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; Misquitta and Patterson, 1999; Sanchez-Alvarado and Newmark, 1999). 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* (Cogoni 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 (Cogoni and Macino, 1999).

Here, we report the identification of *R*NA interference-*de*ficient (*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 (Bohmert 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, eIF2C (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.

Results

Identification of RNAi-Deficient Mutants
In order to screen for mutants defective in RNAi, we sought methods that would permit the large-scale application of dsRNA to mutagenized populations. We have

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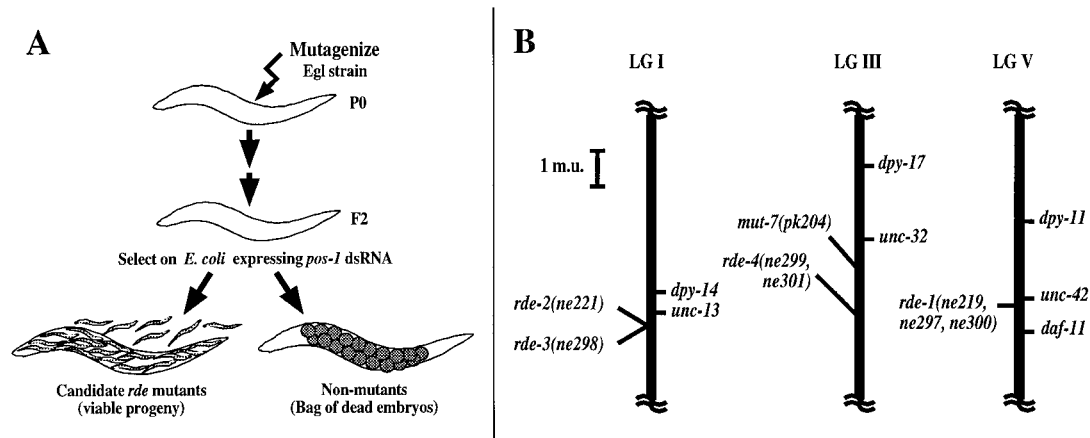
Figure 1. Identification and Linkage Group Analysis of RNAi-Deficient Mutants

(A) Genetic scheme for the identification of *rde* mutants.

(B) Summary of genetic mapping data. The vertical bars represent chromosomes LGI, LGIII, and LGV. Reference genetic markers are indicated at the right of each chromosome, and the relative genetic positions of the *rde* and *mut* alleles are indicated at the left.

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 deliver interfering RNA for an essential gene, *pos-1* (see Experimental Procedures). *C. elegans* hermaphrodites that ingest bacteria expressing dsRNA corresponding to a segment of *pos-1* are themselves unaffected but produce dead embryos with the distinctive *pos-1* embryonic lethal phenotype.

To identify RNAi-resistant strains, we mutagenized wild-type animals and then searched in the F2 generation for rare individuals that were able to produce complete broods of viable progeny. In addition to screens using chemical mutagenesis, we searched for spontaneous mutants using the *mut-6* strain in which Tc1 transposons are activated (Mori et al., 1988). To facilitate these screens, we used a starting strain that was egg laying deficient. In the absence of egg laying, the F3 progeny remained trapped within the mother's cuticle. Candidate mutants had internally hatched broods of viable embryos and were thus easily distinguished from the background population of individuals filled primarily with dead embryos (Figure 1A). Candidates were then retested for resistance to injected dsRNA. We next genetically mapped seven mutant strains identified in this way. These seven mutants defined four complementation groups: *rde-1*, with three alleles; *rde-4*, with two alleles; and *rde-2* and *rde-3*, each with one allele (Figure 1B).

