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ANALYSIS OF RNA INTERFERENCE IN *C. ELEGANS*

A Dissertation Presented

By

ALLA GRISHOK

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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CELL BIOLOGY

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APPROVAL PAGE

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ABSTRACT

RNA interference (RNAi) in the nematode *Caenorhabditis elegans* is a type of homology-dependent post-transcriptional gene silencing induced by dsRNA. This dissertation describes the genetic analysis of the RNA interference pathway and inheritance properties associated with this phenomenon. We demonstrate that the RNAi effect can be observed in the progeny of the injected animal for at least two generations. Transmission of the interference effect occurs through a dominant extragenic agent. The wild-type activities of the RNAi pathway genes *rde-1* and *rde-4* are required for the formation of this interfering agent but are not needed for interference thereafter. In contrast, the *rde-2* and *mut-7* genes are required downstream for interference. These findings provide evidence for germline transmission of an extragenic sequence-specific silencing factor and implicate *rde-1* and *rde-4* in the formation of the inherited agent.

Other forms of homology-dependent silencing in *C. elegans* include co-suppression and transcriptional silencing of transgenes in the germline. We demonstrate that silencing of a germline transgene can be initiated by injected dsRNA, via the RNAi pathway, and then maintained on a different level. This observation indicates that post-transcriptional and transcriptional silencing of homologous genes could be connected.

This dissertation also describes the connection between RNAi and developmental pathways of gene regulation in *C. elegans*. 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* small temporal RNAs that regulate stage-specific development. Our findings suggest that a common processing machinery generates guide RNAs that mediate both RNAi and endogenous gene regulation.

Finally, this study illustrates the detection of small interfering RNAs (siRNAs), intermediates in the RNAi process, and describes requirements for their accumulation. We show that, in the course of RNAi induced by feeding dsRNA, *C. elegans* accumulate only siRNAs complementary to the target gene. This accumulation depends on the presence of the target sequence and requires activities of several RNAi-pathway genes. We show that selective retention or amplification of RNAi-active molecules can create a reservoir of memory antisense siRNAs that prevent future expression of the genes with complementary sequence. This suggests a parallel at the molecular level with the clonal selection of antibody forming cells and in the vertebrate immune system.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Ago	Argonaute
Alg	Argonaute-like gene
Caf	Carpell factory
DDM	deficient DNA methylation
Df	deficiency
Dp	duplication
Dpy	dumpy
Dcr	Dicer related
dsRBP	double-stranded RNA binding protein
dsRNA	double-stranded RNA
Ego	enhancer of Glp-1
GERp95	Golgi Endoplasmic Reticulum protein 95kDa
GFP	green fluorescent protein
HC-Pro	helper component protease
Let	lethal
Lin	lineage abnormal
Mes	maternal effect sterile
Met	methyltransferase
Mom	more mesoderm
Mut	mutator
NMD	nonsense-mediated decay
Par	abnormal embryonic partitioning of cytoplasm
PAZ	Piwi Argonaute Zwille
Pes	patterned expression site
Pie	pharynx and intestine in excess
Pos	posterior segregation
Prg	piwi-related gene
PTGS	post-transcriptional gene silencing
Qde	quelling deficient
Rde	RNAi deficient
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RNAi	RNA interference
Rol	roller
RRF	RdRP family
SDE	silencing deficient
Sgg	shaggy
SGS	suppressor of gene silencing
siRNA	small interfering RNA
Sqt	squat

STM	shoot meristemless
stRNA	small temporal RNA
Unc	uncoordinated
UTR	untranslated region
YAC	yeast artificial chromosome

CHAPTER I

INTRODUCTION

History of RNAi discovery

The natural regulation of gene function by antisense RNAs is documented in diverse organisms, such as bacteria (Stolt and Zillig, 1993; van Biesen et al., 1993; Delihas, 1995), *C.elegans* (Lee at al., 1993; Reinhart et al., 2000), and mammals (Hastings et al., 2000; Li and Murphy, 2000), including regulation of non-coding *Xist* RNA by antisense *Tisx* (Lee at al., 1999). Antisense transcripts have also been detected and implicated in the regulation of gene expression in *Drosophila* (Akhmanova et al., 1997; Aravin et al., 2001) and plants (Terryn and Rouze, 2000).

Antisense RNA was first used experimentally by Izant and Weintraub (1984, 1985) to induce a sequence-specific block of mRNA expression in tissue culture cells. Soon thereafter the technique found applications in a variety of other systems, including *Xenopus* oocytes (Harland and Weintraub, 1985), *Drosophila* embryos (Rosenberg et al., 1985) and mouse oocytes (Strickland et al., 1988). In *C.elegans*, antisense RNAs expressed from transgenes were shown to be effective in blocking the expression of two muscle genes *unc-22* and *unc-54* (Fire et al., 1991). However, inconsistency in the effectiveness of antisense approach from gene to gene or from one application to another in many systems led to the perception that this methodology was somewhat unreliable for inhibiting gene function. This perception was dramatically altered for *C. elegans* in 1995 when Guo and Kemphues found that the microinjection of antisense RNA corresponding

to the *par-1* gene induced a strikingly accurate *par-1* loss-of-function phenotype. Surprisingly, they found that control preparations of *par-1* sense RNA also induced a *par-1* loss of function phenotype (Guo and Kemphues, 1995).

Despite the apparent lack of strand specificity, the use of antisense RNA to inhibit gene function rapidly gained acceptance as the number of *C. elegans* genes that could be silenced with this technique continued to grow. Two additional observations suggested that something more than a simple concentration dependent pairing between the antisense RNA and the mRNA must be involved in the interference process in *C. elegans*. The first of these surprising observations came with the discovery that the interference effect could be inherited for at least two generations after the injection of RNA (Mello, unpublished observations). The second came with the discovery that interference could spread from the site of injection into the other tissues in the organism (Fire at al., 1998). Together these findings led *C. elegans* researchers to coin a new name for the methodology: RNA interference, or simply RNAi.

The mystery of RNAi took on a helical twist with the discovery that double-stranded RNA (dsRNA) was at least ten times more effective than preparations of sense or antisense RNA (Fire et al., 1998). This discovery was of great importance because it rapidly led to applications of dsRNA to silence genes in other organisms, including plants (Waterhouse et al., 1998), trypanosomes (Ngo et al., 1998), flies (Kennerdell and Carthew, 1998), planaria (Sanchez-Alvarado and Newmark, 1999), hydra (Lohmann et al., 1999) and mouse embryos (Wianny and Zernicka-Goetz, 2000).

The improved efficiency of dsRNA made possible new methods for inducing RNAi. These included simply soaking worms in dsRNA solutions (Tabara et al., 1998), feeding worms *E. coli* expressing a dsRNA segment of a target gene (Timmons and Fire, 1998), or driving dsRNA expression from a transgene (Tabara et al., 1999 a; Tavernarakis et al., 2000). These new methods in turn opened new doors for the application of RNAi. The applications included genetic screens for mutants resistant to RNAi (Tabara et al., 1999 a) and, more recently, genome wide applications of RNAi to systematically inhibit *C. elegans* genes (Fraser et al., 2000; Gonczy et al., 2000; Piano et al., 2000).

Studies on the mechanism of RNAi in *C. elegans* have identified similarities to post-transcriptional gene silencing (PTGS) mechanisms previously described in plants and *Neurospora*. The similarities and differences between RNAi and other homology-dependent silencing phenomena, like co-suppression and transgene silencing, have been recognized both within a given organism (Tabara et al., 1999 a; Dernburg et al., 2000; Ketting and Plasterk, 2000) and between different organisms (see reviews by Montgomery and Fire, 1998; Fire, 1999; Grant, 1999; Sharp, 1999, 2001; Wolffe and Matzke, 1999; Hunter, 1999, 2000; Bass, 2000; Bosher and Labouesse, 2000; Cogoni and Macino, 2000; Gura, 2000; Maine, 2000; Marx, 2000; Plasterk and Ketting, 2000; Carthew, 2001; Matzke et al., 2001).

RNAi and other silencing mechanisms in *C. elegans*

RNAi deficient mutants. The remarkable features of RNAi suggest that at least several distinct mechanisms may exist in the animal to facilitate this process. It is likely that specific mechanisms underlie: i) the spreading or transport of dsRNA or a secondary agent within and between tissues, ii) the formation and inheritance of an active interfering agent, and, of course, iii) the interference process itself. To identify genes required for these mechanisms in *C.elegans* Tabara and colleagues (1999 a) cultured populations of mutagenized worms on *E. coli* expressing a dsRNA corresponding to a segment of an essential *C. elegans* gene. Wild type animals feeding on this *E. coli* strain produced inviable embryos due to RNA interference with the essential gene (targeted via the ingested dsRNA). In contrast, mutants resistant to RNAi escaped interference and survived. Mutants identified in this way were named *RNAi def*icient or *rde* mutants.

This screen proved very powerful as a means for selecting mutations in RNAi pathway genes, and three classes of mutants were found. The first and largest class included mutants resistant to RNAi by feeding but sensitive to injected dsRNA, suggesting that these mutants may either be weak mutants or may be defective in the uptake or transport of RNA from the intestine. This class of mutants has yet to be characterized further. The second class consisted of 6 mutants defining two genes, *rde-1* and *rde-4*, which are absolutely required for RNAi. Although *rde-1* and *rde-4* mutants are completely resistant to RNAi, they exhibit no other obvious phenotypes. In contrast,

the third and final class, comprised of 14 mutants and defining 6 complementation groups, was deficient in RNAi targeting germline genes but remained sensitive to RNAi targeting several somatic genes. Members of this third class also exhibited several additional phenotypes including temperature dependent sterility, a high incidence of males and mobilization of transposons in the germline (Tabara et al., 1999 a), which are the features of mutator mutants (Collins et al., 1987; Ketting et al., 1999).

    RNAi and transposon silencing . The observation that transposons are mobilized in several of the RNAi deficient strains was exciting as it suggested a possible *in vivo* function for RNAi, a defense against transposons. Indeed, Ketting and colleagues (1999) showed that many of the mutator strains they had identified in a screen for mutants with increased transposition were also resistant to RNAi. They proposed a model for transposon silencing via RNAi initiated by transposon-derived dsRNA. This simple model was complicated, however, by the fact that the strongest RNAi deficient mutants, *rde-1* and *rde-4*, do not exhibit transposon mobilization (Tabara et al., 1999 a), indicating that there is some mechanistic distinction between the two types of silencing. The simplest interpretation of these findings is that the mutator class of RNAi mutants disrupts a step common to both RNAi and transposon silencing.

    A peculiar aspect of the mutator class of *rde* mutants is that they exhibit only partial loss of RNAi and primarily in the germline. One explanation may be that redundant genes exist that carry out the functions of these genes in other tissues. Alternatively, some of these mutations might disrupt RNAi indirectly by causing an excess of an unrelated dsRNA to accumulate in the germline. Indeed, an unrelated

dsRNA can, under some conditions, render wild type worms resistant to a second dsRNA, suggesting that there is a saturable step involved in the process (Parrish et al., 2000). In a recent study of post-transcriptional gene silencing in *Chlamydomonas* it was shown that both transposon and transgene silencing are affected by a mutation in the *mut-6* gene, which encodes a DEAH-box RNA helicase (Wu-Scharf et al., 2000). The authors demonstrate that aberrant transcripts accumulate in the *mut-6* mutant. Thus, disruption of an unrelated RNA degradation pathway or the upregulation of genes expressing natural dsRNA might indirectly cause mutant strains to become partially resistant to RNAi. Cloning and future analysis of more of the mutator class of *rde* mutants should shed light on whether these genes are direct or indirect effectors of RNAi.

RNAi and co-suppression. Co-suppression was first discovered in plants (Napoli et al., 1990; van der Krol et al., 1990) as a silencing phenomenon in which a transgene bearing an extra copy of a cellular gene initiates silencing of both the transgene and the endogenous copy of the corresponding gene. Co-suppression was subsequently shown to occur in fungi and called quelling (Romano and Macino, 1992). A similar type of homology-dependent gene silencing was found in *Drosophila* (Pal-Bhadra et al., 1997, 1999; Jensen et al., 1999; Chabiossier et al., 1998), *Paramecium* (Ruiz et al., 1998) and mammals (Garrick et al., 1998).

Gene silencing in co-suppression can occur both on transcriptional (Pal-Bhadra et al., 1997) and post-transcriptional (Cogoni and Macino, 1997) levels. Recent work has clearly demonstrated that post-transcriptionally induced co-suppression is related to RNAi. For example, the *qde-2* gene which is essential for co-suppression in *Neurospora*,

is a homolog of *C. elegans rde-1*, and another *rde-1* homolog, *ago1*, is important for post-transcriptional gene silencing in *Arabidopsis* (Fagard et al., 2000).

But how similar are RNAi and co-suppression? For example, do both mechanisms involve a dsRNA trigger? Although in some cases of PTGS in plants the initiation of silencing was correlated with dsRNA expression from the transgenes (Waterhouse et al., 1998; Smith et al., 2000; Chuang and Meyerowitz, 2000; Sijen et al., 2001), the presence of dsRNA has not been documented in most cases. The initiation of systemic PTGS by bombardment of plants with gold particles containing promoterless DNA (Voinnet et al., 1998) also indicates differences in the mechanism as injection of DNA into *C. elegans* does not induce systemic silencing. However, both in plants and in *Neurospora* mutations in genes that encode proteins with homology to RNA-dependent RNA polymerases (RdRP) completely abolish PTGS induced by transgenes (Cogoni and Macino, 1999 a; Dalmay et al., 2000; Mourrain et al., 2000) suggesting that dsRNA might play a role in co-suppression as well. Nevertheless, it is not clear at which step the target RNA gets copied into dsRNA. The prevailing model assumes that full-length mRNA molecules are copied into dsRNA which act as an initiator of mRNA degradation (Dalmay et al., 2000; Voinett et al., 2000). But it is still possible that transgene-encoded aberrant or excess RNA gets into the degradation machinery first and partial products of degradation get copied into dsRNA molecules which mark more transgene-specific RNAs for degradation and ensure amplification and maintenance of the process.

In *C. elegans* it is now clear that co-suppression, which appears to occur only in the germline, is genetically distinct from RNAi. In elegant genetic studies Dernburg and

colleagues (2000) and Ketting and Plasterk (2000) have shown that co-suppression is independent of *rde-1* activity but is dependent on the activity of other members of the mutator class of *rde* mutants. These findings suggest that whatever function *rde-1* provides in RNAi must be provided by some other gene in co-suppression. Considering that *rde-1* homologs are involved in co-suppression in at least two other species it seems likely that a *C. elegans* homolog of *rde-1* will be involved in co-suppression. It also remains possible that *rde-1* is involved in co-suppression in *C. elegans* but that some redundant gene exists that can mediate co-suppression when *rde-1* is not functional. Whatever the specific explanation might be, it is clear that the factors mediating co-suppression are not sufficient for carrying out RNAi in the absence of *rde-1*.

RNAi and transgene silencing. In *C. elegans*, transgenes are readily expressed in somatic tissues and can be transmitted for generations with undiminished expression. In contrast, transgenes that drive expression in the germline are rapidly silenced. Transgene silencing is an extremely interesting phenomenon that is, from a practical perspective, terribly frustrating for researchers who would like to stably express a transgene. Germline silencing of transgenes appears to occur at a transcriptional level as it has been shown to depend on the function of *C. elegans* genes homologous to members of the *Drosophila* Polycomb Group thought to regulate chromatin structure (Holdeman et al., 1998; Kelly and Fire, 1998; Korf et al., 1998).

In plants, co-suppression has been correlated with the methylation of the silenced gene (Wassenegger et al., 1994; Jones et al., 1998). In addition, transcriptional silencing can be triggered in plants by the dsRNA targeting a promoter region (Mette et al., 2000;

Sijen et al., 2001). Although a connection between RNAi and transcriptional silencing has not been firmly established in *C. elegans*, a significant degree of transgene de-silencing does occur when silent transgenes are crossed into strains homozygous for mutator class *rde* mutants (Tabara et al., 1999 a). Interestingly, silencing was not suppressed unless the animals were cultured at an elevated temperature, a culture condition that renders the mutator *rde* mutants nearly completely sterile. Thus, the mutator class *rde* genes seem to function in a temperature-dependent process that is essential for both fertility and gene silencing. It is tempting to speculate that transgene silencing in *C. elegans* involves a post-trancriptional step necessary for initiation and/or maintenance of a silent chromatin state.

Systemic nature of RNAi

One of the most intriguing features of RNAi is its ability to spread to other tissues after exposure to dsRNA via injection, soaking or feeding. Appreciation of this systemic nature of RNAi came with the observation that it is not necessary to inject dsRNA directly into the target tissue. For example, injection of dsRNA into the intestine or body cavity was found to cause interference in the germline and even among the progeny of the injected animal (Fire et al., 1998). In fact, the intestine appears to be the best injection site for dsRNA even when the target mRNA is expressed in the germline (Mello, unpublished observations). If RNAi represents an immunity mechanism against a dsRNA pathogen, the gut would likely be an ideal place to prepare the initial defense.

Viruses encountered while feeding might be ingested, their RNA fragmented and then transported into the body cavity for circulation to potentially infected tissues where they might block viral mRNA expression.

Little is known about the uptake and transport of dsRNA in *C. elegans*. So far, none of the *rde* mutants that have been characterized prohibit the transport of dsRNA or its derivatives from the intestine or body cavity into the germline. Conceivably, mutations in genes involved in transport could be found within the first and largest class of *rde*-mutants (Tabara et al., 1999 a), those resistant to RNAi by feeding but sensitive to injection.

Systemic transport of RNAi in *C.elegans* is often related to a similar phenomenon in plants, wherein PTGS can spread via a sequence-specific agent (Palauqui et al., 1997; Palauqui and Vaucheret, 1998). For plants it has been shown that DNA sequences corresponding to the 5' end of the target gene caused systemic silencing that was able to target the 3' portion of the gene not present in the trigger DNA (Voinnet et al., 1998). This suggests that the systemic signal is produced by copying the target mRNA, possibly via a RdRP. In *C.elegans*, the systemic transport of the injected material (dsRNA or its derivative) and its persistence to the F1 generation occurs in the absence of the target gene (Tabara et al., 1999 a; our unpublished data). Although we can not exclude that long-term persistence of RNAi requires target-dependent amplification of the signal, this has not yet been established in *C. elegans*. It will be very exciting in the future to learn more about the mechanisms involved in the systemic transport of nucleic acids into and out of cells in *C.elegans* and other organisms.

Inheritance of RNAi

As we mentioned above, exposure to dsRNA causes interference both in the exposed animal and, via transport to the germline, can also cause interference in the exposed animal's progeny (Fire et al., 1998). This is a truly remarkable process as in some cases the targeted gene is completely silent in the progeny of an injected animal more than a week after the initial injection of dsRNA. Interestingly, although males respond to RNAi and can inherit RNAi from their mothers, male animals exposed to dsRNA do not transmit interference to their progeny. This suggests that some process that occurs in the hermaphrodite but not in the male is required for uptake of the interfering agent by the germ cells. One could imagine that, because the sperm has a relatively small cytoplasmic compartment, there simply is not enough cytoplasm to store the interfering agent in the sperm. However, as we will describe below, males can pass the interfering agent to their progeny, provided that they inherited the interfering agent from their mother (Grishok et al., 2000). Thus, males can transmit but cannot create the inherited interfering agent.

While inheritance of RNAi for one generation is remarkable enough, for at least several genes, germline transmission of interference is possible for two or more generations (Grishok et al., 2000; our unpublished data). The ability to passage the interfering agent for two generations permits a genetic analysis of the inheritance phenomenon.

