

THE GENETICS OF RNA SILENCING

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■ **Abstract** Although initially recognized as a handy tool to reduce gene expression, RNA silencing, triggered by double-stranded RNA molecules, is now recognized as a mechanism for cellular protection and cleansing: It defends the genome against molecular parasites such as viruses and transposons, while removing abundant but aberrant nonfunctional messenger RNAs. The underlying mechanisms in distinct gene silencing phenomena in different genetic systems, such as cosuppression in plants and RNAi in animals, are very similar. There are common RNA intermediates, and similar genes are required in RNA silencing pathways in protozoa, plants, fungi, and animals, thus indicating an ancient pathway. This chapter gives an overview of both biochemical and genetic approaches leading to the current understanding of the molecular mechanism of RNA silencing and its probable biological function.

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INTRODUCTION

Posttranscriptional Gene Silencing in Plants

In an attempt to deepen the purple color of petunia plants, the groups of Jorgensen and Mol introduced extra copies of the pigment-producing genes: dihydroflavonol-4-reductase or chalcone synthase. Rather than increasing flower pigmentation as expected, the opposite result was observed in many plants: variegated or even completely white flowers (76, 112). These observations [and (68, 101)], the first manifestation of RNA silencing, were originally termed cosuppression because of the apparent communication between unlinked but homologous loci: RNAs derived from both the transgenes and the homologous endogenous genes were degraded, resulting in the loss of pigmentation phenotype. Cosuppression has subsequently been found to occur in many species of plants, fungi, and animals (see below). The silencing does not depend on the presence of endogenously encoded homologous loci; silencing can occur between two related transgenes, and it is not only triggered by transgenes but can also be initiated by viruses (8), known as VIGS, for virus induced gene silencing. Cosuppression can operate at the transcriptional level (TGS for transcriptional gene silencing), in which it appears to involve alterations at the DNA level, e.g., DNA methylation (114), and at the posttranscriptional level: Nuclear run-on experiments demonstrate that the homologous transcripts are produced, but are rapidly degraded in the cytoplasm (28, 111). This process does not affect transcription of the endogenous locus, hence the name posttranscriptional gene silencing (PTGS). In this chapter we focus on the posttranscriptional part of RNA silencing; for studies on TGS, the reader is directed to recent reviews (69,116) and references therein.

Over the past decade much research has been done to unravel the PTGS phenomenon, and at least two observations suggest that RNA molecules constitute the sequence-specific trigger of PTGS: (a) Transcriptionally active genes are better inducers of PTGS than transcriptionally inactive genes (88, 117), and (b) RNA viruses can induce silencing of homologous genes encoded by the host plant (8, 56, 93). Numerous models have been proposed, often invoking a causal presence of single-stranded RNAs that result from aberrant processing of the transgenes, hence the name aberrant RNAs (10). Such RNAs, but with antisense polarity, were hypothesized to account for the sequence-specificity and posttranscriptional nature of PTGS by the ability to pair with the target mRNA, leading to its subsequent destruction. Such antisense species, however, were never detected by conventional RNA analyses. In perhaps one of the biggest breakthroughs in PTGS (and RNA silencing in general), Hamilton & Baulcombe discovered an unexpectedly short RNA species of around 25 nt, corresponding to both the sense and antisense

sequence of the targeted mRNAs, that had a strict correlation with the occurrence of PTGS (44). These species, later designated siRNAs, were found in a broad spectrum of plant systems undergoing PTGS, including transgene and virus-induced silencing, but were not detected in the absence of PTGS. The accumulation of both sense and the antisense siRNAs suggests that double-stranded RNA (dsRNA) is produced prior to formation of such species. The importance of dsRNA in triggering PTGS was also proposed by Waterhouse et al. who demonstrated that constructs producing RNAs capable of duplex formation, either via hairpin/panhandle constructs or by crossing sense- and antisense-producing clones, are more potent inducers of gene silencing than constructs that produce RNAs of only sense or antisense polarity (123). Invoking a dsRNA species in PTGS (see Figure 1) can now explain why silencing is observed more frequently where the introduced transgenic copies are arranged as inverted repeats, compared to transgenes in a direct repeat orientation [(e.g., 88, 102, 103)].

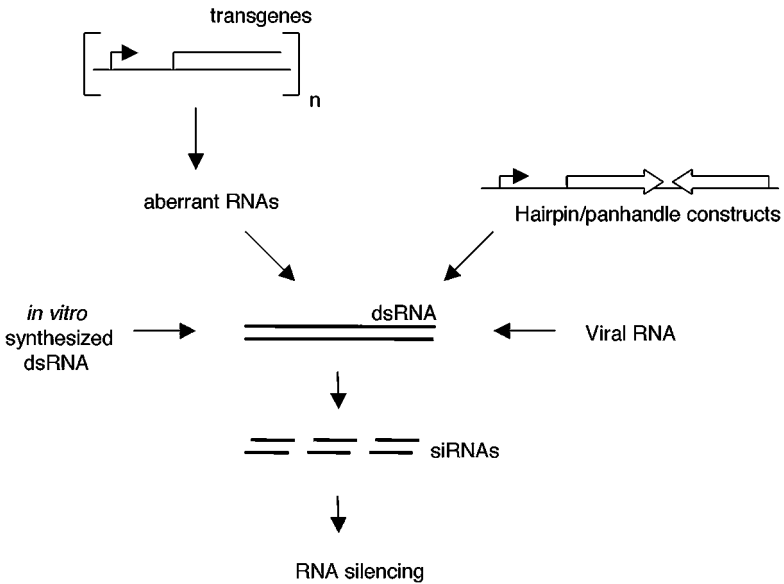


Figure 1 RNA silencing directed by foreign dsRNA. In this model, all triggers that induce RNA silencing operate through a dsRNA intermediate giving rise to the formation of siRNAs. While inversely orientated transgenes, *in vitro* prepared dsRNA, and viruses are direct sources of dsRNA, an additional step is needed to explain the production of dsRNA from multicopy transgenic arrays or highly transcribed single-copy transgenes. This might occur via read-through transcription, either from an endogenous promoter at the site of integration or as a result of head-to-head organization of transcription units in the multicopy array, giving rise to dsRNA directly or to antisense RNA that can pair with the sense to form dsRNA. Alternatively, sense (and possibly antisense) aberrant RNAs are converted into dsRNA via *de novo* RNA synthesis, reminiscent of virus replication.

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RNAi in *Caenorhabditis elegans*

The first direct evidence that dsRNA could lead to RNA silencing came from work in the nematode *C. elegans*. While using an antisense approach to inhibit gene function in *C. elegans*, Guo & Kemphues surprisingly found that molecules with sense polarity were as effective in knocking down target gene expression as molecules with antisense polarity (43). This paradox was resolved by the Fire and Mello labs; they demonstrated that dsRNA is actually the potent inducer of RNA interference (RNAi) (38) and that the outcome of RNAi experiments with single-stranded (antisense) RNAs can largely be explained by modest amounts of dsRNA that contaminate in vitro prepared single-stranded RNA populations. By contrast, pure single-stranded RNA of sense polarity was unable to trigger gene silencing.

Subsequent experiments suggested that RNAi targets a posttranscriptional event in gene expression (38, 71). By increasing the rate of mRNA turnover in a sequence-specific manner, RNAi leads to phenotypes that are either identical to genetic null mutations, or resemble allelic series of mutants. The notion that only a few molecules of dsRNA per cell are sufficient to completely interfere with gene expression suggests a catalytic or amplification component in RNAi (38, 70). dsRNA targets mRNA both in the cytoplasm and in the nucleus of exposed cells, but acts post splicing: Individual mRNAs that are polycistronically transcribed can be targeted separately, and dsRNA directed against promoter sequences and introns are not effective at inducing RNAi. The sequence of the dsRNA is not necessarily identical to the target: dsRNAs that were 88% identical to the target mRNA triggered RNAi, but these triggers still contained significant stretches (of up to 41 nt) that perfectly matched the target mRNA (84). Although dsRNA is required for triggering RNAi, the chemical composition of the antisense strand is more important than the sense strand. Numerous chemical modifications on the dsRNA trigger, either directly on the bases or on the RNA backbone, preferentially block RNAi when carried out on the antisense strand, suggesting that the two strands have different functions in RNAi (84).