In the course of this work, we also examined the RNAi sensitivity of several existing *C. elegans* mutants. 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 Ketting 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
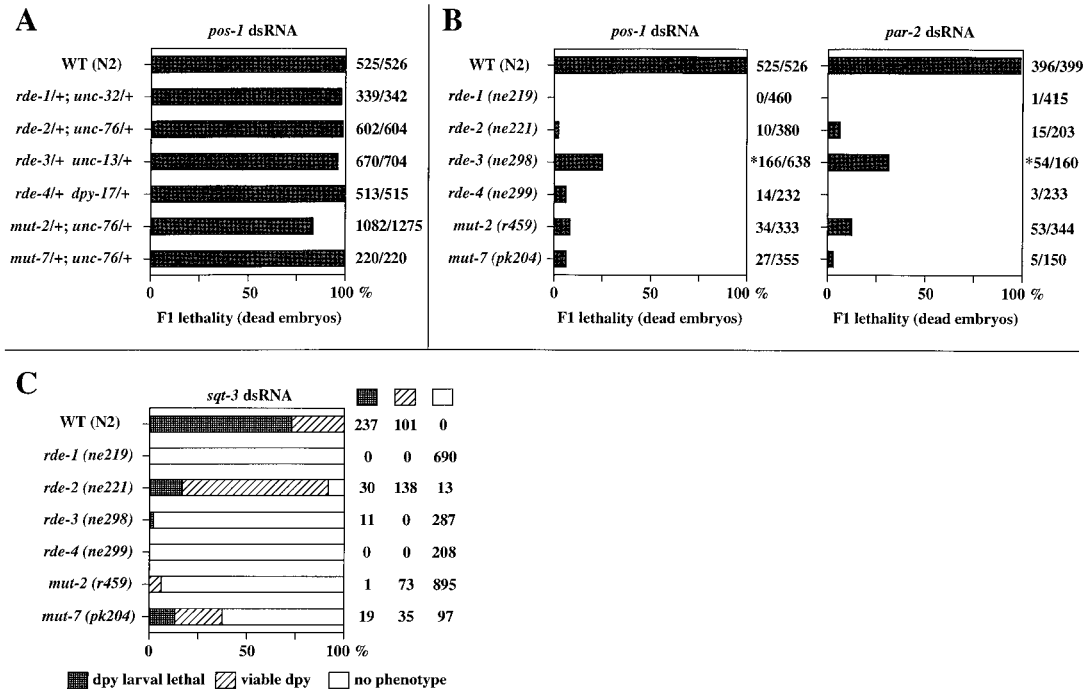
Figure 2. Sensitivity of rde and mut Strains to RNAi by Microinjection

Graphic representations of the sensitivity of rde and mut strains to dsRNA. The RNA species indicated above each graph was injected at high concentration (pos-1, 7 mg/ml; par-2, 3 mg/ml; sqt-3, 7 mg/ml). The strains receiving injection are indicated at the left, and the horizontal bar graphs reflect the percent of progeny that exhibited genetic interference.

(A) rde and mut alleles are recessive. Animals heterozygous for the rde and mut alleles were generated by crossing wild-type males with Unc-Rde or Unc-Mut hermaphrodites. The Unc marker mutants used are also indicated.

(B) Animals homozygous for rde and mut alleles are resistant to RNAi targeting maternally expressed genes, pos-1 and par-2. In (A) and (B), the percent embryonic lethality of F1 progeny was plotted as shaded bars, and the fraction of affected progeny is indicated at the right of each graph. The rde-3 data (asterisk in [B]) includes a 10% nonspecific embryonic lethality present in the rde-3 strain.

(C) rde and mut strains differ from one another in sensitivity of sqt-3 dsRNA. Progeny of injected animals were classified into the following three groups: larval lethality due to strong dumpy (Sqt) phenotype (dark shading), viable dumpy phenotype (cross hatching), and no phenotype (no shading). The total number of animals of each type is indicated in the columns at right.

