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REVIEW

The rest is silence

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PERSPECTIVE

The use of double-stranded (ds) RNA to manipulate gene expression is evolving into an increasingly powerful tool to study gene function in organisms ranging from plants to invertebrates to mammals. Underlying the technological advances enabled by RNA interference (RNAi) is a rich biology. RNAi and related phenomena represent evolutionarily conserved mechanisms, which protect organisms from invasion by both exogenous (e.g., viruses) and endogenous (e.g., mobile genetic elements) genetic parasites. In addition, it is now clear that dsRNA-dependent silencing mechanisms represent a conserved regulatory motif for endogenous programs of gene expression. We are just beginning to understand not only the biological roles of RNAi but also the mechanistic basis of this process. The goal of this review is to familiarize the reader with the diversity of biological responses that may be related to RNA interference and to summarize the current state of knowledge regarding the mechanistic bases of these silencing phenomena.

HOMOLOGY-DEPENDENT GENE SILENCING—A DIVERSITY OF RESPONSES

Homology-dependent gene silencing (HDGS) was first discovered with the introduction of transgenes coding for chalcone synthase into petunia plants. Although the expectation was increased flower pigmentation, in many of the plants, the result was in fact the opposite, white or variegated petunia petals. This was a remarkable observation, suggesting not only that the introduced transgenes were inactive but also that these exogenous genetic elements affected the endogenous chalcone synthase locus. This apparent communication between unlinked but homologous loci was termed cosuppression (Jorgensen, 1990).

It is now recognized that HDGS is a commonly observed outcome of transgenesis in plants. Communication can occur between transgenes and endogenes (e.g., Holtorf et al., 1999), between two related transgenes (e.g., Kanno et al., 2000), and even between silenced and active endogenous loci (e.g., Coen & Carpenter, 1988). A similar phenomenon, called paramutation, describes an interaction between two endogenous alleles, which leads to a directed alteration in transcription that is both mitotically and meiotically heritable (Chandler et al., 2000). It is possible that paramutation and cosuppression are mechanistically related phenomena that differ only in the source of the silencing trigger.

Homology-dependent gene silencing is not restricted to plants, but is instead observed in a wide range of organisms. For example, in the unicellular ciliate, *Paramecium*, injection into the somatic nucleus of DNA homologous to the coding region of a target gene leads to sequence-specific silencing (Ruiz et al., 1998). In the filamentous fungi, *Neurospora crassa*, transgene cosuppression has been demonstrated directly, and this phenomenon has been termed "quelling" (Romano & Macino, 1992).

Homology-dependent silencing can also be accompanied by covalent alterations of the genome, which in some cases result in changes in the DNA sequence. For example, in *Neurospora*, "repeat-induced point mutation" (RIP) describes an exceptionally high rate of mutation, particularly GC → AT transitions, in repeated sequences (Cambareri et al., 1989). Underlying this phenomenon is thought to be the methylation of cytosine residues, which become substrates for deoxycytidine deaminase with consequent conversion to thymidine (Selker, 1997). In *Ascobolus immersus*, another filamentous fungus, duplicated sequences are methylated before meiosis and silenced throughout sub-

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sequent nuclear divisions, a process referred to as "methylation induced premeiotically" (MIP; Goyon & Faugeron, 1989; Rhounim et al., 1992). In some cases, modifications are copied from a methylated allele to an unmethylated allele during meiosis in a homology- and position-dependent fashion (Colot et al., 1996).

The key question regarding all of these HDGS processes is whether they are mechanistically related. In the case of transgene cosuppression, and perhaps also paramutation, we are beginning to formulate mechanistic hypotheses to explain the communication between homologous loci, and these are discussed below. However, for most of the phenomena described above, there is insufficient evidence to determine whether silencing is triggered by interaction between loci at the DNA level, via an RNA intermediate (as for some cases of cosuppression), through proteins, which establish heritable and self-reinforcing gene regulatory circuits, or through a combination of these processes.

TRANSGENE COSUPPRESSION AND POSTTRANSCRIPTIONAL GENE SILENCING

Transgene cosuppression is one form of HDGS that has been observed in numerous organisms. In plants, cosuppression can operate both at the transcriptional and at the posttranscriptional level (e.g., de Carvalho et al., 1992; Meyer et al., 1993). As with RIP and MIP, cosuppression is associated with methylation of the silenced loci (e.g., Elmayan et al., 1998). Methylation within the coding sequence of the gene correlates with destabilization of the mRNA product, whereas methylation within promoter sequences is associated with transcriptional silencing (e.g., Ingelbrecht et al., 1994; Dieguez et al., 1998). Quelling in *Neurospora* may also be associated with methylation of target sequences, even though methylation is dispensable for this response (Cogoni et al., 1996). Although biochemical insights into the precise mechanism of quelling are lacking, genetic evidence suggests a relationship with PTGS in plants and RNAi in animals (e.g., Dalmay et al., 2000; Fagard et al., 2000; Mourrain et al., 2000; Smardon et al., 2000; Hammond et al., 2001a).

The first extension of transgene cosuppression to the animal kingdom occurred in *Drosophila* (Pal-Bhadra et al., 1997). Pal-Bhadra and colleagues demonstrated that the introduction of repeated *white-Adh* fusion transgenes into *Drosophila* leads to the considerable repression of the transgene and also of endogenous *Adh* expression. The degree of silencing was proportional to transgene copy number. The methylation status of cosuppressed genes is unknown in *Drosophila*, in part due to the fact that DNA methylation in flies has only recently been discovered (Gowher et al., 2000; Lyko et al., 2000). However, genetic studies have correlated cosuppression with alterations in chromatin structure. The role of Polycomb proteins in mediating long-term gene repression through effects on higher-order chromatin structure is well established (Pirrotta, 1998). In some cases of *Drosophila* cosuppression, mutation of Polycomb proteins relieves silencing (Pal-Bhadra et al., 1997). In plants, evidence for the involvement of chromatin structure in PTGS has also emerged with the observation that mutations in DNA methylases or chromatin-remodeling proteins (Jeddeloh et al., 1998; Morel et al., 2000) compromise cosuppression (see below).

In *Caenorhabditis elegans*, cosuppression is observed mainly in the germline but also to a limited extent in somatic tissues (e.g., Fire et al., 1991; Dernburg et al., 2000). Methylation of the *C. elegans* genome has yet to be detected; however, altered chromatin structures have been implicated in cosuppression in this system (Kelly & Fire, 1998).