Analogous to PTGS in plants, RNAi in *C. elegans* is associated with the formation of small RNAs of 20–25 nt (siRNAs). The formation of siRNAs from dsRNA precursors was demonstrated by injecting radio-labeled dsRNA into the syncytial gonad of the nematode, thus using the reproductive organ as an in vivo test tube (83, 84). Furthermore, RNase protection assays on RNA isolated from animals fed on bacteria that generate dsRNA homologous to a specific *C. elegans* gene also revealed small RNA molecules of 20–25 nt in length (98).

RNA SILENCING EFFECTOR MOLECULES: siRNAs

Important insight into the generation of siRNAs and their function in RNA silencing came from the biochemical dissection of RNAi when in vitro systems that recapitulate the features of RNAi observed in vivo were developed. In a cell-free system derived from *Drosophila* embryo, dsRNA targets a corresponding mRNA

for degradation during which time RNA species of 21–23 nt are formed that are processed from the input dsRNA (109, 128). Independently, it was found that *Drosophila* S2 cells supported sequence-specific mRNA degradation upon transfection of homologous dsRNA (45). A sequence-specific nuclease activity was partially purified from these cells, which also contained RNA fragments of approximately 25 nt in length, corresponding to the input dsRNA. This led to the hypothesis that such species, by providing sequence-specificity, guide the nuclease to the substrate mRNA (45). This proposed function was further supported by the observation that in the cell-free system, substrate mRNAs are cleaved in regular intervals (of 21–23 nt) but only in the region that is covered by the dsRNA (128). Taken together, these data led to a simple two-step model to explain RNAi: (a) long dsRNA molecules are diced into 21–23nt siRNAs by a dsRNA-specific nuclease; (b) these siRNAs guide a nuclease-containing protein complex, designated RISC (for RNAi-induced silencing complex), to the substrate through conventional base-pairing interactions of the antisense strand of the siRNA to the mRNA, triggering its subsequent destruction (Figure 2). Although it was noted early on that the RNA component of RISC was essential for its activity (45), formal proof that siRNAs are the intermediates in the RNAi reaction and mediate sequence-specific mRNA degradation was only recently obtained by showing that chemically synthesized RNA duplexes with sizes similar to siRNAs can guide target cleavage in the *Drosophila* in vitro system described above (34, 35).

The discrete size of siRNAs hinted at the involvement of an RNase III or related enzyme because these cleave dsRNA substrates (like hairpin structures) into products with a defined length [e.g., (1)]. In a directed approach, Hannon and coworkers tested *Drosophila* RNase III family members for their ability to generate siRNAs from input dsRNA and found that dsRNA dicing activity could indeed be immunoprecipitated with antibodies raised against one particular member, subsequently designated Dicer (14). To address the involvement of Dicer in RNAi in living cells, these authors performed RNAi on a gene whose function is required for the interference. Transfection of *Drosophila* S2 cells with dsRNA homologous to Dicer significantly reduced the ability of these cells to silence a GFP reporter via RNAi.

Dicer is evolutionarily well conserved with homologs present in fungi (*Neurospora crassa*, *Saccharomyces pombe*), plants (*Arabidopsis*), and animals, including *C. elegans*, *Drosophila*, and mammals. Functional conservation of this family of proteins and their requirement in RNAi came from the demonstration that human Dicer was also found to dice dsRNA into siRNA (14), and that *C. elegans* mutants with a genetic defect in the Dicer ortholog (DCR-1) were resistant to RNAi induced by dsRNA (41, 52, 55). It is thus reasonable to assume that all systems that support dsRNA-induced gene silencing depend on a member of this protein family to convert dsRNA into siRNA intermediates that target the mRNA for destruction.

With the rapid progress of biochemical and genetic approaches, a minimal set of proteins that are sufficient to carry out the RNAi reaction in the test tube should soon be identified (see also below). Some of these proteins are or will

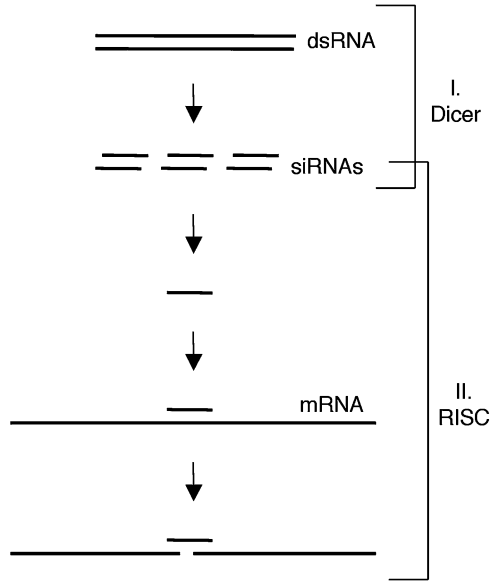


Figure 2 Two-step model to explain RNAi. In the first step, dsRNA is diced by an ATP-dependent ribonuclease (later found to be Dicer) into short interfering RNAs (siRNAs): duplexes of 21–23 nucleotides bearing two-nucleotide 3′ overhanging ends. These siRNAs are subsequently transferred to a second enzyme complex, designated RISC for RNAi-induced silencing complex, which contains an endoribonuclease that is distinct from Dicer. The siRNA guides the endonuclease—the antisense strand of the siRNA is perfectly complementary to the target mRNA—to the target mRNA leading to its destruction. The position of the cleavage site in the target is within the sequences covered by the siRNA (near the center) and is determined by the 5′ end of the guiding molecules (34, 35).

be identified using genetic screens aimed at isolating mutants that are defective in RNA silencing, followed by positional cloning of the mutated loci. Here, we discuss genetic approaches used successfully in several systems (*C. elegans*, *Arabidopsis*, *N. crassa*) that exhibit different manifestations of RNA silencing (RNAi, PTGS, transgene silencing).

GENETIC ANALYSIS OF RNA SILENCING

Quelling in *Neurospora crassa*

RNA silencing, manifested as transgene-induced gene silencing, has also been described in the filamentous fungus *Neurospora crassa*, and has been termed quelling (21, 92). Tandemly inserted transgenic copies containing part of the albino-1 (*al-1*)

gene, essential for the biosynthesis of carotenoids, can invariably silence the endogenous *al-1* locus, leading to an albino phenotype instead of the orange color seen when the *al-1* gene is expressed at wild-type levels. This simple visual reporter system was used to identify components required for silencing, by isolating quelling-deficient (*qde*) mutants followed by the identification of the responsible gene defects (22). All recessive *qde* mutants that were isolated fall into three complementation groups, *qde-1*, *qde-2*, and *qde-3* (loss of transgenic copies also leads to the mutant phenotype). In addition to the release of *al-1* silencing in these mutants, the transgene-encoded selectable marker, which is required for construction purposes, was expressed at elevated levels, suggesting that this transgene was partially silenced in the starting *Neurospora* strain (22).

