ed that these find a corresponding target mRNA that they can immediately hop onto (*26*).

The polymerase required for this amplification is probably different in different tissues. In the germline of *C. elegans*, the *ego-1* gene has been implicated in RNAi; it has sequence homology to a factor previously isolated from the tomato as an RdRP (*29*). In somatic *C. elegans* cells, another RdRP homolog has been implicated: *rrf-1*. Mutation of the *rrf-1* gene results in loss of RNAi and in significant decrease of siRNAs. Inactivation of another RdRP homolog has the opposite effect, of enhancing RNAi [*rrf-3* (*11*)]. The *rrf-3* gene product may be less active and may compete with RRF-1 in the relevant complex. In *Dictyostelium*, three RdRP homologs have been described. Loss of one of them, *rrpA*, resulted in loss of RNAi and of detectable siRNAs (*13*).

The *Arabidopsis thaliana* RdRP homolog SDE1/SGS2 is also required for transitive RNAi (*12*). A significant difference between transitive RNAi in *C. elegans* and plants (*Nicotiana bethamiana* and *Arabidopsis*) is that, in plants, the transitive effect can occur in the 3′ as well as the 5′ direction, and as a consequence, secondary siRNAs are found both 5′ and 3′ of the targeted region. In plants, siRNAs may direct an RdRP to an mRNA, triggering unprimed RdRP activity of the complete RNA molecule. Alternatively, the initial reaction may show polarity, but frequent template jumps may occur.

The combination of siRNA stabilization and transitive RNAi results in a "chain reaction," in which multiple cycles of replication can occur, followed by Dicing, new priming, and a new round of amplification (Fig. 1).

Conclusion

We are beginning to dissect an ancient mechanism that protects the most sensitive part of a species: its genetic code. Like the vertebrate immune system, the machinery recognizes molecular parasites, raises an initial response, and stabilizes and amplifies this response. Given the conservation of parts of the RNAi-silencing machinery [see reviews (*30*, *31*)], this genome defense mechanism should be widespread, although details may differ. It is thus also possible that RNAi silencing refers to a family of mechanisms that are quite different in context and detail. This will almost certainly be the case for more specific aspects of the biology: for example systemic RNAi in *C. elegans* (*21*), spreading of silencing in plants (*22*), and suppression of silencing induced by several plant viruses (*32*).

Just as knowledge of immunology has laid the foundation for (experimental) immune therapy, a thorough understanding of the genome's immune system has great potential for applications in directed gene silencing, in experimental biology, and possibly also in disease therapy.

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VIEWPOINT

Ancient Pathways Programmed by Small RNAs

Phillip D. Zamore

Double-stranded RNA can now be used in a wide variety of eukaryotes to suppress the expression of virtually any gene, allowing the rapid analysis of that gene's function, a technique known as RNA interference. But how cells use the information in double-stranded RNA to suppress gene expression and why they contain the machinery to do so remain the subjects of intense scrutiny. Current evidence suggests that RNA interference and other "RNA silencing" phenomena reflect an elaborate cellular apparatus that eliminates abundant but defective messenger RNAs and defends against molecular parasites such as transposons and viruses.

Virtually any gene can now be disrupted in cultured human cells, flies, worms, and a growing list of other organisms in just a week or two (*1*, *2*) using new tools based on the cellular phenomenon of "RNA silencing" (Fig. 1). These new tools likely will soon be extended to whole mammals (*3*–*5*) and may one day form the basis of a new class of drugs to treat human disease. Knowing only the DNA sequence of a gene, molecular biologists can design potent, sequence-specific inhibitors—a form of double-stranded RNA—that block expression of just that gene. Using such inhibitors, we can now ask for each of the tens of thousands of human messenger RNAs (mRNAs) the central question of genetics: what does this gene do?