CHAPTER II

GENETIC REQUIREMENTS FOR INHERITANCE OF RNAi IN *C. ELEGANS*

Summary

In *Caenorhabditis elegans*, the introduction of double-stranded RNA triggers sequence-specific genetic interference (RNAi) that is transmitted to offspring. The inheritance properties associated with this phenomenon were examined. Transmission of the interference effect occurred through a dominant extragenic agent. The wild-type activities of the RNAi pathway genes *rde-1* and *rde-4* were required for the formation of this interfering agent but were not needed for interference thereafter. In contrast, the *rde-2* and *mut-7* genes were required downstream for interference. These findings provide evidence for germline transmission of an extragenic sequence-specific silencing factor and implicate *rde-1* and *rde-4* in the formation of the inherited agent.

Results and discussion

Gene-silencing mechanisms play an important role in regulating gene expression and cellular differentiation in a wide variety of organisms and are responsible for such diverse phenomena as chromosomal dosage compensation, genetic imprinting in mammals, virus resistance in plants, and transposon silencing in *Drosophila* (Panning and Jaenisch, 1998; Surani, 1998; Ratcliff et al., 1997; Jensen et al., 1999). A variety of mechanisms underlie these diverse silencing phenomena including apparent transcriptional blocks (reviewed in Panning and Jaenisch, 1998; Surani, 1998) and post-transcriptional interference (Ratcliff et al., 1997; 1999). RNA signals have been

implicated in the initiation of gene silencing in both natural (Panning and Jaenisch, 1998; Ratcliff et al., 1999) and experimental contexts (Fire et al., 1998). Recently, double-stranded RNA (dsRNA) has been shown to induce sequence-specific genetic interference in several organisms (Waterhouse et al., 1998; Ngo et al., 1998; Kennerdell and Carthew, 1998; Sanchez-Alvarado and Newmark, 1999). This interference phenomenon has been named RNA interference, or RNAi. The current body of evidence favors a model in which RNAi blocks a post-transcriptional step in gene expression (Fire et al., 1998; Montgomery et al., 1998) and suggests possible similarities with post-transcriptional gene silencing (PTGS) phenomena previously described in plants (reviewed by Baulcombe, 1996) and *Neurospora* (Cogoni and Macino, 1997). In *C. elegans*, potent and long-lasting effects associated with RNAi have led to speculation that amplification of the interfering agent or modification of chromosomal targets might play a role in RNA interference (Fire et al., 1998; Sharp, 1999). To gain insight into the nature of RNAi we examined the inheritance properties associated with this phenomenon.

Transmission of RNAi from the injected hermaphrodite to the first generation (F1) progeny has been observed for several genes (Fire et al., 1998; Montgomery et al., 1998; Tabara et al., 1999 a). In most cases complete recovery of wild-type gene activity occurs in the second (F2) generation post injection (Fire et al., 1998; Montgomery et al., 1998). However, in interference experiments targeting genes expressed in the maternal germline, we observed interference in the F2 generation and to a lesser extent in later generations (Figure 2-1, p.15).[1] In genetic crosses the interference effect was transferred

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[1] dsRNA was synthesized in vitro using T3 and T7 polymerases. Template DNA was removed from the RNA samples by DNAase treatment (30 minutes at 37°C). Equal amounts of sense and antisense RNAs were then mixed and annealed to obtain dsRNA. dsRNA (1-5 mg/ml) was injected into the intestine of animals. In control experiments mixture of linearized template DNA plasmids (0.2 mg/ml) used for synthesizing RNA

with the sperm or oocyte as a dominant factor, resulting in genetic interference in the F1 and F2 generations up to 10 days after the injection of dsRNA (Figure 2-1, p. 15). The persistence of genetic interference raised the possibility that an active genetic process was required for the initiation and transmission of interference.

In other organisms, the inheritance of epigenetic effects can involve reversible alterations of the gene or of the associated chromatin. In some cases these effects can exhibit genetic dominance, reviewed by Henikoff and Comai, 1998. We therefore examined whether the interference effect induced by RNAi exhibited linkage to the target gene. To do this we constructed a strain such that the F1 males that carry the RNAi effect also bear a chromosomal deletion that removes the target gene (Figure 2-1B, p. 15). We then investigated whether the sperm that inherit the deletion, and hence have no copies of the target locus, could carry the interference effect into the F2 generation. The wild-type sperm and deficiency bearing sperm were able to transfer interference to the F2 hermaphrodite progeny (Figure 2-1B). Thus, the target locus was not needed for inheritance of the interference effect. Although males were sensitive to RNAi and could inherit and transmit RNAi acquired from their mothers (Figure 2-1), direct injections into males did not cause transmission of RNAi to F1 for several genes tested.[2]

Thus, the initial transmission of RNAi to F1 progeny may involve a mechanism active only in hermaphrodites, whereas subsequent transmission to the F2 progeny

---

failed to induce interference in P0, F1 or F2 when injected into the intestine of hermaphrodites.

[2] Wild type males were injected with dsRNA targeting body muscle structural gene *unc-22* (Moerman et al., 1986), cuticle collagen gene *sqt-3* (van der Keyl et al., 1994), and maternal genes *pos-1* (Tabara et al., 1999 b) and *sgg-1* (Bei and Mello, manuscript in preparation). Males of *pes-10::gfp* strain were injected with *gfp* dsRNA. Injected males were affected by *unc-22* and *gfp* dsRNA to the same extent as injected hermaphrodites. No RNAi interference was detected in F1 progeny of injected males (40-200 F1 animals scored for each RNA tested].

A

pos-1 dsRNA
or mom-2 dsRNA
or sgg-1 dsRNA

P0

carrier oocytes   unaffected embryos   carrier oocytes

F1 carriers of RNAi

F1 affected   pos-1 (44/88) ♂
mom-2 (6/15) ♀
sgg-1 (12/23)

F1 ♂ pos-1 (19/37) X uninjected ♀
mom-2 (3/9)
sgg-1 (1/16)

F2

dead eggs

F2 affected   ♀ pos-1 (152/225)
♀ mom-2 (4/29)
sgg-1 (7/29)

F3

dead eggs

B

pos-1 dsRNA

P0   ♀ $\frac{mDf3}{+}$ X ♂ $\frac{mDf3}{+}$

F1   ♂ $\frac{mDf3}{+}$ X ♀ $\frac{dpy\text{-}11unc\text{-}42}{dpy\text{-}11unc\text{-}42}$ (Uninjected)

mDf3
pos-1 unc-42
deficient sperm

Unc F2 animals

F2 $\frac{mDf3}{dpy\text{-}11unc\text{-}42}$ ♀ $\frac{+}{dpy\text{-}11unc\text{-}42}$ ♀

↓ Self   ↓ Self

F3

Results:

6/9 mDf3 sperm gave rise to F2 with 100% (n>1000) F3 pos-1 embryos.

3/27 (+) sperm gave rise to F2 with 100% (n>500) F3 pos-1 embryos.

Figure 2-1. Maternal establishment and paternal transmission of RNAi. (A) Schematic diagram showing a wild-type hermaphrodite, (P0), receiving injection of dsRNA. The needle is illustrated inserted in the intestine (the normal target for RNAi injection). [In subsequent figures the injection of dsRNA is indicated by similar schematic needle shown above the genotype of the recipient worm]. Three different species of dsRNA, named above the needle, were delivered into worms in independent experiments. The hermaphrodite gonad with its symmetrical anterior and posterior U-shaped arms

(Figure 2-1 legend continued) is shown. Several fertilized eggs are shown in the centrally located uterus (white ovals). Rectangular mature oocytes are shown queued up in the gonad arms most proximal to the uterus. The embryos present at the time of injection give rise to unaffected F1 progeny. Oocytes in the proximal arms of the gonad inherit the RNAi effect but also carry a functional maternal mRNA (F1 carriers of RNAi). After a clearance period during which carrier and unaffected F1 progeny are produced, the injected P0 begins to produce exclusively dead F1 embryos with the phenotype corresponding to the inactivation of the gene targeted by the injected RNA (Tabara et al., 1998; Rocheleau et al., 1997). Potential F1 and F2 carriers of the interference effect were identified within the brood of the injected animal. In the case of hermaphrodites, carriers were defined as "affected" if the animals produced at least 20% dead embryos with phenotypes corresponding to maternal loss of function for the targeted locus. Male carriers were defined as animals whose cross progeny included at least one affected F2 hermaphrodite. The total number of carriers identified in each generation for each of three dsRNAs injected is given as a fraction of the total number of animals assayed. F2 and F3 dead embryos from the carriers are illustrated as black ovals. (B) Extragenic inheritance of RNAi. Illustration of a genetic scheme to generate F1 males that carry both *pos-1* (RNAi) and a chromosomal deficiency for the *pos-1* locus. F2 progeny of the carrier male include two genotypes: phenotypically wild-type animals that inherit the (+) chromosome, and phenotypically uncoordinated (Unc) progeny that inherit the mDf3 chromosome. The fraction shown (in this and all subsequent figures) represents the number of RNAi-affected F2 hermaphrodites over the total number of cross progeny scored for each genotype class.

appears to involve a distinct mechanism, active in both hermaphrodites and males.[3]

A previous study identified a set of *C. elegans* genes required for RNAi (Tabara et al., 1999 a). One phenotypic class comprised of the *rde-1* and *rde-4* mutants that are deficient in RNAi but have no other phenotypes, and a second class, which includes *rde-2*, *rde-3*, *mut-2* and *mut-7* was deficient in RNAi and also exhibited transposon mobilization, reduced fertility, and high incidence of chromosome loss. Our studies have shown that all mutants in both phenotypic classes are strongly deficient in RNA interference in both the F1 and later generations (Tabara et al., 1999 a).[4] However, these previous experiments did not address whether the activities of these genes might be sufficient in the injected animals to initiate heritable RNAi or are required directly in the F1 or F2 animals themselves for interference, or both.

The activities of *rde-1*, *rde-2*, *rde-4* and *mut-7* may be sufficient in the injected hermaphrodite for interference in the F1 and F2 generations. We designed crosses such that wild-type activities of these genes would be present in the injected animal but absent in the F1 or F2 generations (Figures 2-2, p.19; 2-3, p.20). To examine inheritance in the

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[3] After injection of dsRNA into mated homozygous mutant *rde-1*, *rde-2* and *mut-7* hermaphrodites, interference is observed among their heterozygous *rde* (+) or *mut* (+) F1 cross-progeny. This type of inheritance occurs only in hermaphrodites and may reflect a passive transfer of the injected material into the maternal germline. The activities of *rde-1* and *rde-4* genes are necessary in the ensuing generation for the initiation of interference in response to this inherited material.

[4] Homozygous hermaphrodites of *rde-1* and *rde-2* strains were allowed to mate with males of the same strains and then injected with *pos-1* or *mom-2* dsRNA (5mg/ml). More than 400 F1 hermaphrodites from each strain were picked (10 worms per plate) and their broods examined for occurrence of inviable *pos-1*-like or *mom-2*-like embryos. Similarly, 300 F2 animals from these injections were analyzed. Finally, 60 F1 males from each strain were mated and 300 of their F2 progeny were examined for affected embryos. No *pos-1* affected embryos were observed in any generation.

F1 generation, we injected mothers heterozygous for each mutant, allowed them to produce self-progeny, and examined whether the homozygous mutant progeny exhibited genetic interference (Figure 2-2A, p.19). The *rde-1* and *rde-4* mutant F1 progeny exhibited robust interference comparable to that exhibited by the wild-type, whereas the *rde-2* and *mut-7* F1 progeny did not (Figure 2-2A). In control experiments injection of dsRNA directly into the *rde-1* and *rde-4* mutant progeny of uninjected heterozygous mothers failed to result in interference (Figure 2-2B). Thus, injection of dsRNA into heterozygous hermaphrodites results in an inherited interference effect that triggers gene silencing in otherwise RNAi resistant *rde-1* and *rde-4* mutant F1 progeny whereas *rde-2* and *mut-7* mutant F1 progeny remain resistant.

To examine the genetic requirements for RNAi genes in the F2 generation, we generated F1 male progeny that carry the interference effect as well as one mutant copy of each respective locus, *rde-1*, *rde-2*, and *mut-7* (Figure 2-3A, p.20). We then backcrossed each of these males with uninjected hermaphrodites homozygous for each corresponding mutant (Figure 2-3A). The resulting cross progeny included 50% heterozygotes and 50% homozygotes that were distinguished by the presence of the linked marker mutations. The heterozygous siblings served as controls and in each case exhibited interference at a frequency similar to that seen in wild-type animals (Figure 2-3A). The *rde-2* and *mut-7* homozygous F2 progeny did not exhibit interference, indicating that the activities of these two genes are required for interference in the F2 generation. In contrast, homozygous *rde-1* F2 animals exhibited wild-type levels of F2 interference (Figure 2-3A, p.20). Control *rde-1* homozygotes generated through identical

A Injected P0 F1

$\frac{rde\text{-}1\ unc\text{-}42}{+}$ (pos-1 dsRNA) ⟶ self X rde-1 (-) 11/24
rde-1 (+) 9/72

$\frac{rde\text{-}2\ unc\text{-}13}{+}$ ⟶ rde-2 (-) 0/39
rde-2 (+) 23/78

$\frac{mut\text{-}7\ dpy\text{-}17}{+}$ ⟶ mut-7 (-) 0/15
mut-7 (+) 20/50

$\frac{rde\text{-}4\ unc\text{-}69}{+}$ ⟶ rde-4 (-) 5/15
rde-4 (+) 11/48

B P0 Injected F1

$\frac{rde\text{-}1\ unc\text{-}42}{+}$ ⟶ $\frac{rde\text{-}1\ unc\text{-}42}{rde\text{-}1\ unc\text{-}42}$ (pos-1 dsRNA) 0/37

$\frac{rde\text{-}4\ unc\text{-}69}{+}$ ⟶ $\frac{rde\text{-}4\ unc\text{-}69}{rde\text{-}4\ unc\text{-}69}$ 0/37
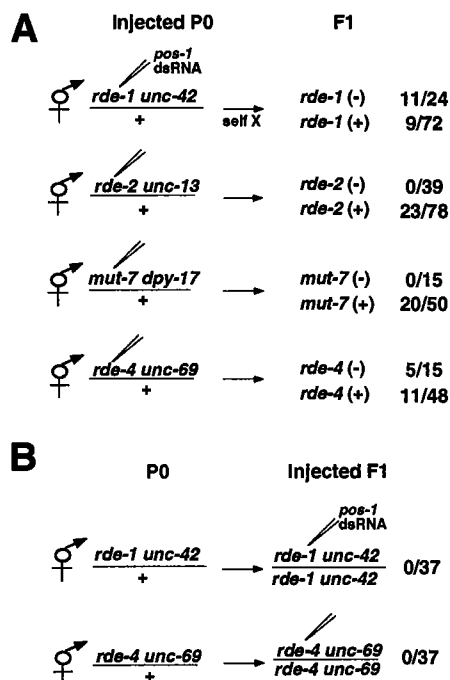
Figure 2-2. Genetic schemes to determine whether the wild-type activities

of *rde-1*, *rde-2*, *mut-7*, and *rde-4* are sufficient in the injected animal

for interference among the F1 self progeny. (A) Heterozygous

hermaphrodites from each genotype class (as shown) were injected

with *pos-1* dsRNA. In each case two types of F1 self-progeny (shown right),

distinguished by virtue of the linked marker mutations, were scored for interference.

(B) Homozygous F1 progeny from heterozygous (uninjected) mothers were

directly injected with *pos-1* dsRNA. The fractions indicate the number of

affected animals out of the total number of animals of each genotype scored.

A

PO ♀ $\dfrac{dpy\text{-}11\ unc\text{-}42}{dpy\text{-}11\ unc\text{-}42}$ X ♂ $\dfrac{rde\text{-}1}{rde\text{-}1}$ *pos-1* dsRNA or *sgg-1* dsRNA

F1 ♂ $\dfrac{dpy\text{-}11\ unc\text{-}42}{rde\text{-}1}$ X ♀ $\dfrac{rde\text{-}1\ unc\text{-}42}{rde\text{-}1\ unc\text{-}42}$

	F2 affected	
	rde-1(-)	rde-1(+)
	$\dfrac{rde\text{-}1\ unc\text{-}42}{rde\text{-}1}$	$\dfrac{rde\text{-}1\ unc\text{-}42}{dpy\text{-}11\ unc\text{-}42}$
pos-1 RNAi	63/76	22/49
mom-2 RNAi	-	-
sgg-1 RNAi	2/14	5/15

PO ♀ $\dfrac{dpy\text{-}5\ unc\text{-}13}{dpy\text{-}5\ unc\text{-}13}$ X ♂ $\dfrac{rde\text{-}2}{rde\text{-}2}$ *pos-1* dsRNA

F1 ♂ $\dfrac{dpy\text{-}5\ unc\text{-}13}{rde\text{-}2}$ X ♀ $\dfrac{rde\text{-}2\ unc\text{-}13}{rde\text{-}2\ unc\text{-}13}$

	F2 affected	
	rde-2(-)	rde-2(+)
	$\dfrac{rde\text{-}2\ unc\text{-}13}{rde\text{-}2}$	$\dfrac{rde\text{-}2\ unc\text{-}13}{dpy\text{-}5\ unc\text{-}13}$
	0/35	17/31

PO ♀ $\dfrac{dpy\text{-}17}{dpy\text{-}17}$ X ♂ $\dfrac{mut\text{-}7}{mut\text{-}7}$ *pos-1* dsRNA or *mom-2* dsRNA

F1 ♂ $\dfrac{dpy\text{-}17}{mut\text{-}7}$ X ♀ $\dfrac{mut\text{-}7\ dpy\text{-}17}{mut\text{-}7\ dpy\text{-}17}$

	F2 affected	
	mut-7(-)	mut-7(+)
	$\dfrac{mut\text{-}7\ dpy\text{-}17}{mut\text{-}7}$	$\dfrac{mut\text{-}7\ dpy\text{-}17}{dpy\text{-}17}$
	1/92*	50/69
	0/31	4/29

B

PO *mut-7* ♂ $\dfrac{mut\text{-}7}{mut\text{-}7}$ $\dfrac{pos\text{-}1}{dsRNA}$ X ♀ $\dfrac{rde\text{-}1\ unc\text{-}42}{+}$

F1 rde-1(-) $\dfrac{mut\text{-}7}{+} ; \dfrac{rde\text{-}1\ unc\text{-}42}{rde\text{-}1\ unc\text{-}42}$ 22/43 affected

PO *rde-2* ♂ $\dfrac{rde\text{-}2}{rde\text{-}2} ; \dfrac{rde\text{-}1\ dpy\text{-}11}{+}$ $\dfrac{pos\text{-}1}{dsRNA}$ X ♂ $\dfrac{rde\text{-}1\ dpy\text{-}11}{+}$

F1 rde-1(-) $\dfrac{rde\text{-}2}{+} ; \dfrac{rde\text{-}1\ dpy\text{-}11}{rde\text{-}1\ dpy\text{-}11}$ 10/45 affected

PO *rde-4* ♂ $\dfrac{rde\text{-}4}{rde\text{-}4} ; \dfrac{rde\text{-}1\ dpy\text{-}11}{+}$ $\dfrac{pos\text{-}1}{dsRNA}$ X ♂ $\dfrac{rde\text{-}1\ dpy\text{-}11}{+}$

