The dsRNA Binding Protein RDE-4 Interacts with RDE-1, DCR-1, and a DExH-Box Helicase to Direct RNAi in *C. elegans*

Hiroaki Tabara,[1,3,5] Erbay Yigit,[1] Haruhiko Siomi,[3]
and Craig C. Mello[1,2,4]
[1]Program in Molecular Medicine
[2]Howard Hughes Medical Institute
University of Massachusetts Medical School
Worcester, Massachusetts 01605
[3]Institute for Genome Research
University of Tokushima
Tokushima 770-8503
Japan

Summary

Double-stranded (ds) RNA induces potent gene silencing, termed RNA interference (RNAi). At an early step in RNAi, an RNaseIII-related enzyme, Dicer (DCR-1), processes long-trigger dsRNA into small interfering RNAs (siRNAs). DCR-1 is also required for processing endogenous regulatory RNAs called miRNAs, but how DCR-1 recognizes its endogenous and foreign substrates is not yet understood. Here we show that the *C. elegans* RNAi pathway gene, *rde-4,* encodes a dsRNA binding protein that interacts during RNAi with RNA identical to the trigger dsRNA. RDE-4 protein also interacts in vivo with DCR-1, RDE-1, and a conserved DExH-box helicase. Our findings suggest a model in which RDE-4 and RDE-1 function together to detect and retain foreign dsRNA and to present this dsRNA to DCR-1 for processing.

Introduction

The introduction of dsRNA can induce sequence-specific posttranscriptional silencing of cognate genes in a variety of organisms (reviewed in Cogoni and Macino, 2000; Vance and Vaucheret, 2001; Waterhouse et al., 2001). The experimental application of dsRNA to induce gene silencing has been termed RNA interference or RNAi (Fire et al., 1998). RNAi and related posttranscriptional gene silencing (PTGS) pathways have been implicated in silencing transposons (Tabara et al., 1999a; Ketting et al., 1999; Wu-Scharf et al., 2000) and/or viruses (Ratcliff et al., 1997; Mourrain et al., 2000; Dalmay et al., 2001), suggesting that these pathways may function as a form of sequence-directed immunity.

PTGS pathways share features with a developmental gene regulatory pathway that involves natural dsRNA-encoding genes, recently named micro-RNA (miRNA) genes (Lau et al., 2001; Lee and Ambros, 2001; Lagos-Quintana et al., 2001). During PTGS, introduced dsRNAs, or dsRNAs synthesized from aberrant transgene transcripts, are processed into small RNAs of approximately 22–26 nucleotides that have been termed small interfering RNAs (siRNAs) because they are thought to guide

the destruction of complementary target mRNAs (reviewed in Baulcombe, 2002). Natural miRNA genes encode RNA products of approximately 70 nucleotides which are predicted to fold into stable stem-loop structures that are processed into mature ~22 nucleotide miRNAs. The founding members of the miRNA gene family, *lin-4* and *let-7*, encode miRNA products that appear to regulate translation during *C. elegans* development by base pairing with complementary sequences located in the 3′ UTRs of their target mRNAs (Lee et al., 1993; Wightman et al., 1993; Reinhart et al., 2000).

Genetic and molecular studies have uncovered similarities in the processing and function of miRNA and siRNA species. For example, both the RNAi pathway and miRNA pathway require related genes. In *C. elegans,* RNAi requires the gene *rde-1*, which encodes a member of a functionally novel but highly conserved eukaryotic gene family (Tabara et al., 1999a) whose members have also been implicated in gene silencing in fungi, plants, and *Drosophila* (Catalanotto et al., 2000; Fagard et al., 2000; Hammond et al., 2001). Homologs of *rde-1* have also been implicated in development in *Drosophila*, *C. elegans,* and *Arabidopsis* (reviewed in Benfey, 1999). A recent study has shown that two *C. elegans* homologs of *rde-1*, named *alg-1* and *alg-2*, are required for the processing and function of the *lin-4* and *let-7* miRNAs (Grishok et al., 2001). Similarly, studies in *Drosophila*, *C. elegans*, and in vertebrate cells have shown that the multifunctional RNaseIII-related enzyme Dicer is required for the proper processing of both the dsRNAs that trigger RNAi (Bernstein et al., 2001; Ketting et al., 2001) and the miRNA precursors whose products mediate developmental gene regulation (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001).

The convergence between PTGS pathways and pathways that control developmental gene regulation raises the questions of what other features these pathways share and, importantly, what factors function uniquely within each pathway to guide RNA recognition and targeting. In the present study we show that *rde-4* encodes a dsRNA binding protein required for RNAi. Interestingly, RDE-4 is not required for other PTGS pathways or for developmental pathways, suggesting that RDE-4 activity may function at a step unique to the RNAi pathway. We show that the stable interaction between RDE-4 and dsRNA requires *rde-1(+)* activity and that RDE-4 interacts with the RDE-1 protein in vivo. RDE-4 protein also interacts in vivo with DCR-1 and with a conserved DExH-box helicase that is required for RNAi in *C. elegans*. Interactions were not detected between RDE-4 and siRNAs, or miRNA precursors. RNA sequences bound to RDE-4 were restricted to regions found within the trigger dsRNA, and thus RDE-4 does not appear to interact with the mRNA or with dsRNA sequences amplified from the target mRNA. Our findings suggest that RDE-4 functions during the initial steps of RNAi to recognize foreign dsRNA and to present this dsRNA to DCR-1 for processing.

[4]Correspondence: craig.mello@umassmed.edu
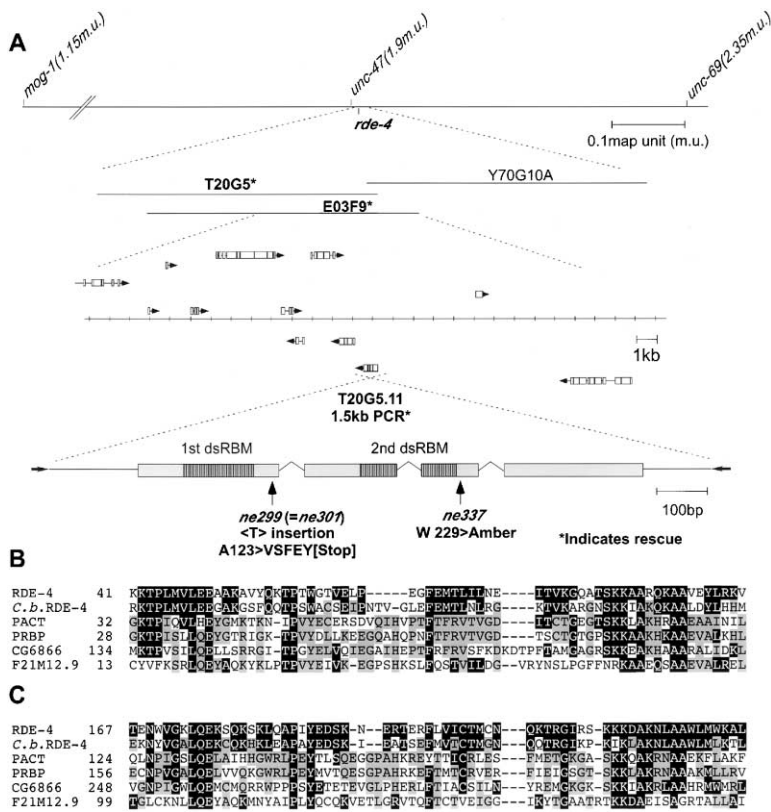[5]Present address: Genome Biology Laboratory, National Institute of Genetics, Mishima 411-8540, Japan.

