, *mut-7*, encodes a protein that shares homology with Werner Syndrome helicase and RNase D (Ketting et al., 1999) and was also isolated in the Mello screen (Tabara et al., 1999).

In worms, RNAi can cross cell boundaries and be inherited in first generation progeny from injected long dsRNA, and genetic studies have provided insights into these characteristics. For example, a screen for mutant worms that showed a defect in RNAi in response to dsRNA introduced through feeding, but not by injection, revealed a loci containing *sid-1* (systemic RNAi deficient), which encodes a transmembrane protein potentially involved in uptake and spreading (Winston et al., 2002). In order to identify how the effects of RNAi are inherited in the worm, and if a mechanism for amplification leading to the observed long-lasting effect existed, the Mello group found that *rde-1* and *rde-4* were required in the initial stages of RNAi and were not required for transposon silencing, while *rde-2* and *mut-7* acted farther downstream (Grishok et al., 2000). Although the few known genes involved in RNAi could now be placed at specific steps in the pathway genetically, the mechanism of amplification in animals is still a matter of controversy (see below).

Previous genetic studies in *Neurospora* in which 15 mutants defective in quelling (Cogoni and Macino, 1997), the post-transcriptional gene silencing pathway in fungi, were isolated shed light onto proteins that may be acting in the RNAi pathway in *Neurospora*. One such protein could be an RNA-dependent RNA polymerase (RdRP), a protein that was shown to play an important role in quelling and is activated in response to expression of transgenes and endogenous genes sharing sequence with the transgene. In fungi, the RdRP homologous gene qde-1 (quelling defective) was isolated in the attempts to identify mutants in which transgene silencing was impaired (Cogoni and Macino, 1999), which is similar to the RdRP found in tomato that plays a role in the post-

transcriptional process known as co-suppression in plants (Schiebel et al., 1998). RdRPs in other organisms were known to play roles in silencing, including SDE1 (Dalmay et al., 2000) and SGS2 (Mourrain et al., 2000) in *Arabadopsis thaliana*. In worms, the gene found in the germ-line that is homologous to qde-1 and the tomato RdRP was identified as *ego-1*, of which some mutants displayed defects in germ-line development and resistance to RNAi (Smardon et al., 2000). Three worm paralogs of *ego-1* were identified and renamed *rrf-1*, *rrf-2*, and *rrf-3*, which may perform redundant functions as indicated by partial insensitivity to RNAi exhibited by some mutants (Smardon et al., 2000), although the RRF-1 protein is found in the soma (Sijen et al., 2001) and EGO-1 is found in the germ-line (Smardon et al., 2000). It appears the *rrf-2* may not play a role in RNAi, but *rrf-3* may be an inhibitor of RNAi (Sijen et al., 2001; Simmer et al., 2002).

There are several lines of reasoning as to why an RdRP would play a role in RNAi through comparison to PTGS in plants and quelling in fungi, as well as observations in flies. First, the tomato RdRP was shown to transcribe RNA from an RNA template in vitro, and therefore may perform a similar function in vivo in PTGS (Schiebel et al., 1993), which has been shown to be induced by the presence of RNA species such as exogenous dsRNA or transgenes, as well as viruses. Additionally, the degree of homology between the putative RdRP in worms and QDE-1 in fungi raises the possibility that these genes function in similar ways (Smardon et al., 2000). Finally, it was observed that only a few molecules of long dsRNA could mediate the silencing (Fire et al., 1998; Kennerdell and Carthew, 1998), suggesting that a method of amplification existed (Fire, 1999), possibly by the worm RdRP *ego-1* (Smardon et al., 2000).

A revised version of the RNAi pathway unfolded, in which worm *rde-1* and *rde-4* were likely to be involved in the processing of long dsRNA into siRNAs, the specificity determinants of the pathway which may recruit the nuclease responsible for mRNA destruction. Later it was found that *rde-1* mutants, which showed no response to dsRNA, produced normal levels of siRNAs (Parrish and Fire, 2001). In contrast, *rde-4* mutants produced much lower levels of siRNAs in response to injections of long dsRNA (Parrish and Fire, 2001). Therefore, it was determined that RDE-1 played a role downstream of siRNA production, while RDE-4 was essential in dsRNA processing (Parrish and Fire, 2001). It was also found that Dicer, which is related to RDE-1 and Argonaute family members, was responsible for the processing of long dsRNA into siRNAs in flies (Bernstein et al., 2001) in an ATP-dependent manner (Nykanen et al., 2001). Later it was found that DCR-1, the ortholog of Dicer in flies, was responsible for dsRNA processing in worms, and is required for functional RNAi (Ketting et al., 2001; Knight and Bass, 2001). DCR-1 is found in a complex with RDE-1 and DRH-1, an RNA helicase, indicating that these proteins may work together to produce siRNAs (Tabara et al., 2002). It was hypothesized that the double-stranded siRNAs may then incorporated into a ribonucleoprotein complex in a step that does not require ATP (Nykanen et al., 2001) and unwound into single strands by an unidentified helicase in an ATP-dependent fashion (Nykanen et al., 2001; Zamore et al., 2000). The active RNP, or RISC, can then go on and mediate target cleavage in an ATP-independent manner (Nykanen et al., 2001), leading to cleavage of the cognate mRNA at a single site (Elbashir et al., 2001b).

Argonaute gene family members, which were identified in various organisms, were also shown to play a role in post-transcriptional gene silencing. In worms, *rde-1* was shown to play a role in the initial steps of RNAi (Tabara et al., 1999), in plants AGO1 was identified in a screen for mutants impaired for PTGS (Fagard et al., 2000), and QDE-2 was isolated in fungi (Catalanotto et al., 2000). In flies, the Hannon group identified AGO2 as a component of RISC through size fractionation of soluble RISC purified to near homogeneity, and co-migration with siRNAs and sequence-specific mRNA degradation activity (Hammond et al., 2001). AGO2, which was identified by mass spectrometry, showed homology with AGO1 in plants (Bohmert et al., 1998; Fagard et al., 2000), eIF2C in humans, QDE-2 in fungi (Catalanotto et al., 2000), and *rde-1* in worms (Tabara et al., 1999). Of the four Argonaute proteins that were known to exist in flies, only AGO2 was identified as a component of RISC (Hammond et al., 2001). Even though AGO2 was identified as a component of RISC, the exact biochemical function had not been deciphered. Previous studies looking into the role of Dicer, the RNase III protein that processes long dsRNA, had identified a domain with unknown function called a PAZ domain (Bernstein et al., 2001). Interestingly, AGO also contained a PAZ domain, and the group hypothesized that it may be essential for interaction between the two proteins (Hammond et al., 2001). Although Dicer was not identified as a component of RISC, the PAZ domain may provide a means for interaction between Dicer and AGO2 to hand off the processed siRNAs to the silencing complex (Hammond et al., 2001).

At this point, several of the key steps in the RNAi pathway were identified, and clues from these findings led to the next big breakthrough. It was known that siRNAs, the silencing intermediates, have a specific architecture which includes 19 nucleotides of double-stranded RNA, 2 nucleotide 3´ overhangs, 3´ hydroxyls and 5´ phosphates (Boutla et al., 2001; Chiu and Rana, 2002; Elbashir et al., 2001b; Nykanen et al., 2001; Zamore et al., 2000). Long dsRNA had been used to mediate silencing in *Drosophila* S2 cells (Caplen et al., 2000; Clemens et al., 2000; Hammond et al., 2000; Ui-Tei et al., 2000), but a problem arose in using long dsRNA in cultured cells because it did not show a specific and potent RNAi knock-down in various cell lines including HEK 293, NIH 3T3, BHK-21, and CHO-K1 (Caplen et al., 2000; Ui-Tei et al., 2000). Instead, the dsRNAs were inducing the protein kinase response (PKR), also commonly known as the interferon response (Clemens, 1997). When dsRNA binds and activates PKR (Manche et al., 1992) and 2´, 5´-oligoadenylate synthatase (Minks et al., 1979), this causes sequence non-specific degradation by stalling translation and eventually leads to apoptosis.

When the Tuschl group found that siRNAs can mediate target mRNA cleavage in vitro, they postulated that these small RNAs could bypass the long dsRNA induced interferon response and be used in cultured cells to target a gene of interest (Elbashir et al., 2001b). Using 21 nucleotide siRNAs directed against firefly and renilla luciferases in HEK 293 , COS-7 and HeLa cells, they found that co-transfections using liposome-mediated technology resulted in a decrease in expression up to 25 fold (Elbashir et al., 2001a). Endogenous genes, including lamin A/C, lamin B1, nuclear mitotic apparatus protein (NuMA), and vimentin were targeted using siRNAs and assayed for knock-down

by Western blotting and some knock-downs were more than 90% complete (Elbashir et al., 2001a).

This finding opened the door to many possibilities, including investigation into gene function through RNAi-mediated knock-down. In addition, together with earlier findings that single nucleotide mismatches between the siRNA and the mRNA may block silencing (Elbashir et al., 2001b), siRNAs showed promise in selectively targeting a mutant form of a gene while keeping the wildtype copy intact. It was known that a central A-form helix was required for siRNAs to function efficiently (Chiu and Rana, 2002), so disrupting this conformation between a mutant siRNA and a wild-type mRNA would block cleavage and allow for normal protein expression from the wild-type mRNA. Several groups have taken advantage of this discovery for targeting mutant genes associated with diseases including amyotrophic lateral sclerosis (ALS) (Ding et al., 2003; Maxwell et al., 2004; Ralph et al., 2005; Raoul et al., 2005), Alzheimer's (Miller et al., 2004), slow channel congenital myasthenic syndrome (SCCMS) (Abdelgany et al., 2003), Huntington's (Harper et al., 2005), spinocerebellar ataxia type 1 (SCA1) (Xia et al., 2004), human immunodeficiency disorder (HIV) (Lee et al., 2005; Li et al., 2005), Machado-Joseph disease/spinocerebellar ataxia type 3 (Miller et al., 2003), and cancer (Martinez et al., 2002b). Specifically, purine:purine mismatches between the siRNA and mRNA increase the chance of discrimination between an siRNA and the wildtype allele so as that it is not degraded by disrupting the base pairing between the siRNA guide strand and mRNA to the greatest degree (Ding et al., 2003). While the molecular details of single nucleotide discrimination are being revealed, the real challenge exists in the

delivery, stability, and long-term potentiation of silencing in an animal model or human trial.

RNAi can also be used to target viruses and viral RNAs to stop infection in a host organism when infected with viruses including Hepatitis C (Kapadia et al., 2003; Korf et al., 2005; Kronke et al., 2004; Prabhu et al., 2005; Sen et al., 2003; Seo et al., 2003; Takigawa et al., 2004; Wilson et al., 2003; Yokota et al., 2003), Hepatitis B (Giladi et al., 2003; Hamasaki et al., 2003; McCaffrey et al., 2003; Moore et al., 2005; Ren et al., 2005; Shlomai and Shaul, 2003; Wu et al., 2005; Ying et al., 2003), Dengue virus (Adelman et al., 2002), HIV encoded RNAs (Capodici et al., 2002; Coburn and Cullen, 2002; Jacque et al., 2002; Lee et al., 2003a; Lee et al., 2002; Novina et al., 2002; Park et al., 2003; Park et al., 2002b; Song et al., 2003a; Surabhi and Gaynor, 2002), polio virus (Gitlin et al., 2002; Gitlin et al., 2005), influenza virus (Ge et al., 2004; Ge et al., 2003), and rotavirus (Dector et al., 2002). siRNAs hold great potential in therapeutics, but currently the limiting factors include stability, delivery, the emergence of mutated viruses that can escape RNAi-mediated silencing, and the establishment of a long-lasting effect. Presently, only one disease, macular degeneration, is being tested in clinical trials using siRNA technology to treat the overgrowth of blood vessels in the retina (for news see:(Check, 2004)).

MicroRNAs - How RNAi Regulates Eukaryotic Development

While the story of RNA interference unfolded, a related set of small, non-coding RNAs were coming to light. Unbeknownst to the scientists, studies revolving around the regulation of the expression of specific genes at particular times in development would be linked to an unknown class of tiny RNAs. The first studies linking tiny RNAs with development began as an investigation into the heterochronic mutants which had lesions in genes that caused the misexpression of proteins at incorrect stages of development resulting in improper execution of cell fates in the developing worm (Ambros and Horvitz, 1984; Chalfie et al., 1981; Horvitz and Sulston, 1980; Sternberg and Horvitz, 1984; Sulston and Horvitz, 1981). Victor Ambros pioneered this study by focusing on one of these heterochronic genes with defects in cell lineage, *lin-14*, which was repressed at certain stages of development (Ambros and Horvitz, 1987), and he witnessed a curious genetic puzzle unravel. He found that during the course of worm development, high levels of *lin-14* gene activity were correlated with expression of early cell fates in larval stage 1 (L1), whereas low levels of *lin-14* gene activity are correlated with late cell fates L2 and beyond (Ambros and Horvitz, 1984; Ambros and Horvitz, 1987). Ambros also observed that another heterochronic mutation in the gene *lin-4* appeared to regulate *lin-14* as if it was epistatic, however, at the time, a clear model explaining how *lin-4* and *lin-14* interacted to regulate *lin-14* activity could not be established.

A glimpse into the mode of regulation by *lin-14* activity came about when the mapping, characterization and cloning of the *lin-14* locus was performed (Ruvkun et al., 1989). Two alleles were isolated in which *lin-14* activity was elevated in late stages, and sequences from the mature mRNA were deleted in these mutants indicating that these

sequences were important in regulation of *lin-14* expression (Ruvkun et al., 1989). Later it was found that sequences within the 3′ untranslated region (UTR) of *lin-14* were responsible for the negative regulation of the translated nuclear protein (Wightman et al., 1991), and this regulation provided the temporal switch determining early and late cell fates (Ruvkun and Giusto, 1989). In an attempt to determine if other heterochronic genes may be involved in the regulation of *lin-14* expression, the Ruvkun lab found that *lin-4* is required for the downregulation in L1, and therefore the *lin-4* gene product is the most likely candidate to interact with the negative regulatory element that resides in the 3′ UTR of the *lin-14* mRNA (Arasu et al., 1991).

When the Ambros lab finally cloned the *lin*-4 gene, which they knew genetically regulated *lin-14*, they were surprised to find that the gene product of *lin-4* was not a protein, but instead two small transcripts of ~22 and 61 nucleotides were identified, the longer of which was the precursor of the small RNA, predicted to fold into a stem loop structure (Lee et al., 1993). These small RNAs encoded by *lin-4* shared partial homology with sequences in the 3′ UTR of the *lin-14* mRNA, suggesting that the regulation of *lin-14* expression and translation occurred via an RNA:RNA interaction (Lee et al., 1993; Wightman et al., 1993). The RNA:RNA interactions were thought to occur at seven sites within the UTR, four of which do not result in perfect pairing, but instead result in a "bulged" interaction at a C nucleotide, illustrating the importance of the RNA secondary structure at these sites (Ha et al., 1996). In addition, developmental profiles of *lin-4* show that accumulation of the RNA correlates with the downregulation of LIN-14 expression (Feinbaum and Ambros, 1999). The developmental expression profiles of the two genes,

the presence of *lin-4* complementary sites in the 3′ UTR of *lin-14*, along with mutational analysis, show that *lin-4* is controlling the timing of *lin-14* expression, although it was still unclear as to the molecular details of regulation.

Finally, approximately eight years after the discovery that *lin-4* may be involved in *lin-14* expression, the Ambros group went on to show that *lin-14* mRNA synthesis, state of polyadenylation, abundance in the cytoplasm, and polysome profiles do not change (Olsen and Ambros, 1999). This suggests that the regulation occurs at a step following translation initiation, including elongation or release of the mature protein (Olsen and Ambros, 1999). *Lin-28*, another heterochronic gene, was later identified as a second target of *lin-4* regulation because it also contained a sequence in the 3′ UTR that is complementary to the *lin-4* RNA (Moss et al., 1997). A similar mechanism of translational repression was observed for *lin-28* regulation by *lin-4*, suggesting that gene expression is fine-tuned at a step following the initiation of translation (Seggerson et al., 2002).

Despite the advances in understanding the regulation of the *lin-4* small RNA, many scientists thought that the *lin-4* story was a curious one found only in worms. However, in 2000, the Ruvkun lab identified a second gene encoding a 21 nucleotide RNA called *let-7* that displayed complementarity to the 3′ UTR of heterochronic genes including *lin-14*, *lin-28*, *lin-41*, *lin-42* and *daf-12* (Reinhart et al., 2000), and may also be derived from a stem-loop precursor RNA like *lin-4* (Pasquinelli et al., 2000). The longer precursor RNA decreased in abundance over time as the mature, 22 nucleotide *let-7* accumulated, indicating that the longer species was a bona fide stem-loop precursor

(Pasquinelli et al., 2000). In addition, *let-7* is capable of negatively regulating *lin-41*, an event that hypothetically relieves inhibition of another heterochronic gene and transcription factor lin-29 (Slack et al., 2000). Now that two small RNAs have been identified that regulate developmental timing and expression during cell fate determination, these molecules were called small temporal RNAs (stRNAs). The Ruvkun lab then showed that *let*-7 is conserved among several species, including vertebrates, hemichordates, annelids, and arthropods (Pasquinelli et al., 2000). This suggested that these small RNAs play large roles in complex regulatory networks, and hinted that many other small RNAs may exist in a variety of organisms.

The proof that more small RNAs existed arrived in 2001, when three groups used cloning and bioinformatics strategies to identify over 100 small RNAs from flies, worms, and cultured human cells (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). These RNAs were ~22 nucleotides in length, the same as *lin-4* and *let-7*, while genomic and Northern analysis indicated that these RNAs could also be derived from stem-loop precursors. However, not all of the small RNAs were temporally regulated like *lin-4* and *let-7*, so a new term, microRNAs (miRNAs) was selected for this new class of non-coding RNAs. Searches revealed that these small RNAs can reside on either arm of the stem-loop precursor encoded in the genome, were conserved over evolution to varying degrees, and were found in various different tissues (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001).

Additional cloning efforts revealed that miRNAs were present in numerous organisms including worms (Ambros et al., 2003; Lim et al., 2003b), human cells (Dostie

et al., 2003; Kim et al., 2004; Lagos-Quintana et al., 2003; Mourelatos et al., 2002), mouse (Lagos-Quintana et al., 2003; Lagos-Quintana et al., 2002), embryonic stem cells (Houbaviy et al., 2003), flies (Aravin et al., 2003), plants (Reinhart et al., 2002; Sunkar and Zhu, 2004), rice (Sunkar et al., 2005; Wang et al., 2004a), zebrafish (Chen et al., 2005; Lim et al., 2003a) and even viruses (Bennasser et al., 2004; Pfeffer et al., 2005; Pfeffer et al., 2004).

These cloning efforts took advantage of the 2001 finding that Dicer, the enzyme responsible for the processing of long dsRNA (Bernstein et al., 2001), was also involved in maturation of miRNAs from the stem loop precursors in worms (Grishok et al., 2001) as well as flies and humans (Hutvagner et al., 2001). In worms genetic studies revealed that *rde-1* homologs *alg-1* and *alg-2*, along with *dcr-1*, are necessary for the maturation of miRNAs *lin-4* and *let-7* (Grishok et al., 2001). Studies in flies showed that the long stem loop RNA that was suspected to be the precursor for *let-7* was the true precursor and that only the *let-7* RNA sequence from the 5′ arm of the stem loop structure was detected as the mature single-stranded miRNA (Hutvagner et al., 2001). Analysis of the termini of the mature miRNAs showed that the 3′ end bore 2′, 3′ terminal hydroxyls and 5′ end possessed a monophosphate, indicative of processing by RNasee III enzymes (Hutvagner et al., 2001). The 2001 efforts to clone additional small non-coding RNAs took advantage of the research showing that stRNAs were processed by Dicer leaving characteristic termini, which was adapted into the small RNA cloning protocols (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001).

miRNAs are derived from longer transcripts that are found in intragenic regions or within introns of pre-mRNAs, and can be found singularly or within a cluster as a primary miRNAs (pri-miRNAs) that may be related (Lagos-Quintana et al., 2001; Lau et al., 2001). Most of the miRNAs are conserved among closely related species (Lagos-Quintana et al., 2003; Lim et al., 2003a; Lim et al., 2003b), but some miRNAs show broad conservation among more distantly related species (Ambros et al., 2003; Aravin et al., 2003; Lagos-Quintana et al., 2003; Lim et al., 2003b). miRNA expression levels can vary from extremely high to very low within an organism, with some miRNAs expressed only in individual cell types such as neurons (Johnston and Hobert, 2003; Lim et al., 2003b).

Although the identification of additional miRNA genes remains an interesting topic, the next burning question in the field was to find target mRNAs that are regulated by all the newly identified miRNAs and to determine if miRNA targets might represent a specific type of mRNA. The mRNA targets of the two founding members of the miRNAs, *lin-4* and *let-7*, had been identified by genetic studies with mutants defective in heterochronic development. Since the finding of the new non-coding RNA family, only a few target/miRNA pairs have been identified experimentally. This includes the fly bantam miRNA that targets the apoptotic gene *hid* (Brennecke et al., 2003), and miR-14 whose target is not identified but is involved in fat metabolism and apoptosis (Xu et al., 2003). In mammals, miR-181 was found to play a role in hematopoietic differentiation (Chen et al., 2004). Two additional miRNAs found in worms, *lsy-6* and miR-273, were found to play a role in left/right neuronal asymmetry by targeting *cog-1* and *die-1*

transcription factors, respectively (Chang et al., 2004; Johnston and Hobert, 2003). The vertebrate miR-196a was shown to be involved in anterior/posterior patterning by targeting *Hoxb8*, *Hoxc8*, *Hoxd8* and *Hoxa7* (Mansfield et al., 2004; Yekta et al., 2004). Although these examples indicate experimental progress in identifying miRNA targets, the number of experimentally verified examples is still low compared to the hundreds of animal miRNAs identified.

In plants, however, the identification of miRNA targets has proceeded much more rapidly than in animals, with several miRNA targets identified to be transcription factors that contribute to leaf, flower, and meristem formation (Achard et al., 2004; Aukerman and Sakai, 2003; Baker et al., 2005; Chen, 2004; Emery et al., 2003; Juarez et al., 2004; Kasschau et al., 2003; Kidner and Martienssen, 2004; Kim et al., 2005; Laufs et al., 2004; Llave et al., 2002b; Mallory et al., 2005; Mallory et al., 2004a; Mallory et al., 2004b; McHale and Koning, 2004; Palatnik et al., 2003; Rhoades et al., 2002; Tang et al., 2003).

Unlike animal miRNAs, plant miRNAs share near perfect complementarity with their targets, making it more straight-forward to identify putative target mRNAs (Jones-Rhoades and Bartel, 2004; Rehmsmeier et al., 2004; Rhoades et al., 2002; Wang et al., 2004b; Zhang, 2005). Some of these predicted plant targets were then verified experimentally or through phylogenetic searches (Kasschau et al., 2003; Llave et al., 2002a; Llave et al., 2002b; Park et al., 2002a; Xie et al., 2003). Whereas most animal miRNA:target pairs identified to date result in translational repression, plant miRNAs predominantly mediate an mRNA cleavage event (Kasschau et al., 2003; Llave et al., 2002a; Rhoades et al., 2002; Tang et al., 2003) typically characteristic of siRNAs. Other

differences between the plant and animal miRNAs exist. First, plant miRNAs are thought to derive from precursor stem loops that are larger than their animal counterparts, and the extent of base pairing along the stem of the plant miRNA precursors is higher (Reinhart et al., 2002). However, while animal pre-miRNAs can be detected by Northern blots, plant pre-miRNAs have been difficult to detect. Second, the majority of plant miRNAs are only 21 nucleotides in length, as compared to the wider size range characteristic of animal miRNAs (Reinhart et al., 2002).

But since animal miRNAs do not perfectly pair with their targets, and it was not known at the time how miRNAs could specifically pair to the 3′ UTR of the mRNAs that they regulate, identifying their targets became more of a challenge because the lower degree of base-pairing reduces the signal-to-noise ratio when comparing potential targets versus randomized sequence controls. In addition, some miRNAs are only expressed at certain stages of development or within specific cell types or tissues (Baskerville and Bartel, 2005; Thomson et al., 2004), making it even more difficult to show if candidate target mRNAs are truly being regulated by a miRNA, since co-expression of a miRNA and an mRNA in the same cell is required for regulation to occur. Scientists then turned to bioinformatics to compliment the experimental data (Enright et al., 2003; Kiriakidou et al., 2004; Lewis et al., 2003; Rajewsky and Socci, 2004; Stark et al., 2003), some of which were verified experimentally following computational predictions (Kiriakidou et al., 2004; 2003; Lai et al., 2003; Lewis et al., 2003; Stark et al., 2003). Several different groups place more importance on certain factors contributing to miRNA-mediated down-regulation of gene expression.

The bioinformatics approaches take several factors into account, including number of recognition sites for a certain miRNA present in a particular 3′ UTR, where some groups consider at least 2 sites to be significant (Lewis et al., 2003) and others require only one response element to be a considered a significant hit (Kiriakidou et al., 2004). Most groups place a high level of importance as to where the response elements are located within the mRNA, with high importance placed on the 3′ UTR for that is where the *lin-4* and *let-7* binding sites were found. If the miRNAs are mediating translational repression at a point following initiation, then a location on the mRNA far from ribosome loading and clearing would be key. This classification may be somewhat premature as a single imperfect site capable of mediating translational repression was identified in a mammalian construct (Saxena et al., 2003).

Bioinformatic analysis of miRNA sequence conservation has yielded an important insight into miRNA and siRNA function. Clustering of related miRNA family members indicated that the base pairs shared in common were in the 5′ end, while some studies examined the degree of miRNA sequence conservation across species (Lewis et al., 2003), or specific conservation of nucleotides (Lewis et al., 2005). It was observed that the conservation at the 5′ end of the miRNA pairing to the target mRNA exhibited the strongest conservation, suggesting that this might represent the most critical aspect of miRNA recognition of its target (Lai, 2002; Lewis et al., 2005; Lewis et al., 2003; Stark et al., 2003). Nucleotides 2 – 8 at the 5′ end of the miRNA are called the 'seed' region, and several lines of experimental evidence have confirmed the functional importance of this region (Brennecke et al., 2005; Doench and Sharp, 2004; Haley and Zamore, 2004;

Lim et al., 2003b). Thus, siRNAs targeting a specific gene may mediate miRNA-like translational repression if the level of base pairing in the 'seed' region is extensive in non-targeted genes. This concept has been proposed to explain the off-target effects where unintended mRNAs get downregulated by an siRNA that simply binds with the 'seed' region (Jackson et al., 2003). In addition, the 5′ end was shown to disproportionately contribute to miRNA (Brennecke et al., 2005; Doench and Sharp, 2004; Lai et al., 2005; Mallory et al., 2004b) or siRNA binding to its target (Haley and Zamore, 2004; Jackson et al., 2003).

Increasing amount of evidence blurs the lines between siRNAs and miRNAs. For example, when siRNAs were designed to imperfectly pair with CXCR4 sites engineered into the 3′ UTR of a luciferase reporter construct, they were able to act like miRNAs and mediate translational repression as measured by the knock-down of renilla luciferase protein, but the levels of mRNA remained fairly constant (Doench et al., 2003). In turn, while an siRNA can be designed to function like a miRNA, a miRNA that is endogenously programmed into an RNP complex is capable of mediating mRNA cleavage when presented with a target that is perfectly complementary to the miRNA sequence (Hutvagner and Zamore, 2002). These results taken together suggest that the extent of complementarity between a target and a small RNA dictates the mode of regulation, either mRNA cleavage or translational repression (Doench et al., 2003; Hutvagner and Zamore, 2002; Zeng et al., 2003). Additional insights came when another group identified numerous different miRNAs in a complex called the miRNP (now collectively referred to as RISC), which contains eiF2C2 (Mourelatos et al., 2002), a

member of the Argonaute family previously shown to be a member of RISC (Hammond et al., 2001). Another member of the RNAi pathway, Dicer, was previously shown to play roles in both siRNA and miRNA production (Grishok et al., 2001; Hutvagner et al., 2001). This information presented the possibility that both silencing pathways have several key factors in common.

However, it was still not known exactly how miRNAs mediated translational repression, at what point translation was affected, and what other proteins may be involved in the process, and the role that miRNAs may be playing in developmental and biological processes as only a few miRNAs have been characterized in which targets have been identified. Two groups came up with a way to determine the function of each individual gene by blocking miRNA function with a $2'$-O-methyl oligo (Hutvagner et al., 2004; Meister et al., 2004a), a method that has been used to look at several miRNAs in development (Leaman et al., 2005). Instead of mutating or knocking out the regions that contain miRNA genes, this method can be used to irreversibly block a RISC that contains a specific miRNA in order to access what biological role it may normally being playing. Now that a method existed to assay miRNA function, the next question addressed how these miRNAs mature.

When looking for the protein that processes long primary miRNAs (pri-miRNAs), the Kim group found that the nuclear protein Drosha, an RNAse III enzyme, cleaved the stem loop pre-miRNA from these longer capped and polyadenylated transcripts (Lee et al., 2003b) which are transcribed by RNA Polymerase II (Bracht et al., 2004; Cai et al., 2004; Lee et al., 2004a). This first cut made by Drosha establishes one end of the

miRNA (Basyuk et al., 2003; Lee et al., 2003b). Later, several groups found that Drosha

required the presence of a dsRNA binding partner, Pasha (DGCR8 in humans), whose

function remains unknown (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004;

Landthaler et al., 2004). Following processing by Drosha, the pre-miRNA is exported to

the cytoplasm by Exportin 5 in a Ran-GTP dependent manner (Bohnsack et al., 2004;

Lund et al., 2004; Yi et al., 2003; Zeng and Cullen, 2004) where the second cut is made

by Dicer (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001).

In flies, Dcr-1 was found to have a dsRNA-binding partner, called Loquacious

(Forstemann et al., 2005) or R3D1 (Jiang et al., 2005), or in humans identified as TRBP

(Chendrimada et al., 2005), that facilitates the function of Dicer in processing miRNAs

from pre-miRNAs. Depletion of these dsRNA-binding partners results in accumulation

of pre-miRNAs, loss of maintenance of germ-line stem cells and sterility in male and

female flies (Forstemann et al., 2005; Jiang et al., 2005). In humans, the Dicer-TRBP

heterodimer was shown to interact with hAgo2 (Chendrimada et al., 2005). To date, all

known RNAse III enzymes that play a role in RNA silencing and production of small

RNAs have been shown to function via interaction with a dsRBD partner.

siRNA Function and RISC Composition

While it was known that siRNAs cleaved homologous targets, several questions still

remained, including which proteins form the final silencing complex and whether

siRNAs contribute to an amplification step in flies and humans. Beyond Argonaute proteins, it was shown that dFXR, the fly homolog of the human fragile X mental retardation protein (FMRP), is another component of RISC which associates with miRNAs, siRNAs, and characteristic nuclease activity (Caudy et al., 2002; Ishizuka et al., 2002). dFXR was also shown to interact with vasa intronic gene (VIG), which contains a domain for RNA binding (Caudy et al., 2002). The exact role of these proteins in RNA silencing still remains under investigation. The Hannon group identified an additional protein, Tudor-SN (TSN), a homolog of Staphylococcus nuclease, found to be associated with RISC (Caudy et al., 2003). Tudor-SN is conserved in humans, worms, and flies, and contains a nuclease domain, which lead researchers to postulate that this protein may contribute to the 'slicer' activity characteristic of RISC (Caudy et al., 2003).

miRNAs and siRNAs were also shown to be associated with the human Argonatue2 homolog, eiF2C2, in addition to Gemin3, a known DEAD-box helicase, and Gemin4, a protein of unknown function (Mourelatos et al., 2002). Finally, it was shown that eiF2C1 and/or eiF2C2 contained single-stranded RNA when double-stranded siRNA was added to lysate, showing that the siRNA must be unwound by an unidentified helicase (Martinez et al., 2002a).

Amplification of siRNAs in flies and humans was once a controversial topic. An RNA-dependent RNA Polymerase (RdRP), a component that had been identified in worms, fungi, and plants, had not been identified in humans or flies. An RdRP would act to amplify the silencing signal, but no evidence of an RdRP existed in the sequenced genome of flies or humans. One group asserted that siRNAs were acting as primers for

synthesis of additional dsRNA molecules, which would then be processed by Dicer into siRNAs to amplify the silencing signal (Lipardi et al., 2001). However, it was shown that replacing the 3´ OH, which would be required for polymerization, on the siRNA with groups that would inhibit addition of nucleotides on the 3´ end had no effect on efficiency of silencing (Schwarz et al., 2002). In fact, single-stranded siRNAs could be added directly to fly embryo lysate, HeLa S100 lysate or transfected into cultured human cells and still elicit RNA cleavage when perfectly paired to the substrate mRNA, although the response was much less robust due to higher instability of unprotected single-stranded RNA (Martinez et al., 2002a; Schwarz et al., 2002). Both groups noted the importance of a 5´ phosphate on the single-strand siRNA to mediate efficient mRNA destruction, with an absolute requirement for a 5´ phosphate in fly lysate (Martinez et al., 2002a; Schwarz et al., 2002), which was previously shown to be a requirement for duplexed siRNAs in fly lysates as well (Nykanen et al., 2001).

But were there any other requirements for efficient assembly of an siRNA into RISC? Previously, it was shown that both strands of a double-stranded siRNA can be incorporated into RISC separately and drive the cleavage of a homologous target, either sense or anti-sense (Elbashir et al., 2001b; Elbashir et al., 2001c; Nykanen et al., 2001). Because siRNAs are double-stranded prior to unwinding and incorporation into RISC as single strands, an equal population of sense-containing and anti-sense-containing RISCs could exist (Elbashir et al., 2001b). However, several groups have discovered that not all siRNAs deliver the same extent of silencing, which should not be the case if each strand of an siRNA has the same chance to incorporate into RISC.

Differences in structure, sequence or target accessibility may play a role in the varied efficiencies of different siRNAs. However, each strand of an siRNA is not equally eligible for entry into RISC, but the relative stabilities of the 5′ ends of the siRNA strands is what determines the ability of each strand to be assembled into RISC (Khvorova et al., 2003; Schwarz et al., 2003). Nearest neighbor calculations allowed for the determination of free energy differences (Borer et al., 1974; Gralla and Crothers, 1973a; Gralla and Crothers, 1973b; Tinoco et al., 1973; Tinoco et al., 1971) between the two ends by calculating the contribution that each of the last four nucleotides contribute to the overall thermodynamic properties of the siRNA (Figure 1). For each siRNA, the strand whose 5′ end is less tightly paired is assembled into RISC while the other strand is degraded (Schwarz et al., 2003). If the difference in free energy between both ends is great enough, siRNAs can be functionally asymmetric with only one strand capable of entry into RISC (Khvorova et al., 2003; Schwarz et al., 2003). By unpairing the 5′ most nucleotide on a guide strand of an siRNA and creating a single nucleotide mismatch at the termini, that strand can enter RISC to the near exclusion of the other strand (Schwarz et al., 2003).

Asymmetric loading of the short double-stranded RNAs into the silencing complex may explain one important step in the biogenesis of the other short RNAs capable of post-transcriptional gene silencing, including miRNAs. miRNAs can derive from either the 5′ or 3 ′ side of the stem loop from which they reside, or in some cases two miRNAs are processed from the same predicted fold-back structure based on the frequency of cloning (Lagos-Quintana et al., 2001; Lau et al., 2001). However, the

Figure 1

5´-CGUACGCGGAAUACUUCGAAA-3´
●●●●●●●●●●●●●●●●●●●●
3´-GUGCAUGCGCCUUAUGAAGCU-5´

5´-CGUACGCGGAAUACUUCGAAA-3´
●●●●●●●●●●●●●●●●●●●●   first 3´ dangle (U next to C/G): – 0.6
3´-GUGCAUGCGCCUUAUGAAGCU-5´

5´-CGUACGCGGAAUACUUCGAAA-3´
●●●●●●●●●●●●●●●●●●●●   C/G pair (next to G/C): – 2.36
3´-GUGCAUGCGCCUUAUGAAGCU-5´

5´-CGUACGCGGAAUACUUCGAAA-3´
●●●●●●●●●●●●●●●●●●●●   G/C pair (next to U/A): – 2.24
3´-GUGCAUGCGCCUUAUGAAGCU-5´

5´-CGUACGCGGAAUACUUCGAAA-3´
●●●●●●●●●●●●●●●●●●●●   U/A pair (next to A/U): – 1.33
3´-GUGCAUGCGCCUUAUGAAGCU-5´

5´-CGUACGCGGAAUACUUCGAAA-3´
●●●●●●●●●●●●●●●●●●●●   A/U pair (next to C/G): – 2.24
3´-GUGCAUGCGCCUUAUGAAGCU-5´

first 3´ dangle (U next to C/G): – 0.6
C/G pair (next to G/C): – 2.36
G/C pair (next to U/A): – 2.24
U/A pair (next to A/U): – 1.33
A/U pair (next to C/G): – 2.24

total = – 8.77 kcal/mole

Figure I - 1. Free energy calculations of siRNA ends. Thermodynamic calculations were based on the last 4 paired nucleotides of an siRNA end plus the free energy contributed by the 3' overhang. Free energy (kcal/mol) was added together to determine the total free energy for a particular siRNA end. Values were based on Xia et al., 'Chapter 2. Thermodynamics of RNA Secondary Structure Formation' in *RNA* pp 21 – 48.

majority of mature miRNA sequences are represented by only one strand from one of the arms of the stem-loop precursor. The precursor miRNA (pre-miRNA) can be viewed as an intermediate that resembles an siRNA if the loop portion is ignored (Hutvagner and Zamore, 2002; Lim et al., 2003b; Reinhart et al., 2002). In this siRNA-like intermediate, the free energy of the 5′ end of the strand that is more weakly paired to its complement can be incorporated into the silencing complex while the other strand is destroyed, explaining why miRNAs accumulate as single-strands in vivo (Khvorova et al., 2003; Schwarz et al., 2003) (Figure 2). If the free energies of the 5′ ends of the intermediate are relatively equal then both strands may be incorporated into the complex, but for each complex only one strand may be incorporated. This concept could also explain why, for some miRNAs, both arms of the precursor can be processed into mature miRNAs (Khvorova et al., 2003; Schwarz et al., 2003) and be detected in cloning experiments (the more abundant miRNA is referred to as miR while the less abundant sequence is called miR*) (Lau et al., 2001).