F1 rde-1(-) $\dfrac{rde\text{-}4}{+} ; \dfrac{rde\text{-}1\ dpy\text{-}11}{rde\text{-}1\ dpy\text{-}11}$ 0/38 affected
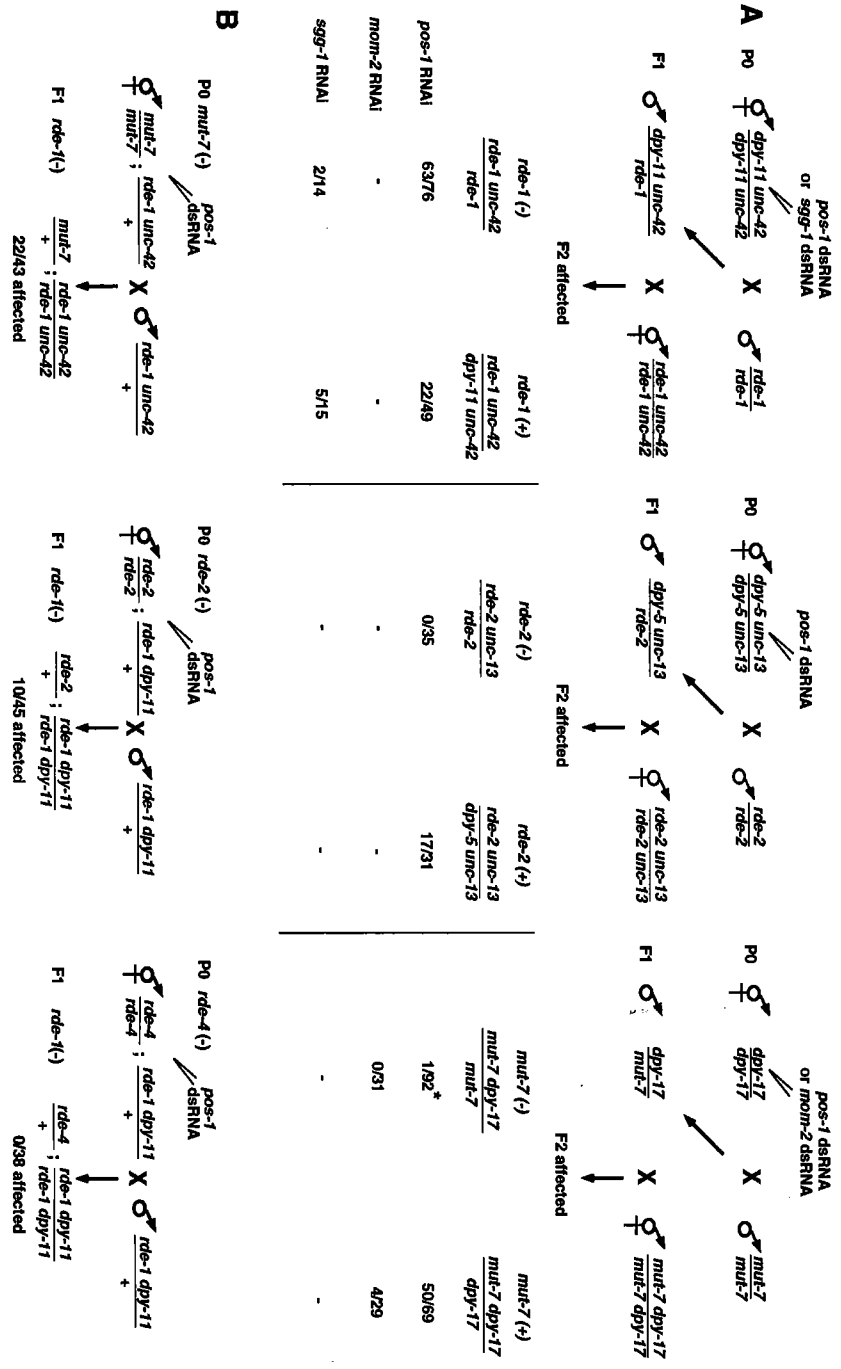
Figure 2-3

Figure 2-3. Genetic crosses designed to follow the requirements for *rde-1*, *rde-2*, *rde-4* and *mut-7* in F2 (A) and F1 (B) interference. (A) The dsRNAs injected are listed above the schematic needle. Recipient hermaphrodites were marked with visible mutations closely linked to wild-type alleles of each RNAi pathway gene. F1 carrier males heterozygous for each mutation were crossed with the homozygous mutant hermaphrodites of the genotype shown. Two types of cross progeny were analyzed for F2 interference. The results are tabulated with the injected dsRNA listed at the left and the genotype inferred from the linked visible marker mutations listed above each column. The fractions indicate the number of affected animals out of the total number of animals of each genotype scored.

The asterisk indicates that the *dpy-17* gene is located 2.7 map units away from *mut-7* whereas *unc-42* and *unc-13* markers are each approximately 0.1 map units from *rde-1*, and *rde-2* respectively. Thus, recombination between *dpy-17* and *mut-7* is likely in F1 males and may explain the occurrence of a single carrier F2 animal (1/92).

(B) Genetic crosses to determine whether *rde-1* activity is sufficient to initiate RNAi in injected animals that lack the wild-type activities of *rde-2*, *mut-7* or *rde-4*. Animals with the genotypes shown were injected with *pos-1* dsRNA and then crossed to generate F1 hermaphrodites homozygous for *rde-1*. The fraction illustrates the number of F1 affected hermaphrodites out of the total number animals of each genotype scored.

crosses were completely resistant to *pos-1 (RNAi)* when challenged *de-novo* with dsRNA

in the F2 generation.[5] Thus, *rde-1* activity in the preceding generations was sufficient to

allow interference to occur in *rde-1* mutant F2 animals while the wild-type activities of

rde-2 and *mut-7* were required directly in the F2 animals for interference.

In the preceding experiments, the expression of *rde-1* (+) and *rde-4* (+) in the

injected animal was sufficient for interference in later generations. In contrast, the wild-

type activities of the *rde-2* and *mut-7* genes were required for interference in all

generations assayed. Thus, *rde-2* and *mut-7* might be required only downstream or might

also function along with *rde-1* and *rde-4*. To examine whether *rde-2* and *mut-7* activities

function along with or downstream of *rde-1* we designed genetic crosses in which the

activities of these genes were present sequentially (Figure 2-3B, p.20). For example, we

injected *pos-1* dsRNA into *rde-1*(+); *rde-2*(-) animals and then crossed these to generate

rde-1(-); *rde-2*(+) F1 progeny. *rde-1*(+) activity in the injected animals was sufficient for

F1 interference even when the injected animals were homozygous for *rde-2* or *mut-7*

mutations (Figure 2-3B); however it was not sufficient when the injected animals were

homozygous for the *rde-4* mutation (Figure 2-3B). Thus, *rde-1* can act independently of

rde-2 and *mut-7* in the injected animal, but *rde-1* and *rde-4* must function together.

These findings indicate that *rde-1* and *rde-4* function in the formation of the inherited

interfering agent while *rde-2* and *mut-7* function at a later step.

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[5] Thirty-five *rde-1* homozygous animals generated through crosses shown in Figure 2-3A
were tested by feeding bacteria expressing *pos-1* dsRNA and 21 similar animals were
tested by direct injections of *pos-1* dsRNA, all animals tested were resistant to *pos-1*
(RNAi).

What is the physiological function of such inherited interfering agents? The *rde-1* and *rde-4* mutations appear to be simple loss-of-function mutations and do not exhibit any overt phenotypes, except for a nearly complete absence of interference in response to dsRNA (Tabara et al., 1999 a). However, *rde-2*, *mut-7* and other RNAi pathway genes have several additional phenotypes, most notably a mobilization of the normally silent transposons in the germline. Because the *rde-1* and *rde-4* appear to initiate RNAi in response to dsRNA but are not required for transposon silencing, it seems reasonable to speculate that other stimuli act upstream of *rde-2* and *mut-7* to initiate transposon silencing. The *rde-1* gene is a member of a highly conserved gene family with 22 homologs in *C. elegans* as well as numerous homologs in plants, other animals and fungi (Tabara et al., 1999). The *Drosophila* gene *sting* encodes a *rde-1* homolog involved in a PTGS-like silencing mechanism that acts on the transcripts of the repetitive X-linked *Stellate* locus (Schmidt et al., 1999). Perhaps gene silencing mediated by *sting* and other *rde-1* homologs involves upstream stimuli distinct from dsRNA (Figure 2-4, p.24). These distinct upstream stimuli might in turn lead to the formation of secondary extragenic agents similar to those induced via dsRNA injection (Figure 2-4). Molecules similar to the small 25 nucleotide RNAs recently found in silenced transgenic plants (Hamilton and Baulcombe, 1999) may constitute the sequence component that confers specificity on these hypothetical secondary interfering agents (Figure 2-4).

Stimulii   ds RNA   Transposons, repetitive genes

Threshold of sense RNA   Gene copy number

Initiators   rde-1, rde-4   X   Y

Secondary extragenic agents
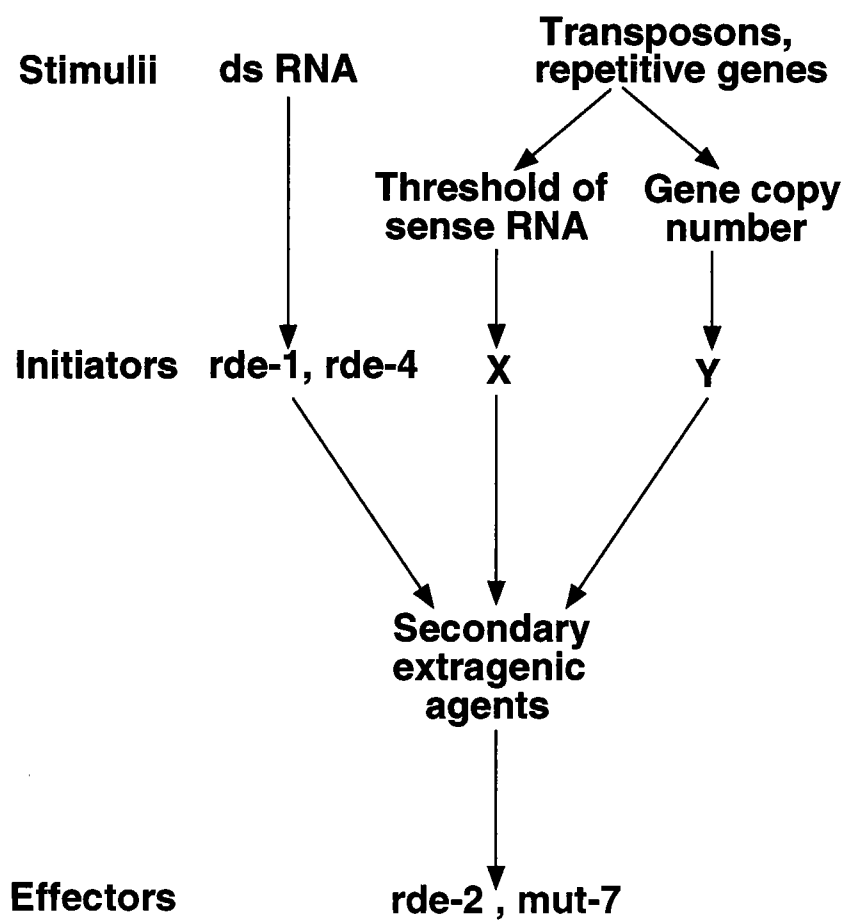
Effectors   rde-2 , mut-7

Figure 2-4. Model for RNAi and other PTGS-like silencing pathways in *C. elegans*.

CHAPTER III

INITIATION OF TRANSCRIPTIONAL GENE SILENCING BY dsRNA

In *C. elegans*, three types of homology-dependent silencing phenomena have been identified to date: RNAi (Fire et al., 1998), co-suppression (Ketting and Plasterk, 2000; Durnberg et al., 2000) and transcriptional silencing of repetitive transgenes in the germline (Kelly et al., 1997; Kelly and Fire, 1998). Despite differences in the nature of these mechanisms, they share a requirement for a common set of genes (Tabara et al., 1999 a). For example, both germline RNAi and transcriptional silencing of germline transgenes require the activity of *rde-2, rde-3* and *mut-7*. However, while RNAi strictly depends on these genes, their role in transgene silencing is only revealed at elevated temperature. We hypothesize that the transcriptional silencing of transgenes might require initiation and maintenance steps. RNAi-pathway genes might be required only for initiation of transgene silencing or re-initiation, while maintenance of silencing might be independent of RNAi genes but sensitive to temperature.

The connection between different silencing phenomena in *C. elegans* can best be explained by the existence of sequence-specific RNA guides as proposed in Figure 2-4, p.24. Indeed, 21-25nt long RNA species have been associated with co-suppression in plants (Hamilton and Baulcombe, 1999) and proven to be sequence-specific guides for targeted mRNA degradation during RNAi in *Drosophila* (Zamore et al., 2000; Hammond et al., 2000; Yang et al., 2000; Elbashir et al., 2001 a). Initiation of transcriptional

silencing of both transgenes and endogenous genes has been induced by dsRNA converted to small RNAs in plants (Mette et al., 2000; Sjien et al., 2001). However, in *C. elegans*, biochemical evidence for the role of small RNAs in gene silencing is documented so far only for RNAi (Parrish et al., 2000; Grishok et al., submitted, Chapter V). Also, there is no evidence reported for the induction of transcriptional silencing by dsRNA in animals.

In this section we describe the initiation of transgene silencing by dsRNA, via the RNAi pathway, and show that after initiation silencing is maintained by a different mechanism. The study of the connection between RNAi and transgene silencing in *C. elegans* represents a challenge since transcriptional silencing of transgenes occurs very rapidly. The expression level of germline transgenes is inversely dependent on their copy number (Kelly et al., 1997), and different methods have been developed for generating less repetitive arrays of introduced transgenes (Kelly et al., 1997; Rocheleau et al., 1999).

The *pie-1::gfp* transgene used in this study was produced using a yeast artificial chromosome (YAC) vector and thus was relatively complex and low copy number. The *pie-1::gfp* construct was made by targeted homologous recombination in yeast using methods described in Rocheleau et al., 1999. Transgenic, GFP-positive worm strains were obtained by injecting the YAC transgene with whole yeast genomic DNA (200µg/ml) along with the co-injection marker plasmid, pRF4 (100µg/ml). Microinjection for DNA transformation was performed as described in Mello et al., 1991. The GFP expression pattern of *pie-1::gfp* closely follows that of endogenous *pie-1*

(Figure 3-1A, p.28), indicating that the transgene is under normal transcriptional/post-transcriptional regulation in the worm.

Second generation *pie-1::gfp* transgenic worms were then injected with *gfp* dsRNA to induce post-transcriptional silencing (Figure 3-1, p.28). Then we followed the expression of *pie-1::gfp* in the progeny of injected and control animals for several generations. Despite the less repetitive nature of the introduced transgene, spontaneous silencing of the GFP expression was observed within the 5 generations analyzed (Figure 3-1C, dark bars). However, the silencing induced by dsRNA persisted independently of spontaneous gene silencing for 4 generations at least (Figure 3-1C, white bars). Thus, RNAi appeared to trigger rapid and complete silencing of a germline transgene compared to partial spontaneous silencing observed in the uninjected control populations.

In our experiment, initiation of stable transgene silencing was induced by the RNAi mechanism since it was dependent on sequence-specific *gfp* dsRNA and was not induced either by *pos-1* dsRNA or by the dsRNA corresponding to the gene Y49E10.14, most adjacent to the *pie-1* and present on the yeast artificial chromosome used to introduce *pie-1::gfp* (data not shown). Also, *rde-1* was required for the initiation of transgene silencing via RNAi (5/5 of *rde-1; pie-1::gfp* animals were resistant to injections of *gfp* dsRNA), whereas spontaneous transgene silencing occurs independently of *rde-1* (Tabara et al., 1999 a).

In our study of RNAi inheritance described in Chapter II we demonstrate that *rde-1* gene is required for the initiation of RNAi in parent animals, but is dispensable in later
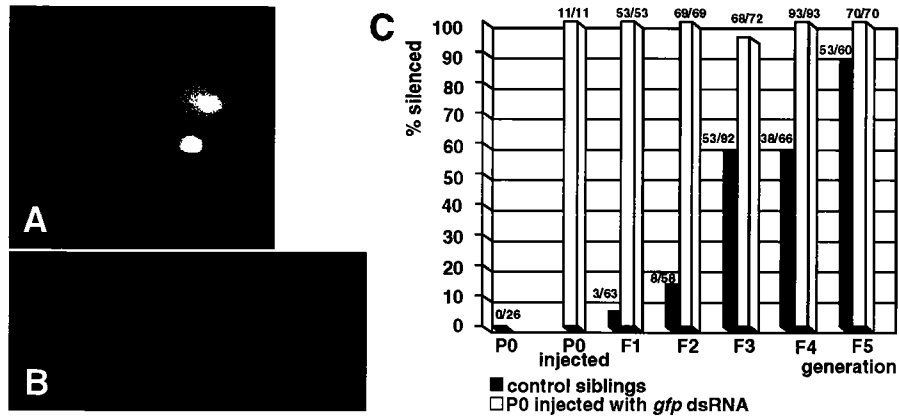
Figure 3-1

Figure 3-1. Persistent silencing of a germline transgene induced by dsRNA.

(A, B) Fluorescence micrographs each showing two embryos from a strain bearing a

germline specific transgene, *pie-1::gfp*. Embryos from uninjected mothers (A) or mother

injected with a non-specific dsRNA (not shown) exhibit distinctive nuclear and

cytoplasmic PIE-1::GFP fluorescence localized in one cell, the germline cell, at each

stage. Embryos from *gfp* dsRNA injected mothers (B) exhibit no GFP fluorescence.

(C) A graph depicting the percentage of transgenic animals expressing PIE-1::GFP in

each generation after microinjection of *gfp* dsRNA (white bars) and in control

populations (shaded bars). The proportion of silenced animals in each population is

indicated by the fractions above each bar with the units [(adults with GFP-negative

embryos) over (total adults scored)]. At the beginning of the experiment, 11 GFP-

positive, P0 generation adults were injected with *gfp* dsRNA. One day after injection, all

11 injected animals produced exclusively GFP-negative embryos indicated by the

fraction 11/11. In each of the following generations (F1, F2, F3, F4 and F5) a number of

adults (indicated by the denominator in the fraction above each bar) were chosen at

random and were scored for silencing.

generations, while *mut-7* and *rde-2* are required both in F1 and F2 animals affected by

inherited RNAi (Figure 2-3, p.20). We therefore tested the requirement for the *mut-7*

gene in the F2 progeny of the *pie-1::gfp* animals injected with *gfp* dsRNA (Table 1,

p.31). In contrast to the inheritance of the *pos-1*(RNAi), the dsRNA- induced silencing of

pie-1:: gfp transgene in the F2 generation did not require activity of the *mut-7* gene

(Table 1). Also, dsRNA-induced silencing of the *pie-1::gfp* was much more penetrant

than the inherited RNAi targeting endogenous genes: 100% silenced F2 for *pie-1:: gfp*

(Figure 3-1C, p.28) versus 20-70% silenced F2 in inherited *pos-1(RNAi)* (Figure 2-1A,

p.15).

These findings demonstrate that dsRNA-induced silencing of *pie-1::gfp* transgene

was initiated by the RNAi mechanism and maintained on a different level. Similarly to

pie-1::gfp silenced by *gfp* dsRNA, spontaneously silenced *pie-1::gfp* and *let-858::gfp*

transgenes did not become desilenced in *mut-7* background at 20° C (Table 1). It was

previously shown that maintenance of the spontaneous silencing of the *let-858::gfp*

transgene required the activity of two *mes* genes homologous to the *Drosophila Enhancer*

of zeste and *extra sex comb* members of Polycomb Group affecting chromatin structure

(Kelly et al., 1998).

The findings described here suggest that the same RNA factor that triggers

mRNA destruction in RNAi may also function in transcriptional gene silencing. While

RNAi is initiated by dsRNA, spontaneous transgene silencing may be initiated by

aberrant transgene RNA. In our *gfp* RNAi experiment, small RNAs derived from the

introduced dsRNA are likely to enter this silencing pathway, enabling the level of *gfp-*

Table 1. Requirements for *mut-7* in RNAi and transgene silencing

Type of silencing initiation	mut-7 (-) F2 affected	mut-7 (+) F2 affected
RNAi (dsRNA):		
pos-1 (RNAi)	0/92	50/69
pie-1::gfp (*gfp* RNAi)	29/30	36/36
Transgene silencing: (extra gene copies)		
let-858::gfp	31/32	35/35
pie-1::gfp	9/9	27/27

specific small RNAs to reach a threshold, and leading to the rapid transcriptional silencing of the germline transgene.