Figure 1. Cloning and Sequence Analysis of rde-4

(A) Maps of the RDE-4 genetic and physical intervals on chromosome III. The structure of rde-4 gene is diagrammed with coding regions boxed. The positions of the dsRBM motifs are shown as striped boxes. The positions and nature of lesions in the ne299, ne301, and ne337 alleles are indicated.

(B and C) Alignments of the first (B) and second (C) dsRBM in RDE-4 and four related proteins. The sequences are RDE-4 (*C. elegans*), an open reading frame from the related nematode (*C. briggsae*), PACT (*Homo sapiens*), PRBP (*Mus musculus*), CG6866 (*Drosophila melanogaster*), and F21M12.9 (*Arabidopsis thaliana*). Identities with RDE-4 are shaded in black, and identities among the related proteins are shaded in gray. Each of these proteins contains two or more dsRBMs and no other recognizable motifs.

Results

Molecular Identification of rde-4

In previous genetic screens for RNAi-deficient mutants, we found that many *C. elegans* mutants deficient in RNAi also exhibit additional phenotypes including activation of transposons in the germline, a low penetrance sterile phenotype, and meiotic chromosome nondisjunction phenotypes (Tabara et al., 1999a). These findings suggested that RNAi may play a role in transposon silencing and in other essential functions in the germline. However, paradoxically, the two strongest mutants, rde-1 and rde-4, were completely deficient in RNAi but failed to exhibit transposon activation or any other discernible phenotypes. Subsequent genetic studies suggested that rde-1 and rde-4 may function upstream in the initiation of RNAi (Grishok et al., 2000).

To shed more light on the role of the RDE-4 protein in RNAi, we set out to clone the corresponding gene. We first mapped the gene to a small genetic interval and then assayed corresponding DNA sequences from the interval for rescue of rde-4 (see Experimental Procedures). rde-4 rescue was mapped to two overlapping cosmid clones, T20G5 and E03F9, and to the single corresponding open reading frame T20G5.11 (Figure 1A). We next sequenced the T20G5.11 gene in each of the rde-4 mutant strains and found point mutations predicted to disrupt the open reading frame of T20G5.11 in each allele. Two alleles, ne299 and ne301, have an identical lesion, a 1 base insertion creating a premature stop codon at the end of the first exon of T20G5.11 (Figure 1A). These two alleles were isolated from a single

large clonal population during a screen for spontaneous mutants (Tabara et al., 1999a) and may thus reflect a single spontaneous lesion (we will henceforth refer to this allele as ne299). A second allele of rde-4, ne337, was identified in a screen using chemical mutagenesis and induces a premature stop codon at amino acid residue 229 of the T20G5.11 product (Figure 1A). The rde-4 gene is predicted to encode a 385 amino acid protein that contains two copies of a conserved motif found in dsRNA binding proteins (Figures 1B and 1C). The genetic lesions in rde-4 appear likely to be null mutations, and consistent with this idea the ne299 allele, when placed in trans to a chromosomal deficiency, exhibited an RNAi-deficient phenotype and no other additional phenotypes (data not shown).

RDE-4 Interacts with dsRNA In Vitro and In Vivo

The RDE-4 protein contains two copies of a dsRNA binding motif (dsRBM) that is found in numerous other proteins that interact with dsRNA in a sequence-non-specific manner (reviewed in Fierro-Monti and Mathews, 2000). Therefore, we first asked whether recombinant RDE-4 could bind to dsRNA by using an in vitro gel shift assay. Labeled dsRNA corresponding to a portion of a GFP cDNA was incubated with GST-fused RDE-4, or with the GST protein alone, and was then run on a native polyacrylamide gel. The GST-fused RDE-4 efficiently bound to the dsRNA, causing a mobility shift, while the GST protein did not (data not shown). In competition assays, dsRNA but not single-stranded RNA efficiently competed for RDE-4 binding (data not shown). Similarly, we asked whether the labeled dsRNA could bind to the
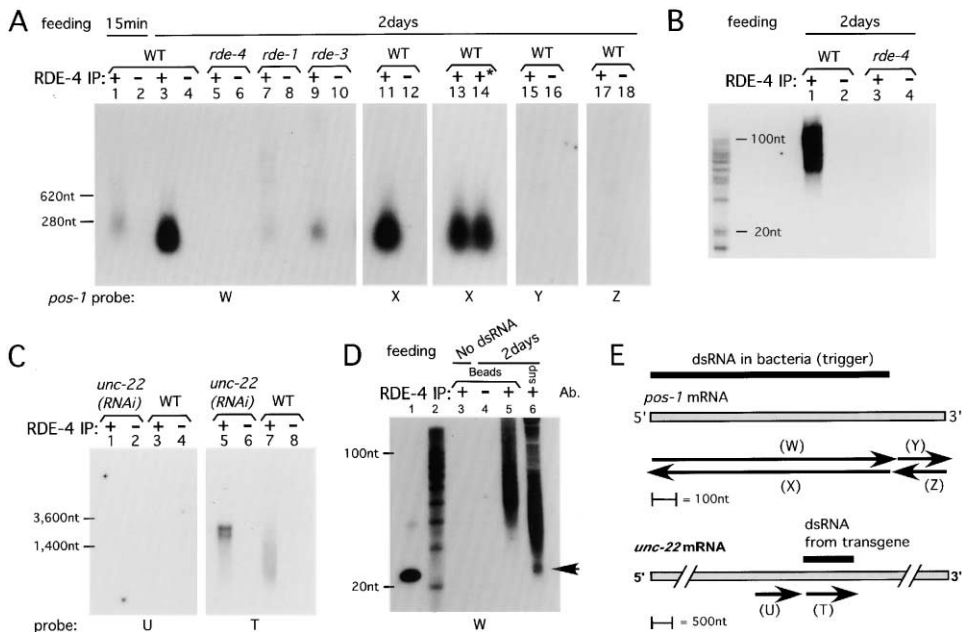
Figure 2. RDE-4 Binds dsRNA In Vivo

(A and B) Wild-type or *rde* mutant animals were exposed to *pos-1* dsRNA by feeding on *E. coli* expressing *pos-1* dsRNA for 15 min or 2 days (as indicated). The *E. coli* feeding strain used expresses a dsRNA, which corresponds to 85% of the *pos-1* mRNA (as indicated in the diagram in [E]). After immunoprecipitation (IP) with anti-RDE-4 antibody (+) or with the IgG fraction obtained from the pre-immune serum (−), coprecipitated RNA was fractionated on 1.8% denaturing agarose gels (A) or on a 15% polyacrylamide sequencing gel (B). RNA species were blotted and analyzed by Northern hybridization with sense RNA probes (W, Y) and antisense RNA probes (X, Z) as illustrated in (E). The hybridizations in (A) and (B) were done with the same stringency. The probes Y and Z were prepared from a region not contained in the bacterial feeding vector. To remove single-stranded RNA, the RNA sample in lane 14 (asterisk) was treated with 10 μg/ml RNaseA and 0.5 μg/ml RNaseT1. The blot in (B) was hybridized with the sense RNA probe (W).

