Genes and Mechanisms Related to RNA Interference Regulate Expression of the Small Temporal RNAs that Control C. elegans Developmental Timing

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Summary
RNAi is a gene-silencing phenomenon triggered by double-stranded (ds) RNA and involves the generation of 21 to 26 nt RNA segments that guide mRNA destruction. In Caenorhabditis elegans, lin-4 and let-7 encode small temporal RNAs (stRNAs) of 22 nt that regulate stage-specific development. Here we show that inactivation of genes related to RNAi pathway genes, a homolog of Drosophila Dicer (dcr-1), and two homologs of rde-1 (alg-1 and alg-2), cause heterochronic phenotypes similar to lin-4 and let-7 mutations. Further we show that dcr-1, alg-1, and alg-2 are necessary for the maturation and activity of the lin-4 and let-7 stRNAs. Our findings suggest that a common processing machinery generates guide RNAs that mediate both RNAi and endogenous gene regulation.

Introduction
In numerous organisms the introduction of dsRNA can induce the sequence specific posttranscriptional silencing of a corresponding gene (reviewed in Cogoni and Macino, 2000). The experimental application of dsRNA to induce gene silencing has been termed RNA interference or RNAi. Genetic studies have linked RNAi to transposon silencing in C. elegans (Ketting et al., 1999; Tabara et al., 1999), while a related posttranscriptional gene silencing (PTGS) mechanism, called cosuppression, has been linked to viral resistance in plants (reviewed by Baulcombe, 1999), raising the possibility that these phenomena represent a form of sequence-directed immunity.

A striking paradigm to emerge from the study of PTGS mechanisms in plants and animals is that of the small RNA guide that can direct an RNA-protein complex to a complementary target sequence. Several studies of PTGS have identified the guide molecule as a species of small RNA of approximately 22 nt, recently termed “small interfering RNAs” (siRNAs) (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000; Elbashir et al., 2001a). For example, in Drosophila cell culture, small RNAs of approximately 22 nt copurify with and provide sequence specificity to an RNase complex that degrades the target mRNA (Hammond et al., 2000). Furthermore, small synthetic dsRNAs of 22–26 nt are sufficient to direct destruction of complementary RNAs both in vitro and in vivo (Elbashir et al., 2001a; Parrish et al., 2000), and duplexes of small 21 nt RNAs have recently been shown to suppress gene expression in cultured mammalian cells (Elbashir et al., 2001b).

It is tantalizing that the size of the siRNAs implicated in RNAi is similar to the approximately 22 nt size of the lin-4 and let-7 small temporal RNAs (stRNAs) that regulate C. elegans developmental timing (Lee et al., 1993; Reinhart et al., 2000). The lin-4 stRNA triggers the transition from larval stage one to larval stage two, whereas the let-7 stRNA controls a later larval to adult transition. stRNAs induce developmental progression by negatively regulating the expression of proteins encoded by mRNAs whose 3' untranslated regions (3'UTRs) contain sites complementary to the stRNAs (Lee et al., 1993; Wightman et al., 1993; Moss et al., 1997; Reinhart et al., 2000; Slack et al., 2000). For example, LIN-14 protein level decreases late in larval stage one when lin-4 stRNA is expressed (Feinbaum and Ambros, 1999; Olsen and Ambros, 1999), and LIN-41 diminishes at later larval stages as let-7 stRNA appears (Slack et al., 2000). Probable orthologs of both let-7 and its target gene, lin-41, have been found in numerous metazoans, including humans (Pasquinelli et al., 2000; Slack et al., 2000); because the temporal regulation of the let-7 orthologs is also conserved, temporal control of development by this 22 nt stRNA may be ancient (Pasquinelli et al., 2000).

Although the 22 nt forms of the let-7 and lin-4 RNAs are more abundant, low levels of larger transcripts of approximately 70 nt can also be detected for each gene (Lee et al., 1993; this paper). These larger forms are predicted to fold into similar stem-loop structures. Human and Drosophila let-7 orthologs are also expressed as larger forms that, likewise, have the potential to fold into stable stem-loop structures (A.E.P. and G.R., unpublished observations), suggesting that this potential secondary structure may have functional importance (Pasquinelli et al., 2000). The existence of possible dsRNA precursors and the 22 nt size of the lin-4 and let-7 stRNAs has fueled speculation about possible...
mechanistic similarities with RNAi (Pasquinelli et al., 2000; Sharp, 2001).

The Dicer protein has been implicated in RNAi in Drosophila where it appears to function in the processing of longer dsRNAs into the siRNAs which subsequently guide mRNA destruction (Bernstein et al., 2001). Dicer belongs to a conserved family of proteins, whose members contain a helicase domain, one or two dsRNA binding domains, and two RNase type III domains (Bass, 2000; Cerutti et al., 2000; Bernstein et al., 2001). Also present in Dicer family members is a PAZ domain (Cerutti et al., 2000), which was identified in the Piwi/Argonaute/Zwille/RDE-1 family of proteins introduced below. The Arabidopsis ortholog of Dicer, Carpel Factory (caf 1), is required for normal plant development (Jacobsen et al., 1999), but has not yet been shown to play a role in PTGS mechanisms.