White Flowers and Silenced Worms

New tools for evaluating gene function (Fig. 1) sprang from the discovery that disparate and bizarre examples of RNA silencing are all man-

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Lazare Research Building, Room 825, 364 Plantation Street, Worcester, MA 01605, USA. E-mail: phillip.zamore@umassmed.edu

ifestations of the same underlying cellular process. Examples of RNA silencing accumulated throughout the 1990s in fungi, worms, flies, and mice. Common to all of these was that the introduction into cells of nucleic acid bearing sequence from a cellular gene decreased the steady-state level of the corresponding cellular mRNA. RNA silencing was first observed in plants. For example, researchers trying to generate more vividly purple petunias created transgenic plants that harbored an extra copy of the enzyme responsible for purple pigment. Yet the resulting plants often produced white flowers. The production of both the transgenic and the petunia's own purple-making genes was switched off or "cosuppressed" (*6*–*8*). The transgenic copies of the gene, intended to produce more gene product than is made in nontransgenic plants, surprisingly made less. This phenomenon, posttranscriptional gene silencing (PTGS), is now used to make genetically modified plants that lack specific endogenous gene products. In parallel, researchers working with the nematode *Caenorhabditis elegans* tried to use antisense technology to block the function of a gene (*9*). They injected antisense RNA for the gene into the worm, with the intent that the antisense RNA would pair with the gene's mRNA transcript and block its translation into protein. The experiment was a qualified success. The protein's concentration was indeed reduced, but injecting sense RNA—that is, the mRNA itself—likewise blocked protein production. How sense RNA could block gene expression was not understood. Fire, Mello, and co-workers brought understanding to these observations when they showed that both the antisense and the sense RNA preparations contained contaminating double-stranded RNA and that, in fact, this double-stranded RNA was the real trigger of gene silencing (*10*). This phenomenon, in which experimentally introduced double-stranded RNA leads to loss of the expression of the corresponding cellular gene, is called RNA interference or "RNAi" (*11*).