(C) Northern analysis of RDE-4::IPs prepared from wild-type animals and transgenic animals expressing an *unc-22*-specific dsRNA segment. The transgenic *C. elegans* line was designed to express a 1400 nt segment of *unc-22*, indicated by the black bar in the diagram (E), as both sense and antisense RNA. After immunoprecipitation with the anti-RDE-4 antibody, coprecipitated RNA was fractionated on a 1.2% denaturing agarose gel. Northern hybridization was performed with sense RNA probes U and T as illustrated in (E). The size of the *unc-22* RNA associated with RDE-4 was approximately 2800 nt, suggesting that the transgenes express dsRNA with a hairpin structure.

(D) RDE-4 does not interact detectably with siRNA. Extracts prepared from wild-type animals either not exposed to dsRNA (lane 3) or exposed to *pos-1* dsRNA by feeding for 2 days (lanes 4, 5, and 6) were subjected to immunoprecipitations as described above. RNA species were recovered from each fraction and were run on a 12% polyacrylamide sequencing gel. The RNA species were analyzed by Northern hybridization with a sense RNA probe (W). Hybridization was done with a moderate stringency to allow detection of ∼25 nt RNA species. As controls, lane 1 contained a synthetic RNA oligo corresponding to 21 nt of antisense *pos-1* sequence, and lane 2 contained size-marker RNA.

(E) Diagrams representing the *pos-1* and *unc-22* mRNAs are shown as gray bars. The regions targeted with dsRNA are indicated by the solid black bars above each diagram. Probes used in the experiments are indicated by arrows beneath the diagrams.

recombinant RDE-4 immobilized on a membrane. This North-Western blot analysis also showed that the dsRNA binds efficiently to the GST-fused RDE-4 but not to the GST protein alone (data not shown).

We next wished to ask if RDE-4 interacts with dsRNA during RNAi in vivo. To address this question, polyclonal antibodies were raised against a recombinant RDE-4 protein. We then exposed animals to dsRNA targeting the gene *pos-1* (Tabara et al., 1999b). We prepared extracts from these animals and from control animals not exposed to dsRNA, and we used RDE-4-specific antibodies to precipitate the RDE-4 protein complex. The precipitates were extracted to purify any associated RNA and analyzed by agarose and acrylamide gel electrophoresis followed by Northern blotting using *pos-1* sense and antisense radiolabeled RNA probes. Both the sense and antisense probes detected approximately equal amounts of *pos-1* RNA that migrated below 350 bases on an agarose gel (Figure 2A, lanes 3 and 11) and migrated in a size range of 50 to 200 bases on an

acrylamide gel (Figure 2B, lane 1). The coprecipitated *pos-1* RNA was resistant to treatment with a mixture of the single-strand-specific RNaseA and RNaseT1 enzymes (Figure 2A, lane 14), suggesting that the *pos-1* RNA is double stranded. Little or no *pos-1* RNA coimmunoprecipitated with RDE-4, which was purified from populations either not exposed to dsRNA or exposed for only 15 min (Figure 2D, lane 3; Figure 2A, lane 1). As expected, *rde-4(ne299)* mutants failed to exhibit *pos-1* dsRNA in the precipitate (Figure 2A, lane 5). The RDE-4 immune complex recovered from the *rde-1(ne300)* and *rde-3(ne298)* mutant strains exhibited greatly reduced quantities of *pos-1* dsRNA (Figure 2A, lanes 7 and 9).

Target mRNA and siRNA Sequences Do Not Coprecipitate with RDE-4

dsRNA is thought to exist and function at multiple steps in RNAi. First, it functions in the initiation step when long dsRNA is recognized as foreign. Second, it functions in the execution step when target mRNA is bound by siRNA
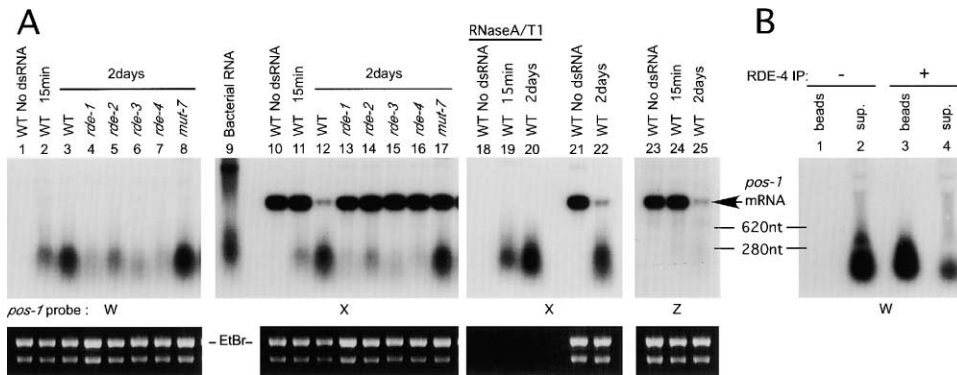
Figure 3. Genetic Analysis of dsRNA Accumulation during RNAi

(A) Genetic analyses of accumulated dsRNA species in total RNA extracts. Mixed-stage populations of wild-type (wt), *rde-1*, *rde-2*, *rde-3*, *rde-4*, and *mut-7* animals were cultured on *E. coli* expressing *pos-1* dsRNA. Subsequently, RNA was extracted from each population and subjected to Northern analyses. The design of probes W, X, and Z was as illustrated in Figure 2E. The RNA samples of lanes 18–20 were treated with a mixture of RNaseA and T1. Ethidium bromide (EtBr) staining of 18S and 28S ribosomal RNA serves as a loading control.

(B) A large portion of the foreign dsRNA in total RNA extracts associates with RDE-4. Extracts from wild-type animals exposed to *pos-1* dsRNA by feeding as described above were subjected to immunoprecipitation with anti-RDE-4 antibody (+) or with the IgG obtained from the preimmune serum (−). RNA species were recovered from each immune complex (beads) and each supernatant (sup) and were fractionated on a 1.8% denaturing agarose gel. The RNA species were analyzed by Northern hybridization with a sense RNA probe (W).

and cleaved. And third, it functions in a potential amplification step in which target mRNA sequences are copied by an RNA-dependent RNA polymerase activity. In the above experiments, the probes and techniques used were designed to detect long dsRNA sequences contained within the trigger dsRNA, and thus could not address the question of whether siRNAs or target mRNA sequences might also coimmunoprecipitate with RDE-4. To address these questions, we first probed for sequences in the 3′ UTR of *pos-1* that were not included in the dsRNA trigger (probes Y and Z, see Figure 2E). These experiments failed to detect significant coprecipitating RNA (Figure 2A, lanes 15 and 17), indicating that sequences within the target mRNA are not stable components of the RDE-4 immune complex.

Two recent studies suggest that RNAi may involve a mechanism for the amplification of the dsRNA signal and provide evidence that sequences in the target mRNA that lie 5′ of the trigger sequence can be amplified (Lipardi et al., 2001; Sijen et al., 2001). In order to ask if the RDE-4 immune complex contains antisense RNA sequences derived from regions of the mRNA located 5′ of the trigger dsRNA, we utilized transgenic animals that express dsRNA corresponding to a portion of *unc-22* gene (see Figure 2E). RNA probes were then used to assay for sequences in the RDE-4 immune complex that were derived either from the trigger region or from the region located just 5′ of the trigger. As expected, the RDE-4 immune complex contained *unc-22* antisense RNA corresponding to the targeted region of *unc-22* (Figure 2C, lane 5). In contrast, no significant hybridization was observed when a sequence derived from the region located just 5′ of the trigger dsRNA was used as a probe (Figure 2C, lane 1). As expected, precipitates prepared from nontransgenic wild-type animals did not exhibit significant hybridization to the probes designed to detect *unc-22* antisense RNA (Figure 2C, lanes 3 and 7).

