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# On the Role of RNA Amplification in dsRNA-Triggered Gene Silencing

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## Summary

**We have investigated the role of trigger RNA amplification during RNA interference (RNAi) in *Caenorhabditis elegans*. Analysis of small interfering RNAs (siRNAs) produced during RNAi in *C. elegans* revealed a substantial fraction that cannot derive directly from input dsRNA. Instead, a population of siRNAs (termed secondary siRNAs) appeared to derive from the action of a cellular RNA-directed RNA polymerase (RdRP) on mRNAs that are being targeted by the RNAi mechanism. The distribution of secondary siRNAs exhibited a distinct polarity (5′→3′ on the antisense strand), suggesting a cyclic amplification process in which RdRP is primed by existing siRNAs. This amplification mechanism substantially augments the potency of RNAi-based surveillance, while ensuring that the RNAi machinery will focus on expressed mRNAs.**

## Introduction

RNA-mediated interference (RNAi) is a conserved gene silencing mechanism that recognizes double-stranded RNA (dsRNA) as a signal to trigger the sequence-specific degradation of homologous mRNA (see Sharp, 2001 for a recent review). Analyses of RNAi and related processes in diverse systems have uncovered several surprising properties, including the double-stranded character of the trigger RNA and a catalytic aspect of the interference reaction. Indeed, a few molecules of dsRNA are sufficient in *C. elegans* or *Drosophila* cells to trigger the decay of a much larger population of target mRNAs (Fire et al., 1998; Kennerdell and Carthew, 1998).

Several features of the RNAi mechanism have been proposed to contribute to the remarkable potency of the reaction. Some degree of amplification is likely to derive from cleavage of the dsRNA trigger into short pieces of 21–25 nt (called siRNAs) by the RNaseIII-like nuclease DICER (e.g., Zamore et al., 2000; Bernstein et al., 2001). For the most commonly used dsRNA triggers (500–1000 bp), this would result in a 20- to 40-fold increase in the molar ratio of trigger to target. A simple (single-use) utilization of the siRNAs would be sufficient to explain the molar efficiency of RNAi in extracts of *Drosophila*, but would be insufficient to account for in vivo potency in *C. elegans*. A multiround mechanism (use of a single siRNA for hundreds or thousands of rounds of target degradation) would be much more efficient.

An additional contribution to the potency of RNA-triggered gene silencing has been proposed to involve physical amplification of an aberrant RNA population through an RNA-directed RNA polymerase (RdRP) activity (Dougherty and Parks, 1995). By producing a large number of copies of a triggering RNA, an RdRP activity might dramatically increase the effectiveness of RNAi. The possibility of RdRP involvement in posttranscriptional gene silencing has been supported by the isolation of an endogenous RdRP activity from tomato (Schiebel et al., 1993a, 1993b, 1998), followed by subsequent demonstrations that factors with protein sequence homology to this RdRP were required for efficient silencing in fungal, nematode, and plant systems (Cogoni and Macino, 1999; Smardon et al., 2000; Dalmay et al., 2000; Mourrain et al., 2000).

A number of apparent constraints on the roles of RdRP activity in RNAi are suggested by experimental observations. Embryonic extracts from *Drosophila* with no measurable RdRP activity can carry out a complete RNAi reaction (Zamore et al., 2000; P. Zamore, personal communication). This, combined with the absence in available *Drosophila* or mammalian genomic sequences of a clear homolog of the RdRP-like genes implicated in other systems, argues that an RNAi reaction can proceed without RdRP. It should be noted, however, that formation of unstable (transient) copy RNAs during the in vitro reaction might be difficult to detect, and that additional enzymes (such as RNA polymerase II and retroviral type reverse transcriptases) are capable of polymerizing RNA in response to certain RNA templates (e.g., Diener, 1991; Filipovska and Konarska, 2000; Modahl et al., 2000). A more limited constraint on possible roles for RdRP in RNAi comes from experiments in which the two trigger strands have been modified differentially prior to injection into *C. elegans* or *Drosophila* (Parrish et al., 2000; Yang et al., 2000). These experiments showed a more stringent requirement for structure and sequence of the antisense strand of the original trigger, as compared to the sense strand. These "strand-preference" experiments do not rule out a role for RdRP in the interference reaction, but do severely limit models in which the RdRP carries out a multiround replication of a double-stranded trigger (e.g., Waterhouse et al., 1998) to produce exponential amplification: this type of exponential amplification would result in loss of memory of the difference between the original two strands and would thus be incompatible with the observed effects of strand-specific modification.

