

# Double-Stranded RNA as a Template for Gene Silencing

## Minireview

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When double-stranded RNA (dsRNA) corresponding to a sense and antisense sequence of an endogenous mRNA is introduced into a cell, in organisms ranging from trypanosomes to mice, the cognate mRNA is degraded and the gene is silenced (reviewed in Fire, 1999; Boshier and Labouesse, 2000). This type of posttranscriptional gene silencing (PTGS) was first discovered in *C. elegans* (Fire et al., 1998) and is called RNA interference, or RNAi. RNAi shows many similarities to the PTGS that is sometimes observed when a transgene is introduced into a cell, and the processes seem to require some of the same gene products (Catalanotto et al., 2000; Ketting and Plasterk, 2000). If transgene-induced silencing of an endogenous gene, or cosuppression, also involves dsRNA, somehow the cell must make both sense and antisense copies of the transgene sequence.

PTGS has captured the interest (and imagination) of geneticists and molecular biologists alike, and now the first clues about its mechanism will certainly bring the biochemists into the fold. As is often the case for biological processes, the first hint about the mechanism comes from the identification of molecules that appear to be reaction intermediates. In particular, several recent papers report the identification of small RNA molecules, 21–25 nucleotides in length (21- to 25-mers), that correspond to sense and antisense pieces of the dsRNA or transgene introduced into the cell.

### *Evidence that Small RNAs Are Required for Certain Types of PTGS*

Consistent with the idea that the sense and antisense 21- to 25-mers are important for transgene-induced PTGS, they are observed in plants containing transgenes that induce silencing but are notably absent from plants whose transgenes are expressed normally (Hamilton and Baulcombe, 1999). A correlation with dsRNA-induced silencing is provided by the recent report of a nuclease activity isolated from cultured *Drosophila* S2 cells that had been transfected with dsRNA to initiate RNAi (Hammond et al., 2000). The partially purified nuclease degrades RNA in a manner consistent with the degradation known to occur during RNAi—it is sequence specific and will only degrade RNAs matching one of the strands of the dsRNA used to transfect the S2 cells. The nuclease activity was partially purified from cells that had been transfected with dsRNA, but dsRNA was not added to in vitro assays of the partially purified nuclease. So, how did the nuclease know which mRNA to degrade? Sure enough, the small 21- to 25-mers copurify with the nuclease, suggesting that these pieces

somehow template degradation of the mRNA. Further, if the S2 extracts are treated with micrococcal nuclease prior to adding the mRNA, RNAi is not observed. Although micrococcal nuclease will degrade both DNA and RNA, treatment of the extract with DNase did not abrogate RNAi, suggesting it was the loss of the small RNAs that led to the loss of RNAi. (Carrier tRNA did not relieve the inhibition, suggesting micrococcal nuclease was not merely competing for nucleic acid binding.)

Further pieces of the puzzle are provided by beautiful work from a collaborative effort of Phil Zamore, Tom Tuschl, Phil Sharp, and David Bartel, published in the March 31, 2000, issue of *Cell* (Zamore et al., 2000). It was these authors who first reported RNAi could work in vitro (Tuschl et al., 1999), and this more recent study continues the characterization of their system, a cell-free extract made from syncytial blastoderm *Drosophila* embryos. In contrast to the S2 extracts, these extracts are prepared from cells that have not been previously treated with dsRNA. Rather, RNAi is performed from start to finish in a cell-free system, allowing the authors to radioactively label the dsRNA and mRNA and monitor the fate of both molecules.

In the presence or absence of the targeted mRNA, the authors find that a portion of the dsRNA is cleaved to the small pieces, and here the length of the molecules is mapped to 21–23 nucleotides. Radioactive 21- to 23-mers are observed when either the sense or antisense strand of the dsRNA is radiolabeled, verifying that both strands are cleaved, and implicating a dsRNA nuclease in the process.