Finally, in order to ask if siRNA sequences precipitate

with RDE-4, we fractionated the RNA species in the RDE-4 immune complex and in the RDE-4-depleted supernatant by acrylamide gel electrophoresis. We then analyzed RNA species smaller than 150 bases by Northern blotting using a *pos-1* sense probe. We found that the RNA species in the supernatant included abundant molecules of less than 150 bases, including a prominent signal corresponding in size to siRNAs, that migrated at approximately 24 to 25 bases (Figure 2D, lane 6). RNA species of 24 to 25 bases were absent in the RDE-4 immune complex, suggesting that RDE-4 preferentially interacts with longer dsRNAs (Figure 2D, lane 5). Taken together, these studies indicate that RDE-4 binds to long dsRNA sequences that are restricted to the region present within the trigger dsRNA. These findings support a model in which RDE-4 interacts with the trigger dsRNA and functions in the initiation step of RNAi.

dsRNA Bound to RDE-4 Accumulates during RNAi

In order to determine what fraction of the total dsRNA was bound to RDE-4 and to examine the requirements for the stability of the dsRNA bound to RDE-4, we next analyzed RNA extracts from wild-type and *rde* mutant strains. Wild-type animals showed an accumulation of antisense and sense RNA corresponding to the *pos-1* trigger dsRNA sequence that appeared to be very similar to the dsRNA sequences found in the RDE-4 immune complex (Figure 3A, lanes 3 and 12). This RNA species was similar in size but slightly smaller, on average, than the major *pos-1* RNA species detected in the bacterial expression strain used to induce RNAi (Figure 3A, lane 9), suggesting that some digestion of the bacterially expressed dsRNA may have occurred during transit through the intestine of the animals. Single-stranded RNA-specific nucleases digested the endogenous *pos-1* mRNA but failed to digest the accumulated RNA species (Figure 3A, lanes 19 and 20), suggesting that the accumulated RNA species is indeed double-stranded. Interestingly, the analysis of total RNA extracts

showed that animals lacking *rde-1*, *rde-3*, and *rde-4* activities exhibited markedly reduced levels of dsRNA. In contrast, animals lacking *rde-2* and *mut-7* accumulated dsRNA to levels that were intermediate (*rde-2*) or similar to wild-type (*mut-7*) (Figure 3A). The finding that the accumulation of this dsRNA species requires some but not all *rde(+)* activities supports the idea that this RNA species is an intermediate in the RNAi process.

To ask what fraction of the accumulating dsRNA in the total RNA extract from wild-type animals is associated with RDE-4, we repeated the immunoprecipitations with RDE-4-specific antibodies or with control nonspecific IgG obtained from preimmune serum and compared the amount of the dsRNA present in the immune complex and supernatant of each lysate. We found that approximately 60% of the *pos-1* dsRNA species present in the total RNA coprecipitates with the RDE-4 immune complex (Figure 3B, compare lanes 3 and 4), indicating that a majority of the accumulating dsRNA is associated with RDE-4.

RDE-4 Interacts with RDE-1 In Vivo

Our previous studies have suggested that RDE-1 and RDE-4 function at an upstream step in the initiation of RNAi (Grishok et al., 2000). This previous observation, and the finding that RDE-1 and RDE-4 are both required for the accumulation of dsRNA, prompted us to ask if RDE-4 forms a complex in vivo with RDE-1. For these studies we generated a transgenic strain expressing RDE-1 tagged with the HA epitope and RDE-4 tagged with the FLAG epitope. We first confirmed that these tagged proteins were functional and able to rescue the corresponding mutants (data not shown). We then immunoprecipitated RDE-4 with RDE-4-specific polyclonal antibodies, and RDE-1 via the epitope tag, and analyzed the precipitates by immunoblotting (Figure 4A). In reciprocal assays, RDE-1 and RDE-4 were found to coprecipitate. Based on a comparison of the RDE-1 present in total extracts and in the RDE-4 immune complex, we estimate that approximately 5% of the RDE-1 molecules stably associate with the RDE-4 complex.

The interaction between RDE-1 and RDE-4 occurred in animals that were not exposed to exogenous dsRNA, and the interaction was not abolished by treatment with either double-stranded RNA-directed nuclease, RNaseV1, or a mixture of RNaseA and T1 that are single-stranded RNA-specific nucleases (data not shown). These findings suggest that RDE-1 and RDE-4 form a complex in the absence of dsRNA, but cannot rule out the possibility that dsRNA promotes the interaction or is required for the interaction but is protected from digestion in the immune complex.

RDE-4 Forms a Complex with DCR-1 In Vivo

In order to identify additional proteins that interact with RDE-4 in vivo, we isolated large quantities of the RDE-4 immune complex and then resolved its components using SDS polyacrylamide gel electrophoresis (SDS-PAGE). For this experiment we used *rde-4(ne299)* mutant animals that were rescued via an RDE-4::GFP fusion protein. We then passed protein extracts prepared from these transgenic animals and from control wild-type animals over a column conjugated with anti-GFP mono-
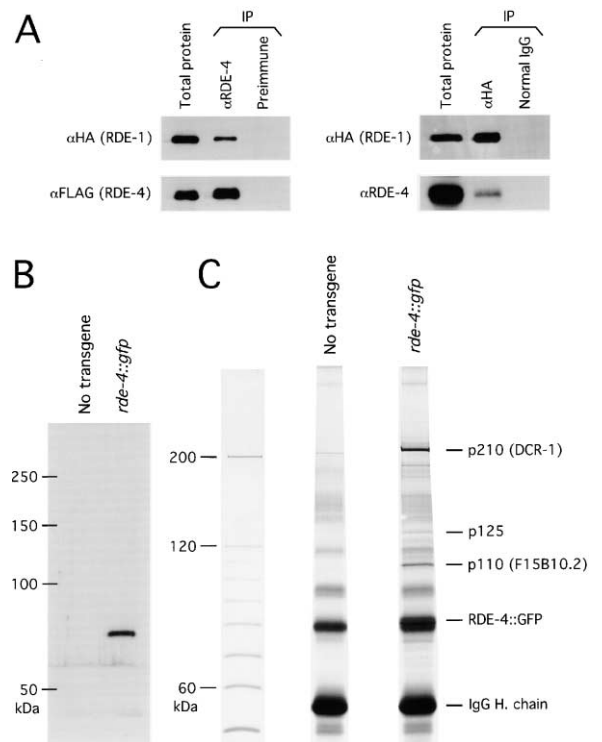

Figure 4. RDE-4 Associates with RDE-1, DCR-1, and an RNA Helicase-Related Protein DRH-1

(A) RDE-1 and RDE-4 form a complex in vivo. Protein extracts were prepared from an *rde-1(ne300)* mutant strain that was rescued by a transgene expressing HA-tagged RDE-1 and FLAG-tagged RDE-4. The total protein extract and immunoprecipitates prepared by incubating the extract with anti-RDE-4 or anti-HA and with control nonspecific sera (preimmune or normal IgG) were run on SDS-PAGE (10%). Western blots were prepared and immunostained, as indicated, with anti-HA (to detect HA::RDE-1) or anti-FLAG or anti-RDE-4 (to detect RDE-4).

