The rde-1 Gene, RNA Interference, and Transposon Silencing in C. elegans

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Summary

Double-stranded (ds) RNA can induce sequence-specific inhibition of gene function in several organisms. However, both the mechanism and the physiological role of the interference process remain mysterious. In order to study the interference process, we have selected C. elegans mutants resistant to dsRNA-mediated interference (RNAi). Two loci, rde-1 and rde-4, are defined by mutants strongly resistant to RNAi but with no obvious defects in growth or development. We show that rde-1 is a member of the piwi/sting/argonaute/zwille/eIF2C gene family conserved from plants to vertebrates. Interestingly, several, but not all, RNAi-deficient strains exhibit mobilization of the endogenous transposons. We discuss implications for the mechanism of RNAi and the possibility that one natural function of RNAi is transposon silencing.

Introduction

Recently, double-stranded (ds) RNA has been shown to be a potent sequence-specific inhibitor of gene function in the nematode C. elegans (Fire et al., 1998). Similar dsRNA-triggered phenomena have now been reported in plants, trypanosomes, Drosophila, and planaria (Kennerdell and Carthew, 1998; Ngo et al., 1998; Waterhouse et al., 1998). The discovery that dsRNA can induce genetic interference in organisms from several distinct phyla suggests a conserved mechanism and perhaps a conserved physiological role for the interference process.

Several observations have suggested that RNAi in C. elegans targets a posttranscriptional event (Fire et al., 1998; Montgomery and Fire, 1998; Montgomery et al., 1998; Tabara et al., 1998). For example, promoter sequences and introns are not effective at inducing interference in C. elegans (Fire et al., 1998), and individual cistrons within a polycistronic message can be interfered with separately (Montgomery et al., 1998). These observations argue against an effect on initiation or elongation of transcription. The steady-state levels of mature cytoplasmic transcript (Fire et al., 1998) as well as a nuclear transcript (Montgomery et al., 1998) are significantly reduced, suggesting that interference may target the nascent mRNA for destruction. Studies of dsRNA-induced interference in plants and trypanosomes have led to similar conclusions (Ngo et al., 1998; Waterhouse et al., 1998). In addition to a mechanism for degrading transcripts, several observations suggest that RNAi involves other active processes. For example, dsRNA delivered by microinjection into the intestine exerts interference effects in tissues throughout both the injected animal and its progeny (Fire et al., 1998), suggesting the existence of activities that transport and perhaps amplify the interfering agent. Similar persistence and spreading effects have been reported for posttranscriptional gene silencing (PTGS) in plants (reviewed in Vaucheret et al., 1998).

Although the phenomenology of gene silencing appears to be remarkably similar in diverse organisms, a direct comparison of the underlying genetic mechanisms is still lacking. Genetic screens have recently been reported for a loss of PTGS in plants and for loss of a related phenomenon called Quelling in Neurospora (Corgini and Macino, 1997; Elmayan et al., 1998). Interestingly, qde-1, the first Quelling-defective mutant identified to date, encodes a gene related to RNA-dependent RNA polymerase, suggesting a possible role for RNA synthesis in this gene silencing process (Corgini and Macino, 1999).

Here, we report the identification of RNA interference-deficient (rde) mutants in C. elegans. Mutations that greatly reduce or abolish RNA interference arise at frequencies expected for simple recessive loss-of-function mutations. rde-1 and rde-4 mutants appear to completely lack an interference response to several dsRNAs tested but are, nevertheless, healthy and viable under laboratory conditions, suggesting that at least some steps in the interference process are nonessential. We show that rde-1 encodes a novel member of an ancient gene family that includes plant, C. elegans, Drosophila, and vertebrate homologs. Genetic studies have implicated several rde-1 family members in germline maintenance and development (Bohmer et al., 1998; Cox et al., 1998; Moussian et al., 1998; Lynn et al., 1999). Interestingly, one family member, the Drosophila gene sting, has been implicated in meiotic drive and genetic silencing of the X-linked repetitive Stellate locus (Schmidt et al., 1999). A rabbit protein from this gene family, elf2C (Zou et al., 1998), has been implicated in translation initiation, suggesting a possible link to translational control. Finally, we show that several RNAi-deficient mutants exhibit mobilization of transposable elements in the germline, raising the possibility that transposon silencing may be one natural function of RNAi.
shown that feeding worms *E. coli* which express a dsRNA, or simply soaking worms in dsRNA solution, are both sufficient to induce interference in *C. elegans* (Tabara et al., 1998; Timmons and Fire, 1998). To carry out a selection, we optimized the feeding method to *mut-2* and *mut-7*. We found that independently outcrossed *mut-2*(*r459*) mutator strains TW410 and MT3126 deliver interfering RNA for an essential gene, *pos-1* (see Experimental Procedures).