As in RNAi in animals and PTGS in plants, quelling is associated with the formation of siRNAs derived from the transgenic copies of the silenced locus (19). [Note that the existence of a dominant diffusible factor in quelling was proposed early on, based on the observation that heterokaryons containing a quelled and nonquelled nucleus exhibit a quelled phenotype (21)]. *qde-1* and *qde-3* mutants fail to produce siRNAs, suggesting that these gene products are involved either in the generation of these silencing intermediates or in steps upstream in the silencing (19). *qde-3* encodes a DNA helicase (24) belonging to the RecQ family of DNA helicases that generally function in DNA repair and recombination; this family includes the human genes involved in Bloom's and Werner's syndromes. It has therefore been proposed that QDE-3 operates at the DNA level, perhaps by remodeling the chromatin status of the tandemly organized transgenic copies to allow the production of the silencing RNA signal. RNase protection assays, however, indicate that sense RNA is still abundantly produced from the transgenic copies in a *qde-3* genetic background (22).

qde-1 strains also fail to produce siRNAs (19) as a result of mutations in a gene with sequence homology to a putative RNA-dependent RNA polymerase isolated from tomato (23, 95, 96). This discovery strengthened the idea that RNA silencing induced by transgenes involves the generation of dsRNA using overexpressed or improperly expressed mRNAs (also termed aberrant RNAs) as a template. The notion that *qde-1* encodes a protein that could, in principle, fulfill this function supports such models, and may explain why siRNAs are not detected: The precursor long dsRNAs are not generated. Thus far, long dsRNA molecules have not been detected in *Neurospora*, and biochemical proof is still lacking that the putative RdRPs, required for RNA silencing, catalyze de novo RNA synthesis on an RNA template.

qde-2 encodes a PAZ/PIWI domain containing protein, and belongs to the Argonaute family of proteins (18). Although the function of these evolutionarily well-conserved protein motifs is not known, their key importance in RNA silencing is illustrated by the notion that several members of this family are implicated in RNA silencing in distinct biological systems (see Table 1). Transgene-dependent siRNA species are still produced in *qde-2* mutants, indicating that QDE-2 acts in a downstream step of RNA silencing perhaps in sequence-specific mRNA

TABLE 1 Proteins implicated in RNA silencing

Domain structure	Protein	Organism	Silencing	Mutant phenotype	Putative function
PAZ- and C-terminal PIWI domain	RDE-1	<i>Caenorhabditis elegans</i>	RNAi	RNAi resistant; not required for cosuppression	Initiation of RNAi, downstream of siRNA production;
	QDE-2 AGO1	<i>Neurospora crassa</i> <i>Arabidopsis thaliana</i>	Quelling PTGS	Quelling defective PTGS deficient, developmental defects	Initiation of silencing
	Ago1	<i>Drosophila melanogaster</i>	RNAi	RNAi deficient	Initiation of silencing
	Ago2	<i>Drosophila melanogaster</i>	RNAi	—	RNA silencing downstream of siRNA production
	Aubergine	<i>Drosophila melanogaster</i>	Stellate silencing	Failure to silence Stellate locus; developmental defects	Component of RISC
RNA-dependent RNA polymerase	QDE-1	<i>Neurospora crassa</i>	Quelling	Quelling defective	Translational repressor
	EGO-1	<i>Caenorhabditis elegans</i>	Germline RNAi	RNAi defective for germline genes; germline-development defects	Generation of dsRNA from aberrant RNAs
	RRF-1	<i>Caenorhabditis elegans</i>	Somatic RNAi	RNAi defective for somatic genes	Generation of dsRNA (germline specific)
	RRF-3	<i>Caenorhabditis elegans</i>	RNAi	Increased sensitivity to RNAi	Generation of dsRNA; secondary siRNA production
	SGS2/SDE1	<i>Arabidopsis thaliana</i>	PTGS	PTGS deficient; abnormal leaf development	Dominantly interfering with EGO-1/RRF-1
	RrpA	<i>Dictyostelium discoideum</i>	RNAi	RNAi defective	Generation of dsRNA

RNA helicase-, PAZ-, RNAse III- and dsRNA-binding-domains	Dicer	<i>Drosophila melanogaster</i>	RNAi	—	Dicing long dsRNA into siRNAs; miRNAs production
		<i>Homo sapiens</i>	RNAi	—	Generation of siRNAs and miRNAs
	DCR-1	<i>Caenorhabditis elegans</i>	RNAi	RNAi defective; developmental timing defects; sterile	Dicing long dsRNA into siRNAs; siRNAs and miRNAs production
dsRNA-binding	RDE-4	<i>Caenorhabditis elegans</i>	RNAi	RNAi defective	Initiation of RNAi; generation of siRNAs
Putative RNA-helicase domains (various types)	<i>Mut6</i>	<i>Chlamydomonas reinhardtii</i>		Deficient in transgene silencing; transposon activation; failure to degrade aberrant RNAs	RNA-unwinding
	SDE3	<i>Arabidopsis thaliana</i>		PTGS deficient; VIGS proficient	RNA-unwinding
	SMG-2	<i>Caenorhabditis elegans</i>	RNAi	Failure to sustain RNAi after initiation	RNA-unwinding
	MUT-14	<i>Caenorhabditis elegans</i>	RNAi	RNAi deficient for germline-expressed genes	RNA-unwinding
	Spinde-E	<i>Drosophila melanogaster</i>	Stellate silencing	Failure to silence <i>Stellate</i> locus; developmental defects; activation of retrotransposons	RNA-unwinding
Rnae D domain	MUT-7	<i>Caenorhabditis elegans</i>	RNAi	RNAi deficient for germline-expressed genes; cosuppression defective	RNA-degradation
RecQ DNA helicase	QDE-3	<i>Neurospora crassa</i>	Quelling	Quelling defective	Generation of aberrant RNAs

degradation (19). In support, QDE-2 was found to copurify with siRNAs. This situation resembles RNAi in *Drosophila*, in which the QDE-2 homolog AGO-2 is part of an siRNA containing RNA-directed ribonuclease complex, termed RISC, that targets mRNA destruction (45, 46). RISC in *Drosophila* is a multiprotein complex, including an AGO-2 unrelated nuclease activity. Such a degree of similarity between different systems suggests that QDE-2 is unlikely to be the only protein required for siRNA-induced mRNA degradation in *Neurospora*. Biochemical approaches are needed to reveal their existence, as the genetic screens suggest that only null alleles of *qde-1*, *qde-2*, and *qde-3* are compatible with life.

Genetic Dissection of PTGS in Plants

Several groups performed genetic screens in *Arabidopsis* to identify components of the PTGS mechanism. Vaucheret and colleagues used a 35S-*GUS* (encoding β -glucuronidase) transgenic system to isolate mutants in at least three genetic loci, *sgs1*, *sgs2*, and *sgs3* (*sgs*: suppressor of gene silencing) (36). Nuclear run-on experiments verified that the defect is in PTGS, not in TGS: Transcriptional activity of the transgene was not affected. Importantly, whereas the defect in PTGS was not limited to the exogenous 35S-*GUS* transgene (transgene-induced silencing of endogenous *Nia* genes was also affected), PTGS in these mutants could still be triggered by transgenes designed to produce dsRNA (13, 75). It has therefore been proposed that the role of these proteins in PTGS is to turn RNA triggers, possibly aberrant RNAs that are produced from certain PTGS-inducing transgenes, into dsRNA. This hypothesis is fueled by the notion that the *SGS2* gene encodes a protein that is homologous to the RNA-dependent RNA polymerases (75) previously found to be mutated in *Neurospora* PTGS and *C. elegans* RNAi (see below and Table 1).

The *SGS3*-encoded protein has no significant similarity to other known proteins in plants or other kingdoms (75), nor does the amino acid sequence provide any insight into its possible function because of a lack of known protein motifs other than a coiled-coil domain, which could point to protein-protein interactions. Both *sgs2* and *sgs3* mutant plants show enhanced susceptibility to cucumovirus (CMV), providing proof for the hypothesis that PTGS reflects an antiviral defense mechanism (75, 113, 118). The observation that a plant-encoded RdRP is able to counteract virus infection may indicate that PTGS targets the virus when it has a single-stranded conformation or that ssRNAs produced from the virus induce PTGS.

Another gene required for PTGS was isolated in a similar but more controlled screen in order to isolate mutants that in addition to this defect might also be compromised in certain stages of development (37). This gene encodes AGO1, which was previously identified to control development—mutations in *AGO1* pleiotropically affect general plant architecture (15, 72)—and is homologous to QDE-2 and *RDE-1*, which are required for quelling in *Neurospora* and RNAi in *C. elegans*, respectively.