The asymmetric loading concept can be utilized to predict which arm of a pre-miRNA could be processed into the mature miRNA, and can be incorporated into new approaches for designing effective siRNAs based on thermodynamics (Khvorova et al., 2003; Reynolds et al., 2004; Schwarz et al., 2003). A difference in a single nucleotide at the end of the siRNA can flip the asymmetry; therefore, a point mutation in a stem loop structure could also alter miRNA expression profiles. Advantages of rational design include increasing the potential to create an effective siRNA, and also the possibility of lowering the concentration of delivery, as well as potentially decreasing unwanted off-

Figure 2
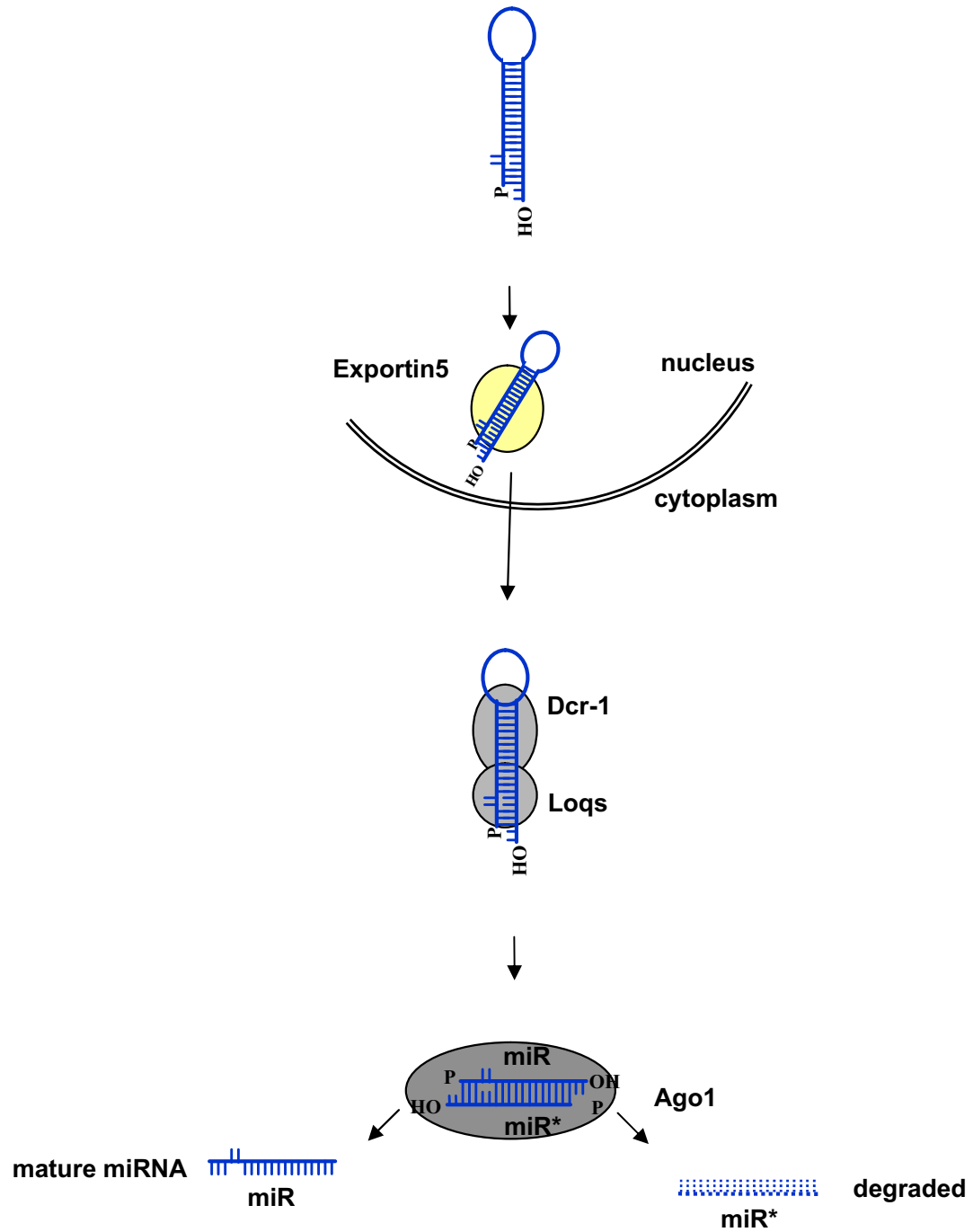
Figure I - 2. microRNA (miRNA) processing in *Drosophila*. miRNAs are processed in the nucleus by the RNase III enzyme Drosha leaving 5' phosphates and 3' hydroxyls with a 2 nucleotide, 3' overhang. The precursor miRNA (pre-miRNA) is exported out of the nucleus to the cytoplasm by Exportin 5 where it is bound by Dcr-1 and Loquacious. Dcr-1 makes the second cut on the stem, near the loop structure, liberating an siRNA-like intermediate called miR/miR*. The siRNA with the end that is less tightly paired will enter the silencing complex and become the mature miRNA whereas the strand with the more tightly paired end will be degraded. Once the mature miRNA is loaded into the Ago1 silencing complex it can then go on mediate translational repression.

target effects by exclusively allowing only one strand of the duplex to enter the silencing complex. While it is clear that there must be an enzyme capable of sensing the asymmetry of the siRNA by determining which end displays weaker pairing, this enzyme had yet to be identified.

A Detailed RISC Assembly Pathway

What protein factors might be good candidates for the enzyme that measures asymmetric unwinding of siRNAs? Flies are known to possess two dicer proteins — Dcr-1, which has been shown to play a role in miRNA maturation, and Dcr-2, which plays a role in siRNA production (Lee et al., 2004b). Dcr-2 has been show to play an additional role beyond long dsRNA processing, which is in loading one of the siRNA strands into RISC (Lee et al., 2004b). The single human Dicer may also function in loading siRNAs into RISC as seen in Dicer knock-down cells that can no longer elicit efficient gene silencing following siRNA transfections (Doi et al., 2003). An additional protein was found to associate with DCR-2 in *Drosophila* S2 cells when scientists were trying to determine how siRNAs are handed off from Dicer to RISC (Liu et al., 2003). This protein was called R2D2 for its two RNA binding domains (R2) and its association with Dcr-2 (D2) (Liu et al., 2003), and is similar to the worm protein RDE-4 (Grishok et al., 2000; Tabara et al., 2002).

Knowing some of the key proteins and steps in the pathway, two groups proposed revised pathways for RISC assembly in *Drosophila* (Pham et al., 2004; Tomari et al.,

2004a) (Figure 3). The siRNA is bound by R2D2, which recognizes the more stable end, and Dcr-2 in a complex known as the RISC Loading Complex (RLC) (Pham et al., 2004; Tomari et al., 2004a; Tomari et al., 2004b). Therefore, this heterodimer acts as a protein sensor in which R2D2 binds to the siRNA end that displays the highest double-stranded character, and forces Dcr-2 to bind the weaker end (Tomari et al., 2004b). The R2D2/Dcr-2 complex then may recruit the holo-RISC, which contains Dcr-1, VIG, dFMR, Tudor-SN (TSN), and Ago2 (Pham et al., 2004). The R2D2/Dcr-2/siRNA complex may bind the PIWI domain of Ago2, which is required for siRNA unwinding (Okamura et al., 2004), through the RNAse III domain, as seen in human Dicer (Tahbaz et al., 2004). Once the siRNA is unwound, by an as-yet-identified protein, the passenger strand is released and degraded (Schwarz et al., 2003), which leads to the formation of mature RISC that contains single-stranded RNA (Martinez et al., 2002a; Pham et al., 2004; Tomari et al., 2004a).

Once mature RISC is formed, it can then go on to mediate mRNA cleavage, but the protein that directs the cleavage had not been identified at that time. Clues as to what protein may be mediating this activity came from the analysis of the cleavage products that resulted from the RNA cleavage. RISC had previously been proposed to act as an endonuclease, but proof came when both a 5′ and 3′ cleavage product resulting from siRNA-directed mRNA cleavage were detected in a single experimental system (Schwarz et al., 2004). Cleavage was shown to occur at a single scissile phosphate, which could be blocked by a phosphorothioate substitution or reduced by a 2′-O-methyl substitution, in a Mg^{2+}-dependent manner liberating a 5′ cleavage product bearing a free 3′ hydroxyl

Figure 3
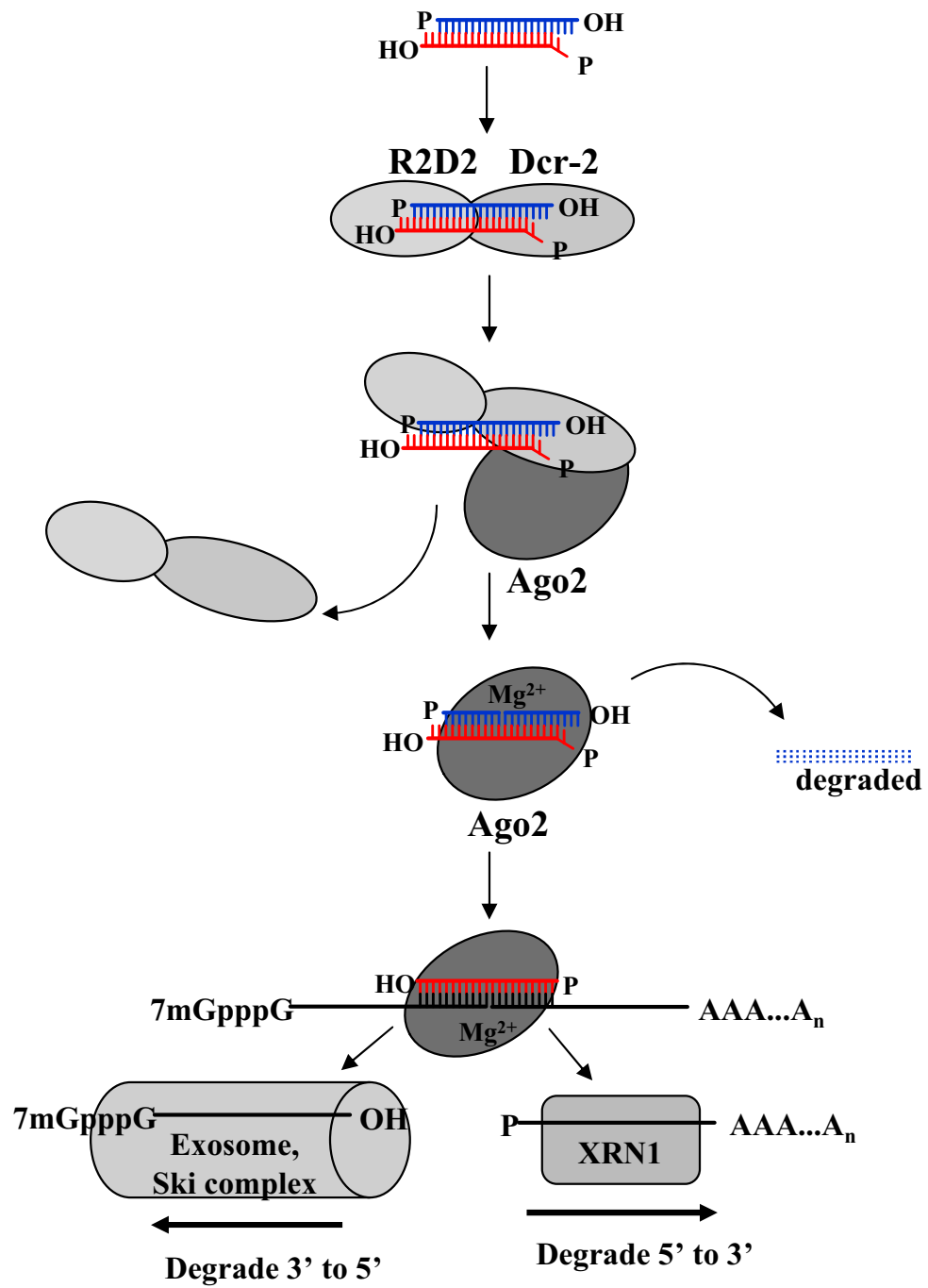
Figure I - 3. siRNA loading in RISC. siRNAs strands whose end is less tightly paired will be bound by R2D2, and the end that is less tightly paired will be bound by Dcr-2. The Dcr-2/R2D2/siRNA complex is called the RISC loading complex, or RLC. Ago2 will bind the siRNA, through an interaction with Dcr-2. It has been postulated that Ago2 can cleave the passenger strand of the siRNA, the strand that is more tightly paired, ultimately leading to the degradation of that strand and incorporation of the guide strand into RISC. Ago2 can then go on and cleave a perfectly paired target in a Mg^{2+}-dependent fashion. The resulting 5' cleavage product is then degraded by the Exosome/Ski complex and the 3' cleavage product is degraded by XRN1.

group, and a 3´ cleavage product bearing a 5´ phosphate (Martinez and Tuschl, 2004; Schwarz et al., 2004). This cleavage occurs at the same scissile phosphate as previously predicted (Elbashir et al., 2001b; Elbashir et al., 2001c).

Although the termini of the mRNA cleavage products were known, the protein that performs the cleavage had yet to be identified. Minimal Human RISC was shown to contain Argonaute family members eiF2C2 and/or eIF2C1, single-strand siRNA, and an unidentified endoribonuclease, although the size of the total complex was only 160 kDa (Martinez et al., 2002a; Martinez and Tuschl, 2004). In flies, RISC was shown to contain Ago2 and siRNA (Hammond et al., 2000; Hammond et al., 2001), although it was possible that an endonuclease could be recruited at a later step. One proposal suggested that Tudor-SN, a homolog of micrococcal nuclease, is the protein acting as the endonuclease that mediates target cleavage (Caudy et al., 2003). However, the presence of 3´ hydroxyls and 5´ phosphates on the termini of the cleavage products, in addition to insensitivity to 2´-deoxythymidine 5´, 3´-bisphosphate (pdTp), a known inhibitor of staphylococcal nucleases (Cuatrecasas et al., 1967), argues against the proposal that Tudor-SN is Slicer (Martinez and Tuschl, 2004; Schwarz et al., 2004). In fact, Tudor-SN was later shown to play a role in the binding of hyper-edited double-strand RNA promoting its cleavage, indicating a potential link between RNA editing and RNA silencing (Scadden, 2005).

It would take the combination of recombinant protein biochemistry and crystal structure analysis of Argonaute proteins to finally reveal which protein possessed the enigmatic Slicer activity. Argonaute proteins are the core components of all identified

RNA silencing complexes (Hammond et al., 2001; Hutvagner and Zamore, 2002; Martinez et al., 2002a; Mourelatos et al., 2002; Tabara et al., 1999). The number of Argonaute homologs identified in different species varies. For example, flies have five Argonaute protein family members (Williams and Rubin, 2002), and humans have eight Argonaute family members (Sasaki et al., 2003). The Argonaute family has been subdivided into two groups based on amino acid sequence called the Piwi and Ago subfamilies (Carmell et al., 2002). Argonaute proteins contain two domains, the PIWI domain (Carmell et al., 2002) and the PAZ domain (Cerutti et al., 2000). Recognition and RNA binding of the 3´ overhang and the base-paired ends of the siRNA are carried out by the PAZ domain (Lingel et al., 2003; Lingel et al., 2004; Ma et al., 2004; Song et al., 2003b; Yan et al., 2003), while it is believed that the PIWI domain is responsible for protein-protein interactions with Dicer, potentially facilitating the hand-off of an siRNA into an Argonaute complex (Doi et al., 2003; Pham et al., 2004; Tahbaz et al., 2004).

Different Argonaute proteins appear to perform different functions in relation to RNA silencing. For example, *Drosophila* Ago1 has been shown to be required for miRNA accumulation, Ago2 is required for RNAi mediated by dsRNA (Okamura et al., 2004). Both Ago1 and Ago2 have been shown to be associated with miRNAs (Caudy et al., 2002; Ishizuka et al., 2002; Williams and Rubin, 2002), but the cleavage activity of these complexes was not tested. Other Argonaute family members, including *aubergine* and *piwi*, have been shown to play a role in RNA silencing (Aravin et al., 2001; Kennerdell et al., 2002; Pal-Bhadra et al., 2002). While it was known in humans that eiF2C1 and eiF2C2, human Argonaute homologs 1 and 2, are associated with RNA

silencing (Hutvagner and Zamore, 2002; Martinez et al., 2002a; Mourelatos et al., 2002), the function of the two other members in the Ago subfamily had not been investigated. Over-expression studies of epitope-tagged Argonautes in human cultured cells suggested that Agos 1 – 4 all bind miRNAs, but only Ago2 possess the ability to mediate siRNA- and miRNA-dependent target cleavage (Meister et al., 2004b). Given the high levels of conservation in the PIWI and PAZ domains of the four human Argonaute family members, one might reason that the distinct residues required for RNA catalysis must reside elsewhere in the protein. However, the Hannon group showed that mutations in three key residues within the Piwi (or RNase H) domain actually abolish the 'slicer' activity of human Ago2 in cultured cells (Liu et al., 2004). In vitro data also confirmed a previous study in which a divalent metal requirement exists for cleavage activity (Liu et al., 2004; Schwarz et al., 2004). Additionally, recombinant hAGO bound to single-stranded siRNA exhibits cleavage activity (Rivas et al., 2005).

Verification of catalytic ability of Ago2 can be drawn from recent structural studies. For example, insights on the nature of mechanism of human Ago2 can be drawn from crystal structures of Ago-like proteins from archaea and eubacteria (Parker et al., 2004; Song et al., 2004). It appears that the Piwi domain (similar to an RNase domain) contains key catalytic residues which could potentially contribute to the 'slicer' activity (Parker et al., 2004; Song et al., 2004), which also show metal dependence (Nowotny et al., 2005; Wintersberger, 1990) and production of 5´ phosphates and 3´ hydroxyls (Wintersberger, 1990). Interestingly, structure studies suggest that the binding of the 3´ end of the siRNA by the PAZ domain would position the mRNA in the Piwi active site

such that the scissile phosphate that is cleaved would be located at close proximity to the putative catalytic residues (Elbashir et al., 2001b; Song et al., 2004). Recently, it has also been postulated that Ago2 is also responsible for cleaving the passenger strand, the strand that is not incorporated into RISC, leading to its degradation (Matranga et al., manuscript submitted).

Finally, structures of a Piwi protein solved with siRNA mimics give further insight into the cleavage reaction. The exclusion of the first base pair from contributing a role in miRNA and siRNA target recognition was first observed bioinformatically due to lack of conservation among miRNAs in different species (Lewis et al., 2005; Lewis et al., 2003), and was observed empirically in siRNA-mediated target cleavage (Haley and Zamore, 2004). The 5′ phosphate of the guide strand has been shown to be anchored into a binding pocket, and hence is not able to interact or base pair with the mRNA at this position (Ma et al., 2005). This finding now explains why the first base pair of miRNAs or siRNAs is excluded from the 'seed' sequence, nucleotides 2-8.

Despite our understanding of the target mRNA cleavage mechanism by siRNAs, and the functional identification of the members involved in the RNAi pathway, including the atomic details of some of these proteins, several questions remained. For example, it is not clear how the target mRNA is degraded following the endonucleolytic cleavage induced by RISC. One group has evidence showing that the 5′ cleavage products are degraded by the exosome and the Ski complex in *Drosophila* in a 3′ to 5′ direction (Orban and Izaurralde, 2005). In addition, the 3′ cleavage products were shown to be degraded by XRN1 in a 5′ to 3′ direction (Orban and Izaurralde, 2005).

Taken together, these results suggest that the mRNA degradation occurs without a decapping or deadenylation step.

Several unresolved questions still remain including how the two cleavage products are released from RISC, what kinase phosphorylates exogenously added siRNAs, how siRNAs are unwound, and what role different Argonaute proteins play in RNAi and if certain miRNAs are pre-destined to be loaded into a specific Argonaute. In addition, it is still unknown what the exact mechanism of translational repression is, or if the mRNA may be regulated at the step of RNA stability. Recent findings suggest that miRNA-mediated regulation may include shuttling of mRNAs to processing bodies, or p bodies, sites of RNA storage and degradation (Liu et al., 2005; Sen and Blau, 2005). These findings may aid in the study of how miRNAs regulated post-transcriptional gene silencing, but as of now that answer is unclear.

RNAi 2001

In 1998 when Fire and Mello determined that dsRNA mediates RNAi and results in a decrease in mRNA levels, several groups started to dissect the pathway in *Drosophila* embryo lysates. Key discoveries were made showing that long dsRNA is processed into 21 nucleotide intermediates called small interfering RNAs (siRNAs) by the RNaseIII enzyme Dicer. Dicer was also shown to be involved in the processing of another class of small RNAs called microRNAs (miRNAs) from longer stem loop RNA precursors. Although siRNAs and miRNAs are related in size, they appeared to be regulating gene expression in very different ways. siRNAs mediate post-transcriptional gene silencing by degrading mRNA, whereas miRNAs function at the level of translational repression. In addition, while siRNAs are double-stranded, mature miRNAs accumulate as single strands.

In 2001 when I joined the Zamore lab, several exciting discoveries were made showing that miRNAs were much more abundant than previously predicted and were detected in plants, worms, humans, and flies. siRNAs, which were known to be the intermediates in the RNAi pathway, found a new role in vivo when the Tuschl lab showed that synthetic siRNAs could be transfected into cultured cells and used to target any gene of interest. Several labs adopted this rapid method for gene knock-down, but it became clear that while some siRNAs were extremely potent others, showed very little silencing potential. In addition, it was still unknown how siRNAs mediated mRNA cleavage and what proteins may be involved.

In worms, it was known that an RNA-Dependent RNA polymerase amplifies the silencing signal through the binding of the single-stranded siRNA to a target RNA and generating dsRNA through the action of an RdRP, which can then be processed by Dicer into a secondary set of siRNAs. One of my first projects in the lab was to determine if an RdRP was functioning in flies and humans by looking for structural requirements of siRNAs that would be conducive to polymerization, specifically the requirement for a 3′ hydroxyl that would be necessary for the addition of NTPs (Chapter 2). We found no requirement for a 3′ hydroxyl, but an absolute requirement for 5′ phosphates, in fly and human systems. In addition we found that single-stranded siRNAs, while not as stable as double-stranded siRNAs, also enter the RNAi pathway and mediate the destruction of complementary mRNA.

The next question that we had concerned the finding that some siRNAs mediated a more robust RNAi response with some clues coming from miRNAs, the other class of small non-coding RNAs. A survey of mature miRNA sequences identified a preference for a 5′ U, leading to an analysis for a potential role of specific sequences in siRNA efficiency (Chapter 3). We found that the thermodynamics of the ends of the siRNAs determine the degree to which each strand incorporates into the active silencing complex, also giving clues as to how miRNAs are processed.

While it was known that siRNA treatment resulted in the destruction of a complementary mRNA, it was not known how this cleavage event occurred, if it was an endonucleolytic process, and if a metal dependence existed. At the time, a Staphylococcal nuclease family member TudorSN was shown to be associated with the

mature silencing complex and would require Ca^{2+}, could be inhibited by a specific Staphylococcal nuclease inhibitor, and would produce specific ends on the RNA products that resulted from the cleavage event. We found that RNAi required Mg^{2+}, could not be inhibited by the SN-specific inhibitor, and that cleavage products possessed 5´ phosphates and 3´ hydroxyls, all of which would be inconsistent with TudorSN mediating cleavage. It was later found that Argonatue2, a known component of the silencing complex, was the elusive "Slicer".

The last part of my research in the Zamore lab revolved around what we knew about the biochemistry of the RNAi pathway and how siRNAs recognized and cleaved target RNAs, and how we could apply siRNAs in the silencing of mutant alleles in dominant disease cases and single nucleotide polymorphisms (Chapters 5 and 6). We found that certain mismatches (such as purine:purine) are capable of discriminating between 2 different alleles that differed at a single nucleotide, and that the position of this mismatch could lead to varying levels of discrimination.

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

The work presented in the following chapter was a collaborative effort. Gyorgy Hutvagner performed experiments demonstrating the requirement for 5′ phosphates on single-stranded and double-stranded siRNAs in *Drosophila* embryo lysates and HeLa S100. I carried out experiments showing that siRNAs are phosphorylated in HeLa S100 lysates, that the 3′ hydroxyl on single-stranded and double-stranded siRNAs of various lengths is dispensable, and that single-stranded siRNAs are degraded in lysates within minutes. Gyorgy Hutvagner and I both performed HeLa transfections demonstrating the requirement for a 5′ phosphate on siRNAs, that single-stranded siRNAs can mediate RNAi, and that 3′ hydroxyls on single-stranded and double-stranded siRNAs are dispensable in cultured cells. Phillip Zamore and I wrote the manuscript.

CHAPTER II

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 in both *Drosophila* and mammalian cell extracts, as well as in vivo in human HeLa cells, a 5´ phosphate is required for siRNA function. 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 multi-domain 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; Elbashir et al., 2001b; Elbashir et al., 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 co-factors 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 post-transcriptional 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.

Figure 1
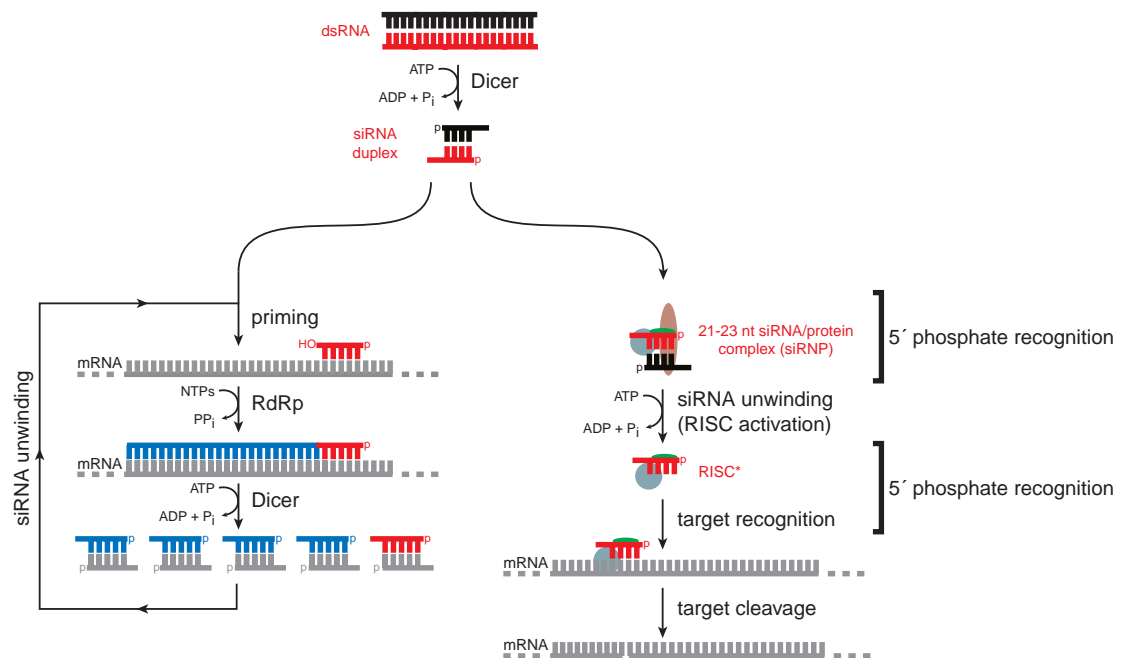
Figure II - 1. Two models proposed for the RNAi pathway in *Drosophila*. Both models postulate that dsRNA is converted to siRNA by the ATP-dependent endoribonuclease Dicer, but the models differ as to the subsequent function of siRNAs. In the 'random degradative PCR' model (at left), siRNAs are postulated 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.

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 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 non-covalent 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 anti-sense 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; Elbashir et al., 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; Elbashir et al., 2002), in mice (McCaffrey et al., 2002; Lewis et al., 2002), and perhaps even 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

Figure 2

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	CH₃O	OH	5´-CH₃O-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 II - 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.

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

Figure 3

A

time (h):   0   0.5   1   2   0   0.5   1   2

target RNA—   *

5´ cleavage —
product

B

time (min):   0   2   5   15   30   60

5´ OH siRNA—
5´ PO$_4$ siRNA—
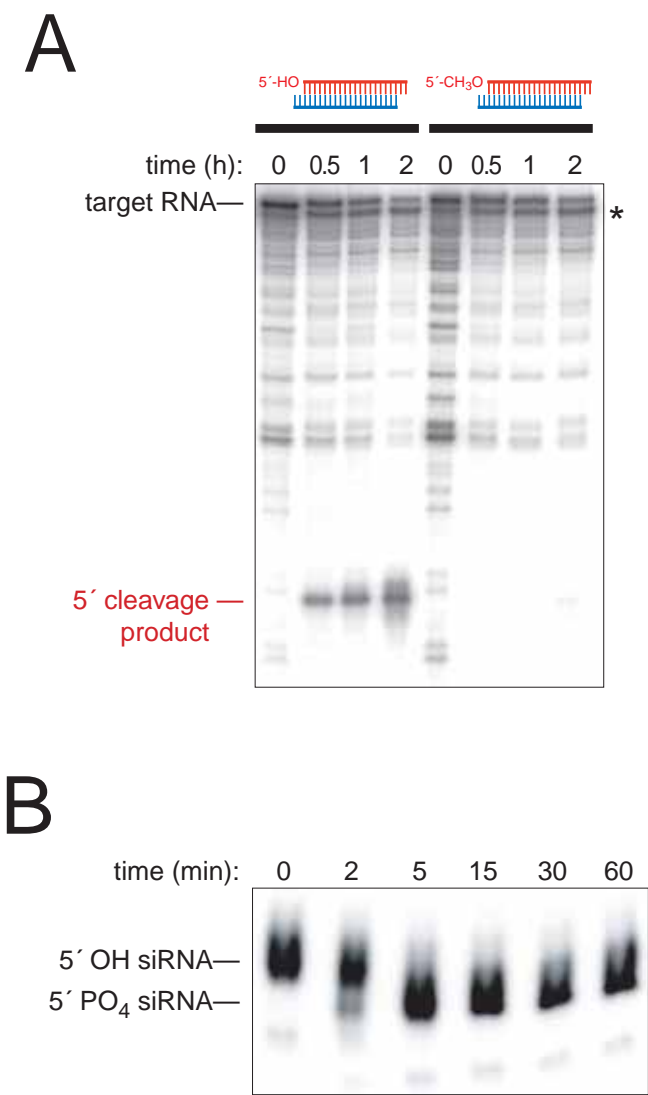
Figure II - 3. The siRNA 5′ phosphate group is required for siRNA-directed target cleavage in HeLa S100 extracts. (A) RNAi in vitro in human HeLa cell S100 extract. At left, a time course of in vitro RNAi for a standard siRNA; at right, for an siRNA duplex bearing a 5′ methoxy guide strand. The asterisk 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.

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

Figure 4

A

B
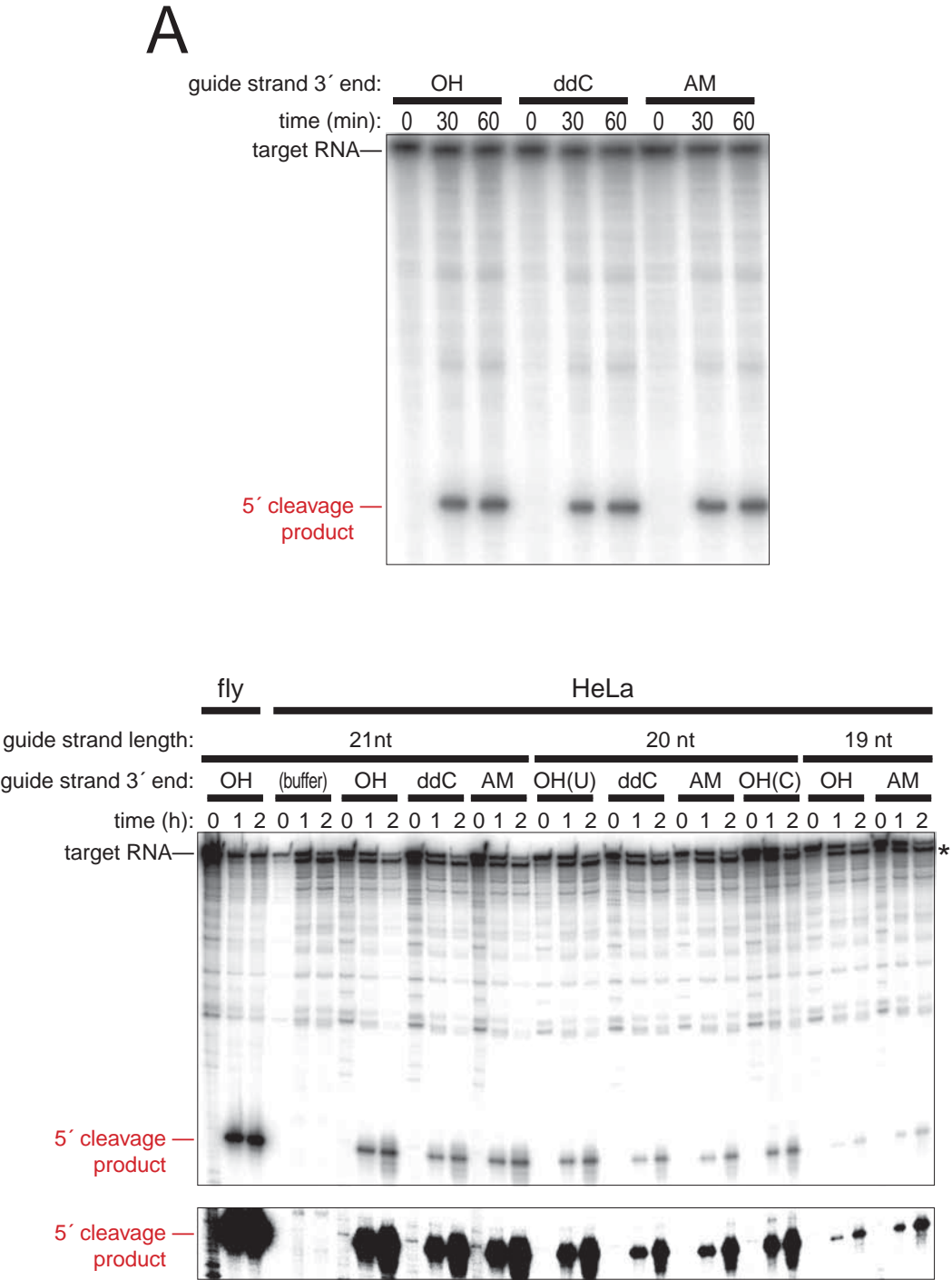
Figure II - 4. The siRNA 3´ hydroxyl is dispensable for siRNA-directed target cleavage in *Drosophila* and human cell extracts. (A) 3´-blocked siRNAs trigger RNAi in *Drosophila* embryo lysates with the same efficiency as 3´-hydroxyl-containing siRNAs. ddC, 2´,3´ dideoxy C; AM, amino modifier. (B) 3´-blocked siRNAs trigger RNAi in HeLa S100 extracts with the same efficiency as standard, 3´-hydroxyl-containing siRNAs. An over-exposure 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.

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 expressivity 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, 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 pre-phosphorylated 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 pre-phosphorylated, 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

Figure 5

A

B

C
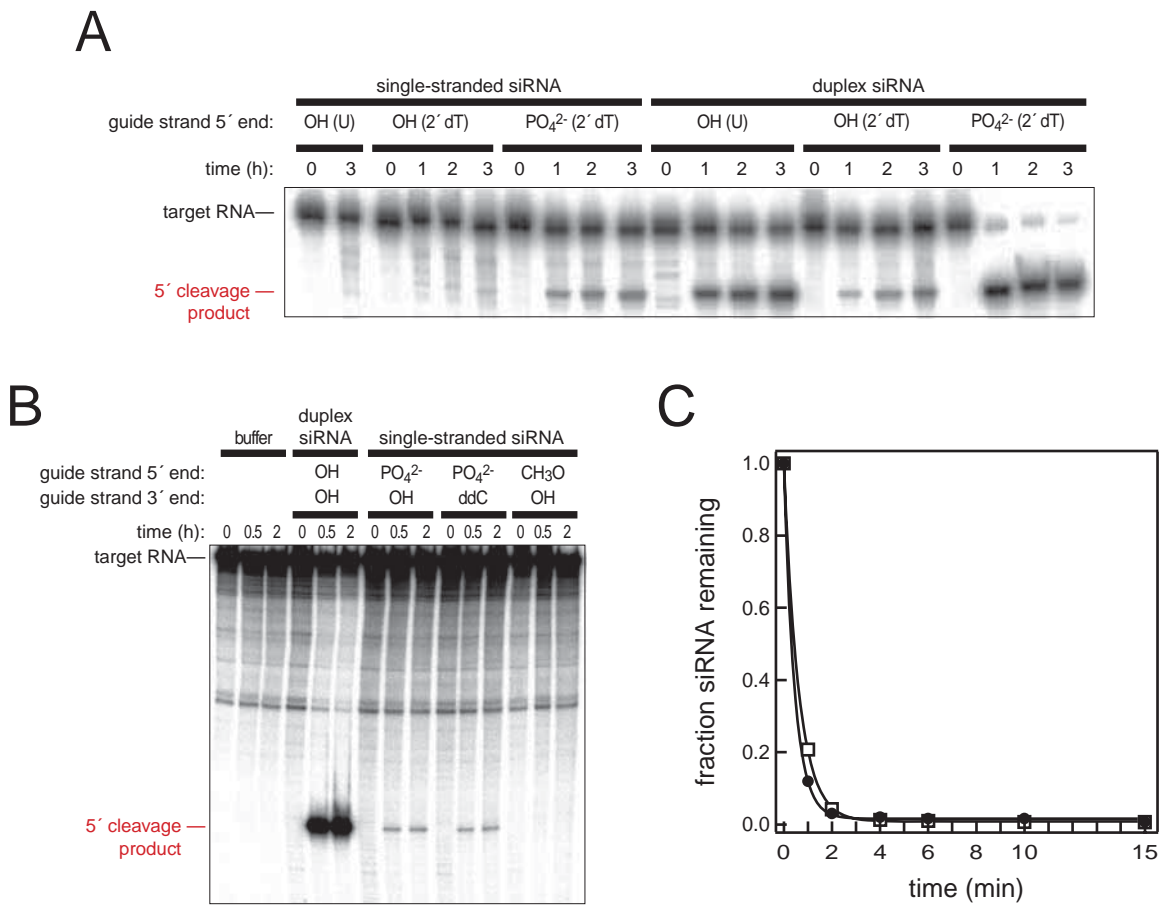
Figure II - 5. Single-stranded siRNA guides target cleavage in *Drosophila* embryo lysates. (A) Single-stranded siRNAs with the sequence of the miRNA *let-7* triggered target cleavage in *Drosophila* embryo lysate, but only if the 5′ end was pre-phosphorylated. (B) Single-stranded siRNAs complementary 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).

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. 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). Pre-phosphorylation 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

Figure 6

A

siRNA 3´ end: (buffer) | OH | ddC
time (h): 0 2 | 0 1 2 0 2
target RNA—
*
5´ cleavage — product

B

siRNA 5´ end: CH$_3$O | PO$_4^{2-}$
time (h): 0.5 1 2 0.5 1 2
target RNA—
*
5´ cleavage — product
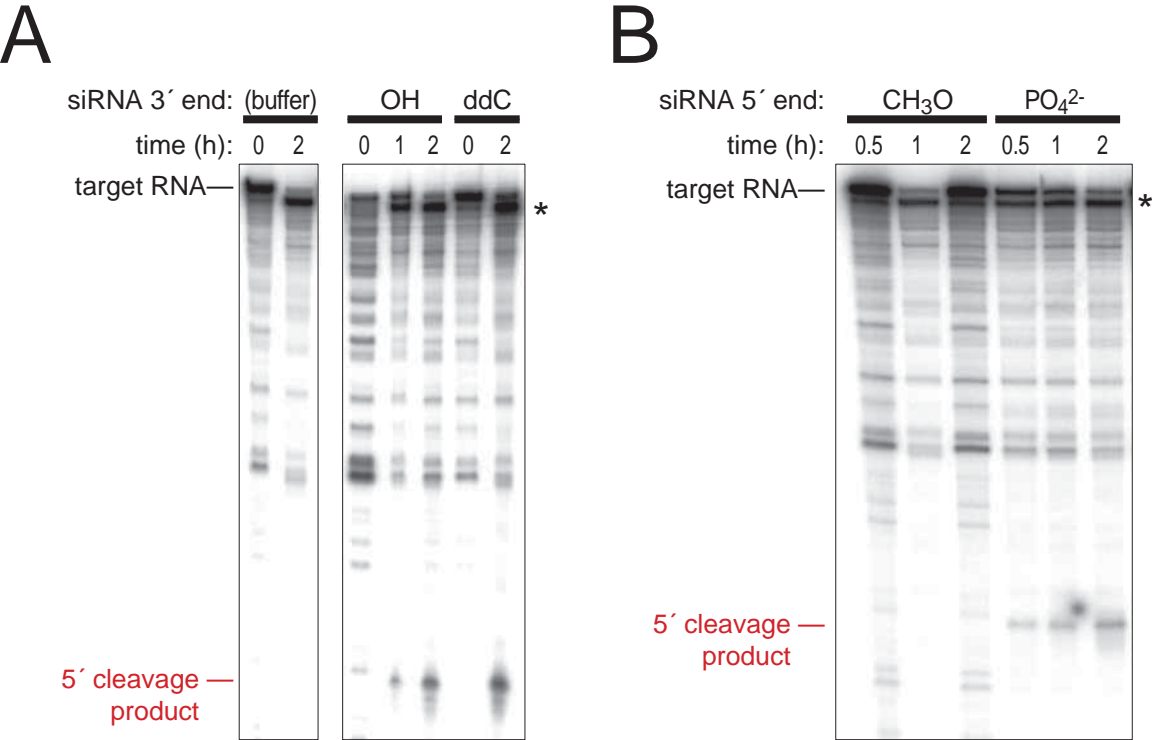
Figure II - 6. A 5′ phosphate, but not a 3′ hydroxyl is required for single-stranded antisense siRNAs to trigger RNAi in HeLa S100 extract. (A) Single-stranded siRNA triggered target cleavage in HeLa S100, even if the 3′ end of the siRNA was blocked (2′,3′ dideoxy). (B) Blocking the 5′ end of the siRNA 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.