We used microinjection to assay the sensitivity of each deficient *C. elegans* mutant. For the most part, these mutant strains were fully sensitive to RNAi (see Experimental Procedures). We did, however, find RNAi resistance in two strains that had previously been shown to exhibit elevated levels of transposon mobilization: *mut-2* (described in Collins et al., 1987) and *mut-7* (described in Ketten et al., 1999 [this issue of Cell]). Another mutator strain, *mut-6(*st702)*, was fully sensitive to RNAi (see Experimental Procedures). Since mutator strains continually accumulate mutations, the resistance of *mut-2* and *mut-7* could conceivably have reflected the presence of secondary mutations. To test this possibility, we examined the genetic linkage between the mutator and RNAi resistance phenotypes of *mut-2* and *mut-7*. We found that independently outcrossed *mut-2*(*r459*) mutator strains TW410 and MT3126 both showed resistance to RNAi. We mapped the RNAi resistance phenotype of *mut-7*(*pk204*) to the center of linkage group III (Figure 1B), the position that had been defined for the mutator activity of *mut-7*(*pk204*) by Ketting and colleagues (1999). Taken together, these observations suggested that the RNAi resistance phenotypes of the *mut-2* and *mut-7* strains were genetically linked to their mutator activities. The *rde* and *mut* mutations appeared to be simple recessive mutations with the exception of *mut-2*(*r459*), which appeared to be weakly dominant (Figure 2A).

Distinct Properties of RNAi-Deficient Mutants

We used microinjection to assay the sensitivity of each strain to several distinct dsRNA species. The *pos-1* and *par-2* genes are expressed in the maternal germline and are required for proper embryonic development (Boyd et al., 1996; Tabara et al., 1999). All *rde* strains tested (as well as *mut-2* and *mut-7*) showed significant resistance to dsRNA targeting these germline-specific genes (Figure 2B), as well as to several other germline-specific genes tested (Experimental Procedures and data not shown).

In order to examine the effect of these mutations on interference targeting somatically expressed genes, we injected dsRNA targeting the cuticle collagen gene *sqt-3* and the body muscle structural gene *unc-22*. *sqt-3* hypomorphic mutants exhibit a short, dumpy body shape (van der Keyl et al., 1994). *unc-22* mutations exhibit severe paralysis with a distinctive body twitching phenotype (Moerman et al., 1986). *rde-1*, *rde-3*, *rde-4*, and *mut-2* strains showed strong resistance to both *sqt-3* and *unc-22* dsRNA, while *rde-2* and *mut-7* strains showed partial resistance (Figure 2C and data not shown). Thus *rde-2* and *mut-7* appeared to be partially...
tissue or gene specific in that they were required for effective RNAi against germline but not somatically expressed genes. The rde-1, rde-3, rde-4, and mut-2 (+) activities appeared to be required for interference for all genes analyzed.

We next asked whether the newly identified rde mutants also exhibited transposon mobilization. We found that two new mutants, rde-2 and rde-3, exhibited a level of transposon activation similar to that of mut-7 (Table 1). In contrast, we did not observe transposon mobilization for rde-1 or rde-4 (Table 1).

Mutator strains (including mut-2, mut-7, rde-2, and rde-3) exhibit a second phenotype: a high incidence of males reflecting an increased frequency of X chromosome loss during meiosis (Collins et al., 1987; Ketting et al., 1999; this work). This phenotype was not observed with the rde-1 and rde-4 strains, which showed a wild-type incidence of males (Table 1).

A previously described gene silencing process appears to act on transgenes in the germline of C. elegans. Although the silencing mechanisms are not well understood, they are known to depend on the products of the genes mes-2, mes-3, mes-4, and mes-6 (Kelly and Fire, 1998). To examine the possibility that the RNAi and germline transgene silencing might share common mechanistic features, we first asked whether the mes mutants were resistant to RNAi. We found normal levels of RNA interference in each of these strains (see Experimental Procedures). We next asked whether RNAi-deficient strains were defective in transgene silencing. Three strains were analyzed: mut-7(pk204), rde-1(ne219), and rde-3(ne299).

### Table 1. Transposon Mobilization and Male Incidence in rde and mut Strains

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Percentage of Non-Unc Revertants</th>
<th>Percentage of Male Animals</th>
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<tbody>
<tr>
<td>unc-22 (r765::Tc4) rde-1 (ne219)</td>
<td>0.0 (0/2000)</td>
<td>0.21 (2/934)</td>
</tr>
<tr>
<td>rde-2 (ne221)</td>
<td>0.0 (0/4000)</td>
<td>0.07 (1/1530)</td>
</tr>
<tr>
<td>rde-3 (ne298)</td>
<td>0.96 (8/830)</td>
<td>3.2 (25/788)</td>
</tr>
<tr>
<td>rde-4 (ne299)</td>
<td>1.6 (35/2141)</td>
<td>7.8 (71/912)</td>
</tr>
<tr>
<td>mut-7 (pk204)</td>
<td>0.0 (0/2885)</td>
<td>0.24 (5/2055)</td>
</tr>
</tbody>
</table>
rde-2(ne221). The mut-7 strain was analyzed most extensively and was found to exhibit desilencing of three different germline transgenes tested (Table 2). The rde-2 strain exhibited a similar level of desilencing for a single transgene (data not shown). In contrast, no transgene desilencing was observed in rde-1 mutants (Table 2). Thus, mut-7 and rde-2, which differ from rde-1 in having transposon mobilization and a high incidence of X chromosome loss, also differ from rde-1 in their ability to partially reactivate silent germline transgenes.

