

REVIEW

# RNA interference—2001

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In the few years since the discovery of RNA interference (RNAi; Fire et al. 1998), it has become clear that this process is ancient. RNAi, the oldest and most ubiquitous antiviral system, appeared before the divergence of plants and animals. Because aspects of RNAi, known as cosuppression, also control the expression of transposable elements and repetitive sequences (Ketjing et al. 1999; Tabara et al. 1999), the interplay of RNAi and transposon activities have almost certainly shaped the structure of the genome of most organisms. Surprisingly, we are only now beginning to explore the molecular processes responsible for RNAi and to appreciate the breadth of its function in biology. Practical applications of this knowledge have allowed rapid surveys of gene functions (see Fraser et al. 2000 and Gönczy et al. 2000 for RNAi analysis of genes on chromosome I and III of *Caenorhabditis elegans*) and will possibly result in new therapeutic interventions.

Genetic studies have expanded the biology of RNAi to cosuppression, transposon silencing, and the first hints of relationships to regulation of translation and development. The possible roles of RNA-dependent RNA polymerase (RdRp) in RNAi have been expanded. Many experiments indicate that dsRNA directs gene-specific methylation of DNA and, thus, regulation at the stage of transcription in plants. Cosuppression may involve regulation by polycomb complexes at the level of transcription in *C. elegans* and *Drosophila*. This article will review these topics and primarily summarize advances in the study of RNAi over the past year.

## Sequence and strand specificity of RNAi

Restriction of virus growth in plants is mediated by post-transcriptional gene silencing (PTGS), which can be initiated by production of dsRNA replicative intermediates. This silencing of expression is gene specific, and Hamilton and Baulcombe (1999) discovered that tissue manifesting PTGS contained small RNAs (25 nt) complementary to both strands of the gene. Using extracts of *Drosophila* embryos that had been shown previously to be active for RNAi (Kennerdell and Carthew 1998), Tuschl

et al. (1999) were able to reproduce RNAi in a soluble reaction. dsRNA added to this reaction is cleaved into 21–23-nt RNAs, which leads to cleavage of the target mRNA at 21–23-nt intervals (Zamore et al. 2000). Hammond et al. (2000) also concluded that small RNAs directed cleavage of mRNAs in *Drosophila* extracts prepared from Schneider cells. These experiments are best explained by a model for RNAi where dsRNA is processed to 21–23-nt RNAs that direct the cleavage of mRNA through sequence complementarity. These RNAs are referred to as siRNAs, or short interfering RNAs (see below).

Fire and Mello have continued their collaboration studying the functional anatomy of dsRNA for induction of RNAi (Parrish et al. 2000). They first concluded, using short RNAs synthesized chemically and assayed by injection into *C. elegans*, that any dsRNA segment greater than ~26 bp can generate RNAi. Thus, the process for generation of siRNAs is probably sequence nonspecific. This was confirmed by the observation that individual short dsRNA formed from sequences that did not contain adenosine, uridine, or cytidine were active for RNAi. Long dsRNAs were more active than short dsRNAs; a 250-fold higher concentration of 26-bp dsRNA generated the equivalent gene silencing activity as an 81-bp dsRNA. dsRNA from a related but not identical gene can be used to target a gene for silencing if the two share segments of identical and uninterrupted sequences of significant length, probably >30–35 nt in length. Silencing was inefficient when the largest uninterrupted segments were 14 and 23 nt in length but efficient when 41 nt of such sequences of identity were shared. These results suggest that silencing will probably occur if long dsRNAs are used and the two related genes are >90% homologous. Assuming that dsRNA is processed to 21–23-nt segments, these results indicate that single basepair mismatches between the siRNA and target RNA dramatically reduce gene targeting and silencing.

In the *C. elegans* assay used by Mello and Fire, it is likely that the injected dsRNA is directly processed to the targeting siRNAs and that these are not replicated by an endogenous RNA-dependent RNA polymerase. This conclusion rests on the effects of asymmetric modifications of the input dsRNA. Substitution of either 2'-amino uracil for uracil or 2'-amino cytidine for cytidine in the sense strand of the dsRNA had little effect on the

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RNAi activity, while the same substitutions in the antisense strand rendered the RNA inactive. If the input dsRNA were replicated before targeting, it would be expected to lose this asymmetry. As the above assays were done in somatic tissue of *C. elegans*, it is possible that the long-term RNAi observed through multiple generations (Grishok et al. 2000) could involve replication in the germ-line tissues. Mutations in a *C. elegans* gene with sequence relationship to RdRp, EGO-1, have been reported to affect some aspects of RNAi (Smardon et al. 2000).