Communication occurring solely between endogenous loci has also been observed in *Drosophila*. For example, a study by Jensen and colleagues showed that crossing flies containing a silenced copy of an I element (a transposon similar to mammalian LINE elements) to flies containing active I elements repressed transposition. Furthermore, such repression was heritable (Jensen et al., 1999). This is similar to the phenomenon of paramutation in plants in which an active locus is repressed by exposure to a silenced locus in a manner that is stable even after alleles are separated by subsequent genetic crosses (e.g., Patterson et al., 1993).

Thus, it is clear that genomic loci that share primary sequence homology communicate in many different systems. Communication can occur between exogenous and endogenous loci (e.g., in transgene cosuppression) or between two endogenous loci that are exposed to each other following mating (e.g., paramutation). In plants and fungi, methylation of either the coding sequence or the promoter region is associated with silencing. However, genetic studies that are discussed below indicate that, in many cases, methylation is associated with but not obligate for inhibition of gene function. In animals, there is ample evidence for the involvement of chromatin structure modification in cosuppression, and in some cases, genetics indicate a dependency on proteins that affect chromatin structure. Finally, suppression can also occur at the posttranscriptional level without apparent alteration of the genomic locus corresponding to the silenced gene.

DIVERSITY OF RESPONSE AND COMMON TRIGGERS

In each of the many manifestations of HDGS, the effects themselves can vary—reversible silencing versus permanent mutation—as can the location and timing of the silencing event. Obviously, HDGS processes that

affect the genome occur in the nucleus. Examples of such phenomena are genomic methylation, paramutation, and possibly some instances of imprinting (an epigenetic phenomenon, which results in the exclusive expression of either a maternal or paternal allele). In contrast, posttranscriptional gene silencing (PTGS) processes, such as RNAi and virus-induced gene silencing, are believed to occur in the cytoplasm. Cosuppression in plants can occur somatically. In fungi, quelling occurs in the vegetative phase, whereas RIP and MIP occur premeiotically during the sexual phase. Despite these differences, HDGS phenomena may be united by the hypothesis that all are triggered by recognition of unusual nucleic acid structures.

Although pairing of homologous genomic loci has commonly been proposed as a trigger for some HDGS processes, such as RIP and MIP, definitive evidence for such an interaction is lacking. Instead, recent studies have pointed to RNA as a common trigger for HDGS processes ranging from transgene cosuppression in plants to quelling in fungi.

The first definitive demonstration that RNA could trigger heritable gene silencing came from the discovery of RNAi in *C. elegans*. In an attempt to use antisense RNA to investigate gene function in *C. elegans*, Guo and Kemphues (1995) observed that injection of either antisense or sense RNAs into the syncitial germline of worms was equally effective at silencing homologous target genes. As an extension of these experiments, Mello and Fire tested whether combination of sense and antisense RNAs might enhance the effect (Fire et al., 1998). The results were startling. Combined sense and antisense, in essence double-stranded RNA, was an incredibly potent silencer of gene expression, at least 10 times more effective than either sense or antisense RNAs alone. Subsequently, much has been learned about the precise requirements for the dsRNA trigger to be functional and the mechanistic basis of this phenomenon (see below). However, the observation that dsRNA could trigger silencing was seminal because it crystallized hypotheses regarding the triggering of HDGS phenomena in other systems.

In retrospect, numerous experiments had hinted that RNA might be a trigger for various HDGS pathways, including cosuppression and virus-induced gene silencing (VIGS). First was the requirement that, to provoke cosuppression, transgenes had to be transcriptionally active (Mette et al., 1999). This alone was not necessarily indicative that dsRNA was the trigger for cosuppression; however, transgenes often form complex repeated structures upon integration into the genome (e.g., Muskens et al., 2000). Studies of these repeats indicated that tail-to-tail (inverted repeat) arrays were much more effective triggers of cosuppression than were head-to-tail (direct repeat) arrays (Waterhouse et al., 1998; Jakowitsch et al., 1999; Stam et al., 2000). Subsequently, the hypothesis that dsRNAs provoke transgene cosuppression has been tested directly. First, a single transgene that directs the transcription of an inverted repeat (hairpin) is a potent trigger of gene silencing, whereas a similar direct repeat transgene is ineffective. A directly repeated transgene that has been integrated into the genome can, however, be activated as a silencer by using recombinase to invert one half of the repeat (Mette et al., 2000). These observations provide compelling evidence that dsRNA produced from transgenes acts as the initiator of cosuppression.

What is much less obvious is how, and indeed whether, dispersed and single-copy transgenes trigger silencing through these same mechanisms. In a number of systems, including *C. elegans*, fungi, and plants, RNA-dependent RNA polymerases (RdRPs) have been implicated in dsRNA-induced gene silencing (Cogoni & Macino, 1999a; Dalmay et al., 2000; Smardon et al., 2000). A viable hypothesis is that these RdRPs may produce dsRNA, using transcripts derived from dispersed transgenes as templates. In plants, genetic evidence supports this notion, considering that the endogenous RdRP is required for some instances of transgene cosuppression. These enzymes are not required for virus-induced gene silencing; however, VIGS requires that the silencing trigger be replication competent (Angell & Baulcombe, 1997). Viruses encode their own RdRPs that generate dsRNA as a replication intermediate (Dalmay et al., 2000).

Missing from this theory is the nature of the primary transcript that might be recognized by the RdRP as an “aberrant” RNA that is to be converted into a dsRNA. Also inconsistent with this proposed mechanism is the absence of an apparent RdRP protein in the *Drosophila* genomic sequence, an organism that both cosuppresses and silences in response to dsRNA. Thus, it remains to be determined how single-copy and dispersed transgenes, as well as endogenes, trigger silencing and whether the trigger is of the same nature as that produced by complex transgene arrays and inverted repeats.

MECHANISM OF dsRNA-INDUCED SILENCING

Based upon the evidence described above, there was an emerging realization that transgene cosuppression, virus-induced gene silencing, and RNA interference may have at their core a common mechanism that is triggered by dsRNA. The question was how information could be extracted from dsRNA as a guide to selecting homologous substrates for silencing. Recent genetic and biochemical studies in plants, fungi, and animals are beginning to illuminate this mystery. In fact, the communion of these two approaches is likely to be required before we fully understand the potentially varied pathways through which dsRNA can affect gene expression.

