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A Short Primer on RNAi: RNA-Directed RNA Polymerase Acts as a Key Catalyst

Minireview

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One of the many intriguing features of gene silencing by RNA interference is the apparent catalytic nature of the phenomenon. New biochemical and genetic evidence now shows that an RNA-directed RNA polymerase chain reaction, primed by siRNA, amplifies the interference caused by a small amount of "trigger" dsRNA.

RNA interference (RNAi) or RNA silencing is the process whereby double-stranded RNA (dsRNA) induces the homology-dependent degradation of cognate mRNA. Although RNAi was discovered first in nematodes (Fire et al., 1998), similar phenomena, "PTGS" (posttranscriptional gene silencing) in plants and "quelling" in fungi, had been known for many years. Repression of sequence-related viral genes occurs in plants undergoing PTGS, whereas quelling (simultaneous silencing or cosuppression) of homologous endogenous genes and transgenes has been observed in fungi. It has become increasingly clear that mechanisms closely related to RNAi underlie both PTGS and quelling. Furthermore, RNAi has been implicated even in more diverse phenomena such as suppression of transposable elements and repetitive sequences and silencing of imprinted genes. Chromatin structure remodeling leading to epigenetic silencing of these genes and sequence elements may be regulated through an RNA-directed DNA methylation that may share a common mechanism with RNAi (Bender, 2001). Not surprisingly, RNAi immediately drew the attention of both geneticists and molecular biologists, and significant progress in understanding its molecular mechanism has been made recently.

The first clue to the biochemical agent directly involved in the RNAi process came from studies of PTGS in plants. Sense and antisense strands of 21–25 nt RNA fragments (denoted short interfering RNA, "siRNA") corresponding to the specific gene undergoing PTGS were detected by Hamilton and Baulcombe (1999). Development of in vitro assay systems (Zamore et al., 2000; Bernstein et al., 2001) has since led to rapid identification of essential steps, and the RNA and protein factors involved. The assay system was essential for identification of a dsRNA-specific endonuclease ("DICER") responsible for processing of the long targeting dsRNA (denoted the "trigger") into siRNA (Bernstein et al., 2001), as well as for demonstration of the active role played by siRNA in guiding homology-dependent destruction of the cognate mRNA (Elbashir et al., 2001). Further details of the progress in this rapidly moving field are more comprehensively described in a recent review (Sharp, 2001).

A particularly fascinating aspect of RNAi is its extreme efficiency—a few trigger dsRNA molecules suffice to inactivate a continuously transcribed target mRNA for long periods of time; the inactivation persists through cell division, spreads to untreated cells and tissues of plants, and is even inherited by subsequent generations of nematodes. Although conversion of the long trigger dsRNA (usually several hundred bp) into many 21–25 nt siRNA fragments results in some degree of amplification, it appears that an additional mechanism is necessary to explain the potency and self-sustaining nature of RNAi observed in *C. elegans* (Fire et al., 1998). Since RNA-directed RNA polymerase (RdRP) was one of the genes identified through genetic screenings for RNAi mutations (Cogoni and Macino, 1999; Dalmay et al., 2000), its involvement in the amplification process has been postulated. In plants, however, the RdRP gene (SDE-1) is required only for transgene-induced PTGS, but not for virus-induced PTGS (Dalmay et al., 2000). Similarly, EGO-1, the SDE-1 homolog of nematodes, is essential for RNAi but only in germline cells (Smardon et al., 2000). In addition, sensitivity of the antisense but not the sense strand of the trigger dsRNA to chemical modification (Parrish et al., 2000) seemed to be at odds with the presumed role of RdRP in replication of trigger dsRNAs or siRNAs, since modification of the original trigger dsRNA, either sense or antisense strand, would be diluted during multiround replication by RdRP, such that the overall RNAi efficacy should not be affected (Sharp, 2001). These previous studies cast doubt on the role played by RdRP in the general RNAi mechanism (Sharp, 2001). New studies described in both recent and current issues of Cell (Lipardi et al., 2001; Sijen et al., 2001 [this issue]) now provide convincing biochemical and genetic evidence that RdRP indeed plays a critical role in amplifying the RNAi effect. In addition, characteristics of siRNA that may be critical for serving as a primer for RdRP have been revealed (Lipardi et al., 2001; Nykänen et al., 2001). A new model for RNAi emerges from these recent studies (Figure 1).