### Genesis of RNAi

The structure of siRNAs is probably the same in all organisms, as the 21–23-nt length of siRNAs seems to be universal. Furthermore, siRNAs might be the best candidates for use in targeted gene silencing because their structure would match the biochemical components of the RNAi system. The complex generating the siRNAs from short dsRNAs primarily recognizes the 3' termini of the duplex (Elbashir et al. 2001). Internal cleavage of the dsRNA occurs at a distance of ~22 nt, and a complex of siRNA and proteins targets cleavage of the complementary target RNA at a position ~10–12 nt from the terminus of the original dsRNA (see top panel of Fig. 1). The siRNA duplex probably remains associated with the initial complex because it asymmetrically targets a strand for cleavage and not its partner (the sense strand in the example illustrated in Fig. 1). This asymmetry was not observed when symmetric siRNAs with 2-nt tails on both strands were added to the reaction. Both strands of the target RNAs were cleaved within the region covered by the siRNA duplex, indicating that the siRNA duplex can bind to the complex responsible for cleavage in either orientation (see bottom panel of Fig. 1). In general, a

siRNA duplex with 2-nt 3' tails is thought to be the primary intermediate of RNAi. In fact, addition of RNAs with this structure to reactions in vitro can silence translation of a target mRNA with a similar efficiency (within 10-fold) on a molar basis to dsRNAs of >50 bp. Addition of either one of the two single strands constituting a siRNA duplex generates no activity.

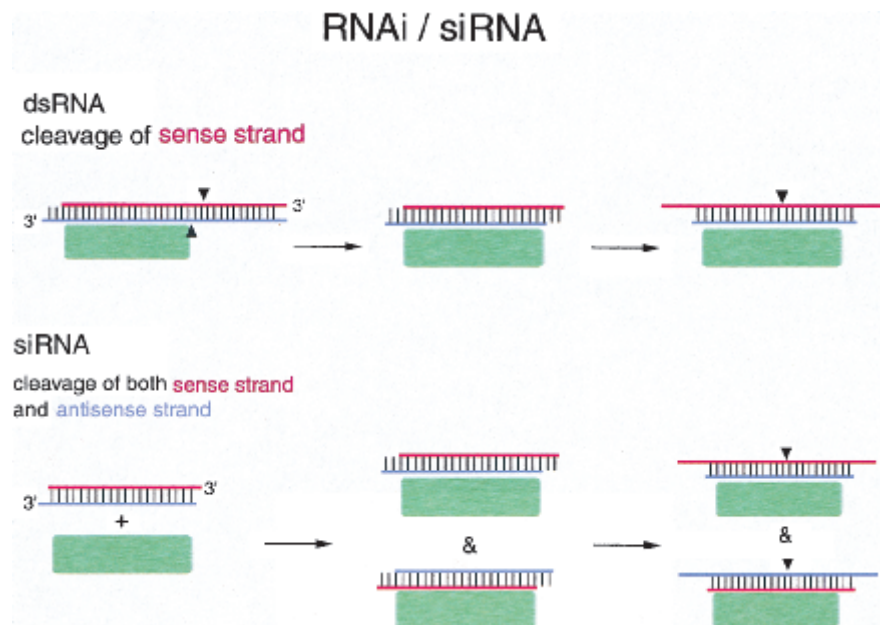
Tuschl's lab developed methods for cloning of siRNAs using T4 RNA ligase to add linker segments to their 5' and 3' termini (Elbashir et al. 2001). The predominant structure is a 19–20-bp duplex RNA with both termini possessing 2-nt 3' single-strand segments, and the total length of each strand is predominantly 21–22 nt. RNase III-type endonucleases cleave dsRNA releasing RNA with 2-nt 3' tails, indicating that this type of activity is probably involved in generating siRNAs (a possibility first suggested by Bass [2000]). Although the results were not described in the paper, Elbashir et al. (2001) reported the cloning of siRNAs that were endogenous to the *Drosophila* extract. This foretells future studies where analysis of the sequence of siRNAs in cells will indicate which genes are naturally silenced by RNAi.

How are the siRNAs related to the site of cleavage on the target mRNA? As shown in Figure 1, the siRNAs direct cleavage of the target RNA in the middle of the paired segments, ~12 bp from the 3' terminus of the siRNA. This positions the site of cleavage of the target RNA about one turn of an A-type duplex helix from the cleavages that generated the siRNAs. This could indicate a rearrangement of the RNase III-type domains contacting the siRNA duplex before the second cleavage.