The putative RdRP-encoding gene *SGS2*, described above, was independently found by Baulcombe and colleagues (26), who used the following experimental approach to screen for mutants in *Arabidopsis* PTGS. Two different GFP-encoding transgenes were used: (a) an initiator of PTGS, a potato virus X:GFP vector-encoding transgene (*35S-PVX:GFP*) that is able to mediate weak PTGS, and (b) a recipient of PTGS: a *35S-GFP*-encoding transgene that, when present alone, is not silenced. When combined in one plant, strong PTGS is observed (25, 26). Isolated mutant alleles (*sde* for silencing defective) were categorized into four complementation groups: *sde1* and *sde2*, which result in the complete loss of PTGS, giving rise to a full green phenotype; *sde3*, which shows a delayed loss of PTGS; and *sde4*, (only one allele in a collection of 64 mutants), which shows transient loss of PTGS only at newly emerging leaves. *SDE1* was cloned and found to encode the identical protein as *SGS2*, described above. The analysis of PTGS-associated siRNAs in these mutant and wild-type transgenic plants revealed some striking clues (26). First, siRNAs detected in the double transgenic wild-type plants could be fully attributed to the target (*35S-GFP*) and not the initiator of PTGS (*35S-PVX:GFP* or replicating *PVX:GFP* RNA), arguing that siRNAs of the targeted transcript are also generated as a consequence of PTGS and not just as a source to trigger PTGS. These data also suggest that siRNAs, or a major fraction in this system, accumulate in a target-dependent fashion. Second, siRNAs were less abundant in *sde1* plants, but in this genetic background, siRNAs were derived from the replicating initiator *PVX:GFP* RNA that was present at elevated levels in *sde1* plants. This suggested that *SDE1* is not required for PTGS induced by a virus. Indeed, when tested directly, GFP expression in *sde1* plants, although resistant to PTGS mediated by a transgene, is still sensitive to silencing triggered by a *Tobacco rattle virus* vector construct (27). This sensitivity was also found for plants defective for *SDE3*, which encodes a putative RNA helicase with sequence similarity to RNA helicase-like proteins conserved in all kingdoms (27).

These observations led to a model in which PTGS is triggered through dsRNA intermediates that are either directly supplied (e.g., by virus infection) or made from a transgene in a process that requires at least *SDE1/SGS2*, *AGO1*, *SGS3*, and *SDE3*, thus placing the action of these proteins upstream of siRNA production (115). This hypothesis was strengthened by the recent finding that *sgs2*, *sgs3*, and *ago1* mutants (isolated because of their resistance to PTGS induced by highly transcribed sense transgenes) are proficient in RNA silencing when triggered by transgene loci producing dsRNA (13, 75).

Transgene and Transposon Silencing in *Chlamydomonas reinhardtii*

In the unicellular green alga *Chlamydomonas reinhardtii*, loss of a DEAH-Box RNA helicase, *Mut6*, not only results in the loss of PTGS (of a transgene) but also leads to enhanced transposition of both the retroelement *TOC1* and the DNA-mediated transposon *Gulliver* (126). The levels of RNAs corresponding to the

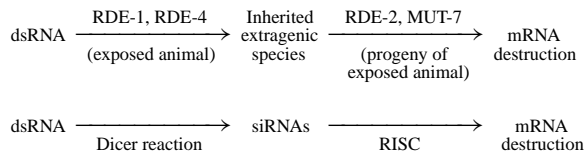
transgene and the retroelement *TOC1* are markedly increased in the *Mut-6* genetic background (in a wild-type genetic background, transgenically derived RNAs are not observed). By inhibiting de novo RNA synthesis, while endogenous mature mRNAs are degraded normally in *Mut-6*, *TOC1* transcripts become stable, suggesting that the degradation of these RNAs depends on the *Mut6* protein. Interestingly, the plasmid insertion into the *Mut-6* locus, causing the defect, results in improperly processed *Mut-6* transcripts with defects in splicing and polyadenylation. These aberrant RNAs disappeared when the *Mut-6* defect was complemented by the introduction of a single copy of the wild-type *Mut-6* allele. This could mean that specifically aberrant RNAs are degraded in a *Mut6*-dependent process that can also silence transgenes, whereas correctly processed mRNAs are left untouched.

Genetic Screens to Identify Components in *C. elegans* RNAi

To identify factors involved in *C. elegans* RNAi, genetic screens were performed that made use of the systemic nature of RNAi, i.e., RNAi can also be triggered by soaking animals in a solution containing dsRNA (104) or by feeding them bacteria that are genetically modified to produce dsRNA homologous to a worm gene (107, 108). Thus, mutagenized animal populations were fed bacteria that produce dsRNA homologous to an essential worm gene, and mutants were selected on their ability to grow on such media. A subset of mutants that were also resistant to injection of dsRNA directly into the animal was further analyzed to determine the molecular nature of the RNAi defect (105). Several complementation groups have been identified this way: *rde-1*, *rde-3*, and *rde-4* are completely RNAi defective, and *rde-2* has a defect in RNAi that appears to be specific for RNAi directed to germline-expressed (or maternally provided) mRNA. Somatically expressed target genes are fully sensitive to dsRNA triggers.

Another genetic screen that yielded RNAi-defective mutants was initially aimed at identifying components of the mechanism that silences DNA transposition in the germline of *C. elegans* (53). Although transposition can readily be detected in somatic tissues of the nematode, the germline is protected from these mutagenic events; worms can be cultured for many years without a single case of transposition. A large fraction of these mutator (*mut*) mutants—loss of silencing leads to a mutator phenotype as a result of frequent gene interruptions by transposon insertions—also have a defect in RNAi (53). Many of these are either allelic to or have similar RNAi characteristics as *rde-2* or *rde-3*, as was also suggested by the reciprocal observation that *rde-2* and *rde-3* lost their defense against DNA transposition. The notion that transposon silencing depends on factors that are also required for dsRNA-induced gene silencing led to the speculation that these DNA elements are tamed via a dsRNA intermediate. This hypothesis, however, is difficult to reconcile with the observation that completely RNAi-deficient *rde-1* and *rde-4* mutant animals (also when the dsRNA is expressed cell autonomously) do not display the mutator phenotype (105).

On the other hand, it is difficult to place the action of the RDE-1 and RDE-4 proteins as central to RNA silencing: Although essential for RNAi, these gene products are not required for transgene-induced cosuppression (29, 54) or RNA silencing via direct administration of short antisense RNAs (106), while for both manifestations of RNA silencing, functionality of the mutator/RNAi type of genes, e.g., *mut-7*, is crucial. Further evidence that the RNAi-specific genes *rde-1* and *rde-4* can be mechanistically separated from the mutator/RNAi genes came from elegantly designed experiments that addressed the inheritance of RNAi (42). It has long been recognized that for a number of target genes, the RNAi phenotype was not restricted to the exposed animals but could also be transmitted to the next generation (F1); in most cases, the F2 generation reverted to a wild-type phenotype. Mello and coworkers investigated this inheritability of RNAi and found clues for the existence of a dominant extragenic agent: For some sensitive targets—genes expressed in the maternal germline—the interference is sustained for more generations and can be passed on to subsequent generations even in the absence of the endogenous gene that is the target of silencing. The authors uncoupled the initiation of this inferred inherited species (in the mother exposed to dsRNA triggers) from the execution step in RNAi (interference of the target mRNA in the F2 offspring), and subsequently analyzed the genetic requirements of both events. They found that the formation of the inherited agent depends on the RNAi genes *rde-1* and *rde-4* but not on the RNAi/mutator genes *rde-2* and *mut-7*. In contrast, execution of silencing in the descendants of the exposed animals required RDE-2 and MUT-7 but did not depend on the wild-type activity of *rde-1* and *rde-4* within these animals. Taken together, these data suggest an order in the mechanism of RNAi: (a) the initiation of RNAi by the generation of a heritable extragenic sequence-specific intermediate; requirement for RDE-1 and RDE-4, followed by (b) the execution of interference downstream of initiation, which depends, at least, on the *rde-2* and *mut-7* genes (42).