The most exciting observations are made when the authors monitor the fate of the mRNA in the presence or absence of cognate dsRNA. The mRNA is degraded only in the presence of the dsRNA and only within the sequences spanned by the dsRNA. Remarkably, cleavage sites within the mRNA occur at specific sites, spaced 21–23 nucleotides apart, again suggesting cleavage was somehow templated by the small pieces of the dsRNA. After electrophoresis on a sequencing gel, a 5' end-labeled mRNA appears as a ladder of bands at 21–23 nucleotide intervals, suggesting that each mRNA in the population is cleaved only once or twice. (Since the mRNA is radiolabeled only at its 5' end, cleavage of each mRNA at every 21–23 nucleotide interval would result in an autoradiogram showing only the most 5' 21–23 nucleotide piece.) The cleavage of the mRNA is unaffected by several translation inhibitors but is ATP dependent.

### *A Model for mRNA Degradation by RNAi*

Figure 1 presents a model for how mRNA is degraded during RNAi. The model is based on the recent observations discussed above and shows how small pieces of dsRNA could direct cleavage of mRNA in a sequence-specific and catalytic manner. As shown, when dsRNA is introduced into a cell it would be targeted by a dsRNA endonuclease to generate short dsRNA pieces, ~23 nucleotides long (Figure 1A: sense strand, blue; antisense, red). Since the short RNAs copurify with the nuclease of S2 cells (Hammond et al., 2000) and are proposed to

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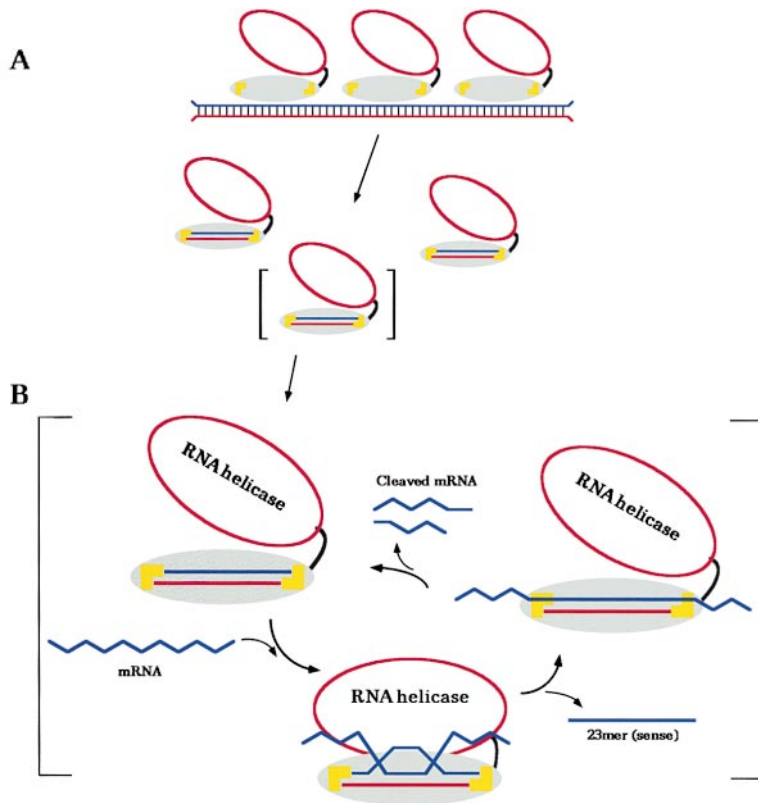


Figure 1. A Model for the Mechanism of mRNA Degradation by RNAi

The reaction is catalyzed by a hypothetical enzyme (RNAi nuclease) that contains a dsRNA binding domain (gray oval), one or more ribonuclease domains (yellow), and an RNA helicase domain (red oval). In the first step (A), the dsRNA that initiates RNAi is bound by the RNAi nuclease and degraded to small dsRNAs that remain stably bound to the RNAi nuclease (blue, sense strands and red, antisense strands). In the second step (B), these small pieces template sequence-specific cleavage of the mRNA. The helicase domain of the protein catalyzes an ATP-dependent strand exchange that replaces the sense strand of the small dsRNA template with the mRNA. The mRNA is then cleaved to regenerate the RNAi nuclease with its small dsRNA. In (A), the enzymes are proposed to coat the dsRNA in a precise register to generate specific fragments that would direct cleavage of the mRNA at specific sites.

serve a templating function, the model depicts the pieces remaining bound to a dsRNA binding domain (gray) of the enzyme. The protein-RNA complex would be in equilibrium with free RNA and protein, but according to the model the complex would be the most stable species and predominate.