New Evidence for Involvement of RdRP in the RNAi Process

An RdRP activity might provide amplification by several routes, such as replication of long trigger dsRNAs (similar to viral RNA replicases) or copying of short siRNAs (similar to the action of a known tomato RdRP) in a primer-independent manner (Dalmay et al., 2000). The new studies by Lipardi et al. (2001) now show that siRNA serves as the primer for the RdRP reaction. The siRNA-primed RdRP reaction converts target mRNA into dsRNA (Figure 1B), as well as possibly replicating trigger dsRNA (Figure 1C). Both products then serve as DICER substrates, initiating the RdRP chain reaction.

Lipardi et al. (2001) investigated the dsRNA-dependent degradation of target GFP mRNA in a cell-free extract of *Drosophila* embryos. In the absence of the target GFP mRNA, GFP trigger dsRNA (corresponding to the full-length GFP target mRNA) was processed into sequence-specific siRNAs, presumably by the DICER-like activity present in the embryo extract. GFP siRNA purified from this reaction mix efficiently targeted the

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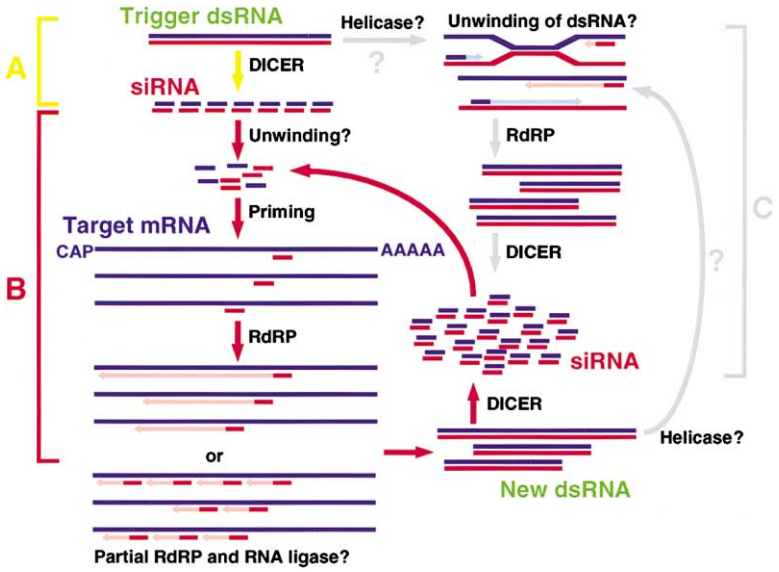

Figure 1. A Model for RNAi

(A) In the "initiation" stage of RNAi, a small amount of trigger dsRNA is processed into siRNA (yellow arrow), which is used as an RdRP primer.

(B) The RdRP reaction converts target mRNAs into new dsRNAs (next generation of trigger dsRNAs), which are then processed into new siRNAs, establishing a self-sustaining cycle of RNAi "maintenance" (red arrows).

(C) Replication of trigger- or newly synthesized-dsRNA by RdRP would amplify the potency of RNAi by further increasing the amount of siRNA, since both sense and antisense strands of trigger dsRNA and siRNA can be now utilized. However, the in vivo significance of this pathway (gray arrows) has not yet been established.