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 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 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 co-transfected 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 two-fold. This small reduction may reflect inhibition of translation, perhaps by an anti-sense 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 non-covalent 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

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 two-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

Figure 7

A

guide strand length (nt):	21	21	21	21	21	21	21	20	20	20	19	19
guide strand 5´ end:	OH	OH	CH₃O	AM	OH	OH	dT	OH	OH	OH	OH	OH
guide strand 3´ end:	OH	OH	OH	OH	AM	ddC	AM	OH	ddC	AM	OH	AM

B
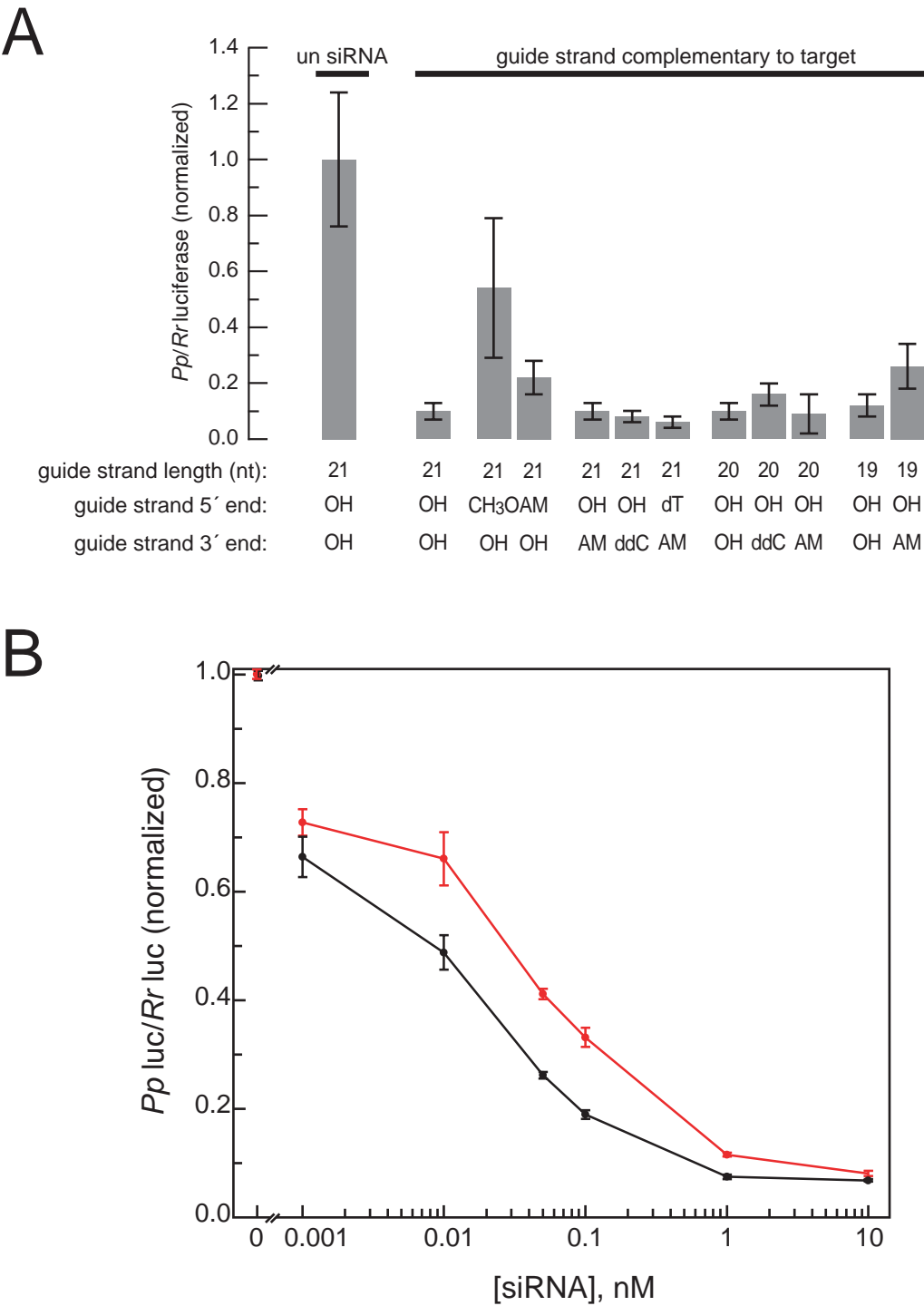
Figure II - 7. A 5´ phosphate, but not a 3´ hydroxyl is required for siRNA duplexes to trigger RNAi in vivo in cultured human HeLa cells. (A) siRNA duplexes were examined for their ability to silence the *Photinus pyralis* (Pp; firefly) luciferase target reporter, relative to the *Renilla reniformis* (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.

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 co-transfections (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 8-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 complex. 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 anti-sense 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. Pre-phosphorylation 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 data support the view that single-stranded siRNAs function in the same pathway as siRNA duplexes, the RNAi pathway.

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

Figure 8
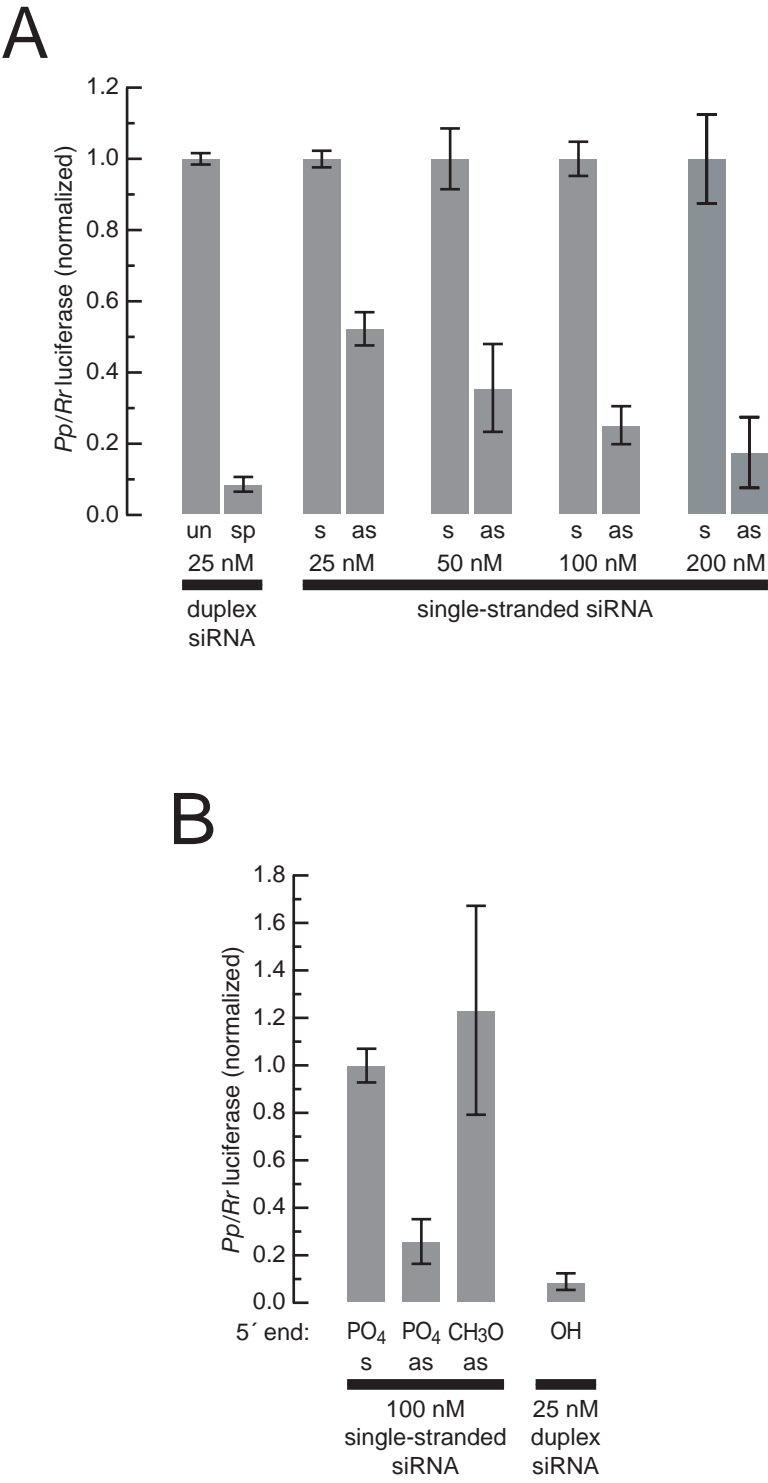
Figure II - 8. Single-stranded siRNA triggers gene silencing in HeLa cells. (A) Single-stranded siRNA silencing as a function of siRNA concentration. (B) Blocking the 5′ end of single-stranded siRNAs prevented their triggering target gene silencing. Gray bars indicate the average ± standard deviation for three trials. Un, siRNA unrelated in sequence to the target RNA; sp, specific siRNA corresponding to the target RNA; s, sense strand; as, anti-sense strand.

encoding canonical RdRPs in the currently available release of either the *Drosophila* or human genome. A hallmark of the involvement of RdRPs in post-transcriptional 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 (Jean-Yves Roignant and Christophe 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; Elbashir et al., 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. (Nykänen et al., 2001). Gels were dried, 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). Briefly, 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 x 10^5 cells/ml in 24 well plates (5 x 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 co-transfected with LipofectAMINE 2000 reagent (Invitrogen) in DMEM (Life Technologies) lacking serum and antibiotics according to manufacturer's instructions. Media was replaced 4 h after transfection with DMEM containing 10% fetal bovine serum (Life Technologies); one 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.

Acknowledgements

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Asymmetry in the Assembly of the RNAi Enzyme Complex

The experiments presented in the following chapter were a collaborative effort. Gyorgy Hutvagner performed RISC pull-out assays, target unwinding assays, and target cleavage assays in Figure 1C - E, 2K - M. Tingting Du carried out the Northern blot analysis in Figure 6. I performed the rest of the experiments in the figures as well as experiments listed as data not shown.

CHAPTER III

Summary

A key step in RNA interference (RNAi) is assembly of the RISC, the protein-siRNA complex that mediates target RNA cleavage. Here, we show that the two strands of an siRNA duplex are not equally eligible for assembly into RISC. Rather, both the absolute and relative stabilities of the base pairs at the 5′ ends of the two siRNA strands determine the degree to which each strand participates in the RNAi pathway. siRNA duplexes can be functionally asymmetric, with only one of the two strands able to trigger RNAi. Asymmetry is the hallmark of a related class of small, single-stranded, non-coding RNAs, microRNAs (miRNAs). We suggest that single-stranded miRNAs are initially generated as siRNA-like duplexes whose structures predestine one strand to enter the RISC and the other strand to be destroyed. Thus, the common step of RISC assembly is an unexpected source of asymmetry for both siRNA function and miRNA biogenesis.

Introduction

Two types of ~21 nt RNAs trigger post-transcriptional gene silencing in animals: small interfering RNAs (siRNAs) and microRNAs (miRNAs). Both siRNAs and miRNAs are produced by the cleavage of double-stranded RNA (dsRNA) precursors by Dicer, a member of the RNase III family of dsRNA-specific endonucleases (Bernstein et al., 2001; Billy et al., 2001; Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001; Paddison et al., 2002; Park et al., 2002; Provost et al., 2002; Reinhart et al., 2002; Zhang et al., 2002; Doi et al., 2003; Myers et al., 2003). siRNAs result when transposons, viruses or endogenous genes express long dsRNA or when dsRNA is introduced experimentally into plant or animal cells to trigger gene silencing, a process known as RNA interference (RNAi) (Fire et al., 1998; Hamilton and Baulcombe, 1999; Zamore et al., 2000; Elbashir et al., 2001a; Hammond et al., 2001; Sijen et al., 2001; Catalanotto et al., 2002). In contrast, miRNAs are the products of endogenous, non-coding genes whose precursor RNA transcripts can form small stem-loops from which mature miRNAs are cleaved by Dicer (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Lagos-Quintana et al., 2002; Mourelatos et al., 2002; Reinhart et al., 2002; Ambros et al., 2003; Brennecke et al., 2003; Lagos-Quintana et al., 2003; Lim et al., 2003a; Lim et al., 2003b). miRNAs are encoded in genes distinct from the mRNAs whose expression they control.

siRNAs were first identified as the specificity determinants of the RNAi pathway, where they act as guides to direct endonucleolytic cleavage of their target RNAs (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000; Elbashir et al., 2001a). Prototypical siRNA duplexes are 21 nt, double-stranded RNAs that contain 19 base pairs, with two-nucleotide, 3′ overhanging ends (Elbashir et al., 2001a; Nykänen

et al., 2001; Tang et al., 2003). Active siRNAs, like miRNAs, contain 5´ phosphates and 3´ hydroxyls (Zamore et al., 2000; Boutla et al., 2001; Hutvágner et al., 2001; Nykänen et al., 2001; Chiu and Rana, 2002; Mallory et al., 2002). Recent evidence suggests that siRNAs and miRNAs are functionally interchangeable, with the choice of mRNA cleavage or translational repression determined solely by the degree of complementarity between the small RNA and its target (Hutvágner and Zamore, 2002; Doench et al., 2003). siRNAs and miRNAs are found in similar, if not identical complexes, suggesting that a single, bifunctional complex—the RNA-induced silencing complex (RISC)— mediates both cleavage and translational control (Caudy et al., 2002; Hutvágner and Zamore, 2002; Martinez et al., 2002; Mourelatos et al., 2002).

Each RISC contains only one of the two strands of the siRNA duplex (Martinez et al., 2002). Both siRNA strands can be competent to direct RNAi (Elbashir et al., 2001a; Elbashir et al., 2001b; Nykänen et al., 2001). That is, the anti-sense strand of an siRNA can direct cleavage of a corresponding sense RNA target, whereas the sense siRNA strand directs cleavage of an anti-sense target. Here, we show that small changes in siRNA sequence have profound and predictable effects on the extent to which the individual strands of an siRNA duplex enter the RNAi pathway, a phenomenon we term siRNA functional asymmetry. We designed siRNAs that are fully asymmetric, with only one of the two siRNA strands forming RISC in vitro. Such highly asymmetric siRNA duplexes resemble intermediates previously proposed for the miRNA biogenesis pathway (Hutvágner and Zamore, 2002; Reinhart et al., 2002; Lim et al., 2003b). Our data suggest that RISC assembly is governed by an enzyme that selects which strand of an siRNA is loaded into RISC. This strand is always the one whose 5´ end is less tightly paired to its complement. We propose that for each siRNA duplex that is unwound, only one strand enters the RISC complex, whereas the other strand is degraded. For miRNAs, it is the

miRNA strand of a short-lived, siRNA duplex-like intermediate that assembles into a RISC complex, causing miRNAs to accumulate in vivo as single-stranded RNAs. Designing siRNAs to be more like these double-stranded miRNA intermediates produces highly functional siRNAs, even when targeting mRNA sequences apparently refractory to cleavage by siRNAs selected by conventional siRNA design rules.

Results and Discussion

Functionally asymmetric siRNA duplexes

To assess if the two strands of an siRNA duplex are equally competent to direct RNAi, we measured the in vitro rates of sense and anti-sense target cleavage for an siRNA duplex directed against firefly luciferase mRNA (Figure 1A). For this siRNA, the anti-sense siRNA strand directed more efficient RNAi against its sense target RNA than the sense siRNA strand did towards the anti-sense target (Figure 1B). (Throughout this paper anti-sense siRNA strands and their sense target RNAs are presented in black, and sense siRNAs and their anti-sense targets, in red.) Control experiments showed that using siRNA duplexes with 5′ phosphates did not alter this result (data not shown), indicating that different rates of 5′ phosphorylation for the two strands cannot explain the asymmetry.

Single-stranded siRNA can direct RNAi, but is >10-fold less effective than siRNA duplexes, reflecting the reduced stability of single-stranded RNA in vitro and in vivo (Schwarz et al., 2002). Surprisingly, the two strands of the luciferase siRNA duplex, used individually as 5′ phosphorylated single-strands, had identical rates of target cleavage (Figure 1C). Thus, the difference in the cleavage rates of the sense and anti-sense strands cannot reflect a difference in the inherent susceptibility of the two targets to RNAi. Instead, the finding that the two siRNA strands are equally effective as single-strands, but show dramatically different activities when paired to each other, suggests that the asymmetry in their function is established at a step in the RNAi pathway before the encounter of the programmed RISC with its RNA target.

Differential RISC assembly and siRNA functional asymmetry

Figure 1

A

Pp luc sense target: 5´-…cgaggugaacauc<u>acguacgcggaauacuucgaaa</u>ugucc…-3´

Pp luc anti-sense target: 3´-…gcuccacuuguagu<u>gcaugcgccuuaugaagcuuua</u>cagg…-5´

B

5´-CGUACGCGGAAUACUUCGAAA-3´
●●●●●●●●●●●●●●●●●●
3´-GUGCAUGCGCCUUAUGAAGCU-5´

C

5´-pCGUACGCGGAAUACUUCGAAA-3´

5´-pUCGAAGUAUUCCGCGUACGUG-3´

D

E

5´-UGUACGCGGAAUACUUCGAAA-3´
○●●●●●●●●●●●●●●●●●●
3´-GUGCAUGCGCCUUAUGAAGCU-5´
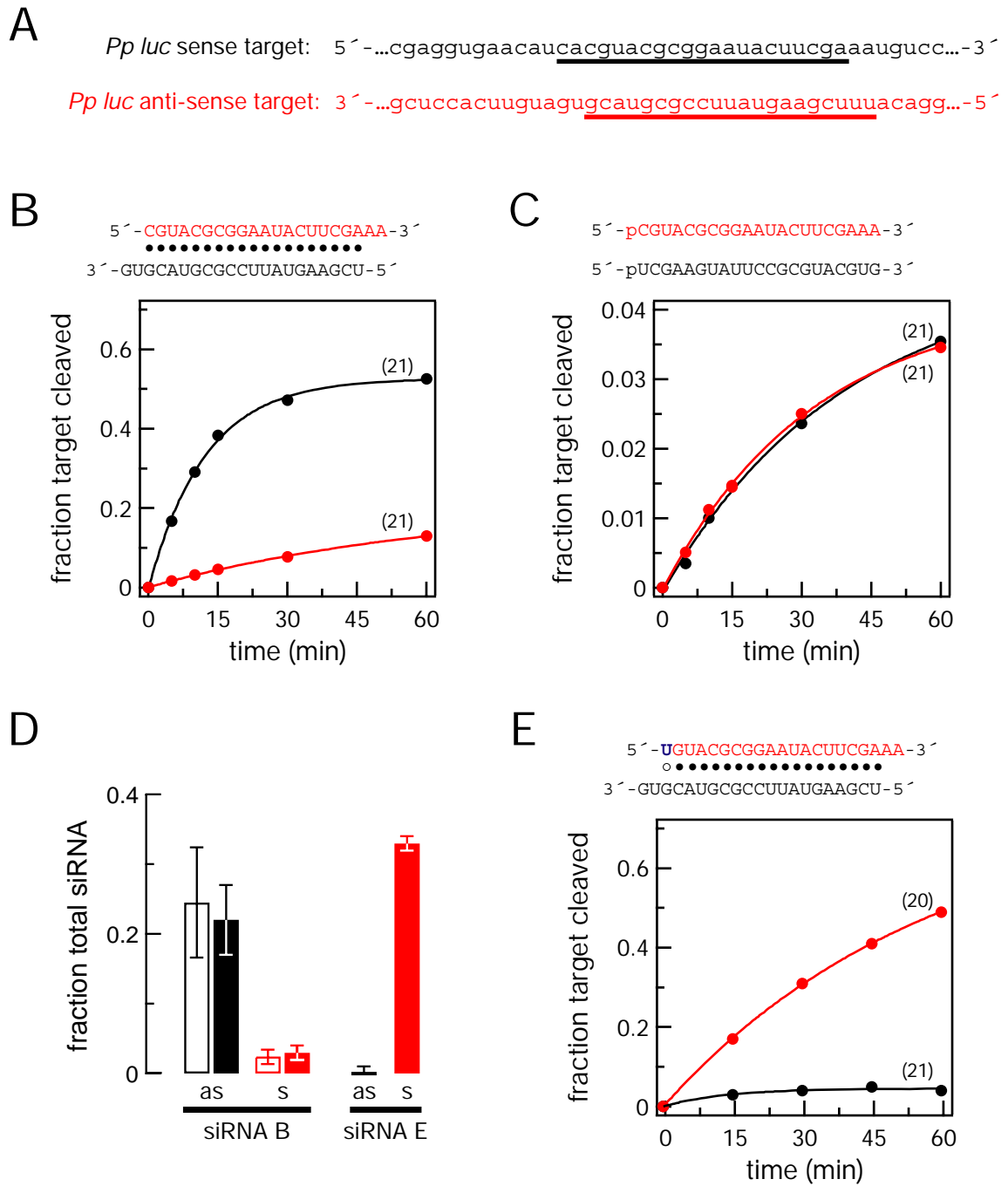
Figure III - 1. The two strands of an siRNA duplex do not equally populate the RISC. (A) Firefly luciferase sense and anti-sense target RNA sequences. (B) In vitro RNAi reactions programmed with the siRNA duplex indicated above the graph. (C) In vitro RNAi reactions as in (B), but programmed with either the anti-sense or sense single-stranded, 5′ phosphorylated siRNAs indicated above the graph. (D) Fraction of anti-sense (black) and sense (red) siRNA strands assembled into RISC (open columns) or present as single-strands (filled columns) after incubation with *Drosophila* embryo lysate for the siRNA duplexes shown in (B) and (E). The average of four trials ± standard deviation is shown. (E) In vitro RNAi reactions programmed with the siRNA duplex indicated above the graph and the target RNAs in (A). Throughout the figures, the number of Watson-Crick base pairs formed between the siRNA guide strand and the target RNA is indicated in parentheses and siRNA bases that mismatch with the target RNA are noted in blue.

siRNA unwinding correlates with siRNA function (Nykänen et al., 2001; Martinez et al., 2002), likely because siRNA duplex unwinding is required to assemble a RISC competent to base pair with its target RNA. We measured the accumulation of single-stranded siRNA from the luciferase siRNA duplex after 1 h incubation in an in vitro RNAi reaction in the absence of target RNA. In this reaction, ~ 22% of the anti-sense strand of the luciferase siRNA was converted to single-strand (Figure 1D, 'siRNA B,' solid black bar). Remarkably, we did not detect a corresponding amount of single-stranded sense siRNA (Figure 1D, 'siRNA B,' solid red bar). Since the production of single-stranded anti-sense siRNA must be accompanied by an equal amount of single-stranded sense siRNA, the missing sense-strand of the siRNA must have been destroyed after unwinding.

We also used a novel 'RISC-capture assay' to measure the fraction of each siRNA strand that was assembled into RISC (GH, Martin Simard, Craig Mello, and PDZ, manuscript in preparation). Double-stranded siRNA was incubated in an RNAi reaction for 1 h, then we added a complementary 2′-*O*-methyl RNA oligonucleotide tethered to a magnetic bead via a biotin-streptavidin linkage. 2′-*O*-methyl oligonucleotides are not cleaved by the RNAi machinery, but can bind stably to complementary siRNA within the RISC, so the amount of radioactivity stably associated with the beads is a direct measure of the amount of RISC formed. The assay was performed with siRNA duplexes in which either the sense or the anti-sense strand was 5′-^{32}P-radiolabeled. All RISC activity directed by the siRNA strand complementary to the tethered oligonucleotide was captured on the beads; no RISC was captured by an unrelated 2′-*O*-methyl oligonucleotide (data not shown). The RISC-capture assay recapitulated our unwinding measurements: ten-fold more anti-sense siRNA-containing RISC was detected than sense-strand RISC (Figure 1D, 'siRNA B,' open bars). The simplest explanation is that

the two strands of the siRNA duplex are differentially loaded into the RISC and that single-stranded siRNA not assembled into RISC is degraded.

siRNA structure and RISC assembly

The finding that the two siRNA strands can have different capacities to form RISC when paired suggests that some feature unique to the duplex determines functional asymmetry. For the siRNA in Figure 1B, the 5′ end of the anti-sense siRNA strand begins with U and is thus paired to the sense siRNA strand by an U:A base pair (2 hydrogen bonds). In contrast, the 5′ nucleotide of the sense siRNA strand is linked to the anti-sense strand by a C:G base pair (3 hydrogen bonds). A simple hypothesis is that the siRNA strand whose 5′ end is more weakly bound to the complementary strand more readily incorporates into RISC.

As an initial test of this idea, we changed the first nucleotide of the siRNA sense strand from C to U, replacing a C:G pair with a less stable U:G wobble, because the sequence of the anti-sense siRNA was not altered (Figure 1E). This single nucleotide substitution increased the rate of cleavage directed by the sense strand, and virtually eliminated RNAi directed by the anti-sense strand (Figure 1E). That is, the single C-to-U substitution inverted the functional asymmetry of the siRNA. Assembly of the two strands of the siRNA into RISC was also reversed: nearly 30% of the sense siRNA strand was converted to single-strand after 1h incubation, but no single-stranded anti-sense strand was detected (Figure 1D, 'siRNA E').

We calculated the stability of the initial four base pairs of the siRNA strands in Figure 1 using the nearest-neighbor method and the mfold algorithm (Mathews et al., 1999; Zuker, 2003). The 5′ end of the sense siRNA strand in Figure 1E, but not that in 1B, is predicted to exist as an equilibrium of two conformers of nearly equal energy. In

one conformer, the 5′ nucleotide of the sense strand is bound to the anti-sense strand by a U:G wobble pair, whereas in the other conformer the 5′ end of this siRNA strand is unpaired (Figure 2A-C). The analysis suggests that RISC assembly favors the siRNA strand whose 5′ end has a greater propensity to fray.

To test our hypothesis, we examined the strand-specific rates of cleavage of sense and anti-sense human *Cu, Zn-superoxide dismutase* (*sod1*) RNA targets (Figure 3A) triggered by the siRNA duplexes shown in Figure 3. In Figure 3B, the 5′ ends of both siRNA strands of the duplex are in G:C base pairs and the two strands are similar in their rates of target cleavage. In Figure 3C, the C at position 19 of the sense strand was changed to A, causing the anti-sense strand to begin with an unpaired nucleotide. This change, which was made to the sense-strand of the siRNA, caused the rate of target cleavage guided by the anti-sense siRNA strand to be dramatically enhanced and the sense strand rate to be suppressed (Figure 3C). Because the enhancement of sense target cleavage was caused by a mutation in the sense siRNA strand, which does not participate in the recognition of this target, the effect of the mutation must be on a step in the RNAi pathway that is spatially or temporally coupled to siRNA unwinding. However, the suppression of anti-sense target cleavage might have resulted from the single-nucleotide mismatch between the sense strand and its target RNA generated by the C-to-U substitution.

To exclude this possibility, we used a different strategy to unpair the 5′ end of the anti-sense strand. In Figure 3D, the sense-strand is identical to that in Figure 3B, but the first nucleotide of the anti-sense strand was changed from G to U, creating a U-C mismatch at its 5′ end, in place of the G-A of Figure 3C. This siRNA duplex still showed pronounced asymmetry, with the anti-sense strand guiding target cleavage to the nearly complete exclusion of the sense strand (Figure 3D). Thus, the suppression of the

Figure 2

A

sod1 sense target: 5´-…agagaggcauguuggagacuugggcaaugugacugcugacaa…-3´

sod1 anti-sense target: 3´-…cuccguacaaccucugaacccguuacacugacgacuguuuc…-5´
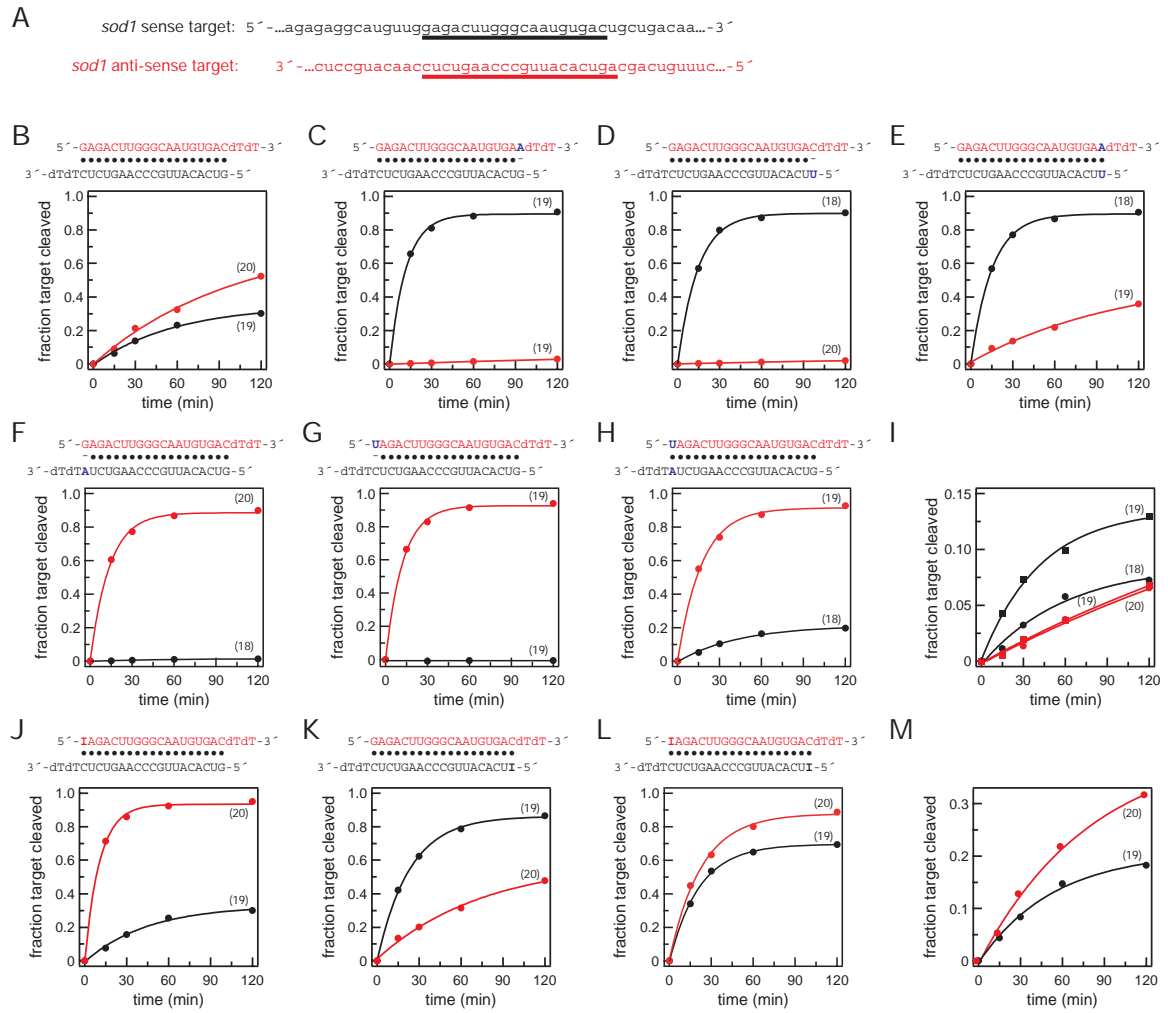
Figure III - 2. The relative thermodynamic stability of the first four base pairs of the siRNA strands explains siRNA functional asymmetry. (A-C) Thermodynamic analysis of siRNA strand 5′ ends for the siRNAs in Figures 1B and 1E. ΔG (kcal/mole) was calculated in 1M NaCl at 37°C. (D-M) Altering the functional asymmetry of siRNA duplexes. (D) Sense and anti-sense *sod1* target RNA sequences. (E) Thermodynamic analysis of siRNA strand 5′ ends for the siRNA duplex in (F). ΔG (kcal/mole) was calculated in 1M NaCl at 37°C. (F) In vitro RNAi reactions programmed with the siRNA indicated above the graph using the target RNAs in (D). (G) In vitro RNAi reactions programmed with the siRNA indicated above the graph, in which the 5′ terminal C of the anti-sense strand in (F) was changed to U. (H) In vitro RNAi reactions programmed with anti-sense or sense single-stranded, 5′ phosphorylated siRNAs as indicated. (I) Sense and anti-sense *huntingtin* (*htt*) target RNA sequences. (J) Thermodynamic analysis of siRNA strand 5′ ends for the siRNA duplex in (K). ΔG (kcal/mole) was calculated in 1M NaCl at 37°C. (K) In vitro RNAi reactions programmed with the siRNA indicated above the graph using the target RNAs in (I). (L) In vitro RNAi reactions programmed with the siRNA indicated above the graph, in which C19 of the sense siRNA strand and G1 of the anti-sense strand were changed to A and U, respectively. (M) In vitro RNAi reactions programmed with the siRNA indicated above the graph, in which the sense strand was that used in (L) and the anti-sense strand was that used in (K).

Figure 3

A

5´-CGUACGCGGAAUACUUCGAAA-3´
  ●●●●●●●●●●●●●●●●●
3´-GUGCAUGCGCCUUAUGAAGCU-5´

ΔG = -8.7 kcal/mol

B

5´-UGUACGCGGAAUACUUCGAAA-3´
  ○●●●●●●●●●●●●●●●●
3´-GUGCAUGCGCCUUAUGAAGCU-5´

ΔG = -7.4 kcal/mol (U/G unpaired)

ΔG = -7.2 kcal/mol (U/G paired)

C

5´-CGUACGCGGAAUACUUCGAAA-3´

3´-GUGCAUGCGCCUUAUGAAGCU-5´

ΔG = -8.4 kcal/mol

D

sod1 sense target: 5´-...agagaggcauguuggagacuugggcaaugugacugcugacaa...-3´

sod1 anti-sense target: 3´-...cuccguacaaccucugaacccguuacacugacgacuguuuc...-5´

E

5´-UGGAG
  ●●●●●
3´-UUACCUC   ΔG = -10.4

5´-CACAU
  ●●●●●
3´-UUGUGUA   ΔG = -8.2

F

5´-UGGAGACUUGGGCAAUGUGdTdT-3´
  ●●●●●●●●●●●●●●●●●●●
3´-dTdTACCUCUGAACCCGUUACAC-5´

G

5´-UGGAGACUUGGGCAAUGUGdTdT-3´
  ●●●●●●●●●●●●●●●●●○
3´-dTdTACCUCUGAACCCGUUACAU-5´

H

5´-UGGAGACUUGGGCAAUGUGdTdT-3´ (●)
5´-CACAUUGCCCAAGUCUCCAdTdT-3´ (●)
5´-UACAUUGCCCAAGUCUCCAdTdT-3´ (■)

I

htt sense target: 5´-...ugcagcugaucaucgaugugcugacccugaggaacaguuc...-3´

htt anti-sense target: 3´-...acgucgacuaguagcuacacgacugggacuccuugucaag...-5´

J

5´-UGUGC
  ●●●●●
3´-CUACACG   ΔG = -10.5

5´-GUUCC
  ●●●●●
3´-GACAAGG   ΔG = -10.5

K

5´-UGUGCUGACCCUGAGGAACAG-3´
  ●●●●●●●●●●●●●●●●●●
3´-CUACACGACUGGGACUCCUUG-5´

L

5´-UGUGCUGACCCUGAGGAAAAG-3´
  ●●●●●●●●●●●●●●●●●●
3´-CUACACGACUGGGACUCCUUU-5´

M

5´-UGUGCUGACCCUGAGGAAAAG-3´
  ●●●●●●●●●●●●●●●●●●●
3´-CUACACGACUGGGACUCCUUG-5´

Figure III - 3. 5′ terminal, single-nucleotide mismatches make siRNA duplexes functionally asymmetric. (A) The sequences at the cleavage site of the 560 nt *sod1* RNA sense or 578 nt *sod1* anti-sense target RNAs. The siRNAs in this figure and in Figure 3 cleave the sense target to yield a 320 nt 5′ product and the anti-sense target to yield a 261 nt 5′ product. (B-H) In vitro RNAi reactions programmed with the siRNA indicated above each graph using the target RNAs diagrammed in (A). (I) In vitro RNAi reactions programmed with anti-sense or sense single-stranded, 5′ phosphorylated siRNAs (the single nucleotide mismatch with target RNA is underlined): black squares, 5′-pGUC ACA UUG CCC AAG UCU CdTdT-3′; black circles, 5′-pUUC ACA UUG CCC AAG UCU CdTdT-3′; red squares, 5′-pGAG ACU UGG GCA AUG UGA AdTdT-3′; red circles, 5′-pGAG ACU UGG GCA AUG UGA CdTdT-3′. (J-M) A single hydrogen bond difference can cause the two strands of an siRNA duplex to assemble differentially into RISC. (J-L) In vitro RNAi reactions programmed with the siRNA indicated above each graph using the target RNAs in (A). (M) In vitro RNAi reactions as in (J-L), but programmed with anti-sense or sense single-stranded, 5′ phosphorylated siRNAs: black circles, 5′-IUC ACA UUG CCC AAG UCU CdTdT-3′; red circles, 5′-IAG ACU UGG GCA AUG UGA CdTdT-3′.

cleavage rate of the sense-strand in Figure 3C was not a consequence of the position 19 mismatch with the anti-sense target. This finding is consistent with previous studies that suggest that mismatches with the target RNA are well tolerated if they occur near the 3′ end of the siRNA guide strand (Amarzguioui et al., 2003). When we paired the sense strand of Figure 3C with the anti-sense strand of Figure 3D to create the duplex in Figure 3E, the resulting siRNA directed anti-sense target cleavage significantly better than the siRNA in Figure 3C, although the two siRNAs contain the same sense strand (Figure 3E).

Figures 3F, G, and H show a similar analysis in which the 5′ end of the sense strand or position 19 of the anti-sense strand of the siRNA in Figure 3B was altered to produce siRNA duplexes in which the 5′ end of the sense strand was either fully unpaired (Figures 3F and G) or in an A:U base pair (Figure 3H). Again, unpairing the 5′ end of an siRNA strand—the sense strand, in this case—caused that strand to function to the exclusion of the other strand. When the sense strand 5′ end was present in an A:U base pair and the anti-sense strand 5′ end was in a G:C pair, the sense strand dominated the reaction (Figure 3H), but the anti-sense strand retained activity similar to that seen for the original siRNA (Figure 3B). We conclude that the relative ease with which the 5′ ends of the two siRNAs can be liberated from the duplex determines the degree of asymmetry.

Additional data supporting this idea is shown in Figure 2. Figure 2F shows an siRNA that cleaved the two *sod1* target RNAs (Figure 2D) with modest functional asymmetry that reflects the collective base pairing strength of the first four nucleotides of each siRNA strand (2E; see below). Asymmetry was dramatically increased when a G:U wobble was introduced at the 5′ end of the anti-sense strand of the siRNA (Figure 2G), but no asymmetry was seen when the individual single-strands strands were used to trigger RNAi (Figure 2H), demonstrating that differential RISC assembly, not target accessibility, explains the functional asymmetry of the siRNA duplex.