Genetic studies in C. elegans have identified several genes essential for RNA interference (Tabara et al., 1999; Ketting et al., 1999). Probable null mutations in rde-1 (for RNAi defective) cause a complete lack of RNAi but no other discernible phenotypes (Tabara et al., 1999). rde-1 encodes a 1020 amino acid protein that is a member of a large family of proteins found in a wide range of eukaryotes. Members of the RDE-1 family have two conserved domains of unknown biochemical function. The 300 amino acid PIWI domain located in the C-terminal region of these homologs shows the highest degree of sequence conservation (Cox et al., 1998; Cerutti et al., 2000). The 110 amino acid PAZ domain is located N-terminal to the PIWI domain and is also found in the Dicer family of proteins. RDE-1 homologs in the fungus, Neurospora, and the plant, Arabidopsis, have also been implicated in PTGS mechanisms (Catalanotto et al., 2000; Fagard et al., 2000) suggesting that RDE-1 family members not only share conserved structures but also have conserved functions in gene silencing in three kingdoms of eukaryotic organisms. Mutations in rde-1 homologs have also been shown to have developmental consequences. For example, in Drosophila, the ago1 gene is required for embryogenesis (Kataoka et al., 2001), the piwi gene is required for the maintenance of the germline stem cell population (Cox et al., 1998), and aubergine is required for the proper expression of the germline determinant Oskar (Wilson et al., 1996). Additionally, aubergine (also known as Sting) has been implicated in the PTGS-like suppression of the repetitive Stellate locus in the Drosophila germline (Schmidt et al., 1999). In Arabidopsis two very similar genes, argonaute (ago1) and pinhead/zwille, are required for stem cell patterning of the plant meristem (Bohmert et al., 1998; Moussian et al., 1998; Lynn et al., 1999). argonaute is also necessary for PTGS in Arabidopsis (Fagard et al., 2000). The C. elegans genome contains 23 homologs of rde-1 including orthologs of both piwi and ago1. Previous studies have shown that the C. elegans piwi and ago1 orthologs have germline and possibly additional developmental functions (Cox et al., 1998; Cikuluk et al., 1999). The pleiotropic nature of the defects associated with loss-of-function mutations in members of this family could reflect discrete regulatory functions in numerous developmental events or alternatively might reflect a more general misregulation of silencing mechanisms that are necessary to insure proper stem cell maintenance and differentiation.

In this paper we provide evidence for the involvement of RNAi related genes and mechanisms in the expression and activity of the stRNA genes, lin-4 and let-7. We show that the activities of two C. elegans homologs of rde-1, alg-1 and alg-2, are essential for the proper function of the lin-4 and let-7 stRNA pathway. alg-1 and alg-2 activities are necessary for efficient processing of the lin-4 stRNA but may be less important for let-7 stRNA expression. Further, we demonstrate that the C. elegans ortholog of Drosophila Dicer, dcr-1, is an essential gene and is required for both RNAi and for processing of the lin-4 and let-7 precursor RNAs. Inhibition of dcr-1 or alg-1 and alg-2 causes heterochronic phenotypes that are consistent with their effects on lin-4 and let-7 RNA processing. These findings suggest that natural dsRNAs are processed into small regulatory RNAs via a mechanism analogous to that involved in processing the double-stranded RNAs that trigger RNAi. Thus, a common processing machinery may produce natural small guide RNAs that regulate the activities of endogenous mRNA targets as well as the small interfering RNAs implicated in RNA interference and viral surveillance.

Results

alg-1 and alg-2 Function in Embryogenesis and Larval Development

There are 23 C. elegans homologs of rde-1 (Figure 1). cDNA clones for 14 rde-1 homologs, indicated by asterisks in Figure 1, were tested for developmental functions by RNAi (see Experimental Procedures). dsRNAs derived from two closely related genes, F48F7.1 and T07D3.7, which we have named alg-1 and alg-2 (for argonaute like genes), induced developmental phenotypes in the progeny of injected animals, including a tendency to burst at the vulva (Figure 2A), and a lack of the adult specific alae, longitudinal stripes that run the length of the cuticle on both sides of the adult animal (Figure 2E). In addition these dsRNAs induced incompletely penetrant slow growth and germline abnormalities (Figure 2D and data not shown). The other 12 genes assayed did not exhibit discernable developmental phenotypes.

The alg-1 and alg-2 DNA sequences are 80% identical at the nucleotide level, suggesting a recent duplication of these genes, although they map to distinct chromosomes. This level of similarity is within the range where partial cross-interference is expected in RNAi assays (Parish et al., 2000; Schubert et al., 2000). To target only alg-1 or alg-2, we prepared dsRNAs from short 5’ unique segments of each gene (see Experimental Procedures). The dsRNA prepared from the unique segment of alg-1 produced the same vulval bursting phenotype, although at a reduced frequency relative to that observed with longer dsRNAs (data not shown). No RNAi phenotype was observed after injections of the unique segment of alg-2.

We obtained a deletion allele of alg-2 from the C. elegans gene knockout consortium. This allele, alg-2(ok304), is an out-of-frame deletion that removes the nucleotides encoding amino acids 34–374, including the...
Figure 1. Phylogenetic Tree Grouping the RDE-1/AGO1/PIWI Protein Family Members

The PIWI domain defined by Cerutti et al., 2000 was used for alignment by CLUSTAL W (Thompson et al., 1994) and tree building using GrowTree in the Genetics Computer Group (GCG) Program. The asterisk indicates that RNAi was performed on this gene and the double asterisk indicates that, in addition, dsRNA was coinjected with alg-1 dsRNA (see Results).

Finally, we assayed alg-1 and alg-2 for possible roles in RNAi. The alg-2(ok304) homozygotes were fully sensitive to RNAi, and likewise the inhibition of alg-1 or alg-2 by RNAi did not suppress RNAi targeting a second gene (data not shown). These findings suggest that alg-1 and alg-2 are not necessary for RNAi. Nevertheless, it remains possible that these genes might have some redundant function in RNAi with rde-1 or with other members of this gene family (see Discussion).