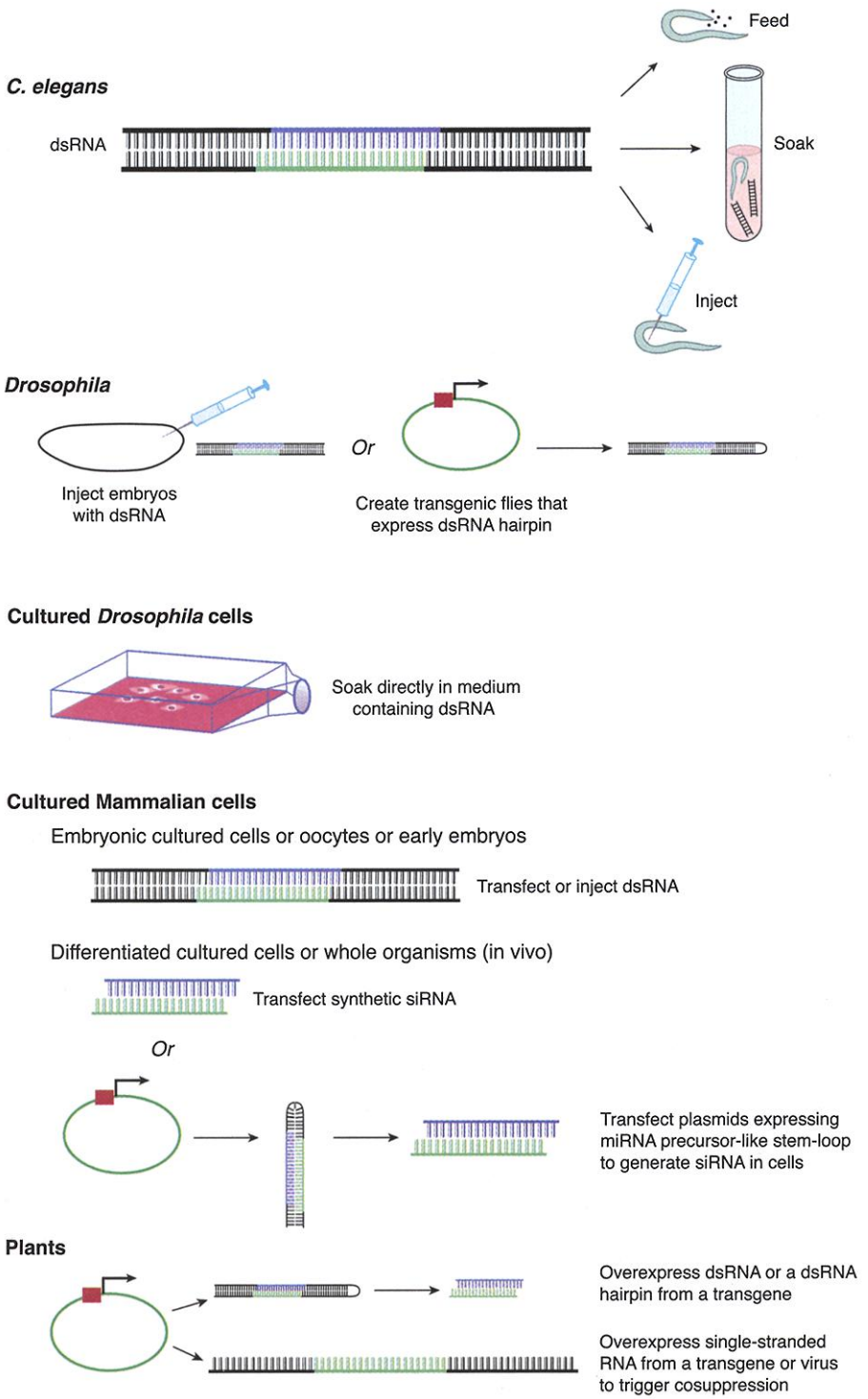

Fig. 1. Gene silencing tools. dsRNA, double-stranded RNA.

We now understand that both PTGS and RNAi are manifestations of a broader group of posttranscriptional RNA silencing phenomena common to virtually all eukaryotes, except perhaps the baker's yeast, *Saccharomyces cerevisiae* (*12*). In all these RNA silencing phenomena, transcription of the silenced gene is unperturbed, yet the mRNA transcript for the gene fails to accumulate to its normal cytoplasmic concentration. That is, the gene is copied into mRNA in the nucleus, but the mRNA is destroyed—probably in the cytoplasm—as quickly as it is made. RNA silencing pathways in protozoa, plants, fungi, and animals require a set of related proteins, suggesting that the common aspects of the pathways are quite ancient (*13*–*24*). Archea and prokaryotes lack these proteins, so RNA silencing is probably a eukaryotic innovation.

Understanding Started Small

Our understanding of the mechanism of RNA silencing was shaped in large part by the discovery that silenced plants always contain small RNAs, about 25 nucleotides (nt) long, derived from the sequence of the silenced gene. Such small RNAs are never found in plants that do not display silencing (*25*). The small RNAs include both sense and antisense fragments of the silenced gene's sequence. Similar small RNAs are found as part of a ribonuclease complex in extracts of insect cells pretreated with double-stranded RNA and in *Drosophila* embryo lysates that reproduce most, if not all, of the RNAi pathway in a test tube (*26*, *27*). We now know that these "small interfering RNAs" or "siRNAs" are double-stranded and that they are chopped from longer double-stranded RNA by an ATP-dependent ribonuclease called "Dicer" (*21*, *28*). In flies and humans, siRNAs are