The gradual progression of spontaneous transgene silencing in succeeding generations which we observed in *C. elegans* (Figure 3-1B, p.28) is reminiscent of the progressive silencing of *Drosophila* retrotransposons, I-elements (Jensen et al., 1999 a). Progressive silencing of the I-elements introduced by crosses can be initiated by the expression of any portion of the I-element from the transgenes either in sense or antisense orientation (Jensen et al., 1999 b). The speed of I-elements' silencing progression in succeeding generations is dependent on the copy number of initiating transgenes. It has been proposed that gradual accumulation and inheritance of the RNA molecules mediate this type of co-suppression in *Drosophila* (Jensen et al., 1999 a).

The transient nature of the initiation step in transgene silencing maintained at the transcriptional level represents the biggest challenge for the biochemical studies of such a process. We were not able to detect significant accumulation of small RNAs corresponding to the silenced transgene in both completely silenced or partially silenced populations of *pie-1::gfp* transgenic animals (data not shown). However, our result with the induction of transgene silencing by dsRNA indicates that an RNA intermediate must play an important role in this process, although the levels of such an intermediate might be low.

Studies on the interconnection between the different levels of gene silencing will remain a fascinating area for future investigations. These studies might ultimately

provide insight into the complex interplay between different silencing and gene

regulatory mechanisms during development where RNA molecules might play a

significant role (see Chapter IV and General discussion).

CHAPTER IV

GENES AND MECHANISMS RELATED TO RNA INTERFERENCE REGULATE EXPRESSION OF THE SMALL TEMPORAL RNAs THAT CONTROL *C. ELEGANS* DEVELOPMENTAL TIMING

Summary

RNAi is a gene-silencing phenomenon triggered by double-stranded (ds) RNA, and involves the generation of 21 to 26 nucleotide 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 post-transcriptional 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 a), while a related post-transcriptional gene silencing (PTGS) mechanism, called co-suppression, 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 co-purify 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 nts 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 21nt 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 nucleotide 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.Pasquinelli and G.Ruvkun, 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 a ; Ketting et al., 1999). Probable null mutations in *rde-1*, (for *R*NAi *de*fective) cause a complete lack of RNAi but no other discernible phenotypes (Tabara et al., 1999 a). *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, 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; Cikaluk 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 chapter 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 4-1, p.40). cDNA clones for 14 *rde-1* homologs, indicated by asterisks in Figure 4-1, 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 *a*rgonaute *l*ike *g*enes), induced developmental phenotypes in the progeny of injected animals, including a tendency to burst at the vulva (Figure 4-2A, p.44), 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 4-2E). In addition these dsRNAs induced incompletely penetrant slow growth
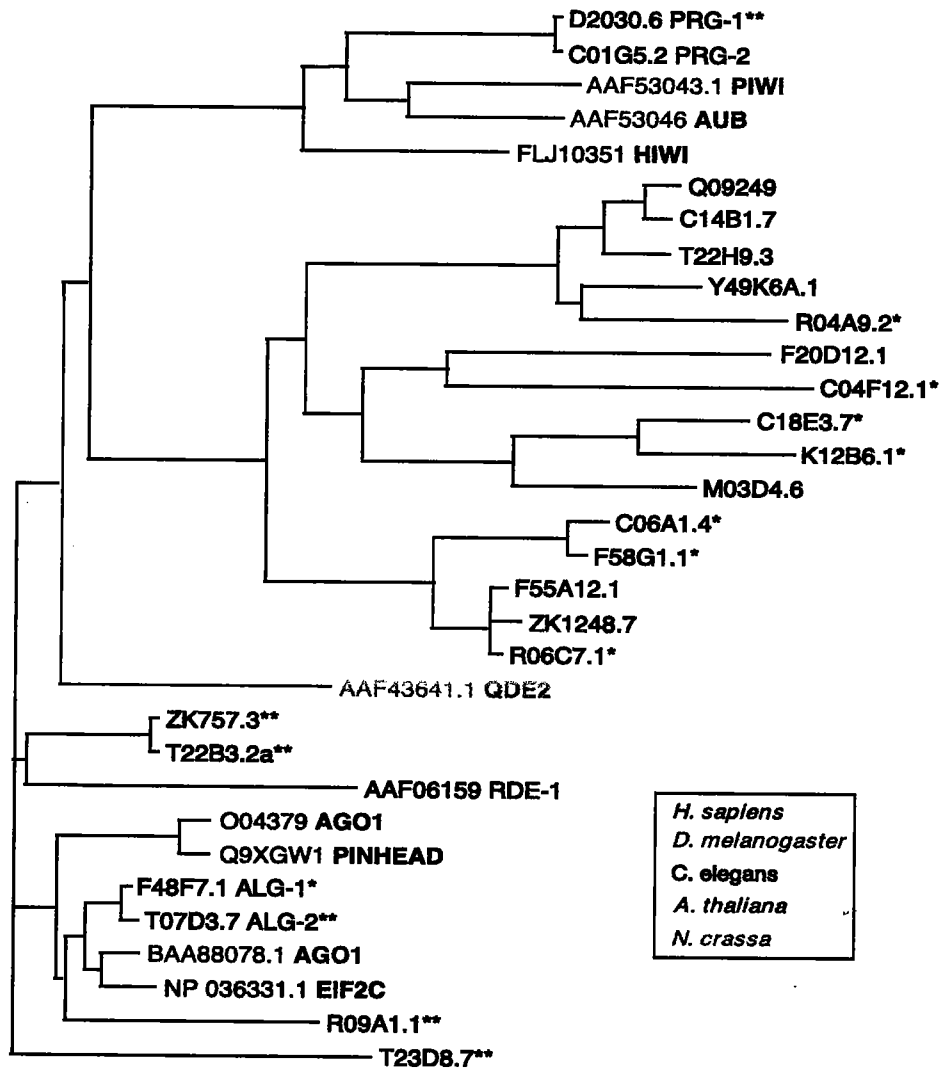
Figure 4-1

Figure 4-1. 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 co-injected with *alg-1* dsRNA (see Results).

and germline abnormalities (Figure 4-2D 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 (Parrish 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 knock-out consortium. This allele, *alg-2(ok304)*, is an out of frame deletion that removes the nucleotides encoding amino acids 34-374 including the PAZ domain and terminates after encoding 8 additional amino acids from reading frame two (Figure 4-2H, p.44) and is therefore likely to be a null allele of *alg-2*. The RNAi experiments above suggest that *alg-2* may be a non-essential gene, and consistent with this finding the *alg-2(ok304)* homozygotes are viable and show, at most, subtle defects in fertility and development (data not shown).

We next asked if *alg-1* and *alg-2* might have overlapping functions by co-injecting dsRNAs prepared from both genes and by injecting *alg-1* dsRNA into *alg-2(ok304)* homozygotes. Consistent with a shared function, co-injection of *alg-1* and *alg-*

2 dsRNAs caused enhanced larval lethality and also induced an embryonic lethal phenotype (Figure 4-2C, and data not shown). Injection of *alg-1* dsRNA into *alg-2(ok304)* homozygous animals resulted in a fully penetrant embryonic lethal phenotype identical to that observed in the double RNAi experiment (Figure 4-2C, and data not shown). No such synergy was observed when *alg-1* dsRNA was injected with dsRNAs prepared from other *rde-1* family members (Figure 4-1, double asterisks, see Experimental Procedures). These findings indicate that *alg-1* and *alg-2* have overlapping functions in both embryogenesis and larval development. Efficient induction of the larval developmental phenotypes described below required the injection of full-length *alg-1* dsRNA, a procedure that appears to partially inhibit *alg-2*. Therefore, we refer to animals produced in such experiments as "*alg-1/alg-2*" RNAi animals.

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 (Jacobson et al., 1999) proteins implicated in RNAi and regulation of development, respectively. A

E

RNAi used	Absence of alae in F1		Defective alae if present	
	Fraction	(% no alae)	Fraction	(% defective alae)
alg-1/alg-2 (RNAi)	94/105	(91%)	2/9	(22%)
dcr-1(RNAi)	10/63	(19%)	46/53	(87%)

F

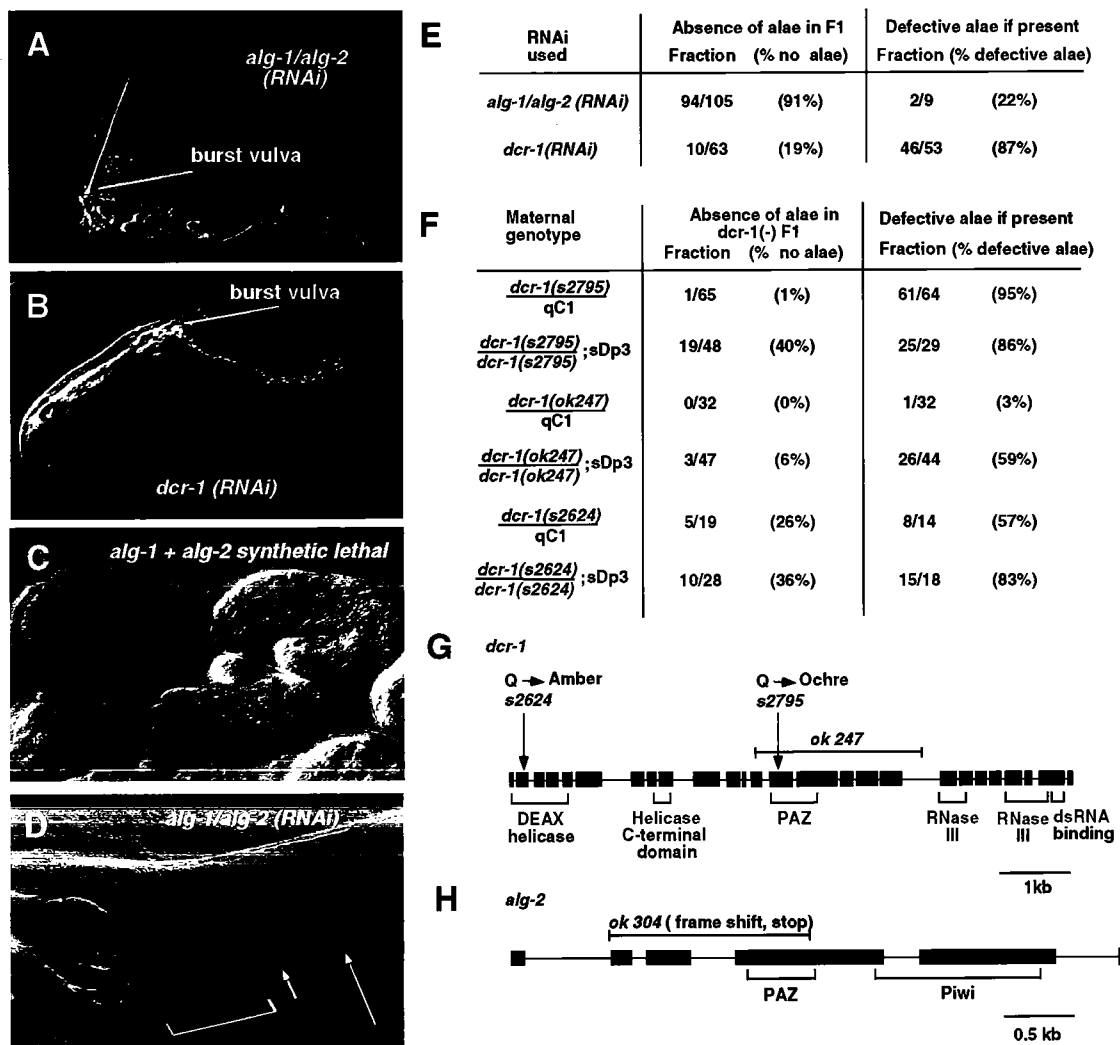| Maternal genotype	Absence of alae in dcr-1(-) F1		Defective alae if present	
	Fraction	(% no alae)	Fraction	(% defective alae)
dcr-1(s2795)/qC1	1/65	(1%)	61/64	(95%)
dcr-1(s2795)/dcr-1(s2795) ;sDp3	19/48	(40%)	25/29	(86%)
dcr-1(ok247)/qC1	0/32	(0%)	1/32	(3%)
dcr-1(ok247)/dcr-1(ok247) ;sDp3	3/47	(6%)	26/44	(59%)
dcr-1(s2624)/qC1	5/19	(26%)	8/14	(57%)
dcr-1(s2624)/dcr-1(s2624) ;sDp3	10/28	(36%)	15/18	(83%)

Figure 4-2

Figure 4-2. Genetic and RNAi analysis of *dcr-1* and *alg-1/alg-2*.

(A-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 two to three 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-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-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.

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 4-2B, p.44). In addition, *dcr-1(RNAi)* animals frequently exhibited faint or

missing adult-specific alae (Figure 4-2E).

Although the phenotypes induced by *dcr-1(RNAi)* 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 4-2E). 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 non-complementing 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 free duplication

sDp3 (Stewart et al., 1998). The third allele, *dcr-1(ok247)*, was made by the *C. elegans*

gene knock-out consortium. The *let-740(s2624)* and *let-740(s2795)* mutations result in

premature stop codons while *dcr-1(ok247)* is an out-of-frame deletion allele removing

residues 708 through 1321 (Figure 4-2G, p.44) and terminating after expression of 15

amino acid residues from intronic 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 4-

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 4-2F). 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 4-2C,

see Experimental Procedures). These findings suggest that maternal *dcr-1(+)* activity

rescues essential functions of *dcr-1* in the homozygous 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

homozyous 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* homozygotes are strongly rescued by one

maternal dose of *rde(+)* activity (Tabara et al., 1999 a ; 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 2, p.49). These

Table 2. Reduced sensitivity to RNAi in *dcr-1* (RNAi) background

dsRNA I	dsRNA II	Resistance to dsRNA II
		non–Unc
none	unc-22	0% (n=434)
mes-2	unc-22	0% (n=824)
dcr-1	unc-22	29% (n=604)
		non–Sqt
none	sqt-3	2% (n=51)
gfp	sqt-3	6% (n=67)
dcr-1	sqt-3	89% (n=309)

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 4-3A, p.52).

RNAi of either *dcr-1* or *alg-1/alg-2* resulted in adults with extra seam cells

(Figure 4-3B,C) 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

observed 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

A uninjected (16)
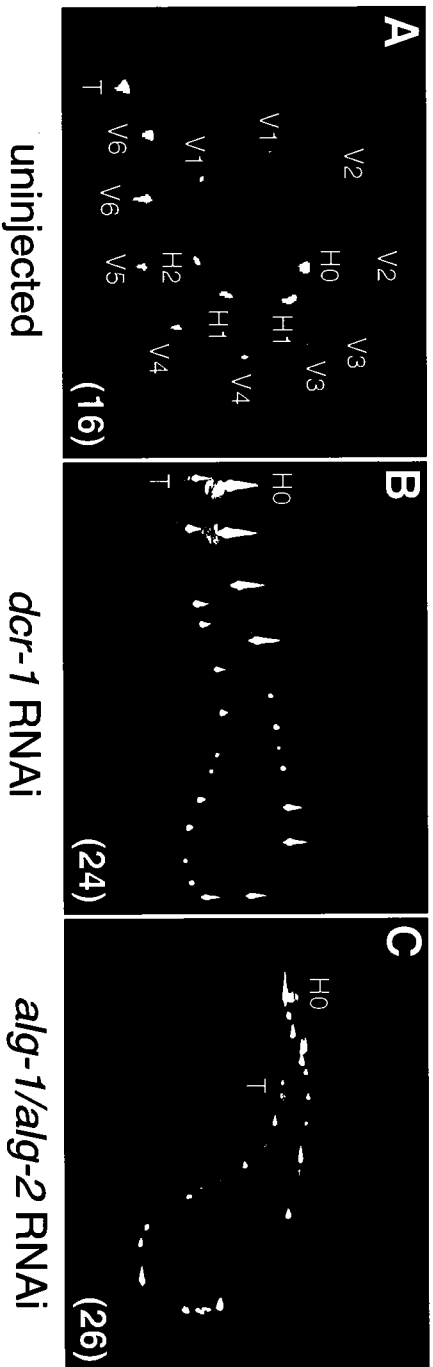
B dcr-1 RNAi (24)

C alg-1/alg-2 RNAi (26)

Figure 4-3

Figure 4-3. Extra seam cells in *dcr-1(RNAi)* and *alg-1/alg-2(RNAi)* animals.

A strain carrying a nuclear localized GFP reporter, expressed specifically in the lateral

seam cells, was injected with *dcr-1* or *alg-1* dsRNA. The GFP-positive seam cell nuclei

were counted in adult progeny of the injected animals. The 16 normal seam cells are

indicated by name for (A) the uninjected control. (B-C) The anterior H0 and posterior T

cells are indicated for (B) the *dcr-1(RNAi)* animal and (C) *alg-1/alg-2 (RNAi)* animal.

The number of seam cells present is indicated in parentheses.

co-injecting 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, 1989; 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)* non-null mutations (Figure 4-4, p.55). In addition, the penetrant germline phenotype associated with *alg-1/alg-2(RNAi)* was partially suppressed by the *lin-41* and *lin-14* mutations (Figure 4-4, see also Figure 4-2D, p.44), but the synthetic lethal phenotype associated with double *alg-1/alg-2(RNAi)* was not suppressed (data not
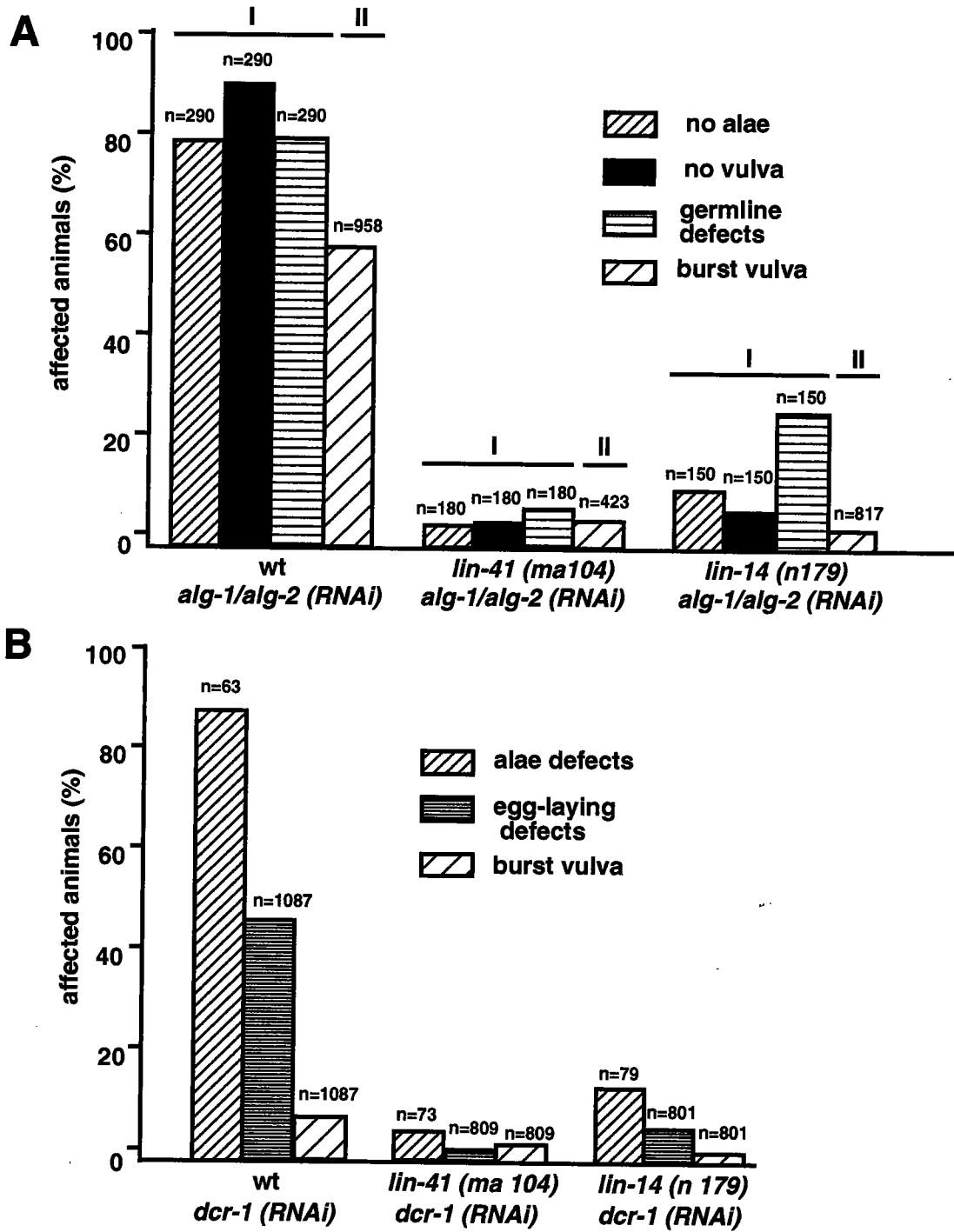
A

B

Figure 4-4

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

mutants. (A-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 micrsocope,

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 Panel 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.