rde-1(-) and rde-4(+) Activities Appear to Be Required in the Target Tissue

The above experiments suggest that rde-1 and rde-4 differ from other RNAi-deficient strains both in their lack of transposon mobilization and lack of chromosome loss. We considered whether these differences might reflect a role for these genes in upstream events, such as dsRNA uptake, transport, or stability. Such events could be required for interference induced by exogenous trigger RNAs but might be dispensable for natural functions of RNAi. To evaluate these upstream events, we exposed rde-1 and rde-4 homoyzgotes to dsRNA and then waited until the next generation to score for interference. dsRNA targeting the unc-22 gene was injected into the intestinal cells of homozygous rde-1 and rde-4 hermaphrodites, and the injected animals were then mated to wild-type males (Figure 3). The self-progeny for both strains exhibited no interference with the targeted gene. However, we found that rde-1(-) and rde-4(+) cross-progeny exhibited potent interference (Figure 3). These observations indicated that rde-1 and rde-4 mutants have intact mechanisms for transporting the interference effect from the site of injection (the intestine) into the embryos of the injected animal and then into the tissues of the resulting progeny. The stability of the resulting interference also appeared to be normal in rde-1 and rde-4, as the homozygous injected mothers continued to produce affected cross-progeny for several days after the time of injection.

Next we asked whether rde-1 and rde-4 mutants could block interference caused by dsRNA expressed directly in the target tissue. We used the muscle-specific promoter from the myo-3 gene (Dibb et al., 1989) to drive the expression of both strands of the muscle structural gene unc-22 in the body wall muscles (Moerman et al., 1986; Fire et al., 1991). Wild-type animals bearing this transgene exhibit a strong twitching phenotype consistent with unc-22 interference. We found that this twitching phenotype was strongly suppressed by both rde-1 and rde-4 mutants (Table 3). The mut-7 and rde-2 mutants, which are both sensitive to unc-22(RNAi) by microinjection, were also sensitive to promoter-driven unc-22 interference in the muscle (Table 3). Taken together, these findings suggest that rde-1(-) and rde-4(+) activities are not necessary for uptake or stability of the interfering RNA and may function directly in the target tissue.

**Table 2. Reactivation of Silenced Transgenes in the Germline of mut-7(pk204)**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Transgene Array</th>
<th>Percentage of Germline Desilencing</th>
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<tbody>
<tr>
<td>+/-</td>
<td>ccEx7271</td>
<td>8.3 (4/48)</td>
</tr>
<tr>
<td>mut-7/+</td>
<td>ccEx7271</td>
<td>14.5 (7/48)</td>
</tr>
<tr>
<td>mut-7/mut-7</td>
<td>ccEx7271</td>
<td>91.0 (71/78)</td>
</tr>
<tr>
<td>+/-</td>
<td>jhEx1070</td>
<td>3.9 (2/51)</td>
</tr>
<tr>
<td>mut-7/mut-7</td>
<td>jhEx1070</td>
<td>86.5 (32/37)</td>
</tr>
<tr>
<td>+/-</td>
<td>cclin4810</td>
<td>6.3 (2/34)</td>
</tr>
<tr>
<td>mut-7/mut-7</td>
<td>cclin4810</td>
<td>73.3 (33/45)</td>
</tr>
<tr>
<td>rde-1/rde-1</td>
<td>ccEx7271</td>
<td>0 (0/34)</td>
</tr>
</tbody>
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GFP reporter transgenes were introduced into different genetic backgrounds as described in Experimental Procedures. Activation of GFP transgene expression in germ cells was assayed at 25°C by fluorescence microscopy. ccEx7271 is a highly repetitive array carrying >100 copies of pBK48 (GFP-tagged version of a ubiquitously expressed gene, let-850). jhEx1070 is a low-copy, “complex” array carrying pJH3.92 (GFP-tagged version of the maternal gene pie-1). cclin4810 is a low-copy, “complex” array that has been integrated into the X chromosome; this array carries plasmid pJ K380.4 (GFP-tagged version of the C. elegans nuclear laminin gene lam-1).
likely to contain the gene. We then used yeast artificial chromosome clones (YACs) containing C. elegans DNA from this interval to rescue the rde-1 mutant phenotype. To facilitate this analysis, we cojected the candidate rescuing YACs along with plasmids designed to express unc-22(RNAi). We found that two overlapping YAC clones provided rde-1 rescuing activity as indicated by unc-22 genetic interference with characteristic body paralysis and twitching in the F1 and F2 transgenic animals. In contrast, a nonoverlapping YAC clone failed to rescue, resulting in 100% nonwitting transgenic strains (Figure 4 and data not shown). The rescuing activity was further localized to two overlapping cosmid clones and finally to a single 4.5 kb genomic PCR fragment predicted to contain a single gene, designated K08H10.7. The K08H10.7 PCR product gave strong rescue when amplified from wild-type genomic DNA. This rescue was greatly diminished using a PCR fragment amplified from any of the three rde-1 alleles and was abolished by a 4 bp insertion at a unique NheI site in the rde-1 coding region (data not shown). A wild-type PCR product from an adjacent gene, C27H6.4, also failed to rescue. Finally, we sequenced the K08H10.7 gene from each of the rde-1 mutant strains and found distinct point mutations predicted to alter coding sequences in K08H10.7 (Figure 4). Based on these findings, we conclude that rde-1 is the K08H10.7 gene.