(B and C) Immunopurification of proteins associated with RDE-4 in vivo. The *rde-4(ne299)* mutant was rescued by an integrated transgene expressing an RDE-4::GFP fusion protein. In (B), total protein extracts prepared from this strain and from a wild-type nontransgenic strain were resolved on SDS-PAGE and immunostained with anti-GFP polyclonal antibody (Molecular Probes). The expression of RDE-4::GFP was detected as a single band. In (C), total protein extracts were first precipitated with anti-GFP-specific antibodies, and proteins associated with the RDE-4::GFP immune complex were resolved by SDS-PAGE (8%) followed by silver staining.

clonal antibody. After electrophoresis, the gel was silver stained to detect components of the RDE-4::GFP immune complex (Figure 4C). These experiments identified prominent bands migrating at 210 kilodaltons (kDa) and 110 kDa as well as several additional polypeptides that were present in the RDE-4::GFP immune complex but absent in wild-type control animals. A minor band at 125 kDa was identified, corresponding in size to RDE-1, but contained insufficient protein for further analysis (Figure 4C). The 210 kDa and 110 kDa bands were subjected to tryptic digestion, and the resulting fragments were then analyzed by mass spectrometry. The peptide mass fingerprints of p210 and p110 were compared with in silico-digested sequences from protein databases (see Experimental Procedures). A total of 21 peptides from the tryptic digestion of p210 matched DCR-1 (Table

Table 1. Peptide Mass Fingerprinting of p210 (DCR-1) and p110 (DRH-1)

Data of p210	MH$^+$ Matched	Peptide Sequences	Amino Acid Position in K12H4.8 (DCR-1)	Data of p110	MH$^+$ Matched	Peptide Sequences	Amino Acid Position in F15B10.2
1049.59	1049.52	FDRPLDMR	478–485	1026.53	1026.53	YFTPTEIR	996–1003
1067.58	1067.54	QLGPWAAWR	289–297	1067.63	1067.48	SEWMSGLNK	732–740
1085.67	1085.65	IPPFPVFLR	695–703	1081.51	1081.51	NSPYSNIMR	438–446
1134.60	1134.54	ADLQCFNPR	6–14	1111.66	1111.59	FYQIINASR	216–224
	1134.54	TGWDIGGDVSK	1458–1468	1192.63	1192.61	TPNFTVNMIR[b]	654–663
1160.66	1160.61	VTVEVVNNMR	1797–1806	1195.43	1195.61	LKMFADGEIR	759–768
1214.72	1214.63	YLHQIEQQR	1828–1836	1208.66	1208.61	TPNFTVNMIR[b]	654–663
1231.71	1231.63	QYSPGVLTDLR[a]	1638–1648		1208.66	TRYEATILNK	708–717
1248.75	1248.66	QYSPGVLTDLR[a]	1638–1648	1295.67	1295.61	STDGPIGMFTNR	527–538
1313.80	1313.74	VNQLLLTDELR	957–967	1371.68	1371.69	VDNIVQENSTPR	264–275
1317.79	1317.72	FREAELTLNPK	660–670	1490.80	1490.80	TVQYIVEQNLQR	686–697
1370.79	1370.73	YLHQIEQQRR	1828–1828	1536.84	1536.82	TALRNEHIGIEQR	552–564
1380.79	1380.69	FVNPDYVVGASGR	416–428				
1412.78	1412.72	NLASSDSQGLHKR	429–441				
1428.91	1428.80	SIEALRPYVPQR	346–357				
1446.96	1446.80	VMNWMGLKVIQK	1531–1542				
1490.84	1490.70	ECFIYAFELER	649–659				
1498.89	1498.80	AEHLSAIIFVDQR	382–394				
1638.87	1638.76	YVSEVVANMENMPR	780–793				
1778.02	1777.92	EYGVQLFAPLDQGGKR	49–64				
1807.89	1807.86	NLDTTWQVIFHMMR	1742–1755				
1868.04	1867.95	AYLVQAFTHASYINNR	1593–1608				

The masses of the tryptic peptides were measured with a MALDI-TOF mass spectrometer. The values for mass/charge ratio of the protonated molecular ions corresponding to 21 fragments of p210 were matched to the sequence of K12H4.8 (DCR-1) with mass errors of 0.002%–0.011%. Eleven fragments of p110 were matched to the sequence of F15B10.2 (DRH-1) with mass errors of 0.001%–0.015%.
[a] The difference between these two originates in a possible modification of glutamine (Q).
[b] The difference between these two originates in a possible modification of methionine (M).

1), while 11 peptides from p110 matched a DExH-box RNA helicase-related protein F15B10.2 (Table 1, see below).

The finding that RDE-4 forms a complex in vivo with DCR-1 suggests that RDE-4 may function to present the foreign trigger dsRNA to DCR-1 for processing. DCR-1 also acts to process the stem-loop precursors of the developmental regulators *lin-4* and *let-7* (Grishok et al., 2001; Ketting et al., 2001). However, *rde-4* mutants do not exhibit developmental defects (Tabara et al., 1999a), and thus are not required for the activities of *lin-4* or *let-7*. Furthermore, we did not observe *lin-4* or *let-7* precursor RNAs associated with the RDE-4 immune complex (data not shown). Thus, although RDE-4 interacts strongly with DCR-1 in vivo, RDE-4 is not essential for the developmental functions of DCR-1 and may rather serve as an adaptor protein that recruits DCR-1 to the RNAi pathway.

A Conserved DExH-Box Helicase Required for RNAi

The RDE-4 protein interacts with a 110 kDa protein, which as described in the previous section corresponds to the predicted gene F15B10.2 (Table 1), one of two closely related *C. elegans* DExH-box RNA helicase genes. F15B10.2, along with its homolog C01B10.1, map directly adjacent to one another on chromosome *IV* and appear likely to be expressed together from a single promoter. Consistent with this idea, we found that some F15B10.2 cDNAs were spliced to the *trans*-spliced leader sequence (sl2), which is often spliced onto the 5′ end of transcripts encoded by downstream genes in an operon (reviewed in Blumenthal, 1995). We found

that the C01B10.1 gene and a cDNA clone (yk226c6) both appear to encode a frame-shifted open reading frame predicted to terminate the protein prior to the helicase domain, raising the question of whether or not this represents a functional gene. An alternative conceptual splicing of C01B10.1, or utilization of an alternative initial methionine, could encode a protein with overall 74% identity to F15B10.2 (Figures 5A and 5B).

These *C. elegans* genes have close homologs in mammals, including a gene named RHIV-1 induced in response to viral infection in pigs (Figures 5A and 5B; Zhang et al., 2000). Notably, these helicase proteins are more similar to the DCR-1 helicase domain than to any other helicase-related proteins in *C. elegans* or other organisms (Figures 5A and 5B). Consequently, we have named these genes *drh-1* and *drh-2* (for F15B10.2 and C01B10.1, respectively) which stands for dicer-related helicase.