A single hydrogen bond can determine which siRNA strand directs RNAi

How small a difference in siRNA base pairing can the RISC-assembly machinery sense? To explore this question, we altered the siRNA in Figure 3B by introducing inosine (I) in place of the initial guanosines of the siRNA strands. These siRNAs cleave the same sites on the two target RNAs as the siRNA in Figure 3B, but contain I:C pairs instead of G:C. An I:C pair is similar in energy to an A:U (Turner et al., 1987). When the sense strand began with I, it directed target cleavage more efficiently than the anti-sense strand (Figure 3J). An inosine at the 5′ end of the anti-sense strand had the opposite effect (Figure 3K). Thus, a difference of a single hydrogen bond has a measurable effect on the rate of RISC assembly. When both siRNA strands began with I, the relative efficacy of the two siRNA strands (Figure 3L) was restored to that measured for the individual single strands (Figure 3M). Thus, the small difference in rates in Figure 3L reflects a difference in the intrinsic capacity of the two strands to guide cleavage, not a difference in their assembly into RISC. We note that the absolute rates are faster for the siRNA in Figure 3L than that in Figure 3B, suggesting that production of RISC from an individual strand is governed not only by the relative propensity of the two 5′ ends to fray but also by their absolute propensities to fray.

We hypothesize that siRNA end fraying provides an entry site for an ATP-dependent RNA helicase that unwinds siRNA duplexes (Figure 5A). The involvement of a helicase in RISC assembly is supported by previous observations: (1) both siRNA unwinding and production of functional RISC require ATP in vitro (Nykanen et al., 2001) and (2) several proteins with sequence homology to ATP-dependent RNA helicases have been implicated in RNA silencing (Wu-Scharf et al., 2000; Dalmay et al., 2001; Hutvágner and Zamore, 2002; Ishizuka et al., 2002; Kennerdell et al., 2002; Tabara et al.,

2002; Tijsterman et al., 2002). However, other mechanisms are possible, including strand selection by an ATP-dependent nuclease or the concerted action on the siRNA of an ATPase and single-stranded RNA-binding proteins and/or nucleases.

Four-to-six bases of single-stranded nucleic acid are bound by the well-studied helicases PcrA (Velankar et al., 1999) and NS3 (Kim et al., 1998). Therefore, we tested the effect of single-nucleotide mismatches in this region of the siRNA, using a series of siRNAs containing a mismatch at the second, third, or fourth position of each siRNA strand. We also analyzed siRNAs bearing G:U wobble pairs at the second, third, or both second and third positions (Figure 4). These siRNAs were again based on the siRNA in Figure 3B and targeted the *sod1* sense and antisense RNAs in Figure 3A. The results of this series demonstrate that mismatches, but not G:U wobbles, at positions 2-4 of an siRNA strand alter the relative loading of the two siRNA strands into RISC. Mismatches at position five have very modest effects on the relative loading of the siRNA strands into RISC (data not shown). In contrast, the effects of internal mismatches at positions 6-15 cannot be explained by their influencing the symmetry of RISC assembly (data not shown). In sum, these data are consistent with the action of a non-processive helicase that can bind about four nucleotides of RNA.

Implications of siRNA asymmetry for miRNA biogenesis

miRNAs are derived from the double-stranded stem of hairpin precursor RNAs by cleavage catalyzed by the double-stranded RNA-specific endonuclease, Dicer (Lee et al., 1993; Pasquinelli et al., 2000; Reinhart et al., 2000; Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Lagos-Quintana et al., 2002; Reinhart et al., 2002). pre-miRNA processing by Dicer may generate a product with the essential structure of an siRNA

Figure 4
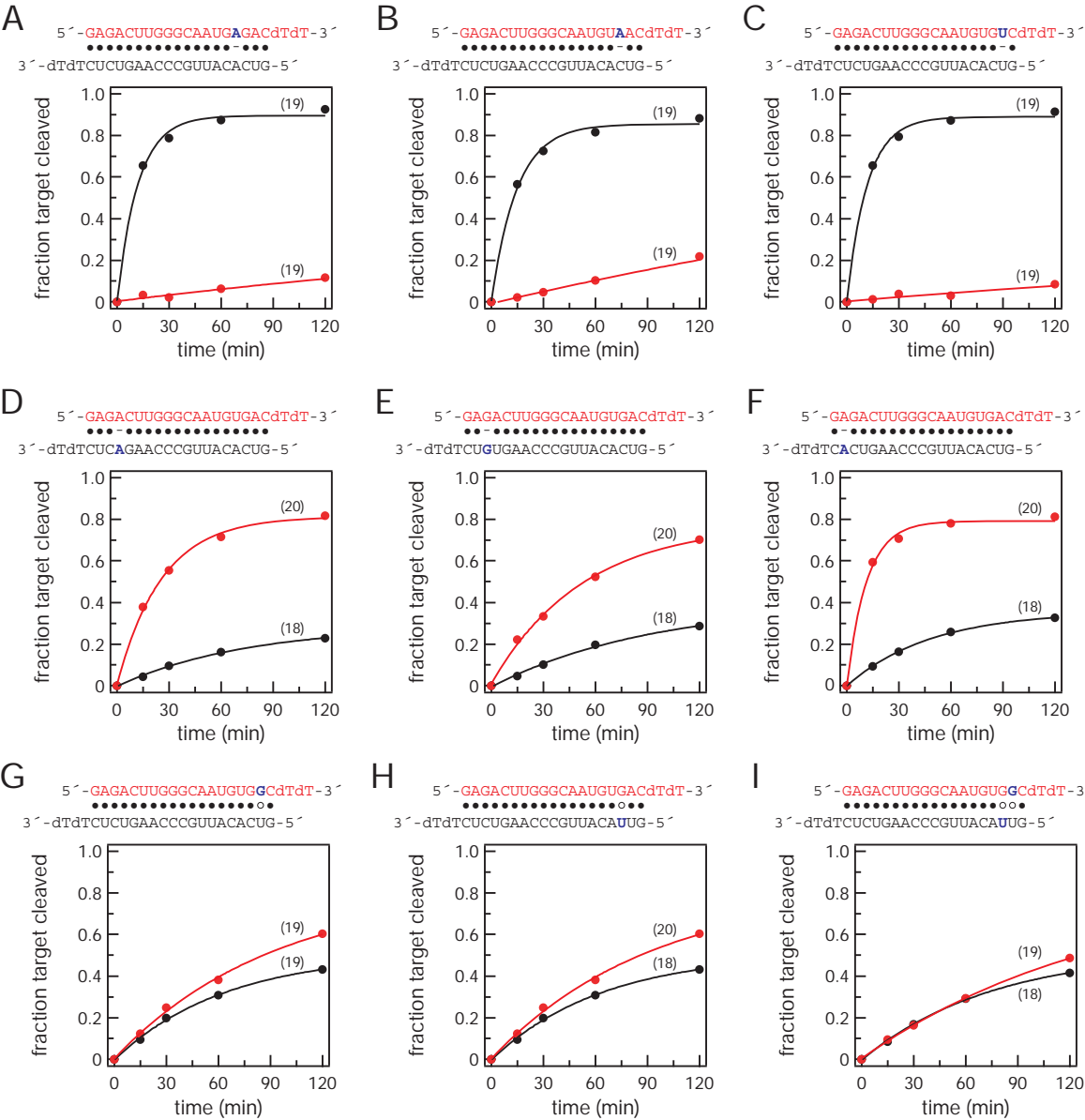
Figure III - 4. The first four base pairs of the siRNA duplex determine strand-specific activity. Internal, single-nucleotide mismatches (A-F) near the 5´ ends of an siRNA strand generate functional asymmetry, but internal G:U wobble pairs (G-I) do not. Target RNAs were as in Figure 3A.

duplex, as first suggested by Bartel and colleagues (Reinhart et al., 2002; Lim et al., 2003b). Using a small RNA cloning strategy to identify mature miRNAs in *C. elegans*, they recovered small RNAs corresponding to the non-miRNA side of the precursor's stem (Lim et al., 2003b). Although these 'miRNA*' sequences were recovered at about 100 times lower frequency than the miRNAs themselves, they could always be paired with the corresponding miRNA to give 'miRNA duplexes' with 2 nt overhanging 3´ ends (Lim et al., 2003b). Their data suggest that miRNAs are born as duplexes, but accumulate as single-strands because some subsequent process stabilizes the miRNA, destabilizes the miRNA*, or both.

We propose that incorporation of miRNA into RISC is this process. Our results with siRNA suggest that preferential assembly of a miRNA into the RISC would be accompanied by destruction of its * strand (Figure 5A). To favor miRNA accumulation, miRNA duplexes would present the miRNA in a structure that loads the miRNA strand, but not the miRNA*, into RISC.

Is this idea plausible? We deduced the miRNA duplex that might be generated by processing of pre-*let-7* ('conceptual dicing'; Figure 5B). pre-miRNA stems are only partially double-stranded; the typical animal pre-miRNA contains mismatches, internal loops, and G:U base pairs predicted to distort an RNA helix. As a consequence, miRNA duplexes should also contain terminal and internal mismatches and G:U base pairs. For pre-*let-7*, the 5´ end of *let-7* is unpaired in the predicted miRNA duplex, whereas the 5´ end of the * strand is paired. The results presented in Figures 1 and 3 predict that this structure should cause the *let-7* strand to enter the RISC and the *let-7** strand to be degraded. Emboldened by this thought experiment, we extended the analysis to other *Drosophila* miRNA genes (Lagos-Quintana et al., 2001). For each, we inferred from its precursor structure the double strand predicted to be produced by Dicer. These

Figure 5
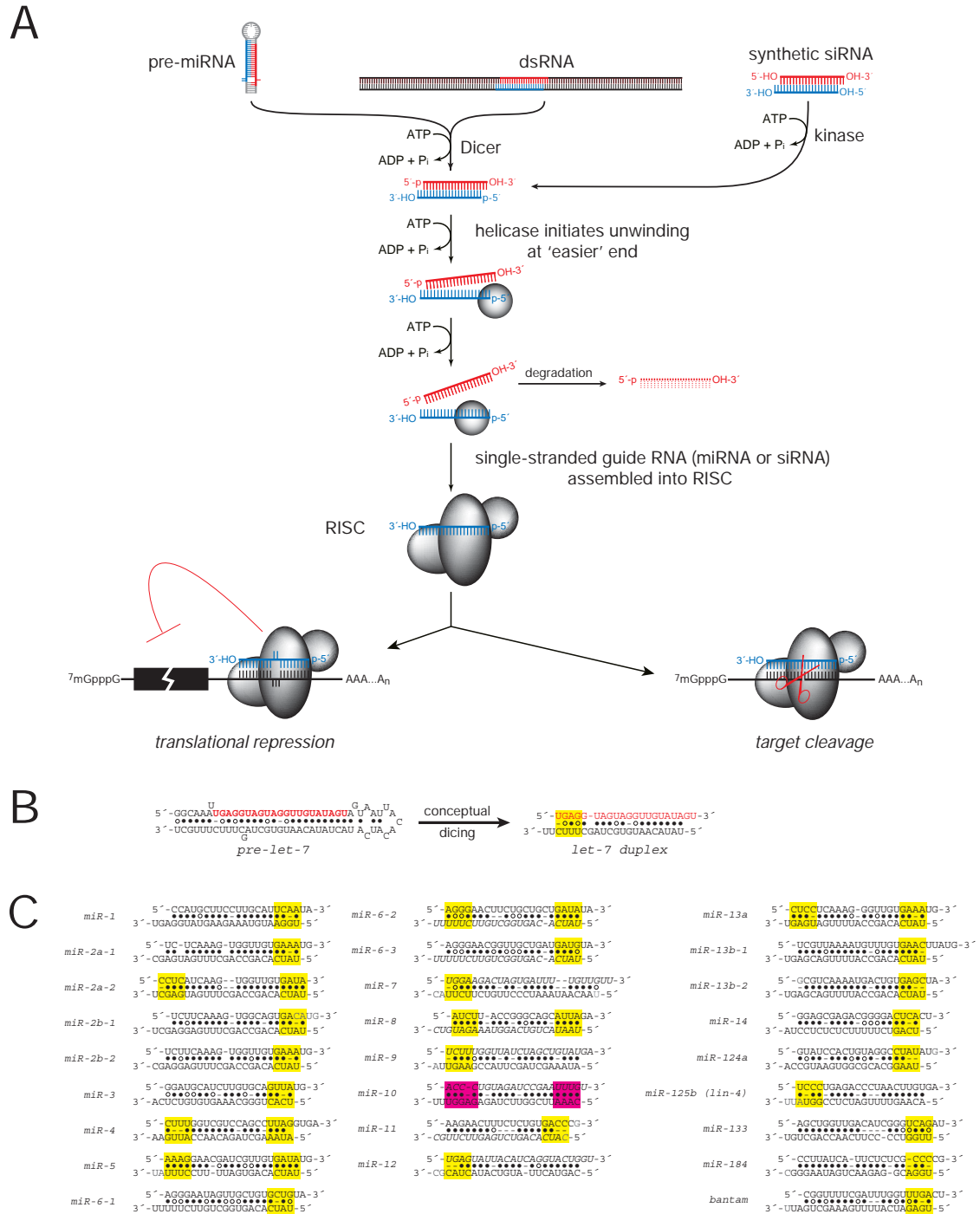
Figure III - 5. Asymmetric RISC assembly can explain siRNA and miRNA strand choice. (A) A model for RISC assembly. Dicing of both pre-miRNAs and dsRNA is proposed to generate a duplex intermediate that is a substrate for an ATP-dependent RNA helicase that directs only one of the two strands into RISC; the other strand is degraded. (B, C) Asymmetric RISC assembly from double-stranded intermediates explains why miRNAs accumulate in vivo as single-strands. (B) pre-*let-7* might be processed by Dicer into a miRNA duplex in which the 5′ end of *let-7*, but not that of *let-7** is unpaired. (C) The miRNA duplexes predicted to result from Dicer cleavage of *Drosophila* miRNA precursors. The end bearing features predicted to promote asymmetric siRNA strand incorporation into RISC is highlighted in yellow, and the mature miRNA sequence is in italics. Analysis of the predicted miR-10/miR-10* duplex, for which both ends are highlighted in purple, provides little information as to why miR-10 would predominate in vivo. miRNA sequences are from Lagos-Quintana et al. (2001) and Brennecke et al. (2003), with minor sequence corrections from Aravin et al. (2003); miRNA* sequences for miR-2a-2, miR-4, miR-8, miR-10, and miR-13a are as reported by Aravin et al. (2003).

conceptually diced miRNA duplexes are shown in Figure 5C. For 20 of the 27 duplexes analyzed (including pre-*let-7*), the difference in the base pairing of the first five nucleotides of the miRNA versus the miRNA* strand accurately predicted the miRNA, and not the miRNA*, to accumulate in vivo. The analysis succeeded irrespective of which side of the pre-miRNA stem encoded the mature miRNA. In this analysis, we relied on our observations that single mismatches in the first four nucleotides of an siRNA strand, an initial G:U wobble pair, but not internal G:U wobbles, directed the asymmetric incorporation of an siRNA strand into RISC (Figures 1, 2, 3, and 4). However, our experiments with siRNA predict that both the miRNA and the miRNA* strand should accumulate for miR-2a-2, miR-4, miR-5, one of the three miR-6 paralogs, miR-8, miR-10, and miR-13a. Recently, Tuschl and colleagues reported an exhaustive effort to clone and sequence miRNAs from *Drosophila* (Aravin et al., 2003). They found that miR-2a-2*, miR-4*, miR-8*, miR-10*, and miR-13a* are all expressed in vivo. We have confirmed by Northern hybridization that both miR-10 and miR-10* are expressed in adult *Drosophila* males and females, and in syncitial blastoderm embryos (Figure 6). Thus, of the seven miRNAs we predict to accumulate as both miRNA and miRNA* species, five have now been confirmed experimentally. No miRNA* species were cloned by Tuschl and colleagues for any of the miRNAs we predicted to accumulate asymmetrically (Aravin et al., 2003). These data strengthen our proposal that pre-miRNAs specify on which side of the stem the miRNA resides by generating miRNA duplexes from which only one of the two strands is assembled into RISC. When these double-stranded miRNA intermediates do not contain structural features enforcing asymmetric RISC assembly, both strands accumulate in vivo. It is tempting to speculate that pre-miRNAs such as pre-miR-10, which generates roughly equal amounts of small

Figure 6
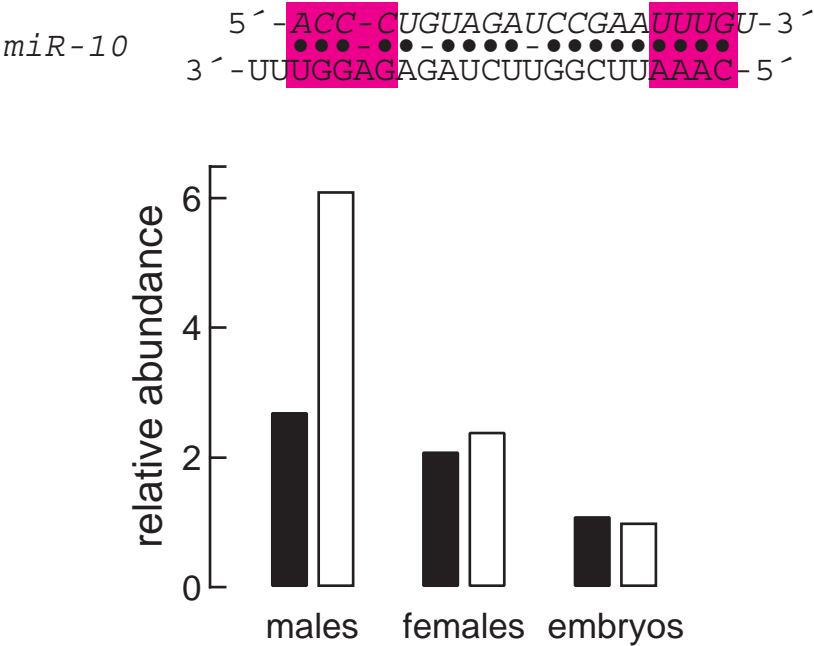
Figure III - 6.

Northern analysis of *Drosophila* miR-10 (filled columns) and miR-10* (unfilled columns) in adult males or females, or in syncitial blastoderm embryos shows that both miR-10 and miR-10* RNAs accumulate in vivo.

RNA products from both sides of the precursor stem, regulate target RNAs with partial complementary to either small RNA product.

Implications for RNA silencing

Our observations have dramatic implications for the design of functional siRNAs for mammalian RNAi. We have shown that siRNA structure can profoundly influence the entry of the anti-sense siRNA strand into the RNAi pathway. A review of the published literature suggests that the structure of the siRNA duplex, rather than that of the target site, explains most reports of ineffective siRNAs duplexes. Such inactive duplexes may be coaxed back to life simply by modifying the *sense* strand of the siRNA. An example of this is shown in Figure 2 for an ineffective siRNA directed against the *huntingtin* (*htt*) mRNA (Figure 2K). Changing the G:C (Figure 2K) to an A:U pair (Figure 2L) or a G-A mismatch (Figure 2M) dramatically improved its target cleavage rate in vitro and its efficacy in vivo (Eftim Milkani, NA, and PDZ, unpublished observations). Because RNAi is a natural cellular pathway, siRNAs should be designed to reflect the biological requirements for entry of the anti-sense strand into RISC. In cultured HeLa cells, siRNAs designed according to the mechanism-based rules presented in this paper show maximum suppression of target mRNA expression at concentrations ~ 100-fold lower than those typically used in mammalian RNAi studies (Schwarz et al., 2002 and our unpublished data). Khvorova and colleagues have similarly found that a low base-pairing stability at the 5′ end of the antisense strand, but not the sense strand, characterizes functional siRNAs in cultured cells (Anastasia Khvorova, Angela Reynolds, and Sumedha D. Jayasena, this issue). siRNAs designed to function asymmetrically may also be used to enhance RNAi specificity. Expression profiling studies show that the sense strand of an siRNA can direct off-target gene silencing (Jackson et al., 2003). A

potential remedy for such sequence-specific, but undesirable effects is to redesign the siRNA so that only the anti-sense strand enters the RNAi pathway.

Our observations also suggest a need to revise the current design rules for the construction of short hairpin RNA (shRNA) vectors, which produce siRNAs transcriptionally in cultured cells or in vivo (Brummelkamp et al., 2002; McManus et al., 2002; Paddison et al., 2002; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002). We suggest that shRNAs be designed to place the 5′ end of the anti-sense siRNA strand in a mismatch or G:U base pair. Moreover, a recent report suggests that some shRNAs may induce the interferon response (Bridge et al., 2003). Mismatches and G:U pairs could be designed into these shRNAs simultaneously to promote entry of the correct siRNA strand into the RNAi pathway and to diminish the capacity of the shRNA stem to trigger non-sequence specific responses to double-stranded RNA. Redesigning shRNAs to more fully reflect the natural mechanism of miRNA incorporation into RISC should make them more effective, allowing lower levels of shRNA to silence target mRNAs in vivo.

Experimental Procedures

General methods

In vitro RNAi reactions and analysis was carried out as previously described (Tuschl et al., 1999; Zamore et al., 2000; Haley et al., 2003). Target RNAs were used at ~ 5 nM concentration so that reactions were mainly under single-turnover conditions. Target cleavage under these conditions was proportionate to siRNA concentration. siRNA unwinding assays were as published (Nykänen et al., 2001).

siRNA preparation

Synthetic RNA (Dharmacon) was deprotected according to the manufacturer's protocol. siRNA strands were annealed (Elbashir et al., 2001a) and used at a final concentration of = 50 (Figures 1B, 2F - H, 3, 4) or = 100 nM (Figures 1D,1E and 2K-M). siRNA single strands were phosphorylated with polynucleotide kinase (PNK; New England Biolabs) and 1 mM ATP and used at 500 nM final concentration.

Target RNA preparation

Target RNAs were transcribed with recombinant, histidine-tagged, T7 RNA Polymerase from PCR products as described (Nykänen et al., 2001; Hutvágner and Zamore, 2002), except for sense *sod1* mRNA, which was transcribed from a plasmid template (Crow et al., 1997) linearized with Bam HI. PCR templates for *htt* sense and anti-sense and *sod1* anti-sense target RNAs were generated by amplifying 0.1 ng/μl (final concentration) plasmid template encoding *htt* or *sod1* cDNA using the following primer pairs: *htt* sense target, 5′-GCG TAA TAC GAC TCA CTA TAG GAA CAG TAT GTC TCA GAC ATC-3′ and 5′-UUCG AAG UAU UCC GCG UAC GU-3′; *htt* anti-sense

target, 5′-GCG TAA TAC GAC TCA CTA TAG GAC AAG CCT AAT TAG TGA TGC-3′ and 5′-GAA CAG TAT GTC TCA GAC ATC-3′; *sod1* anti-sense target, 5′-GCG TAA TAC GAC TCA CTA TAG GGC TTT GTT AGC AGC CGG AT-3′ and 5′-GGG AGA CCA CAA CGG TTT CCC-3′.

Immobilized 2′-*O*-methyl oligonucleotide capture of RISC

The 5′ end of the siRNA strand to be measured was ^{32}P-radiolabeled with PNK. 10 pmol biotinylated 2′-*O*-Methyl RNA was immobilized on Dynabeads M280 (Dynal) by incubation in 10 µl lysis buffer containing 2mM DTT for 1 h on ice with the equivalent of 50 µl of the suspension of beads provided by the manufacturer. The beads were then washed to remove unbound oligonucleotide. 50 nM siRNA was pre-incubated in a standard 50 µl in vitro RNAi reaction for 15 min at 25°C. Then, all of the immobilized 2′-*O*-Methyl oligonucleotide was added to the reaction and the incubation continued for 1 h at 25°C. After incubation, the beads were rapidly washed three times with lysis buffer containing 0.1% (w/v) NP-40 and 2 mM DTT followed by a wash with the same buffer without NP-40. Input and bound radioactivity were determined by scintillation counting (Beckman). The 5′-biotin moiety was linked via a six-carbon spacer arm. 2′-*O*-methyl oligonucleotides (IDT) were: 5′-biotin-ACA UUU CGA AGU AUU CCG CGU ACG UGA UGU U-3′ (to capture the siRNA sense strand) 5′-biotin-CAU CAC GUA CGC GGA AUA CUU CGA AAU GUC C-3′ (to capture the anti-sense strand).

Northern hybridization

Northern hybridization was essentially as described (Hutvágner et al., 2001). 50 µg total RNA was loaded per lane. 5′ ^{32}P-radiolabeled synthetic RNA probes

(Dharmacon) were: 5′-ACA AAU UCG GAU CUA CAG GGU-3′ (to detect miR-10) and 5′-AAA CCU CUC UAG AAC CGA AUU U-3′ (to detect miR-10*). The amount of miR-10 or miR-10* detected was normalized to the non-specific hybridization of the probe to 5S rRNA. Normalizing to hybridization of the probe to a known amount of a miR-10 or miR-10* synthetic RNA control yielded essentially the same result.

Acknowledgements

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The RNA-Induced Silencing Complex is a Mg^{2+}-dependent Endonuclease

The work in the following chapter was a collaborative effort. Yukihide Tomari performed phosphorothioate substitution experiments in Figure 3 and Figure 6. I performed all other experiments, wrote the manuscript and prepared the figures.

CHAPTER IV

Summary

In the *Drosophila* and mammalian RNA interference (RNAi) pathways, target RNA destruction is catalyzed by the siRNA-guided RNA-induced silencing complex (RISC). RISC has been proposed to be an siRNA-directed endonuclease, catalyzing cleavage of a single phosphodiester bond on the RNA target. Although 5′ cleavage products are readily detected for RNAi in vitro, only 3′ cleavage products have been observed in vivo. Proof that RISC acts as an endonuclease requires detection of both 5′ and 3′ cleavage products in a single experimental system. Here, we show that siRNA-programmed RISC generates both 5′ and 3′ cleavage products in vitro; cleavage requires Mg^{2+} but not Ca^{2+}, and the cleavage product termini suggest a role for Mg^{2+} in catalysis. Moreover, a single phosphorothioate in place of the scissile phosphate blocks cleavage; the phosphorothioate effect can be rescued by the thiophilic cation, Mn^{2+}, but not by Ca^{2+} or Mg^{2+}. We propose that, during catalysis, a Mg^{2+} ion is bound to the RNA substrate through a non-bridging oxygen of the scissile phosphate. The mechanism of endonucleolytic cleavage is not consistent with the mechanism of the previously identified RISC nuclease, Tudor-SN. Thus, the RISC-component that mediates endonucleolytic cleavage of the target RNA remains to be identified.

Results and Discussion

The RISC is an endonuclease

In vitro, 5′ cleavage products are readily detected for both siRNA-programmed (Chiu and Rana, 2003; Elbashir et al., 2001a; Elbashir et al., 2001b; Hutvagner and Zamore, 2002; Martinez et al., 2002; Nykanen et al., 2001; Schwarz et al., 2002; Tuschl et al., 1999; Zamore et al., 2000) and microRNA-programmed (Hutvagner and Zamore, 2002; Tang et al., 2003) RISC. In contrast, only stable 3′ cleavage products are detected in vivo in plants (Llave et al., 2002; Palatnik et al., 2003; Xie et al., 2003) and in cultured mammalian cells (Holen et al., 2002). Although the proposal that RISC is an endonuclease is appealing (Elbashir et al., 2001a), current evidence is also consistent with a highly processive 5′-to-3′ or 3′-to-5′ exonuclease that stops at the center of the siRNA. In fact, a Staphylococcal family exonuclease, Tudor-SN (TSN) has been identified as a component of RISC in *Drosophila melanogaster*, mammals, and *Caenorhabditis elegans* (Caudy et al., 2003). The cysteine-alkylating agent, *N*-ethylmaleimide (NEM) reduces non-sequence specific ribonuclease activity present in *Drosophila* embryo lysates (Figure 1A). NEM also blocks the assembly, but not the activity, of RISC (Nykanen et al., 2001; Tomari et al., 2004). We assembled siRNA into RISC in *Drosophila* embryo lysate, treated the reactions with NEM, then added either a 5′ or a 3′ ^{32}P-radiolabeled luciferase target RNA and monitored target RNA cleavage. The 3′-radiolabeled target RNA contained a 7-methylguanosine "cap" at its 5′ end and was 3′ end-labeled with α-^{32}P, 3′-deoxyadenosine 5′ triphosphate (3′-dATP) and yeast poly(A) polymerase (Figure 1A). The 3′-radiolabeled RNA corresponded to the antisense sequence of the 5′-radiolabeled RNA target. The siRNA used in this study

generates both sense- and antisense-strand RISCs. The 5′ cleavage product for this antisense target RNA and siRNA has been characterized previously (Elbashir et al., 2001a; Nykanen et al., 2001).

If RISC is an endonuclease, then the 5′ cleavage product of the 5′-radiolabeled sense target RNA and the 3′ cleavage product of the 3′-radiolabeled antisense target RNA should be the same length. By using the 5′-radiolabeled sense and 3′-radiolabeled antisense target RNAs, we detected both 5′ and 3′ products of siRNA-directed target RNA cleavage. No 3′ cleavage products were detected in the absence of NEM treatment or if dithiothreitol, which quenches NEM, was added before, rather than after, NEM (Figure 1A, "– NEM" and "mock"), likely because both the 3′-radiolabeled target RNA and the 3′ cleavage product are unstable in these conditions. Because the 5′ cleavage product of the sense target and the 3′ cleavage product of the antisense target co-migrate, we conclude that RISC is an endonuclease that cleaves at a single, unique site, as first proposed by Tuschl and colleagues (Elbashir et al., 2001a). A 3′ cleavage product was also detected for a different siRNA:target pair (Figure 2).

The termini of the products of nucleases often provide clues to the mechanisms of nuclease catalysis. We purified each cleavage product by polyacrylamide gel electrophoresis, and then the purified cleavage products were enzymatically probed to determine if their termini contained hydroxyl or phosphate groups (Figures 1B and 1C). To identify the nature of its 3′ end, the 5′ cleavage product was treated with T4 polynucleotide kinase (PNK) or poly(A) polymerase and 3′-dATP. When treated with PNK, the gel mobility of the 5′ cleavage product was indistinguishable from the untreated sample, suggesting that it does not contain a 3′ phosphate (Figure 1B). (In addition to its 5′ kinase activity, PNK is a 3′ monophosphatase.) In contrast, incubation of the 5′ cleavage product with poly(A) polymerase and 3′-dATP converted it to a slower

Figure 1

A

5´-³²P-sense target
3´-³²P-anti-sense target

− NEM − NEM mock NEM + NEM

400 nt —
300 nt —
200 nt —
100 nt —
72 nt —

target RNA

cleavage product

time (min): 0 15 30 60 0 15 30 60 0 15 30 60 0 15 30 60

B

⁷mG(5´)ppp(5´)G 2´,3´ ?

input PNK PAP + 3´-dATP

3´ = pA₃´H²´OH —
3´ = OH —

C

5´ ? pA₃´H²´OH

input CIP PNK CIP + PNK

5´ = OH —
5´ = PO₄²⁻ —
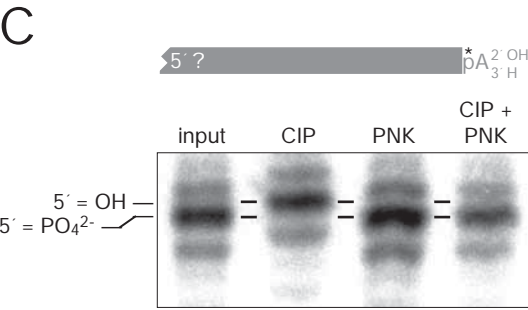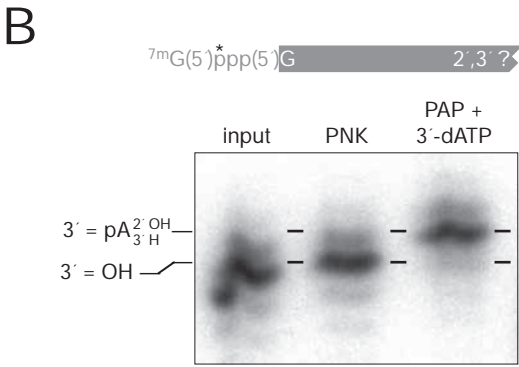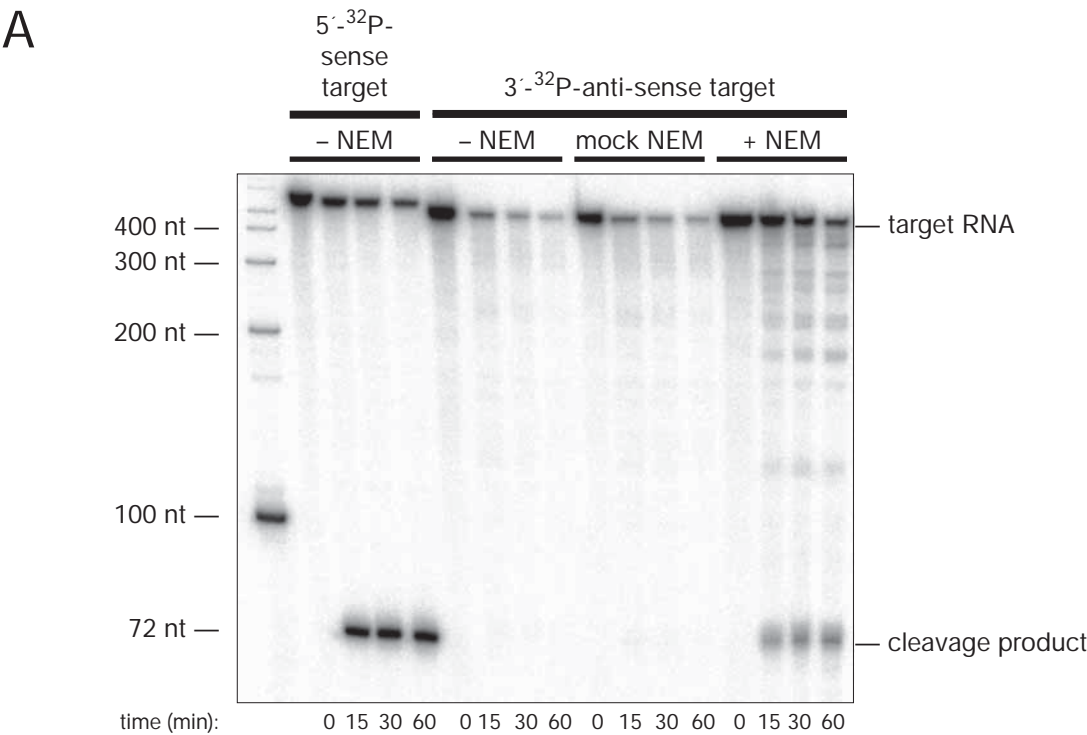
Figure IV - 1. RISC is an endonuclease. (A) In vitro RNAi reactions programmed with a firefly luciferase-specific siRNA. As shown previously (Elbashir et al., 2001a; Nykanen et al., 2001), this siRNA directs cleavage of a $5'$-^{32}P-radiolabeled sense luciferase target RNA to yield a 72 nt $5'$ cleavage product. When the same reaction was performed using a ^{7m}G($5'$)ppp(G) capped, $3'$-^{32}P-radiolabeled antisense luciferase target RNA, no cleavage product was detected (– NEM). When the lysate was treated with NEM after RISC assembly, a $3'$ cleavage product was readily detected. This $3'$ cleavage product was 72 nt long, the length predicted if RISC is an endonuclease. (B) The $5'$ cleavage product was gel isolated and analyzed to determine the structure of its $3'$ terminus. The $5'$ cleavage product could be extended one nucleotide by treatment with poly(A) polymerase (PAP) and $3'$-dATP; its mobility was unaltered by treatment with PNK. Therefore, it contains a $3'$ hydroxy terminus. (C) The $3'$ cleavage product was gel isolated and analyzed to determine the structure of its $5'$ terminus. Treating the $3'$ cleavage product with calf intestinal phosphatase (CIP), produced a species with a slower gel mobility. The mobility of this RNA was restored to that of the original $3'$ cleavage product after further treatment with PNK and ATP. The mobility of the $3'$ cleavage product was unaltered by treatment with PNK alone. Thus, the $5'$ end of the $3'$ cleavage product must bear a monophosphate.

Figure 2
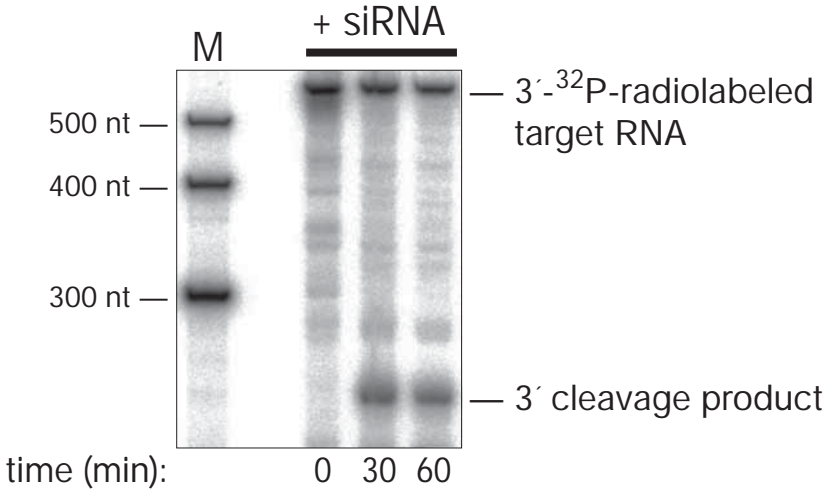
Figure IV - 2. When the RISC was treated with NEM before addition of target RNA, a 3´ cleavage product was also detected for a 3´-^{32}P-radiolabeled *sod1* target RNA. The 3´ cleavage product has the size expected for endonucleolytic cleavage.

migrating form, consistent with the addition of a single adenosine to its 3′ end. This result implies the presence of a free 3′ hydroxyl group on the 5′ cleavage product (Figure 1B). We note that while poly(A) polymerase can add 3′-dATP to the 3′ end of an RNA chain, it cannot extend the 3′ hydroxyl group present on the 7-methyl guanosine(5′)ppp(5′) cap (data not shown).

The 3′ cleavage product was treated with calf intestinal phosphatase (CIP), PNK, or CIP then PNK. CIP treatment alone converted the 3′ cleavage product to a slower migrating form, compared to the untreated RNA, consistent with the original 3′ cleavage product containing one or more 5′ phosphate groups. Treatment of the dephosphorylated sample with PNK and ATP restored its mobility to that of the untreated sample. No change was observed when the RNA was treated with PNK alone. Together, these results indicate that the 3′ cleavage product begins with a single 5′ phosphate group and the 5′ cleavage product ends with a 3′ hydroxyl group.

These termini are not consistent with a 2′ hydroxyl group on the target RNA acting as a nucleophile to attack the scissile phosphate; such a mechanism would be expected to leave a 2′,3′ cyclic phosphate and a 5′ hydroxyl. Control experiments demonstrate that 2′,3′cyclic phosphate and 5′ hydroxyl termini are stable in the in vitro RNAi reaction but are nonetheless not found on the cleavage products of RISC (data not shown). After 1 h incubation in NEM-treated lysate in standard RNAi conditions, an 80 nt, 5′-7-methyl-guanosine cap-radiolabeled RNA bearing a 2′,3′-cyclic phosphate at its 3′ terminus was unaltered. Similarly, a 3′-radiolabeled RNA bearing a 5′ hydroxyl group, but otherwise identical to the 3′ cleavage product described in this manuscript, was not converted to a 5′ phosphorylated form. Thus, the 5′ phosphate and 3′ hydroxy termini we observed on the cleavage products of RISC are unlikely to result from modification after the initial cleavage reaction.