### Genetic analysis of RNAi

Several groups are actively pursuing the identification and characterization of enzymes implicated in RNAi and

**Figure 1.** Comparison of the cleavage patterns on sense and antisense target strands when either a short double strand RNA (*top*) or a siRNA (*bottom*) are added to a reaction in vitro. The dsRNA generates a siRNA complex which only cleaves the sense strand. Processing of siRNAs from the opposite end of the dsRNA would only cleave the antisense strand (not shown). Addition of a siRNA with the same structure as that processed from the dsRNA generates cleavage of both the sense and antisense strands, suggesting that the siRNA can bind the complex in either orientation.



cosuppression. In *C. elegans*, initial mutant screens have generated ~80 candidates, of which five have been specifically identified: RDE-1, RDE-2, RDE-3, RDE-4, and Mut-7 (Ketting et al. 1999; Tabara et al. 1999; Ketting and Plasterk 2000; Grishok et al. 2000). Selection of mutations in cosuppression in *Arabidopsis* have identified homologs of the same genes (Dalmay et al. 2000; Fagard et al. 2000; Mourrain et al. 2000). Testing of previously identified mutations for defects in RNAi in *C. elegans* and other organisms has expanded this list.

## Enzymes of RNAi

### *RNase III proteins and RNAi*

What type of RNase III-like activity might be active in RNAi? Bacterial RNase III and its homologs in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* function in processing of rRNA and other structural RNAs (Chanfreau et al. 2000). There are two general families of RNase III homologs in plants and animals. One family is represented by the drosha *Drosophila* gene, which is composed of two RNase III domains and one dsRNA binding domain (Filippov et al. 2000). Antisense experiments suggest that a ubiquitously expressed human family member closely related to drosha is important for rRNA processing (Wu et al. 2000). The second family of RNase III proteins contains an N-terminal ATP-dependent helicase-type domain as well as two RNase III-type domains and a dsRNA motif (Filippov et al. 2000). Perhaps these represent the best candidates for the RNase III activity in RNAi (Elbashir et al. 2001). Recent results from Bernstein et al. (2001) describe the cleavage of dsRNA into 22-nt segments by a *Drosophila* protein of the RNase III type. Furthermore, RNA interference was used to indicate that this protein is important for RNAi activity. Mutations in an *Arabidopsis* gene in this family result in unregulated cell division in floral meristems (Jacobsen et al. 1999). This would be consistent with a relationship between RNAi and development. Interestingly, the presence of two RNase III domains in this family of proteins suggests that it might cleave dsRNA as a monomer. The dsRNA-binding domain could position the enzyme on the substrate, and the two catalytic domains could hydrolyze bonds in both strands.

### *RNA-dependent RNA polymerase*

Mutations in genes encoding a protein related to RNA-dependent RNA polymerase (RdRp) affect RNAi-type processes in *Neurospora* (QDE-1), *C. elegans* (EGO-1), and plants (SGS2, Mourrain et al. 2000; and SDE-1, Dalmay et al. 2000). It has been generally assumed that this type of polymerase would replicate siRNAs as epigenetic agents permitting their spread throughout plants and between generations in *C. elegans*. This may still be the case; however, results from *Arabidopsis* indicate that SDE-1 is important for gene silencing mediated by the presence of transgenes but not for posttranscriptional gene silencing (PTGS), induced by a replicating RNA vi-

rus (Dalmay et al. 2000). The efficient generation of siRNAs from transgenes was dependent upon SDE-1, whereas siRNAs were generated in SDE-1 mutant plants by viral replication, which generates dsRNA. The authors conjecture that aberrant RNAs from the transgenes are recognized by the RNA-dependent RNA polymerase, SDE-1, generating dsRNA that is processed to siRNAs.

### *RNA-dependent RNA helicase*

Another type of RNA helicase of the DEAH-box helicase super family has also recently been shown to be important for RNAi or PTGS in *Chlamydomonas reinhardtii* (Wu-Scharf et al. 2000). Mutations in this gene, Mut-6, relieve silencing by a transgene and also activate transposons. Helicases of the same family are important for RNA splicing in yeast; however, Mut-6 is not thought to be involved in RNA splicing. A closely related yeast gene that is involved in RNA splicing, PRP16, has been shown to have ATP-dependent RNA helicase activity (Wang et al. 1998). Perhaps Mut-6 unwinds duplex RNA in some step of RNAi.