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'UTR 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 down-regulation 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 down-regulation 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-41* 3'UTRs are up-regulated 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 *lin-4* 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. Because *alg-1/alg-2 (RNAi)* causes a similar heterochronic

phenotype but acts at an unknown step in the pathway, we also monitored *lin-4* and *let-7*

processing in *alg-1/alg-2 (RNAi)* animals.

Both *dcr-1* and *alg-1/alg-2(RNAi)* animals exhibited a marked accumulation of the *lin-4* long form at both L3-L4 and adult stages (Figure 4-5A, upper panel, p.60). The same RNA preparations from the *dcr-1* or *alg-1/alg-2 (RNAi)* animals were probed for the expression of *let-7*. We found that, as with *lin-4*, *let-7* processing depends on *dcr-1* activity (Figure 4-5A, bottom panel) but, in contrast, did not appear to depend on *alg-1/alg-2* activity. We next monitored *lin-4* and *let-7* stRNA processing in *dcr-1(ok247)* homozygotes and in animals specifically depleted for either *alg-1* or *alg-2*. In this experiment RNAs prepared from each population were simultaneously probed for expression of *lin-4* and *let-7* RNA (Figure 4-5B, p.60). As with *dcr-1(RNAi)*, the *ok247* homozygotes exhibited a significant accumulation of both *lin-4* and *let-7* long forms (Figure 4-5B, lanes 3-4). A gene specific dsRNA targeting *alg-1* induced accumulation of the pre-*lin-4* RNA but not pre-*let-7* (Figure 4-5B lane 4), and similarly, *alg-2(ok304)* animals exhibited a slight accumulation of pre-*lin-4* and little or no accumulation of pre-*let-7* (Figure 4-5B, lane 5).

The quantity of the short forms of the *lin-4* and *let-7* stRNAs consistently appeared to be reduced in RNA populations prepared from *alg-1/alg-2(RNAi)*, *dcr-1(RNAi)* and *dcr-1(ok247)* animals (Figures 4-5A and 4-5B), while control RNA population prepared from animals undergoing RNAi of the cuticle collagen gene *rol-6* exhibited normal levels of *lin-4* and *let-7* stRNAs (Figure 4-5B, compare lanes 1 and 2). This apparent reduction in *let-7* stRNA level was observed even in *alg-1/alg-2(RNAi)* populations where no significant accumulation of pre-*let-7* was observed. These findings suggest *alg-1/alg-2* activities may be more important for the stability or function of *let-7*
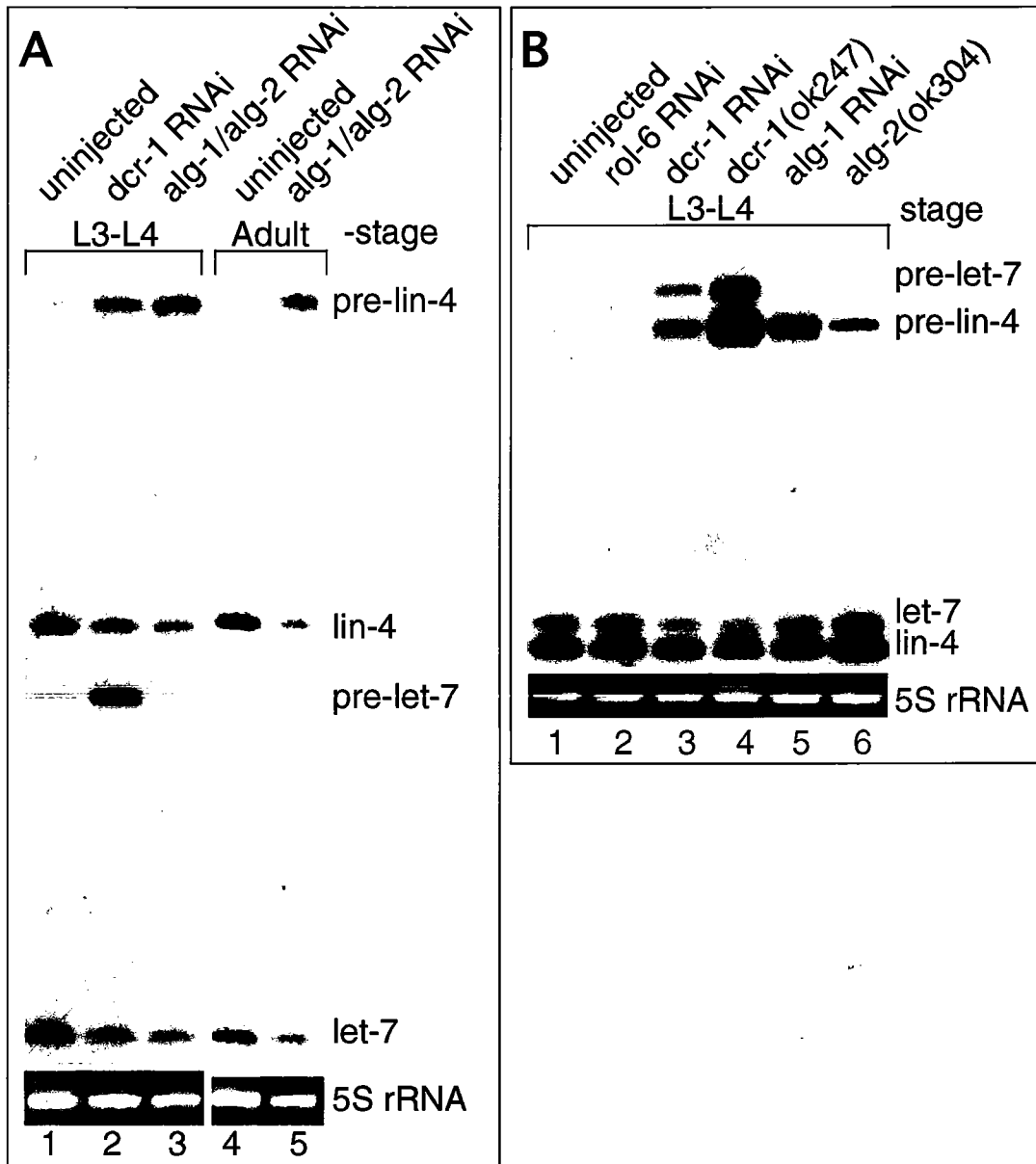
Figure 4-5

Figure 4-5. *dcr-1* and *alg-1/alg-2* activities are required for efficient expression of *let-7*

and *lin-4* stRNAs. (A-B) Northern blot of total RNA isolated from staged populations of

worms as indicated. (A) The top panel shows a blot probed for *lin-4* RNA while the

bottom panel shows the same blot after stripping and re-probing for *let-7* RNA. (B) A

second experiment in which the blot was probed simultaneously to detect both *lin-4* and

let-7 RNAs. The precursor and mature forms of each stRNA are indicated. 5S rRNA

serves as a loading control.

stRNA than for its processing from the larger form. Alternatively, *alg-1/alg-2* might also be involved in *let-7* processing but the *let-7* long form may be less stable, so that unprocessed *let-7* does not accumulate in the absence of *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 *lin-4* and *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, *lin-4* and *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 *lin-4* and *let-7* RNAs 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 *lin-4* and *let-7* stRNAs from larger precursors depends on the activity of DCR-1, a *C. elegans* homolog of the *Drosophila* multifunctional RNase III related protein, Dicer, that has been shown in *Drosophila* cell extracts to process dsRNA into siRNAs that can mediate RNAi (Bernstein et al., 2001). Further, we have shown that *alg-1* and *alg-2*, two homologs of

the RNAi pathway gene *rde-1*, are required for efficient stRNA expression, and along with *dcr-1* function to promote *lin-4* and *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 4-6, p.64). 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
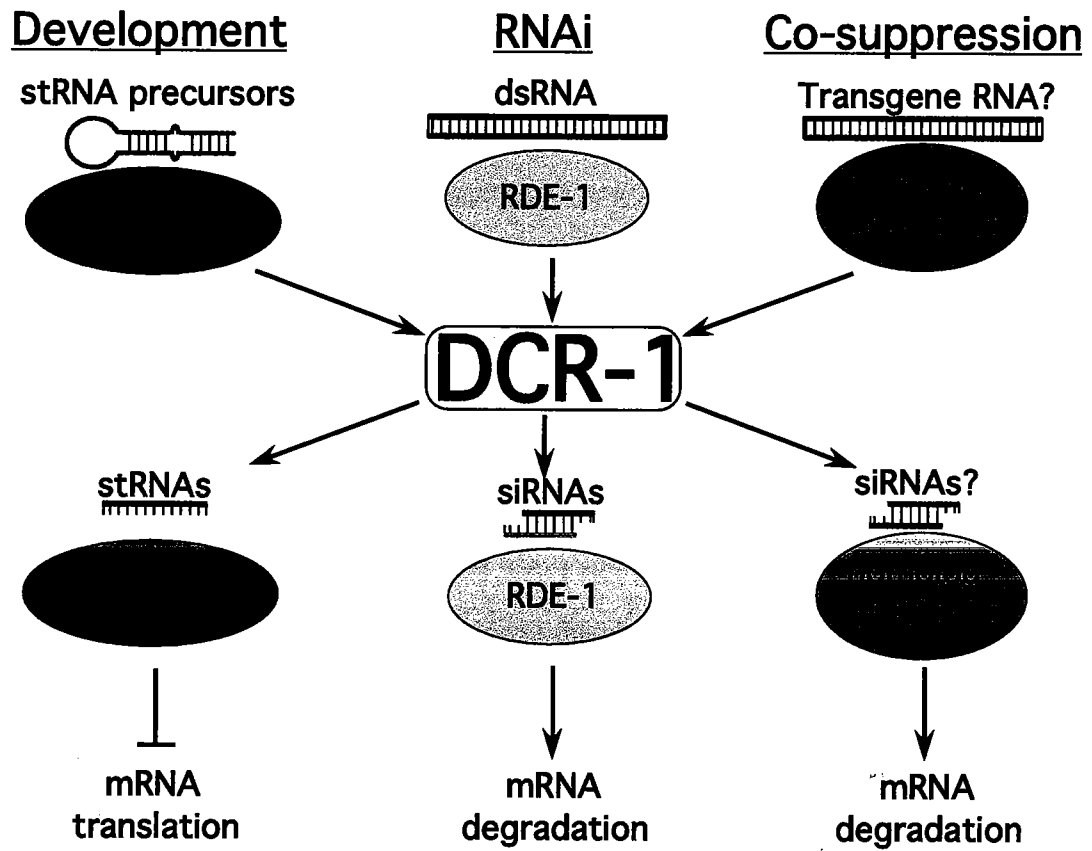
64

Figure 4-6. Model.

(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-41 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 are 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 co-factors in development and PTGS. There are 24 members of the RDE-1/AGO1/PIWI family in *C. elegans* (Figure 4-1, p.40). 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 4-1). 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 *ago1* 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 co-factors 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 co-factors 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 homologs 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 co-suppression in *C. elegans* (Figure 4-6, p.64). 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 insure that the small

RNAs produced are targeted to the appropriate downstream complex, for example to

mediate mRNA destruction versus translation inhibition (Figure 4-6).

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 affect on *lin-4*

and *let-7* function while 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(+)* 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 4-6).

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 4-6, p.64). 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*(+) 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

temporal transitions mediated by the founding members of the class, the *lin-4* and *let-7* stRNAs.

A recent study (Hutvagner et al., 2001) 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 co-evolved 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 (*prg-1*); yk87d2, ZK757.3b; yk359b5, T22B3.2; yk36g4, R06C7.1; yk102d8, yk358e1, R09A1.1; yk20f1, 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'-GGC GAT TCG CTG ACA TCG-3' and 5'-GGC AAA ATA CAT GAC GTT GTT C-3' for full-length *alg-2*,

5'-GGC GAT TCG CTG ACA TCG-3' and 5'-GCA AAA TGA TTG GCT CGC A-3'

for unique 200bp fragment of *alg-2*, 5'-GGC GG CCG CAA TAT TTG-3' and 5'-GGT

TCT CCA ATT GAG ACA CT-3' for unique 300bp fragment of *alg-1*. 5'-GGC TTT

GCT TTC TTT GCT GCT-3' and 5'-GGT AAT GAT GAT ATC TCT CCA CTT-3' for

dcr-1. 5'-G CCC AGC ACA TCA ACT CCC TCA GG-3' and 5'-C ACC CCA ATT

CGG TGC TCT CCG GCG-3' for *mes-2*. The *alg-1* full-length dsRNA was made from

the plasmid pCCM508.

Combinations of full-length *alg-1* dsRNA with *prg-1* dsRNA (yk397e11) or with

dsRNAs corresponding to predicted genes, T23D8.7, 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=235) *dcr-1* GFP (-) adult homozygous animals.

After injection of 13 *dcr-1(ok247)/hT2::GFP* mothers, zero GFP(-) animals were

observed (n=144). Embryonic lethalilty after *dcr-1(RNAi)* was measured using the *dcr-1(ok247)/qC1* strain, which uninjected segregates 3% (n=521) arrested embryos. After

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 except hybridization and wash steps were performed at 50°C. Oligonucleotides used as Northern probes were *let-7* : 5'-AACTATACAACCTACTACCTCACCGGATCC-3' and *lin-4* : 5'-ATAGTACACTCACACTTGAGGTCTCAGGG. 5S rRNA was detected by ethidium bromide staining of polyacrylamide gels prior to transfer.

Analysis of the *let-7* regulated lacZ::*lin-41* 3' UTR reporter gene. Worms containing the mgEx540 reporter array were injected with dsRNA against *alg-1* and transgenic progeny as well as uninjected controls were collected for fixing and staining with X-gal to detect expression of lacZ::*lin-41* .

CHAPTER V

TARGET-DEPENDENT SELECTION AND ACCUMULATION OF SMALL INTERFERING RNAs DURING RNAi IN *C. ELEGANS*

Summary

Surveillance mechanisms that can be entrained to recognize and silence specific nucleic acid sequences have been identified in a variety of organisms. These mechanisms include RNA interference (RNAi) (Fire et al., 1998), triggered by exposure to double-stranded RNA(dsRNA), and co-suppression (Napoli et al., 1990; van der Krol et al., 1990), triggered by introduction of extra copies of a gene. Mutations that disrupt RNAi and co-suppression have been correlated with increased transposon activity (Ketting et al., 1999) and decreased viral resistance (Mourrain et al., 2000), supporting the idea that these post-transcriptional gene silencing (PTGS) mechanisms are important for sequence-directed immunity. The silencing agent in PTGS is a species of small interfering RNA (siRNA) of 21-26 nt that guides the destruction of cognate RNAs (Hamilton and Baulcombe, 1999; Zamore et al., 2000). We have explored the accumulation of siRNAs during RNAi in *Caenorhabditis elegans*. While the trigger dsRNA is processed directly into siRNAs including both strands of the trigger (Elbashir et al., 2001 a; Elbashir et al., 2001 b; Yang et al., 2000; Sijen et al., 2001), we find that only segments complementary to a target RNA are retained and accumulate. This target-dependent accumulation of antisense but not sense siRNAs requires the activities of several RNAi pathway genes. We show that selective retention or amplification of RNAi-active molecules can create a

reservoir of memory antisense siRNAs which prevent future expression of the genes with complementary sequence. This suggests a parallel at the molecular level with the clonal selection of antibody forming cells and in the vertebrate immune system.

Results and discussion

siRNAs are thought to be essential intermediates in PTGS in both plants and animals. siRNAs were first discovered in plants undergoing transgene or virally induced PTGS (Hamilton and Baulcombe, 1999). In cultured *Drosophila* cells, siRNAs co-purify with a nuclease complex that degrades the target mRNA (Hammond et al., 2000). Long dsRNA is processed into siRNAs in *Drosophila* embryo lysates (Zamore et al., 2000), cultured cells (Hammond et al., 2000), and embryos (Yang et al., 2000), as well as in *C. elegans* (Parrish et al., 2000). In cultured *Drosophila* cells, processing of long dsRNA into siRNAs and RNAi itself requires the RNase III enzyme Dicer (Bernstein et al., 2001). Furthermore, synthetic RNA duplexes that mimic the siRNA products of the Dicer reaction initiate RNAi both *in vitro* and in cultured *Drosophila* and mammalian cells (Elbashir et al., 2001 a; Elbashir et al., 2001 b). The *C. elegans* homolog of *dicer*, *dcr-1*, has been recently shown to be involved in RNAi as well (Grishok et al., 2001; Knight and Bass, 2001).

The nematode *C. elegans* is remarkably sensitive to dsRNA, exhibiting a potent PTGS response when exposed to dsRNA administered by injection (Fire et al., 1998), by ingestion of dsRNA-expressing *E. coli* (Timmons and Fire, 1998), or by soaking in a solution of dsRNA (Tabara et al., 1998). *C. elegans* mutants resistant to RNAi (Tabara et

al., 1999 a) provide useful reagents to dissect the RNAi process. For example, the wild-type activities of two genes, *rde-1* and *rde-4*, are required for the initiation of RNAi but not for the inheritance and function of a sequence-specific interfering agent (Grishok et al., 2000). Conversely, *rde-2* and *mut-7* are required for interference but not for the formation of the inherited agent. These studies are consistent with models in which siRNA production mediated by *rde-1* and *rde-4* corresponds to an initiation step, distinct from the subsequent *rde-2-* and *mut-7*-dependent destruction of the target mRNA. Such a model predicts that these two classes of mutant strains might exhibit distinct phenotypes with respect to siRNA accumulation.

To examine the accumulation of siRNAs in wild-type and mutant *C. elegans* strains we fed each strain *E. coli* expressing *pos-1* dsRNA and then assayed the production of small RNAs corresponding to the *pos-1* dsRNA sequence. In this assay, wild-type worms fed *pos-1* dsRNA produce dead eggs that lack intestinal and germline cells, a phenotype identical to *pos-1* mutants, whereas the RNAi deficient mutants, *rde-1, rde-2, rde-4* and *mut-7*, produce only viable progeny (Tabara et al., 1999 a). In multiple independent experiments, wild-type worms, but not the mutant strains, consistently produced ~23 nt antisense *pos-1* RNA (Figure 5-1A, B; p.76). The ~23 nt RNAs detected in wild-type worms were not merely byproducts of *pos-1* mRNA degradation, since they included antisense siRNAs and must therefore derive from the ingested dsRNA.