21- to 23-nt-long double-stranded RNAs bearing two-nucleotide, 3′ overhanging ends (*2*, *29*). The peculiar structure of siRNAs reflects the enzymatic mechanism by which Dicer, a member of the RNase III family of double-stranded RNA-specific endonucleases, cleaves double-stranded RNA (*30*). Synthetic siRNAs with the structure of Dicer products are now routinely used to trigger silencing in cultured human cells, providing an alternative to the time-consuming process of making somatic cell knockouts (*1*, *2*). Data from in vitro studies in *Drosophila* suggest that the siRNAs produced by Dicer are then transferred to a second enzyme complex, the RNA-induced silencing complex (RISC), which contains an endoribonuclease that is distinct from Dicer (*20*, *26*, *28*). The endoribonuclease uses the sequence encoded by the antisense siRNA strand to find and destroy mRNAs of complementary sequence. The siRNA thus acts as a guide, restricting the ribonuclease to cleave only RNAs complementary to one of the two siRNA strands. How the RNA-degrading capacity of the ribonuclease is constrained by the siRNA guide is not yet understood, but current evidence suggests that it cuts the mRNA partner of an siRNA-mRNA duplex across from the center of the siRNA (*29*, *31*). It remains to be determined whether the RISC is a bona fide enzyme in which a single siRNA molecule directs multiple rounds of mRNA cleavage.

So has the once bizarre collection of disparate RNA silencing phenomena been stripped of its mystery? In a way, yes. RNA silencing in plants (PTGS or cosuppression), fungi ("quelling"), and animals (RNAi) now share a common intellectual framework, united by common genes (discovered, for the most part, by classical genetics) and organized around a stepwise pathway that continues to emerge from biochemical studies of RNAi in *Drosophila* and human cells. But the mystery has also intensified, as the molecular dissection of RNA silencing phenomena reveals differences in mechanism between organisms and between different "triggers" of silencing, of which double-stranded RNA is but one. The mystery has also intensified as we identify—but do not yet understand—the connections between RNA silencing and the normal functions of the eukaryotic cell, especially between RNA silencing and animal development [reviewed in (*32*, *33*)].

One Mechanism or Variations on a Theme?

Experiments in *C. elegans* suggest that RNAi requires a target RNA copying step, without which siRNAs fail to reach sufficient concentration to accomplish target mRNA cleavage (*34*). These studies, as well as similar studies in plants and fungi (*13*, *18*, *35*), demonstrate a clear genetic role for a family of RNA-dependent RNA polymerases (RdRPs) in the mechanism of RNA silencing. Furthermore, the *Arabidopsis* RdRP, *SDE-1/SGS-2*, is required for PTGS but is dispensable for the silencing of viruses that encode their own RdRP proteins. Copying of the target mRNA into double-stranded RNA could also explain why stable accumulation of siRNA duplexes in *Dictyostelium discoideum* requires the presence of both the target mRNA and a gene encoding a putative RdRP (*23*). A high concentration of siRNA may be achieved in vivo by copying the target RNA into new double stranded RNA, which is then diced into a new crop of siRNAs. In this view, exogenous double-stranded RNA does not produce enough siRNA-programmed RISC complexes to accomplish silencing. Instead, the exogenous double strand is proposed to be diced into "primary" siRNAs that function as primers for new double-stranded RNA synthesis. Such synthesis is likely catalyzed by the RdRP using the target mRNA as a transcription template. However, a direct role for primers in the function of the RdRP has not been demonstrated in silencing in any organism, and other mechanisms of RdRP action are possible. The double-stranded RNA synthesized by the RdRP would then be cleaved by Dicer to generate a new crop of "secondary" siRNAs, amplifying the silencing signal and leading to enough RISC complex to establish silencing. Remarkably, secondary—that is, RdRP-dependent—siRNA production in *C. elegans* [but not in *Neurospora* (*36*), *Dictyostelium* (*23*), or *Arabidopsis* (*25*)] is asymmetric, where only the target-complementary siRNA strand can be detected (*37*). At present, we cannot answer the question, why are secondary siRNAs required to eliminate target mRNA in worms, but primary siRNAs appear to suffice in flies and humans?