One obvious class of candidate molecules to constitute the extragenic inherited agents are siRNA species (see the parallels between the *C. elegans* genetic data and the model to explain RNAi in vitro in the scheme depicted above). Fire and coworkers demonstrated that siRNAs are produced upon injection of dsRNA into the gonadal syncytium, and although wild-type animals were able to dice the dsRNA into molecules of sizes reminiscent of siRNAs, this activity was almost completely abolished in *rde-4* mutant animals, which suggests that RDE-4 is directly involved in this processing step (83). In contrast, phenotypically similar *rde-1* mutant animals were fully proficient in dicing dsRNA, suggesting that this protein acts downstream of siRNA production. In agreement with this interpretation,

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direct injection of short dsRNA molecules into the animal could bypass the requirement for RDE-4, but not RDE-1, in triggering gene silencing (83).

The involvement of these genes in the Dicer reaction was also analyzed *in vitro* by assaying *C. elegans* extracts prepared from wild-type and various mutant animals for their ability to produce siRNAs from long double-stranded molecules, an activity attributed to *C. elegans* DCR-1, the ortholog of the *Drosophila* RNase III enzyme Dicer; siRNA-producing activity can be immunoprecipitated with antisera raised against DCR-1 (52). Although extracts from the RNAi mutants *rde-1*, *mut-7*, and *mut-14* were fully proficient (106), extract prepared from *rde-4* animals failed to generate siRNAs (unpublished observations), supporting the proposed role for RDE-4 in the first step of RNAi: dicing dsRNA into siRNAs. Using RNase protection assays, this study also analyzed the production of siRNAs *in vivo* in wild-type and mutant animals that were exposed to dsRNA by feeding on bacteria that generate dsRNA homologous to a specific *C. elegans* gene. Whereas in wild-type animals, RNA molecules of 20–25 nt could easily be detected, most mutants, including those that are proficient in siRNA production *in vitro*, failed to produce detectable levels of small RNA species (106). The observation that only RNA species of antisense polarity, not of sense polarity, are detected in wild-type animals suggests that siRNAs that are directly diced of the input dsRNA were not visualized; if so, one would expect species of both sense and antisense polarity, as was found in the *C. elegans* *in vitro* assay. The species that are detected in a wild-type genetic background more likely result either from stabilization (presumably on the target mRNA, which would explain why only antisense siRNAs are seen) and/or amplification. These more downstream steps in the RNAi reaction probably require the action of several genes that have been isolated in mutant screens.

mRNA DESTRUCTION

As mentioned above, biochemical experiments in *Drosophila* and mammalian cells demonstrated that siRNAs are the effector molecules in RNAi (33, 34). However, most of the genes isolated in the genetic screens described above can be placed either upstream of the formation of dsRNA or in the process of dicing the dsRNA into siRNAs; relatively little is known about the protein components involved in the execution step of RNAi: destruction of the mRNA. The genetic data from *C. elegans* suggest that the mutator/RNAi genes, e.g., *mut-7* and *rde-2*, might be involved, but biochemical support is lacking. In the short term, further identification of proteins that copurify with RISC activity should help to elucidate this issue; identification of additional RISC components is under way in several laboratories. The only RISC factor thus far published is *Drosophila* AGO2 (46), a homolog of RDE-1, QDE-2, and Arabidopsis Ago1, which are required for RNA silencing in genetic systems (20). Another *Drosophila* member of this family, AGO1, was recently found also to be required for RNAi *in vivo* (124). Remarkably, RDE-1 and Arabidopsis Ago1 are thought to operate in initiating stages of RNA silencing, and

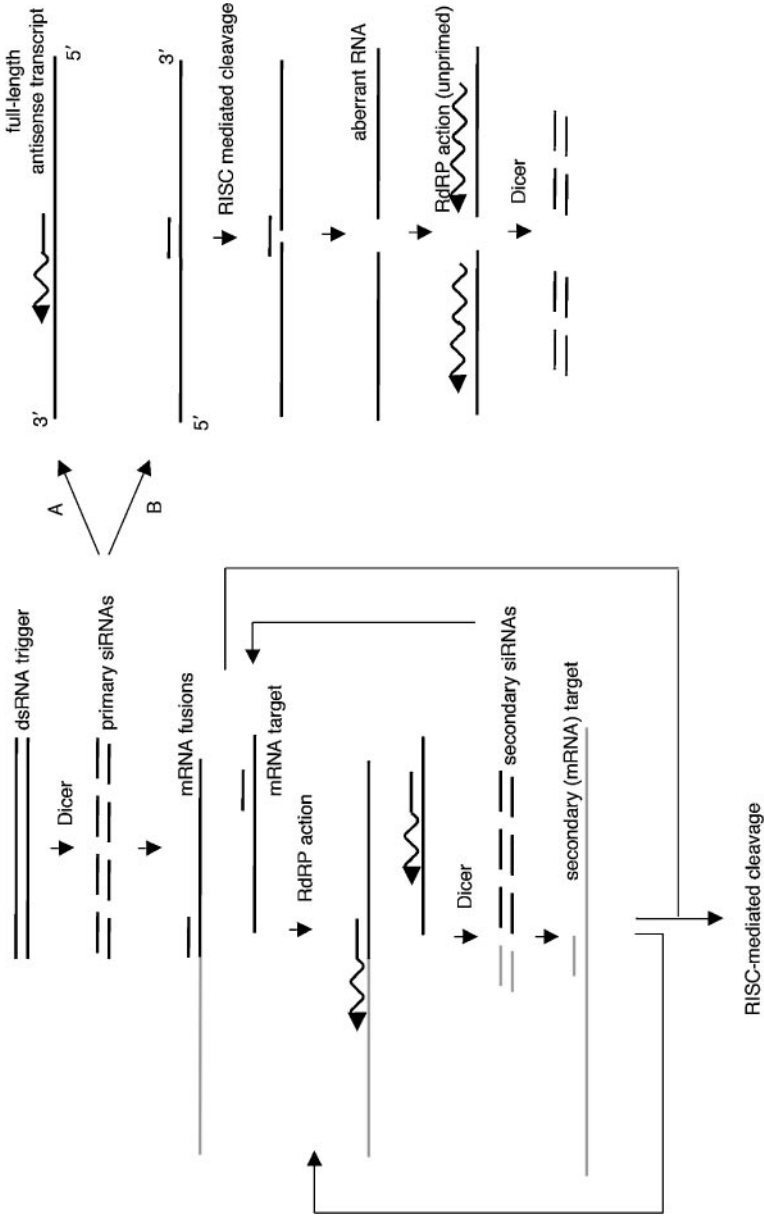
not in the more downstream steps. In these systems, other family members, not yet identified genetically, [*C. elegans* encodes at least 24 members of this family (41, 97)] may be required, perhaps because these are essential for viability. Support for a scenario in which downstream components in RNA silencing, possibly of RISC, might not be isolated in genetic screens (as a result of an associated essential function) comes from the notion that most, if not all, PTGS mutants isolated in plant systems are still proficient when RNA silencing is triggered directly by dsRNA. This suggests that only upstream components can be found.

Alternatively, the RNA silencing pathways might be more diverged between species than initially anticipated: In *Drosophila* and also in mammalian cells, RISC activity in RNA silencing has been well established, including the ability to bypass the upstream part of RNAi by direct administration of siRNAs in vivo and in vitro. However, in *Neurospora*, plants, and *C. elegans*, evidence for RISC activity has not been found to date.