C. elegans dcr-1 Functions in Development and RNAi

The C. elegans gene K12H4.8, which we have named dcr-1, is predicted to encode a protein related to the Drosophila Dicer (Bernstein et al., 2001) and the Arabidopsis Carpel Factory (Jacobsen et al., 1999) proteins implicated in RNAi and regulation of development, respectively. A previous study has shown that RNA interference of Drosophila Dicer can induce a partial loss of RNAi (Bernstein et al., 2001). We used RNAi of C. elegans dcr-1 to assess its role in developmental control and RNA interference. dcr-1(RNAi) induced developmental abnormalities during larval growth that were very similar to those induced by alg-1/alg-2(RNAi). These included a protruding and non-functional vulva, and a tendency to burst at the vulva shortly after the molt from the larval to the adult stage (Figure 2B). In addition, dcr-1(RNAi) animals frequently exhibited faint or missing adult-specific alae (Figure 2E).

Although the phenotypes induced by dcr-1(RNAi)
Figure 2. Genetic and RNAi Analysis of dcr-1 and alg-1/alg-2

(A and B) Burst vulva phenotype among young adult animals after (A) alg-1/alg-2 (RNAi) induced by injection of dsRNA prepared from the partial alg-1 cDNA yk403g7, that contains short regions of perfect nucleotide identity with alg-2, and (B) dcr-1(RNAi).

(C) Nomarski image of three embryos arrested at the 2- to 3-fold stage of morphogenesis after simultaneous injection of both alg-1 and alg-2 dsRNAs (see Results).

(D) An adult animal with germline defects induced by alg-1/alg-2(RNAi); undifferentiated germline cells (bracket) are observed more proximal to the uterus (black arrow) than are the sperm (short arrow) and oocytes (long arrow); approximately 50% of F1 RNAi animals exhibit this defect in one or both gonad arms.

(E and F) Cuticle defects among animals obtained from (E) RNAi targeting dcr-1 and alg-1/alg-2 and from (F) various dcr-1 mutant strains.

(G and H) Schematic box and line diagrams indicating the exon-intron structure, conserved domains, and the lesions in (G) the predicted dcr-1 gene, and (H) the predicted alg-2 gene.

were similar to those induced by alg-1/alg-2(RNAi), dcr-1(RNAi) phenotypes were less penetrant. For example, 91% of the alg-1/alg-2(RNAi) animals lack the adult-specific alae while only 19% of the dcr-1(RNAi) animals completely lack the alae (Figure 2E). This finding could indicate that dcr-1 has only a relatively minor role in the specification of the alae; alternatively, it might reflect a difficulty in inhibiting dcr-1 function via RNAi. For example, if dcr-1 is required for RNAi in C. elegans as it appears to be in Drosophila, then the use of RNAi to target dcr-1 may, at best, diminish its activity.

We therefore compared the dcr-1(RNAi) phenotype to the phenotype of animals homozygous for mutations in dcr-1. We obtained three noncomplementing mutant strains that define the dcr-1 locus. Two of these, let-740(s2624) and let-740(s2795), were identified in an extensive genetic screen for mutations balanced by the indistinguishability of the let-740(s2624) and let-740(s2795) mutations result in premature stop codons appears to be in Drosophila, then the use of RNAi to target dcr-1 may, at best, diminish its activity.

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moving residues 708 through 1321 (Figure 2G) and terminating after expression of 15 amino acid residues from intrinsic sequences. All of these lesions are likely to severely disrupt DCR-1 protein expression; the s2624 allele would encode a protein of only 59 amino acids lacking all of the recognizable functional motifs, while the latter two alleles would encode truncated proteins lacking the PAZ, RNase III, and dsRBP domains. All three mutant dcr-1 strains exhibit a similar, fully penetrant, sterile phenotype. Homozygous hermaphrodites produce germ cells, including both sperm and oocytes, but for unknown reasons fail to produce embryos. In addition, all three strains exhibit adult cuticle and vulval defects identical to the defects induced by dcr-1(RNAi), including a protruding vulva and occasional vulval bursting as well as faint or missing alae (Figure 2F, and data not shown). Because the let-740 mutations are allelic to dcr-1(ok247), we will henceforth use the more descriptive name dcr-1, to refer to this gene.

The severity of the phenotypes observed in the dcr-1 homozygous mutants was dependent on the maternal genotype, suggesting that dcr-1(+/+) activity is provided maternally (Figure 2F). If dcr-1(+/+) activity is provided maternally, then RNAi of dcr-1 into a dcr-1 heterozygous mother might be expected to enhance the cuticle defects or cause additional phenotypes in the homozygous mutant progeny of the injected animal. Consistent with this possibility, the homozygous mutant class of progeny from dcr-1 heterozygous mothers injected with dcr-1 dsRNA arrested as embryos at a developmental stage similar to that observed in the double RNAi targeting alg-1 and alg-2 (Figure 2G, see Experimental Procedures). These findings suggest that maternal dcr-1(+/+) activity rescues essential functions of dcr-1 in the homzygous embryos and larvae and that RNAi of dcr-1 depletes this maternal activity. Because RNAi of dcr-1 efficiently inhibits dcr-1 activities required for larval development without inducing sterility or embryonic lethality, we use dcr-1(RNAi) for the subsequent developmental studies described here.