To ask if these genes might function in RNAi, we used RNAi to target their inactivation. Previous work on *dcr-1* has shown that RNAi targeting *dcr-1* causes developmental defects and can also partially inhibit RNAi (Grishok et al., 2001). Although the helicase domain of DCR-1 is similar to DRH-1 and DRH-2 at the protein level, the *dcr-1* gene does not contain regions of extended nucleotide identity required for crossinterference in an RNAi assay. The *drh-1* and *drh-2* genes, on the other hand, are more than 81% identical at the nucleotide level and contain several regions of 40 to 200 nucleotides with 100% identity, and are thus expected to crossinterfere in the RNAi assay. There are no extensive regions predicted to be unique to either helicase gene and thus it is doubtful that effective single RNAi is possible. We
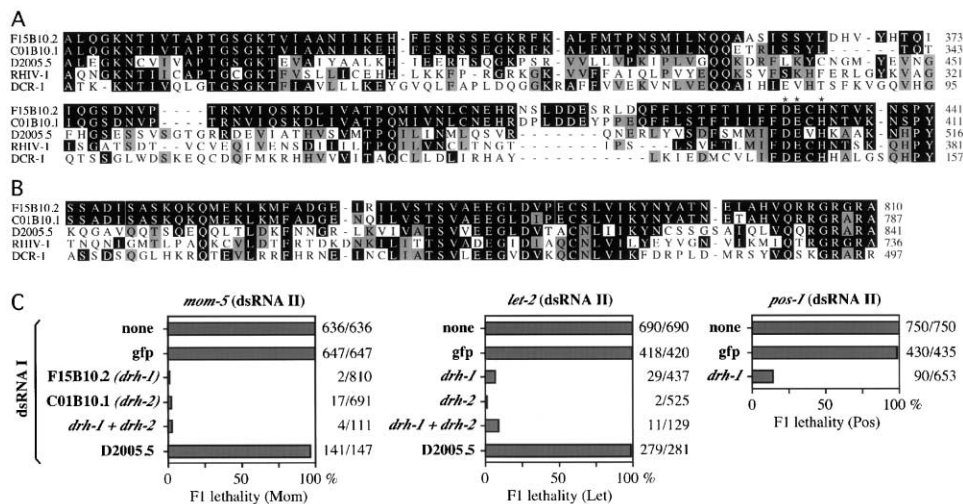
Figure 5. DRH-1 and DRH-2 Are Helicase-Related Proteins Required for RNAi

(A and B) Alignment of DRH-1 (F15B10.2), DRH-2 (C01B10.1), D2005.5, RHIV-1, and DCR-1. (A) Alignment of DExH/D domains, and (B) alignment of the helicase conserved C-terminal domains. Identities shared with DRH-1 are shaded in black, and identities among the other proteins are shaded in gray. Asterisks above residues in (A) indicate the location of DExH/D motif.

(C) Reduced sensitivity to RNAi following *drh-1* and *drh-2(RNAi)*.

also targeted D2005.5, which, other than DCR-1, is the next most similar *C. elegans* helicase-related gene (Figures 5A and 5B).

We did not observe any developmental abnormalities in animals exposed to *drh-1* or *drh-2* dsRNA, injected either singly or in combination. However, we found that injection of either *drh-1* or *drh-2* (or both) dsRNAs rendered the injected animals resistant to RNAi targeting a second gene (Figure 5C). Both germline and somatic RNAi was dramatically reduced in the helicase RNAi animals. RNAi targeting D2005.5 did not induce developmental or RNAi defects (Figure 5C and data not shown). These results suggest that the DExH-box helicase DRH-1 (and perhaps DRH-2) not only interact with RDE-4 but are also required for RNAi in *C. elegans*.

Discussion

The RDE-4 Protein and the Initiation of RNAi

Recent work in numerous organisms has shown that RNAi and other forms of sequence-directed immunity share features both with each other and with natural developmental regulatory pathways. For example, both the foreign dsRNAs that trigger RNAi and the endogenous miRNA precursors that function in development are processed into small RNAs by a multifunctional RNaseIII-related enzyme, Dicer (DCR-1). These small RNAs (siRNAs and miRNAs) are thought to be incorporated into silencing complexes that mediate, respectively, mRNA destruction during RNAi or translational control during development. These findings raise two kinds of interesting question. First, what additional genes and mechanisms do these pathways share? And second, what factors function uniquely within each pathway to guide RNA recognition and targeting? In the present study we have described the dsRNA binding protein RDE-4, which appears to be dedicated to dsRNA

recognition within the RNAi gene-silencing pathway. We have shown that RDE-4 forms a complex in vivo with DCR-1. However, the RDE-4 protein is not required for development and does not interact detectably with miRNAs in vivo. These findings suggest a model in which RDE-4 functions as a sensor that recognizes foreign dsRNA and brings this RNA to DCR-1 for processing (Figure 6A).

The RDE-4 protein contains two copies of the dsRNA binding motif (dsRBM) and no other recognizable functional domains. The crystal structure of a dsRBM bound to dsRNA reveals extensive contacts between this protein motif and the backbone of the double-helical RNA (Ryter and Schultz, 1998). Thus, dsRBMs are expected to interact nonspecifically with dsRNAs of any sequence. Several groups of dsRBM-containing proteins can be distinguished by the presence of additional conserved motifs (reviewed in Fierro-Monti and Mathews, 2000). Members of one such group, the dsRNA-activated protein kinases (PKRs) in mammals, contain a kinase motif and two dsRBMs. PKRs have previously been shown to be involved in the response to foreign dsRNA (reviewed in Proud, 1995). PKRs are activated by binding dsRNA, and they trigger signal transduction pathways that direct a general repression of translation and ultimately induce apoptosis. Thus, PKRs are thought to provide a first line of defense for clearing viral infection in mammals. Like PKRs, RDE-4 appears to play an upstream role in the recognition of foreign dsRNA. However, instead of a general translation arrest, RDE-4 initiates the sequence-specific destruction of complementary mRNAs.

The RDE-1 Protein and the Initiation of RNAi

We have shown that the RDE-4 protein interacts with RDE-1 in vivo. The RDE-1 protein has numerous homologs in *C. elegans* and in other organisms, and members
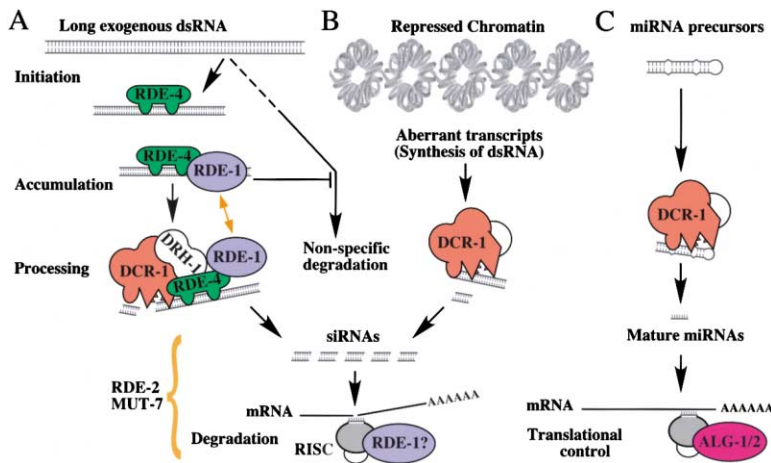
Figure 6. Model
(A) RNAi.
(B) Cosuppression and transposon silencing.
(C) miRNA-mediated translational regulation. The unlabeled shapes in the diagram indicate unknown proteins. The yellow arrows and brackets in (A) indicate potential interactions.

of the RDE-1 family have been implicated in gene silencing and developmental pathways in plants, fungi, and animals. RDE-1 homologs in *Drosophila* and *Neurospora* are found in complexes that contain siRNA molecules (Hammond et al., 2001; Catalanotto et al., 2002). Furthermore, RDE-1 homologs are required for miRNA function in *C. elegans* (Grishok et al., 2001) and have recently been found in a complex with miRNAs in vertebrate cells (Mourelatos et al., 2002). Thus, RDE-1-related proteins appear to function in a variety of homology-dependent mechanisms that involve pairing between small guide RNAs and mRNAs.