We next determined a full-length cDNA sequence for rde-1 from the cDNA clones yk296b10 and yk595h5 (see Experimental Procedures). The rde-1 cDNA sequence was used to generate a conceptual translation product, referred to as RDE-1, consisting of 1020 amino acids. This predicted protein was used to query GenBank and identified numerous related genes in C. elegans as well as other animals and plants. This gene family includes at least 23 predicted C. elegans genes, several of which appear to be members of conserved subfamilies. Within subfamilies, conservation extends throughout the protein, and all family members have a carboxy-terminal region that is highly conserved (Figure 4).

There are no defined functional motifs within this gene family, but members, including RDE-1, are predicted to be cytoplasmic or nuclear by PSORT analysis (Nakai and Horton, 1999). Furthermore, one family member named elf2C has been identified as a component of a cytoplasmic protein fraction isolated from rabbit reticulocyte lysates. The RDE-1 protein is most similar to the rabbit elf2C. However, two other C. elegans family members are far more similar to elf2C than is RDE-1 (Figure 4 and data not shown).

The rde-1 mutations appear likely to reduce or eliminate rde-1(+) activity. Two rde-1 alleles, ne219 and ne297, are predicted to cause amino acid substitutions within the RDE-1 protein and were identified at a frequency similar to that expected for simple loss-of-function mutations (see Experimental Procedures). The rde-1(ne219) lesion alters a conserved glutamate to a lysine (Figure 4). The rde-1(ne297) lesion changes a nonconserved glycine, located four residues from the end of the protein, to a glutamate (Figure 4). The third allele, ne300, contains the strongest molecular lesion and is predicted to cause a premature stop codon prior to the most highly conserved region within the protein (Q > Ochre in Figure 4). Consistent with the idea that rde-1(ne300) is a strong loss-of-function mutation, we found that when placed in trans to a chromosomal deficiency the resulting deficiency trans-heterozygotes were RNAi deficient but showed no additional phenotypes (see Experimental Procedures). These observations suggest that rde-1 alleles are simple loss-of-function mutations affecting a gene required for RNAI that is otherwise nonessential.

Discussion

Genetic Analysis of RNAI in C. elegans

Double-stranded RNA has been shown to be a potent activator of sequence-specific genetic interference in C. elegans as well as several other organisms. In C. elegans, this interference process is so robust that exposure of the animals to dsRNA in their environment is sufficient to induce genetic interference (Timmons and Fire, 1998; Tabara et al., 1998). We have taken advantage of this remarkable sensitivity of C. elegans to dsRNA to select mutant strains that are resistant to RNAI interference. In this study, we have described seven rde mutants that define four loci.

rde-1 and rde-4 mutants are strongly deficient in RNAI but exhibit no other apparent phenotypes. The mutants grow normally and are fertile. Alleles of rde-1 were identified at “knockout frequency”, and genetic and molecular analysis of rde-1(ne300) suggests that this allele may represent a null allele. These findings suggest that at least some genes involved in RNAI are nonessential. Two other rde loci are defined by single alleles, indicating that our forward genetic screens are far from saturation. RNAI in C. elegans, PTGS in plants, and “Quelling” in Neurospora appear to share several features and may represent related phenomena (for review, Montgomery and Fire, 1998; Fire, 1999; Sharp, 1999). Genetic screens for a lack of PTGS in Arabidopsis (Elmayan et al., 1998) and for a lack of “Quelling” in Neurospora (Cogoni and Macino, 1997) have also resulted in viable mutants that apparently fail to carry out the silencing process. Our findings suggest that RNAI, like PTGS and Quelling, is dispensable at least under the conditions of laboratory growth.