Finally, we asked if homozygous dcr-1 mutants were sensitive to RNAi. The conceptually straightforward experiment of assaying RNAi in the complete absence of dcr-1 is, unfortunately, not feasible since dcr-1 is required for viability of the animal. The best experiments that can be done are to assay for sensitivity to RNAi in animals where dcr-1 activity has been decreased. We first tested dcr-1(ok247) homozygous animals for sensitivity to dsRNA delivered by injection into their mother or directly into the homozygous L4 larvae. In both assays we observed nearly normal levels of RNAi (data not shown). This observation could indicate that maternal dcr-1(+/+) activity can rescue RNAi in dcr-1 homozygous mutant progeny just as it appears to rescue the developmental and alae defects described above. Consistent with this idea, other RNAi pathway mutants including rde-1 and rde-4 homoyzgotes are strongly rescued by one maternal dose of rde(+/+) activity (Tabara et al., 1999; and data not shown). Because dsRNA targeting dcr-1 induces strong larval developmental defects, we next asked if dcr-1(RNAi) might sufficiently reduce dcr-1 activity to cause an RNAI-deficient phenotype. For this assay, we injected dcr-1 dsRNA into adult hermaphrodites and then assayed for sensitivity to RNAi targeting a second gene. In experiments targeting two different genes, we observed a significant reduction of RNAi among the progeny of dcr-1(RNAI) animals but not among control animals injected with unrelated dsRNAs (Table 1). These results support the findings from Bernstein et al. (2001) that implicate Drosophila Dicer in RNAi and suggest that DCR-1 may have a similar activity in C. elegans.

dcr-1(RNAI) and alg-1/alg-2(RNAI) Cause Retarded Heterochronic Defects

The combination of vulval and adult cuticle maturation defects caused by RNAi of alg-1/alg-2 and dcr-1 is reminiscent of phenotypes resulting from mutations in the genes lin-4 and let-7 (Lee et al., 1993; Reinhart et al., 2000). The lin-4 and let-7 genes promote transitions from earlier to later cell fates and, thus, mutations in these genes cause reiteration of cell divisions typical of earlier larval stages, a hallmark of genes that regulate developmental timing (such genes have been termed “heterochronic genes”). For example, loss-of-function mutations in let-7 result in a failure of larval seam cells in the hypodermis to progress to the adult-specific program of terminal differentiation indicated by the production of the adult-specific alae and, instead, the cells repeat the late larval type of divisions. These reiterated divisions contribute to an unstable vulval structure and failure to form a cuticle with adult alae.

We determined that the developmental defects in alg-1/alg-2 and dcr-1 RNAI animals also result from temporal misspecifications in the seam cell lineages. To aid in the observation of seam cell divisions, we utilized a transgenic strain that drives GFP expression specifically in the seam cell nuclei (see Experimental Procedures). Normally, the ten seam cells present at hatching divide to generate 16 cells during the second larval stage. Although these 16 cells divide at the succeeding third and fourth larval transitions, only one daughter cell maintains the seam cell fate (Sulston and Horvitz, 1977), so that the total number of GFP-expressing seam cells in the adult is 16 (Figure 3A).

RNAi of either dcr-1 or alg-1/alg-2 resulted in adults with extra seam cells (Figures 3B and 3C) that arise from reiterated L2 type divisions. This observation is specific for RNAi of dcr-1 or alg-1/alg-2 because control RNAi of mes-2, a gene not involved in developmental timing, did not affect the seam cell division pattern (data not shown). Most progeny of dcr-1 and alg-1/alg-2 dsRNA-injected parents had normal seam cell divisions until the L3 stage, when reiterations of L2 type divisions were common. Many animals showed mixed patterns of stage-specific divisions, a phenotype similar to that ob-

Table 1. Reduced Sensitivity to RNAi in dcr-1 (RNAI) Background

<table>
<thead>
<tr>
<th>dsRNA I</th>
<th>dsRNA II</th>
<th>Resistance to dsRNA II</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>unc-22</td>
<td>0% (n = 434)</td>
</tr>
<tr>
<td>mes-2</td>
<td>unc-22</td>
<td>0% (n = 824)</td>
</tr>
<tr>
<td>dcr-1</td>
<td>unc-22</td>
<td>29% (n = 604)</td>
</tr>
</tbody>
</table>

| none | sqt-3  | 2% (n = 51) |
| gfp  | sqt-3  | 6% (n = 67) |
| dcr-1| sqt-3  | 89% (n = 309) |
served previously in heterochronic mutants (daf-12, for example; Antebi et al., 1998). The number of seam cells observed in dcr-1(RNAi) adults ranged from 16 to 33 with an average of 21, and only 15% showed the normal number of 16 seam cells (n = 52); alg-1/alg-2(RNAi) adults exhibited 18–36 seam cells with an average of 25 (n = 81). The dcr-1 and alg-1/alg-2 (RNAi) progeny also repeated L3 or L4 seam cell division programs into adulthood, when normally these cells would stop dividing and become terminally differentiated (data not shown).

We consistently observe inappropriate seam cell division patterns in L3 through later stages in dcr-1(RNAi) and alg-1/alg-2(RNAi) animals. However, because of the likely incomplete RNAi of dcr-1 and the redundancy of alg-1 and alg-2, it is not possible to establish the precise point in larval development where these genes are first required. Additional support that these genes may act earlier in larval development comes from the seam cell division pattern displayed by the more strongly affected animals obtained by coinjecting dsRNAs targeting portions of both alg-1 and alg-2. In these experiments, reiterations of L1-type divisions were observed, in addition to repetition of later stage patterns (data not shown).