RISC exhibits dependence on divalent cations

Instead of the 2′ hydroxyl group, a water or hydroxide ion may serve as a nucleophile for endonucleolytic cleavage by RISC. Such enzyme mechanisms often use divalent cations to assist in catalysis. To test whether a divalent metal ion is required for RISC endonuclease function, siRNA was incubated with *Drosophila* embryo lysate to assemble RISC, and then RISC assembly was inactivated with NEM. Finally, the siRNA-programmed RISC was mixed with target RNA and EGTA (to chelate calcium), EDTA (to chelate magnesium), or 1,10-phenanthroline (to chelate zinc). Compared to a reaction with no chelator added, the reactions containing either EGTA or 1,10-phenanthroline showed no decrease in cleavage efficiency. In contrast, reactions that contained EDTA showed a marked decrease in cleavage efficiency at 2 mM chelator; at 5 mM and 10 mM EDTA, no cleavage was detected (Figure 3A).

RISC function could be rescued by adding additional Mg^{2+} after EDTA, demonstrating that inactivation of RISC by EDTA reflected Mg^{2+} chelation, not an unrelated effect (Figure 3B). Furthermore, EDTA did not cause RISC disassembly, because RISC could be incubated with the RNA target in the presence of EDTA for 1 hr prior to the addition of Mg^{2+} (Figure 3C). RISC was assembled, the assembly was quenched with NEM, then EDTA and target RNA added. The reaction was incubated for 1hr; no target cleavage occurred. Next, Mg^{2+} was added, and the incubation continued for another hour. Addition of Mg^{2+} resurrected RISC activity. We note that RISC could not have fully disassembled in the presence of EDTA and then reassembled when Mg^{2+} was added because the initial NEM treatment inactivates RISC assembly (Nykanen et al., 2001; Tomari et al., 2004). Of course, these data alone cannot exclude that RISC was

Figure 3
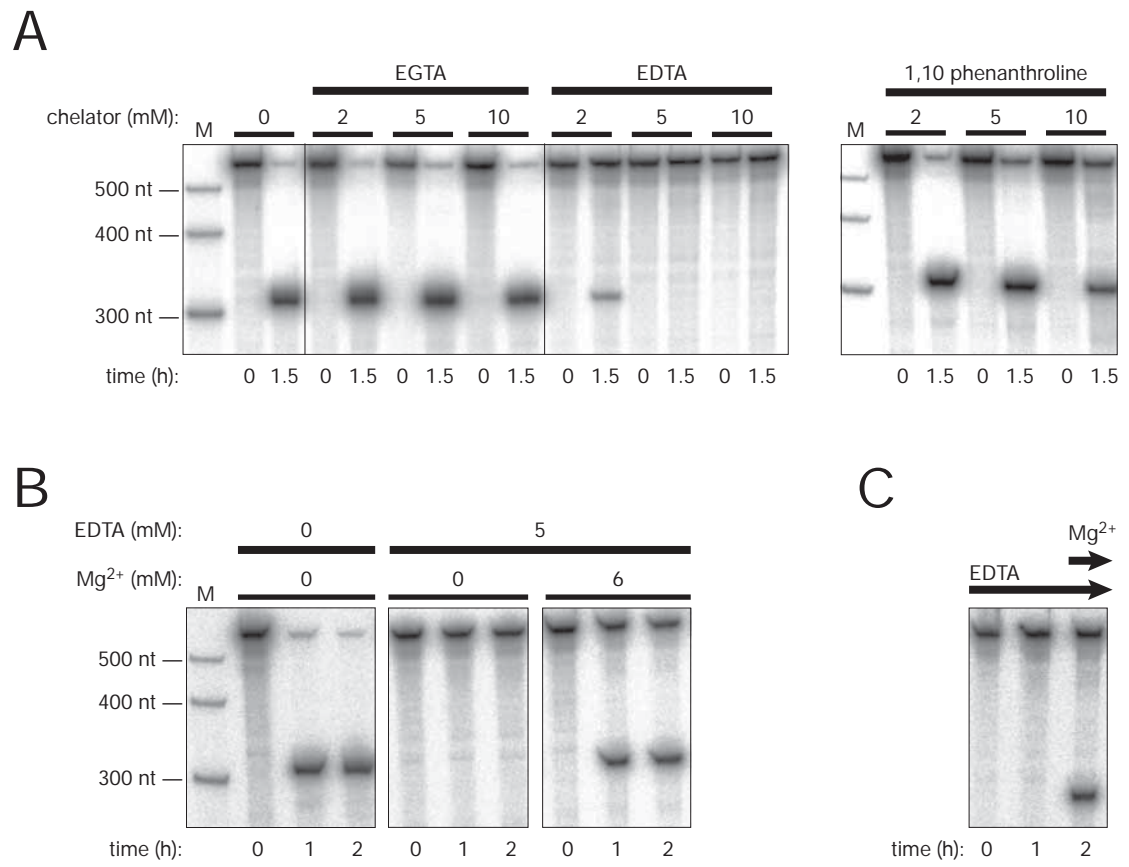
Figure IV - 3. Endonucleolytic cleavage by siRNA-programmed RISC requires Mg^{2+}. (A) Standard in vitro RNAi reactions were assembled and pre-incubated for 1 hour at 25°C, treated with NEM to block further RISC assembly, then 2 mM, 5 mM, or 10 mM chelator was added together with 5′-radiolabled target RNA. Reactions were incubated for an additional 1.5 h. (B) Reactions were assembled as in (A) but in one set, additional magnesium acetate was added. (C) Reactions were assembled as in (A) with 5 mM EDTA. After 1 h incubation of RISC with target RNA in the presence of EDTA, magnesium acetate was added and incubation continued for another hour.

partially disassembled to an intermediate state whose maturation does not require any NEM-sensitive factors and then reassembled to the active form upon Mg^{2+} addition. However, such a possibility is inconsistent with our observation below that a nonbridging oxygen of the scissile phosphate is likely a ligand for at least one divalent cation.

The scissile phosphate is a Mg^{2+} ligand

RISC cleaves its cognate mRNA target across from siRNA nucleotides 10 and 11, measured from the 5′ end of the siRNA guide strand (Elbashir et al., 2001a; Elbashir et al., 2001b). That is, the scissile phosphate of the target RNA lies between nucleotides 11 and 12, where nucleotide 1 is the target base paired to the twenty-first nucleotide of the siRNA guide strand (Figure 4A). To test whether one or more phosphate groups on the target RNA might bind Mg^{2+} during endonucleolytic cleavage, we prepared five different target RNAs, each bearing a single phosphorothioate substitution (Figure 4A). Phosphorothioate linkages contain a sulfur atom in place of one of the two nonbridging oxygens of the phosphodiester bond (Eckstein, 1985) (Figure 4B). Sulfur, unlike oxygen, does not bind well to Mg^{2+} (Eckstein, 1985; Pecoraro et al., 1984). Although phosphorothioate linkages are chiral, we did not resolve the Rp and Sp isomers but, instead analyzed the racemic mixture for each substrate RNA. Of the five singly substituted phosphorothioate target RNAs, only the RNA with a phosphorothioate between nucleotides 11 and 12 was detectably impaired for cleavage (Figure 4C). This position corresponds to the scissile phosphate originally identified by Tuschl and colleagues (Elbashir et al., 2001a; Elbashir et al., 2001b). Although the phosphorothioate-substituted target RNA contained both Rp and Sp isomers, cleavage was blocked by much more than 50%. Therefore, our data suggest that both the *pro*-Rp

Figure 4

A


```
              13 12 11 10 9  8
siRNA: 3´-UGAUAUGUU G  G  A  U  GAUGGAGU-5´
              • • • • • • • •  •  •  •  •  • • • • • • •
target: 5´-⁷mGpppG...ACUAUACAApCpCpUpApCUACCUCA...-3´
              9  10 11 12 13 14
```

B

C

phosphorothioate between:

| – | 9 & 10 | 10 & 11 | 11 & 12 | 12 & 13 | 13 & 14 |

target RNA —
5´ cleavage product —

time (h): 0 1 2   0 1 2   0 1 2   0 1 2   0 1 2   0 1 2

D

fraction target cleaved at 1 h

- ● Mn²⁺
- ▲ Mg²⁺
- ■ Ca²⁺

metal concentration (mM)

E

+ siRNA

Mn²⁺ added (mM): 0   0 0.1 0.5 1 2 5

target RNA —
5´ cleavage product —

time (h): 0   1

– siRNA

Mn²⁺ added (mM): 0   0 0.1 0.5 1 2 5

target RNA —
5´ cleavage product —

time (h): 0   1

Figure IV - 4. A nonbridging oxygen of the scissile phosphate binds at least one Mg^{2+} ion. (A) Scheme for single phosphorothioate substitution. The phosphorothioate-substituted phosphates in the target are indicated by 'p' and the scissile phosphate is in bold. (B) An Sp phosphorothioate linkage. (C) In vitro RNAi reactions were programmed with a *let-7* siRNA duplex. The target RNA contained a 21 nt sequence with complete complementarity to *let-7*. The effect on target cleavage of substituting each phosphate indicated in (A) was assessed. Only when the scissile phosphate was replaced by a phosphorothioate was target cleavage impaired. (D) The inhibition of cleavage observed when the scissile phosphate was replaced with a phosphorothioate could be rescued by Mn^{2+}, but not by Ca^{2+} or additional Mg^{2+}. Mn^{2+} and Ca^{2+} reactions also contained 1.2 mM Mg^{2+}; indicated Mg^{2+} concentrations are the concentration added in addition to the 1.2 mM basal level contained in a standard RNAi reaction. Triangles, Mg^{2+}; circles, Mn^{2+}; squares, Ca^{2+}. (E) Rescue of cleavage by Mn^{2+} requires siRNA.

and *pro*-Sp nonbridging oxygens of the scissile phosphate of the RNA target play a role in siRNA-directed endonucleolytic cleavage of mRNA.

Substitution of a nonbridging oxygen with sulfur might block RISC activity simply because sulfur is larger than oxygen. Alternatively, one or both nonbridging oxygens may be a ligand for Mg^{2+}. Such a Mg^{2+} ion might play a role in generating the nucleophile (e.g., hydroxide ion) at the active site or in stabilizing the transition state. If a nonbridging oxygen acts as a Mg^{2+} ligand, then sulfur substitution should be rescued by Mn^{2+}, which binds more strongly to sulfur than does Mg^{2+} (Eckstein, 1985; Pecoraro et al., 1984). Addition of Mn^{2+}, but not Mg^{2+} or Ca^{2+}, partially rescued the effect of phosphorothioate substitution at the scissile phosphate (Figure 4D). Rescue by Mn^{2+} was specific for the phosphorothioate-substituted RNA target; addition of 2 mM Mn^{2+} did not increase the rate of cleavage of a target RNA containing only phosphodiester linkages (Figure 5). Efficient cleavage occurred only in the presence of a complementary siRNA; in the absence of siRNA, Mn^{2+} did not induce target cleavage (Figure 4E). The simplest explanation for our results is that RISC is a Mg^{2+}-dependent endonuclease in which at least one nonbridging oxygen of the scissile phosphate directly interacts with the divalent cation.

Effects of pdTp inhibitor on endonucleolytic cleavage

The Tudor-SN (TSN) protein is the only purified protein component of RISC that displays single-stranded, RNA-specific ribonuclease activity. TSN is a component of the RISC in *Drosophila*, *C. elegans*, and mammals (Caudy et al., 2003) and is present in *Drosophila* embryo lysates (György Hutvágner and PDZ, unpublished observations). Three lines of evidence suggest that the nuclease activity of TSN does not mediate

Figure 5
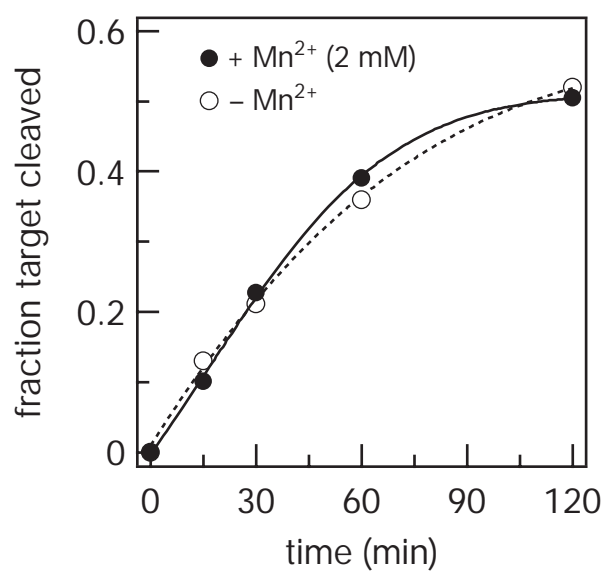
Figure IV - 5. Mn^{2+} does not affect the rate of cleavage of a target RNA containing only phosphodiester linkages. 2 mM Mn^{2+} was added to a standard target cleavage reaction after RISC assembly, together with the target RNA. RISC was programmed with *let-7* siRNA duplex, and the target RNA, which had no phosphorothiate linkages, contained a 21 nt sequence complementary to *let-7* (as in Figure 4C). Open circles, reaction without Mn^{2+}; filled circles, reaction with 2 mM Mn^{2+}. Both reactions contained 1.2 mM Mg^{2+}.

siRNA-directed endonucleolytic cleavage of target RNA. First, Staphylococcal nuclease homologs are not known to be endonucleases, nor are they expected to leave 3′ hydroxy and 5′ phosphate termini after cleavage. In fact, RNA or DNA hydrolysis by members of this class of nucleases yields 3′-phosphomononucleotides and dinucleotides (Cunningham et al., 1956; Reddi, 1958; Reddi, 1960), inconsistent with the 3′ hydroxy terminus we observe for the 5′ cleavage product of RISC. Second, as a member of the Staphylococcal nuclease family, TSN is expected to require Ca^{2+} for activity (Cuatrecasas et al., 1967a). Our data show that Mg^{2+}, but not Ca^{2+}, is required for siRNA-directed endonucleolytic cleavage. Third, 2′-deoxythymidine 5′,3′-bisphosphate (pdTp), a general inhibitor of staphylococcal nucleases (Cuatrecasas et al., 1967a), inhibits TSN activity (Caudy et al., 2003), but does not inhibit endonucleolytic cleavage by RISC (Figure 6). We assembled siRNA into RISC and then incubated it with target RNA in the presence of 50 µM or 100 µM pdTp, 50 µM or 100 µM 2′-deoxythymidine 3′-monophosphate (dTp), or no inhibitor (Figure 6). 5′ cleavage products were efficiently formed in all conditions, even though the pdTp concentration was more than 2,000-fold higher than RISC and ~ 2-fold greater than the K_i reported for inhibition of Staphylococcus nuclease (Cuatrecasas et al., 1967b). 100 µM pdTp was shown previously to block TSN activity and RISC-directed target RNA degradation in vitro (Caudy et al., 2003). Our data suggest that if the nuclease activity of TSN functions in target RNA destruction, the protein must act after the siRNA-directed, Mg^{2+}-dependent endonucleolytic cleavage of the target RNA.

Figure 6

Figure IV - 6. Endonucleolytic cleavage by siRNA programmed RISC is unaltered by the TSN-inhibitor pdTp (2´-deoxythymidine 5´,3´-bisphosphate). Standard RNAi reactions were carried out in the absence or presence of 50 µM or 100 µM pdTp, or in the presence of 50 or 100µM dTp (2´-deoxythymidine 3´-monophosphate). An *sod1*-specific siRNA and *sod1* target RNA were used.

Materials and Methods

General methods

In vitro RNAi reactions using *Drosophila* embryo lysate were as described previously (Haley et al., 2003). Synthetic RNAs (Dharmacon) were deprotected according to manufacturer's instructions and annealed as described previously (Elbashir et al., 2001a). The luciferase target RNAs were transcribed with recombinant histidine-tagged T7 RNA polymerase from a PCR-generated DNA template as described (Haley et al., 2003; Hutvagner and Zamore, 2002; Nykanen et al., 2001). *sod1* sense target RNA was transcribed from a plasmid template (Crow et al., 1997) linearized with BamHI (New England Biolabs) as described previously (Schwarz et al., 2003). Target RNAs were used at ~5 nM final concentration and siRNAs were used at 50 nM final concentration. Chelating agents were prepared with acetate as the counter ion.

siRNA

The luciferase-specific siRNA was composed of the antisense strand 5′-UCG AAG UAU UCC GCG UAC GUG-3′ and the sense strand 5′-CGU ACG CGG AAU ACU UCG AUU. For the *sod1* target RNA, the siRNA was composed of the antisense strand 5′-CAC AUU GCG CAA GUC UCC AdTdT-3′ and the sense strand 5′-UGG AGA CUU GCG CAA UGU GdTdT-3′. The siRNA corresponding to the sequence of the miRNA, *let-7*, was as described previously (Hutvagner and Zamore, 2002): *let-7* strand, 5′-UGA GGU AGU AGG UUG UAU AGU-3′ and passenger strand, 5′-UAU ACA ACC UAC UAC CUC AUU-3′.

Analysis of cleavage product termini

5´ and 3´ cleavage products were isolated from an 8% denaturing polyacrylamide gel. Reactions were performed with T4 Polynucleotide Kinase (PNK) (New England Biolabs) and Calf Intestinal Phosphatase (CIP) (New England Biolabs), according to manufacturer's instructions. 3´-dATP (Sigma) was used to 3´ end-label RNA (Martin and Keller, 1998) using yeast poly(A) polymerase (USB) according to manufacturer's instructions.

Construction of target RNAs bearing a single phosphorothioate substitution.

Target RNAs containing a single phosphorothioate substitution were prepared by three-way splinted ligation (Moore and Query, 2000; Moore and Sharp, 1992). The 5´ fragment was in vitro transcribed from a PCR template generated using a plasmid containing the human *sod1* gene (pcDNA3SOD1) (Ding et al., 2003) and the primers 5´-AGC TTG GTA CCG AGC TCG-3´ and 5´-TGT ATA GTC CAA GTC TCC AAC ATG CCT CT-3´ (the underlined nucleotides correspond to the first 8 nucleotides of the target site for the *let-7* siRNA). The central (5´-pACC UAC UAC CUC A-3´) and 3´ (5´-pUCG AAG UAU UCC GCG UAC GUG AUG UUC ACC-3´) fragments were synthesized (Dharmacon) and deprotected according to the manufacturer's protocol. RNA fragments were ligated using high concentration T4 DNA ligase (New England Biolabs) and a DNA splint (5´-CGC GGA ATA CTT CGA TGA GGT AGT AGG TTG TAT AGT CCA AGT-3´) (IDT). For example, phosphorothioate substitution between the first and second bases of the central fragment created an RNA bearing a phosphorothioate linkage between the ninth and tenth bases of the target site of *let-7* siRNA. The ligation product was subsequently 5´ cap ^{32}P-radiolabeled.

Synthesis of 2´-deoxythymidine 5´,3´-bisphosphate (pdTp)

pdTp was synthesized in a standard kinase reaction containing 1 mM 2´-deoxythymidine 3´-monophosphate (dTp; Sigma), T4 Polynucleotide Kinase (3´-phosphatase free; Roche), and 1mM ATP. dTp controls correspond to reactions carried out in the absence of dTp, to which 1mM dTp was added after the kinase was heat inactivated at 65°C. Synthesis was confirmed by thin-layer chromatography PEI-cellulose plates (Baker) in 0.2M ammonium sulfate. In this solvent system, the R_f for dTp was 0.64, and the R_f for pdTp was 0.34.

Acknowledgements

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Selective silencing by RNAi of a dominant, disease-causing ALS allele

The work in the following chapter was a collaborative effort among several lab members. I performed all in vitro RNAi reactions in Figure 1 showing the efficiency of the SOD siRNAs and their ability to discriminate between a mutant and wild-type sequence. Hongliu Ding, Alex Keene, El Bachir Affar, Laura Fenton, and Xugang Xia performed the transfections, Western blots, and carried out the mouse work.

CHAPTER V

Abstract

RNA interference (RNAi) can achieve sequence-selective inactivation of gene expression in a wide variety of eukaryotes by introducing double-stranded RNA corresponding to the target gene. Here we explore the potential of RNAi as a therapy for amyotrophic lateral sclerosis (ALS) caused by mutations in the Cu, Zn superoxide dismutase (SOD1) gene. Although the mutant SOD1 is toxic, the wild-type SOD1 performs important functions. Therefore, the ideal therapeutic strategy should be to selectively inhibit the mutant, but not the wild-type, SOD1 expression. Because most SOD1 mutations are single nucleotide changes, to selectively silence the mutant requires single nucleotide specificity. By coupling rational design of small interfering RNAs (siRNAs) with their validation in RNAi reactions in vitro and in vivo, we have identified siRNA sequences with this specificity. A similarly designed sequence, when expressed as small hairpin RNA (shRNA) under the control of a RNA polymerase III (pol III) promoter, retains the single-nucleotide specificity. Thus, RNAi is a promising therapy for ALS and other disorders caused by dominant, gain-of-function gene mutations.

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that causes motor neuron degeneration, skeletal muscle atrophy and paralysis. This disease is progressive and invariably fatal, resulting in the death of the patient within 1 to 5 years after diagnosis. At present there is no cure (Rowland and Shneider, 2001). A fraction of ALS is caused by mutations in the Cu, Zn superoxide dismutase (SOD1) gene (Rosen et al., 1993). These mutations cause motor neuron degeneration because the mutant protein has acquired some toxic property (Cleveland and Rothstein, 2001). Neither the molecular basis of this toxic property nor the way in which the toxic protein triggers motor neuron degeneration is understood. In mice, expression of mutant SOD1, but not complete elimination of SOD1, causes ALS. Nonetheless, SOD1-knockout mice show reduced fertility (Matzuk et al., 1998), motor axonopathy (Shefner et al., 1999), age-associated loss of cochlear hair cells (McFadden et al., 2001) and neuromuscular junction synapses (Flood et al., 1999), as well as enhanced susceptibility to a variety of noxious assaults on the nervous system, such as axonal injury (Reaume et al., 1996), ischemia (Kawase et al., 1999; Kondo et al., 1997), hemolysate exposure (Matz et al., 2000) and irradiation (Behndig et al., 2001). Given the toxicity of the mutant protein and the functional importance of the wild-type, the ideal therapy for ALS would selectively block expression of the mutant while retaining expression of wild-type protein.

Sequence-selective inactivation of gene expression can be achieved in a wide variety of eukaryotes by introducing double-stranded RNA corresponding to the target gene, a phenomenon termed RNA interference (RNAi) (Hannon, 2002; Hutvagner and Zamore, 2002; McManus and Sharp, 2002). Early mechanistic studies of the RNAi pathway (Elbashir et al., 2001b; Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000) led to its extension to cultured mammalian cells (Caplen et al., 2001; Elbashir et al., 2001a). In this approach, small interfering RNAs (siRNAs), 21 nt, double-stranded RNA intermediates in the RNAi pathway, are used to trigger the destruction of an mRNA containing the siRNA sequence. More recent experiments indicate that small hairpin RNAs (shRNAs) transcribed in vivo can trigger degradation of a corresponding mRNA, because shRNAs are processed into siRNAs in cells (Brummelkamp et al., 2002b; Jacque et al., 2002; Lee et al., 2002; McManus et al., 2002; Miyagishi and Taira, 2002; Paddison et al., 2002; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002; Zeng et al., 2002). These technical advances raise the possibility that siRNA duplexes or viruses expressing shRNA may be used to block the expression of a mutant gene.

Because the vast majority of ALS-causing SOD1 mutations are single-nucleotide point mutations that alter a single amino acid in the protein (http://www.alsod.org/), the first step in developing RNAi therapy is to identify siRNA and shRNA sequences that can selectively silence the expression of mutant, but not wild-type, protein with single-nucleotide specificity. RNAi is a promising strategy for allele-specific silencing, but the design of siRNAs with single-nucleotide specificity is not straightforward. siRNAs that

differ from the sequence of their target RNA at one or more nucleotides retain efficacy in some cases (Boutla et al., 2001; Holen et al., 2002) and lose activity in others (Boutla et al., 2001; Brummelkamp et al., 2002a; Brummelkamp et al., 2002b; Elbashir et al., 2001c; Yu et al., 2002). Here we coupled rational design with validation in RNAi reactions in vitro and in vivo, and developed siRNA and shRNA sequences that selectively silence two mutant SOD1 alleles but not the wild-type. These sequences can be developed further to treat ALS caused by these SOD1 mutations.

Results

Mechanistic studies suggest that formation of an A-form helix between the siRNA and its mRNA target is required for mRNA cleavage (Chiu and Rana, 2002). We reasoned that mismatches at or near the site of target cleavage would disrupt the required A-form helix. We targeted an allele of SOD1 in which guanosine 256 (G256, relative to the start of translation) is mutated to cytosine, generating a glycine-to-arginine mutation (G85R). We placed the mismatch at positions 9, 10, and 11 from the 5′ end of the siRNA. The G256C mutant/wild-type pair produces the largest possible clash (purine:purine) between the mutant siRNA and the wild-type gene and the greatest hydrogen-bonding (G:C) between the mutant siRNA and the mutant SOD1 allele (Fig. 1A). As controls, we synthesized comparable siRNAs to target wild-type but not mutant SOD1 mRNA (Fig. 1A). In the controls, the siRNAs contain a G:C base pair at the selective site, but the mismatch between wild-type siRNA and mutant allele is a smaller pyrimidine:pyrimidine clash (C:C). The selectivity of each siRNA was tested in a cell-free RNAi reaction containing *Drosophila* embryo lysate (Tuschl et al., 1999; Zamore et al., 2000) (Fig. 1B and 1C).

Each of the six siRNAs cleaved the corresponding target RNA, although with dramatically different efficiency (Fig. 1B). For example, neither mutant nor wild-type p11 siRNAs cut their respective RNA targets with a rate expected to be effective in vivo. On the other hand, the p10 mutant siRNA efficiently cleaved the mutant SOD1 mRNA. In all cases, destruction of full-length target mRNA was accompanied by a corresponding

accumulation of a ~288 nt 5′ cleavage product, demonstrating that the siRNAs trigger RNAi, rather than non-specific RNA degradation (Fig. 1B). In the absence of siRNA or in the presence of an unrelated siRNA, the mutant SOD1 target RNA was stable in the *Drosophila* embryo lysate (data not shown). Data for both the destruction of target RNA and the accumulation of 5′ cleavage product fit well to a single exponential equation, indicating that the reaction follows pseudo first-order kinetics (Fig. 1C).

To determine the selectivity of the six siRNAs, each siRNA corresponding to the mutant SOD1 sequence was tested for its ability to cleave wild-type SOD1 mRNA, and each wild-type siRNA was tested for its ability to cleave mutant mRNA. Some but not all of the siRNA duplexes effectively discriminated between the target to which they were perfectly matched and the target with which they had a single-nucleotide mismatch (Fig. 1B). We observed two types of defects for a subset of siRNAs. Both wild-type and mutant p11 siRNA did not trigger efficient target cleavage of either the perfectly matched or the mismatched RNA target (Fig. 1B). Thus, these siRNA sequences are inherently poor triggers of RNAi. The p9 and p10 wild-type siRNAs not only triggered rapid cleavage of their corresponding wild-type target, but also produced significant cleavage of the mutant RNA (Fig. 1B). These siRNAs are good triggers of RNAi but show poor selectivity. In contrast, the p10 mutant siRNA showed both efficient RNAi and robust discrimination between mutant and wild-type SOD1 RNAs, cleaving the mutant far more efficiently than the wild-type RNA in the cell-free reaction (Fig. 1B and 1C). Because this siRNA showed nearly complete discrimination between mutant and wild-type SOD1 mRNA targets (Fig. 1B and 1C), it is an ideal candidate for therapeutic application.

Figure 1

A

```
mutant siRNA p9     5´-UGGAGACUUGCGCAAUGUGdTdT-3´
                    3´-dTdTACCUCUGAACGCGUUACAC-5´
             p10      5´-GGAGACUUGCGCAAUGUGAdTdT-3´
                    3´-dTdTCCUCUGAACGCGUUACACU-5´
             p11        5´-GAGACUUGCGCAAUGUGACdTdT-3´
                    3´-dTdTCUCUGAACGCGUUACACUG-5´
sod1 wild type 5´-...GAGAGGCAUGUUGGAGACUUGGGCAAUGUGACUGCUGACAAAGAUGGU...-3´
sod1 mutant    5´-...GAGAGGCAUGUUGGAGACUUGCGCAAUGUGACUGCUGACAAAGAUGGU...-3´

wild-type siRNA p11     5´-GAGACUUGGGCAAUGUGACdTdT-3´
                    3´-dTdTCUCUGAACCCGUUACACUG-5´
             p10      5´-GGAGACUUGGGCAAUGUGAdTdT-3´
                    3´-dTdTCCUCUGAACCCGUUACACU-5´
             p9     5´-UGGAGACUUGGGCAAUGUGdTdT-3´
                    3´-dTdTACCUCUGAACCCGUUACAC-5´
```
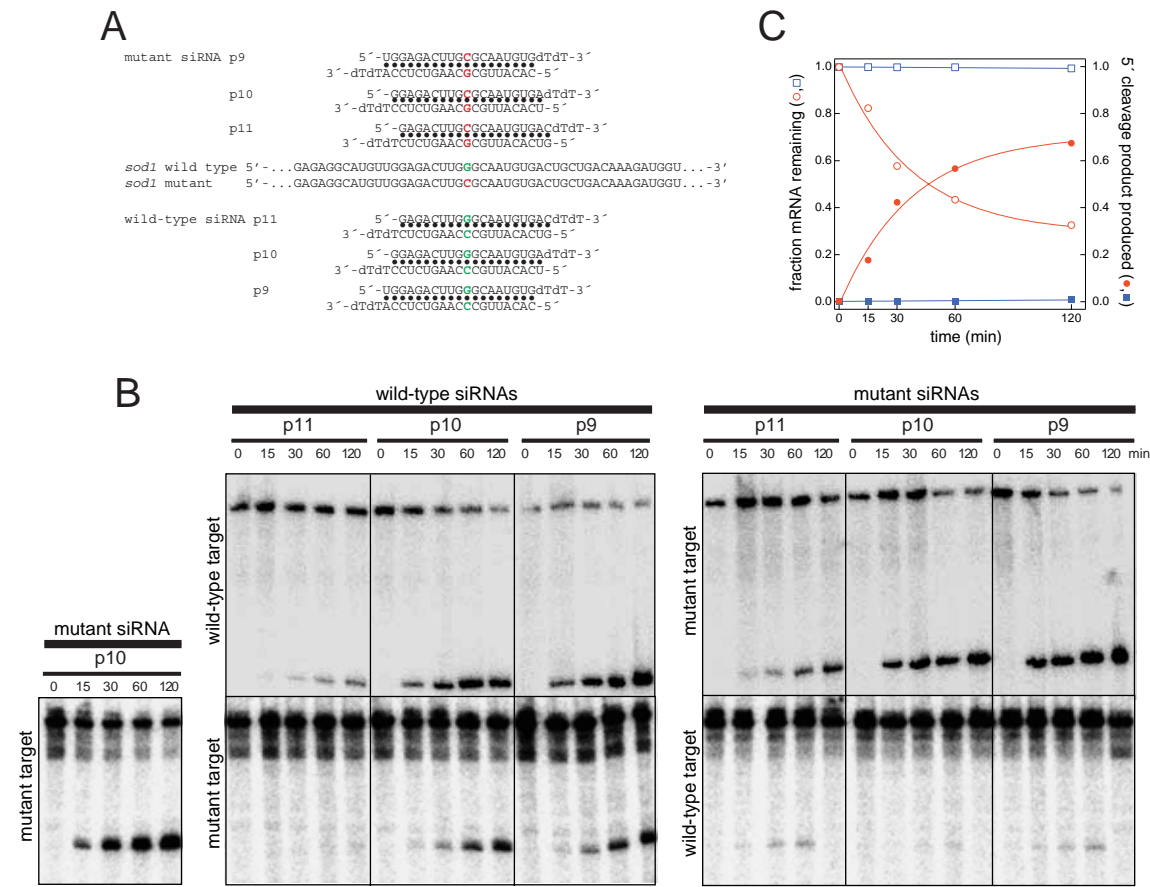
C

B

Figure V - 1. siRNA duplexes can discriminate between mutant and wild-type SOD1 in vitro. (A) siRNA duplexes used. (B) In vitro RNAi experiments targeting mutant or wild-type SOD1 mRNA with mutant or wild-type siRNAs. (C) Mutant siRNA p10 targets mutant (red curves) but not wild-type SOD1 (blue curves) mRNA for destruction by the RNAi pathway.

To test whether the cell-free reactions accurately predict siRNA efficacy and selectivity in mammalian cells, we analyzed the siRNAs in a HeLa cell assay. We prepared plasmids that expressed either SOD1^{WT} or SOD1^{G85R} with GFP fused to their carboxyl terminus. Each construct was transfected into HeLa cells together with both siRNA and a dsRed-expressing vector that served as a transfection control. The expression of either mutant or wild-type SOD1 was monitored by fluorescence-activated cell sorting (FACS). Transfection of p9, p10 and p11 siRNAs with their corresponding mutant or wild-type targets suppressed gene expression, although with distinctly different efficiency and selectivity (Fig. 2). Co-transfection with an siRNA complementary to firefly luciferase did not suppress either SOD1 allele (Fig. 2). As observed in the cell-free reactions, the p10 siRNA against wild-type SOD1 showed no selectivity and suppressed both wild-type and mutant SOD1 mRNA (Fig. 2). The other siRNAs all showed some degree of selectivity, but the p10 siRNA directed against the SOD1 mutant mRNA showed both the greatest efficacy and selectivity, in agreement with the results of the cell-free reactions. Thus, some but not all siRNAs can efficiently discriminate between mRNA targets with a single-nucleotide difference.

Recently, it has been shown that shRNAs can trigger RNAi in vivo. To test whether shRNA against mutant SOD1 can selectively block the expression of the mutant, but not the wild-type, SOD1 expression, we constructed a plasmid that synthesizes a shRNA homologous to another disease-causing mutant SOD1^{G93A} (Sui et al., 2002). This mutant was examined because, like the SOD1^{G85R}, it is a guanosine to cytosine change at 281st neucleotide, thus placing a G:G mismatch at the selective site between the shRNA

Figure 2

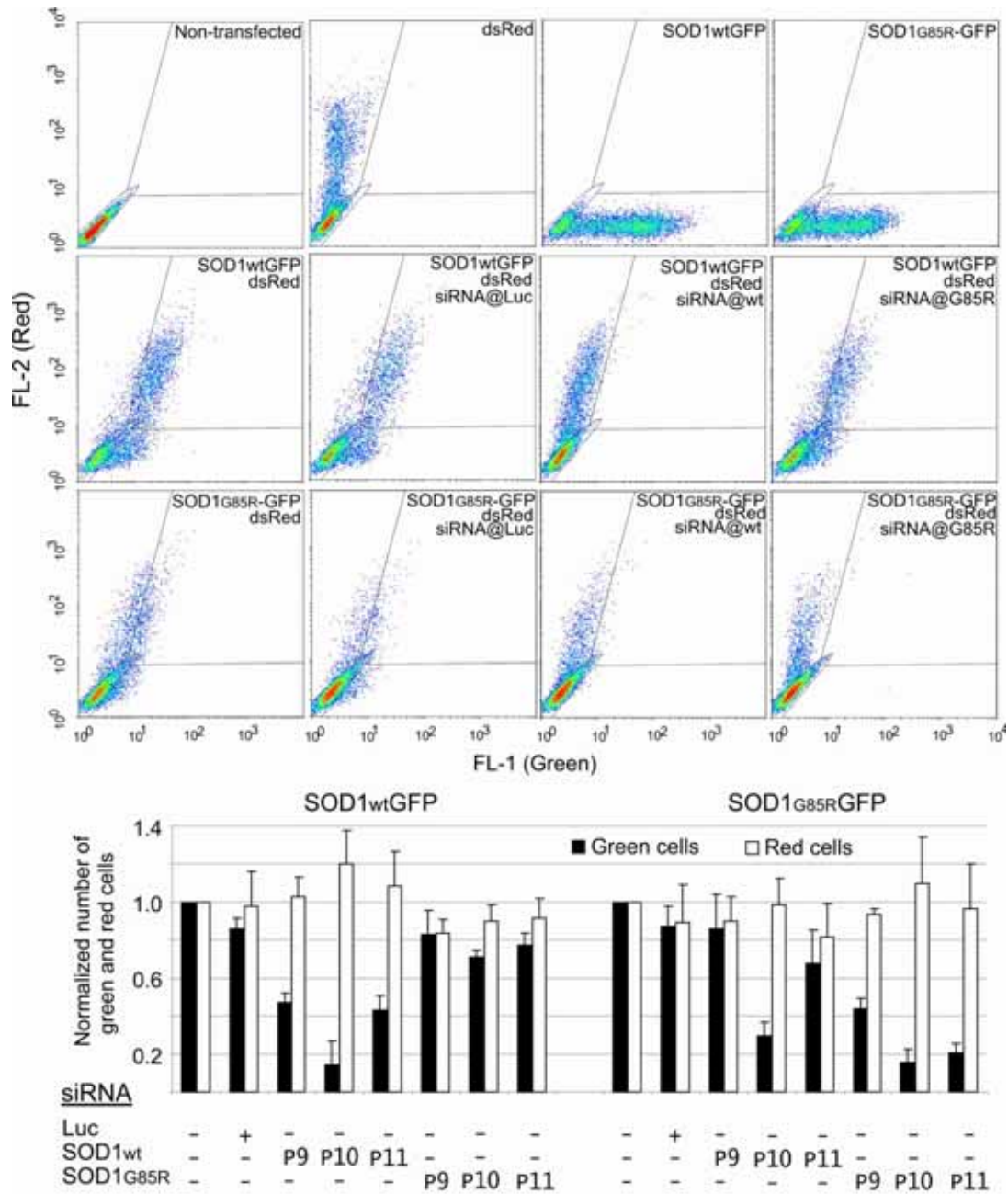**Figure V - 2.** Selective inhibition of mutant SOD1^{G85R} expression by siRNA in Hela cells. SOD1^{WT}-GFP or SOD1^{G85R}-GFP was cotransfected with various siRNAs. DsRed was cotransfected as a transfection control. Green and red fluorescent cells were quantified using FACS. (A) Raw FACS cell counts, (B) relative number of green and red cells in the transfections (n=3). Error bars represent standard deviation.

and wild type SOD1 (Fig. 3A). When co-transfected separately with mutant SOD1-GFP

or wild-type expressing plasmid, this hairpin construct inhibited mutant, but not the wild-

type, expression (Fig. 3). Thus, hairpin constructs can be used to trigger single-

nucleotide selective RNAi of mutant SOD1 in cultured cells. To test if mutant-selective

inhibition can be achieved in neuronal cells, we separately transfected the wild-type or

mutant SOD1-GFP constructs with either siRNA p10 against SOD1^{G85R} or shRNA-

synthesizing vector against SOD1^{G93A} into the neuroblastoma cell line N2a. As in HeLa

cells, both synthetic siRNAs and shRNA constructs directed the selective inhibition of

mutant SOD1 expression in N2a cells (Fig. 4A, B).