No member of this nearly ubiquitous family of RdRPs has been detected by BLAST searching the nearly complete genome sequences of *Drosophila melanogaster* or humans. Furthermore, a variety of experiments argue against a role for an RdRP in the RNAi pathway in *Drosophila* (*20*, *27*, *28*, *38*, *39*). In humans, the most compelling evidence against the involvement of an RdRP is the recent finding that siRNAs that cannot act as primers for an RdRP because they contain blocked 3′ termini nonetheless trigger efficient RNAi in vivo (*40*). Why might an RNA-copying enzyme be essential for RNAi in some organisms (*C. elegans*, *Arabidopsis*, *Neurospora*, *Dictyostelium*) but not in others (*Drosophila*, humans)? To begin to answer this question requires an understanding of how cells sense the various RNA-silencing triggers, rather than how they dispatch mRNA targets.

A Diversity of Silencing Triggers

There is strong evidence that RNA silencing phenomena share a common biochemical machinery, but that this machinery likely lies downstream of a more diverse array of sensors that detect different silencing "triggers" (*41*). Double-stranded RNA is but one of several RNA molecules that induce silencing. For example, the white-flowered petunias that overexpressed the purple pigment–making gene did so in response to the introduction of a transgene designed to produce a high level of single-stranded, sense RNA. So why did the flowers silence the transgenic and the endogenous genes? The standard explanation is that the transgene made "aberrant" RNA. It is tempting to view this aberrant RNA as simply unanticipated double-stranded RNA that triggers silencing by the standard mechanism proposed for RNAi (Fig. 2A). But other evidence suggests that aberrant RNA may be single-stranded and that it is converted into double-stranded RNA by cellular enzymes designed to detect its aberrancy. What makes single-stranded RNA aberrant is an unresolved question in our understanding of RNA silencing. Premature termination of transcription, inappropriate pre-mRNA splicing, failure to associate with the appropriate hnRNP proteins, lack of a poly(A)$^+$ tail, or failure to be translated may all make an mRNA aberrant. Common to all of these may be increased access of the RNA to an RdRP that could convert aberrant single-stranded RNA into double-stranded RNA, which could then enter the RNAi pathway through its conversion by Dicer into siRNAs. Thus, the chief candidate for an aberrant RNA sensor is the RdRP. Perhaps *C. elegans* and other organisms that require an RdRP for silencing sense double-stranded RNA by a mechanism that cannot directly load siRNAs into the RISC complex (Fig. 2B). In these organisms, silencing is probably not triggered by creating siRNAs from the exogenous double-stranded RNA, but rather by using the primary siRNAs to activate the pathway that normally senses aberrant RNA, the cosuppression pathway. One testable prediction of this model is that all genes required for cosuppression in worms will be required for RNAi, but not vice versa (*42*, *43*). A second prediction is that cosuppression in flies and, perhaps, mammals will not use the same RdRP-based mechanism thought to operate in worms or plants.

Why should RdRPs be required for RNA silencing in some organisms, such as *C. elegans*, *Arabidopsis*, and *Neurospora*, but not *Drosophila* and human cells? An obvious answer is that an RNA polymerase of similar biochemical activity but different sequence fulfills this function in flies and mammals. A noncanonical RdRP has been proposed to play a role in RNAi in *Drosophila* (*44*), but biochemical evidence does not support an obligatory role for such an enzyme in flies (*28*, *38*) or in human cells (*40*). siRNA-mediated RNAi in human cells is transitory, with cells recovering from a single treatment with siRNAs in 4 to 6 days (*40*, *45*), suggesting that the original siRNAs are not amplified or copied. siRNAs may simply be less stable in some organisms than others. In those organisms in which