dcr-1 and alg-1/alg-2 Regulate Stage-Specific Gene Expression

The similarity of phenotypes described above to those of the heterochronic genes lin-4 and let-7 raised the possibility that alg-1, alg-2, and dcr-1 might act upstream of the lin-4 or let-7 stRNAs or might be necessary for their regulatory activities. The targets of lin-4 and let-7 include the lin-14 and lin-41 mRNAs. Genetic studies suggest that lin-4 and let-7 stRNAs directly regulate lin-14 and lin-41 through complementary sequences in their 3’UTRs (Lee et al., 1993; Wightman et al., 1993; Slack et al., 2000; Reinhart et al., 2000). Because the retarded phenotypes of lin-4 and let-7 are caused in part by failure to downregulate their target genes, mutations in lin-14 and lin-41 partially suppress the lin-4 and let-7 mutant phenotypes (Ambros, 1988; Reinhart et al., 2000; Slack et al., 2000). To determine if alg-1/alg-2 and dcr-1 RNAi animals exhibit a similar genetic relationship with lin-14 and lin-41 mutants, we performed dsRNA injections in the lin-14 and lin-41 mutant backgrounds. We found significant suppression of the RNAi-induced alg-1/alg-2 and dcr-1 heterochronic phenotypes, including alae and vulval defects, by the lin-14(n179) and lin-41(ma104) nonnull mutations (Figure 4). In addition, the penetrant germline phenotype associated with alg-1/alg-2(RNAi) was partially suppressed by the lin-14 and lin-41 mutations (Figure 4, see also Figure 2D), but the synthetic lethal phenotype associated with double alg-1/alg-2(RNAi) was not suppressed (data not shown). In control RNAi experiments, the lin-14 and lin-41 mutant strains were fully sensitive to RNAi. These findings are consistent with the idea that the retarded heterochronic phenotypes induced by alg-1/alg-2 and dcr-1 (RNAi) are caused, at least in part, by misregulation of lin-14 and lin-41.

Elements in the 3’UTRs of lin-14 and lin-41 mRNAs are responsible for negative regulation mediated by the lin-4 and let-7 stRNAs. If alg-1, alg-2, and dcr-1 are necessary for lin-4 and let-7 function, then we would expect misregulation of reporter genes that carry the lin-14 and lin-41 3’UTER elements. To test for misregulation of the lin-14 3’UTR, we used a transgene containing a dominant mutation in the cuticle collagen gene rol-6(su1006) fused to the lin-14 3’UTR. In wild-type animals the lin-14 3’UTR downregulates the expression of the dominant rol-6 reporter gene in a lin-4-dependent fashion, leading to a non-Rolling phenotype in 100% of animals bearing the transgene (n = 825). In contrast, 54% of lin-4(e912) animals bearing the same transgene exhibit a Rolling phenotype (n = 253). While injection of this strain with control mes-2 dsRNA produced virtually no rolling progeny (n = 256), injection of dcr-1 dsRNA caused rolling in half of the progeny (n = 296), indicating a marked interference with downregulation of the rol-6/lin-14 3’UTR reporter gene.

We tested alg-1/alg-2(RNAi) animals for misregulation of the lin-41 3’UTR by using a transgene bearing a LacZ:lin-41/3’UTR gene fusion, which is expressed early in larval development but then undergoes let-7-dependent downregulation prior to adulthood (Reinhart
et al., 2000; Slack et al., 2000). Only 12% (n = 25) of control adult worms expressed LacZ from the lin-41 3' UTR fusion gene, while 48% (n = 23) of alg-1/alg-2(RNAi) adult animals expressed the fusion gene, consistent with derepression of the LacZ::lin-41/3' UTR gene fusion. This 4-fold increase in the number of adults expressing the LacZ::lin-41/3' UTR gene fusion after alg-1/alg-2(RNAi) is similar to the effect of a let-7(−) mutation (Reinhart et al., 2000). The findings that reporter genes bearing the lin-14 and lin-4 3' UTRs are upregulated by dcr-1 and alg-1/alg-2 inhibition, together with the observation that lin-14 and lin-41 mutations suppress the retarded heterochronic phenotypes caused by dcr-1 and alg-1/alg-2 RNAi, are consistent with the model that dcr-1, alg-1, and alg-2 function in the lin-4 and let-7 pathway to regulate larval development.

**dcr-1 and alg-1/alg-2(RNAi) Animals Exhibit Defects in stRNA Processing**

**lin-4 and let-7** are expressed as longer, approximately 70 nt RNAs that are predicted to fold into structures containing regions of double-stranded RNA. Because *Drosophila* Dicer cleaves introduced dsRNAs into fragments of approximately 22 nt (Bernstein et al., 2001), we hypothesized that the heterochronic phenotypes caused by dcr-1 (RNAi) may be due to a defect in the processing of the larger, potentially dsRNA, forms of control adult worms expressed LacZ from the lin-41 and let-7 into the 22 nt stRNAs. To test this idea we collected progeny from mothers subjected to dcr-1(RNAi) and performed Northern blot analyses to monitor the size and abundance of the lin-4 and let-7 RNAs. The findings that reporter genes in alg-1/alg-2 (RNAi) animals bearing the LacZ::lin-41/3' UTR gene fusion after alg-1/alg-2(RNAi) are upregulated by dcr-1 and alg-1/alg-2 inhibition, together with the observation that lin-14 and lin-41 mutations suppress the retarded heterochronic phenotypes caused by dcr-1 and alg-1/alg-2 RNAi, are consistent with the model that dcr-1, alg-1, and alg-2 function in the lin-4 and let-7 pathway to regulate larval development.