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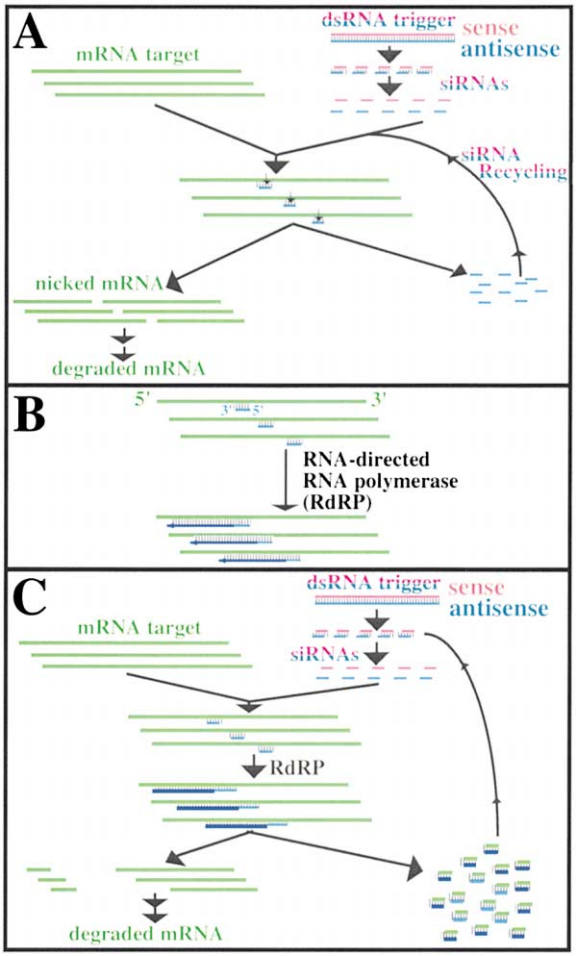



Figure 1. Could siRNA-Primed Copying of Target RNAs by an RNA-Directed RNA Polymerase Contribute to RNAi?

(A) A current model of the nucleic acid alterations during RNA interference based primarily on in vitro studies of RNAi in *Drosophila* extracts (e.g., Zamore et al., 2000; Hammond et al., 2001; Bernstein et al., 2001; Elbashir et al., 2001). After cleavage of the dsRNA trigger into short siRNA segments, the individual antisense siRNAs pair with complementary mRNAs, with degradation of mRNA and (eventual) recycling of siRNAs.

(B) shows that at the heart of the working model is an intermediate with the antisense strand of an siRNA hybridized to an mRNA target. Since the siRNAs possess a 3′-terminal hydroxyl group, the resulting intermediate might function as a template for elongation by an RdRP activity.

(C) shows a possible consequence of the reactions proposed in (A) and (B), with the sequential activity of RdRP and a dsRNA-specific nuclease (e.g., DICER) leading to a target-dependent amplification of the siRNA population.

Of the numerous roles proposed for RdRP during gene silencing, we were most intrigued by the possibility (Figure 1) that antisense siRNAs that have annealed to a ssRNA target might be elongated by RdRP to produce longer stretches of dsRNA (Sijen and Kooter, 2000). This model is particularly attractive in that (1) siRNAs are known to have a 3′ hydroxyl group (Elbashir et al., 2001), which would be poised for elongation by an RNA polymerase, (2) cleavage of the RdRP-elongated regions of dsRNA to produce short siRNAs would result in a net amplification of the initial population of siRNAs at the expense of target transcripts, and (3) this mode of amplification utilizes the two input strands of the RNA trigger differentially; thus, there is no inconsistency with earlier results which had shown more stringent chemical requirements for the antisense strand of the initial trigger RNA (Parrish et al., 2000; Yang et al., 2000).