GENETIC APPROACHES TO UNDERSTANDING RNAi

Genetic screens in *Arabidopsis*, *Neurospora*, *C. elegans*, and *Chlamydomonas* have implicated a number of genes in the process of dsRNA-induced gene silencing. Links between these and the emerging biochemical mechanism of RNAi are beginning to be forged. However, the mechanistic defect underlying the RNAi resistance of most genetic mutants remains to be determined.

Caenorhabditis elegans

Numerous RNAi-resistant mutants have been isolated in *C. elegans*, and the potential roles of two such genes have been reported thus far. The MUT-7 protein of *C. elegans*, which is required for RNAi in the germline, has homology to both RNAse D and to Werner Syndrome helicase (Ketting et al., 1999). As such, this protein is a good candidate for the effector component of RNAi, which destroys targeted mRNAs. Interestingly, the mut-7 mutant also shows mobilization of transposons in the germline, a property that is shared by other RNAi-deficient mutants including rde-2, rde-3, and mut-2 (Ketting et al., 1999; Grishok et al., 2000). However, not all RNAi-deficient worms mobilize transposons, suggesting that the machinery required for transposon silencing and for RNAi may share common elements but may not be completely overlapping.

In a separate screen for RNAi-resistant animals, the rde-1 locus emerged as a gene required for RNAi-resistance in the germline and soma (Tabara et al., 1999). This protein is a member of the Argonaute family, a group of related proteins, which has been highly conserved throughout evolution. Relatives of RDE-1 have been studied mainly in plants and in *Drosophila*. *Arabidopsis* containing mutations in members of the Argonaute family (e.g., Argonaute-1 or Pinhead/Zwille) both show pleiotropic developmental abnormalities (Bohmert et al., 1998; Moussian et al., 1998). In *Drosophila,* three genes homologous to rde-1, namely *Argonaute 1*, *Sting*, and *Piwi*, have been investigated. The *Sting* gene plays a role in the silencing of the X-linked, repetitive stellate locus and in meiotic drive, and recent evidence suggests that this silencing proceeds through an RNAi-like mechanism (Aravin et al., 2001). *Piwi* has been implicated in the maintenance of stem cell fate (Cox et al., 1998; Schmidt et al., 1999), which is reminiscent of the role of Argonaute and Zwille proteins in the control of cell proliferation in the floral meristem (see below). The *Drosophila ago1* mutant also shows developmental abnormalities, most prominently in the developing nervous system (Kataoka et al., 2001).

It was these genetic studies that first raised the possibility that RNAi-based mechanisms might control the expression of endogenous protein coding genes. This hypothesis has recently been strengthened by the finding that a component of the RNAi machinery produces small RNAs that regulate developmental timing (see below).

Neurospora crassa

As described above, the filamentous fungus *N. crassa* is highly resistant to the introduction of exogenous transgenes, which are silenced by quelling. The relationship between quelling and RNAi has become apparent from genetic studies in which quelling-deficient mutants have been isolated (Cogoni & Macino, 1997). For example, the *qde-2* gene encodes a member of the Argonaute family (Catalanotto et al., 2000). Interestingly, like rde-1 and unlike Argonaute family mutants in flies and plants, *qde-2* mutants fail to show phenotypic abnormalities beyond their silencing defects. However, it should be noted that selection for defects in RNAi or quelling is often accompanied in genetic screens by selection for viable and fertile offspring. As is MUT-7 in *C. elegans*, *qde-3* is homologous to the RecQ DNA helicase family of proteins, which includes the Werner's and Bloom's syndrome helicases (Cogoni & Macino, 1999b). However, unlike mut-7, qde-3 lacks a recognizable nuclease homology, suggesting that these two proteins play different roles in the silencing process. Finally, *qde-1* encodes an RNA-dependent RNA polymerase, which may be involved in generating or propagating the dsRNA silencing trigger (Cogoni & Macino, 1999a; see above).

Plants

Genetic studies in *Arabidopsis* have also expanded the pantheon of genes that have been linked to RNAi. In a study to identify loci required for PTGS, Baulcombe and colleagues and Vaucheret and colleagues have described five such genes, two of which have been identified and partially characterized. *SDE1*/*SGS2* is an RdRP homolog, which is required for transgene-induced PTGS (Dalmay et al., 2000; Mourrain et al., 2000). In contrast, virus-induced gene silencing is *SDE1*-independent. *SDE3* is also required for transgene silencing but is irrelevant to triggering of PTGS via recombinant tobacco rattle virus. The SDE3 gene product contains homology to RNA helicases (Dalmay et al., 2001). Thus, in contrast to *SDE1*, a simple explanation for the differential requirement for *SDE3* in VIGS and TIGS is unclear. *SGS3* is unrelated to any known gene (Mourrain et al., 2000).

Genetic studies in the algae *Chlamydomonas reinhardtii* have also implicated RNA helicases in RNAi/PTGS. Wu-Scharf and colleagues (2000) used a genetic strategy based upon induction of silencing by an RbcS2 transgene to isolate PTGS mutants. The gene they isolated, *Mut6*, encodes a member of DEAH-Box RNA helicase family. Analysis of the effects of *mut6* on trans-

poson silencing revealed that, like some other RNAi-deficient mutants, *mut6* cells show increased transposon activity. An interesting observation was that in *mut6* cells, the level of "aberrant" RNAs (based on a PCR assay) increases. This raises the possibility that *Mut6* thus may be required in some way for production of silencing triggers from abnormal RNA transcripts.