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**Figure 4. Genetic Suppression of dcr-1 and alg-1/alg-2(RNAi) by lin-41 and lin-14 Mutants**

(A and B) Adult progeny of injected wild-type, lin-41(ma104), and lin-14(n179) animals were assayed for RNAi induced phenotypes as indicated. (A) alg-1/alg-2(RNAi) and (B) dcr-1(RNAi). The number of animals scored (n) for each phenotype category is indicated. The alae, vulva, and germline were observed using the compound microscope, while egg-laying and vulval bursting phenotypes were scored in the dissecting microscope. Two different dsRNAs were used for alg-1/alg-2(RNAi), indicated by the Roman numerals above the bars in (A): (I) Full-length alg-1 dsRNA, used in the alae, vulva, and germline assays, induces a high percentage of vulvaless animals that preclude scoring the vulval bursting phenotype. Therefore, dsRNA prepared from (II) the alg-1 partial cDNA clone yk403g7 was used to induce the weaker vulval bursting phenotype.
targeting \textit{alg-1} induced accumulation of the pre-\textit{lin-4} RNA but not pre-\textit{let-7} (Figure 5B lane 4), and similarly, \textit{alg-2(ok304)} animals exhibited a slight accumulation of pre-\textit{lin-4} and little or no accumulation of pre-\textit{let-7} (Figure 5B, lane 5).

The quantity of the short forms of the \textit{lin-4} and \textit{let-7} stRNAs consistently appeared to be reduced in RNA populations prepared from \textit{alg-1/alg-2(RNAi)}, \textit{dcr-1(RNAi)}, and \textit{dcr-1(ok247)} animals (Figures 5A and 5B), while control RNA populations prepared from animals undergoing RNAi of the cuticle collagen gene \textit{rol-6} exhibited normal levels of \textit{lin-4} and \textit{let-7} stRNAs (Figure 5B, compare lanes 1 and 2). This apparent reduction in \textit{let-7} stRNA level was observed even in \textit{alg-1/alg-2(RNAi)} populations where no significant accumulation of pre-\textit{let-7} was observed. These findings suggest that \textit{alg-1/alg-2} activities may be more important for the stability or function of \textit{let-7} stRNA than for its processing from the larger form. Alternatively, \textit{alg-1/alg-2} might also be involved in \textit{let-7} processing but the \textit{let-7} long form may be less stable, so that unprocessed \textit{let-7} does not accumulate in the absence of \textit{alg-1/alg-2} activity.

Discussion

RNAi and Natural Tiny RNAs

RNAi shares several features with developmental gene regulation mediated by the stRNA encoding genes \textit{lin-4} and \textit{let-7}. In RNAi, experimentally introduced double-stranded RNA is processed into small RNAs of approximately 22 nt. These small RNAs have been termed small interfering “siRNAs” because they appear to guide a nuclease in the destruction of complementary target mRNAs (Elbashir et al., 2001a). The developmental regulators, \textit{lin-4} and \textit{let-7}, are expressed as RNAs of approximately 70 nt that are predicted to fold into stable stem-loop structures that may be the precursors of the small temporal “stRNAs.” Thus, the folded 70 nt \textit{lin-4} and \textit{let-7} stRNAs may be analogous to the dsRNAs that trigger RNAi while the stRNA products may be analogous to the siRNAs that direct mRNA destruction. Genetic and molecular evidence presented here extend this analogy, linking stRNA production and function to a processing machinery and to regulatory proteins related to those that mediate RNAi. Specifically, we have shown that the efficient processing of the \textit{lin-4} and \textit{let-7} stRNAs from larger precursors depends on the activity of DCR-1, a \textit{C. elegans} homolog of the \textit{Drosophila} multifunctional RNase III related protein, Dicer, that has been shown in \textit{Drosophila} cell extracts to process dsRNA into siRNAs that can mediate RNAi (Bernstein et al., 2001). Further, we have shown that \textit{alg-1} and \textit{alg-2}, two homologs of the RNAi pathway gene \textit{rde-1}, are required for efficient stRNA expression, and along with \textit{dcr-1} function to promote \textit{lin-4} and \textit{let-7} activities in temporal development. Thus, the expression of the tiny RNAs that mediate RNAi and developmental gene regulation appear to share a requirement for DCR-1 activity, while RDE-1 and its homologs provide parallel functions in these pathways (Figure 6). Our findings are consistent with a model in
which members of the RDE-1 and DCR-1 families act not only in gene silencing but also with naturally expressed dsRNAs to execute cellular and developmental gene regulatory events.

**Differences between RNAi and stRNA Mechanisms**

Although there are compelling similarities between RNAi and developmental regulation by lin-4 and let-7 there are also several important differences. In RNAi, the dsRNAs utilized, typically contain long stretches of perfect base pairing (Parrish et al., 2000). The stRNA precursors, however, are predicted to contain at most 6, for lin-4, and 13, for let-7, uninterrupted Watson-Crick base pairs. Whereas cleavage of the perfectly base-paired RNAs that initiate RNAi yields both sense and antisense, or potentially double-stranded siRNAs (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000; Elbashir et al., 2001a), only one strand of the lin-4 and let-7 stRNAs is detected (A.E.P. and G.R., unpublished observations). Thus, after generation of the mature stRNA, the remaining sequences must undergo rapid degradation.

The RNAi and stRNA pathways also appear to induce distinct outcomes: RNA destruction versus translation inhibition. In RNAi the target mRNA is rapidly degraded (Montgomery et al., 1998; Tuschl et al., 1999). Although the RNase responsible for target RNA destruction is not yet known, it is thought that the antisense strand of the siRNA acts as a guide in mRNA destruction, by base-pairing with the target mRNA. The stRNAs also specifically downregulate the expression of their target genes. Although details of the mechanism by which stRNAs cause decreased expression are unknown, the regulation of lin-14 by lin-4 occurs at the translational level. Upon expression of lin-4 RNA, the levels of LIN-14 protein rapidly decline, but lin-14 mRNA levels remain constant and appear to remain associated with polyribosomes (Wightman et al., 1993; Olsen and Ambros 1999). Because let-7-mediated regulation of LIN-4 protein expression may only occur in a subset of cells (Slack et al., 2000), it is, as yet, unclear if the mRNA levels or polyribosome loading of this target is affected by the expression of let-7 RNA.