The model in Figure 1C leads to a number of testable predictions; in particular, we would expect to observe a population of secondary siRNAs after RdRP-mediated synthesis of duplex RNAs followed by cleavage by RNaseIII/DICER activity. These secondary triggers would be derived primarily from sequences upstream of the initial trigger region on the target mRNA and would be expected to induce a secondary RNA interference reaction directed to any homologous target RNA.

In this paper, we demonstrate the production and biological activity of RdRP-dependent secondary triggers during RNA interference in *C. elegans*.

## Results

### Biochemical Evidence for Secondary siRNAs

We first sought to demonstrate the existence of secondary siRNAs through direct analysis of RNA populations. Although the appearance of short RNAs in the 21–25 nt range has universally been observed in studies of RNA-triggered gene silencing, the abundance of such RNAs varies considerably between systems. In particular, siRNAs observed during RNAi are apparently much less abundant in *C. elegans* than in plants and *Drosophila* (e.g., Hamilton and Baulcombe, 1999; Parrish et al., 2000; Yang et al., 2000). In order to characterize populations of siRNA from *C. elegans* in detail, we used RNase protection assays. $^{32}$P labeled ssRNA molecules (used as probes) were hybridized to denatured cellular RNA, and the resulting material treated with ssRNA-specific ribonucleases to degrade any unhybridized probe. We used single-stranded probes from the sense strand in order to detect the siRNA signal while avoiding a background due to breakdown products of the cellular mRNA target. To generate a large mass of *C. elegans* actively performing RNAi, we used a procedure in which animals are grown on bacteria engineered to express high levels of a specific dsRNA (Timmons and Fire, 1998; Fraser et al., 2000).

Each RNase-protection experiment involves two segments: a dsRNA trigger produced in bacteria and a probe RNA used to detect siRNA molecules. Figure 2 shows results for two target genes: the muscle-specific gene *unc-22* and the germline-specific gene *pos-1*. In each case, the strongest siRNA signals were obtained when the trigger and probe sequences corresponded. This population of siRNAs would be expected from models in which a dsRNA-specific nuclease cleaves the original dsRNA trigger to produce siRNA segments. In addition to the trigger-coincident siRNAs, we also detected populations of small antisense RNAs that correspond to regions of the target gene outside the original trigger. We tentatively refer to these as secondary siRNAs. The secondary siRNAs were generally detected at levels substantially below those of the trigger-coincident siRNAs, but were reproducibly observed using several