The observation that all four of the RNAi pathway genes assayed are required for siRNA accumulation suggests that, despite their different genetic properties, each of
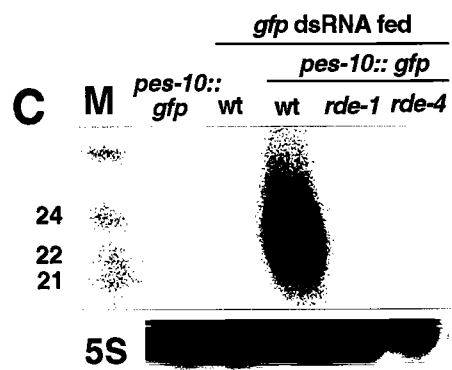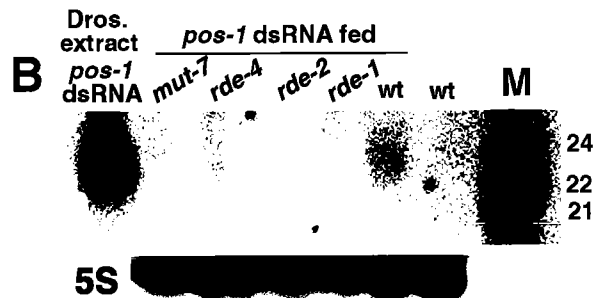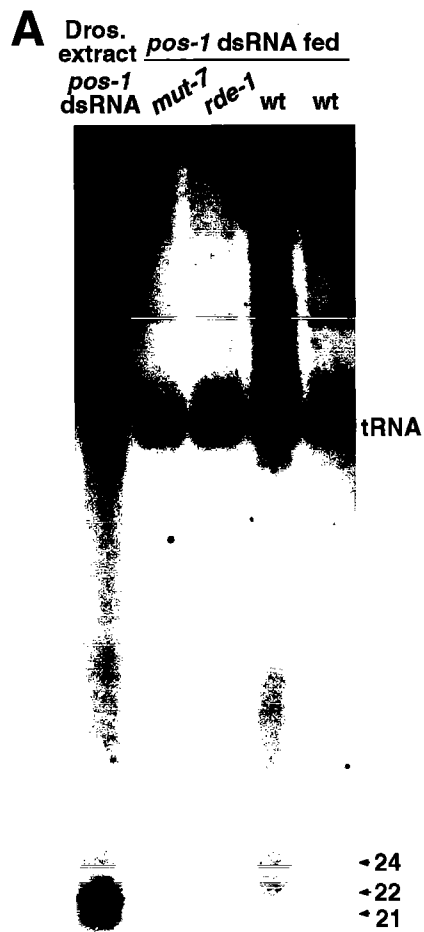
Figure 5-1

Figure 5-1. Accumulation of siRNA in *C. elegans* requires *rde* genes, the trigger dsRNA

and the target mRNA. (A-C) Northern blot analysis siRNA accumulation in wild-type

and mutant strains cultured as indicated. tRNA and ribosomal 5S RNA serve as loading

controls, P^{32} - labeled sense RNA was used as a probe in each experiment. (A, B)

Accumulation of siRNA depends on *rde*- pathway genes and exposure to trigger dsRNA.

The left-most lane in each Northern contains trigger dsRNA processed in *Drosophila*

embryo lysates; in B, size markers are shown in the lane marked "M."

(C) Accumulation of siRNA requires *rde*-pathway genes and the target mRNA.

these genes might be required for the processing of dsRNA. An alternative explanation is that the initial production of siRNAs may occur in the absence of some or all of these RNAi pathway genes, but successful destruction of a target RNA may be necessary for a secondary feedback mechanism that triggers the preservation or amplification of successful siRNA species. If this latter model is correct, then the accumulation of siRNAs should be dependent on the presence of a target mRNA. To test these ideas, we fed *C. elegans* strains *E. coli* expressing dsRNA for the jellyfish green fluorescent protein (GFP) and assayed for siRNA accumulation in strains with and without a GFP transgene. In these experiments, detectable accumulation of ~23 nt antisense GFP RNA depended on the presence of the GFP transgene (Figure 5-1C, p.76). Furthermore, as for *pos-1* RNAi, the RNAi deficient mutants, *rde-1* and *rde-4*, failed to accumulate ~23 nt antisense RNAs (Figure 5-1C). We could not assay *rde-2* and *mut-7* because they are germline-specific (Tabara et al., 1999 a), whereas the *GFP* used in this assay is expressed in the soma.

siRNA production during co-suppression in plants (Hamilton and Baulcombe, 1999) or after the introduction of dsRNA into cultured *Drosophila* cells (Hammond et al., 2000), *Drosophila* embryos (Yang et al., 2000), or *Drosophila* embryo lysates (Zamore et al., 2000; Elbashir et al., 2001 a) leads to the equal production of both sense and antisense strands of the siRNA. Surprisingly, we found that only antisense siRNAs detectably accumulate both in *gfp* and *pos-1* feeding assays (Figure 5-2 A,B; p.79). These data reveal a significant difference between co-suppression in plants and RNAi in flies, on the one hand, and RNAi triggered by dsRNA-feeding in worms on the other. The differential
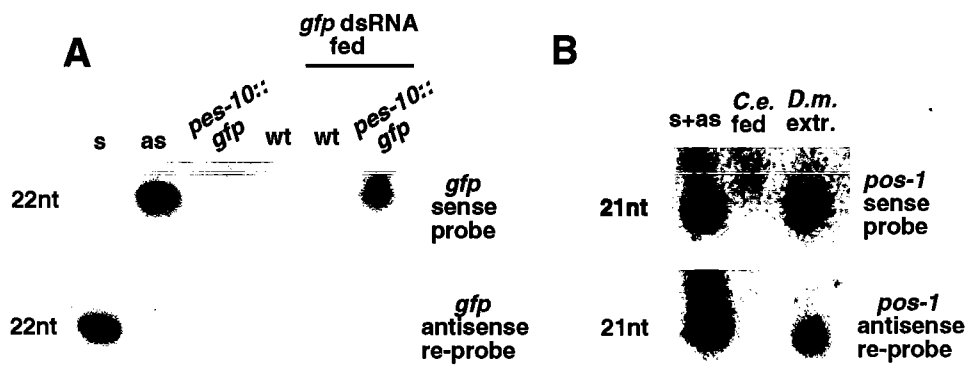
Figure 5-2

Figure 5-2. Antisense but not sense siRNA accumulates during RNAi in *C. elegans*.

(A, B) Northern blot analysis of siRNA accumulation in wild type strains cultured as indicated, upper panels are probed with sense and lower panels are probed with antisense P^{32} -labeled RNA. Synthetic RNA oligos are loaded as controls either in separate lanes (left two lanes panel A), or mixed (left lane panel B). (A) Lanes labeled *"pes-10::gfp"* contain RNA prepared from a wild type transgenic strain cultured with or without *E. coli* expressing *gfp* dsRNA.

(B) The center lane contains RNA prepared from a wild type *C. elegans* strain cultured on *E. coli* expressing *pos-1* dsRNA.

accumulation of antisense and sense ~23 nt RNAs observed in the presence of a target transgene may point to a novel step in the RNAi pathway: the selective retention or amplification of one of the two siRNA strands. This step may not be detected in plants and flies, where the conversion of dsRNA to siRNA duplexes is thought to be highly efficient (Hamilton and Baulcombe, 1999; Sijen et al., 2001; Hutvágner et al., 2000) but is revealed in worms fed dsRNA, were only small quantities of siRNA duplexes may be produced in the initial phase of the RNAi reaction. PTGS in plants is thought to involve the continuous production of dsRNA (Morrain et al., 2000; Waterhouse et al., 1998; Dalmay et al., 2000). Perhaps the symmetric processing of an abundant dsRNA species generated in this way overwhelms or renders unnecessary any mechanism for the target-dependent retention of active siRNAs. The introduction of dsRNA through feeding, on the other hand, is likely to be inefficient, producing a much smaller pool of dsRNA for the symmetric processing reaction.

The results presented here suggest a model in which small amounts of siRNA derived directly from the ingested dsRNA initiate mRNA destruction but are then retained or amplified to initiate successive rounds of interference (Figure 5-3, p.82). The retention of only those molecules that successfully engage a target mRNA would be an efficient mechanism to insure that the RNAi machinery does not become saturated with non-productive siRNA species. Whether this phenomenon is specific to worms, or a more general, but as yet undetected, feature of PTGS in plants and animals, remains to be determined.

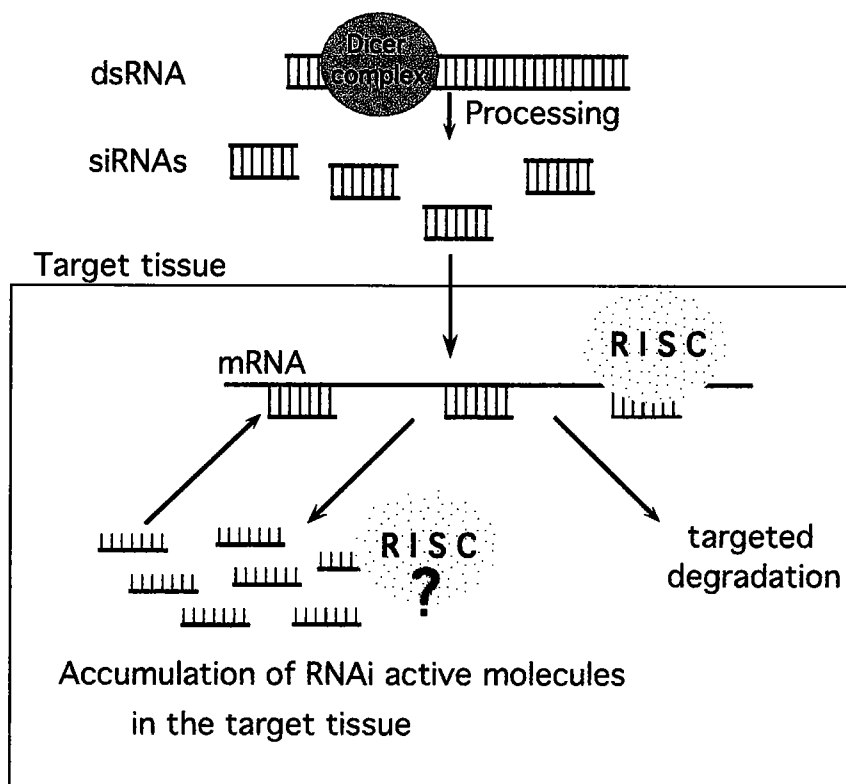A natural precedent exists for the selective retention of antisense versus sense

Figure 5-3. Catalytic model of RNAi in *C. elegans*.

RNAs produced by an RNAi-related mechanism (Grishok et al., 2001; Hutvágner et al., 2001). The *lin-4* and *let-7* small temporal (stRNAs) are natural RNAs in *C. elegans* (Lee et al., 1993; Reinhart et al., 2000) that are expressed as double-stranded hairpins and are processed in a mechanism that involves *C. elegans* Dicer, *dcr-1*, and homologs of *rde-1* (Grishok et al., 2001). Similarly, in flies and humans, Dicer is required for the maturation of *let-7* from a stem-loop precursor RNA (Hutvágner et al., 2001). While theoretically the Dicer processing reaction can produce both strands of the processed stRNA precursor, only one strand is detected in animals. Perhaps related mechanisms operate in the asymmetric accumulation of siRNA and stRNA species.

The findings reported here suggest that RNAi may represent a nucleic-acid based form of acquired immunity. In this model, exposure to abnormal RNA species in the environment initiates formation of ~23nt interfering RNAs that are retained or amplified only if a complementary target RNA sequence is present in the organism. Although both sense and antisense siRNAs are likely present during the initiation phase, only the antisense siRNA accumulates, perhaps as a consequence of engaging the target mRNA. The remarkable potency of RNAi suggests that the siRNAs are not only retained but also reused (Figure 5-3, p.82). The feeding method for RNAi delivers a constant, if small, supply of dsRNA, and thus efficient retention of active siRNA species could explain the accumulation of antisense siRNAs. It is also possible that the target mRNA or the sense siRNA strand is used as a template for the asymmetric amplification of antisense siRNA via an RNA-dependent RNA polymerase. The retention or amplification of active siRNA species would serve to create a reservoir of siRNAs (Figure 5-3, p.82) that may

facilitate the response to future challenge.

We tested this prediction of our model in the following experiment. Worms either expressing the target gene (*pes-10::gfp*) or not expressing *gfp* were fed *gfp* dsRNA-expressing bacteria for 48 h and then challenged by the expression of a *gfp* transgene from the tightly controlled heat-shock promoter. The promoter we used, *hsp16-2* (Stringham et al., 1992), drives expression of GFP in the intestine, the tissue where *pes-10::gfp* is expressed, and in the pharynx, where there is no significant expression of GFP in the *pes-10::gfp* strain. By the end of the 48 h period of *gfp* dsRNA feeding, expression of GFP in the intestine of *pes-10::gfp* transgenic worms was totally suppressed, and accumulation of antisense *gfp* siRNA corresponds to this time (Figure 5-1, p.76). We predict that there was no significant accumulation of antisense *gfp* siRNAs in the *hsp16-2::gfp* transgenic line as the target gene was not expressed at the time of the exposure to dsRNA. Then we induced the expression of *hsp16-2::GFP* in *hsp16-2::gfp* transgenic worms and *hsp16-2::gfp; pes-10::gfp* double transgenic worms either fed or not fed *gfp* dsRNA (Figure 5-4, p.85). While induction of *hsp16-2::GFP* expression was not reduced in the *hsp16-2::gfp* worms exposed to dsRNA (Figure 5-4 A,C), presence of the active *pes-10::gfp* transgene in the double transgenic worms during the time of dsRNA feeding prevented the induction of *hsp16-2::GFP* expression in the intestine (Figure 5-4 B,C). Significantly, expression of the *hsp16-2::GFP* in the pharynx of double transgenic worms fed *gfp* dsRNA was not reduced (Figure 5-4 B,C). Since *pes-10::gfp* is expressed in the intestine, but not pharynx, target-dependent accumulation of siRNAs during RNAi should take place in the intestine. Therefore, silencing of *hsp16-2::GFP* expression

Figure 5-4

A

hsp16-2::gfp

pharynx / embryos

pharynx / intestine

gfp dsRNA(-)

gfp dsRNA(+)

B

hsp16-2::gfp; pes-10::gfp

pharynx / embryos / intestine

pharynx / embryos / intestine

gfp dsRNA(-)

gfp dsRNA(+)

C

animals with silenced GFP (%)

0   20   40   60   80   100

☒ hsp16-2::GFP in the intestine

■ hsp16-2::GFP in the pharynx

gfp dsRNA fed

hsp16-2::gfp   0/30 0/30 2/45 2/45
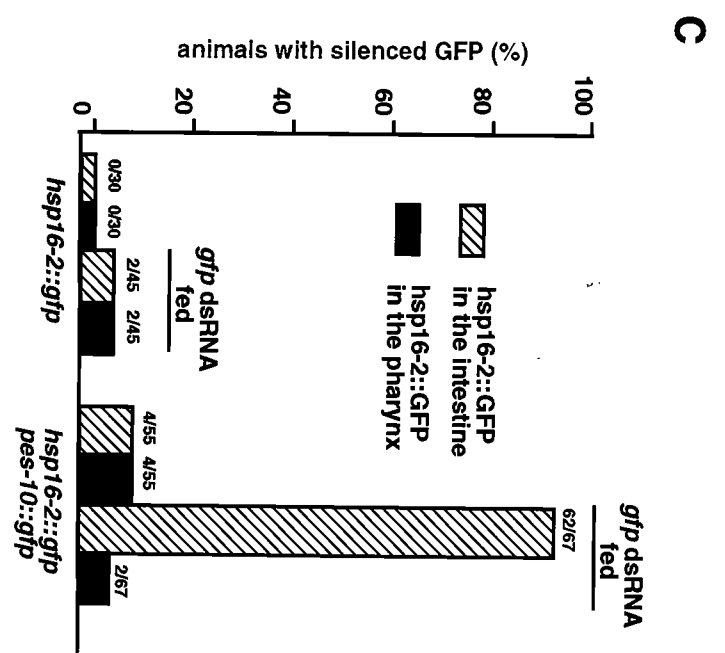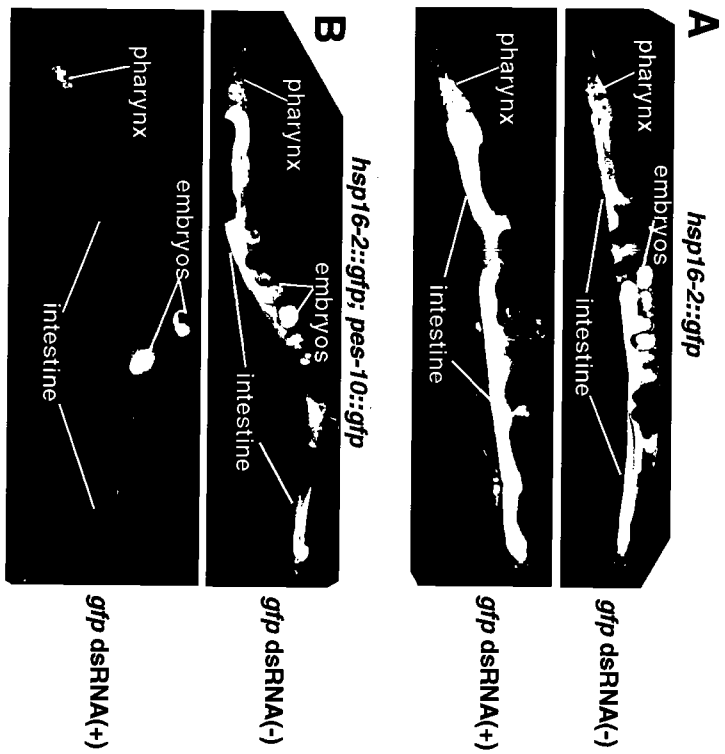
hsp16-2::gfp pes-10::gfp   4/55 4/55 62/67 2/67

Figure 5-4. Target-dependent accumulation of *gfp* antisense siRNAs prevents expression of the heat-shock inducible GFP transgene. (A, B) Fluorescent micrographs showing expression of *hsp16-2::GFP* in *hsp16-2::gfp* transgenic worms (A) and *hsp16-2::gfp; pes-10::gfp* transgenic worms (B) either not exposed (A, B, upper panels) or exposed (A, B, lower panels) to *gfp* dsRNA food. (C) Fractions of animals with silenced *hsp16-2::GFP* expression in the intestine and pharynx after the heat-shock (strains and *gfp* dsRNA treatment is indicated).

occurred precisely in the tissue where target-dependent accumulation of antisense *gfp* siRNAs was predicted to occur. From both our molecular analysis and *in-vivo* data we conclude that selected and accumulated siRNAs complementary to the particular mRNA prevent future expression of the genes with the similar sequence. We also predict that accumulated siRNAs are not likely to get transported outside of the tissue where the target gene is expressed. It appears that sequence-specific immunity in our case is provided by an agent different from the ones mediating systemic spread of PTGS in plants and RNAi in *C. elegans*. Our finding of tissue-specific accumulation of antisense siRNAs able to initiate silencing might explain why the rare cases of long-term inheritance of RNAi in *C. elegans* are observed mostly for the genes expressed in the germline (Grishok et al., 2000; our unpublished observations). When the germline is a tissue where the target gene is expressed, it becomes enriched with the antisense siRNAs and they are likely to be inherited with the germ cells to the next generation. However, the inheritance of RNAi to the F1 generation from the worms injected by dsRNA is more general and independent of the presence of target gene in the injected worms (Grishok and Mello, unpublished observations). It is likely to occur by the same mechanism as systemic transport of RNAi.

Thus, the uncovered clonal selection in PTGS surveillance provides a new level of sophistication and versatility rivaling the protein based immunity mechanisms in vertebrates. Also, the conservation and ancient origin of the cellular factors that underlie PTGS mechanisms (Catalanotto et al., 2000; Cerutti et al., 2000; Fagard et al., 2000), the ability of interference to spread systemically in the organism (Fire et al., 1998), and even

to be transmitted via the germline (Grishok et al., 2000), support the view that PTGS mechanisms represent highly evolved forms of acquired sequence-directed immunity.

Methods

Total RNA preparation from *C.elegans* fed dsRNA. RNAi by feeding *E. coli* expressing dsRNA was induced in large populations of worms (50 000- 100 000) as described (Tabara et al., 1999 a). Total RNA was extracted using guanidine-isothiocyanate followed by phenol extraction (pH 4.7) and ethanol precipitation. Equal amounts of RNA from different samples were run on 1.5% agarose gels and the amount of low molecular weight RNA estimated in the samples.