To be therapeutically relevant, single-nucleotide selective siRNAs must

discriminate between mutant and wild-type SOD1 when both mRNAs are present in the

same cell. We transfected HeLa cells with p10 siRNAs and mutant SOD1^{G85R}-GFP, and

analyzed SOD1 protein expression by immunoblotting with anti-SOD1 antibody that

recognizes both the transfected SOD1^{G85R}-GFP fusion protein and endogenous wild-type

SOD1. As expected, p10 siRNA against wild-type SOD1 inhibited both the endogenous

wild-type SOD1 and the transfected SOD1^{G85R} –GFP (Fig. 5). The near 50% inhibition

of the endogenous wild-type SOD1 expression probably reflected the transfection

efficiency, which was ~50%. In contrast to the p10 wild-type siRNA, at two different

doses, p10 siRNA against the mutant inhibited expression of the mutant, but had no effect

on the expression of endogenous wild-type SOD1 (Fig. 5). No additional selectivity was

seen with a 3´-blocked siRNA, consistent with reports that siRNAs do not function as

primers to trigger the production of 'secondary siRNAs' in human cells (Chiu and Rana,

Figure 3
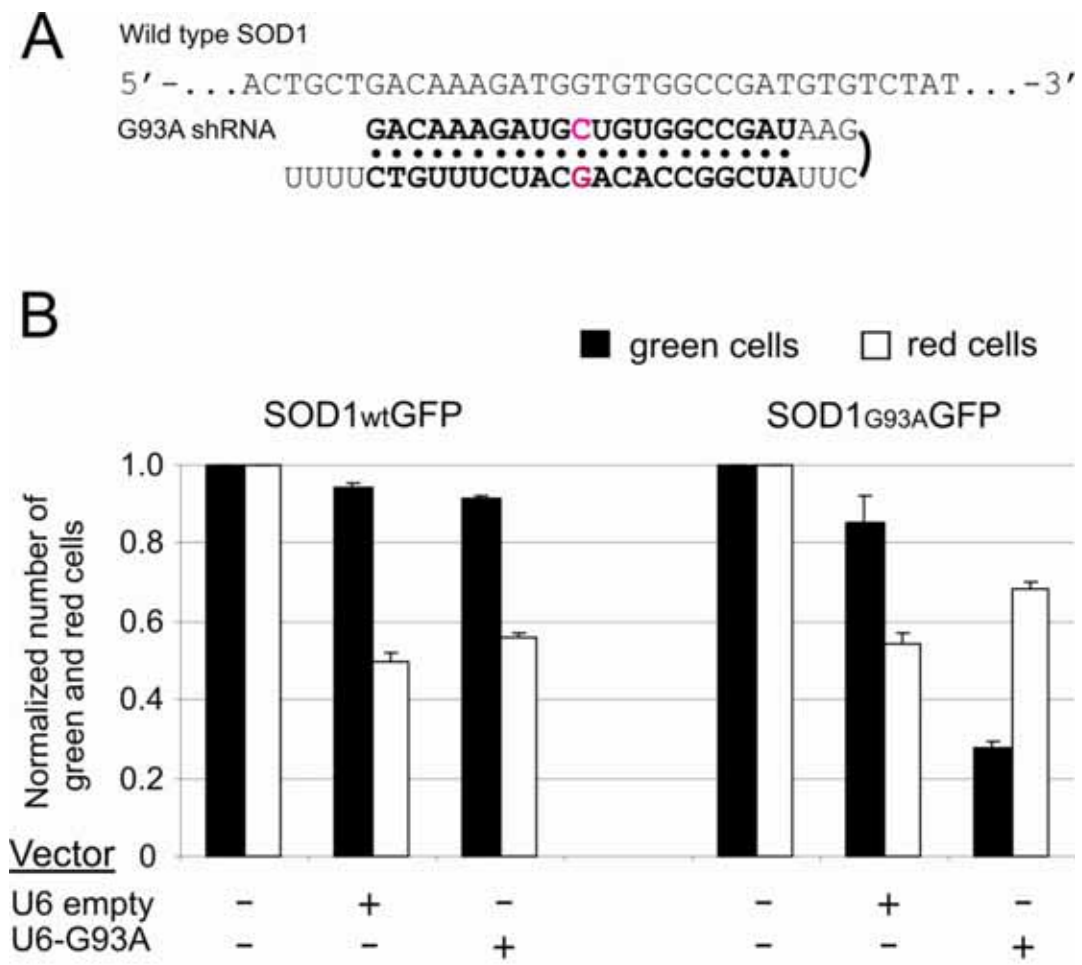
Figure V - 3. Selective inhibition of mutant SOD1^{G93A} expression by U6-G93A vector in Hela cells. (A) Design of the G93A shRNA. Bold letters mark nucleotides that match SOD1 sequence. The pink letter marks the mismatched nucleotide. (B) SOD1^{WT}-GFP or SOD1^{G93A}-GFP was cotransfected with U6-empty or U6-G93A (U6 constructs:SOD1-GFP = 5:1). DsRed was cotransfected as a transfection control (SOD1-GFP:DsRed = 4:1). Green and red fluorescent cells were quantified using FACS. Results from four experiments were averaged. Error bars represent standard deviation.

Figure 4
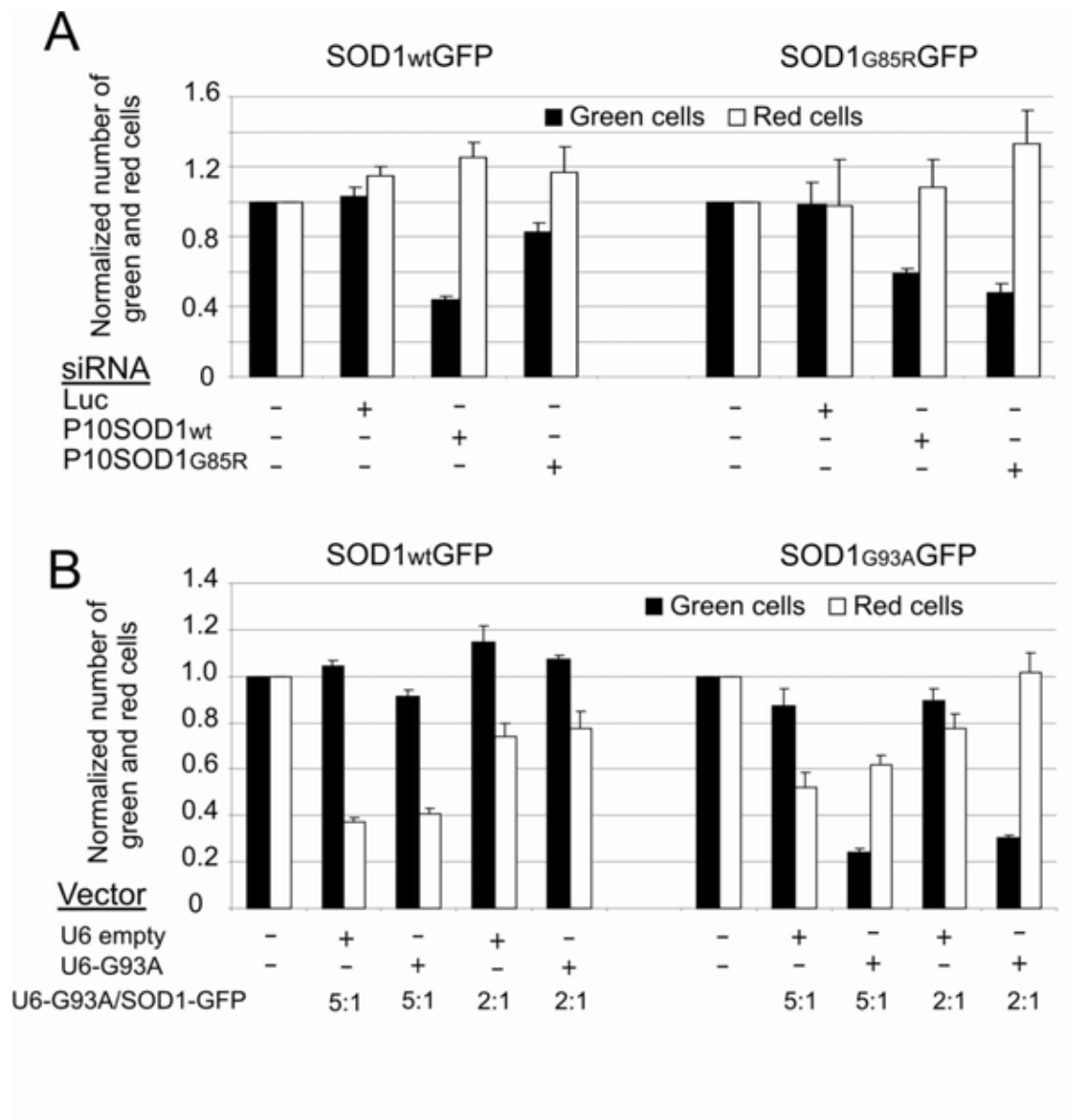
Figure V - 4. Selective inhibition of mutant SOD1 expression by siRNA and U6-G93A vector in neuroblastoma N2a cells. Green and red fluorescent cells were quantified using FACS. (A) siRNA against SOD1^{G85R} (n=4), (B) U6-G93A vector (n=3). Error bars represent standard deviation.

Figure 5
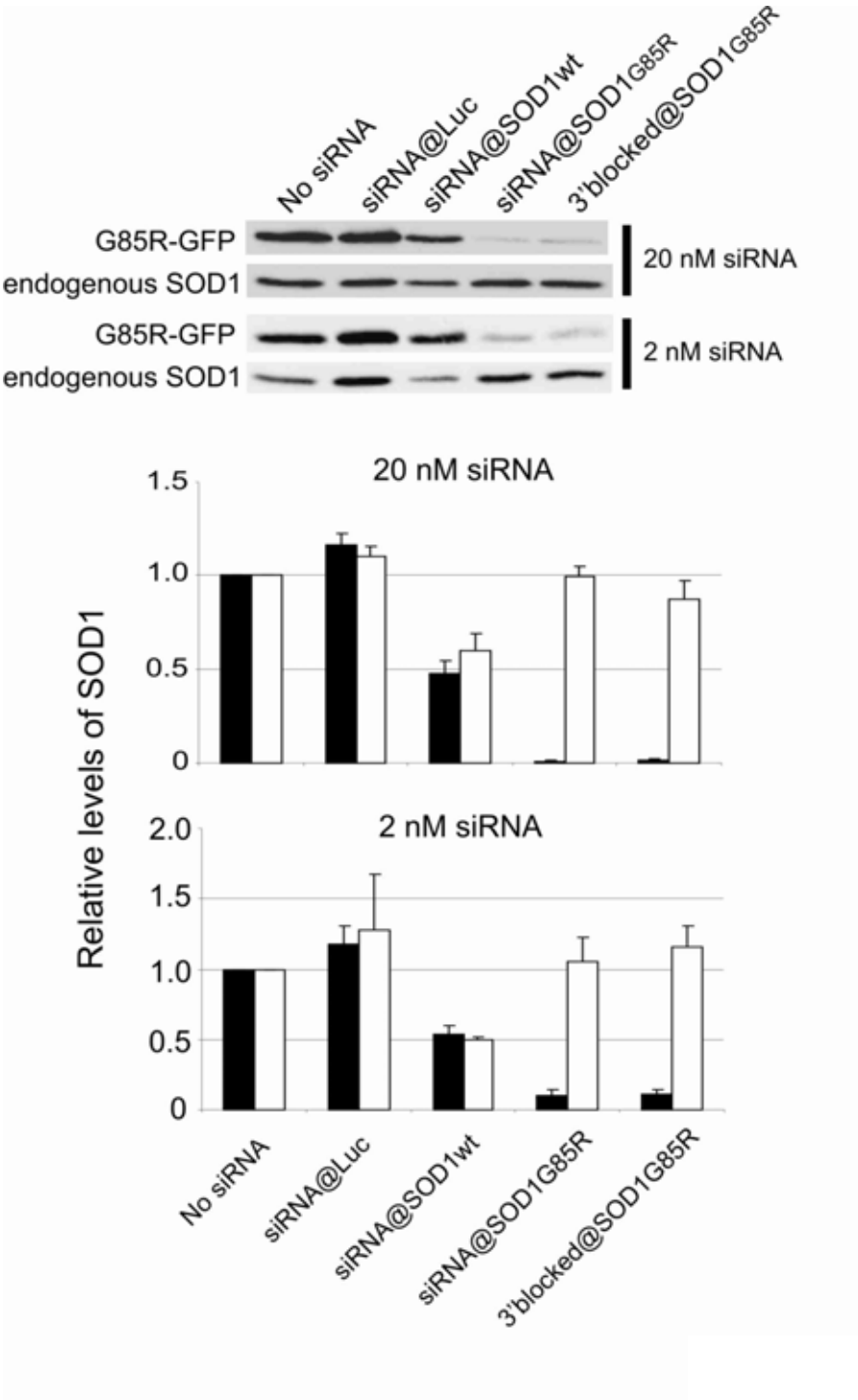
Figure V - 5. Selective inhibition of mutant SOD1 G85R but not the wild type SOD1 expression by siRNA in the same cells. (A) Protein blots of the transfected Hela cells detecting mutant SOD1^{G85R}-GFP and endogenous human SOD1. (B) Relative levels of SOD1 measured from the protein blots (average of 4 transfections). Error bars are standard error.

2002; Holen et al., 2002; Schwarz et al., 2002), as they do in nematodes (Sijen et al., 2001).

Finally, to test whether this selective inhibition can occur in vivo, we transfected SOD1 reporters and shRNA plasmid into mice using the hydrodynamic transfection protocol. Mutant SOD1^{G93A}-GFP and myc-tagged wild-type human SOD1 expression plasmids were used, enabling detection of mutant and wild-type human SOD1 proteins, as well as the endogenous mouse SOD1 by immunoblotting. We analyzed SOD1 expression in liver, a tissue readily transfectable by the hydrodynamic method. Under these conditions, the shRNA-expressing plasmid selectively decreased mutant but not wild-type human SOD1 expression (Fig. 6).

Figure 6
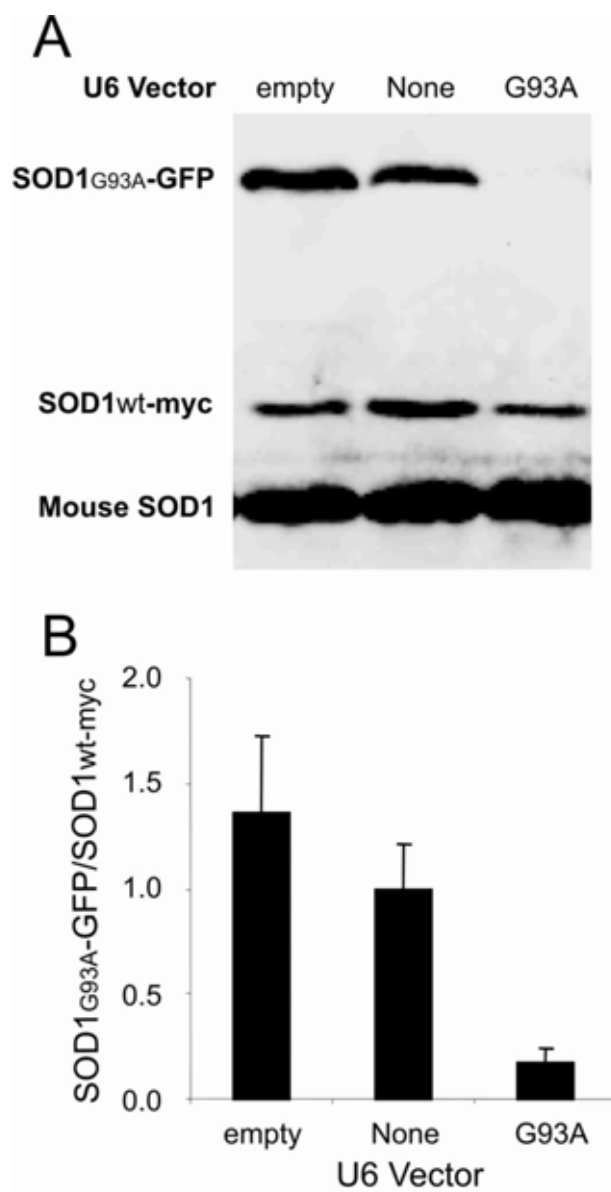
Figure V - 6. Selective inhibition of mutant SOD1 expression by U6-G93A vector in vivo. (A) SOD1^{G93A}-GFP was cotransfected with a C-terminal myc tagged wild type human SOD1 in mice using the hydrodynamic transfection method. Liver proteins were resolved on SDS-PAGE and detected using anti-SOD1 antibody. (B) The relative band intensities were quantified. The ratio of SOD1^{G93A}-GFP to wild type SOD1myc is shown. Eight animals were used in each group. The U6-G93A group is significantly different from the other two groups ($p < 0.05$) by Hohm's modified Bonferroni procedure for multiple t tests.

Discussion

We have found siRNA and shRNA sequences that selectively silence two dominant mutant SOD1 genes. Using multiple siRNAs matching either wild-type or mutant SOD1, we show that a subset of siRNAs against mutant SOD1 cleave the mutant, but not the wild-type SOD1 RNA efficiently in vitro (Fig. 1). Those siRNAs that show both efficacy and selectivity in vitro also selectively inhibit mutant but not wild-type SOD1 protein expression in mammalian cells (Fig. 2, 4), even when both the mutant and the wild-type proteins are present in the same cells (Fig. 5). Furthermore, a vector expressing shRNA similarly designed according to the optimal siRNA also selectively inhibited mutant but not wild-type SOD1 expression in mouse liver (Figs. 3, 4, 6). These results demonstrate that selective inhibition of dominant mutant SOD1 alleles can be achieved using RNAi and the optimal siRNA and shRNA sequences can be identified by a preclinical screen in vitro and in vivo.

Our search for siRNA sequences optimized for selective silencing of the mutant but not the wild-type SOD1 reveal that single nucleotide discrimination is not guaranteed. Some siRNAs can discriminate between alleles that differ at a single nucleotide, whereas others cannot. Our results point to two different types of deficiencies for siRNAs designed to target mutant, disease-causing alleles. First, not all siRNAs silence with the same efficiency. Among the siRNAs directed against wild-type SOD1, p9 and p10 cleave the target more efficiently than p11 (Fig. 1). As predicted by analysis in cell-free RNAi reaction, the p10 siRNA inhibited target gene expression most efficiently in

mammalian cells (Fig. 2). Among the siRNAs against the mutant SOD1^{G85R}, p9 and p10 cleave the mutant far more than P11 (Fig. 1). As in the cell-free assay, p10 was the most efficient siRNA in inhibiting the mutant SOD1 expression in mammalian cells (Fig. 2). It is intriguing that displacing the siRNA along the target sequence by a single nucleotide results in such a dramatic change in silencing efficiency. Second, significant differences in selectivity between the perfectly matched target RNA and the RNA bearing a single-nucleotide mismatch were observed among the six siRNAs used. For example, wild-type p10 siRNA had poor selectivity; it cleaved both wild-type and mutant SOD1 RNA in the cell-free assay and efficiently inhibited the expression of both alleles in mammalian cells (Figs 1, 2, 4, 5). In contrast, the p10 siRNA directed against mutant SOD1 showed nearly perfect selectivity. It cleaved mutant SOD1 RNA but not wild-type, in the cell-free assay, and inhibited mutant but not wild-type SOD1 expression in mammalian cells (Figs 1, 2, 4, 5).

Our results raise questions regarding the rules in designing optimal siRNAs or shRNAs for single nucleotide discrimination. Among the contributing factors is the type of mismatch at the critical site p10. We predict that a purine:purine mismatch disrupts the A-form helix that is required between the anti-sense strand of the siRNA and its mRNA target (Chiu and Rana, 2002). In contrast, a pyrimidine:pyrimidine mismatch may more readily be accommodated within an A-form helix. Thus, the G:G clash between the siRNA and the wild-type target RNA discriminates against the wild-type target, producing greater selectivity for the mutant target, whereas the presence of a G:C basepair between the mutant siRNA and the mutant target mRNA at the selective site

may serve to maximize the energy difference between mismatch and perfect pairing (see Figs. 1A, 3A). Consistent with this view, in *Drosophila* embryos, an siRNA having a pyrimidine:purine mismatch (C:A) with its target mRNA was only slightly less effective than the perfectly matched siRNA (Boutla et al., 2001). Moreover, an siRNA directed against firefly luciferase failed to produce detectable RNAi in vitro when it contained at position 9 or 10 of its guide strand a purine:purine (A:A) mismatch with its target RNA(Elbashir et al., 2001d). Likewise, an siRNA that showed good selectivity for a mutant Ras mRNA created a purine:purine (A:G) clash with the wild-type allele (Elbashir et al., 2001c). Similarly, an siRNA that showed good selectivity for a mutant Ras mRNA created a purine:purine (A:G) clash with the wild-type allele (Brummelkamp et al., 2002a). Arguing against this view, one experiment using siRNA against hTF suggests that a G:G mismatch can still mediate RNAi, albeit with reduced efficiency (Holen et al., 2002). It is possible that this was due to high concentration of siRNA used. Another experiment using shRNA against CDH-1 suggest that a U:C or a U:G mismatch abolished RNAi (Boutla et al., 2001). In light of our demonstration that small differences in siRNA sequence can produce dramatic differences in efficacy, rather than selectivity, it remains to be shown if these inactive shRNAs were active against a perfectly matched target, and not merely poor triggers of RNAi in general. Clearly, further work is required to clarify the rules in designing siRNA and shRNA sequences optimized for selective silencing of mutant alleles with single nucleotide specificity.

Taken together, we have identified siRNA and shRNA sequences that can selectively down-regulate the expression of mutant but not the wild type SOD1, even

when the mutant mRNA differs from wild-type by a single nucleotide. Because the shRNA-synthesizing plasmid construct can be readily incorporated into viral vectors (Brummelkamp et al., 2002a; Devroe and Silver, 2002; Xia et al., 2002), these siRNA and shRNA sequences can be readily placed in virus-based delivery systems to treat ALS caused by mutant SOD1 expression. In broad terms, our results show the promise of RNAi as a therapeutic strategy to diseases caused by dominant, gain-of-function gene mutations.

Materials and Methods

RNA and DNA constructs

Twenty one nucleotide single strand RNAs (Fig. 1) were purchased from Dharmacon Research, deprotected according to manufacturer's instructions, and annealed as described (Nykanen et al., 2001). The 3'-block siRNA was synthesized a 2',3'-dideoxy cytidine at the 3' terminus of the antisense strand. To create wild type and mutant SOD1-GFP fusion proteins, SOD1^{WT}, SOD1^{G85R} and SOD1^{G93A} cDNAs (kind gifts of Dr. Joseph Beckman) were PCR cloned between the PmlI and PstI sites of pCMV/myc/mito/GFP (Invitrogen). This cloning step deleted the mitochondrial targeting sequence. To create myc tagged wild type SOD1, SOD1wt cDNA was PCR cloned between the PstI and XhoI sites of pCMV/myc/mito/GFP. The mitochondrial targeting sequence was then deleted by digestion with BssHII and PmlI and blunt ligation. All constructs were verified by sequencing. DsRed (pDsRed2-C1) was purchased from Clontech (Palo Alto, CA). U6-G93A was constructed as described (Sui et al., 2002) (Fig. 3).

In vitro RNAi assay

Drosophila embryo lysates were prepared as previously described (Tuschl et al., 1999; Zamore et al., 2000). Five hundred and sixty nucleotide human SOD1 target RNAs containing either wild-type or mutant SOD1 G85R coding sequence were cap-labeled using Guanylyl transferase as described previously (Zamore et al., 2000). In vitro

RNAi reactions were carried out in *Drosophila* embryo lysate by incubating ~5 nM of the 5′, ^{32}P-cap-radiolabeled target RNA with 100 nM duplex siRNA at 25°C in a standard reaction (Tuschl et al., 1999; Zamore et al., 2000). Cleavage products were analyzed on 5% denaturing acrylamide gels, dried, and exposed on image plates (Fuji). Plates were scanned using a Molecular Imager FX (Biorad), and images were analyzed using Quantity One version 4.0.3 (Biorad).

Cell culture and transfection

Hela cells were cultured in DMEM and N2A cells in DMED and Opti-MEM (1:1), both supplemented with 10% fetal bovine serum (FBS), 100 units ml^{-1} penicillin, and 100 ug ml^{-1} streptomycin. Twenty-four hours before transfection, cells (70-90% confluency) detached by trituration, transferred to 6-well plates and cultured in fresh medium without antibiotics. Transfection was carried out using lipofectamine 2000 (invitrogen) according to manufacturer's instructions. The amounts of the constructs used in transfections are 4 µg each of mutant or wild type SOD1-GFP and DsRed plasmids, 4×10^{-11} or 4×10^{-12} mole siRNAs, and 20 or 8 µg U6-G93A, unless stated otherwise.

In vivo transfection

Twenty four mice 6-8 weeks old were divided into three groups. The first group received no shRNA vector, the second group received 20 µg empty vector and the third group received 20 µg U6-shRNA vector against SOD1 G93A. All groups received both 20 µg of myc tagged human wild type SOD1 and 20 µg GFP tagged SOD1. The vectors

were diluted in Ringer's solution so that the total volume equaled 2.5 ml per mouse. Mice were anaesthetized with avertin (240mg/kg) and the vectors were injected into the tail vein using a 26-gauge needle in less than 10 seconds. Forty eight hours following injection animals were perfused with 5ml PBS in order to remove blood from the liver. Livers were dissected and quickly frozen on dry ice. Samples were placed in 25 mM PBS buffer (pH 7.2) containing 1% SDS, 1 mM DTT, 1 mM phenylmethylsufonyl fluoride (PMSF), and protease inhibitor cocktail (Sigma, diluted 1:100) and homogenized using a hand held polytrone (Pro-scientific).

Western blot analysis

Protein concentrations were determined using a BCA protein assay kit (Pierce; Rockville IL). Twenty five µg Hela cell proteins or 100 µg liver proteins were separated on a 15% SDS-PAGE gel and transferred onto Genescreen Plus membrane (Perkin Elmer). Rabbit anti-SOD1 (Biodesign) or Sheep anti-SOD1 was the primary and HRP-labeled goat anti-rabbit IgG (Amersham) or donkey anti-sheep IgG was the secondary antibodies. The protein bands were visualized using SuperSignal kit (Pierce) and Kodak Digital Image Station 440CF. The intensity of the bands was quantified using Kodak 1D software.

Acknowledgements

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Application of Functionally Asymmetric siRNA Design

to Single Nucleotide Discrimination

The work presented in Chapter 5 was a collaborative effort among three labs. Specifically, Hongliu Ding in Zuoshang Xu's lab conducted HEK293T cells transfections to study the SOD1 point mutations. Lori Kennington and Jess Moore performed the HeLa cell transfections to look at the siRNAs targeting Huntingtin, as well as artificial point mutations designed to study additional siRNAs. Rosetta performed real-time and microarray experiments assaying wildtype SOD1 levels. I performed all *in vitro* RNAi experiments, as well as data analysis, experimental design, writing of the manuscript and figure preparation.

CHAPTER VI

Summary

The discovery of RNA interference (RNAi), a sequence-specific post-transcriptional silencing pathway found in eukaryotes, offers a means to target mutant alleles of genes causing dominant, gain of function human disease. Specifically, small interfering RNAs (siRNAs) can be used to knock-down the expression of an allele that differs by a single nucleotide. Although this approach is promising, the exact details of single nucleotide discrimination have not been studied on an extensive basis. Here we identify several siRNAs that are capable of discriminating between a single nucleotide mismatch in the wild-type and mutant alleles in the human Cu, Zn superoxide dismutase (SOD1) gene, which contributes to the progression of amyotrophic lateral sclerosis (ALS) through the gain of a toxic property, as well as single nucleotide polymorphisms in the huntingtin (htt) gene which may be linked to CAG-repeat expansion leading to Huntington's disease. In addition, by designing artificial point mutations we show that siRNAs in which the mismatch is located in the region responsible for binding display lower levels of selectivity. In contrast, when the mismatch is located in the region critical for catalysis or in the 3´ region of the siRNA, it results in a higher probability of discriminating between alleles.

Introduction

In principle, RNA interference (RNAi), a conserved, sequence-specific pathway found in eukaryotes, provides a means to selectively inhibit expression of a mutant form of a gene while leaving expression of the wild-type copy unperturbed. RNAi is mediated by small interfering RNAs (siRNAs), 21–22 nt double-stranded RNAs. Synthetic siRNAs provide a straightforward means to knock-down gene expression in cultured cells (Elbashir et al., 2001a) through incorporation as single strands (Martinez et al., 2002a) into a protein-RNA complex known as the RNA-Induced Silencing Complex (RISC) (Hammond et al., 2000; Martinez et al., 2002a; Nykanen et al., 2001). When the siRNA is complimentary to its mRNA target, the siRNA will direct endonucleolytic cleavage of the mRNA at a single scissile phosphate across from nucleotides 10 and 11 in the siRNA, triggering mRNA destruction (Elbashir et al., 2001b; Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000). Both strands of an siRNA can be competent in directing endonucleolytic cleavage if a cognate mRNA is present (Elbashir et al., 2001b; Harborth et al., 2001; Nykanen et al., 2001; Schwarz et al., 2003).

However, it has recently been shown that the thermodynamics of the 5´ ends of the siRNA strands determine the degree to which each of the siRNA strands can enter RISC leading to target mRNA destruction (Khvorova et al., 2003; Schwarz et al., 2003). Some siRNAs exhibit near absolute asymmetry, termed functional asymmetry, in which only one strand of the siRNA is capable of entry into the RISC with the exclusion of the other strand (Schwarz et al., 2003).

It has been shown that certain mismatches between an siRNA and an mRNA can block mRNA cleavage by RISC (Amarzguioui et al., 2003; Boutla et al., 2001; Brummelkamp et al., 2002a; Brummelkamp et al., 2002b; Elbashir et al., 2001c; Harborth et al., 2001; Holen et al., 2002; Yu et al., 2002). Thus, siRNAs can discriminate between mRNAs that differ at a single base pair, suggesting the potential application of this mechanism in suppressing dominant mutant genes in diseases including ALS (Ding et al., 2003; Maxwell et al., 2004; Ralph et al., 2005; Raoul et al., 2005), Huntington's (Harper et al., 2005), Alzheimer's (Miller et al., 2004), human immunodeficiency disorder (HIV) (Lee et al., 2005; Li et al., 2005), slow channel congenital myasthenic syndrome (SCCMS) (Abdelgany et al., 2003) and cancer (Martinez et al., 2002b). Because these siRNAs target single nucleotide polymorphisms, their design is limited to the region surrounding the mutation.

Previous studies have examined whether mismatches at the central region of the siRNA, positions 9 to 11 (Ding et al., 2003), can prevent cleavage of the target mRNA by RISC through potential disruptions to the A-form helix (Chiu and Rana, 2002). However, the understanding of the selectivity of these siRNAs was unclear because even perfectly paired siRNAs (mutant siRNA sequence targeting a mRNA harboring the mutant sequence) displayed varying degrees of silencing. Thus, comparing discrimination among siRNAs capable of varying levels of silencing under perfectly paired conditions complicated the analysis. In addition, another subset of small non-coding RNAs known as microRNAs (miRNAs), normally pair with their target genes in an imperfect fashion leading to translational repression (Olsen and Ambros, 1999). One

group reported that siRNAs displaying imperfect pairing to target mRNAs lead to translational repression (Saxena et al., 2003). Therefore, it was also unclear at what level single nuncleotide mismatched siRNA:mRNA target pairs were disrupting gene expression.

Amyotrophic lateral sclerosis (ALS) is an age-dependent neurodegenerative disease that can be caused by sporadic or dominantly inherited point mutations in the Cu, Zn superoxide dismutatse gene (SOD1) (Rosen et al., 1993) which prevents cellular damage by destroying free oxygen radicals released from metabolic processes (Kinouchi et al., 1991). Point mutations in the SOD1 gene have been linked to the acquisition of a toxic property of the mutant protein, the mechanism of which is not yet understood (Cleveland and Rothstein, 2001). With several point mutations clinically identified, one possible therapy to ameliorate the symptoms of ALS would be to selectively eliminate the mutant copy of the SOD1 gene. It is imperative to only target and destroy the mutant allele, because destruction of the wild-type gene has been shown to lead to developmental and neuronal defects (Flood et al., 1999; McFadden et al., 2001; Shefner et al., 1999), as well as reduced fertility (Matzuk et al., 1998), as seen in knockout mice. Huntington's disease is a neurodegenerative disorder characterized by motor and cognitive symptoms, and cell death in the cortex and striatum (Vonsattel and DiFiglia, 1998). The onset of the disease is linked to the expansion of a CAG repeat in exon 1, which is postulated to be linked to point mutation within the coding region of the htt gene (1993).

By examining various siRNA parameters (sequences, positions, and mismatches), we have identified siRNAs that silence the G85R point mutation in SOD1 which causes

the familial form of ALS, but leave the wild-type copy intact. In addition, siRNAs were designed to target known *htt* SNPs that may be correlated with the CAG repeat expansion. In this study we utilize frayed siRNAs that incorporate more efficiently into RISC to compare the ability of each siRNA to discriminate between alleles. In addition, we examine different nucleotide mismatches (purine:purine, purine:pyrimidine, or pyrimidine:pyrimidine), either based on sod1 or htt mutant alleles, as well as engineered point mutations, between an siRNA and its target mRNA to determine the level of discrimination that can be achieved based on sequence content in both cultured cells and *Drosophila* embryo lysate. By placing the mismatch at every position along the siRNA:target RNA pair, we show that the selectivity of the siRNA is based on the region in which that mismatch is located, in addition to the nature of the mismatch. Regions contributing to binding of RISC to the target RNA are not optimal for designing discriminating siRNAs as these siRNAs can be capable of silencing either allele. However, regions critical for catalysis and maintenance of a central A-form helix display a higher probability of support single nucleotide discrimination. We conclude that the nature of the mismatch, as well as the location of the siRNA mismatch with the target mRNA, determines the level of discrimination that can be achieved between alleles differing in a single nucleotide.

Results

A tiled set of siRNAs targeting mutant SOD1

A set of 19 siRNAs was designed to recognize the G85R point mutation of human SOD1 (Figure 1A). Each siRNA was shifted one nucleotide down across the region of the mutation site to create a tiled set such that the region each siRNA targeted was shifted over 1 nucleotide. Since siRNA strands incorporate into RISC asymmetrically (Khvorova et al., 2003; Schwarz et al., 2003), we designed the siRNA strands in this study to have "frayed", or unpaired, 5´ ends such that they require less energy to unwind and facilitate the entry of that strand into RISC. This allows for high levels of RISC to be made which contain the strand of the siRNA complementary to the mRNA, or the guide strand, and to minimize any differential effects of RISC entry when comparing the efficacies of different siRNAs.

Using this strategy we were able to make each siRNA containing the mutant sequence effective at targeting a perfectly matched mutant target, and in turn allows for the assessment of each siRNA in its ability to discriminate between alleles that differ at a single nucleotide (Figure 1B). These siRNAs were specifically designed to target the mutant sequence, which contains a C, as opposed to the wild-type sequence which contains a G at that position. These siRNAs perfectly match the mutant target, but contain a G:G mismatch when introduced to the wild-type target. We have previously shown that mismatches placed at the central position of the siRNA, nucleotides 9, 10, and 11, display allele-specific discrimination (Ding et al., 2003). Here, using the highly

Figure 1

A

```
                    P1: 5´ GCAAGUCUCCAACAUGCCUdTdT  3´
                   P2: 5´ CGCAAGUCUCCAACAUGCCdTdT  3´
                  P3: 5´ GCGCAAGUCUCCAACAUGCdTdT  3´
                 P4: 5´ UGCGCAAGUCUCCAACAUGdTdT  3´
                P5: 5´ UUGCGCAAGUCUCCAACAUdTdT  3´
               P6: 5´ AUUGCGCAAGUCUCCAACAdTdT  3´
              P7: 5´ CAUUGCGCAAGUCUCCAACdTdT  3´
             P8: 5´ ACAUUGCGCAAGUCUCCAAdTdT  3´
            P9: 5´ CACAUUGCGCAAGUCUCCAdTdT  3´
           P10: 5´ UCACAUUGCGCAAGUCUCCdTdT  3´
          P11: 5´ GUCACAUUGCGCAAGUCUCdTdT  3´
         P12: 5´ AGUCACAUUGCGCAAGUCUdTdT  3´
        P13: 5´ CAGUCACAUUGCGCAAGUCdTdT  3´
       P14: 5´ GCAGUCACAUUGCGCAAGUdTdT  3´
      P15: 5´ AGCAGUCACAUUGCGCAAGdTdT  3´
     P16: 5´ CAGCAGUCACAUUGCGCAAdTdT  3´
    P17: 5´ UCAGCAGUCACAUUGCGCAdTdT  3´
   P18: 5´ GUCAGCAGUCACAUUGCGCdTdT  3´
  P19: 5´ UGUCAGCAGUCACAUUGCGdTdT  3´
3´···UGGUAGAAACAGUCGUCAGUGUAACGCGUUCAGAGGUUGUACGGA···5´ mutant
3´···UGGUAGAAACAGUCGUCAGUGUAACGGGUUCAGAGGUUGUACGGA···5´ wildtype
```

B
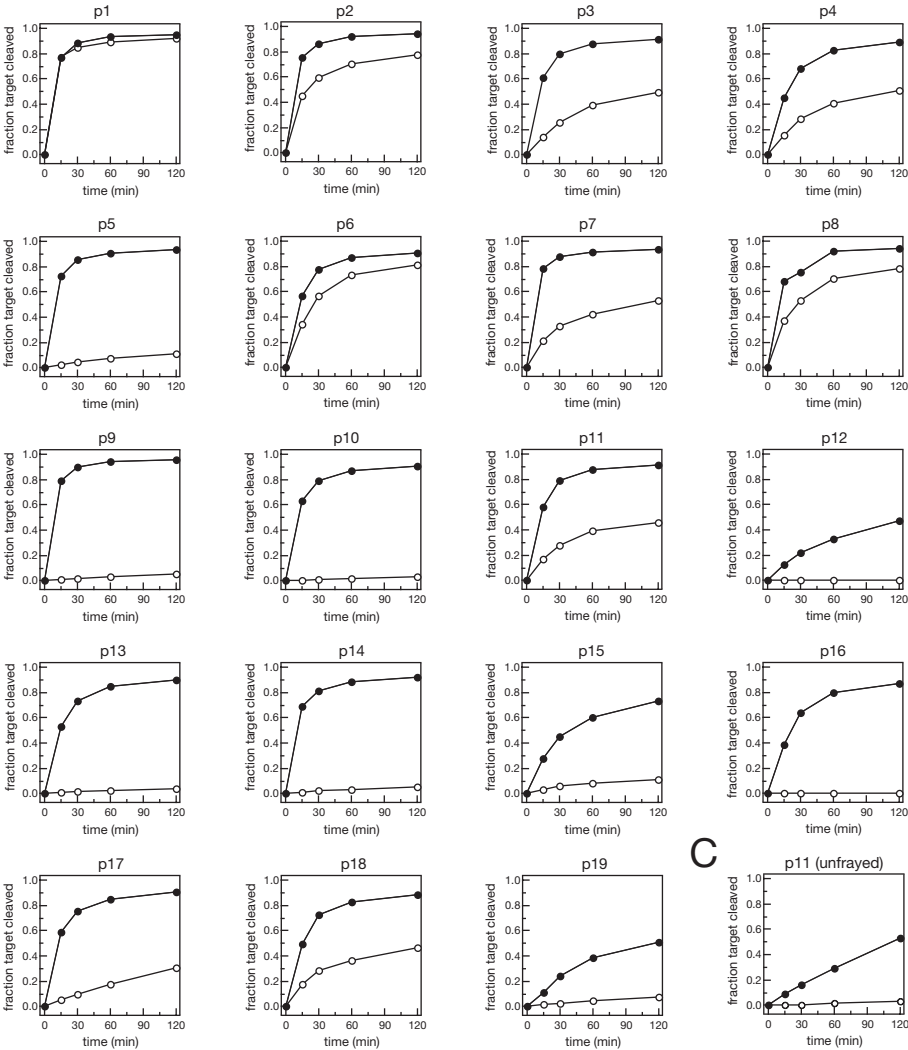
C

Figure VI - 1. *In vitro* **analysis of tiled siRNAs.** (A) Sequences of the guide strands of the siRNAs used. The third nucleotide from the 3′ end of the sense strand of the siRNA (not shown) was altered to result in a frayed siRNA facilitating the entry of the guide strand into RISC. If the 5′ end of the guide strand was a U, the sense strand was changed to a C and vise versa. If the 5′ end of the guide strand was a G, the sense strand was changed to an A and vise versa. Each siRNA is shifted down one nucleotide across the site of the point mutation resulting in a set of 19 siRNAs. The mutant and wild-type mRNA sequences that are targeted by the siRNAs are shown at the bottom. Mutant siRNAs match mutant targets, but mutant siRNAs resulting a G:G mismatch when presented to the wild-type target mRNA. (B) Rates of cleavage as determined by the fraction target cleaved by each siRNA when introduced to a mutant target (filled circle) or a wild-type target (open circle) in *Drosophila* embryo lysate. (C) Comparison of an unfrayed p11 siRNA and frayed p11 siRNA as a means to determine levels of single nucleotide discrimination.

effective frayed siRNAs we show that additional positions can also display discrimination using our in vitro *Drosophila* embryo lysate system.