rde-1 Is a Member of a Large Gene Family

The rde-1 gene family includes members in plants and animals and in the fission yeast S. pombe. Genetic studies in Arabidopsis and Drosophila have implicated rde-1 homologs in developmental regulation and germline maintenance (reviewed in Benfey, 1999). One Arabidopsis homolog, named zwille(= pinhead), acts to maintain shoot apical meristem as undifferentiated stem cells (Moussian et al., 1998). Another Arabidopsis homolog, argonaute 1, has overlapping functions with zwille (Bohmert et al., 1998; Lynn et al., 1999). One Drosophila homolog, sting, was identified as a mutant locus that causes derepression of the normally silent repetitive Stellate locus (Schmidt et al., 1999). The sting mutants also exhibit male sterility and maternal effect lethality. The sting phenotype could reflect a role for this gene in a PTGS mechanism that acts on Stellate transcripts and perhaps other transcripts whose silencing must be maintained for normal male and female fertility (Schmidt et al., 1999). The Drosophila gene sting is most similar to two C. elegans genes, D2030.6 and C01G5.2, and to
Figure 4. Cloning and Sequence Analysis of rde-1

(A) Physical map of rde-1 region. Overlapping clones of C. elegans YAC and cosmid DNA were tested for rescue. Positive clones are indicated by an asterisk. A minimal, 25 kb rescuing interval defined by the overlap between cosmids T10A5 and C27H6 is shown expanded beneath the YAC and cosmid map. Predicted genes within this sequenced interval are illustrated above and below the hatched line. The sequence information was used to predict PCR primers for amplifying individual genes in the interval. A single, rescuing, 4.5 kb PCR fragment containing the K08H10.7 predicted gene is shown enlarged. Exon and intron (box/line) boundaries are shown as well as the positions of rde-1 point mutation in the predicted coding sequences.

(B) Alignment of RDE-1 protein with four related proteins. The sequences are RDE-1 (C. elegans), F48F7.1 (C. elegans), eIF2C (rabbit), ZWILLE (Arabidopsis), and Sting (Drosophila). Identities within these sequences are shaded in gray. Other related genes, including ARGONAUTE 1 (Arabidopsis), SPCC736.11 (S. pombe), and Piwi (Drosophila), also share the homology (data not shown). A portion of the N-terminal region of RDE-1 shows no significant similarity and is not shown in this figure.
a second Drosophila gene, piwi. In a previous study, the piwi gene was shown to be required for germline maintenance in Drosophila, and RNAi targeting the C. elegans gene D2030.6 was shown to cause a mild defect in fertility of C. elegans hermaphrodites (Cox et al., 1998). The observation that sti1 and rde-1 genes both appear to be required for gene silencing mechanisms raises the interesting possibility that members of this novel gene family have conserved cellular functions. It will be important in the future to determine whether the role of several family members in germline maintenance in diverse organisms reflects a conserved biochemical function in germline-specific genetic suppression mechanisms.

The analysis of one homolog of rde-1, rabbit elf2c, provides a possible connection between RNAi and translation initiation. The activity of the elf2c protein is not known. However, elf2c was isolated as a major component of a cytoplasmic protein fraction that stimulates the formation of a ternary complex between the Met-tRNA, GTP, and the eukaryotic peptide chain initiation factor 2 (eIF2). The protein fraction that includes elf2c (previous name: Co-elf2-2A) also stabilizes this ternary complex in the presence of mRNA (Roy et al., 1988; Zou et al., 1998). In vertebrates, the introduction of dsRNA induces a general repression of mRNA translation through a signal cascade that activates an elf2c kinase (reviewed in Proud, 1995). This kinase in turn phosphorylates and inactivates elf2c, causing a rapid and general shutdown of translation in cells exposed to dsRNA. One possibility is that RDE-1 provides an avenue for the sequence-specific inhibition of translation initiation in response to dsRNA. For example, RDE-1 may be brought to a target mRNA via association with the interfering sequence; it might then displace elf2c or directly block the association of the translation initiation complex, preventing translation of the target mRNA. Of course, several other possibilities exist, including the possibility that RDE-1 and elf2c utilize their conserved domains to interact with factors whose functions are unrelated to the control of mRNA translation.

RNAi and Transposon Silencing

In this study, we have shown that mutations in four genes, mut-2, mut-7, rde-2, and rde-3, reduce RNAi while increasing mobilization of transposons. This observation is tantalizing, as it suggests a possible biological role for RNAi in transposon silencing. Consistent with this hypothesis, results from several organisms suggest that gene silencing mechanisms may have evolved to suppress viral and transposon pathogens. For example, a recent report has linked transposon silencing in Drosophila to a posttranscriptional silencing process known as cosuppression (Jensen et al., 1999; reviewed in Birchler et al., 1999). In addition, studies in plants have implicated viral protein products as inhibitors of the plant’s cosuppression mechanism (Anandalakshmi et al., 1998; Beclin et al., 1998; Brigueti et al., 1998; Kasschau and Carrington, 1998). It is therefore reasonable to speculate that RNAi could have evolved (at least in part) as a defense mechanism to protect the germline from the accumulation of transposons (see also Ketting et al., 1999).