## Processes related to RNAi

### *Nonsense-mediated decay of mRNA*

A link between RNAi and nonsense-mediated decay was revealed by screening of mutants in the latter process (Domeier et al. 2000). mRNAs containing nonsense mutations upstream of an intron are rapidly degraded in organisms as diverse as worms and vertebrates. Seven genes, SMG 1–7, are important for this process in *C. elegans* (Page et al. 1999). Surprisingly, mutants of *C. elegans* with lesions in either smg-2, smg-5, or smg-6 failed to efficiently maintain RNAi over the course of 4 d following injection of dsRNA. Both mutant and wild-type animals showed equivalent levels of RNAi on the first day, and this level was essentially unchanged in the wild-type animals over the same 4-d interval. Smg-1, and probably smg-3 and smg-4, are not important for maintenance of RNAi over the 4-d interval. Smg-2, based on homology, is thought to encode an ATPase with RNA binding and helicase activity (Page et al. 1999). Its specific role in nonsense-mediated decay of mRNA is unknown.

### *Regulation of translation during development*

RDE-1, which is important for RNAi in *C. elegans*, is a member of a family of 23 related genes in this organism (Tabara et al. 1999). There are four family members in *Drosophila* and several in humans. In *Drosophila*, two of the most closely related genes have unknown functions, whereas the other two, *piwi* and *aubergine* (*aub*) function in oogenesis (Wilson et al. 1996; Cox et al. 1998). Specifically, *aub* is required for translation of two mRNAs, *oskar* and *gurken*. *Arabidopsis* encodes eight genes related to RDE-1. Mutations in two of these genes, Argonaute 1 (AGO1) and ZWILLE/PINHEAD (ZLL/PNH), result in defects in development. Mutations in the two genes have distinct phenotypes although they are ex-

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pressed in many of the same tissues. A relationship between RNAi and development is suggested by the observation that mutants of AGO1 are also defective for cosuppression (Fagard et al. 2000). These results strongly suggest that multiple RDE-1 family members are likely to be involved in RNAi, perhaps in different tissues and in a redundant fashion. They also suggest that RNAi will share some processes in common with regulation of development.

Interestingly, the *C. elegans* small RNAs *lin-4* and *let-7*, which are 22 and 21 nt long, respectively, are known to regulate translation during development in *C. elegans*. These RNAs are possibly processed from dsRNA regions of a precursor RNA and are thought to pair with the 3' UTR of their targets in regulation of translation. The *let-7* RNA is conserved between *C. elegans*, *Drosophila*, and humans (Pasquinelli et al. 2000). The similarity in lengths of siRNAs and *lin-4* and *let-7* suggests that these systems might share components.