GFP mRNA, but not a negative control luciferase mRNA, when added to fresh embryo extract. Most importantly, incorporation of ^{32}P-labeled GFP siRNAs into new full-length cognate dsRNA was noted at early time points of the experiment, demonstrating for the first time the presence of RdRP activity in *Drosophila* embryo extract. Both ssRNA (equivalent to target mRNA) as well as dsRNA (equivalent to trigger) served as templates for copying by RdRP. New full-length dsRNA was formed rapidly ($<$10 min), and then cleaved by DICER-like activity into new siRNA over the next several hours. When a 21 nt synthetic GFP siRNA duplex was used, nascent dsRNA products extended in the 5′ direction from the antisense strand of the siRNA were also observed, establishing the role of siRNA as primer in the RdRP reaction. In addition, the experiment indicated that a single primer present in the natural siRNA population would be capable of priming the entire GFP dsRNA. Only the antisense strand of the siRNAs can initiate the first and thus critical cycle of the "chain reaction" on the target mRNA (Figure 1B). The sensitivity to chemical modification of the antisense but not sense strand trigger (Parrish et al., 2000) may now be understood in the context of its more significant role as the RdRP initiation primer.

Certain structural features of siRNA appear to be critical (Lipardi et al., 2001). A free 3′-hydroxyl group is required for siRNA to act as primer for the RdRP reaction. A strict requirement for a 5′ phosphate group on siRNA has been reported also (Nykänen et al., 2001). Could this indicate involvement of siRNA in an RNA-ligation step where its 5′ phosphate may be required for the RNA ligation mechanism (England et al., 1977)? Full-length dsRNA appears to be the dominant product formed from the siRNA-primed RdRP reaction, instead of the expected mixture of different size products initiated at various locations along the template mRNA. One possible explanation is that multiple, partial length RdRP products are ligated to a full-length antisense strand RNA by an RNA ligase-like activity as shown in Figure 1B (Lipardi et al., 2001). Although genetic screens have not identified RNA ligase genes essential for RNAi, it should be noted that lethal mutations are selected against during screening.

Secondary siRNAs and Transitive RNAi

Further evidence of RdRP involvement has been obtained by cleverly designed genetic studies in *C. elegans* (Sijen et al., 2001 [this issue of *Cell*]). The polarity of the RdRP reaction predicts that the newly synthesized dsRNA may extend beyond the sequence complementary to the initial trigger dsRNA, into upstream regions of the target mRNA. Furthermore, a new population of secondary siRNA might be generated from the extended dsRNA (Figure 2). To test this idea, Sijen et al. (2001) conducted RNase protection experiments to detect secondary siRNAs corresponding to either a muscle-specific *unc-22* or a gemline-specific *pos-1* as target gene. Both primary siRNAs and secondary siRNAs were indeed detected with region-specific RNA probes. As anticipated, synthesis of secondary siRNAs was limited to the region upstream of the trigger sequence. Furthermore, these secondary siRNAs were also able to induce secondary RNA interference, a phenomenon termed "transitive RNAi." Transitive RNAi was observed when a primary trigger dsRNA, specific for the lacZ region of a target mRNA encoding GFP-lacZ fusion protein, also suppressed expression of a separate GFP mRNA. (In this experiment, the GFP-lacZ protein was nuclear, while GFP was mitochondrial.) In addition, the transitivity faded as the secondary target distance between lacZ and GFP was increased, becoming insignificant when the secondary target was placed more than a few hundred bases from the primary target sequence (Sijen et al., 2001). In contrast, RdRP primed with a synthetic 21 nt siRNA led to robust synthesis of a dsRNA 690 bp in length in the *Drosophila* embryo extract (Lipardi et al., 2001). Thus, the extent of transitive RNAi effects may differ in different systems.

Finally, Sijen et al. also demonstrate a requirement for RRF-1 in the generation of secondary siRNA and detection of transitive RNAi. RRF-1 is one of four known *C. elegans* genes with sequence homology to RdRP. Another homolog is EGO-1, required for RNAi, but only