Secondary siRNAs Produced by Transitive RNAi

Some light on possible differences in executing mRNA degradation was shed recently when it was found that in addition to siRNAs that could be attributed to the input dsRNA, siRNAs were also detected from sequences outside of the targeted area (98). This could mean that siRNAs are also produced from the targeted mRNA transcript (and not just from the input dsRNA), as secondary products of mRNA degradation. These secondary siRNAs must be produced by de novo RNA synthesis because the 20–25-nt molecules of antisense polarity that comprise one strand of the double-stranded siRNAs are not present in unexposed animals nor in the trigger dsRNA molecules. Subsequent analysis indicates that secondary siRNAs are functional. An endogenous gene could be targeted with noncomplementary dsRNA as long as the animals also express an engineered transcript that has sequences of the target mRNA fused to sequences that are homologous to the input dsRNA (see also Figure 3). In agreement with the polarity seen in the production of secondary siRNAs, in these experiments RNAi transits (hence called transitive RNAi) in one direction. Interference of the endogenous locus was observed only when the homologous sequence was positioned 5' of the sequences corresponding to the dsRNA input. These data suggest that mRNAs are not only targets but also amplifiers of the original signal, which may explain earlier observations that only traces of dsRNA can wipe out an excess of mRNAs, and the fact that RNAi can persist for more than one generation.

In plants such a unidirectional generation of secondary siRNAs is not seen: Secondary siRNAs are also detected for sequences downstream of the input trigger and their generation is dependent on a putative RdRP protein. Possible explanations include (a) the presence of undetectable levels of full-length antisense transcripts: The sense strand of the siRNA can then prime RNA synthesis identical to its antisense counterpart on the sense mRNA, thus giving rise to siRNAs that can be assigned to both upstream and downstream sequences. (b) The sense transcript



is cleaved by the primary siRNAs, but the mRNA degradation products are seen as aberrant RNAs that are turned into dsRNA in a process that likely involves primer-independent RdRP action. At present, biochemical data exist only from a member of this family isolated from Tomato: This protein can produce RNA products by extending primers on RNA templates but is also capable of initiating RNA synthesis in the absence of a primer.

Generation of secondary siRNAs requires the action of a protein capable of de novo RNA synthesis using the mRNA as a template, which would result in dsRNA that can be a substrate for Dicer-mediated cleavage. Likely candidates for this function are RNA-dependent RNA polymerases (RdRPs), especially since in *C. elegans* putative RNA-dependent RNA polymerases have also been implicated in gene silencing phenomena. Based on the observation that gene silencing in *Neurospora* requires a putative RdRP (Qde-1), Maine and coworkers investigated RNAi in animals mutated in *ego-1*, one of the four homologous genes encoded by the *C. elegans* genome, and found that this protein is involved in RNAi, but the requirement appears specific for germline tissue (100). Recently, Sijen et al. demonstrated that loss of *rrf-1*, a second member of this family of four (and in the genome positioned immediately downstream of *ego-1*), also results in an RNAi-defective phenotype, but in this case, only somatic cells are resistant to RNAi (98). This could indicate that both proteins fulfill the same function but in different tissues.

Additional support for the involvement of RdRP action in *C. elegans* RNAi comes from the observation that short RNA of antisense polarity can also trigger RNA silencing when administered in close proximity to the target mRNA (106). These antisense species have a less strict size requirement than double-stranded

←

Figure 3 RNAi amplification, transitive RNAi, and the generation of secondary siRNAs. The left part of the figure depicts a model to explain how transitive RNAi can lead to amplification of RNAi in *C. elegans*: Primary siRNAs diced from the input dsRNA anneal with RNA molecules that contain complementary sequences. This primer-template substrate can then be used to support de novo RNA synthesis by an RNA-dependent RNA polymerase resulting in dsRNA 5' to the sequences that are within the input dsRNA. The detection of siRNAs corresponding to such upstream regions suggests that Dicer cuts the dsRNA leading to "secondary" siRNAs that, in a second round, can anneal to a novel target that does not share any sequence homology with the initial input dsRNA, but shares homology with the intermediate bridging RNA molecules. Another round can now, in principle, be initiated, degrading the mRNA in the process, thus completing RNAi. Note that this mechanism does not require, nor exclude, the action of a RISC activity: cutting the mRNA within the siRNA-covered region. The notion that RNAi is not initiated at all in RdRP mutants suggests that either mRNA is degraded via this mechanism or that an amplification of the original signal is required to completely wipe out mRNA.

siRNAs—siRNAs only work when they are of 21–23 nt in length (35, 78)—suggesting that these molecules do not enter RISC-mediated mRNA degradation directly. Instead, as RNAs might directly act as RNA primers allowing extension of the 3' end by an RdRP, thereby creating dsRNA that can subsequently be a substrate for Dicer action (106). Together, these observations led to the following model (Figure 3) (2, 86, 127). Although the model includes the involvement of a RISC complex in degradation of the mRNA, the presumed action of an RdRP on the mRNA target resulting in dicer substrates will automatically lead to its destruction thus creating the amplification products. In such a scenario, a separate parallel RISC activity is superfluous.

Mutations in a third member of this RdRP gene family, *rrf-3*, make *C. elegans* supersensitive to RNAi (98), a phenotype that might be explained if the RFF-3 protein dominantly interferes with the action of EGO-1 and RRF-1.

Although the term transitive RNAi was first coined in *C. elegans*, the phenomenon was initially observed for PTGS in plants. In *N. benthamiana* plants that express full-length GFP (as a presumed bridging molecule), GFP DNA fragments containing only part of the GFP sequence can “transitively” silence the expression of nonoverlapping GFP sequences that are independently expressed from a virus-based vector (122). A putative RdRP (*SGS2/SDE1*) is also required, although in contrast to *C. elegans*, no directionality is observed: siRNAs are produced both 5' and 3' of the original trigger, indicating bidirectional spreading from the initiator region into adjacent regions of the target gene (Figure 3) (110).

Independently, Nellen and coworkers, in *Dictyostelium discoideum*, showed that hairpin constructs can silence expression of homologous transgenes or endogenous genes. Here too, silencing required the action of an enzyme with presumed RdRP activity (67). Of the three RdRPs encoded by the *Dictyostelium* genome, RrpA, RrpB, and RrpC, only RrpA was required to induce RNA silencing; however, RrpA was also essential for the detection of siRNAs, despite the fact that extracts of *rrpA* in vitro were proficient in dicing dsRNA into siRNAs. This could indicate that secondary siRNAs rather than primary siRNAs (coming from the initiating dsRNA hairpin) are visualized in the in vivo assay. This hypothesis is further supported by the observation that siRNAs were not detected in the absence of a target locus, suggesting that the target gene is involved in siRNA generation and amplification. This situation resembles observations made in *C. elegans* in which most siRNAs that are detected in vivo are thought to be secondary (as discussed above).

Does this mean that in these species RNA silencing operates via an RdRP dependent mechanism, whereas *Drosophila* and mammals execute RNAi via RISC? Although homologs of the RdRP genes described above have not been found in BLAST searches in the *Drosophila* and human genome, in vitro experiments using *Drosophila* cell extracts suggest that siRNAs might prime RdRP action on complementary RNA targets (63). Perhaps the most convincing finding against a role for obligatory polymerase action on the siRNA is that in human cells, siRNAs that cannot act as primers because their 3' terminus is blocked by a FITC moiety are still potent inducers of RNA silencing (47).

SYSTEMIC NATURE OF RNA SILENCING

Systemic Propagation of PTGS

An amplification step has also been implicated in the systemic spread of RNA silencing throughout exposed organisms. RNA silencing is not restricted to individual cells but can spread from the site of infection to more distant tissues. The systemic nature of PTGS in plants has been termed SAS, for systemic acquired silencing, and was most convincingly demonstrated in grafting experiments (81). Sequence-specific silencing was transmitted unidirectionally from silenced stocks (lower tissues and root system) to target scions (vegetative upper tissues), suggesting the presence of a diffusible silencing agent. Noteworthy in these experiments is that the expression level of the target gene needed to be elevated compared to wild-type plants to observe systemic PTGS (82). Non-silenced scions that either contain extra transgene-encoded copies or are overexpressing the endogenous target gene as a result of metabolic derepression, are sensitive to PTGS upon grafting, although systemic PTGS was not observed when wild-type scions were grafted onto silenced stocks. This could mean that only a limited number of triggers reach the distant tissues and some sort of target-dependent amplification is required to completely degrade all mRNAs. The notion that transmission of PTGS also occurred between a silenced stock and an engrafted scion through a stem of a non-transgenic wild-type plant suggests that such amplification is not required during long-distance transport of the proposed, but as yet unidentified, RNAi signaling molecule.