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

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

	Percentage of Non-Unc Revertants
unc-22 (r765::Tc4)	0 (0/2000)
rde-1 (ne219) ; unc-22 (r765::Tc4)	0 (0/4000)
rde-2 (ne221) ; unc-22 (r765::Tc4)	0.96 (8/830)
rde-3 (ne298) ; unc-22 (r765::Tc4)	1.6 (35/2141)
rde-4 (ne299) ; unc-22 (r765::Tc4)	0 (0/2885)
mut-7 (pk204) ; unc-22 (r765::Tc4)	1.0 (40/3895)

	Percentage of Male Animals
Wild type (N2)	0.21 (2/934)
rde-1 (ne219)	0.07 (1/1530)
rde-2 (ne221)	3.2 (25/788)
rde-3 (ne298)	7.8 (71/912)
rde-4 (ne299)	0.24 (5/2055)

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

Genotype	Transgene Array	Percentage of Germline Desilencing
+/+	ccEx7271	8.3 (4/48)
mut-7/+	ccEx7271	14.5 (7/48)
mut-7/mut-7	ccEx7271	91.0 (71/78)
+/+	jhEx1070	3.9 (2/51)
mut-7/mut-7	jhEx1070	86.5 (32/37)
+/+	ccIn4810	4.3 (2/46)
mut-7/mut-7	ccIn4810	73.3 (33/45)
rde-1/rde-1	ccEx7271	0 (0/34)

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-858*). *jhEx1070* is a low-copy, "complex" array carrying pJH3.92 (GFP-tagged version of the maternal gene *pie-1*). *ccIn4810* is a low-copy, "complex" array that has been integrated into the X chromosome; this array carries plasmid pJKL380.4 (GFP-tagged version of the *C. elegans* nuclear laminin gene *lam-1*).

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* homozygotes 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.

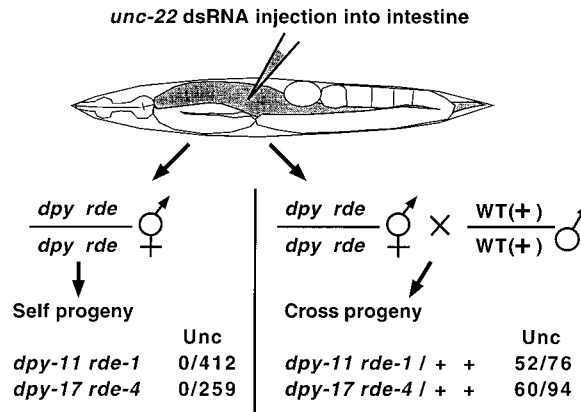Next we asked whether *rde-1* and *rde-4* mutants could

unc-22 dsRNA injection into intestine

Figure 3. *rde-1*(+) and *rde-4*(+) Activities Are Not Needed for dsRNA Uptake, Transport, or Stability

Schematic representation of homozygous *rde-1(ne219)* and *rde-4(ne299)* mutant mothers receiving injections of dsRNA targeting the body muscle structural gene *unc-22*. Injected animals were allowed to produce self-progeny (left arrow) or instead were mated after 12 hr to wild-type males (right arrow) to produce heterozygous *rde/+* cross-progeny. Each class of progeny was scored for the *unc-22* twitching phenotype as indicated by the fraction (Unc progeny/total progeny).

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.

Molecular Identification of the *rde-1* Gene

In order to clone the *rde-1* gene, we used standard genetic mapping to define a physical genetic interval

Table 3. Sensitivity of *rde* and *mut* Strains to Transgene-Driven Interfering RNA

	Unc Animals in Transgenic F1	Unc F2 Lines in Inherited Lines
Wild type (N2)	26/59	10/11
rde-1 (ne219)	0/25	0/3
rde-2 (ne221)	35/72	14/14
rde-3 (ne298)	1[a]/38	1[a]/9
rde-4 (ne299)	0/51	0/4
mut-7 (pk204)	9/13	3/3

A mixture of three plasmids was injected: [*myo-3* promoter::*unc-22* antisense], [*myo-3*::*unc-22* sense], and a marker plasmid (pRF4[*rol-6(su1006gf)*] [Mello et al., 1991]). Frequencies of Unc transgenic animals were followed in F1 and F2 generations.
[a] Unc phenotype was weak.