While it is tempting to speculate that transposon silencing is a natural target of RNAi, two observations suggest a degree of additional complexity. First, RNAi is intact in the mut-6 mutator strain (this work) and in several mutator strains described by Ketting et al. (1999). The mut-6 strain differs from mut-2 and mut-7 in that transposon activation appears to be restricted to the Tc1 transposon. Furthermore, the mutator activity of mut-6 may itself be mobile, raising the possibility that the mut-6 gene encodes a mutated Tc1 element (Mori et al., 1988). In contrast, mut-2 and mut-7, which activate not only Tc1 but also other transposons (Collins et al., 1987; Yuan et al., 1991; Collins and Anderson, 1994; Ketting et al., 1999), may represent mutations that damage a general host mechanism for suppressing all transposons. If the mut-6 lesion involves a change in a Tc1 transposon, then the question remains whether and how the mutated transposon could escape the hypothetical RNAI-based host surveillance mechanism.

Additional complexity in the connection between RNAi and transposon silencing comes from the observation that rde-1 and rde-4 mutants do not exhibit a detectable mobilization of transposons. The rde-1 and rde-4 mutants are strongly defective in RNAi, and their wild-type activities appear to be required directly in the target tissue. Thus, RNAI appears to be neither sufficient (in mut-6) nor necessary (in rde-1 and rde-4 mutants) to prevent transposon activity. The fact that rde-1 is a member of a large gene family points to further potential complexity. For example, although rde-1 and rde-4 appear to be necessary for interference with all genes analyzed in the present study, it is possible that specialized or redundant activities, perhaps including distinct rde-1 family members, have evolved to mediate transposon silencing. The mut-2, mut-7, rde-2, and rde-3 genes might then encode factors required for other upstream or downstream steps that mediate both the general and transposon-specific interference mechanisms.

Cross-Talk between Gene Silencing Mechanisms

Transgene silencing in C. elegans exhibits properties consistent with silencing at a transcriptional level (Kelly et al., 1997), while RNA interference appears to occur at a posttranscriptional step (Fire et al., 1998; Montgomery et al., 1998). Thus, the observation that mut-7 activity is required both for RNAi and for transgene silencing in the germline of C. elegans could indicate a mechanistic connection between these otherwise seemingly distinct gene silencing pathways. Consistent with this idea, studies of transcriptional and PTGS mechanisms in plants, fungi, and animals have suggested possible connections or feedback between these pathways (reviewed in Elmayan et al., 1998; Wassenneger and Pelissier, 1998; Selker, 1999; Sharp, 1999). In light of the current study, it is interesting to note that mutants in the Drosophila rde-1 homolog, sting, appear similar to mut-2, mut-7, rde-2, and rde-3. All of these strains exhibit defects in gene silencing and also cause increased chromosome loss (Collins et al., 1987; Ketting et al., 1999; Schmidt et al., 1999; this study). These findings suggest that direct or indirect connections exist between the activities of these genes and the regulation of chromosome behavior.

While it is possible that posttranscriptional silencing could directly trigger chromosomal effects including
transcriptional silencing, it is also possible that these are mechanistically unrelated pathways that exert indirect effects upon one another. For example, chromosomal mechanisms that suppress transcription of repetitive elements in the genome (for review, Henikoff, 1998) might be required to prevent an accumulation of non-specific dsRNA that would otherwise saturate machinery needed for targeted RNA interference. Alternatively, DNA- or chromatin-based gene silencing mechanisms might suppress many types of transposons or viral pathogens. Once activated, a family may in turn express gene products that block RNA-dependent gene silencing mechanisms. As discussed above, studies in plants have already indicated that viral pathogens have evolved in competition with host silencing mechanisms. It is likely that host defense strategies have also evolved and may include several distinct mechanisms with partially overlapping sets of target pathogens. Perhaps invertebrates, plants, and fungi have evolved sophisticated RNA- and DNA-based mechanisms that provide pathogen-specific immunity. Considering the selective pressure that DNA and RNA parasitoids are likely to exert on all organisms, we may find that sequence-based immunity mechanisms are as sophisticated and as highly evolved as the antigen/receptor-based immunity mechanisms found in vertebrates.

Ketting and colleagues have shown that mut-7 encodes a protein with similarity to the exonuclease domain of ribonuclease D. This homology could indicate that mut-7 functions in RNAi by directly degrading the target mRNA. However, as discussed by Ketting et al. (1999), the motif found in mut-7 is also present in many other proteins, including proteins with DNA exonuclease activity (Moser et al., 1997). Thus, mut-7 might be involved in the RNA-based or DNA-based mechanisms discussed above. The molecular identification of additional rde and mut genes as well as genes with related functions in other organisms will likely lead to a much better understanding of the mechanism and physiological role of RNA interference.