The distinction between mRNA destruction by RNAi and inhibition of translation by the lin-4 regulatory RNA could reside in the target mRNA sequence or in the particular region of the mRNA targeted. Whereas siRNAs can target sequences anywhere in the mature mRNA, stRNAs pair with specific sites in the 3′ UTRs of their target genes. And just as the precursors of the stRNAs have imperfect internal complementarity, the stRNAs contain imperfect complementarity to their target sequences. Imperfect pairing could permit access to RNA nucleotides by sequence-specific RNA binding proteins or conversely might reduce the affinity with which a nuclease could cleave the mRNA/stRNA hybrid. Alternatively, both siRNAs and stRNAs may induce similar modifications of their target mRNAs while flanking sequences provide for context dependent interactions that cause inhibition of translation in the case of lin-14 but promote destruction of other mRNAs.

**RDE-1 Family Members and Small RNA Cofactors in Development and PTGS**

There are 24 members of the RDE-1/AGO1/PIWI family in C. elegans (Figure 1). The degree of conservation between certain members of this family is striking. For example, ALG-1 and ALG-2 exhibit 41% identity with AGO1 from Arabidopsis and 67%–69% identity with AGO1 relatives in animals. The common ancestor of worms and humans appears to have had both an AGO1 ortholog and a second, already-divergent family member that has given rise to the PIWI family of genes (Figure 1). The fact that divergent members of this family, including rde-1, qde-2, and ago-1, all function in gene silencing suggests that PTGS mechanisms represent an important ancestral function of genes within this family.

Developmental functions have also been reported for members of the piwi and ago-1 families in both animals and plants (Bohmert et al., 1998; Moussian et al., 1998; Cox et al., 1998; Cikaluk et al., 1999; Kataoka et al., 2001). One feature that emerges from studies of these developmental phenotypes is that many of these genes appear to regulate germ cell and stem cell functions. Perhaps germ cells and stem cells have developed PTGS mechanisms for suppressing viral and transposon pathogens that might otherwise degrade the genome and, thus, the totipotency of these cells. The developmental phenotypes associated with mutations in members of the rde-1 gene family could thus reflect a general loss of gene silencing important for stem cell maintenance or differentiation. However, the findings reported here suggest an alternative possibility. We have shown that rde-1-related genes, alg-1 and alg-2, function with natural small RNA cofactors in specific developmental gene regulation events. Thus, we speculate that the Drosophila genes piwi, aubergine, and ago1, the Arabidopsis gene ago1, and perhaps many other members of this family in C. elegans and other organisms may similarly have small endogenous RNA cofactors with which they function to regulate specific target mRNAs.

While there are 24 members of the rde-1/Argonaute gene family in C. elegans, there are fewer in Arabidopsis, humans, and Drosophila. Only the Piwi and Argonaute subtypes are conserved in many species, while RDE-1 as well as most of the other C. elegans family members...
are more divergent. Perhaps the family of tiny RNAs that may act with these proteins has also undergone expansion in C. elegans. Whether the ancestral function of RDE-1-related genes was in developmental control or sequence-directed immunity, it is clear that a great potential exists for exploiting these proteins, along with small RNAs as guides, to direct the regulation of specific gene targets in the cell.

Previous work has indicated that RDE-1 plays an upstream role in the initiation of interference in response to dsRNA in C. elegans (Grishok et al., 2000). Findings described here suggest that ALG-1 and ALG-2 may play a similar upstream role in the lin-4 and let-7 stRNA pathways. Thus, distinct members of the extended family of RDE-1 homologues in C. elegans may play specific roles in RNAi and stRNA pathways. We speculate that one or more of the other C. elegans RDE-1 family members may provide a similar function in cosuppression in C. elegans (Figure 6). One attractive possibility is that these diversified factors provide specificity to their respective pathways. This might involve a role in the recognition of the distinct trigger sequences or in insuring that the processed small RNAs are assembled into distinct downstream complexes. Perhaps members of the RDE-1 family remain associated with the RNA sequences throughout processing and provide specificity needed to ensure that the small RNAs produced are targeted to the appropriate downstream complex, for example, to mediate mRNA destruction versus translation inhibition (Figure 6).

A role for RDE-1 family members in both small RNA production and targeting could explain why the inhibition of alg-1/alg-2 induces such a dramatic effect on lin-4 and let-7 function at best reducing but not eliminating the processed stRNA. Similarly, recent studies of small RNA accumulation during RNAi suggest that rde-1 is not essential for small RNA production after exposure to dsRNA (S.P. and A.F.; A.G. and C.C.M., unpublished observations) and yet rde-1(+ or -) activity is absolutely required for interference. Conceivably, dsRNA processing might still occur in the absence of RDE-1 or its homologs but the resulting siRNAs or stRNAs may not be assembled into the appropriate downstream complexes and therefore fail to function. Nevertheless, the finding that alg-1/alg-2(RNAi) dramatically affects the accumulation of the lin-4 precursor supports a role for these factors either upstream of, or at the same step as DCR-1 (Figure 6).

The Role of DCR-1 in RNAi and stRNA Processing

We have shown that dcr-1 is an essential gene and is also required for RNAi in C. elegans. In the model proposed above, dcr-1, which appears to be a single copy gene in C. elegans, could play a role in dsRNA processing important in many gene silencing and developmental pathways. DCR-1 has several motifs that might be expected in a dsRNA processing enzyme, including a helicase, a dsRNA binding domain, and two RNase III type dsRNA exonuclease domains. Thus, we propose that DCR-1 functions in multiple pathways important for developmental and PTGS mechanisms, and may be guided in its processing of distinct substrates by members of the RDE-1 family (Figure 6). Consistent with a relatively specific role for dcr-1, we found that mature ribosomal RNAs, which are also produced by RNase III type processing, accumulate to normal levels in animals with reduced dcr-1 activity (data not shown).