The interaction we detect between RDE-4 and RDE-1 could reflect a transfer of siRNA products from the RDE-4/DCR-1 complex to an RNA-induced silencing complex (RISC) that contains RDE-1 (Figure 6A). However, we have shown that RDE-1 activity is required for the accumulation of dsRNA bound to RDE-4. Thus, if RDE-1 does function downstream in the execution step of RNAi, it must also function upstream at a step required for the retention of dsRNA (Figure 6A). It is also possible that both the initiation of RNAi and the subsequent destruction of target mRNA occurs within a single complex and that completion of the targeting steps may be required for the continued retention and accumulation of the trigger dsRNA molecules engaged by RDE-4.

The activities of RDE-1 and RDE-4 appear to protect the foreign dsRNA from entering a nonspecific dsRNA destruction pathway (Figure 6A). This finding suggests that the initiation complex containing RDE-4 may collect dsRNA faster than DCR-1 or other downstream factors can process the dsRNA. Conceivably, RDE-1 and RDE-4 function as adaptor proteins that regulate the entry of dsRNA into the RNAi pathway so as not to saturate the cell's essential dsRNA-processing machinery (Figure 6A).

A DExH-Box RNA Helicase Involved in RNAi
We found a 110 kDa RNA helicase-related protein, DRH-1, in a protein complex with RDE-4 in vivo. Our analysis, using peptide mass fingerprinting, specifically identified DRH-1, but we cannot rule out the possibility that a small portion of the 110 kDa band contains a second very similar predicted protein named DRH-2. While numerous proteins related to DRH-1 are present

in eukaryotes, DRH-1 is most similar to an unstudied human protein and to a porcine protein, RHIV-1. Interestingly, the *rhiv-1* gene was identified as a locus whose expression is activated in pigs infected with porcine reproductive and respiratory syndrome virus (PPRSV) (Zhang et al., 2000). PPRSV is a positive-strand RNA virus that forms dsRNA structures during its life cycle. The finding that DRH-1 is most similar to a family of vertebrate helicase proteins that include a protein activated in response to viral infection suggests that this family of proteins may have ancestral functions in the response to foreign dsRNA.

Up to the present, studies in other organisms have revealed several RNA helicases required for PTGS pathways. Mut6 in *Chlamydomonas* is a DEAH-box RNA helicase involved in PTGS and is most similar to the *C. elegans* MOG-1 helicase required for sex determination in the germline (Wu-Scharf et al., 2000). SDE3 in *Arabidopsis* is required for PTGS and is similar to Upf1-type RNA helicases (Dalmay et al., 2001). A biochemical study of RNAi in *Drosophila* suggests that RNA helicase activity may be required downstream of siRNA formation (Nykanen et al., 2001). And another recent study in *C. elegans* has identified MUT-14 as a DEAD-box helicase required downstream of siRNA formation for target mRNA destruction during RNAi (Tijsterman et al., 2002). The Dicer protein also contains an RNA helicase domain, which notably is most similar to the helicase domain of DRH-1.

We have found that RNAi targeting *drh-1* results in inhibition of RNAi in both the soma and germline but causes no other phenotypes. In contrast, mutations in *dcr-1* or RNAi targeting *dcr-1* inhibit RNAi but also result in lethal phenotypes (Grishok et al., 2001; Knight and Bass, 2001; Ketting et al., 2001). Thus, DRH-1 does not appear to be required for developmental functions of DCR-1 and may instead function closely with RDE-4 in a step specifically required for RNAi (Figure 6A). There are a several possibilities for the role of DRH-1. For example, the helicase activity of DRH-1 may change the conformation of the dsRNA in order to facilitate the translocation of the protein complex along the dsRNA. Or it may facilitate transfer of the dsRNA from RDE-4 to the active site of DCR-1. It is conceivable that this additional helicase activity is necessary in order to pro-

cess the long-contiguous helices of the foreign dsRNAs that initiate RNAi. This activity may be less critical in processing miRNA precursors in which only short-contiguous helices are present (Lau et al., 2001; Lee and Ambros, 2001; Lagos-Quintana et al., 2001).

Distinct Initiation Steps in Related Gene Silencing Pathways

In *C. elegans,* the RNAi pathway overlaps genetically with the transposon-silencing and cosuppression pathways (Tabara et al., 1999a; Ketting et al., 1999; Ketting and Plasterk, 2000; Dernburg et al., 2000). Cosuppression is a PTGS silencing phenomenon that occurs when extra copies of a gene are introduced into an organism, resulting in the simultaneous silencing of both the transgene and homologous endogenous genes (reviewed in Cogoni and Macino, 2000). Transposon silencing may represent one in vivo function of the cosuppression pathway, as no genetic or experimental data provides evidence for a distinction between these pathways.

A previous study on the genetics of RNAi pathway genes suggested that *rde-1* and *rde-4* function upstream in a step that is unique to the RNAi pathway, while *rde-2* and *mut-7* function together at a downstream step common to the RNAi, transposon silencing, and cosuppression pathways (Grishok et al., 2000). Consistent with these findings, we have shown that during the initiation of RNAi, foreign dsRNA is recognized by RDE-4 and accumulates in tissues in a process that requires both RDE-4 and RDE-1. Furthermore, we have shown that RDE-2 and MUT-7, which appear to function in multiple silencing pathways, are not required for the accumulation of dsRNA during RNAi, consistent with a downstream function for these gene products (Figure 6).

The finding that RDE-4 is a dsRNA binding protein that interacts with foreign dsRNAs raises the question of why this protein does not also recognize and initiate silencing in response to dsRNAs that are postulated to function in the transposon silencing and cosuppression pathways (reviewed in Plasterk and Ketting, 2000; Vance and Vaucheret, 2001; Waterhouse et al., 2001). One attractive possibility is that transposon silencing and cosuppression are triggered when transcription occurs within partially repressed chromatin (Figure 6B). Conceivably, abnormal transcripts produced from the repressed chromatin are converted into dsRNAs through the activity of an RNA polymerase dedicated to this silencing pathway. Perhaps DCR-1 resides in a complex with this polymerase and thus binds the nascent dsRNAs efficiently without the need for RDE-4. Alternatively, the initiation of RNAi might occur when dsRNA is encountered in the cytoplasm, while the initiation of transposon silencing and cosuppression could involve nuclear dsRNA triggers. However, this latter model seems unlikely, as RDE-1 and RDE-4 are both absolutely required for dsRNA-induced silencing, even when the trigger dsRNA is expressed from a transgene directly within the nuclei of target tissues (Tabara et al., 1999a).