An unfrayed siRNA (p11) that was used in our previous studies appeared to exhibit high levels discrimination because the perfectly paired mutant mRNA is cleaved, while non-cognate wild-type mRNA that results in a single nucleotide mismatch is not cleaved (Figure 1C). However, this mutant siRNA was not highly active when it was introduced to the perfectly matched, mutant target. This result complicates the analysis of siRNA discrimination because siRNA specificity was masked by the unfavorable incorporation of the guide strand into RISC. Here, when the 5´ end of the guide strand is frayed, facilitating the incorporation of the guide strand into RISC to a greater extent, it becomes clear that this siRNA does not display high levels of discrimination. By analyzing the complete tiled set of frayed siRNAs, specifically nucleotides 5, 9, 10, 13, 14, 15, and 16, siRNAs that show high levels of discrimination can be identified (Figure 1B). Additionally, nucleotides 12 and 19 display discrimination, but these siRNAs do not show robust silencing against a perfectly matched mutant target even when the 5´ end of the guide strand is frayed.

Using the same tiled set of siRNAs, we analyzed the initial rate of cleavage, in the linear range, using single turnover conditions (Figure 2). This results in a more quantitative measure of activity, or rate of reaction, because most siRNAs are near the point of saturation (Figure 1) by 15 minutes. In addition, shorter time points performed in enzyme excess gives an indication of the active amounts of RISC formed from each

Figure 2
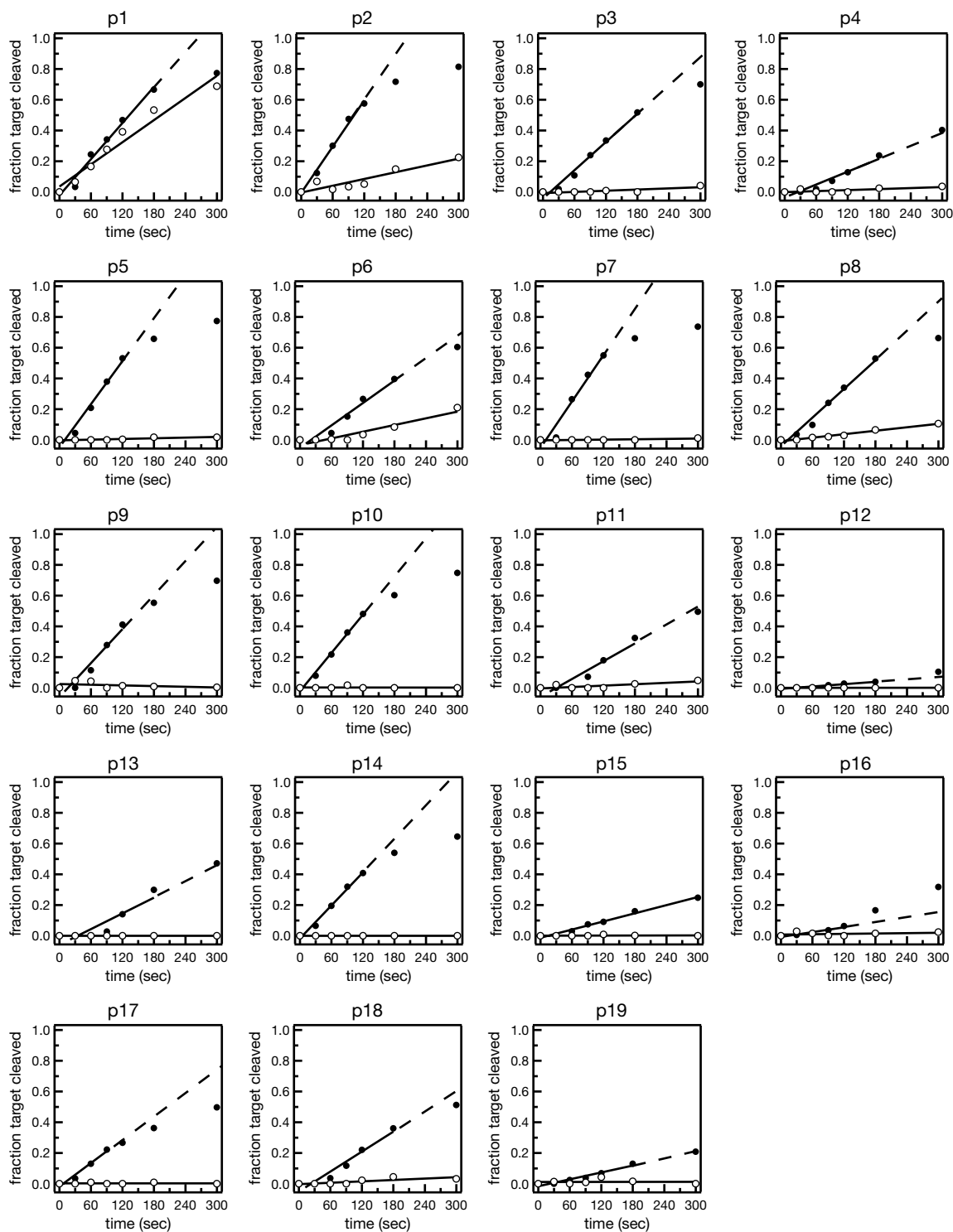
Figure VI - 2. Initial rates of siRNA-mediated cleavage. *In vitro* reactions were performed in vast excess of RISC over substrate RNA in order to ensure single turnover conditions. The linear rate of the reaction (solid line) was determined and the slopes, or rate of reaction, are displayed. Filled circles indicate mutant siRNA and mutant target; open circles indicate reactions in which mutant siRNAs were incubated with a wild-type target. In these conditions, the concentration of RISC was in great excess over substrate, or target RNA, with concentration determined by the first 300 seconds of the reaction.

individual siRNA. In this analysis, p12, p15, p16 and p19 exhibited slow initial rates of reaction. Of these siRNAs, p12 and p19 also showed poor cleavage in Figure 1B. However, p15 and p16, although silenced well over time, also showed a slower rate of initial cleavage as reflected in Figure 1B.

A rigorous test for siRNA selectivity

While some of the siRNAs assayed in this study exhibit high levels of discrimination over the original reaction time of 2 hours, we decided to analyze the discrimination over an extended reaction time as some of these siRNAs may give misleading results in the short term and cleavage may be detected if given ample time (Figure 3). RISC was present in great excess over target RNA in order to establish the level of sequence discrimination. Over a 24 hour period, some of the siRNAs which originally showed high levels of discrimination start to show low levels of cleavage of the wild-type, mismatched target, but certain siRNAs, in particular p12 and p16, still do not exhibit cleavage of the wild-type target. Although p12 did not show high levels of activity as a perfectly paired siRNA, and therefore may result in a misleading conclusion, p16 was extremely effective in recognizing and cleaving a perfectly paired mRNA.

Analysis of tiled siRNAs in cultured cells

Does our *in vitro* analysis using *Drosophila* embryo lysate accurately predict the behavior of these siRNAs in cultured cells? The siRNAs were co-transfected into HEK 293 cells with a plasmid expressing a Firefly (Pp) luc-SOD1 fusion target. A co-transfected
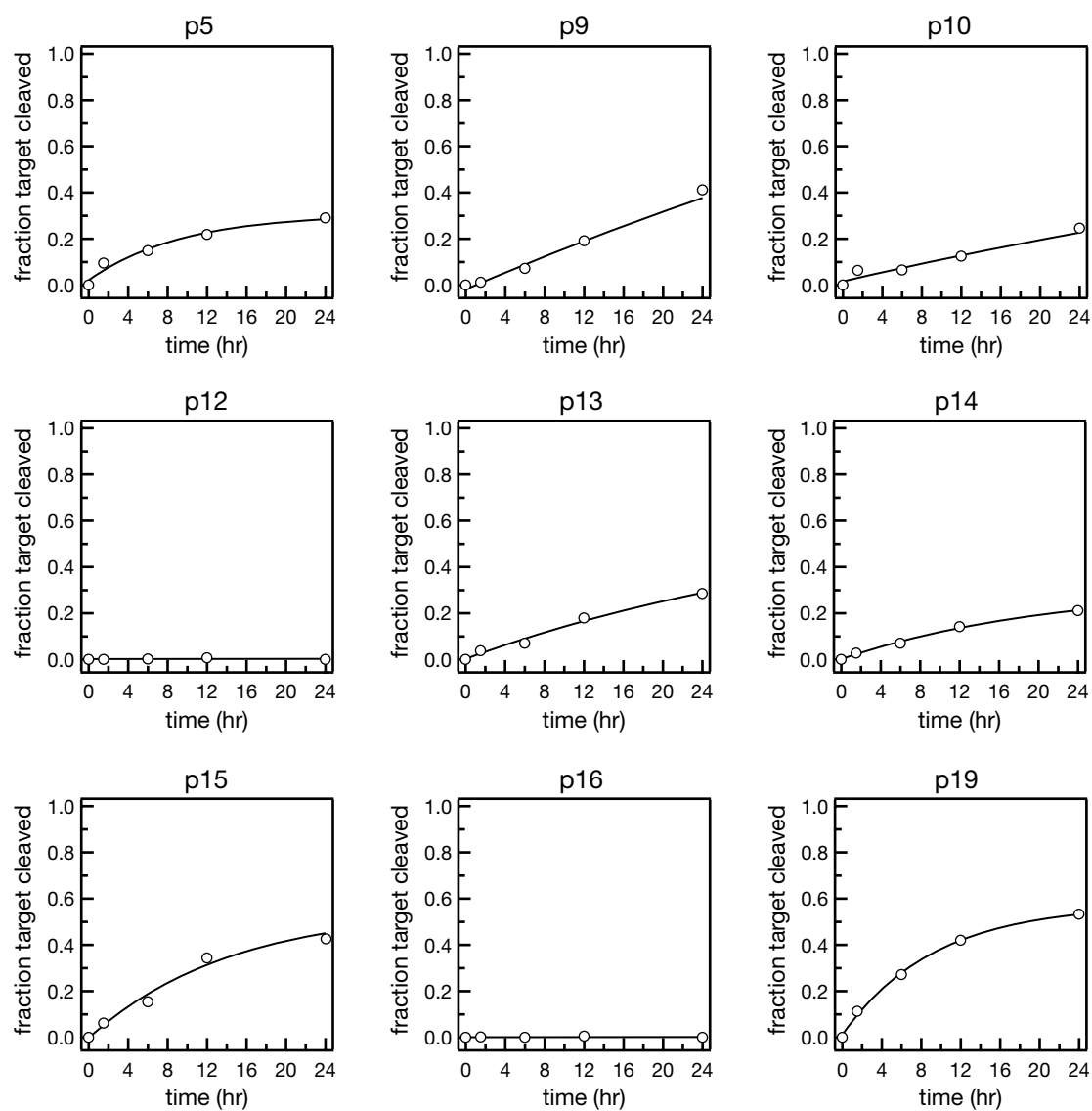
Figure 3

Figure VI - 3. Analysis of discrimination over time. RISC was present at high levels over target RNA in order to increase the probability that the target mRNA would be recognized. siRNAs (p5, p9, p10, p12, p13, p14, p15, p16, and p19) that showed high levels of discrimination were used in this extended analysis.

Renilla (Rr) luciferase-expressing plasmid served as an internal control. Efficiency and discrimination were determined by assaying for the presence of the Pp luciferase protein, normalized to Rr luciferase, following co-transfection with either 2 nM or 20 nM siRNA (Figure 4). In addition, siRNAs were introduced to a third target containing a uridine residue in place of the G (wild-type) or C (mutant) in the SOD1 mRNA sequence. Previous studies show that G:U wobbles are not tolerated at certain positions along the siRNA:mRNA helix (Doench and Sharp, 2004). As seen in Figure 4A, siRNAs that perfectly match the target RNA (mutant siRNA and mutant target sequence) are effective, albeit some more than others. For example, p13, p14, and p18 do not exhibit as high a level of silencing efficiency as the rest of the set of siRNAs, even though these siRNAs displayed a high level of activity *in vitro*.

When the same set of siRNAs was introduced to a wild-type sequence, creating a G:G clash, several of the siRNAs exhibited high levels of discrimination (Figure 4B). siRNAs capable of discrimination in which less than 40% of the mismatched target was down-regulated included p3 – p6, p8, p10 – p13, and p16. When compared to the results obtained in the *in vitro* system, it appears that most of the siRNAs that exhibit high levels of discrimination in *Drosophila* also discriminate in cultured human cells, including p5, p9, p10, p12 – p14, and p16. In addition, when the same set of siRNAs were co-transfected into cells with a plasmid that contains a U at this position, some of the siRNAs show increased levels of mRNA cleavage and show only moderate levels of discrimination (Figure 4C). For example, siRNAs that were previously shown to be selective (Figure 4B) now appear to display a higher degree of silencing and less

Figure 4
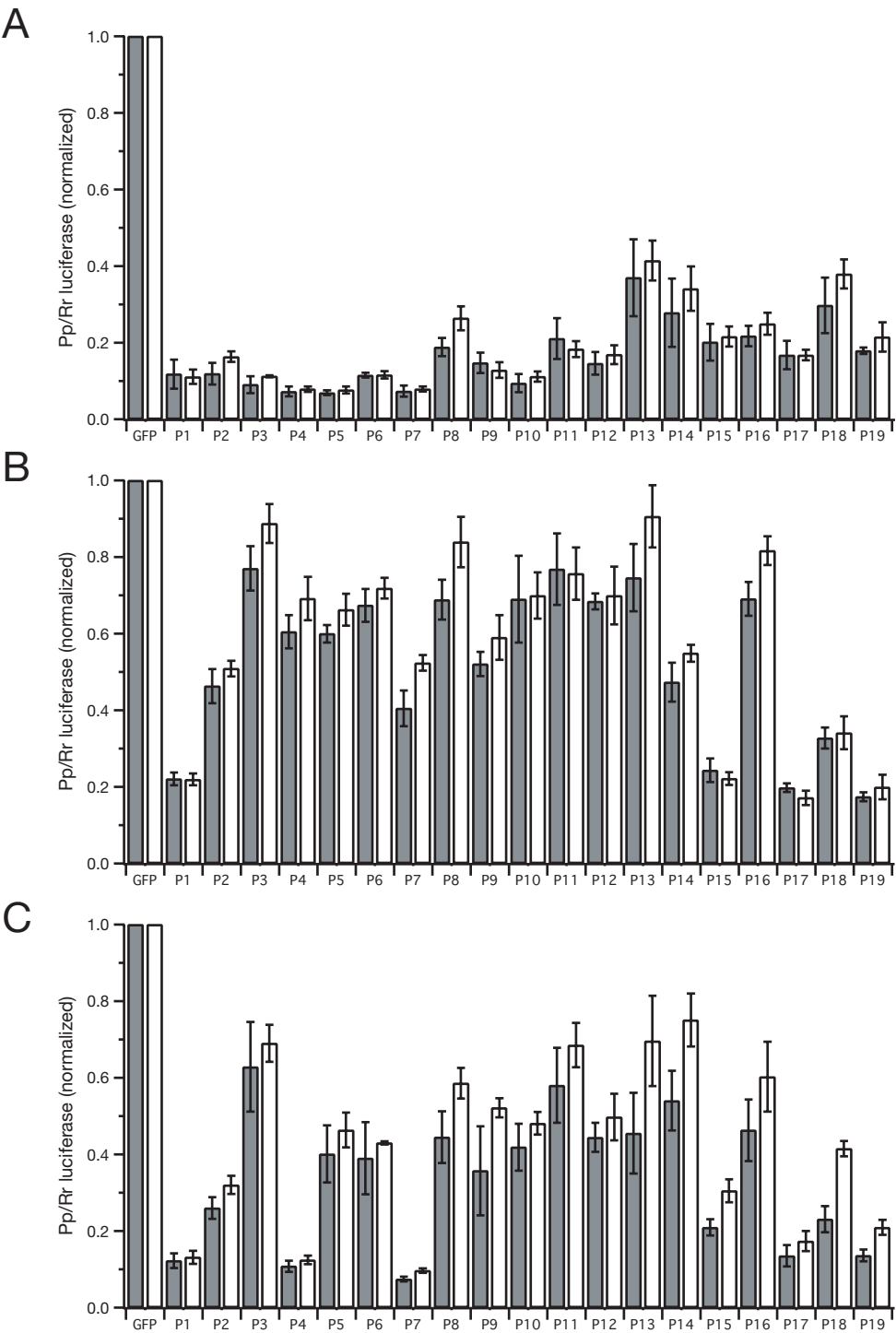
Figure VI - 4. Analysis of tiled siRNAs against a SOD1-luciferase fusion in mammalian cells. (A) siRNAs (Figure 1A) were co-transfected at either 2nM (gray bar) or 20nM (white bar) in conjunction with plasmids containing the mutant, or perfectly matched, SOD1 sequence fused to the luciferase coding sequence. The level of silencing was determined by measuring the levels of firefly luciferase expression. (B) The same set of siRNAs were co-transfected with plasmids containing the wild-type sequence, creating a G:G clash, or (C) a U at that position creating a G:U wobble. Each experiment was performed in triplicate along with an unrelated GFP siRNA and an untransfected control.

discrimination, in particular p4 – p7, and p10 and p12 to a lesser extent. Therefore, mismatches that are less disruptive to the overall conformation of the central helix, particularly in the 'seed' region appear to lose the ability to discriminate among alleles when confronted with a GU mismatch. In contrast, p3, p8, p11, p13, p14 and p16 retained the ability to discriminate and therefore may be regions less tolerant of mismatches. In addition, p13, p14, and p18 also show low levels of efficiency, but when these siRNAs encountered a perfectly matched mRNA they showed lower levels of silencing than expected (Figure 4A).

Specificity and off-target effects of tiled, frayed siRNAs against mutant SOD1

The frayed siRNAs utilized in Figures 1 - 4 were transfected at 100 nM final concentration into HeLa cultured cells to maximize off-target effects, which allows for the identification of the active strand in RISC by analyzing targets that share homology with the seed sequence of the siRNAs. Total RNA was isolated from these cells and hybridized to microarrays (Figure 5). While the siRNAs are only 1 nucleotide apart, the off-target signatures are different for each siRNA.

In addition, this experiment can predict which siRNA is not capable of silencing the wild-type gene that is present on the array, even at concentrations more than 5 times the standard concentration of siRNAs for transfection, and 50 times the lowest concentration (2 nM) used in Figure 3. For example, p8, p9, and p16 are not capable of cleaving the wild-type mRNA as seen by the gray area on the array (Figure 5). These siRNAs were also shown to have either the guide strand of the frayed siRNA

Figure 5

A

B
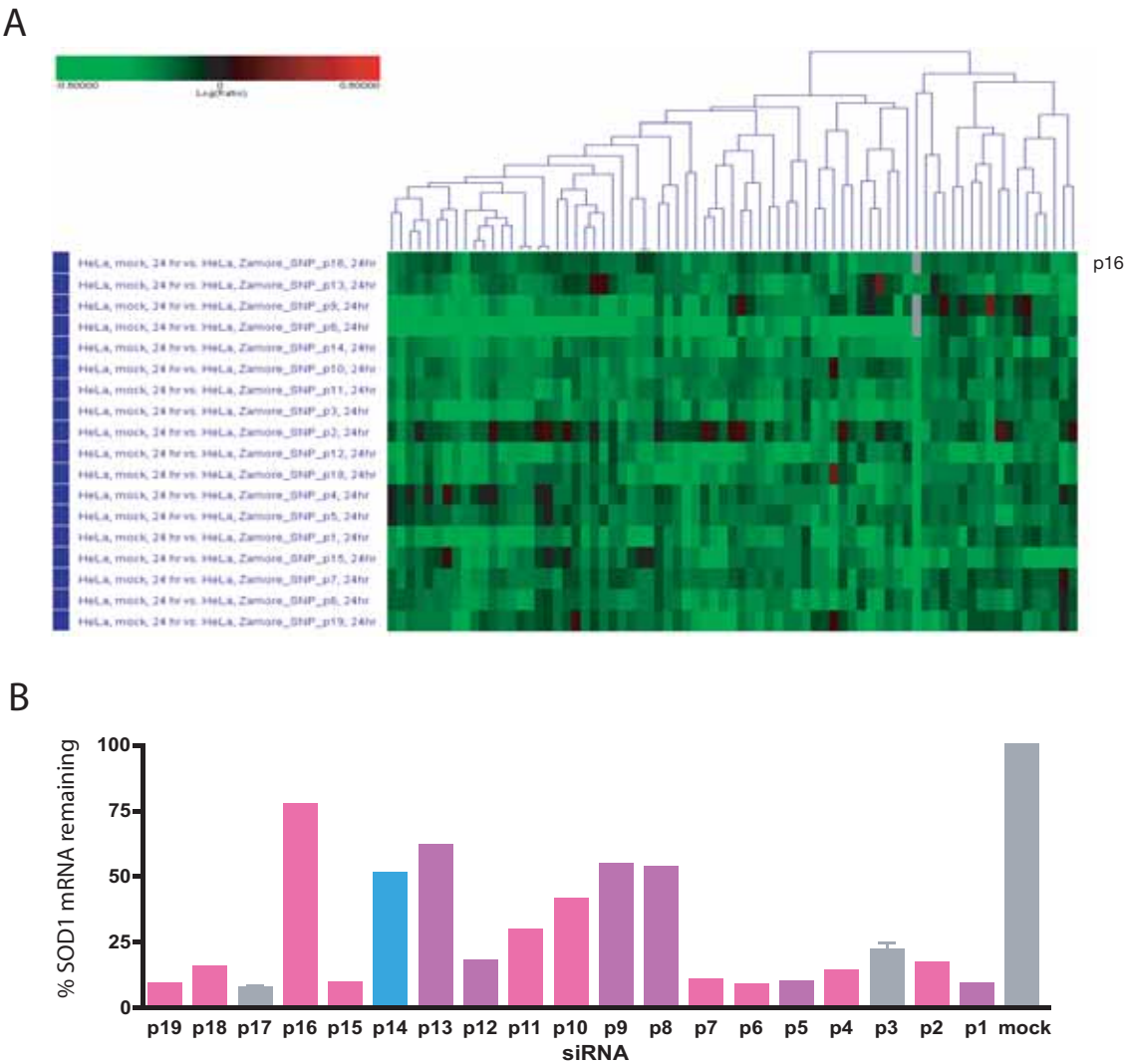
Figure VI - 5. Microarray analysis of frayed SOD1 siRNAs. siRNAs from Figure 1A were transfected into HeLa cells at 100 nM final concentration. (A) Total RNA was isolated and hybridized to the microarray Green represents a decrease in gene expression level, red indicates an increase in gene expression level, both as compared to a mock HeLa cell transfection. (B) Total RNA was isolated from transfections of the tiled, frayed set of siRNAs as described. Real-time PCR was performed using AP Biosystems Taqman.

incorporated into RISC (p9, p16), or mixed sense and guide strand incorporation into RISC (p8) (data not shown). These three siRNAs all show high levels of activity against the perfectly matched mRNA in fly embryo lysate (Figures 1-3) and HEK 293 co-transfections (Figure 4), although p8 only showed moderate selectivity in the lysate and cultured cell experiments. In addition, p16 was not capable of silencing the wild-type mRNA in the lysate system over a long incubation and retained the ability to discriminate in various examples and contexts (Figure 4, Figure 7, Table 1). Notably, the pattern of discrimination observed in the real-time assays (Figure 5) reflect some of the patterns seen when this tiled set of frayed siRNAs were co-transfected with the luc-SOD1 fusion targets (Figure 4) when observed at the RNA level (real-time), and not the protein level (luciferase assay). Specifically, p8, p10, p13, and p16 siRNAs in the tiled set that did not down-regulate the mismatched target in the HEK 293 cells did not mediate high levels of target RNA destruction as indicated in the real-time assay from HeLa transfections. Of note, the transfections in Figure 5 were carried out at concentrations of at least 5 times the highest level used in HEK 293 co-transfections, which contained plasmids driving high levels of the Pp luc-SOD1 fusion constructs instead of targeting an endogenous gene as in the microarray and real-time experiments.

Analysis of additional mismatches using the p10 siRNA

In the case of the G85R SOD1 point mutation, the mismatch between the mutant siRNA and the wild-type mRNA sequence results in a G:G clash. In order to understand which other mismatches could also potentially display discrimination in other contexts, we
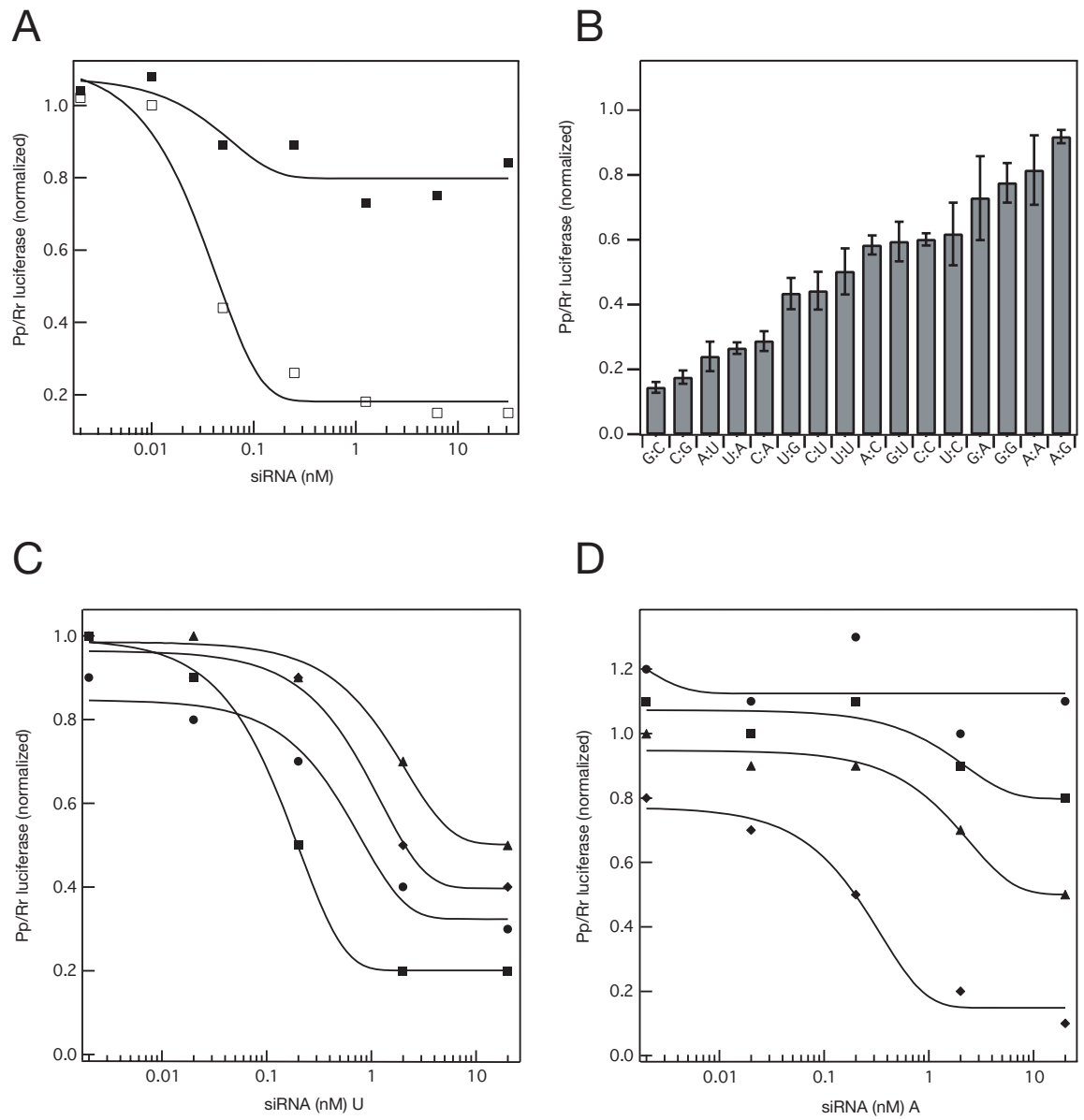
Figure 6

Figure VI - 6. Purine:Purine mismatches are most disruptive. (A) The p10 siRNA is intrinsically asymmetric. Here, the same siRNA was introduced to a sense target (to measure the ability of the guide strand of the siRNA to enter RISC and mediate target mRNA destruction in cells) or an anti-sense target (containing the complementary sequence and therefore to measure the ability of the sense strand to enter RISC and mediate target mRNA destruction). Even at high concentrations of siRNA, only the sense target (open squares) is cleaved at appreciable levels, compared to the anti-sense target (filled squares), and therefore the guide strand of the p10 siRNA is entering RISC. (B) Luciferase activity was measured for each siRNA:mRNA pair. Perfectly complementary siRNA:mRNA pairs show high levels of silencing, purine:pyrimidine mismatches display intermediate levels of silencing, and therefore intermediate levels of discrimination, but purine:purine mismatches show the highest levels of discrimination. (C) Over a range of concentrations, the pyrimidine:purine mismatches show moderate levels of discrimination compared to the perfectly matched siRNA:mRNA pair. U:C mismatches, triangles; U:U mismatches, diamonds; U:G mismatches, circles; U:A matched pair, squares. (D) Purine:purine mismatches can not be overcome by high concentration of siRNAs and is therefore not an artifact of low concentration. A:G mismatches, circles; A:A mismatches, squares; A:C mismatches, triangles; A:U matched pair, diamonds.

designed four siRNAs based on the original p10 siRNA sequence (Figure 1A) with either

a G, a C, a U, or an A at position 10. In addition, we created four targets containing each

possible nucleotide across from p10 in the siRNA. This allowed us to study the effects of

the various combinations of mismatches that might occur in a disease and to analyze

which mismatches result in discrimination between two alleles. In this experiment we

used a non-frayed siRNA because this sequence is intrinsically asymmetric (Figure 6A)

as shown by the silencing potential against a sense and anti-sense target RNA. This

shows that only the guide strand of the siRNA enters RISC and is capable of mRNA

destruction. Since we are only comparing among p10 siRNAs that all share the same free

energies at the 5′ ends of the siRNA we decided to use the non-frayed siRNAs.

When these p10 siRNAs were co-transfected with plasmids containing every

possible nucleotide at p10, resulting in every possible nucleotide combination, the

perfectly matched siRNAs (G:C, C:G, A:U, and U:A) displayeded high levels of

silencing. Conversely, the purine:purine mismatches (A:G, A:A, G:G, and G:A) showed

high levels of discrimination, whereas all other mismatches (pyrimidine:pyrimdine,

pyrimidine:purine, or purine:pyrimidine) displayed intermediate levels of discrimination

(Figure 6B). In addition, when the pyrimidine:purine mismatches were analyzed over a

range of siRNA concentrations, the level of silencing increased (Figure 6C), but when the

siRNA:mRNA pairs that result in the various purine:purine clashes are analyzed over the

same concentration range the discrimination remains constant (Figure 6D circles and

squares), and therefore can not be overcome by high concentrations of siRNA.

Analysis of purine:purine mismatches across the siRNA sequence

After discovering that purine:purine mismatches result in the highest level of discrimination, we decided to determine which position along the siRNA elicited the best discrimination. Again the original p10 sequence was utilized, but this time mismatches were placed at each position within the siRNA and SOD1 targets were constructed that would result in purine:purine mismatches (Figure 7A). siRNAs displaying the highest level of knock-down, or were unaffected by the mismatch, were more prevalent in the 3′ half or middle of the siRNA. In this analysis, p4, p7, p9 – p11, p13 and p16 exhibited selectivity with less than 40% silencing of the mismatched, or wild-type, target.

Similar analysis was employed when testing purine:purine mismatches in another context, this time utilizing frayed siRNA design rules applied to the original p4 siRNA (Figure 1). Again, luciferase activity was determined and, in general, siRNAs retaining the ability to cleave even in the presence of purine:purine mismatches are located in the middle or 3′ half of the siRNA (Figure 7B). For the p4 analysis, p3 – p5, p9 – p13 and p16 showed discrimination. Of note, p10 G:G and A:G mismatches were more selective than the A:A and G:A mismatches, potentially indicating that the nature of the mismatch may also be affected by surrounding nucleotide composition and may depend on which nucleotide is present in the mRNA or siRNA sequence. Combined, Figure 7 shows that p4, p9 – p11, p13 and p16 were capable of discrimination.

In depth analysis of p16 mismatches

Based on results from Figures 1 – 7, we decided to take a closer look at the p16

Figure 7
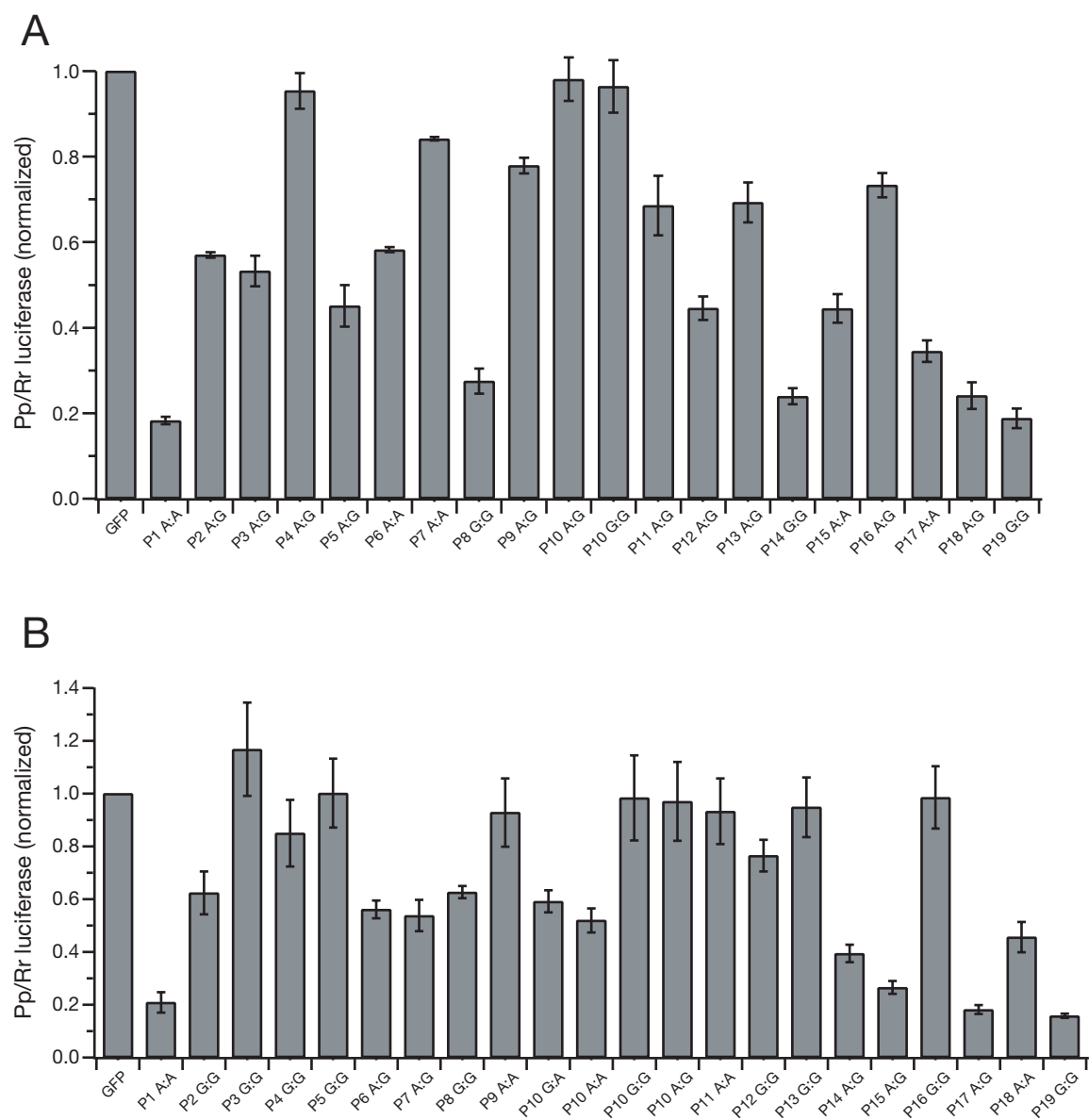
Figure VI - 7. Purine:purine mismatches display varying degrees of specificity. (A) Purine residues were placed at each position along the p10 siRNA. Targets were constructed so that siRNA:mRNA target pairs would result in purine:purine mismatches. Luciferase activity was measured for each pair. (B) The original p4 siRNA sequence was the basis for analysis with the 5′ end of the siRNA guide strand frayed to ensure entry into RISC. Targets were constructed such that siRNA:mRNA target pairs would result in purine:purine mismatches.

mismatches in co-transfection experiments. Through fly embryo lysate, HEK 293 transfections, and microarray and real-time data from HeLa transfections, p16 was most consistent in its ability to discriminate. Ten separate siRNA-mRNA pairs bearing G:G, A:G, or A:A mismatches against the wildtype sequence were designed to target sod1 or htt naturally occurring point mutations or SNPs, or point mutations engineered into the 3′ UTR of Renilla firefly luciferase (Table 1). siRNAs were transfected over a concentration range from 0.001nM to 20nM, and renilla/firefly ratios were normalized to an unrelated control (for htt), or firefly/renilla ratios were normalized to an unrelated control (for sod1). IC 50's were determined for the match or mismatched siRNAs by fitting the curves to a Hill equation with a coefficient of 1. For siRNAs in which the half maximal concentration for silencing was not reached at the highest concentration tested, IC 50 values were estimated as > than the highest concentration.

For the ten siRNAs tested, 6 had an IC 50 of greater than 20nM for the mismatched siRNA and one had an IC 50 of greater than 10nM. All of the mismatches displayed greater IC 50's than the perfectly paired siRNA:mRNA pair. siRNAs bearing 3′ dTdT tails appeared to have a greater ability to discriminate, but the siRNAs that consisted of all ribonucleotides were designed to contain dTdT tails and showed very little (< 0.1 nM) difference between dTdT and all RNA (data not shown). In addition, we tested whether the level of discrimination was dependent on the cell line and saw no difference between HeLa and 293 cells for the siRNAs in this study (data not shown).