The combination of a maternally provided dcr-1 activity and zygotic sterility make it difficult to unambiguously answer the question of whether this protein is absolutely essential for RNAi and stRNA pathways. Nevertheless, the reiteration of L2 fates revealed by the seam cell lineage analysis of dcr-1(RNAi) animals, and the suppression of those phenotypes by mutations in lin-14 or lin-41 are unique phenotypic and genetic signatures that strongly support the model where lin-4 and let-7 processing is dependent on dcr-1(+ or -) activity. Perhaps the embryonic and larval lethal phenotypes associated with dcr-1 inhibition and the developmental phenotypes associated with the Arabidopsis homolog, caf 1, reflect a role for members of this gene family in the processing of other as yet unidentified small regulatory RNAs. Thus, tiny RNAs may function in a broader range of gene regulatory and developmental events than the transient translocations mediated by the founding members of the class, the lin-4 and let-7 stRNAs.

A recent study (P. Zamore, personal communication) has shown that a human homolog of dcr-1 is important for processing of the let-7 stRNA precursor in cultured human cells, suggesting that the regulatory interactions observed in C. elegans are conserved. A prediction from the current study is that human let-7 processing or function will also require a human member of the RDE-1/AGO1/PIWI family of genes. Indeed, it is likely that the ramified family of RDE-1/AGO1/PIWI related proteins has coevolved with numerous small RNA encoding genes analogous to lin-4 and let-7, and that many such genes await discovery in plant and animal genomes.

Experimental Procedures

RNA Interference Assays

RNAi methods were as described in Grishok et al., 2000. cDNA clones and corresponding targeted genes were as follows: yk403g7, F48F7.1 (alg-1); yk199g3, yk433a5, T07D3.7 (alg-2); yk397e11, D2030.6 (alg-1); yk87d4, ZK757.3b; yk359b5, T22B3.2; yk36g4, R06C7.1; yk102d8, yk358e1, R09A1.1; yk201f, T23D8.7; yk21a5, C16C10.3; yk448d6, R04A9.2; yk548f2, F58G1.1; yk249a12, C06A1.4; yk550c3, C18E3.7; yk307e2, K12B6.1; yk240e7, C04F12.1.

PCR was used to generate templates for dsRNA synthesis using primers with T7 promoter sequences and gene-specific sequences as follows: 5'-GGG GAT CCT CGA ACA TCG-3' and 5'-GGG AAA ATA CAT GAC CCT GTT CTT C-3' for full-length alg-2; 5'-GGG GAT CCT CGA ACA TCG-3' and 5'-GGG AAA TGA TGG CTG CCG ACA A-3' for unique 200 bp fragment of alg-2; 5'-GGG GCC GCG CAA TAT TTG-3' and 5'-GGT CCT CCA ATT GAG ACA CT-3' for unique 300 bp fragment of alg-1; 5'-GGG TCC TTC TCC GGT GGC CTC-3' for dcr-1, 5'-GGG CCC AGC ACA TCA ACT CCC TCA GG-3' and 5'-GGT CCT CCA ATT GAG ACA CT-3' for dcr-1 mes-2. The alg-1 full-length dsRNA was made from the plasmid pCCM508.

Combinations of full-length alg-1 dsRNA with pgr-1 dsRNA (yk397e11) or with dsRNAs corresponding to predicted genes T23D6.7, T22D3.2, and ZK757.3 did not result in phenotypes more severe than single alg-1 (RNAi).

dcr-1(RNAi) of dcr-1 Heterozygous Animals

Injections of dcr-1 dsRNA were performed into animals heterozygous for dcr-1(ok247) and a GFP marked hT2 balancer chromosome. This strain had 21% (n = 239) dcr-1(–) adult homozygous animals. After injection of 13 dcr-1(ok247)/hT2-GFP mothers, zero GFP(–) animals were observed (n = 144). Embryonic lethality after
The injection of dcr-1 dsRNA, 12 dcr-1/qC1 mothers, produced 36% (n = 481) F1 embryos arrested at the 2-fold to 3-fold stage of morphogenesis.

**Determination of Seam Cell Numbers**

The SCM:GFP strain provided by Joel Rothman, which contains a nuclear localized GFP marker expressed specifically in seam cells, was injected with dsRNAs for mes-2, dcr-1, alg-1, and alg-1 + alg-2. Populations of F1 progeny from uninjected controls and dsRNA injected animals were staged and representative animals were examined for seam cell number and division patterns by observing the GFP marker under UV light at the L1, L2, L3, L4, and adult stages.

**Northern Analyses of let-7 and lin-4 RNAs**

Homozygous mutant worms or F1 progeny of uninjected and dsRNA injected worms were collected at the L3-L4 and adult stages. Total RNA isolation and Northern analyses were performed as previously described in Lee et al., 1993, hybridization and wash steps were performed at 50°C. Oligonucleotides used as Northern probes were let-7: 5′-AATATACCTACTACACTTGAGGTCTCAGGG. SS RNA was detected by ethidium bromide staining of polyacrylamide gels prior to transfer.

**Analysis of the let-7 Regulated lacZ::alg-1 3′ UTR Reporter Gene**

Worms containing the ngEx540 reporter array were injected with dsRNA against alg-1 and transgenic progeny as well as uninjected controls were collected for fixing and staining with Xgal to detect expression of lacZ::alg-1.

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**References**