RDE-1 and RDE-4 are required for the initiation of RNAi in animals exposed to dsRNA, but not for interference inherited by progeny (Grishok et al., 2000). Thus, if an amplification mechanism functions downstream in the RNAi pathway (Lipardi et al., 2001; Sijen et al., 2001),

RDE-4 is not likely to function in the recognition of the amplified dsRNAs. Perhaps amplification in the RNAi pathway involves the same hypothetical RNA polymerase complex postulated to function in the production of dsRNA transcripts from partially repressed chromatin (Figure 6). A good candidate for such a polymerase has been described as a factor required for cosuppression in both fungi and plants (Cogoni and Macino, 1999; Mourrain et al., 2000; Dalmay et al., 2000). However, surprisingly, members of this protein family are also required for RNAi in *C. elegans* (Smardon et al., 2000; Sijen et al., 2001; D. Conte and C.C.M., unpublished). These findings could indicate that amplification is required for RNAi even when abundant dsRNA is present. Alternatively, it is possible that members of this protein family do not function in dsRNA synthesis, or function at multiple steps, both in producing dsRNAs that trigger cosuppression and in later steps that occur downstream of the convergence between these silencing pathways.

Our study sheds light on the upstream events that distinguish RNAi from related gene-silencing pathways. A future challenge will be to elucidate the events unique to the initial steps in the cosuppression and microRNA pathways. These studies will no doubt lead to new insights into the world of small regulatory RNAs and should in turn lead to improved methods for controlling gene expression.

Experimental Procedures

Strains, Genetic Analysis, and Mapping

The Bristol strain N2 was used as a standard wild-type *C. elegans* strain. Genetic mapping placed *rde-4(ne299)* mutation to the right end of the interval between *sqv-3(n2842)* and *unc-69(e587)* mutations on chromosome III. Rescue experiments were performed by coinjection of the rescuing construct with a mixture of plasmids that express sense and antisense *unc-22* RNA as well as *rol-6* transformation marker pRF4 as described in Tabara et al. (1999a).

rde-1(ne300), *rde-2(ne221)*, *rde-3(ne298)*, *rde-4(ne299)*, and *mut-7(pk204)* mutants were used to analyze the accumulation of dsRNA species during feeding RNAi. *rde-4(ne337)* was isolated by this work and utilized for the detection of a *rde-4* mutation.

RNA Interference Assays

RNAi by microinjection was performed as described in Fire et al. (1998) and Rocheleau et al. (1997). RNAi by feeding was performed as described in Timmons and Fire (1998) and Tabara et al. (1999a).

Cosmid and cDNA Clones

Cosmid clones around *rde-4* locus were obtained from A. Coulson. *rde-4* cDNA clone yk333g4, *drh-1* cDNA clone yk447b12, and *drh-2* cDNA clone yk226c6 were obtained from Y. Kohara and used in this work.

Transgenic Worms Used for Immunoprecipitation

rde-1 and *rde-4* genes were tagged with epitope sequences and cloned into plasmids. Instead of 145 lysine in RDE-1, two copies of HA-tag sequences were inserted into the *rde-1* coding region. FLAG-tag or GFP-tag sequences were introduced at the carboxyl terminus of *rde-4* coding region.

Transgenic lines were produced with microinjection as described in Mello and Fire (1995). The HA-tagged *rde-1* gene, the FLAG-tagged *rde-4* gene, and pRF4 were coinjected into *rde-1(ne300)* mutant. The GFP-tagged *rde-4* gene and pRF4 were coinjected into *rde-4(ne299)* mutant. Animals whose transgenes were stably integrated into chromosomes were selected following UV irradiation or identified as a spontaneous event.

Antibody Production and Immunoprecipitation

A carboxy-terminal region (amino acid residues 218–385) of RDE-4 was tagged with 6× His and expressed in bacteria. Polyclonal rabbit antibodies were raised against the purified recombinant protein. The antibody was affinity purified using the recombinant protein immobilized to metal-chelated resin.

Before immunoprecipitation, animals were separated from the bacteria with sucrose flotation method and agitated in M9 buffer for 30 min to digest the bacteria remaining in guts. The animals were homogenized in lysis buffer (25 mM HEPES-NaOH [pH 7.4], 150 mM NaCl, 0.2 mM DTT, 10% glycerol, 1% Triton X-100 and complete protease inhibitors [Roche]). For RNA coprecipitation experiments, 2% Superasein (Ambion) was included in the lysis buffer. The affinity-purified anti-RDE-4 antibody, anti-HA antibody 3F10 (Roche), and anti-FLAG antibody M2 (Sigma) were used for immunoprecipitation and immunoblotting. To recover RNA from RDE-4 immunoprecipitates, the precipitates were incubated in 0.2 mg/ml Proteinase K, 0.7× TBS, 5 mM EDTA, and 0.1% SDS for 30 min at 50°C and were extracted with phenol and chloroform. The recovered nucleic acids were analyzed by Northern hybridization essentially as described below.

Protein Identification by Peptide Mass Fingerprinting

To identify proteins interacting with RDE-4, the transgenic animals expressing RDE-4::GFP fusion protein were homogenized in the lysis buffer as described above. Anti-GFP monoclonal antibody 3E6 (Q-BIOgene) was crosslinked onto Protein G-Sepharose beads with dimethyl pimelimidate. RDE-4::GFP immune complex was precipitated with the anti-GFP beads and was analyzed with SDS-PAGE. The gels were visualized by silver staining or Coomassie staining.

After Coomassie staining, 210 kDa and 110 kDa bands were excised from the gel and digested in gel with trypsin. The masses of the tryptic peptides were measured with matrix-assisted laser desorption ionization-reflectron time-of-flight (MALDI-TOF) mass spectrometry (Shimazu Biotech, Inc., Japan). Searching of the mass values against the database was performed with the program MS-Fit at http://prospector.ucsf.edu (Clauser et al., 1999).

Northern Blot Analysis

For analyses of total RNA, animals exposed to *pos-1* dsRNA by feeding were purified from the plates and homogenized in guanidine solution (4 M guanidine thiocyanate, 50 mM Tris [pH 7.4], 10 mM EDTA, 0.65% lauroylsarcosine, and 1% 2-mercaptoethanol). Nucleic acids were extracted from the homogenates with phenol and chloroform and were precipitated with sodium acetate and isopropanol. The precipitates were resuspended in water and were subsequently acidified with sodium acetate (330 mM [pH 4]). DNA was removed from the nucleic acids by extraction with phenol and chloroform under this acidic condition.

RNA samples were diluted with formamide/EDTA gel-loading buffer and were heat denatured before loading. Approximately 20 μg of total RNA was run per each lane on agarose/formaldehyde gels. RNA species were transferred onto plus-charged nylon membranes from the gels with capillary blotting. Strand-specific riboprobes were synthesized using T7 or T3 RNA polymerase and [α-³²P]UTP (380 Ci/mmol). Hybridization was performed at 57°C in 50% formamide, 2× SSC, 1% SDS, 5% dextran sulfate, and 150 μg/ml Torula yeast RNA. The blots were washed basically with 0.1× SSC/0.1% SDS at 65°C.

To detect low-molecular-weight RNA, RNA species were run on polyacrylamide/urea gels and transferred to membranes via semidry electroblotting. For riboprobes used for the detection of 25 nt RNA species, the radiolabeled probe was partially fragmented by boiling for 60 min in a hybridization buffer containing formamide. Subsequently, blots were hybridized with riboprobe in 50% formamide, 3× SSC, 1% SDS, 5% dextran sulfate, and 150 μg/ml yeast RNA at 50°C and washed with 0.8× SSC/0.1% SDS at 50°C. To detect *lin-4* and *let-7* RNAs, DNA oligonucleotides were end-labeled and used for the hybridization as described in Reinhart et al. (2000).

Acknowledgments

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