Genetic links to other cellular processes

RNA interference is a regulatory pathway in which nucleases destroy targeted mRNAs. This is reminiscent of another process that eliminates mRNAs containing premature nonsense mutations, called "nonsense mediated decay" (NMD). Both of these pathways utilize nucleases for RNA turnover and require the action of ATP-dependent RNA helicases. NMD is a quality control mechanism that prevents the synthesis of proteins from mRNAs that prematurely terminate translation, ultimately leading to errors in gene expression (e.g., Maquat & Carmichael, 2001). Recently, NMD has been linked to RNAi in *C. elegans*. On the premise that both processes involve RNA degradation, Domeier and colleagues examined how RNAi is affected by mutations in the *smg* genes, a set of seven genes required for NMD. The result of these studies suggested that the persistence of RNAi relies on the *smg* genes. Worms containing mutations in *smg-2,5* and *6* initially silenced expression of targeted genes in response to dsRNA but expression was quickly recovered, in contrast to wild-type worms in which silencing persisted (Domeier et al., 2000). In *Arabidopsis*, the gene *SDE3* encodes an RNA helicase that is required for silencing (see above). This protein is similar to, but distinct from proteins in yeast (Upf1p) and *C. elegans* (SMG-2), which are required for NMD (Dalmay et al., 2001). The evidence thus far is not concrete; however, it seems likely that NMD and RNAi have a connection that needs to be elucidated.

Genetic studies in several model systems have begun to identify a common set of proteins that may be involved in homology-dependent silencing. These have also revealed a complicated web of interactions that implicate other cellular processes, such as NMD and chromatin remodeling in maintenance of silencing. What now becomes essential is to understand the precise mechanisms by which loci emerging from genetic studies contribute to silencing. To accomplish this, we must begin to fit the proteins encoded by these loci into a biochemical model of RNAi/PTGS and related silencing processes.

BIOCHEMICAL DISSECTION OF RNAi

A separate track toward understanding dsRNA-induced silencing has been the development of cell-free extracts that reproduce elements of the silencing process in vitro. Thus far, such studies have been carried out exclusively in *Drosophila*; however, progress is now being made toward the development of similar cell-free systems in plants, *C. elegans,* and fungi.

The first extracts that were reported to respond to dsRNA by repressing the expression of cognate genes were developed from *Drosophila* embryos (Tuschl et al., 1999). In this system, RNAi was initiated by the addition of dsRNA to the embryo extract. A complementary system has emerged from the finding that dsRNA is a potent trigger of gene silencing in cultured *Drosophila* cells (Hammond et al., 2000). In the S2 system, RNAi is provoked in vivo by treatment of cells with dsRNA, and RNAi-related activities are assayed in extracts derived from these cells.

RISC, the effector complex

The first insight to emerge from in vitro studies was that silencing via RNAi was enforced by mRNA degradation (Tuschl et al., 1999; Hammond et al., 2000). Of course, it was previously known that cognate mRNAs were lost upon treatment of organisms with dsRNA triggers; however, numerous mechanistic explanations were possible. Work from both *Drosophila* embryo and S2 cell-free systems indicated that treatment with dsRNA induced the assembly of a nuclease (termed RISC, RNA-induced silencing complex) with the ability to specifically target mRNAs homologous to the trigger for degradation.

The outstanding question was how RISC incorporated information from the dsRNA trigger as a guide to substrate selection. A clue arose from studies of Hamilton and Baulcombe, who were searching for antisense nucleic acids that appeared specifically in plants that were undergoing either transgene cosuppression or virus-induced gene silencing. They found the consistent appearance of small, ~25-nt RNAs that were homologous to the gene that was being silenced (Hamilton & Baulcombe, 1999). This prompted a search for small RNAs that might associate with RISC, as it was clear from in vitro studies that this enzyme had an essential nucleic acid component (Hammond et al., 2000). Indeed, small RNAs of a discrete size (~25 nt) cofractionated with the RNAi-effector nuclease, strongly suggesting that these might be the integral nucleic acid that guided the enzyme to substrate selection. Simultaneously, Zamore and colleagues (2000) made two observations that solidified the model. First, they noted that in their in vitro RNAi reactions, substrates were cleaved with a periodicity that matched the size of the small RNAs (which they correctly sized as ~22 nt). Second, they identified an activity in their extracts that was capable of specifically processing dsRNA triggers into ~22-nt RNAs, which they termed siRNAs (small interfering RNAs). Considered together, these studies sparked the mechanistic model for RNAi that is de-

picted in Figure 1, and led to the realization that the mechanistic basis of dsRNA-induced gene silencing is conserved in evolutionarily diverse organisms.

Biochemical purification of the RNAi-effector enzyme, RISC, has indicated that this is a multiprotein complex of ~500 kDa. It contains both essential protein and nucleic acid subunits (most likely the siRNAs). One protein component of this complex has recently been identified as *Drosophila* Argonaute-2, one of the four Argonaute family members in flies (Hammond et al., 2001b). This finding begins the process of merging the biochemical studies from insects with the genetic studies from a variety of systems.

Dicer, the initiator of silencing

Careful analysis of the siRNAs generated in vitro (and later in vivo) suggested that dsRNA triggers might be processed into ~22-nt RNAs by an RNAse III family enzyme (Bass, 2000; Cerutti et al., 2000; Elbashir et al., 2001). This knowledge, combined with the availability of complete genome sequences (or nearly so) for *Drosophila*, *C. elegans*, *Arabidopsis*, and *Neurospora* allowed biochemical purification, comparative genomics, and candidate gene approaches to the siRNA-generating enzyme. This ultimately led to the identification of a particular family of RNAse III proteins, the Dicer family, as the enzyme responsible for initiating RNAi by processing dsRNA triggers (Bernstein et al., 2001).

Dicer family proteins have an unusual structure comprising an amino-terminal helicase domain, and a carboxy-terminal segment containing dual RNAse III domains and one or more dsRNA-binding motifs. These proteins are evolutionarily conserved and are represented in fungi (*N. crassa*, *Schizosaccharomyces pombe*), plants (*Arabidopsis*), and metazoans (*C. elegans*, *Drosophila*, mammals). Dicer family members also generally contain a PAZ domain (Bernstein et al., 2001).