In the *in vitro* system of Zamore and Tuschl, the targeted mRNA is cleaved at sites that are also ~23 nucleotides apart, so the model assumes the dsRNA and mRNA are cleaved by the same enzyme. As shown in Figure 1B, in the next step the mRNA (blue zigzag) must exchange with the 23 nucleotide "sense" strand (blue line) of the short dsRNA. During strand exchange, the 23 nucleotide sense strand dissociates from the enzyme and is replaced by the mRNA. The mRNA is positioned just like the original sense strand of the dsRNA and cleaved at the same sites by the ribonuclease active site(s) (yellow). Importantly, cleavage of the mRNA regenerates the nuclease just as it was when the cycle began, bound to the short sense and antisense pieces. Thus, in the model of Figure 1, the nuclease is able to carry out endless rounds of strand exchange and cleavage, perhaps explaining why RNAi appears to act catalytically (see below).

Although strand exchange between a dsRNA and one of its strands will occur slowly without a catalyst, to occur on a biological time scale the reaction probably requires catalysis. Because Zamore et al. (2000) find that cleavage of the mRNA requires ATP, the model shown in Figure 1 invokes an RNA-dependent ATPase, or RNA helicase (reviewed in de la Cruz et al., 1999). For reasons discussed below, Figure 1B shows strand

exchange catalyzed by a helicase domain of the same protein that catalyzes cleavage, but the two activities could exist in separate molecules.

In theory, there are two ways that strand exchange could occur. Strand exchange could occur by a dissociative mechanism, where the dsRNA strands first dissociate completely, making the antisense strand accessible for subsequent hybridization with the mRNA (e.g., see Figure 7, Zamore et al., 2000). Alternatively, strand exchange could occur by an associative mechanism, where the mRNA somehow forms a close association with the base-paired dsRNA and invades the duplex to allow annealing. Figure 1B shows the associative type of strand exchange, since it seems most consistent with the observation that both sense and antisense strands copurify with the nuclease of *Drosophila* S2 cells, as well as the fact that this type of strand exchange appears to be operative *in vitro* (Homann et al., 1996).

#### Does PTGS by dsRNA Involve an RNase III-Like Enzyme?

Although the identity of the RNAi nuclease has not been determined, the characteristics of the short 21–25 nucleotide RNA pieces suggest they were generated by RNase III or a highly related enzyme (see Rotondo et al., 1997; Abou Elela and Ares, 1998, and references therein). RNase III is the only characterized nuclease known to cleave dsRNA at specific sites to generate dsRNA fragments of discrete sizes. For RNase III to stably bind a dsRNA, it must be at least two helical-turns in length, consistent with the observation that RNAi and transgene-induced silencing yield stable fragments of ~22 base pairs. RNase III can produce fragments

<22 base pairs from a longer dsRNA, but these shorter fragments themselves do not bind well to RNase III. In the context of the model of Figure 1, fragments less than 21–23 base pairs would not have been observed in the recent experiments because they would not remain stably bound to the enzyme and thus would be more accessible to degradation by other cellular nucleases.

Given the similarities between the cleavage products of RNase III and the RNAi nuclease, I have incorporated properties of the RNase III enzymes into the model of Figure 1. For example, RNase III makes staggered cuts that leave 3' overhangs of two base pairs, as shown for the 23-mers of Figure 1. If RNAi involves an RNase III-like enzyme, it might explain why the small RNAs observed by Zamore and Tuschl range from 21–23 nucleotides. The initial cleavage might produce dsRNAs comprised of sense and antisense 23-mers, but the 3' overhangs would be more accessible to single-strand-specific nucleases present in the extract, and trimmed to 21 and 22 nucleotide pieces. Zamore and Tuschl observe that cleavage of the dsRNA, unlike mRNA cleavage, does not absolutely require ATP. However, dsRNA cleavage is faster in the presence of ATP, and without ATP the pieces are predominantly the longer 23-mers. Certainly this is a clue to the role of ATP in this *in vitro* reaction, but at present its meaning is unclear.