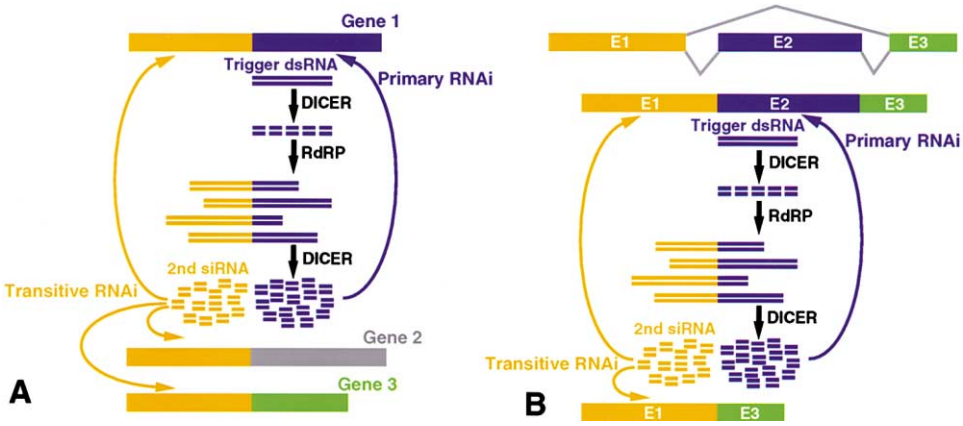

Figure 2. Transitive RNAi by Secondary siRNA

Secondary siRNAs, generated from the dsRNA extended (orange) upstream of the primary target region (blue) by RdRP, promote transitive RNAi against their sequence homologous gene family members (A) or alternatively spliced mRNAs (B).

in germline cells (Smardon et al., 2000). RNAi, including production of secondary siRNAs and transitive RNAi, is detected in germline cells but completely absent in somatic tissues of *rrf-1* null mutant worms. Importantly, synthesis of primary siRNAs derived from the original trigger dsRNA does occur in somatic cells of the *rrf-1* null mutant worm. Furthermore, injection of a preformed synthetic siRNA into the *rrf-1* null mutant does not lead to significant RNAi effects. These new observations establish a two-step mechanism of RNAi, previously predicted from analysis of SDE-1 (RRF-1 homolog of plants) null mutant phenotypes of *Arabidopsis* (Dalmay et al., 2000). The initial step in gene silencing by RNAi, processing of the trigger dsRNA into siRNAs through the action of DICER and other factors ("initiation"), is separable from the following amplification step involving RdRP ("maintenance"), but both steps are required for RNAi.

A New RNAi Model: Amplification by Degradative PCR?

The model for RNAi depicted in Figure 1 focuses on the processes involving RdRP described in this review (Lipardi et al., 2001; Sijen et al., 2001 [this issue of *Cell*]). A separate pathway leading to degradation of the target mRNA through an endonuclease(s) (DICER or another RNase) guided by the antisense strand of siRNA is not shown in this model for the sake of simplicity, but of course that degradation pathway must exist. The degradation steps have been extensively studied, and certain characteristics, such as specificity of the cleavage site of the target mRNA relative to the siRNA sequence, have been revealed (Zamore et al., 2000; Elbashir et al., 2001). The most recent "siRNA guide model" for the target mRNA degradation pathway is found in the studies by Nykänen et al. (2001).

Trigger dsRNA introduced into cells is first cleaved into siRNA by the RNase III-like endonuclease DICER (Bernstein et al., 2001). This cleavage reaction and the production of siRNAs require at least ATP (Zamore et al., 2000) and RDE-4, a protein containing two dsRNA binding domains (Sijen et al., 2001). These intermediates of RNAi, siRNAs, are double stranded (Lipardi et al., 2001; Nykänen et al., 2001), and a 2 nt 3′-overhang is present in each sense and antisense strand of siRNA, most likely due to the cleavage characteristics of DICER (Figure 1A). The 5′ phosphate group of siRNA is maintained by a specific kinase (Nykänen et al., 2001), whereas the free 3′ hydroxyl group is essential for priming of the subsequent RdRP reaction (Lipardi et al., 2001).