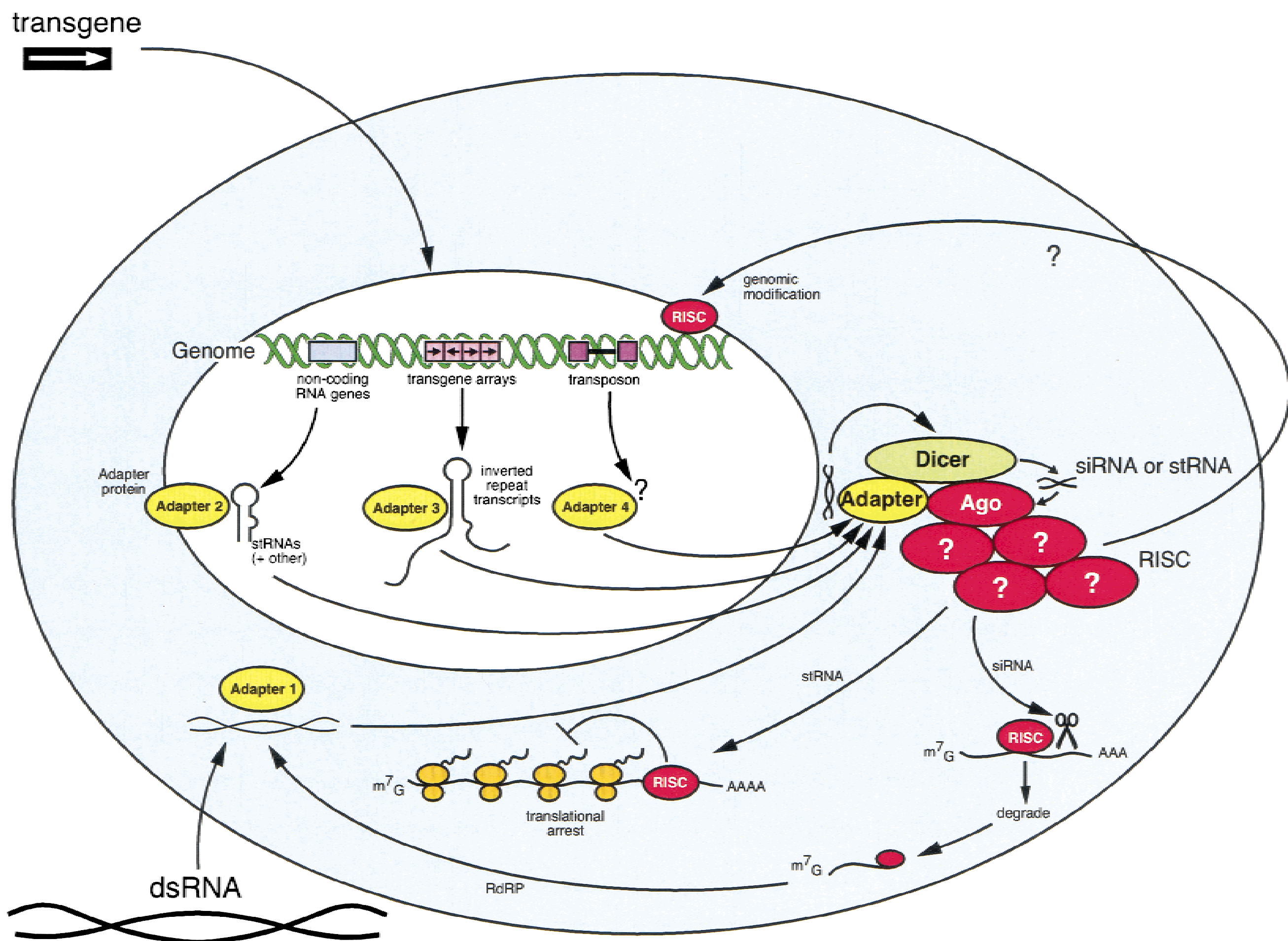

FIGURE 1. A schematic representation of RNAi-related gene silencing pathways is shown. The silencing trigger can be generated by a number of different circumstances (as discussed in the text). A possible explanation for different types of triggers leading to different outcomes is that each may be delivered to Dicer-containing complexes by specific adapter proteins, which interact with specific Argonaute family members. The precise adapter-Argonaute interactions may determine the type of RISC complex that is generated and, thus, the silencing mode—either RNA degradation as in standard RNAi, translational suppression as for stRNAs, or perhaps also genomic modification.

This motif is found in only one other protein family, the Argonaute proteins. This has led to the suggestion that perhaps Argonaute and Dicer interact physically via their PAZ domains, and such an interaction has recently been detected in vivo (Hammond et al., 2001b). Based on this, it has been suggested that Argonaute proteins in RISC transiently recruit Dicer to the effector complex, facilitating incorporation of siRNAs into RISC and effectively link the first and second steps of RNAi (Fig. 1).

These proteins have been examined biochemically in *Drosophila*, humans (Bernstein et al., 2001), and more recently in *C. elegans*, tobacco, and other insect cells (Ketting et al., submitted; A.M. Denli & G.J. Hannon, unpubl.), and all are capable of generating ~22-nt siRNAs from long dsRNAs (although the precise size is species specific). Curiously, Dicer is an ATP-dependent nuclease. This led to the proposal that Dicer may act processively, with the helicase domain using the energy of ATP hydrolysis for translocation along the dsRNA substrate (Bernstein et al., 2001). This model would be consistent with the observed periodic cleavage of mRNA targets in *Drosophila* embryo extracts (Zamore et al., 2000). Consistent with this prediction, examination of Dicer processing products and intermediates reveals a ladder with an ~22 bp periodicity. In the absence of ATP, only the first decrement of the ladder is formed, supporting the hypothesis that ATP drives processive cleavage (Ketting et al., submitted)

Definitive evidence that this protein family is integral to RNAi has come recently from the development of *C. elegans* mutants that contain deletions within their single Dicer gene (Grishok et al., 2001; Knight & Bass, 2001; Ketting et al., submitted). These animals are RNAi-deficient and also display a number of other phenotypes that led to the discovery of a biological function for the RNAi machinery in the regulation of development (see below).

MANY MYSTERIES REMAIN

In many ways, the contributions of genetics and biochemistry to the mechanistic model for RNAi that is presented in Figure 1 have served to highlight our naiveté concerning many of the most interesting aspects of the silencing process. Among these are the ability of silencing to spread throughout some organisms, particularly worms and plants, the heritable nature of silencing in some systems, the mechanisms by which silencing signals are amplified, and the interactions between dsRNA and the genome.