Northern blotting. 60-100 μg of total RNA per lane was resolved on 15% denaturing polyacrylamide gels in 0.5x TBE and electoblotted to Hybond N+ nylon membrane (Amersham) in 0.5x TBE at 400 mA for 1h, using semi-dry apparatus (BioRad). RNA was crosslinked to the membrane using UV crosslinker (Stratagene). ^{32}P-labeled strand specific riboprobes were made in T7 transcription reactions containing α^{32}P-ATP (6000Ci/mmole; 40mCi/ml; ICN). After synthesis probes were partially hydrolyzed in 80mM NaHCO$_3$, 160mM Na$_2$CO$_3$ at 60°C for 1h followed by neutralization to pH 7.0. Hybridization was performed at 50°C for 24h in 50mM Na-phosphate buffer (pH 7.2) containing 300mM NaCl, 7% SDS, 1x Denhardt solution and 250μg/ml denatured salmon sperm DNA. Membrane was washed with 2xSSC 5%SDS buffer twice followed

by several washes in 1xSSC 1%SDS at 50°C. Blots were exposed to phosphoimaging

screens (Fuji), images were analyzed using BioRad phosphoimager and QuantityOne

software (BioRad). Size markers were generated by complete T1 digest of α^{32}P-ATP-

labeled 1kb *pos-1* sense RNA. *pos-1* dsRNA was processed in *in vitro* RNAi reactions

using *Drosophila* embryo lysates as described (Tuschl et al., 1999). Synthetic RNA

oligonucleotides used as hybridization controls (1pmole/lane) were purchased from

Dharmacon Research Inc..

Heat-shock assay. *hsp16-2::gfp* transgenic lines were made by transformation of

pCMM 317 construct provided by Yingdee Unhavaithaya along with the pRF4

transformation marker. The *pes-10::gfp* integrated strain JH 103 was provided by

Geraldine Seydoux. It was crossed with *hsp16-2::gfp* to obtain *hsp16-2::gfp; pes-10::gfp*

strain. *hsp16-2::gfp, pes-10::gfp* and *hsp16-2::gfp; pes-10::gfp* worms were cultured

starting from L2-L3 for 48 h on bacteria expressing *gfp* dsRNA. Control populations of

the same stage were cultured under regular conditions. pes-10::GFP expression in worms

exposed to dsRNA was monitored and was completely silenced by the end of the 48h

period. Then *gfp* dsRNA fed strains and controls were heat-shocked for 4 hours at 33°C

and hsp16-2::GFP expression was scored using fluorescent microscopy (Zeiss).

CHAPTER VI

GENERAL DISCUSSION

Molecular mechanism of RNAi

<u>Initiation of PTGS: dsRNA and RNAi.</u> There is a confusion in the literature

concerning the distinctions between different types of post-transcriptional silencing

mechanisms, such as co-suppression and RNAi. And yet the genetic analysis of these

phenomena in *C. elegans* suggests that they must have distinct mechanisms and employ

distinct but overlapping sets of genes (Dernberg et al., 2000; Grishok et al., 2000; Ketting

and Plasterk, 2000). The confusion, no doubt, reflects the fact that all of these

mechanisms are likely to obtain their sequence specificity through base pairing between a

guide RNA and the target mRNA. Indeed, once targeting occurs, the mechanisms may

thereafter be indistinguishable, perhaps involving self- renewing reactions that produce

more targeting complexes with each round of mRNA degradation (Figure 5-3, p.82;

Figure 6-1, p.103).

RNAi in *C.elegans* is by definition initiated by dsRNA (injected, fed or expressed

from transgenes) (Fire et al., 1998). The most upstream components responding to

dsRNA are *rde-1* and *rde-4* (Grishok et al., 2000) and the activities of these two genes are

totally dispensable for the initiation of other silencing phenomena in *C.elegans*, such as

transposon silencing (Tabara et al., 1999 a), co-suppression (Dernburg et al., 2000;

Ketting and Plasterk, 2000) and transcriptional silencing of germline transgenes (Tabara

et al., 1999 a). The work in *C. elegans* clearly suggests that co-suppression and transposon silencing must have initial triggers other than dsRNA. However, it is likely that dsRNA functions downstream as a common factor involved in the renewal, amplification and targeting of all PTGS phenomena. Thus, the defining feature of each form of PTGS is likely to be the initiation signal (or trigger). These triggers could be aberrant RNA, RNA accumulated above a threshold, the repetitive nature of the DNA, or the modification of DNA or chromatin structure. Involvement of the RecQ DNA helicase family member, *qde-3*, in post-transcriptional gene silencing in *Neurospora* may indicate that initiation of this process starts with DNA-DNA interactions facilitated by the activity of *qde-3* (Cogoni and Macino, 1999 b). Recent study in *Arabidopsis* reinforces the idea of the connection between methylation of DNA and chromatin structure on one hand and degradation of mRNA on the other (Morel et al., 2000). This work shows that mutants impaired in a SWI2/SNF2 chromatin component (*ddm1*; Jeddeloh et al., 1999) or in the DNA methyltransferase (*met1*; Finnegan and Dannis, 1993) cause stochastic release of PTGS. Clearly an area of considerable importance for future study will be defining the sequence of events that initiates RNAi and other forms of PTGS.

Role of small 21-22 nt RNA species in RNAi. In 1999, Hamilton and Baulcombe discovered what seems likely to be the common currency of PTGS mechanisms, a species of small 21-25 nt RNAs associated with PTGS in plants (Hamilton and Baulcombe, 1999). These small interfering RNAs (siRNAs; Elbashir et al., 2001 a) have now been found associated with RNAi in *Drosophila* (Hammond et al., 2000; Zamore et al., 2000; Yang et al., 2000; Elbashir et al., 2001 a) and RNAi in *C.elegans*

(Parrish et al., 2000; Grishok et al., submitted). Moreover, siRNAs derived from a transgene expressing promoter-specific dsRNA have been correlated with transcriptional gene silencing in plants (Mette et al., 2000; Sijen et al., 2001). Conceivably, siRNAs serve as sequence-specific guide RNAs that direct distinct protein complexes to the target RNA or DNA.

Production of siRNA has been shown to occur independently of the initiation of mRNA degradation *in vitro* (Zamore et al., 2000). However, accumulation of siRNAs during PTGS *in vivo* seems to occur only in plants actively involved in the process of target RNA degradation, similarly to our study in *C. elegans* described in Chapter V. The detected accumulation of siRNAs in plants could, therefore, result from a secondary mechanism aimed at the stabilization or amplification of the small RNA species that successfully initiate mRNA degradation. This possibility of a feedback mechanism leading to siRNA accumulation in plants has been discussed by Llave and collegues (2000). The mechanism for the accumulation of siRNAs in plants might be different from the one operating in *C. elegans* as both sense and antisense siRNAs are detected in plants (Hamilton and Baulcombe, 1999) while only antisense siRNA accumulate in *C. elegans* (Grishok et al., submitted). Alternatively, the difference could be explained by the fact that antisense RNA as well as mRNA is used as a target in plant PTGS, and, therefore, target-dependent accumulation of siRNA of both polarities takes place.

The best evidence for a direct role for small RNAs in the initiation of RNAi comes from biochemical studies. It has been shown *in vitro* that dsRNA is processed into small 21-22mer RNAs prior to targeted mRNA degradation (Zamore et al., 2000). In

cultured *Drosophila* S2 cells siRNAs were shown to co-purify with a nuclease activity that degrades the target mRNA (Hammond et al., 2000). Furthermore, in *Drosophila* embryos the appearance of small RNAs derived from a dsRNA trigger were correlated with disappearance of the target RNA (Yang et al., 2000). Direct evidence for the sufficiency of 21-22 nt RNA fragments as mediators of RNAi has been obtained using *Drosophila* lysates wherein chemically synthesized 21-22 dsRNA molecules with overhanging 3′ ends were shown to be efficient triggers for target mRNA cleavage (Elbashir et al., 2001 a). The observation that 3′ overhangs stimulate RNAi is consistent with the idea that an RNase III -like processing reaction is involved in processing the trigger RNA. Indeed, as discussed below, a recent study has identified an RNAse III type enzyme from *Drosophila* that is able to process dsRNA into the small RNAs involved in RNAi (Bernstein et al., 2001). Duplexes of small 21nt RNAs have been recently shown to suppress gene expression in cultured mammalian cells (Elbashir et al., 2001b; Caplen, et al., 2001).

Biochemical studies analyzing the degradation of target RNA have shown that the target RNA is cleaved with a periodicity roughly corresponding to 20-23 nt (Zamore et al., 2000), consistent with the idea that siRNAs may guide degradation. In experiments with synthetic small dsRNA triggers, the target RNA cleavage site was located near the center of the region covered by the 22 nt guide RNA and there was no nucleotide preference for this reaction (Elbashir et al., 2001 a). Although both strands of small 21-22 nt guide RNAs have been identified in targeting complexes, each complex appears to have an asymmetric character and either contains only one of the strands or allows only

one of the strands to guide target RNA cleavage (Elbashir et al., 2001 a).

Dissection of the trigger RNA. Several studies have examined structural and sequence requirements for the trigger RNA. In *C.elegans*, dsRNA of several hundred base pairs in length is normally used to achieve optimal knock-out phenotypes, and similar length requirements have been observed in other systems (Ngo et al., 1998; Tuschl et al., 1999; Hammond et al., 2000). In a recent study examining length requirements for the RNAi trigger in *C.elegans*, dsRNA molecules as short as 26 bp were found to induce interference but showed much higher concentration requirements (Parrish et al., 2000). However, this study did not examine small RNAs with 3' overhangs similar to those shown to be effective in *Drosophila* extracts. Perhaps this structure will improve the efficiency of targeting via small synthetic RNAs *in vivo*. Indeed, it would be interesting to see if such small RNAs might bypass certain of the *rde* mutants in *C. elegans* to cause interference or cause interference in the organisms that apparently lack RNAi.

The degree of similarity required between the dsRNA and target RNA in *C.elegans* was also examined by Parrish and colleagues (2000). Efficient degradation of a target RNA required 96% sequence identity with the trigger dsRNA. Perhaps not surprisingly, targets lacking segments of at least 23 nucleotides of perfect identity were not efficiently degraded. To determine the relative importance of sense and antisense strands in the trigger, dsRNA with mismatches or chemical modifications have been tested in *Drosophila* (Yang et al., 2000) and *C.elegans* (Parrish et al., 2000). These studies concluded that modifications in the antisense strand of dsRNA are less well

tolerated than are modifications of the sense strand. These findings strongly support the notion that the antisense strand determines the target specificity. These findings also suggest that direct amplification of the introduced dsRNA is not necessary for induction of interference.

Targets of RNAi. In the initial studies of RNAi in *C.elegans* it has was shown that intron and promoter sequences are not effective in causing RNAi (Fire at el. 1998), which argued against transcription or RNA processing as the targets for RNAi. In most cases RNAi seems to target only sequences present in the mature mRNA. For example, individual cistrons within a multicistronic gene can be targeted separately (Montgomery et al., 1998). However, in one study, two genes in an operon were inactivated by injection of dsRNA corresponding to one of the genes and also by dsRNA corresponding to an intron within the operon (Bosher et al., 1999). This finding suggests that the pre-mRNA can be targeted by RNAi. Indeed, *in-situ* hybridization studies in *C.elegans* have shown that both nuclear and cytoplasmic levels of target RNA can be reduced by RNAi (Montgomery et al., 1998). These studies suggest that RNAi may occur in the nucleus or in both the nucleus and cytoplasm.

Several studies suggest that RNAi in other systems can target mature maternal mRNAs. For example, in the early mouse embryo the *c-mos* mRNA was susceptible to RNAi (Wianny and Zernicka-Goetz, 2000). Also, mRNAs corresponding to *bicoid* and *hunchback* homologs were successfully knocked out in the fly, *Megaselia abdita* (Stauber et al., 2000). Finally, in *Drosophila* extracts, mature capped and polyadenylated mRNAs are efficiently degraded (Tuschl et al., 1999). These studies imply that the

mature cytoplasmic mRNA can serve as a target for RNAi. Interestingly however, in *C. elegans* mature maternal mRNAs can apparently co-exist and remain functional even when sufficient interfering RNA is present in the oocyte to direct interference in the next generation (Grishok et al., 2000). This observation indicates that either mature maternal RNA in *C. elegans* are protected from RNAi or that some component necessary for RNAi initiation is not present in the oocyte.

Another intriguing aspect of RNAi in *C. elegans* is that certain genes are relatively resistant to interference (Rappleye et al., 1999; Shin et al., 1999; Mello unpublished observations). Although this seems to be especially true for genes expressed in the nervous system (Fire, 1999), it is not clear that this is a problem of tissue specificity as in some cases genes expressed in the same cell exhibit different sensitivities to RNAi. It will be interesting in the future to examine what factors render certain genes resistant to RNAi.

Components of the RNAi pathway

The RDE-1 gene family . The *rde-1* gene is a member of a large gene family with members in plants, fungi, *D. melanogaster* and mammals (Tabara et al., 1999 a). Members of this family have the greatest degree of similarity within the carboxy-terminal 300 amino acids of the protein, which has been referred to as the PIWI box (Cox et al., 1998) or PIWI domain (Cerutti et al., 2000) after a *Drosophila* member of the gene family. Cerutti and colleagues (2000) identified a second, apparently transportable motif

in the proteins of RDE-1 family- Piwi, Argonaute and Zwille- and called the motif PAZ. They also found this motif in *Arabidopsis* gene CAF (Carpel Factory); (Jacobsen et al., 1999). The CAF protein is related to proteins in *Drosophila* and *C. elegans* including the *Drosophila* Dicer protein discussed in Chapter IV and below. The PAZ domain consists of two small regions of similarity that extend from amino acid 390 to 421 in RDE-1. Although the functions of the PAZ and PIWI domains are not known it is likely that these domains are important for RDE-1 function. The existing alleles of *rde-1* include three stop codon alleles that truncate the protein before or within the PIWI domain. The allele (*ne219*) contains a single amino acid substitution that changes a highly conserved glutamate residue to a lysine within the PAZ domain. The N-terminal domain of *rde-1* is the least conserved region in the protein but some members of the family exhibit sequence similarity that extends to the very N-terminus.

To date, the biochemical function of *rde-1* gene family members has not been elucidated. One homolog, eIF2C, was purified as a possible translation factor (Zou et al., 1998) from rabbit reticulocyte lysates. One out of four *Drosophila* RDE-1 homologs, AGO2, has been recently identified as a component of a dsRNA induced silencing complex (RISC) (Hammond et al., 2001) acting at the second step of RNAi. Nuclease activity degrading mRNA has been previously associated with this complex (Hammond et al., 2000). This finding seems to contradict our genetic analysis of the RNAi pathway in *C. elegans* where *rde-1* is required at the initial step of RNAi and dispensable later (Grishok et al., 2000). It is possible that, while *rde-1* acts upstream in the RNAi pathway

in *C. elegans* , there is another *rde-1* homolog acting similarly to *ago2* in the downstream complex.

As mentioned above (Chapter IV), members of *rde-1* family have been implicated in development and silencing in diverse organisms. In *D. melanogaster,* a homolog of *rde-1, aubergine /sting* is implicated in regulation of embryonic development (Schupbach and Wieschaus, 1991; Schmidt et al., 1999). It plays role in the post-transcriptional silencing of the *Stallete* locus (Schmidt et al., 1999; Aravin et al., 2001) and also in activating the translation of the *oskar* mRNA (Wilson et al., 1996). Another *Drosophila rde-1* homolog, *piwi,* is important for stem-cell maintenance (Cox et al., 1998). The important role for *Drosophila ago1* in fly development has recently been reported by Kataoka et al., 2001.

In *A. thaliana,* mutations in the gene of the same family, *argonaute1 (ago1),* lead to small size plants and defects in plant architecture (Bohmert et al., 1998). *Ago1* has recently been shown to play a role in PTGS (Fagard et al., 2000). Another member of *rde-1* family in *Arabidopsis, pinhead/zwille,* has been shown to have role in the formation of central shoot meristem (Moussian et al., 1998). This gene appears to have a partially overlapping function with *ago1* in controlling expression of the *shoot meristemless* (STM) homeobox-containing gene required for shoot apical meristem formation and maintenance (Lynn et al., 1999). It has been proposed that Pinhead and Argonaute might promote translation of specific mRNAs such as STM (Lynn et al., 1999). In the rat, a close homolog of Argonaute1, GERp95, is localized to the cytoplasmic side of the ER and Golgi (Cikaluk et al., 1999). Finally, the *Neurospora* gene, *qde-2,* is required for a

co-suppression (Catalanotto et al., 2000). QDE-2, like RDE-1 does not have any obvious essential functions for viability or development.

In the genome sequence of *C.elegans* there are at least 23 sequences related to *rde-1*, with both the PAZ and PIWI domains. Inactivation by RNAi in *C. elegans* of the two *rde-1* homologs most similar to *Drosophila piwi* (*prg-1* and *prg-2*) leads to defects in germline stem cell production and reduced brood size similarly to defects reported for *Drosophila piwi* mutants (Cox et al., 1998).

Our work described in Chapter IV extended analysis of the developmental roles of the members of *rde-1* family and identified two very closely related genes, *alg-1* and *alg-2*, that play crucial role in the embryonic and larval development of *C. elegans* and also affect fertility of the animals. Supporting the broad role of these proteins, we found that ALG-1 and ALG-2 proteins fused to a GFP tag show expression in numerous tissues, most notably hypodermis, neurons and vulva (data not shown). Our analysis concentrated on the role of *alg-1* and *alg-2* in the heterochronic pathway (Grishok et al., 2001). However, ALG-1 and ALG-2 are likely to have multiple interactions with components of different developmental pathways.

An interesting question remaining unanswered is which of 23 *rde-1* homologs in *C. elegans* is required for co-suppression. *ago1* is required for co-suppression in *Arabidopsis* (Fagard et al., 2000), while it is not needed for PTGS induced by transgenes expressing dsRNA, which is most analogous to the initiation of RNAi in *C. elegans* (Vance and Vaucheret, 2001). *alg-1* and *alg-2* are most homologous to *ago1* and, similarly, they are not required for RNAi in *C. elegans* (Grishok et al., 2001). Thus, *alg-*

1 and *alg-2* are the best candidates for the role in a co-suppression in *C. elegans*. Also, a plant member of the *rde-1* family required for dsRNA-induced PTGS remains to be found.

Role of Dicer in RNAi . The *dicer* gene of *Drosophila* is a recent newcomer to the list of cloned genes involved in RNAi (Bernstein et al., 2001). Dicer appears to be a multifunctional protein with a helicase domain, two dsRNA binding domains and two RNAse III domains. Interestingly, this protein also contains the PAZ motif found in members of the *rde-1* family and in the *Arabidopsis* gene CAF (Jacobsen et al., 1999; Cerutti et al., 2000). Bernstein and colleagues (2001) have shown that Dicer can process dsRNA into the small 22 nucleotide RNAs which are thought to be the sequence-specific factors involved in both co-suppression in plants (Hamilton and Baulcombe, 1999) and RNAi in *Drosophila* (Zamore et al., 2000). Previous work in *Drosophila* cultured cells demonstrated that these small RNAs co-purify with an enzyme complex (RISC) that can degrade a target RNA, and, moreover, the RNA moiety was necessary for the sequence specificity of the complex (Hammond et al., 2000). The *Drosophila* RDE-1 homolog, AGO2, has been recently shown to be a component of the downstream RISC complex and also shown to be able to interact with Dicer (Hammond et al., 2001). Since Dicer exists in a complex acting on the first step of RNAi which is different from RISC (Bernstein et al., 2001), AGO2 might act as a component shared by two complexes and determine the direction of the RNAi process (Baulcombe, 2001). This idea is consistent with the role proposed for RDE-1 family members in Chapter IV (Grishok et al., 2001; Figure 4-6, p.64).