siRNAs are acutely unstable, an RdRP might be essential to amplify the original silencing signal, generating secondary siRNAs. It is important to note that this amplification may not involve the primed synthesis of new RNA. Rather, the initial double-stranded RNA may yield a small number of siRNA-programmed RISC endonuclease complexes that cleave the target mRNA. The resulting mRNA fragments, in particular the relatively stable, capped 5′ fragment, might constitute aberrant mRNA, which would be copied into double-stranded RNA by an RdRP in an unprimed reaction (Fig. 2B). A high concentration of such aberrant RNA may be required to activate the RdRP, with the normal products of mRNA turnover at too low a concentration to provoke RdRP-mediated copying. As concentration-dependent sensors of aberrant RNA, RdRP enzymes should be mediocre polymerases, with relatively low affinity for RNA templates and modest processivity, allowing them to ignore healthy, cellular mRNAs. Consistent with this view, Han and Grierson recently showed that, in tomatoes, siRNAs were preferentially produced from the 3′ end of a transgene that triggered silencing but not from the endogenous target RNA that is silenced (*46*). This suggests that the RdRP initiates primer-independent copying at the 3′ end of an abundant but aberrant transcript from the transgene but does not copy the nonaberrant, and presumably less abundant, endogenous mRNA. The double-stranded RNA resulting from RdRP copying of an aberrant transcript would then be converted by Dicer into siRNAs, which, as part of a RISC complex, could destroy additional aberrant RNA from the transgene, as well as transcripts from an endogenous gene of corresponding sequence, leading to the silencing of both transgene and endogenous gene. This explains the observed decline in siRNA levels that accompanies the establishment of silencing in tomatoes (*46*). Furthermore, both a 5′ fragment lacking a poly(A)$^+$ tail and a 3′ polyadenylated fragment of the endogenous, silenced mRNA were detected, additional evidence that the RISC-based pathway of siRNA-directed endonucleolytic cleavage operates in plants, too (*46*). Short antisense RNA fragments may also be silencing triggers, eliciting silencing by recruiting an RdRP to convert an mRNA into double-stranded RNA. Plasterk and co-workers have shown that in *C. elegans* exogenous single-stranded RNA oligomers of as long as 40 nucleotides can trigger silencing (*37*). Remarkably, the genetic requirements for this type of silencing resemble those of cosuppression, not RNAi. An alternative view, of course, is that the pathway worked out in *Drosophila* does not exist in all organisms. It is sobering to recall that RISC activity has not yet been demonstrated in *C. elegans* or *Neurospora*. Nevertheless, recent evidence suggests that siRNAs direct endonucleolytic cleavage of the target RNA in human cells, indicating the presence of a RISC in mammals (*40*).

In plants and in animals, RNAi-like mechanisms defend against viral infection (*47*–*49*). Thus, viral infection is another distinct trigger of silencing. For some RNA viruses, double-stranded intermediates in the viral life cycle may provoke RNAi, but for others, such as DNA viruses, the molecular species that induce viral silencing are yet unidentified. Transposons and repetitive DNA sequences are also kept in check in eukaryotic cells by RNAi-like mechanisms. In *C. elegans*, silencing of such parasitic DNA requires downstream components of the RNAi pathway but does not use the same upstream sensors. For example, the *mut-7* gene is required for RNAi, and worms defective in *mut-7* show increased transposition (*14*, *50*). MUT-7, a putative 3′-to-5′ exonuclease, may actually function far downstream in the pathway, degrading the initial endonucleolytic fragments produced by the RISC. *mut-7* function may be especially important when RISC-mediated cleavage leaves abundant, translatable mRNA fragments. In contrast, no increase in transposition occurs in *rde-1* mutants, which are nonetheless completely refractory to RNAi elicited by exogenous double-stranded RNA (*14*). If double-stranded RNA produced from transposons triggers their silencing, why do