Systemic silencing

One of the most provocative and least understood aspects of dsRNA-induced gene silencing is its ability to spread throughout certain organisms. This property is most evident in *C. elegans* and in plants. In the former case, systemic silencing was initially observed upon injection of dsRNA into the gut; however, it is now clear that exposure of the worms to dsRNA either in solution or by feeding worms with *Escherichia coli* engineered to express dsRNA can provoke a systemic effect (Timmons & Fire, 1998). In worms, little is known about the precise mechanisms that underlie systemic silencing or the mechanisms by which dsRNA is recognized and absorbed from the gut. However, the latter is likely to be an active process, as genetic mutants have been isolated that dissociate uptake from silencing (i.e., worms that can silence following direct injection but that cannot silence upon feeding; R.H.A. Plasterk, pers. comm.).

In plants, the ability of the silencing agent to move within the plant is called systemic acquired silencing (SAS; Fagard & Vaucheret, 2000). As an example of this response, tobacco plants transgenic for the green fluorescent protein (GFP) can be infiltrated with *Agrobacterium tumefaciens* carrying a GFP reporter construct. This results in rapid suppression at the infiltration zone, and, by 18 days postinfiltration, the upper leaves of the plant also silence the GFP transgene (Voinnet & Baulcombe, 1997). However, systemic transmission of silencing was perhaps most strikingly demonstrated by the grafting of a nonsuppressed scion (the upper vegetative tissues) onto a cosuppressed stock (lower tissues and the root system), which resulted in the scion becoming cosuppressed. In fact, in a three-way graft, silencing can be passed between a silenced stock and an engrafted scion through a central stock that completely lacks sequences corresponding to the targeted gene (Palauqui et al., 1997).

The systemic silencing signal from plants has yet to be isolated, or even assayed in a cell-free system (e.g., by using plant extracts to transfer silencing to a nonsilenced recipient). However, it is logical to assume that the systemic, sequence-specific effect results from the migration of a nucleic acid. Obvious candidates are the dsRNA trigger, a replicated and amplified form thereof, or the ~22-nt siRNAs. It remains unknown whether the systemic signal is transported via an active machinery or whether it migrates passively through the plant vasculature. Indeed, it is well established that other types of RNAs, such as viroids, can be disseminated throughout the plant via the phloem (Sijen & Kooter, 2000). Furthermore, plant cells are interconnected via cell–cell junctions known as plasmodesmata. These junctions allow passage of both protein and RNA molecules (e.g., mRNAs) between cells, and are a likely route for local spread of silencing (Lucas, 1995; Lucas et al., 1995).

Although the precise nature of the systemic signal remains unclear, plant viral inhibitors of silencing have provided tools to probe this process. Hc-Pro is a protein inhibitor of PTGS that is expressed by potyviruses (Anandalakshmi et al., 1998). The accumulation of small

RNAs is greatly decreased in the presence of Hc-Pro, but the local and systemic silencing signaling remains (Mallory et al., 2001). This suggests a model in which systemic signaling is independent of siRNA production and raises the possibility that the dsRNA trigger is itself replicated and/or disseminated as the systemic silencing trigger.

The heritable nature of RNAi

The classification of RNAi/PTGS as an epigenetic phenomenon rests largely upon its ability to provoke heritable changes in gene expression. Inheritance of silencing could derive from either of two sources. The first is persistence of the signal. The second is persistence of the silenced state. The former case refers to instances such as stable incorporation of transgene arrays into the genome, the presence of endogenous repetitive elements such as transposons, or the enforced expression of hairpin RNAs. Such cases require no additional mechanisms to explain heritable silencing because the trigger is expressed from an endogenous and heritable genetic element. The latter case is more provocative and requires consideration of mechanisms that propagate either the signal or the silenced state independently of the silencing trigger.

The classical example of silencing that is inherited after a transient introduction of the silencing trigger comes from *C. elegans*. Worms that have been injected with dsRNA can impart the silenced state to the next generation, and this has been demonstrated for numerous genes (Fire et al., 1998). Experiments targeting genes that are expressed in the maternal germline demonstrated interference in the F2 generation; however this waned in later generations (Grishok et al., 2000). Thus far, no genetic mutants have emerged that specifically affect the heritability of silencing without affecting the interference process itself, although alterations in NMD pathways do alter persistence (see above).

Amplification of the silencing signal

One of the striking observations in the original reports of RNAi in *C. elegans* was that silencing could be provoked by very small amounts of dsRNA (Fire et al., 1998). In fact, calculations indicated that complete silencing could be effected with what amounted to only a few molecules of dsRNA per cell in F1 worms. This strongly suggests that the silencing signal is amplified in the course of creating heritable silencing. As discussed above, genetic studies in a number of organisms have identified a candidate enzyme for signal amplification: RNA-dependent RNA polymerases.

In principle, signal amplification could occur via replication of the dsRNA trigger or via replication of the siRNAs. In both *C. elegans* and *Drosophila*, heteroduplex silencing triggers have been used to test the contribution of the sense and antisense strands of the dsRNA to RNAi (Parrish et al., 2000; Yang et al., 2000). In both cases, silencing was much more dependent on the sequence of the antisense strand of the trigger. If either the trigger or the siRNAs were replicated, the expectation would have been that silencing could be provoked equally well by heteroduplex triggers containing mismatches in either strand. Thus, these results indicate that silencing depends upon the homology of the trigger itself and argue against amplification of the signal by replication of the dsRNA or siRNAs. An alternative possibility is that the degradation products that are formed as a result of PTGS could be recognized as aberrant RNAs and formed by RdRPs into silencing triggers. In this scenario, the targeted RNAs themselves form part of the amplification loop.

DOUBLE-STRANDED RNA AND THE GENOME

Thus far, our discussion of dsRNA-induced gene silencing has been confined largely to the posttranscriptional arena. However, it has long been clear from studies in plant systems that phenomena related to RNAi—VIGS and cosuppression—also produce effects at the transcriptional level. Recent studies have supported interactions between dsRNA and the genome, which could serve as the basis for such silencing phenomena. Similar mechanisms may also underlie RIP and/or MIP in fungi; however, evidence addressing this question is lacking. Furthermore, the ability of dsRNA to exert effects on the genome has yet to be discovered in animals.

Double-stranded RNA-induced genomic methylation

Perhaps the most striking evidence that dsRNA can communicate with the genome is that production of dsRNA in plant cells induces methylation of homologous DNA sequences. RNA-directed DNA methylation (RdDM) was first discovered in analyses of plants infected with recombinant viroids (Wassenegger et al., 1994). By introduction of potato spindle tuber viroid vectors into tobacco plants, Pelissier and Wassenegger (2000) found that genomic targets with as few as 30 bp of sequence complementary to the viroid RNA are methylated during infection. In fact, genomic methylation commonly accompanies PTGS. However, if cells are exposed to dsRNA that is homologous to the promoter region, rather than the expressed region of the gene, methylation is also evident and silencing occurs at the transcriptional level.