The bacterial and yeast RNase III enzymes have similar open reading frame (ORF) structures, with a ribonuclease domain followed by a C-terminal motif known as a dsRNA binding motif (dsRBM). Database searches show that sequences encoding this ORF structure are also present in metazoa, and intriguingly, reveal a second group of ORFs with an RNA helicase domain, N-terminal of the ribonuclease domain and dsRBM (as noted in Rotondo et al., 1997, and references therein). Genes encoding ORFs with such a structure exist for many organisms, including *Caenorhabditis* (K12H4.8, L14331), *Drosophila* (CG4792, AE003740), humans (AB028449), and *Arabidopsis* (AF187317). Such proteins would be ideal for catalyzing the reactions shown in Figure 1 and formed the basis for the hypothetical enzyme invoked in the model.

#### *Is RNAi Self-Propagating, and If So Why Doesn't It Last Forever?*

PTGS by dsRNA has a remarkable ability to cross cell boundaries (Fire et al., 1998) and can even be passed to subsequent generations in a process that occurs via a dominant extragenic agent, possibly the small dsRNA molecules described here (Grishok et al., 2000). However, dsRNA-induced gene silencing is not maintained forever, presumably because the extragenic agent is diluted by cell division and degradation. Calculations that take into account the dilution of injected dsRNA by cell division suggest that small amounts of dsRNA can target degradation of many mRNAs (Fire et al., 1998). In the mechanism shown in Figure 1B, the enzyme bound to its small dsRNA template is regenerated with each round of annealing and cleavage, which could explain how RNAi appears to act catalytically.

RNAi may also involve amplification of the dsRNA signal by an RNA-dependent RNA polymerase (RdRP), and genes encoding proteins with sequence similarity to RdRPs are important for PTGS in both *C. elegans* (Smardon et al., 2000) and *Neurospora* (Cogoni and

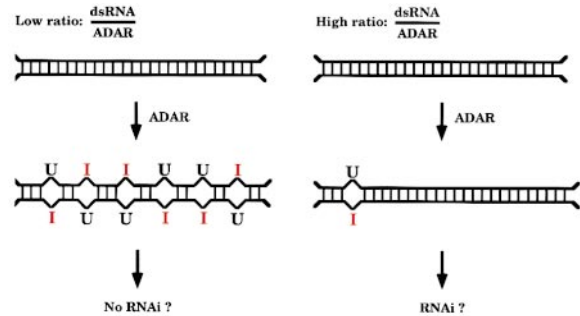


Figure 2. High Levels of ADARs Are Predicted to Antagonize RNAi

Macino, 1999). The model proposed in Figure 1 does not at all preclude amplification by an RdRP, and the involvement of such a polymerase could also help explain how sense and antisense transcripts might derive from a single transgene.

Regardless of whether there are cellular mechanisms that allow amplification of the signal, RNAi works better, *in vivo* and *in vitro*, when more dsRNA is used to initiate the process. Assuming the small 21- to 25-mer dsRNAs are the extragenic agent that propagates PTGS from one cell to the next, and to offspring, this makes sense, at least for *in vivo* experiments. More dsRNA would produce more 21- to 25-mer dsRNA and allow the effect to better survive dilution by cell division.

Another reason why more dsRNA might work better is that putative dsRNA binding proteins (dsRBPs) required for RNAi would have to compete with other cellular dsRBPs for the dsRNA. dsRBPs have little sequence specificity, and although they often bind very tightly to dsRNA, they will bind to any dsRNA; a substrate for one dsRBP is a substrate for all dsRBPs. In fact, Zamore and Tuschl demonstrate that their extracts contain adenosine deaminases that act on RNA (ADARs), dsRBPs that convert adenosines to inosines within dsRNA (see Morse and Bass, 1999, and references therein). Full-length dsRNA molecules that remain after incubation in the extract show 3%–6% of their adenosines deaminated, while only 0.4%–0.7% of the adenosines in the 21- to 23-mers appear as inosine. Although ADARs will deaminate 50%–60% of the adenosines in long, completely base-paired RNA of the type typically used in RNAi experiments, they are very sensitive to substrate inhibition (Hough and Bass, 1994). Given the high concentrations of RNA used by Zamore et al., as well as in most RNAi experiments, the low amounts of deamination are as expected.