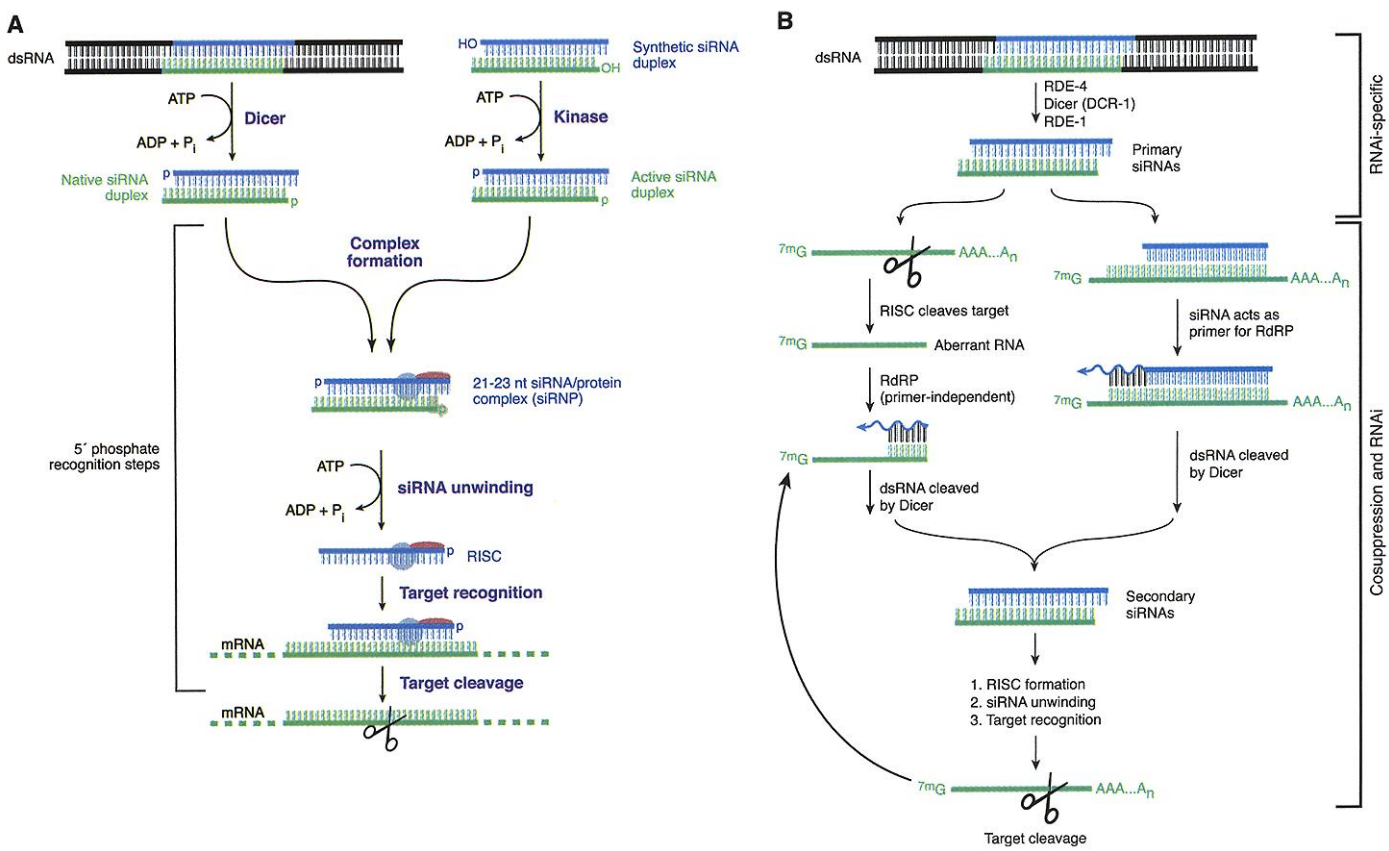

Fig. 2. Mechanisms for RNA silencing. (**A**) The "standard" model for RNAi in *Drosophila*. (**B**) A proposed but untested mechanism for RNAi and cosuppression in *C. elegans*.

they not require *rde-1* to activate the RNAi machinery? One possibility is that some other molecular abnormality triggers transposon silencing. In this view, transposon silencing might be triggered by aberrant RNA rather than double-stranded RNA. RDE-1 is also not required for cosuppression in worms (*42*, *43*).