Mutations in MET1, the major maintenance methylase of *Arabidopsis*, compromises inheritance of dsRNA-induced methylation and persistence of TGS, but does

not affect initial dsRNA-induced methylation or silencing. In plants, virus-induced PTGS is not heritable, whereas transcriptional gene silencing (TGS) is heritable and is correlated with the inheritance of methylation (Jones et al., 2001). In this study, Baulcombe and colleagues analyzed the inheritance of dsRNA-induced silencing. PTGS and TGS were induced in GFP transgenic plants using viral constructs that carried portions of either the GFP coding region (PTGS) or the 35S promoter, which directs GFP expression (TGS). Progeny from these plants were analyzed for GFP silencing and for methylation of the GFP transgene. TGS was inherited in the progeny, whereas PTGS was not. Monitoring the methylation status of the GFP transgene revealed that symmetric methylation was inherited and correlated with heritable promoter silencing. Methylation of the targeted gene in response to dsRNA did not require MET1; however, both heritable silencing and maintenance of methylation in progeny did require an intact MET1 gene. These findings suggest a model in which dsRNA initiates PTGS, and independently, methylation of the genome in a MET1-independent manner. Heritable silencing occurs when methylases, such as MET1, maintain the methylated state following DNA replication through preferential recognition and modification of hemi-methylated DNA.

In most systems, genomic methylation in the promoter region causes silencing via alterations in chromatin structure. Chromatin remodeling complexes are recruited to methylated promoters, ultimately preventing transcription. The mechanisms by which chromatin alterations suppress transcription have been reviewed extensively and will not be discussed here (e.g., Meyer, 2000; Dobosy & Selker, 2001; Finnegan et al., 2001; Rice & Allis, 2001). However, genetic evidence has linked chromatin-remodeling factors to dsRNA-induced transcriptional gene silencing. DDM1 encodes a SWI2/SNF2-related protein (Jeddeloh et al., 1999). This was originally isolated in a screen for hypomethylation of centromeric repetitive DNA. Mutations in this locus show epigenetic anticipation for developmental abnormalities (Kakutani et al., 1995). Initially, effects are mild; however, propagation of homozygous plants ultimately results in severe defects. Phenotypic abnormalities are correlated with loss of methylation at both repetitive and single-copy loci, with the former occurring much more rapidly. Several other studies have shown that ddm1 mutants are defective in genomic imprinting (Vielle-Calzada et al., 1999), in silencing of transposons (Hirochika et al., 2000; Miura et al., 2001; Singer et al., 2001), and in cosuppression of transgenes (Furner et al., 1998).

Clearly, genomic methylation is essential for TGS; however, significant evidence also suggests a role for genomic methylation in PTGS in plants. In fact, mutations that block methylation of silenced loci compromise systemic silencing. Conversely, mutations in components of the PTGS machinery, such as *sgs2* and *ago1* in *Arabidopsis* cause decreased methylation of cosuppressed transgenes (Fagard et al., 2000).

Considered together, the results presented above draw important links between dsRNA and genomic modification in plants, and similar interactions have been proposed in other systems, such as *Neurospora* (e.g., RIP). These have been detected so far exclusively in organisms in which such changes can be marked by genomic methylation. However, similar effects may also exist at the chromatin level in systems such as *Drosophila* and *C. elegans*, in which genomic methylation systems are less active or have yet to be detected.

BIOLOGICAL ROLE OF RNAi

It has always seemed unlikely that dsRNA-induced gene silencing evolved as a convenient tool for biologists. Therefore, since the discovery of this evolutionarily conserved phenomenon, there has been a major push to illuminate the biological function(s) of RNAi. To date, three distinct roles for this process have emerged. First, RNAi clearly acts as an antiviral defense. Second, genetic evidence has implicated RNAi as a geno-protective mechanism. Third, recent findings have demonstrated a role for components of the RNAi machinery in the regulation of cellular gene expression.

Antiviral response

In mammals, there exist well-characterized responses to dsRNA that act as an antiviral defense. Therefore, one obvious role for the RNAi/PTGS machinery was as a functional homolog of such systems. Indeed definitive evidence for the use of RNAi as a viral defense comes from genetic studies in plants. *Arabidopsis* mutants that lose the ability to mount a PTGS response are hyper-susceptible to virus infection. For example, in a study by Vaucheret's group, *sgs2* and *sgs3* mutant plants have been shown to be extremely sensitive to the cucumovirus (CMV; Mourrain et al., 2000).

Just as plants have evolved a defense against viral invasions, viruses have evolved a counterattack. For example, proteins such as cucumber mosaic virus 2b and p25 of potato virus X are able to inhibit the spread of silencing within the plant (see above; Voinnet et al., 2000). As expected if PTGS is considered as a primary defense mechanism against such viruses, these inhibitors are essential determinants of virulence.

Genome defense

In all complex genomes, a significant fraction of sequence is formed by endogenous repetitive elements, including numerous copies of defective and intact transposons. Suppression of these elements contributes to genetic stability in two ways. First, intact transposons

are potential mutagens. Second, both defective and intact transposons provide potential sites for nonhomologous crossovers that could occur during DNA repair. As such, genomic stability requires that they be packaged into heterochromatin. As should be clear from the foregoing discussion, RNAi is often triggered by and is used to silence repetitive transgenes. This raised the possibility that this same mechanism might function to silence endogenous repetitive sequences.

Clear evidence in favor of this hypothesis has come from studies in *C. elegans* (see above) in which RNAi-deficient worms show high rates of transposition. In *Drosophila*, I elements (similar to mammalian LINE elements) can be silenced by previous introduction of transgenes expressing a small region of the transposon (Jensen et al., 1999). Furthermore, in an effort to identify endogenous targets of RNAi in *Drosophila*, sequencing of ~22-nt siRNAs has uncovered guide sequences corresponding to endogenous transposons that move via both RNA and DNA intermediates (Elbashir et al., 2001; A. Caudy & G.J. Hannon, unpubl. data).