Zamore and Tuschl previously showed that a large excess of dsRNA is required for optimal RNAi in their *Drosophila* cell-free system (Tuschl et al., 1999), and possibly this is due to the presence of ADARs in the extract. Since inosines, like guanosines, prefer to base pair with cytidine, ADARs change RNA sequence (A→I), as well as RNA structure (AU base pair→IU mismatch; Figure 2). At low concentrations of dsRNA, ADARs would promiscuously deaminate the dsRNA so it would no longer be homologous to the targeted mRNA; further, because of its increased single-stranded character, it would not be recognized by dsRBPs involved in RNAi.

Assuming RNAi requires the small 21- to 23-mers to bind a dsRBM as shown in Figure 1, it would make sense that few inosines would be present in this population. Although the tissue specificity of ADARs in *C. elegans* is not yet known, if like other organisms the enzyme is highest in neural tissues, this may explain why many worm neuronal genes are refractory to RNAi.

#### Concluding Remarks

The in vitro observations of Zamore et al. (2000) have not yet been directly connected to RNAi in vivo. In fact, given the intrinsic activities of RNase III and helicases, if the dsRNA and mRNA used in the Zamore et al. study were simply mixed with these enzymes, similar cleavage products might be observed. In this light, it could be argued that the reactions occurring in the extract are unrelated to RNAi and merely reflect that *Drosophila* embryos contain RNase III and helicases. However, since similar 21–25 nucleotide RNA pieces have now been observed in multiple systems and shown to strongly correlate with PTGS in plants, these worries seem unjustified. Regardless, future studies, perhaps using extracts made from strains containing mutations known to affect RNAi, will be important to verify the relevance of the in vitro observations.

Existing studies suggest that RNAi represents a complex set of phenomena, and the data discussed here focus on only one of these—the dsRNA-induced degradation of mRNA. As mentioned, genes encoding proteins with sequence similarity to RdRP have been implicated in PTGS; the model of Figure 1 does not preclude the involvement of these proteins. Several other genes have been identified that appear to be required for RNAi (Ketting et al., 1999; Tabara et al., 1999; reviewed in Bosher and Labouesse, 2000). The identity of only a subset of these is known, and so far, none are the RNase III enzymes discussed here. Although the idea that RNase III-type enzymes are involved in RNAi is compelling, the basic mechanism proposed in Figure 1 could be catalyzed by other proteins. In fact, among the genes identified as important for RNAi are those with homologs to other ribonucleases (*C. elegans mut-7*, Ketting et al., 1999) and helicases (*Neurospora qde-3*; see citations in Zamore et al., 2000). It even seems possible that different cells and tissue types carry out RNAi with overlapping but distinct factors, as already suggested by comparisons of PTGS in germline and soma.

The model of Figure 1 predicts that introducing 21- to 25-mer dsRNAs into a cell should trigger gene silencing, but so far all RNAi systems require dsRNAs greater than ~100 base pairs for efficient inhibition of gene expression. (Although possibly the 21- to 23-mer RNAs work in vitro [Zamore et al., 2000]). A given molar amount of a shorter dsRNA would yield fewer 21- to 25-mers, and this may explain their lower efficacy. However, it is also possible that the length requirement derives from another factor involved in RNAi, for example the putative RdRP.

There are still many mysteries about the mechanism of PTGS, but we know enough to consider the tantalizing possibility that dsRNA is an important signaling molecule in this process. While studies of PTGS usually involve introducing an exogenous sequence into a cell, either a transgene or dsRNA, it seems likely that PTGS reflects a natural biological process. Some of the genes

identified as important for RNAi are also important for silencing transposon hopping in the germline (Ketting et al., 1999; Tabara et al., 1999), raising the possibility that dsRNA plays a more general role in gene silencing.

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