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 coinjected 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% nontwitching 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 eIF2C 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 eIF2C. However, two other *C. elegans* family members are far more similar to eIF2C 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*-heterozyotes 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 RNA 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
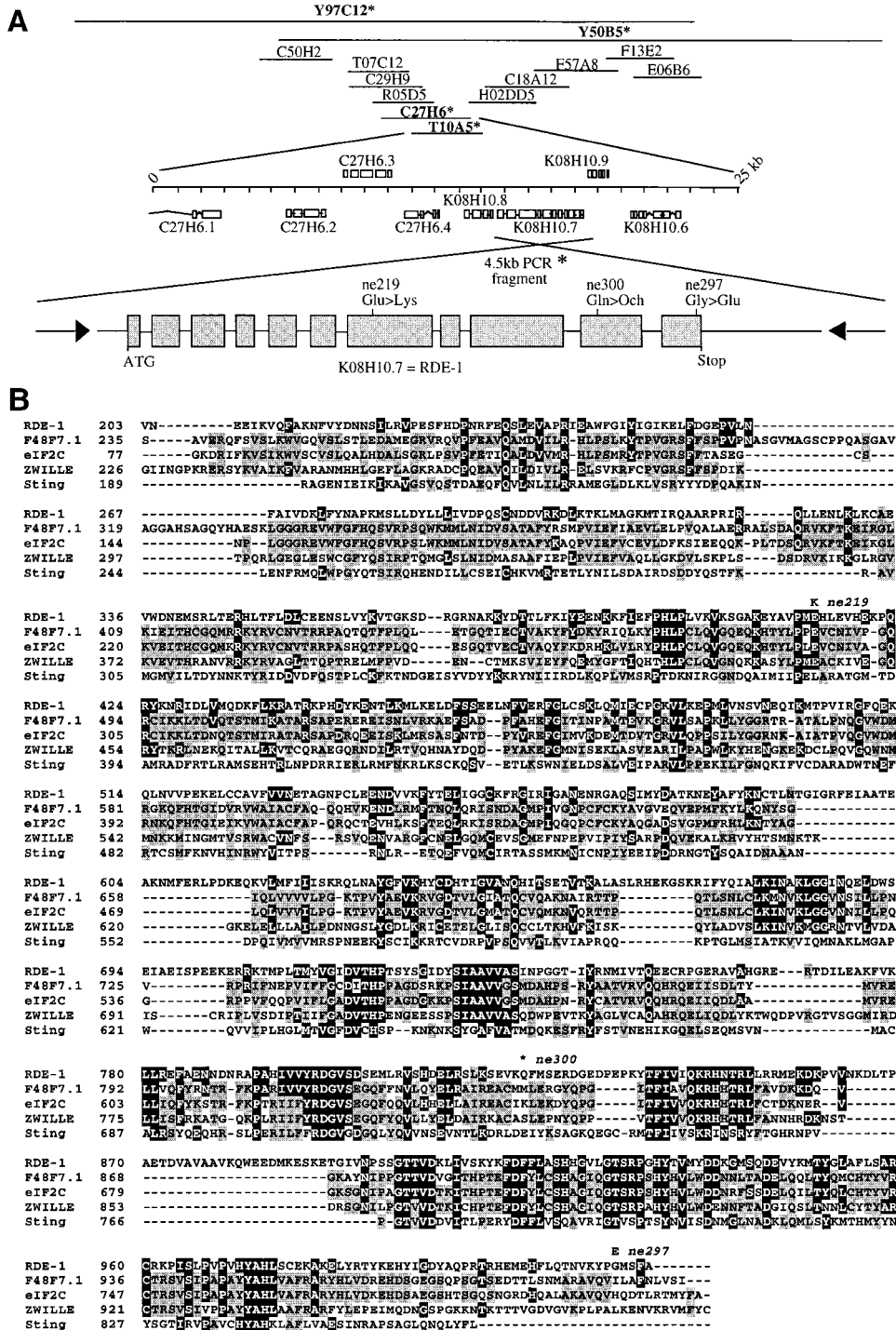
A