At present, it is not known if Dicer is involved in both trigger dsRNA processing and in degrading the template RNA. The Dicer homolog in *C. elegans*, DCR-1, has been implicated in RNAi by our study (Grishok et al., 2001) and by the work of Knight and Bass, 2001. It is not known yet if Dicer plays role in co-suppression in *Drosophila* or if Dicer homologs are crucial components of PTGS pathways in plants, as well as co-suppression, transcriptional transgene silencing and transposon silencing in *C. elegans*. These questions are likely to open the doors to extensive future investigations.

Role of MUT-7, EGO-1 and other components of RNAi. Another cloned gene important for RNAi as well as transposon and transgene silencing is *mut-7*. The MUT-7 protein has homology to a 3'-5' exonuclease motif found in RNAseD and the Werner syndrome helicase (Ketting et al., 1999). Its homology to ribonuclease D has led to models wherein MUT-7 functions in RNAi to degrade the target RNA (Ketting et al., 1999; Lin and Avery, 1999; Bosher and Labouesse, 2000). If MUT-7 is the enzyme that degrades the target RNA then there must be a related function encoded by another gene, as MUT-7 does not seem to be necessary for RNAi in somatic cells.

The *ego-1* gene encodes a protein essential for fertility in *C. elegans* and is related to RNA-dependent RNA polymerase. The *ego-1* mutant is defective in RNAi for only a small number of genes expressed in the germline (Smardon et al., 2000). Both in *Neurospora* and *Arabidopsis* genes encoding proteins with homology to RdRP were among the first discovered to play role in the PTGS phenomena (Cogoni and Macino, 1999 a; Dalmay et al., 2000; Mourrain et al., 2000). The apparently limited role of *ego-1*

in RNAi may be explained by the possible existence of redundant genes as there are three more genes homologous to RdRP in the *C.elegans* genome. Indeed, in a recent screen for mutants impaired in promoter-driven *unc-22* RNAi, a new category of the *rde* mutants have been discovered: those resistant to RNAi targeting somatic genes, but susceptible to RNAi specific for the germline-expressed ones (Conte et al., submitted). The first cloned gene among this new class of mutants, *rde-9*, previously known as *rrf-1* encodes a protein homologous to RdRp (Conte et al., submitted). *ego-1* and *rde-9* mutants complement each other, consistent with their tissue-specific roles in RNAi. However, when expressed in the muscle, EGO-1 can rescue *rde-9* mutant indicating that proteins have identical molecular function (Conte et al., submitted).

This recent discovery indicates that RdRp proteins are necessary for RNAi. It also poses the question: what RNA is amplified by RdRp during RNAi in *C. elegans*? Models of PTGS in plants consistently put the RdRp- encoding gene *sde1/sgs2* upstream in the pathway implicating its role in the production of dsRNA from the abberant transgene-encoded mRNA (Dalmay et al, 2000 a; Voinnet, 2001; Vance and Vaucheret, 2001). The requirement for the RdRp encoding genes during RNAi in *C. elegans* is surprising since dsRNA is provided as an initiator of RNAi. It is even more surprising that requirement for *rde-9* was found in a screen where RNAi is induced by sense and antisense RNAs expressed from the promoter. Although further amplification of dsRNA in the RNAi process via EGO-1 or RDE-9 can not be excluded, it seems more likely that the step involving RNA amplification is downstream in the pathway. Our discovery of the target-dependent accumulation of only antisense siRNAs during RNAi described in
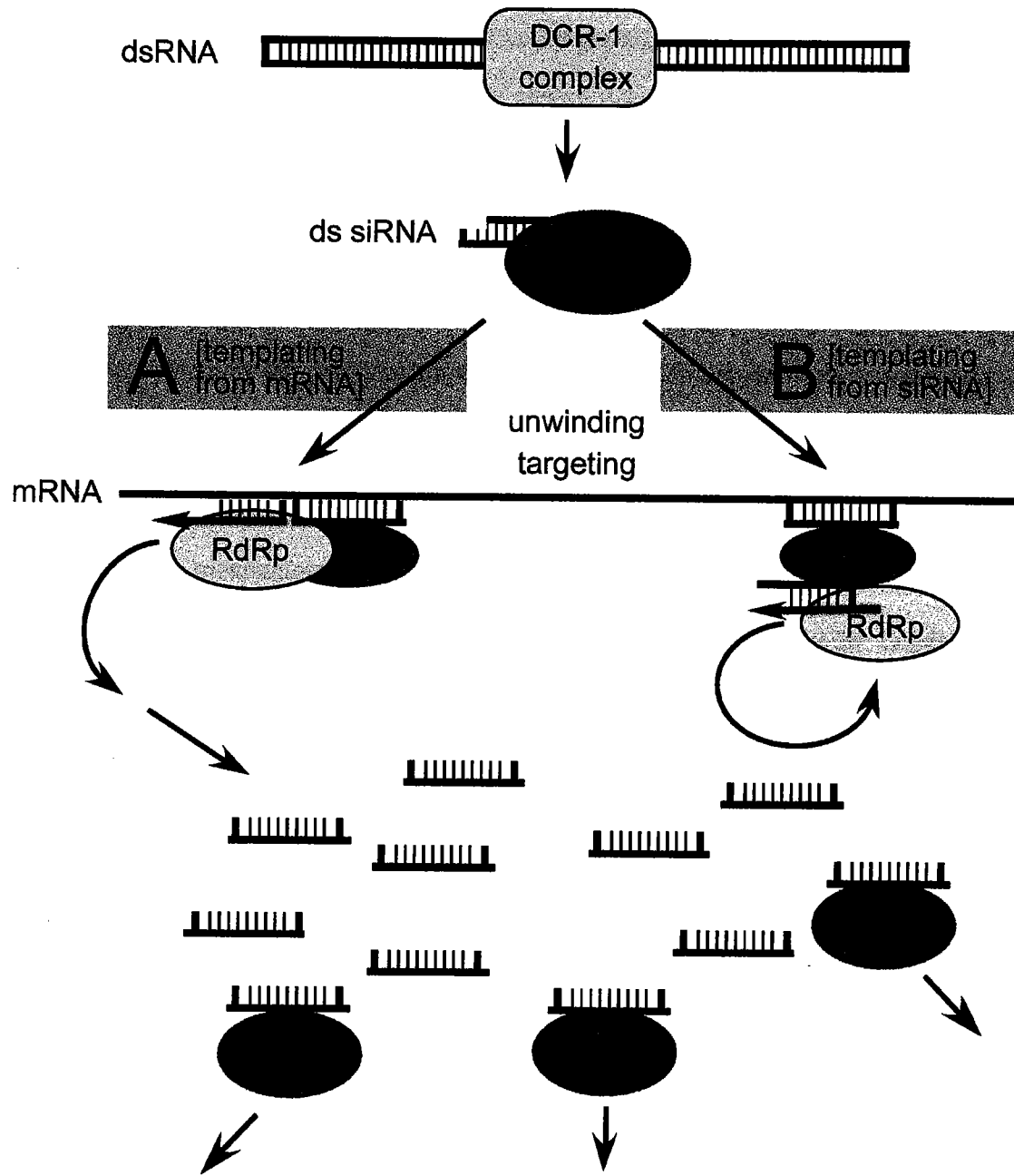
Figure 6-1. Model proposing the role of RdRp in the asymmetric production of siRNAs.

Chapter V might indicate that this is the step in the RNAi pathway where RdRp proteins are involved. It is possible that upon successful engagement in the target mRNA degradation step more antisense siRNAs are produced by RNA-dependent RNA synthesis where either mRNA or sense strand of siRNA serves as a template (Conte et al., submitted ; Figure 6-1, p.103).

The contradiction between plausible roles for RdRp proteins in RNAi in *C. elegans* and transgene-induced PTGS in plants can be resolved in light of the recent finding that an RdRp protein different from SDE1/SGS2 is involved in mediating resistance against viruses in tobacco (Xie et al., 2001). It appears that virus-induced gene silencing in plants is more similar to RNAi in *C. elegans*, while there are independent co-suppression mechanisms initiated by extra copies of the transgenes both in plants and animals, as we proposed earlier (Grishok et al., 2000; Figure 2-4, p.24; Grishok et al., 2001; Figure 4-6, p.64). Consistent with this model, different members of multigene families can function in different branches of the silencing pathways.

The involvement of the RNA polymerase in RNAi in *C. elegans* could explain the remarkably robust and long lasting interference response. Future analysis of the molecular mechanism of RNAi amplification might reveal the nature of the heritable extragenic factor described in Chapter II.

One study has linked components of nonsense-mediated decay in *C.elegans* to the persistence of the interference effect (Domeier et al., 2000). Nonsense-mediated decay (NMD) is a mechanism conserved from yeast to vertebrates wherein messages with premature stop codons are degraded. Domeier and colleagues (2000) have reported that

several, but not all, of the *C. elegans* NMD mutant strains recover more rapidly from RNAi. Since all of the strains are initially sensitive to RNAi this finding suggests that some components of the NMD pathway play a role in the maintenance of RNAi.

It seems that new factors involved in RNAi and other silencing mechanisms are now being reported at a steadily increasing pace. In *C. elegans* there is much more work to do. The screens performed to date have clearly not identified all the components involved in RNAi in *C.elegans*. Indeed, gene products important for fertility or viability were excluded by the design of the original screens for *rde* mutants. New genetic screens as well as protein interaction screens and biochemical studies will be necessary to identify all the factors that mediate RNAi.

RNAi and development

Considering the sophisticated mechanism and apparently ancient origin of RNAi one would expect to find related mechanisms employed in natural developmental or cellular functions. However, initially identified mutants deficient in RNAi did not exhibit gross developmental abnormalities (Tabara et al., 1999 a). Mutants specifically defective for RNAi targeting germline genes often are sterile. The mutator class of *rde* mutants shows temperature-dependent sterility and mobilization of different transposons in the germline of *C. elegans* (Tabara et al., 1999 a ; Ketting et al., 1999). These defects might arise as a consequence of deregulation of the natural silencing process in the germline affecting both endogenous genes and transposable elements. The molecular mechanism

of this regulation and the point of its intersection with the RNAi pathway are yet to be found. Another germline-specific RNAi-deficient mutant, *ego-1*, has multiple abnormalities in germline development, including defects in gametogenesis, proliferation and meiosis (Smardon et al., 2000). Mutations in the *C. elegans* homolog of *Drosophila* *Dicer*, *dcr-1*, implicated in RNAi in both organisms lead to a sterile phenotype with abnormal oogenesis and fertilization (Knight and Bass, 2001). Germline phenotypes of *ego-1* and *dcr-1* might have a similar origin arising from the disruption of an RNAi-like mechanism regulating the timing of germline development.

A precedent for the involvement of an RNAi-like mechanism in the control of the germline gene *Stellate* exists in *Drosophila* (Aravin et al., 2001). A high level of *Stellate* expression in male germline leads to sterility. The downregulation of *Stellate* expression in males is maintained by a dsRNA homologous to *Stellate* and produced from a degenerate, highly repeated *Stellate* paralog, *Suppressor of Stellate*, located on the Y chromosome. Appearance of a small ~25nt RNA species is correlated with the silencing of *Stellate*. Silencing is dependent on the *Drosophila* RDE-1 homolog, Aubergine, and the putative ATP-dependent RNA helicase, Spindle E (Aravin et al., 2001).

Another parallel between the mechanism of RNAi and the production of small regulatory RNAs playing role in the development of *C. elegans* has been discovered recently and described in Chapter IV (Grishok et al., 2001). Briefly, it has been shown that RNAi-like mechanism involving DCR-1 operates in the production and function of the *let-7* and *lin-4* small temporal RNAs regulating developmental timing in *C. elegans*. A recent study by Zamore and co-workers implicated *Drosophila* Dicer and its

mammalian homolog in the processing of *let-7* precursor (Hutvágner et al., 2001).

Multiple developmental phenotypes associated with the downregulation of *dcr-1* as well as *alg-1* and *alg-2*, including embryonic defects, suggest that more genes might be regulated by small RNAs in *C. elegans*. Also, the similarity of developmental abnormalities in *ago1* and *caf* mutants in *Arabidopsis* suggest that a pathway including small regulatory RNAs may also exist in plants. It seems that much of the regulatory role of RNA is yet to be discovered both in plant and animal kingdoms.

RNAi and immunity

Both *rde-1* and *rde-4* null mutants are completely deficient for RNAi and apparently have no other phenotypes (Tabara et al., 1999 a). If animals lacking these genes are wild type in appearance and development, what then might be the physiological relevance of these genes? The most attractive model is that these genes (and RNAi in general) may be part of an immunity mechanism. In the wild, *C. elegans* may encounter dsRNA viruses that are absent under laboratory culture conditions; *rde-1* and *rde-4* could confer immunity against such pathogens.

The role of PTGS as a sequence-specific antiviral response is an area of extensive investigation in plant pathology (reviewed in Baulcombe, 1999; Vance and Vaucheret, 2001; Voinnet, 2001). There are numerous DNA and RNA viruses inducing host PTGS mechanisms. However, plant PTGS does not provide absolute protection from viruses as they evolved the means of suppressing PTGS. Several viral suppressors of PTGS in

plants have been characterized so far. Helper component protease (HC-Pro) was shown to reverse PTGS induced by viruses and transgenes in different plant species (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998) and to interfere with the maintenance step in PTGS and the accumulation of siRNA (Llave et al., 2000). Another viral protein, 2b, prevents the initiation of gene silencing (Brigneti et al., 1998). 2b was shown to localize to the nucleus and to possibly affect transcription of plant genes important for the initiation of PTGS response (Lucy et al., 2000). Interestingly, there is continuing competition between the plant defense system and viruses as 2b protein affecting host PTGS system represents a target for yet another independent host resistance mechanism (Li et al., 1999). The spreading of plant PTGS via a sequence-specific silencing signal is also targeted by viruses. Particularly, the viral movement protein has been shown recently to prevent the spread of silencing signal (Voinnet et al., 2000).

The idea that RNAi might represent a form of acquired sequence specific immunity in animals as well is very attractive. *C. elegans* adults live only a few days and, thus, immunity developed in an individual animal may not be of much benefit. However, if an infected animal could pass immunity to its offspring as can occur with RNAi, this would likely confer a very considerable selective benefit. It seems likely that co-suppression, transposon silencing and transgene silencing may all represent related forms of sequence-directed immunity that share a mechanism but have evolved to respond to independent triggers. It will be interesting in the future to identify potential

pathogens of *C. elegans* and challenge *rde* mutant strains to ask if they are compromised

in the resistance to such pathogens.

CHAPTER VII

CONCLUSIONS AND PERSPECTIVE

The natural role of RNA molecules in the regulation of various aspects of gene expression in development was predicted more than two decades ago (Dickson and Robertson, 1976; Davidson and Britten, 1979). However, until recently, natural regulatory RNAs were considered more of a rare curiosity than a rule. Researchers were busy creating antisense RNAs with regulatory potential by their own design. Ironically, the discovery of RNA interference (Fire et al., 1998), which originated from the antisense technology, lead back to the appreciation of the natural forms of RNA regulation (Tabara et al., 1999 a ; Ketting et al., 1999; Grishok et al., 2001; Hutvágner et al., 2001; Aravin et al., 2001). At the beginning, the view of the RNAi phenomenon was that of an accidentally found magic wand which could be used but whose mechanism was a complete mystery. With the efforts of many scientists, the mystery of RNAi began to unfold and the phenomenon grew bigger, from the curious form of gene regulation in worms to the ancient gene silencing mechanism common to plants and animals.

No doubt, discovery of the signature silencing RNA mediators, siRNAs, associated with co-suppression in plants (Hamilton and Baulcombe, 1999) and RNAi in *Drosophila* (Zamore et al., 2000; Hammond et al., 2000; Elbashir et al., 2001a), *C. elegans* (Parrish et al., 2000; Grishok et al., submitted) and mammals (Elbashir et al., 2001 b; Caplen et al., 2001), strongly argues for the similarity of the mechanisms. The main point of the most current reviews on the topic remains the similarity of PTGS

silencing mechanisms and the conserved nature of the genes involved, namely the RdRp and AGO1/ RDE-1/QDE-2 families (Cogoni and Macino, 2000; Carthew, 2001; Matzke et al., 2001).

Although the uncovered common ground of RNA-silencing phenomena is exciting enough, I think that the diversity within the field of RNA-regulation is much more interesting. Clearly, solving the mystery of RNAi is not equal to finding the key biochemical component degrading target mRNA. The complexity of interconnection between different pathways, both in development and in sequence-specific immunity, is very far from being solved and will develop into a large field of scientific investigation.

The work I described here was done in the early days of RNAi research and probably provides more questions than answers. Genetic analysis of the RNAi pathway presented in Chapter II predicted the upstream role of the *rde-1* and *rde-4* genes as well as the production of a sequence-specific RNAi intermediate dependent on the function of both genes. The exact biochemical nature of this heritable factor is still a mystery despite the identification of the role of siRNAs in RNAi. Since siRNAs are produced in *rde-1* mutant worms (Parrish and Fire, in press; Grishok and Mello, unpublished), but the functional heritable RNAi factor is not, it remains to be determined whether RDE-1 directly binds to siRNAs forming the functional complex, facilitates modifications of the siRNA duplex, or recruits factors necessary for siRNA unwinding.

As a result of our RNAi inheritance studies and the progress of RNAi research at that time we proposed the branched model for the existence of diverse initiators of sequence-specific silencing pathways in *C. elegans* (Figure 2-4, p.24) and predicted that

different members of RDE-1 family of proteins will determine the specificity of diverse pathways. While potential RDE-1 homologs playing role in the initiation of co-suppression and transcriptional transgene silencing still remain to be found, we were able to include additional branch in our model with the identification of ALG-1 and ALG-2 as the partners of stRNAs regulating development of *C. elegans* (Figure 4-6, p.64). Again, precise biochemical role of ALG-1 and ALG-2 is still unknown as is as the identity of their additional RNA partners playing roles in development. The diverse and severe developmental phenotypes resulting from *alg-1*, *alg-2* and *dcr-1* downregulation in *C. elegans* indicate that we have only touched the tip of the huge hidden iceberg of RNA-regulated pathways. There is little doubt that similar pathways exist in other systems as *let-7* stRNA is widely expressed in animals (Pasquinelli et al., 2000) and *dcr-1* is involved in its processing both in *Drosophila* and mammals (Hutvágner et al., 2001). Although *let-7* has not been found in plants, future studies are likely to identify numerous natural targets for Dicer-like nucleases in this kingdom as well.

Finally, our biochemical study described in Chapter V revealed previously unpredicted requirements for accumulation of siRNAs, indicating the possibility of asymmetric amplification of siRNAs and selection of the species complementary to the target. This study complements recent identification of an additional RdRp encoding gene as a necessary component of RNAi pathway (Conte et al., submitted) and suggests that it functions downstream in the RNAi pathway. However, since both sense and antisense siRNAs were usually detected during RNAi and co-suppression in other systems, it remains to be determined if asymmetric target-dependent accumulation of

siRNAs is a general feature of PTGS in all organisms. One way or another, the parallel between the clonal selection of antibody-producing cells in vertebrate immunity and selection of siRNAs in more ancient form of sequence-specific immunity in *C. elegans* is very intriguing and deserves further investigation.

Aside from the value of the RNAi field for the increase of basic knowledge of living systems and diversity of natural forms of gene regulation, RNAi has always been primarily viewed as a tool for gene inactivation. RNAi technology has been successfully applied recently to the mammalian tissue culture (Elbashir et al., 2001 b; Caplen et al., 2001) which opens new perspectives for both basic research and possible drug development, as well as gene therapy applications. This new advance would have not been possible without the investigation of RNAi mechanism itself and identification of siRNAs as intermediates. Thus, basic research of RNAi and its practical application are intertwined. It is already possible to predict development of a new strategies for gene inactivation based on the recent progress in the field of RNA regulation to which the study presented here belongs, and I feel happy to be able to work in this field.

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