After reaching some threshold concentration, antisense strands of siRNA (or its complex with other factors, see below) hybridize to the target mRNA and prime the RdRP reaction. The newly synthesized dsRNA created by RdRP is then subjected to the next cycle of DICER digestion and production, thus further amplification, of siRNAs (Figure 1B). A fraction of newly synthesized dsRNA might be unwound by an RNA helicase activity or by RdRP itself. In this scenario, both sense and antisense strands of the newly synthesized dsRNA now can be primed by antisense and sense strands of siRNA, followed by the next RdRP cycle (Figure 1C). This would initiate an exponential amplification of not only siRNAs but also template RNAs, which may explain the extremely potent and efficient nature of RNAi, aptly described as "degradative PCR" (Lipardi et al., 2001).

There is no information available on the complex containing RdRP nor on the process by which siRNA recognizes and hybridizes to the target mRNA. RISC (RNA-induced silencing complex) is a multicomponent-nuclease containing siRNA, AGO-2/RDE-1 (genetically implicated in RNAi and related to eIF2C), and presumably additional factors that degrade the target mRNA (Bernstein et al., 2001). Interestingly, a complex different from RISC in which the two strands of the siRNA duplex are unwound has been detected (Nykänen et al., 2001). It remains to be established whether any of these complexes also contain the RdRP activity. Alternatively, a separate complex specific to RdRP may exist.

Pitfalls for Reverse Geneticists

In the last several years, RNAi has become the technique of choice for creating null mutant phenotypes of a specific gene, and has even been utilized for large-scale functional genomics of *C. elegans*. One previously unforeseen limitation of this popular tool of research has now become clear. The elongation of the dsRNA up-

stream of the original trigger dsRNA results in generation of secondary siRNAs derived from this newly extended region of dsRNA and transitive RNAi triggered by subsequent RdRP reactions primed with the secondary siRNAs (Figure 2). This may lead to creation of phenotypes due to suppression of a different gene that happens to have a region of sequence highly homologous to the region attacked by transitive RNAi. One may choose a target region unique to one member of a gene family, aiming to create a phenotype specific to the selected gene, but end up inadvertently knocking out a family of many genes because of a highly conserved domain present upstream of the trigger dsRNA (Figure 2A). RNAi of an alternatively spliced mRNA by designing the trigger dsRNA specific to its unique exon sequence may also lead to silencing of other mRNAs of the same gene, again due to transitive RNAi effects (Figure 2B). Obviously, design of trigger dsRNA now needs to be done carefully to take into account the possibility of transitive RNAi.

Prospects and Problems

Although an RdRP-like activity has now been clearly demonstrated in the *Drosophila* embryo extract (Lipardi et al., 2001), an RdRP homolog has not yet been identified in the *Drosophila* or human genome, suggesting the presence of a separate enzyme capable of primer-dependent replication of an RNA template. As pointed out by Sijen et al. (2001 [this issue of *Cell*]), RNA-dependent replication of hepatitis delta virus anti-genome RNA strand by an unknown cellular RNA polymerase has been reported in human hepatoma cells (Modahl et al., 2000). Curiously, the RdRP activity in hepatoma cells is resistant to α-amanitin at high concentration, as is the RdRP activity found in the *Drosophila* embryo extract (Lipardi et al., 2001).

A number of issues remain unclear. The combination of transitivity, amplification, and the rather small minimum length of perfect homology needed for RNAi would seem to put a significant fraction of the genome at risk in every RNAi experiment. What keeps this process from spreading out of control? Are we getting a hint of the presence of a control system from the much smaller extent of transitivity observed in vivo (Sijen et al., 2001) than extent of secondary siRNA generation in vitro (Lipardi et al., 2001)? To understand the complete phenomenon of gene silencing by RNA interference, we must have answers to these and many other questions that cannot be dealt with in this short review. What are the processes that permit maintenance of gene silencing through successive cell divisions, propagation from one cell to another, and inheritance by succeeding generations? What components of the RNAi mechanism overlap with normal controls of gene expression, particularly in epigenetic controls such as imprinting and X chromosome inactivation? The answers to these questions may be uncertain, but there is no doubt that this quest will continue to generate surprises and excitement for all biologists.

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