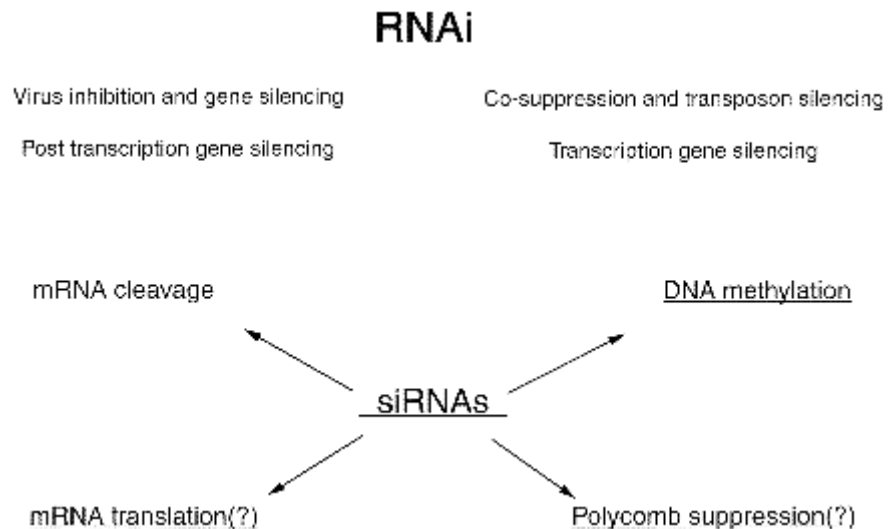
### Regulation of transcription

Three gene-silencing phenomena, cosuppression, transposon silencing, and DNA methylation, are related to RNAi by dependence on a common set of genes. For example, in *C. elegans*, both transposon silencing and cosuppression depend on RDE-2, RDE-3, and Mut-7, which are critical for RNAi (Ketting et al. 1999; Tabara et al. 1999; and Ketting and Plasterk 2000). Cosuppression is generally defined as suppression of an endogenous locus following introduction of homologous transgenes. This trans-suppression requires transcription of the transgenes but is independent of the specific-promoter sequence used to direct transcription (Dernburg et al. 2000). Loss of a transgene array from the germ line of *C. elegans* by deletion results in reactivation of the endogenous locus after a few generations. Thus, the endogenous locus is not mutated during silencing by cosuppression as it is during a related phenomenon, called

quelling, in *Neurospora*. There is no evidence for pairing of the transgenic array and the endogenous locus during cosuppression in *C. elegans* (Dernburg et al. 2000). Thus, the silencing of the endogenous locus is probably mediated by a trans-acting factor that is sequence specific and dependent on transcription. This, and its dependence upon the RNAi related genes RDE-2, RDE-3, and Mut-7, strongly indicates that cosuppression is mediated by trans-acting RNA, probably siRNAs (see Fig. 2).

**Cosuppression and the polycomb complex** The silencing of tandem arrays in *C. elegans* is dependent on the set of *mes* genes (*maternal-effect sterile*; Holdeman et al. 1998; Kelly and Fire 1998; Korf et al. 1998). Two of these genes are homologs of enhancer of zeste and extra sex-combs in *Drosophila* and are in the polycomb group of genes. In *Drosophila*, endogenous loci silenced by cosuppression are bound by a polycomb complex (Pal-Bhadra et al. 1997, 1999), indicating that this process directs the gene-specific binding of this epigenetic regulatory machine. Polycomb complexes are thought to silence genes at the stage of transcription by forming inactive chromatin. Once associated with a gene, the polycomb complex and the transcriptionally suppressed state are stable through DNA replication and cell division. This suggests a model where siRNAs target specific genomic DNA sequences, probably by base pairing, thus directing the binding of the polycomb complex to adjacent sites, resulting in silencing of the locus. This attractive but speculative model awaits direct evidence that dsRNA or siRNAs can silence endogenous genes at the stage of transcription with concomitant association of polycomb complexes.

**Double-strand RNA-directed methylation of DNA** Double-strand RNA-initiated gene-specific methylation of endogenous loci is a well-established phenomenon in plants. An early observation of the specific methylation of chromosomal DNA dependent on RNA replication in plants was described in Wassenegger et al. (1994). This work has been extended to demonstrate that genomic



**Figure 2.** Proposal that siRNAs might be a regulatory intermediate in mRNA cleavage, mRNA translation, DNA methylation, and suppression of transcription by the polycomb group. See text for discussion of evidence for these potential relationships.



sequences as short as 30 bp can be specifically methylated when present in cells with replicating viral RNA containing homologous sequences (Pélissier and Wessnegger 2000). Replicating recombinant viral RNA vectors containing different segments of an expressed gene have been used to demonstrate homology-based RNA-directed methylation (Jones et al. 1999; Merrett et al. 2000). Methylation was directed to different portions of either the body of the gene or to the promoter when the corresponding segment was part of the replicating RNA. This would be consistent with conversion of the dsRNA of the replicating intermediate into siRNAs and targeting of methylation by these short RNAs (Merrett et al. 2000). Interestingly, a viral protein (Hc-Pro) that suppresses PTGS (RNAi) when introduced into cells inhibited the maintenance of siRNAs, and a concomitant decrease in methylation of the corresponding specific genome sequence was observed (Llave et al. 2000).

DNA methylation results in suppression of transcription probably by recruitment of histone deacetylases. The modified and silenced state is epigenetically transmitted, reducing expression of the gene in daughter cells. This is strikingly similar to the conjectured role of polycomb proteins in cosuppression in *C. elegans* and *Drosophila*. At present, there is no known relationship between polycomb suppression of gene expression and subsequent DNA methylation, but the possibility does not seem unreasonable. The analysis to date of cosuppression, RNAi, and PTGS strongly indicates that RNAs can specify regulation of transcription of genomic sequences. These processes probably account for suppression of expression of repetitive sequences in genomes, such as transposons and retroelements. RNAi/cosuppression has been demonstrated to be active in germ-line tissue and should be considered a ubiquitous process shaping the sequence content and structure of the genome of eukaryotic organisms.

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