The RDE-1 protein is a member of the PPD (PAZ and Piwi domain) family. A PPD protein is required for posttranscriptional RNA silencing in every organism where the function of this family has been tested genetically or biochemically. Another member of the PPD protein family, the RDE-1 ortholog Ago-2, is a component of the *Drosophila* RISC complex (*20*), and the PPD protein Qde-2 is associated with an siRNA-containing complex, likely a RISC, in *Neurospora* (*36*). Perhaps some PPD proteins are coupled to RdRPs that sense aberrant RNA, whereas others like RDE-1 are linked instead to proteins that bind directly to double-stranded RNA. In *C. elegans*, the RDE-1–associated protein RDE-4 is a good candidate for such a partner (*51*). Thus, RDE-4 might sense double-stranded RNA, recruit Dicer to generate primary siRNAs, then pass the primary siRNAs to downstream components of the RNAi pathway through RDE-1. Reinforcing this view, *rde-4* mutants fail to make either primary or secondary siRNAs, whereas primary siRNA levels are normal but secondary siRNAs are not made in *rde-1* mutants (*37*, *52*). In contrast, fungi mutant for the related *qde-2* gene show normal levels of siRNAs (*36*). Consistent with the idea that in worms *rde-4* is required to convert double-stranded RNA into siRNAs, whereas *rde-1* acts to shunt primary siRNAs to the cosuppression pathway, injection of short synthetic RNA duplexes partially bypasses the requirement for *rde-4*, but not *rde-1*, but only if the RNAs have the characteristic end-structure of siRNAs (*52*).

siRNAs and Other Types of Gene Silencing

In *Drosophila* and in human cells, synthetic or purified siRNA duplexes can replace double-stranded RNA as an RNAi trigger both in vitro and in vivo (*1*, *2*, *28*, *29*, *31*, *53*). Thus, siRNAs are true intermediates in the RNAi pathway in these organisms. Although siRNAs were first detected in plants, they have not yet been shown to be efficient triggers of silencing in nematodes, plants, or fungi (*52*, *54*). Is the RNAi pathway that seems to be essentially identical in flies and humans conserved more broadly? Do siRNAs serve as specificity determinants in silencing pathways other than RNAi, PTGS, and quelling? In plants, transcriptional silencing can be triggered by the introduction of transgenes that generate double-stranded RNA corresponding to the sequence of a gene's promoter. Such transcriptional silencing is accompanied by (and perhaps mediated by) methylation of the DNA sequences in the promoter region of the silenced gene (*55*, *56*). The gene is silenced because it is no longer transcribed, unlike RNAi or PTGS, in which the mRNA is transcribed at normal levels but then destroyed. Even in such promoter-based transcriptional silencing, the double-stranded RNA is converted to siRNA-like small RNAs. Determining whether these siRNAs are part of the transcriptional silencing pathway or merely reflect the nonproductive entry of a bit of the double-stranded RNA into the RNAi pathway is unknown. Support for a connection between transcriptional and posttranscriptional silencing comes from recent experiments by Birchler and colleagues, who find that the protein Piwi plays a role in the silencing of endogenous genes by homologous transgenes by both posttranscriptional and transcriptional routes (*24*).

In addition to their roles in RNAi, PTGS, and quelling, RdRPs also function in a surveillance mechanism in *Neurospora* that silences unpaired DNA at meiosis. Meiotic silencing by unpaired DNA, or "MSUD," blocks the expression of genes not found at two identical chromosomal locations during the diploid phase of the *Neurospora* life cycle (*57*). Because genes normally exist in pairs, each at the same location on sister chromosomes, unpaired genes are likely to be foreign DNA sequences, such as transposons, that pose a threat to the cell. Silencing by MSUD has been proposed to be posttranscriptional, but it is conceivable that MSUD is a form of transcriptional silencing in which specialized sensors convert the DNA sequences of unpaired genes into double-stranded RNA, which can then trigger siRNA production. Consistent with this alternative model, proteins that associate with chromatin are required for PTGS in plants (*58*), quelling in fungi (*59*), and RNAi in *C. elegans* (*60*). It is tantalizing to speculate—but harder to test—that siRNAs function both in posttranscriptional RNA silencing and in various forms of transcriptional silencing. Thus, siRNAs might not only direct the endonucleolytic destruction of a corresponding mRNA but also direct the modification of chromatin structure or the methylation of DNA, thereby turning off transcription.

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