Experimental Procedures

Strains and Alleles

The Bristol strain N2 was used as standard wild-type strain. The marker mutations and deficiencies used are listed by chromosomes as follows. LGI: dpy-14(e188), unc-13(e51); LGII: dpy-17(e164), unc-32(e189); LGV: dpy-11(e224), unc-42(e270), daf-11(m87), eDF1, mDF3, nDF31, sDF19, sDF35, unc-76(e911). The C. elegans strain DR13 was used to generate hybrids for STS linkage mapping (Williams et al., 1992). Sensitivity to RNAi was tested in the following strains during the course of this work. MT3126: mut-2(4r59); dpy-19(n3147), TW410: mut-2(4s59) semi-4(n1378), NL917: mut-7(pk204), S5552: mes-2(bn76) rol-1(e911)/rmC1, S5449: mes-3(bn88) dpy-1(e611); HDP20, S5268: dpy-11(e224) mes-4(bn23) unc-76(e911)/nt1, S5360: mes-6(bn66) dpy-20(e1282)/nt1, CB789: him-1(e879). A non-unc mut-6 strain used was derived from RW7096, mut-6(st702) unc-22 (st192::Tc1), due to the loss of Tc1 insertion in unc-22. Strains S5449 and S5552 were gifts from S. Strome.

Homozygous mutants of mut-6, mes-2, mes-3, mes-4, mes-6, and him-1 showed sensitivity to RNAi by injection of pos-1 dsRNA. The dose of injected RNA was about 0.7 mg/ml, and this dose is lower than that used in Figure 2 and lies within the range where reduced concentration leads to reduced interference effects. The results (dead embryos/F1 progeny) were as follows: mut-6, 422/437; mes-2, 781/787; mes-3, 462/474; mes-4, 810/814; mes-6, 900/1002; him-1, 241/248; N2 (control), 365/393.

To test mutator activity, we used TR1175: unc-22(r765; Tc4) that was caused by Tc4 transposon insertion. Strains TW410 and TR1175 were gifts from Q. Boese and J. Collins.

RNA Interference Assay

RNAi by microinjection was performed as described in Fire et al. (1998) and Rocheleau et al. (1997). pos-1 dsRNA clone yk61H1, par-2 cDNA clone yk96H7, and sq-3 cDNA clone yk75F2 were used to prepare dsRNA in vitro. These cDNA clones were obtained from the C. elegans cDNA project (Y. Kohara).

RNAi by feeding was performed as described in Timmons and Fire (1996). pos-1 dsRNA was cloned into a plasmid that contains two T7 promoter sequences arranged in head-to-head configuration. The plasmid was transformed into an E. coli strain, BL21(DE3), and the transformed bacteria were seeded on NGM plates containing 60 μg/ml ampicillin and 80 μg/ml IPTG. The bacteria was grown overnight at room temperature to induce pos-1 dsRNA. Seeded plates stored at 4°C remained effective for inducing interference for up to 2 weeks. To test RNAi sensitivity, C. elegans larvae were transferred onto BL21(DE3)[dsRNA] plates, and embryonic lethality was assayed in the next generation.

Transgenic lines expressing interfering RNA for unc-22 were engineered using a mixture of three plasmids: pPD[L4218] (unc-22 antisense segment, driven by myo-3 promoter); pPD[L4218] (corresponding unc-22 sense segment, driven by myo-3 promoter); and pRF4 (semidominant transformation marker). DNA concentrations in the injected mixture were 100 μg/ml each. Injections were as described (Mello et al., 1991; Mello and Fire, 1995).

Isolation of RNAi-Defective Mutants

The genetic screen used was similar to one designed by J. James R. Preiss for the identification of maternal effect mutants (Kemphues et al., 1988). An Egl strain, lin-2(e1309), was mutagenized with EMS, and the F2 generation was cultured on a bacterial lawn expressing pos-1 dsRNA. Mutagenized population were then screened for rare individuals that were able to produce complete broods of viable progeny forming a distinctive “bag of worms” phenotype. To make sure that the animals were truly resistant to RNAi, candidate strains were next assayed for resistance to RNAi by injection. Independent EMS-induced alleles of rde-1 were found in two separate pools of mutagenized animals at a frequency of approximately one allele in 2000 to 4000 haploid genomes.

In addition, we searched for spontaneous mutants using a mut-6 strain in which Tc1 transposons are activated (Mori et al., 1988). One hundred thousand mut-6; lin-2 animals (Mello et al., 1994) were cultured on bacteria expressing pos-1 dsRNA. After one generation of growth, surviving animals were transferred again to plates with bacteria expressing the dsRNA and screened for resistant mutants. Three resulting strains were genetically mapped. One of these strains (ne300) mapped to LGV and failed to complement each other, defining the rde-1(ne219). Two strains, ne299 and ne301, mapped to LGII and define the rde-4 complementation group. Because the screen was clonal in nature and involved rounds of enrichment, it remains possible that both rde-4 strains are related.