B

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 with RDE-1 are shaded in black, and identities among the homologs 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 *sting* 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 eIF2C, provides a possible connection between RNAi and translation initiation. The activity of the eIF2C protein is not known. However, eIF2C 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 eIF2C (previous name: Co-eIF-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 eIF2-α kinase (reviewed in Proud, 1995). This kinase in turn phosphorylates and inactivates eIF2-α, 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 eIF2C 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 eIF2C 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; Brigneti 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., 1989; 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; Wassenegger 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 specific pathogen 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 parasites 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 intereference.

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)*; LGIII: *dpy-17(e164)*, *unc-32(e189)*; LGV: *dpy-11(e224)*, *unc-42(e270)*, *daf-11(m87)*, *eDf1*, *mDf3*, *nDf31*, *sDf29*, *sDf35*, *unc-76(e911)*. The *C. elegans* strain DP13 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(r459)*; *dpy-19(n1347)*, TW410: *mut-2(r459)* *sem-4(n1378)*, NL917: *mut-7(pk204)*, SS552: *mes-2(bn76) rol-1(e91)/mnC1*, SS449: *mes-3(bn88) dpy-5(e61)*; *hDp20*, SS268: *dpy-11(e224) mes-4(bn23) unc-76(e911)/nT1*, SS360: *mes-6(bn66) dpy-20(e1282)/nT1*, CB879: *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 SS449 and SS552 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* cDNA clone yk61h1, *par-2* cDNA clone yk96h7, and *sqt-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 (1998). *pos-1* cDNA 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 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 *rde-1(ne219)*. Two strains, *ne299* and *ne301*, mapped to LGIII 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*. Mapping with visible marker mutations was 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 recombinants analyzed). The *rde-1(ne300)* allele complemented the chromosomal deficiency *sDf29* 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* 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 homozygous 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).

YAC, Cosmid, and cDNA Clones for *rde-1* Locus
YAC and cosmid clones around *rde-1* locus were obtained from A. Coulson. *rde-1(ne219)* was rescued by YAC clones Y97C12 and Y50B5 and cosmid clones C27H6 and T10A5.

cDNA clones for *rde-1* were obtained from Y. Kohara. The cDNA sequence of coding region and 3′UTR was determined on yk296b10, except that the sequence of 5′UTR was determined on yk595h5.

Analysis of Transgene Silencing in *mut-7* Germ Cells
Homozygous *mut-7* lines carrying various GFP reporters transgenes (as described below) were generated as follows: N2 (Bristol strain) males were mated to *mut-7 (pk204) unc-32 (e189)* hermaphrodites; cross-progeny males were then mated to strains carrying the GFP transgenes. *mut-7 unc-32/++* cross-progeny from these matings were cloned, and *mut-7 unc-32* homozygous 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) pJH3.92 is an in-frame fusion of GFP with the *pie-1* gene kindly provided by M. Dunn and G. Seydoux (unpublished data). *jhEx1070* carries pJH3.92 in a "complex" extrachromosomal array generated by the procedure of Kelly et al. (1997). (3) pJKL380.4 is a fusion of GFP with the *C. elegans* nuclear laminin gene, *lam-1*, which is expressed in all tissues (J. Liu and A. Fire, unpublished data). *ccIn4810* carries pJKL380.4 in a complex array that has been integrated into the X chromosome by γ irradiation (J. Liu, unpublished data).

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GenBank Accession Number

The accession number for *rde-1* cDNA is AF180730.