The systemic propagation of PTGS was also demonstrated by Voinnet et al. (119, 122). These authors show that in non-silenced GFP transgenic tobacco plants, silencing can be initiated by localized introduction of an additional GFP transgene, either via *Agrobacterium* infiltration or biolistic transformation. Although GFP silencing is initially localized to the exposed tissues, after some time silencing starts to spread—long-distance movement of the signal occurs through the phloem, and cell-to-cell movement is through plasmodesmata—eventually leading to PTGS even in the upper leaves of the plant. A system that amplifies the initiating silencing event has been proposed in this case also.

Systemic RNAi in *C. elegans*

In *C. elegans*, silencing is also not restricted to those cells that are exposed to the original dsRNA trigger. GFP expression can be silenced in the germline and in almost all somatic cells by feeding animals on dsRNA directed against GFP (50, 107, 108). Apparently, the dsRNA is taken up from the environment and the signal spreads throughout the organism to exert its effect in differentiated tissues. Only neuronal cells appear to be less sensitive to incoming triggers. RNAi works when the dsRNA is expressed within these cells, but silencing is not complete when the dsRNA is administered via food. A possible explanation is that neurons are less proficient in the uptake of such nucleic acids from their surroundings.

Because genetic screens to isolate mutants in the mechanism of RNAi made use of this systemic nature of RNAi, mutants with defects in the uptake of dsRNA or transport of RNAi signals have also been found. Mutagenized animals were selected on bacteria producing dsRNA homologous to genes essential for embryonic development (thus resulting in dead embryos), which are expressed in the maternal germline. Thus, animals with a defect in the uptake of dsRNA or in the transport of trigger molecules to the target tissue would survive this selection. To obtain true RNAi-deficient (*rde*) mutants, a second filter was applied: Only those mutants that were also RNAi defective when the dsRNA was directly injected into the target tissue (thus bypassing the routing of RNAi signals) were further analyzed.

Genetic screens have recently been performed directly aimed at identifying components required for systemic RNAi (125). Mutants were isolated that lost dsRNA-induced silencing of a GFP marker gene but only when the dsRNA triggers come from outside the silenced cells: to control for an intact RNAi machinery, GFP was also silenced by transgene-induced expression of GFP dsRNA in a limited number of cells. Mutants that fail to silence GFP triggered by environmentally produced dsRNA, but that had a wild-type response to dsRNA expressed cell autonomously, were further analyzed and three complementation groups were identified: *sid-1*, *sid-2*, and *sid-3* (for systemic RNAi deficient). *sid-1* has been cloned and encodes a transmembrane domain-containing protein that is required cell autonomously, perhaps for the uptake of dsRNA or RNAi intermediates.

Notably, in this experimental system, some systemic spread is seen from cells that express GFP dsRNA to cells that only have the recipient GFP mRNA target, but this spread was very limited. One explanation might lie in a limited intercellular transport between the tissues that were analyzed (muscle cells). However, expression of hairpin constructs within intestinal cells (which take up food from the lumen of the gut and are thus the likely entry point of environmentally provided dsRNA) silences GFP expression within these cells but does not trigger silencing throughout the organism (our unpublished observations). Perhaps environmentally provided dsRNA (i.e., by feeding or soaking) is immediately endocytosed into specialized cellular compartments for further transport. It is unclear whether there are distinct routes by which RNAi signaling or effector molecules travel through the organism or whether these molecules are hitchhiking along existing pathways to take up nutrients. Many mutants that show some sort of defect in transitive RNA are viable and do not have a significant growth delay, which a priori would not be expected if the defect compromises the intake of food.

BIOLOGICAL FUNCTIONS OF RNA SILENCING

Interplay Between PTGS and RNA Viruses

The discovery that mutant plants defective in PTGS are also hypersensitive to the infection of certain viruses strengthened the hypothesis that PTGS participates in a defense mechanism to protect the plant (and other species) against infectious

agents. Several observations had pointed to a complex interplay between PTGS and virus infection. For example, plants that show transgene-induced PTGS of a virus sequence are immune to the cognate virus [e.g., (66)]. Also in some cases that involve wild-type plants, recovery from virus infection is associated with specific degradation of virus RNA (3, 90); these wild-type plants have now become immune to that virus and to viruses that have been genetically modified to contain part of the virus used for the initial infection (89, 90).

Not surprisingly from an evolutionary point of view, in response to this defense strategy, plant viruses produce proteins that counteract PTGS. This idea followed from the observed synergistic effects in disease symptoms when plants were infected with two viruses. Because the helper component proteinase (Hc-Pro) from potyvirus suppressed the host defense mechanism, the pathogenicity and accumulation of two other viruses, *Cucumber mosaic virus* and *Tobacco mosaic virus*, were enhanced (87). Subsequently, it was shown that HC-Pro and also the 2b protein of the CMV virus function as effective suppressors of PTGS (7, 16, 51), further strengthening the proposed role of PTGS as a natural mechanism against viruses. Several virally encoded suppressors of PTGS have subsequently been identified that target PTGS at different stages: initiation, systemic spread, or maintenance (12, 121). For example, HC-Pro inhibits PTGS in all symptomatic tissues, and this suppression appears to act at a step preceding accumulation of the PTGS-associated siRNAs: No siRNAs were observed in plants in which PTGS has been suppressed by HC-Pro (64). Remarkably, overexpression of a tobacco gene, *rgs-CaM*, which was identified because the encoding protein interacts with Hc-Pro, also inhibits PTGS (6). Possibly, this type of virus-induced suppression of PTGS is established through activation of the plant-encoded *rgs-CaM* gene: *rgs-CaM* mRNA levels are increased by virus infection or in plants expressing HC-Pro. The biological function of this calmodulin-related protein in wild-type plants is not yet known.

Other types of virally encoded suppressors, including the CMV 2b and TBSV 19K proteins, have a more restricted inhibitory effect: Initiation of PTGS was inhibited only at the growing points of the plants, but no effect was observed in tissues where PTGS has already been established (16). In yet another interesting case, the 25-kDa viral movement protein (p25) of *Potato virus X*, although originally thought not to interfere with PTGS (16), was shown to target the spread of silencing. Grafting experiments showed that systemic PTGS was absent in the presence of functional p25 (120).

Virally encoded suppressors of RNA silencing are not restricted to plants. In animal cells, RNA silencing can also act as an antiviral defense: *Flock house virus* (FHV), an RNA virus that can infect both vertebrate and invertebrate hosts and is both an initiator and target of RNA silencing in *Drosophila* host cells, encodes a protein, B2, that also operates as an RNA-silencing suppressor (61). Interestingly, this protein was investigated not because of sequence similarity to known suppressors, but because of its similar location in the viral genome to the CMV RNA silencing-suppressor 2b (62).

RNAi and Repression of DNA Transposition

In addition to protecting organisms against viruses, RNA silencing can also protect the organisms from DNA transposition, as observed for *C. elegans* and *Chlamydomonas reinhardtii* in which mutations in RNA silencing can also result in increased rates of transposition (53, 126). In another example of coordinate loss of RNA silencing and loss of transposition repression, Aravin et al. observed that mutations in the RNA helicase spindle-E not only relieved silencing of the Stellate genes—Stellate expression is sensitive to dsRNA-mediated silencing—but also resulted in derepression of retrotransposition in the *Drosophila* germline (9). A further indication that the RNA silencing machinery might directly target retrotransposon transcripts comes from the detection of retrotransposon-derived siRNAs in *Trypanosoma brucei*, where dsRNA-induced RNA silencing has been shown (77), indicating that cognate dsRNA intermediates are generated in this parasite that can be processed through Dicer action (30).