Table 1

siRNA guide strand	IC 50 (nM)	
	match	mismatch
5´ UCACAUUGCCCAAGU**A**UCCdTdT 3´	< 1.0	> 20
5´ UGCCCAAGUCUCCAA**G**AUGdTdT 3´	< 0.2	> 20
5´ CAGCAGUCACAUUGC**G**CAAdTdT 3´	< 1.0	> 20
5´ AGUCACAUUGCCCAA**G**UCUdTdT 3´	< 0.4	> 20
5´ CCAAGUCUCCAACAU**G**CCUdTdT 3´	< 1.0	> 20
5´ UGAAGUGCACACAGU**G**GAUGA 3´	< 0.2	> 0.4
5´ UGAAGUGCACACAGU**A**GAUGA 3´	< 0.2	> 0.3
5´ GAUGAAGUGCACACA**G**UGGAU 3´	< 0.4	> 2
5´ GUGCACACAGUGGAU**G**AGGGA 3´	< 0.2	> 10
5´ AGGGUCAAGAUGACA**A**UGGAC 3´	< 1.0	> 20

Table VI - 1. Mismatches at position 16 confer high levels of discrimination.
siRNAs at the top of the table are targeted to sod 1, while siRNAs at the bottom of the table are targeted to htt. Mismatches result in a G:G, A:G, or A:A mismatch with the wildtype sequence. SOD1 siRNAs were transfected into HEK293T cells, n=4. HTT siRNAs were transfected into HeLa cells, n=9 for siRNAs 1, 2, and 5; n=6 for siRNA 4; n=15 for siRNA 3. siRNAs targeting sod1 were designed with the mutant sequence and were targeted to plasmids bearing either the mutant or the wildtype sequence. siRNAs targeting htt were designed either the same as sod1 or 2 siRNAs were designed bearing either the SNP sequence or the wildtype sequence to target a plasmid bearing mutant sequence. Luciferase ratios were normalized to controls containing an unrelated GFP siRNA. IC 50's were determined by plotting the concentration gradient versus percent knock-down and fit to a Hill equation with a rate = 1. IC 50's for siRNAs that did not reach half-maximal silencing were estimated from the graph to be greater than the highest concentration of siRNA transfected.

Discussion

The use of RNAi to knock-down the expression of single mutant alleles holds promise in the treatment of various dominant negative diseases caused by point mutations in specific genes. The data presented here brings us closer to the identification of parameters for designing optimal siRNAs that suppress specific deleterious alleles. Mismatches between siRNAs and target mRNAs where a purine:purine mismatch occurs leads to the greatest level of discrimination, probably the result of a greater disruption in the central A-form helix that is required between the siRNA and mRNA (Chiu and Rana, 2002; Haley and Zamore, 2004). Therefore, a point mutation that is the result of a pyrimidine to a purine change increases the probability of discrimination through siRNA-mediated silencing.

In addition, while the region in the target RNA in which one can design siRNAs against a point mutation is limited, the level of discrimination can be increased based on where the mismatch is placed along the RNA:RNA helix between siRNA and target mRNA. The combination of *in vitro* systems and cultured cell transfections shows that certain positions can increase the chances of discrimination between two alleles that differ at a single nucleotide. This finding is consistent through three strategies of analysis (Figures 1 - 7) using frayed and unfrayed siRNAs, and by tiling the siRNA sequences or by changing each position of a fixed siRNA sequence. In general, more of the positions located in the 5′ region of the siRNA, which has been shown to responsible for binding and recognition (Doench and Sharp, 2004; Haley and Zamore, 2004), are only moderately affected by the presence of a single mismatch and can still mediate silencing,

but this depends on the nature and context of the mismatched nucleotides. When the mismatch is located in the 3′ region of the siRNA, which has been demonstrated to contribute to catalysis and maintenance of the required central A-form helix (Haley and Zamore, 2004), specifically positions 10 and 16, the loss of silencing of the wild-type gene is observed and the discrimination is fairly constant through all experiments (Figures 1 – 5, 7, Table 1, and data not shown). Our lab has previously shown that the first 2 nucleotides (p1 and p2) and the last 5 nucleotides (p17 – p21) contribute little to the binding of an siRNA to its target mRNA (Haley and Zamore, 2004). Specifically, bioinformatic analysis predicted that the first nucleotide of a miRNA does not contribute to conservation (Lewis et al., 2005; Lewis et al., 2003), and a first nucleotide mismatch between an siRNA and mRNA as observed in an *in vitro* target cleavage assay indicated that the first nucleotide does not contribute to silencing (Haley and Zamore, 2004). The crystal structure of an Archae Piwi protein indicates that the 5′ phosphate of the first nucleotide in the siRNA is anchored in a binding pocket of the protein and does not contribute to siRNA function (Ma et al., 2005). Therefore, current evidence in the field, combined with results presented here, show that the first nucleotide probably plays a role in positioning the siRNA within the silencing complex and should not be a position selected for targeting single nucleotide polymorphisms.

The findings presented here are slightly inconsistent with the results that siRNAs designed to function as miRNAs are more affected by mismatches in the 5′ end of the molecule (Doench and Sharp, 2004), and in fact are the same bases that are used to predict miRNA targets by computational approaches (Enright et al., 2003; Lewis et al.,

2003; Rajewsky and Socci, 2004; Stark et al., 2003). But it should be noted that the actual mode of translational repression as mediated by miRNAs is still a source of debate in the RNAi field, and the proteins and interactions may be functioning differently than previously expected, therefore strict comparison to siRNA function may be complicated.

While purine:purine mismatches display this selectivity, G:U wobbles display a slightly different mode of regulation where G:U wobbles placed in the 5′ region of the siRNA mediate gene silencing to a greater degree (Figure 4C). This result is inconsistent for G:U wobble pairs in which recent findings suggest that G:U wobbles, which are widely found in miRNAs, are capable of eliminating activity in miRNA:mRNA pairing (Doench and Sharp, 2004), but this may be distinct in that respect to siRNA-mediated silencing.

Additional experiments conducted using high concentrations of siRNAs transfected into HeLa cells and hybridizing total RNA isolated from those cells shows that specific siRNAs are capable of not destroying the wild-type SOD1 mRNA, including p16. Even at high levels of siRNA, some sequences are capable of discriminating alleles that differ by a single nucleotide as these siRNAs were shown to be active against a cognate, mutant G85R SOD1 sequence in transfections with luciferase readout analysis (Figure 4). In addition, it is important to note that when the discrimination was analyzed at the level of RNA from these same experiments, the pattern of silencing observed for p16 in particular was consistent with the results obtained from the co-transfections with the luc-SOD1 expressing plasmids. The real-time results show that greater than 75% of the wild-type mRNA remained, even under conditions of high siRNA concentration.

This shows that the level of silencing occurs at the RNA level, and that siRNA:mRNA target pairs that differ at a single nucleotide probably result in the blockage of RNA cleavage, not in the repression of translation by acting as miRNAs (Figure 5), although the siRNAs, and miRNAs for that matter, are capable of mediating target cleavage and can enter RISC that contains human Ago2 (Meister et al., 2004), the Argonaute that functions as the endonuclease (Liu et al., 2004; Meister et al., 2004).

Through multiple modes of analysis, p16 was selected as the best candidate for an in depth analysis because it consistently showed high levels of discrimination in various contexts. It is important to note that while some position of mismatches show discrimination in one context, they may not be as efficient when the mismatch is incorporated into another siRNA sequence. It is important to note that differences did exist between the analysis of sod1 and htt point mutations and SNPs, including transfections in different cell lines, the use of siRNAs that consisted entirely of RNA or siRNAs that has 3? dTdT tails, and cloning of the fragment of interest into firefly or renilla luciferase vectors. The cell line into which these siRNAs were transfected, as well as the RNA or dTdT-containing siRNA design, did not affect silencing and discrimination (data not shown).

Another group found that using a fixed target region, with each position mutated to each of the four nucleotides, with a single siRNA that positions 5 – 11 did not tolerate mismatches (Du et al., 2005), although they did not test various different sequences in different contexts as we did in this analysis. While they tested mismatches along the length of the siRNA, they were limited to what nucleotide was in the siRNA, therefore, if

a pyrimidine was present in the siRNA they were not able to test that position in the context of a purine:purine mismatch as we did, noting that this type mismatch is the most disruptive to the helix.

The information presented here greatly aids in the design of siRNAs that are capable of discriminating among alleles. Therefore, an siRNA that can cleave a mutant mRNA but leave the wild-type copy intact would result in the expression of functional protein, but prevent the mutated form of the protein to be synthesized, potentially decreasing the symptoms of the disease. Specifically, through three different experimental systems, position 16 relative to the 5′ end of the siRNA guide strand provided the greatest chance of discrimination. Mismatches that result in a purine:purine clash displayed the greatest possibility of discrimination. Therefore, placement of the mismatch, and the nature of the mismatch should be considered when designing SNP-specific siRNAs. These findings can hopefully be translated to other dominant negative diseases where allele specific differentiation is required to ameliorate symptoms.

Methods

General methods

Preparation of *Drosophila* embryo lysate, target RNA preparation, cap labeling, siRNA (Dharmacon) annealing, and in vitro RNAi reactions were carried out as previously described (Haley et al., 2003; Tuschl et al., 1999; Zamore et al., 2000). *sod1* mutant and wild-type RNAs were transcribed from BamHI-linearized plasmids (Crow et al., 1997) with recombinant histidine-tagged T7 RNA polymerase. Target RNAs and siRNAs were used at ~5nM and 50nM final concentrations, respectively (Figure 1) or ~0.5nM and 100nM, respectively, for single turnover conditions (Figure 2). Gels were dried and exposed to phosphorimager plates (Fuji) and developed using a FLA-5000 phosphorimager (Fuji). Gels were analyzed and quantitated using Image Guage version 3.45 (Fuji), results were analyzed in Excel X (Microsoft) and graphed using Igor Pro 5.01 (Wavemetrics).

Cell culture, transfections, and luciferase assays

HeLa cells were propagated and maintained as previously described (Schwarz et al., 2002). HEK 293 cells were maintained in Dulbecco's Modified Eagle Media (DMEM) (Invitrogen), supplemented with 10% Fetal Bovine Serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. htt sequences were engineered into the 3′ UTR of the pRLTK Renilla luciferase vector (Promega) using 55 nucleotide DNA oligos annealed in 1x lysis buffer (100 mM potassium acetate, 30 mM Hepes-KOH, pH 7.4, 2 mM magnesium acetate) to create 5′ overhangs and inserted into the XbaI site. Proper orientation was

verified by sequencing in both directions. Sod1 sequences were cloned into the 3′ UTR of the Firefly lusicferase vector (pGL2 control, Promega) into NdeI and SpeI sites engineered into the plasmid by annealing two 39 nucleotide DNA oligos and ligating them into the vector.   Transfections were carried out using Lipofectamine 2000 (Invitrogen) in 24 well plates using 0.25 μg pGL2 Firefly luciferase (Promega) and 0.1 μg Renilla-Htt constructs or in 96 well plates using 2 μg/ml firefly fusion vector and 0.1 μg/ml renilla vector. Cells were washed in 1x PBS (Invitrogen) and harvested 24 hours post-transfection in 2x passive lysis buffer (Promega). Luciferase levels were determined using the Dual Luciferase kit (Promega) and read on a Veritas Microplate Luminometer (Turner Biosystems).   Renilla luciferase/firefly luciferase ratios were normalized to a sample containing an unrelated GFP siRNA (Qiagen) in addition to the luciferase vectors for htt experiments, and Firefly luciferase/Renilla luciferase ratios were calculated for sod1 experiments. Results were analyzed in Excel (Microsoft) and graphed in IGOR Pro 5.0 (Wavemetrics). Curves were fit to a Hill Equation with a coefficient of 1.

HeLa cell transfections, RNA isolation, microarray hybridization, real-time PCR
HeLa cell transfections were carried out at 100 nM final concentration as described (Jackson et al., 2003). RNA isolation, microarray hybridization, and real-time assays were carried out as described (Jackson et al., 2003).

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Why do miRNAs live in the miRNP?

This perspective was written based on the article:

Mourelatos, Z., J. Dostie, S. Paushkin, A.K. Sharma, B. Charroux, L. Abel, J. Rappsilber, M. Mann, and G. Dreyfuss. 2002. miRNPs: a novel class of Ribonucleoproteins containing numerous microRNAs. *Genes Dev* **16**: 720-728.

The purpose of this perspective was to summarize what was known about the recently identified class of small, non-coding RNAs called microRNAs (miRNAs), and why various members of this class, including previously unidentified miRNAs, might reside in a protein RNA complex. In addition, this perspective gives insight into what biochemical function this protein complex contributes to cellular and developmental regulation.

APPENDIX

Do tiny RNAs now have a home? Non-coding RNAs function in diverse pathways—dosage compensation, gene imprinting, transcriptional regulation, pre-mRNA splicing, and the control of mRNA translation—and they carry out these roles from within specific RNA-protein complexes that ensure each non-coding RNA is in the right cellular compartment with the appropriate proteins needed to accomplish its biochemical function. Thus, identifying the ribonucleoprotein complex (RNP) associated with a non-coding RNA gives clues to its cellular function and biochemical mechanism by revealing the proteins whose company it keeps. The discovery by Dreyfuss and coworkers that microRNAs reside in a ~550 kDa (15S) particle provides new clues toward the functions of this novel and surprisingly large class of tiny, non-coding RNAs (Mourelatos et al. 2002).

The first microRNA (miRNA), *lin-4*, was identified in 1983 (Lee et al. 1993). Ambros and coworkers positionally cloned the *lin-4* gene, a locus required for the correct timing of development in *Caenorhabditis elegans*, only to find that the gene encodes no protein. Instead, *lin-4* comprises two small non-coding RNAs, one 22 nt long, and a longer form, *lin-4L*, that can fold into a hairpin structure. Seven years later, Ruvkun and colleagues discovered that *let-7*, which likewise regulates developmental timing in worms, is also a tiny, non-coding RNA (Reinhart et al. 2000). Because *lin-4* and *let-7* control developmental timing, they have been dubbed small temporal RNAs (stRNAs). Recently, three laboratories succeeded in cloning additional stRNA-like RNAs from

worms, flies, and human cells (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). These efforts uncovered a wealth of 19-25 nt RNAs, including *lin-4* and *let-7*, that are collectively known as miRNAs (reviewed in Moss 2001; Ruvkun 2001; Banerjee and Slack 2002). These efforts added an additional 100 tiny RNAs to the original pair of stRNAs. As anticipated by Ambros, stRNAs and miRNAs derive from longer stem-loop precursor RNAs. Thus, the longer *lin-4L* is the precursor of mature *lin-4*. While many of the new miRNAs are produced constitutively, some are temporally regulated or expressed only in specific tissues. A few appear to be transcribed in coordinately regulated operons, suggesting that they are cleaved from their stem-loop precursors from within a long, common transcript. Others are found only in the germ line or in the early embryo, where translational control dominates the hierarchy of regulatory mechanisms.

The 21-25 nt size of miRNAs is remarkably similar to that of small interfering RNAs (siRNAs), the 21-25 nt double-stranded RNAs that mediate RNA interference (reviewed in Bernstein et al. 2001b; Carthew 2001; Sharp 2001; Vaucheret et al. 2001; Waterhouse et al. 2001). siRNAs are generated by the endonucleolytic cleavage of long double-stranded RNA by the multi-domain RNase III enzyme, Dicer (Bernstein et al. 2001a). siRNAs are then incorporated into a ~500 kDa RNP complex, the RISC, where they provide the specificity determinants that direct an as yet unidentified protein nuclease to cleave mRNAs complementary to the siRNA (Hammond et al. 2000). *lin-4* and *let-7*, as well as the new miRNAs, are encoded by ~70 nt stem-loop structures (Lee et al. 1993; Pasquinelli et al. 2000) whose stems are also substrates for processing by Dicer

(Grishok et al. 2001; Hutvágner et al. 2001; Ketting et al. 2001). Dicer liberates miRNAs from the larger stem-loop precursors in much the same way it generates siRNAs from long dsRNA, leaving the signature 3' hydroxyl and 5' phosphate termini of an RNase III cleavage reaction. Both siRNA and stRNA production by Dicer requires ATP, consistent with the presence of an ATP-dependent helicase domain at the N-terminus of Dicer (Zamore et al. 2000; Bernstein et al. 2001a; Hutvágner et al. 2001; Nykänen et al. 2001). Mature *lin-4* and *let-7* are thought to bind partially complementary sequences in the 3′ untranslated regions of their target mRNAs (Lee et al. 1993; Reinhart et al. 2000). Unlike the binding of siRNAs, which triggers target RNA destruction, binding of the stRNA *lin-4*, and likely *let-7*, leads to translational repression of their natural mRNA targets (Olsen and Ambros 1999; Reinhart et al. 2000; Slack et al. 2000). In worms, translational repression of *lin-4* and *let-7* target mRNAs is required for the progression from one stage of development to the next.

In addition to Dicer, two members of the PPD family of proteins, ALG-1 and ALG-2, are required for the biogenesis or function of *lin-4* and *let-7* in worms (Grishok et al. 2001). PPD proteins, so named because they contain 'PAZ' and 'Piwi' domains, protein sequence motifs of unknown biochemical function, are required for diverse array of developmental functions in plants and animals. *alg-1/alg-2* mutants accumulate *lin-4* and *let-7* precursors and display striking defects in developmental timing (Grishok et al. 2001). Worms lacking *alg-2* also fail to form a normal germ line (Cikaluk et al. 1999). A role for PPD proteins in the biogenesis or function of miRNAs has only been

demonstrated for the PPD proteins ALG-1 and ALG-2 in worms, but it seems likely that PPD proteins will be needed for miRNA biogenesis in other organisms.

PPD proteins function not only in miRNA maturation, but are also required in animals, plants, and fungi for a variety of RNA silencing phenomena, including RNAi and cosuppression, the RNAi-like silencing of an endogenous gene by a transgenic copy of the same sequence (Tabara et al. 1999; Catalanotto et al. 2000; Fagard et al. 2000; Hammond et al. 2001; Pal-Bhadra et al. 2002). In *C. elegans*, the PPD protein RDE-1 is required for RNAi (but not cosuppression; Tabara et al. 1999; Dernburg et al. 2000; Ketting and Plasterk 2000); QDE2 is required for cosuppression in the fungus *Neurospora crassa* (Catalanotto et al. 2000); and Argonaute is required both for RNA silencing and for normal meristem function in plants (Bohmert et al. 1998; Fagard et al. 2000). In flies, the PPD protein, Piwi, is required for the maintenance of germ line stem cells, for the post-transcriptional silencing of endogenous genes by transgenes encoding the same mRNA, and even for some aspects of transcriptional silencing (Cox et al. 1998; Pal-Bhadra et al. 2002). Intriguingly, Piwi localizes to the nucleoplasm, not the cytoplasm, in *Drosophila* ovaries and testes, but disperses to the cytoplasm during mitosis (Cox et al. 2000). The role of Piwi in post-transcriptional silencing—a phenomenon that all current evidence suggests occurs in the cytoplasm—implies that at least a subpopulation of Piwi functions outside the nucleus. Might Piwi associate with miRNAs in the cytoplasm, then move as a Piwi-containing miRNP to the nucleus to regulate transcription or nuclear mRNA stability?

The human homolog of Piwi, Hiwi, plays a role in maintaining hematopoetic stem cells, and the mouse homolog, Miwi, is expressed in primordial germ cells, suggesting its function in mammals may parallel that of Piwi in flies (Kuramochi-Miyagawa et al. 2001; Sharma et al. 2001). Another *Drosophila* PPD protein, Aubergine, is required for the RNAi-like silencing of the *Stellate* locus in testes, for antero-posterior patterning in the developing embryo, where it is required for the translation of the developmental regulator Oskar, and for the formation of pole cells, the progenitors of both male and female germ cells (Wilson et al. 1996; Aravin et al. 2001; Harris and Macdonald 2001). Perhaps all of the phenotypes observed for PPD protein mutants arise from their failure to produce specific sets of miRNAs required for germ line development, stem cell maintenance, or patterning. Reinforcing this view, worms mutant for Dicer (*dcr-1*), like those lacking ALG-2, show germ line defects (Knight and Bass 2001). Dreyfuss and coworkers now find that many human miRNAs are present in a ~550 kDa complex, the miRNP, and that this complex contains the PPD protein eIF2C2 (Mourelatos et al. 2002).

Current evidence supports the view that in metazoans, both the RNAi and miRNA pathways require the activity of Dicer and PPD proteins. The RNAi and miRNA pathways are clearly related, but there are features that differentiate them. First, siRNAs are processed from perfectly complementary, long dsRNA into double-stranded siRNAs that guide the destruction of a target mRNA (Hamilton and Baulcombe 1999; Hammond et al. 2000; Zamore et al. 2000; Bernstein et al. 2001a; Elbashir et al. 2001a; Elbashir et al. 2001b; Nykänen et al. 2001). In contrast, miRNAs are single-stranded, processed from ~70 nucleotide precursors that have the ability to form stem loop structures

containing loops and bulges of unpaired nucleotides. Only one strand of the stem of the miRNA precursor accumulates, indicating that the other strand must either not be produced or is differentially degraded. The fact that multiple PPD proteins are found in various organisms that exhibit RNAi may be an indication that different classes of these proteins play a specialized role in the two pathways. But reality must be more complex than this simple model, which predicts that two PPD proteins—one for RNAi and one for miRNAs—would suffice. Instead, the number of PPD proteins is large and varies greatly in different organisms (Figure 1): flies have five, humans, four, and *Arabidopsis*, six, but worms have at least 24!

Why so many PPD proteins? One explanation is that not all PPD proteins function in the RNAi or miRNA pathways. This seems unlikely, since three of the five *Drosophila* family members have already been implicated in one or another RNA silencing phenomenon. Perhaps different subclasses of miRNAs require distinct PPD proteins for their production, stability, or function. Such a requirement might reflect the individual peculiarities of a miRNA's sequence or precursor structure, or perhaps the subcellular localization of its target mRNAs. Consistent with this notion, human eIF2C1 (also known as GERp95) is associated with the golgi and ER, where it might play a specialized role in mediating miRNA-based translational regulation of secreted proteins (Cikaluk et al. 1999; Tahbaz et al. 2001). Alternatively, all PPD proteins might be biochemically interchangeable, but be distinguished by distinct patterns of developmental or tissue-specific expression. For example, in worms, a subset of PPD proteins are more abundant in the germ line than the soma (Reinke et al. 2000). The finding that PPD
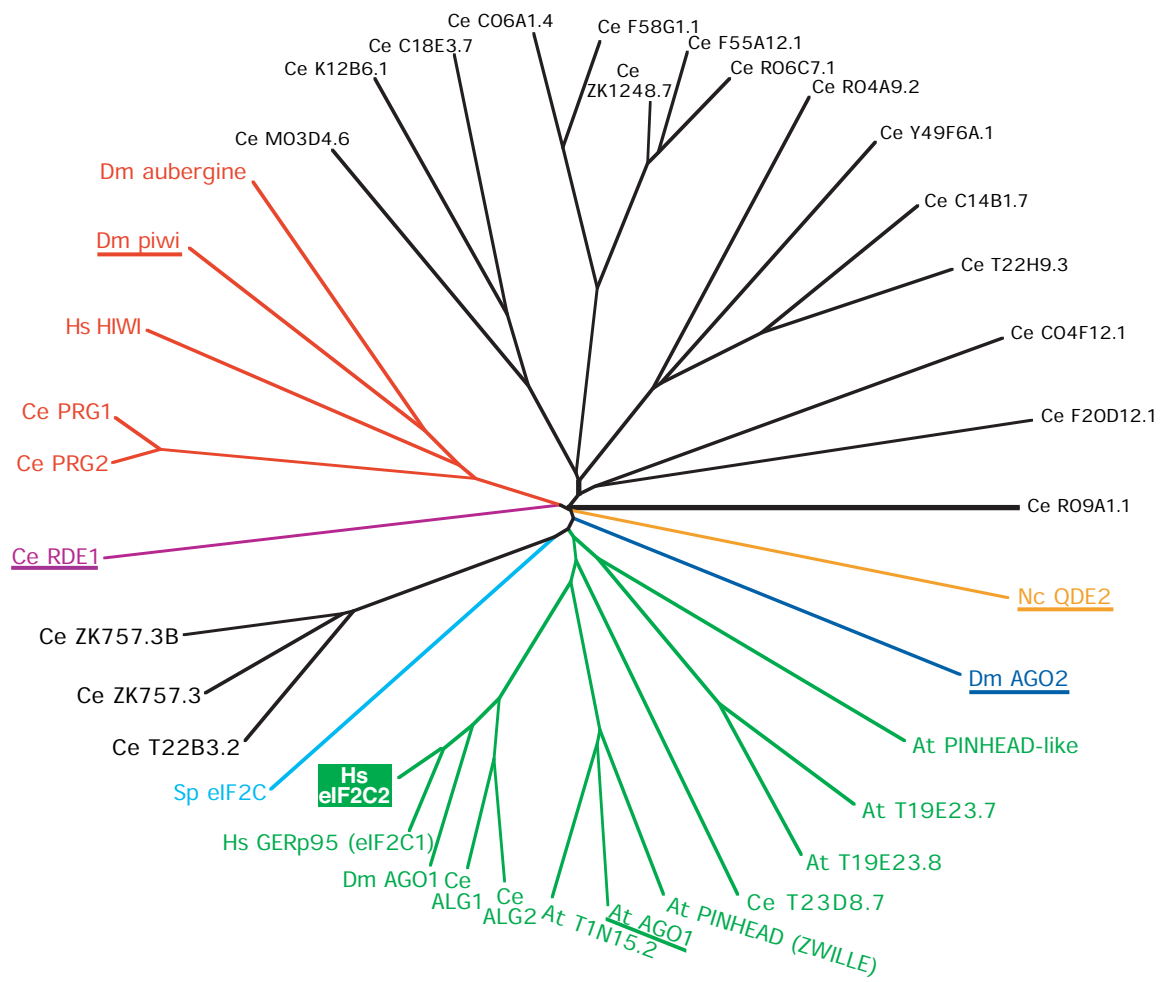
Figure 1

Figure A - 1. Sequence relationship among PPD proteins. At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Nc, *Neurospora crassa*; Hs, *Homo sapien*; Sp, *Schizosaccharomyces pombe*. Red, PIWI subfamily; green, AGO1 subfamily. Protein alignments were performed using ClustalX and illustrated by TreeView.

proteins play important roles in stem cell production, gametogenesis and patterning may be an indication that different classes of tissue-specific miRNAs regulate gene expression in these key developmental events.

In addition to the PPD protein eIF2C2, the recently discovered miRNP contains at least two more proteins, Gemin3 and Gemin4 (Mourelatos et al. 2002). These proteins may interact directly with eIF2C2, since in vitro translated Gemin3 and Gemin4 bind an eIF2C2-glutathione-S-transferase fusion protein (and vice versa). These interactions may be direct or they may be bridged by proteins present in the reticulocyte lysate used for translation. Gemin3 and Gemin4 were previously identified as part of a multi-protein complex containing the Survival of Motor Neurons (SMN) protein, Gemin2 (Charroux et al. 1999; Charroux et al. 2000; Mourelatos et al. 2001), Gemin5 (Meister et al. 2001; Gubitz et al. 2002), and Gemin 6 (Pellizzoni et al. 2002). The SMN-containing complex is distinct from the miRNP. The SMN complex—comprising SMN, Gemin2, Gemin3, Gemin4, Gemin5, and Gemin6—is found in the nucleus in discrete foci known as 'gems.' The SMN complex functions in the assembly and restructuring of diverse RNP particles, including spliceosomal snRNPs. SMN protein is defective in the neurodegenerative disease, spinal muscular atrophy (SMA). It is, therefore, presumed that the underlying cause of SMA is a failure in snRNP biogenesis or recycling. The sequences of the proteins in the SMN complex has thus far failed to reveal their biochemical function, with the exception of Gemin3, a 105 kDa member of the DEAD-box family of putative ATP-dependent RNA helicases.

Gemin3 may provide a catalytic function in the assembly of RNPs (Charroux et al. 1999). Previously, Gemin3 was detected together with Gemin4 in a second complex of approximately 550 kDa (Charroux et al. 1999). In their present work, Dreyfuss and coworkers characterized this second, less abundant complex through a series of co-immunoprecipitations, identifying it as the miRNP (Mourelatos et al. 2002). Two anti-Gemini3-specific monoclonal antibodies were used to characterize Gemin3-containing complexes in total lysates prepared by sonnicating human HeLa cells. One antibody recognizes Gemin3 in the SMN complex, while the second detects only Gemini3 protein that is not associated with SMN (Mourelatos et al. 2002). It is this second antibody that immunoprecipates the miRNP. In addition to Gemin3 and 4, this antibody co-immunoprecipitates two proteins with apparent molecular masses of 115 kDa and 95 kDa, neither of which is found in the SMN complex. Mass spectrometry revealed the 95 kDa protein to be the PPD protein eIF2C2, making the first tentative link between the ~550 kDa complex and the miRNA and RNAi pathways. Since the SMN complex contains non-coding RNAs (e.g., snRNA), the ~550 kDa complex was scrutinized for associated non-coding RNA. Remarkably, miRNAs were found to be tightly associated with the non-SMN, Gemin3-containing complex. In fact, this miRNP appears to be home to at least 40 different miRNAs, all but nine of them not identified in the original screen to clone and sequence human miRNAs (Lagos-Quintana et al. 2001). Like the original tiny RNAs, *lin-4* and *let-7*, each of the new miRNAs is encoded in genomic sequence that can fold to form a ~70 nt precursor RNA.

In addition to co-localizing with the SMN protein in nuclear 'gems,' Gemin3 exhibits a diffuse cytoplasmic staining pattern that may correspond to the miRNPs. In the recent Dreyfuss study employed total cell lysates, so the intracellular localization of miRNPs could not be inferred, but Dicer is a cytoplasmic protein in human cells (Billy et al. 2001), suggesting that miRNA precursors are processed in the cytoplasm by Dicer, which then pass the single-stranded miRNAs to the miRNP. In extracts from cultured *Drosophila* cells, the PPD protein Ago-2 binds Dicer, directly or indirectly, suggesting that the transfer of mature miRNA from Dicer to the miRNP might be mediated by PPD proteins (Hammond et al. 2001). Dicer does not appear to be a stable component of the miRNP, since no appropriately sized protein (~250 kDa) co-immunoprecipitates with either anti-eIF2C2 or Gemin3 antibodies, although as an enzyme Dicer might be present in the miRNP in substoichiometric amounts (Mourelatos et al. 2002). In fact, antibodies to both Gemin3 and eIF2C2 immunoprecipitate ~76 nt long RNA in addition to the miRNAs, suggesting that miRNA precursors might be present in the miRNP. Might the miRNPs therefore be miRNA birthing centers (Figure 2)? Like siRNAs, miRNAs are generated by cleavage of a structured RNA precursor by the double-stranded RNA-specific endonuclease Dicer. However, siRNAs are double-stranded, whereas miRNAs are single-stranded. Are miRNAs single-stranded because Dicer cleaves miRNA precursors only at the 5′ and 3′ ends of the mature miRNA sequence? Or are miRNAs initially double-stranded like siRNAs, but then the anti-sense miRNA strand is rapidly destroyed? In this second model, the miRNP might play a role in the dissociation of the two strands, perhaps catalyzed by the putative ATP-dependent helicase Gemin3, with the
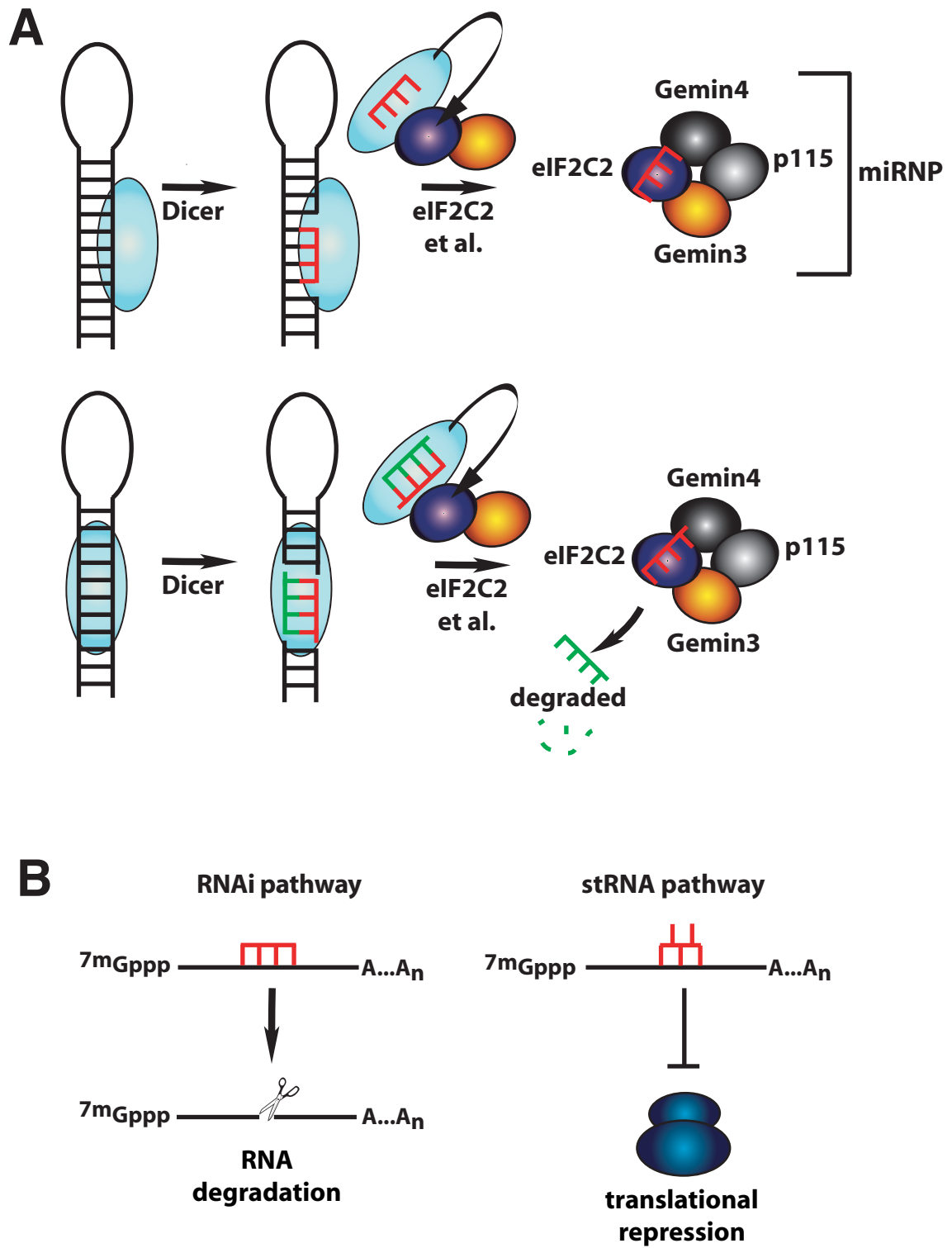
Figure 2

Figure A - 2. Models for miRNA biogenesis and function. (A) Dicer may cleave a single-stranded miRNA directly from its stem-loop precursor RNA, then transfer the mature miRNA to components of the miRNP. Alternatively, Dicer may initially generate a double-stranded siRNA, then transfer it to the miRNP. Protein components of the miRNP would then unwind the siRNA, select one strand of the siRNA to become the mature, single-stranded miRNA and catalyze degradation of the other siRNA strand. (B) In the RNAi pathway, a perfectly complementary siRNA targets mRNA for endonucleolytic cleavage. In contrast, miRNAs pair only imperfectly with sequences in the 3′ untranslated regions of their target RNAs and are believed to repress mRNA translation without altering mRNA stability.

PPD protein eIF2C2 acting to stabilize the mature miRNA against degradation. The miRNP might even promote degradation of the non-miRNA strand and the rest of the precursor.

What do miRNAs do? One-hundred thirty-three distinct miRNAs have been identified thus far in worms, flies, or humans, yet the regulatory function and mRNA targets are known for only *C. elegans lin-4* and *let-7*. The presence of a putative ATP-dependent RNA helicase suggests that the miRNP might catalyze miRNA target recognition. In this regard, it is important to note that target recognition by siRNAs requires no ATP (Nykänen et al. 2001). siRNA-mRNA interactions encompass 21 perfectly complementary base pairs, whereas *lin-4* and *let-7* form only 14 to 17 Watson-Crick base pairs with their target mRNAs. Therefore, the weaker stRNA-mRNA and putative miRNA-mRNA interactions may require the aid of an ATP-dependent helicase to remove local secondary structure in the target mRNA or perhaps even to zipper the miRNA to its target sequence. Finally, a helicase might in principle act to recycle miRNAs, although if miRNAs function as translational regulators, they would likely form a stoichiometric complex with their regulatory targets and turnover infrequently. If, like *lin-4* and *let-7*, miRNAs are sequence-specific translational repressors that bind their targets through 3′ UTR sequences, the miRNP might correspond to the actual mediator of translational repression. In support of this idea, a significant fraction of human eIF2C2, Gemin3, and Gemin4 co-sediment in a sucrose gradient with the ribosomal pellet (Mourelatos et al. 2002), as has also been reported for the RNAi-associated PPD protein Ago-2 from extracts of cultured fly cells (Hammond et al. 2001). It has not yet been

determined if this co-sedimentation reflects an actual association of either eIF2C2 or Ago-2 with ribosomes, although the miRNA *lin-4* has been shown to be directly associated with polysomal *lin-14* mRNA, whose translation it represses (Olsen and Ambros 1999). Furthermore, eIF2C2 is 85% identical to eIF2C1, which was originally identified as a translational initiation cofactor (Zou et al. 1998; Koesters et al. 1999). It is important to note that although purified fractions containing the eIF2C1 protein were shown to enhance translational initiation in vitro, no biochemical activity has yet been demonstrated for the protein that was cloned as eIF2C1 and that mRNA repression by *lin-4* occurs at a step after translational initiation. It is also important to recall that outside of *C. elegans*, no function has been ascribed to any miRNA, including *Drosophila* and human *let-7*. Thus, miRNAs may act not only as translational regulators, but also to modulate mRNA stability or to direct mRNA localization. It is conceivable that some may even provide sequence-specificity to regulators of transcription.

Of the 133 miRNAs identified thus far, none is perfectly complementary to any known mRNA in the worm, fly, or human genome. It is therefore unlikely that miRNAs act in the destruction of mRNA in vivo, since effective RNAi requires a high degree of complementarity between the siRNA and the target RNA (Elbashir et al. 2001b). Since the RNAi pathway is thought to defend eukaryotic cells against colonization by parasitic DNA, it is surprising that not a single miRNA corresponding to a transposon has been cloned from *C. elegans* or human cells. In contrast, small RNA cloning from trypanosomes, which also contain the RNAi machinery, revealed abundant siRNAs corresponding to retrotransposons (Djikeng et al. 2001). Why have miRNA screens from

higher organisms failed to detect such siRNAs? One possibility is that the RNAi pathway plays a major role in silencing transposons only at a highly specific time in development or in a specialized tissue such as germ cell progenitors. In support of this idea, Tuschl and colleagues reported cloning a small number of siRNAs derived from retrotransposon sequences from syncitial blastoderm stage *Drosophila* embryos, a stage in development when germ cell progenitors are formed (Elbashir et al. 2001a).

There are several tantalizing similarities between the miRNP discovered by Dreyfuss and colleagues and the RISC, the RNP that mediates RNAi. First, the sizes of the miRNP (~550 kDa; Mourelatos et al. 2002) and the RISC (~500 kDa; Hammond et al. 2000) are quite similar. Second, a helicase like Gemin3 must play a role in RNAi, because ATP-dependent unwinding of an siRNA duplex is a prerequisite for formation of an active RISC (Nykänen et al. 2001). The putative DEAD-box helicase protein, Spindle E, is required for the RNAi-like silencing of the endogenous Stellate locus in *Drosophila* testes (Aravin et al. 2001; Stapleton et al. 2001), and it will be important to determine if Spindle E is a component of either a *Drosophila* miRNP or the RISC or both. Finally, PPD proteins are found in both the miRNP (human eIF2C2) and the RISC (*Drosophila* Ago-2). Might the miRNP and the RISC be one-and-the-same, a single RNP with multiple functions? If so, it is tempting to predict that the as-yet-unidentified 115 kDa miRNP protein is the elusive 'Slicer,' the ribonuclease postulated to cleave target RNA in the RNAi pathway. Alternatively, the miRNP and the RISC may be distinct complexes containing pathway-specific proteins drawn from the same protein families. In either

case, the search for what miRNAs do and how they do it is easier now that we know where they live.

Acknowledgements

The authors thank members of the Zamore lab for helpful discussions and for comments on the manuscript.

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