Genetic Analysis and Mapping of RNAi-Defective Mutations

To map the RNAi-defective mutations, the RNAi-resistant phenotype was assayed either by feeding bacteria expressing pos-1 dsRNA or by injection of a dsRNA mixture of pos-1 and unc-22. The same assays were used for complementation tests. In vivo expression of unc-22 dsRNA was also used for mapping of rde-1 using visible marker mutations that were performed as described in Brenner (1974), and mapping with STS marker was performed as described in Williams et al. (1992).

ne219, ne297, and ne300 failed to complement each other, defining the rde-1 locus. rde-1 mutations mapped near unc-42. V Three-factor mapping was used to locate rde-1(ne300) one-eighth of the distance from unc-42 in the unc-42/daf-11 interval (3/24 Unc-non-Daf-11 recombinants analyzed). The rde-1(ne300) allele complemented the chromosomal deficiency S29 and failed to complement edF1, mDF3, nDF31, and sDF35. rde-2(ne221) and rde-3(ne298) mapped...
near unc-13 I. rde-2 complemented rde-3, rde-4(ne299) and ne301 mapped near unc-69 III and failed to complement each other. ne299 complemented mut-7(pk204).

The rde-1(+) activity is sufficient maternally or zygotically. To test the maternal sufficiency, animals heterozygous for rde-1(ne219) were injected with dsRNA targeting the zygotic gene, sqt-3, and self-progeny were assayed for the Sqt phenotype. One hundred percent of the self-progeny, including rde-1 homozygous progeny, were found to exhibit the Sqt phenotype. Thus, maternally provided rde-1(+) activity is sufficient to mediate interference with a zygotic target gene. Zygotic sufficiency was assayed by injecting homozygous rde-1(ne219) activity is sufficient to mediate interference with a zygotic target gene. Zygotic sufficiency was assayed by injecting homozygous rde-1 mothers with dsRNA targeting the zygotic unc-22 gene (see Figure 3). The injected animals were then mated with wild-type males. Self-progeny from homzygous injected mothers were unaffected; however, 68% of the cross-progeny were Unc. This result indicates that zygotically provided rde-1(+) activity is also sufficient. However, both maternal and zygotic rde-1(+) activity contribute to zygotic interference, as 100% of progeny from wild-type-injected mothers exhibit unc-22 interference (606/606).

Analysis of Transgene Silencing in mutant-7 Germ Cells
Homologous mut-7 lines carrying various GFP reporter transgenes (as described below) were generated as follows: N2 (Bristol strain) males were mated to mutant-7 (pk204) unc-32 (e189) hermaphrodites; cross-progeny males were then mated to strains carrying the GFP transgenes. mutant-7 unc-32(+) cross-progeny from these matings were cloned, and mutant-7 unc-32 homologous animals carrying the transgenes were isolated from their self-progeny. The GFP reporter transgenes that were tested were each active in some, or all, somatic tissues but had become silenced in the germline. The plasmids used and transgene designations are as follows. (1) pBK48 is an in-frame insertion of GFP into a ubiquitously expressed gene, let-858 (Kelly et al., 1997), cceXPD7271 contains pBK48 in a high-copy repetitive array that is carried extrachromosomally. (2) pJ H3.92 is a in-frame fusion of GFP with the pie-1 gene kindly provided by M. Dunn and G. Seydoux (unpublished data). jhEx1070 carries pJ H3.92 in a “complex” extrachromosomal array generated by the procedure of Kelly et al. (1997). (3) pKL380.4 is a fusion of GFP with the C. elegans nuclear lamin gene, lam-1, which is expressed in all tissues (J. Liu and A. Fire, unpublished data). ccn4810 carries pKL380.4 in a complex array that has been integrated into the X chromosome by q irradiation (J. Liu, unpublished data).

Acknowledgements

We thank Hubert Lam for technical assistance; R. Ketting and R. Plasterk for sharing mutant-7(pk204) and for communicating unpublished results; Q. Boese and J. Collins for several mut-2 strains and for helpful discussions; S. Strom for several mes strains; A. Coulson for YAC and cosmide clones; Y. Kohara for several cDNA clones; and members of our laboratories for support and many discussions. Some strains were obtained from the C. elegans Genetic Stock Center, which is funded by a grant from the National Institutes of Health National Center for Research Support. Research support was provided in part by a PEW scholarship, a University of Massachussetts DERC pilot and feasibility grant, and National Institutes of Health grants to C. C. M. (GM37706), A. F. (GM37706), and L. T. (HD03533).

Received July 12, 1999; revised September 3, 1999.

References


Brigoli, G., Gia/effects of RNAi and Transposon Silencing in C. elegans


GenBank Accession Number

The accession number for rde-1 cDNA is AF180730.