Cleaning Up Aberrant RNAs

Yet another function of the RNA silencing mechanism, or at least of some of its components, might be to remove aberrant nonfunctional RNAs from the nuclear or cellular pool of RNAs to fulfill a role in mRNA surveillance. If aberrant RNAs are turned into dsRNA to trigger RNA silencing, they are destroyed in the process. This would impose a serious problem to the cell if a single aberrant molecule is sufficient to trigger the reaction, sweeping the cell clean of any homologous mRNA. One would assume that only if these aberrant RNAs are produced above a certain threshold level, RNA silencing is triggered. Strong evidence for a threshold level in RNA silencing is seen in *Drosophila* undergoing PTGS (80). Although the strongest support comes from the work in *C. reinhardtii* (11, 126), a possible role for RNA silencing components in mRNA surveillance is also fueled by the discovery that mutations in certain *smg* genes, which are required for mRNA surveillance in *C. elegans*, show a defect in the attenuation of RNAi (31). Mutant animals recover much faster from dsRNA treatment than the wild-type controls [see also (65) for a comprehensive review on the RNAi/mRNA surveillance connection]. In addition, the Arabidopsis gene SDE3, which when mutated renders plants resistant to PTGS, is homologous to the *C. elegans smg-2* gene (27). Such implications could undermine the interpretation of mutants defective in the RNAi response: Any mutation resulting in the abundant production of aberrant RNAs might saturate the RNA silencing machinery and falsely be considered as defective in the RNAi pathway. Goldstein and coworkers (32) raised this possibility as a possible explanation for why mutations in chromatin condensation factors also resulted in a diminished capacity to respond to dsRNA.

RNA Silencing and Development: siRNAs, stRNAs, miRNAs

Some components of the silencing machinery are interconnected with gene regulatory mechanisms that control developmental programs. Following up on in

vitro data in *Drosophila* demonstrating that the RNase III-type nuclease DICER is responsible for processing dsRNA into siRNA species, several research groups independently reported the involvement of the orthologous gene, *dcr-1*, in *C. elegans* RNAi (41, 52, 55). In addition to an RNAi-defective phenotype, i.e., proof for a role of *dicer* in RNAi in vivo, DCR-1-defective animals are sterile; their oocytes are abnormal and no fertilized eggs were detected. They also display several developmental abnormalities during larval growth, including a protruding vulva and a tendency to burst at the vulval region before reaching the adult stage. In addition, seam cells fail to fuse during the L4-to-adult transition. In some cases, additional rounds of cell division are observed that result in animals without adult-specific alae (41, 52). These developmental defects are reminiscent of phenotypes resulting from mutations in the heterochronic genes *lin-4* and *let-7* (60, 91). These genes produce noncoding RNAs that regulate the timing of developmental events (hence named small temporal RNAs or stRNAs) via regulation of translation of downstream target genes. This overlap in phenotypes together with the notion that stRNAs, which are of similar length as siRNAs, can be produced by processing of larger precursors predicted to form a dsRNA hairpin structure, triggered investigation of the role of *dcr-1* in the generation of such stRNAs. Loss of DCR-1 led to accumulation of the *let-7* and *lin-4* “unprocessed” precursor molecules and reduced levels of mature stRNAs (41, 52). Dicer fulfills this function in other animal systems, where the *let-7* stRNA is conserved across much of animal phylogeny with a 100% sequence conservation between *C. elegans*, *Drosophila*, and humans (85): Immunopurified *Drosophila* DICER as well as *Drosophila* embryo extracts could convert the *let-7* precursor RNA into 21-nt-sized stRNA (48, 52), and down-regulation of human Dicer in HeLa cells results in the accumulation of *let-7* RNA precursor (48).

The *let-7* and *lin-4* stRNAs are just the tip of the iceberg. Several laboratories have recently cloned many additional stRNA-like RNAs from *C. elegans*, *Drosophila*, and human cells, subsequently termed micro-RNAs or miRNAs (57–59, 74). Like the stRNAs, all of these miRNAs are predicted to be processed from duplexed precursor molecules and are therefore likely processed by Dicer, which was indeed shown for two of them (59). The eminent role of the *dicer* enzyme in catalyzing the maturation of such a plethora of miRNAs might explain why development in *C. elegans dcr-1* animals is completely compromised. The fact that first-generation *dcr-1* homozygotes are viable (but subsequently fail to produce progeny) is likely the result of maternal contribution of DCR-1. Also, dramatic developmental defects are observed in Arabidopsis that are mutated in the Dicer homolog CARPEL FACTORY(49).

None of the miRNAs is perfectly complementary to any known protein-encoding transcripts in the genome of the cognate species, which suggests that miRNAs do not negatively regulate expression via the RNAi pathway, i.e., destruction of the homologous transcript. Also, vice versa, animals defective for the RNAi protein RDE-4, which is thought to cooperate with Dicer in siRNA production, do not display developmental phenotypes. Instead, miRNAs may interfere with the translation machine, as for *lin-4* and *let-7* stRNAs, by binding the 3'UTR of the

downstream target genes (4, 99). The regulatory potential of miRNAs is further illustrated by the observation that many miRNAs, including those found in *Drosophila* and mammalian cells, have a very strict time- or tissue-specific expression pattern. No function has yet been ascribed to any of the novel miRNAs. A big challenge for today's molecular biologist is to define the potential role of miRNAs (in gene regulation) and identify their target genes.

FUTURE DIRECTIONS

The tremendous pace in which our understanding of RNA silencing progressed over the past four years, despite many unanswered questions, makes it likely that most protein factors required for RNA silencing will be identified through combined biochemical and genetic approaches, and that their specific function in the silencing mechanism will be addressed. A stronger link is needed between the biochemical and genetic data, e.g., by setting up in vitro systems for the genetic systems. Although the outline of the RNA silencing pathways is coming into focus, much interest will drift away from the core silencing mechanism into related areas of research. As an example, the recent identification of a mechanistic link between RNA silencing and gene regulation via stRNAs/miRNAs, together with the notion that such miRNAs are abundantly present throughout the animal kingdom, opened up a completely new area of research into how and to what extent these small noncoding RNA molecules regulate cellular or developmental processes (5, 73, 94).

One of the most interesting aspects of understanding how RNA silencing works is its practical use: the ability to reduce gene expression in a manner that is highly sequence specific as well as technologically facile and cheap. In the *C. elegans* community, RNAi is now routinely used in reverse genetic approaches studying particular gene functions. Moreover, large-scale RNAi screens have been performed in *C. elegans* using RNAi as a forward mutagenesis tool (39, 40). Currently, several laboratories are scanning the complete genome for specific phenotypes using libraries of bacteria that produce dsRNA directed against each ORF encoded by the *C. elegans* genome. Similar tools are about to be applied to higher eukaryotes, including human cells. Upon the demonstration that siRNAs can be used to reduce gene expression in mammalian cells (33), bypassing the PKR response that is triggered when larger dsRNA molecules enter the cells, many laboratories designed vector-based DNA systems that express siRNAs or siRNA-precursor hairpin cell-autonomously, thus providing a stable source of RNA silencing molecules [e.g. (17, 79)]. This tool can be easily scaled to set up a library to systematically target all ORFs encoded by the human genome. However, caution is needed here. Although thus far siRNAs have been shown to be potent, not all siRNAs are equally efficient. In practice, this could mean that several siRNAs must be tested empirically to find one that reduces mRNA levels sufficiently to draw definite conclusions in analysis of gene function. For *C. elegans*, not all genes are targeted efficiently; what constitutes the parameters for success is not yet known.

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