These results may indicate that RNAi has evolved in part to protect organisms against both exogenous invaders and from endogenous parasitic nucleic acids. Thus, the evolutionary conservation of RNAi mechanisms may indicate a critical role in maintaining genome stability.

Regulation of developmental timing

Numerous clues have hinted that RNA interference and developmental control may be intimately linked. The first indication came from *Arabidopsis* mutants with mutations in homologs of genes involved in RNAi. For example, alteration of *Carpel Factory* (homologous to *Drosophila* Dicer) and *Argonaute* (homologous to *Drosophila* Ago-2 and *C. elegans* rde-1 genes) both cause developmental abnormalities or embryo lethality, depending upon the allele. In particular, these mutations cause stem cell defects. *Argonaute* mutants have defects in axillary meristem development and infertile flowers, while *Carpel Factory* mutants have floral meristems that are converted to an indeterminate state (Bohmert et al., 1998; Jacobsen et al., 1999). Furthermore, the *Pinhead/Zwille* gene in *Arabidopsis*, also a member of the *Argonaute* family, is required for formation of primary and axillary shoot apical meristems as well as proper floral development (Lynn et al., 1999). Although these genetic studies were suggestive, a concrete link between the RNAi machinery and developmental control has come from studies in *C. elegans*.

Recently, three groups have studied the phenotypic consequences of mutation of the Dicer enzyme, which initiates RNAi by processing long dsRNA triggers into ~22-nt siRNA (Grishok et al., 2001; Knight & Bass, 2001; Ketting et al., submitted). In *C. elegans*, Dicer is represented by a single gene, K12H4.8, and mutations in this gene were identified by screening libraries of randomly mutagenized worms.

The Dicer homozygous mutant (*dcr-1*) in *C. elegans* shows numerous developmental phenotypes including abnormal oocytes and the inability to fertilize eggs; hence these worms are sterile. The production of seam cells at the L4 to adult transition is altered, resulting in animals without alae. Other phenotypes include a protruding and nonfunctional vulva, which tends to burst after the molt from larval to adult stage. Interestingly, Dicer-mutant worms can perform RNAi in the soma. However, this is likely due to the fact that maternal *dcr-1* is sufficient to rescue the inactive zygotic gene in the F1 mutant soma. However, one would predict an absence of Dicer in the germline of adult *dcr-1* homozygotes. Indeed RNAi is compromised in the mutant germline, suggesting that organs devoid of Dicer protein are incapable of performing RNAi (Grishok et al., 2001; Knight & Bass, 2001; Ketting et al., submitted).

Grishok and colleagues also implicated two *Argonaute* family members, *alg-1* and *alg-2* (Argonaute-like genes), in developmental control. Suppression of expression of either of these genes by RNAi produces defects that are very similar to those of *dcr-1*, including burst vulva and a lack of alae. These results suggest that both *Dicer* and *alg* genes act in the same pathway to regulate development.

Worms defective in either Dicer or ALGs are phenotypically similar to worms carrying a mutation in *let-7*. The *let-7* gene produces a noncoding RNA that regulates developmental timing. This precursor RNA forms a stem-loop structure (in essence double-stranded RNA) of approximately 70 bp, which is processed into a mature form that is 21 nt in length. Both let-7 and another such RNA, *lin-4*, have been termed small temporal RNAs, stRNAs. Both are thought to interact with their target genes, including *lin-41* and *lin-28* (which direct developmental progress), at the 3′ UTR and to negatively regulate gene expression at the translational level.

Both *dcr-1* and *alg* mutant animals have increased levels of the unprocessed let-7 RNA and lower levels of mature stRNAs. Biochemical experiments have demonstrated that both *Drosophila* embryo extracts and immunoaffinity purified Dicer protein are capable of processing the let-7 precursor RNA into the mature 21-nt stRNA (Hutvagner et al., 2001; Ketting et al., submitted).

Although these studies make it clear that the RNAi machinery participates in developmental control via processing of small temporal RNAs, they raise a paradox. RNAi exerts its effect via degradation of targeted mRNAs, whereas stRNAs function via regulation of translation (Lee et al., 1993; Reinhart et al., 2000). Thus, we must now strive to understand how similar inputs, full or partial dsRNAs, are processed by a common initiator enzyme, Dicer, to produce distinct effects. One possibility is that stRNAs and siRNAs join distinct effector complexes. Argonaute family members are com-

ponents of the effector complex for RNAi (Hammond et al., 2001b) and are clearly involved in both stRNA and siRNA-mediated repression (Grishok et al., 2001). Perhaps different types of triggers are targeted to specific Argonaute-containing effector complexes by adaptor proteins, which mediate differential recognition of, for example, dsRNAs that trigger RNAi and stRNA precursors. Alternatively some intrinsic feature of the stRNA or siRNA could determine the mode of inhibition. In this regard, whereas siRNAs are perfectly homologous to their targets, stRNAs are not (Reinhart et al., 2000). Biochemical studies of the effector complexes that house both of these types of small regulatory RNAs will be required to provide illumination.

Small temporal RNAs are conserved from worm to fly to humans. This is very suggestive that the RNAi machinery in each of these organisms plays a role in developmental timing. Hutvagner and colleagues (2001) have shown that targeted degradation of human Dicer leads to the accumulation of the let-7 precursor in cultured human HeLa cells. It now becomes incumbent to determine whether the RNAi machinery indeed regulates development in mammals.

Ironically, small temporal RNAs have not yet been identified in plants, in which mutations in RNAi components first led to the hypothesis that this process controlled development. Thus, it must now be investigated whether the use of small endogenous RNAs as regulators of developmental processes extends to these organisms. In addition, the possibility remains open that let-7 represents the tip of the iceberg and that numerous other small RNAs will emerge as regulators of cellular gene expression programs.

SUMMARY

Over the past several years, RNAi and its related phenomena have emerged not only as a powerful experimental tool but also as a new mode of gene regulation. Through a combination of genetic and biochemical approaches we have learned much about the mechanisms underlying dsRNA responses. However, many of the most intriguing aspects of dsRNA-induced gene silencing have yet to be illuminated. What has become abundantly clear is that the complex and highly conserved biology underlying RNA interference is critical both for genome maintenance and for the development of complex organisms. However, it seems probable that we have only begun to reveal the diversity of biological roles played by RNAi-related processes.

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