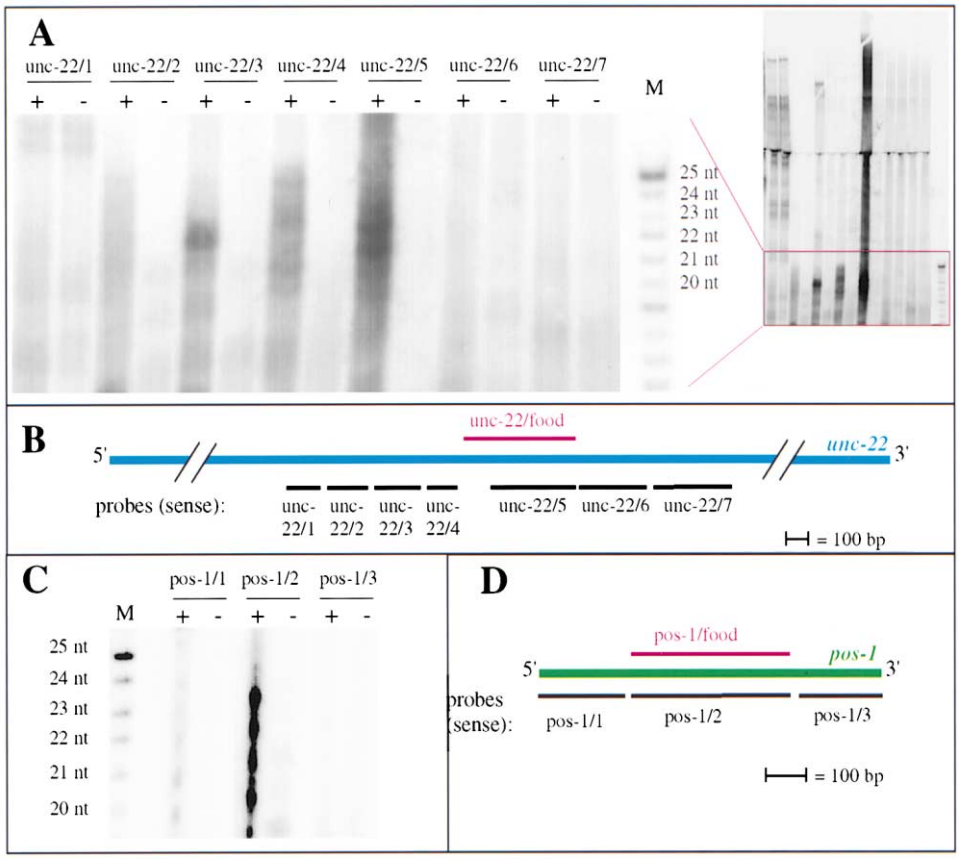



Figure 2. Biochemical Detection of Secondary siRNAs

Analysis of small RNAs from wild-type animals grown on *E. coli* expressing dsRNA segments of *unc-22* or *pos-1*. Total RNA was isolated and RNase protection assays were performed using various *unc-22* or *pos-1* specific probes (all of sense polarity).

(A) Products of RNase protection assay (right: protected fragments of probe resolved on polyacrylamide-urea gel; left: detail of 16–30 nt portion of gel). Feeding on *unc-22* dsRNA yielded siRNAs from the dsRNA segment comprising the food, but also produced siRNAs mapping upstream of this region. Lanes designated "+": RNA from animals fed *unc-22* dsRNA. To determine levels of probe-derived background, negative controls ("−") were carried out by performing RNase protections with yeast tRNA as input RNA. A similar background in the siRNA size range was observed in RNase protection assays on RNA from animals grown on induced bacteria containing the feeding vector L4440 with no insert (data not shown). RNase protection assays have also been carried out using RNA from IPTG-induced *E. coli* producing *unc-22* dsRNA; these showed some level of probe protection but no protected fragments in the siRNA size range (data not shown). Labels above the lanes indicate probes. "M": $^{32}$P-labeled 25 nt RNA oligonucleotide marker.

(B) Map of *unc-22* mRNA with positions of probes and bacterially produced dsRNA.

(C) Secondary siRNAs are also produced upon feeding with *E. coli* producing *pos-1* dsRNA. Since *pos-1* is a germline-specific gene, RNA was isolated from egg preparations. "+": *C. elegans* populations fed with *E. coli* producing *pos-1* dsRNA; "−": equivalent RNA preparations from animals grown on *E. coli* containing the empty L4440 vector.

(D) Map of *pos-1* mRNA with positions of probes and bacterially produced dsRNA.

different combinations of trigger and probe sequences. Although the detection limits of the system preclude a definitive measurement of siRNA levels for each trigger/probe combination, two points emerge rather clearly from the analysis. First, occurrence of a detectable secondary antisense population was limited to cases in which the probe sequence was upstream (closer to the 5′ end of the target mRNA) as compared with the trigger sequence. Second, the abundance of secondary siRNA molecules appeared to decrease as a function of distance from the primary trigger.

## Transitive RNAi

Secondary siRNAs might be expected to act as functional RNAi triggers, targeting any homologous mRNA sequences for degradation. To test this hypothesis, it is necessary to distinguish between targeting by the initial dsRNA trigger and by the secondary siRNAs. This is most conveniently carried out by means of a "transitive RNAi" assay. Essentially, such an assay entails a cell with two populations of target RNA: the first population (primary target) has a segment which matches the dsRNA trigger; the second population has no homology to the initial dsRNA trigger, but has a segment which is identical to the primary target.

Figure 3 shows an example of transitive RNAi in which both primary and secondary target RNAs are transgene-derived transcripts carrying *gfp*. The primary target in this experiment encodes a nuclear-targeted GFP-LACZ fusion protein (NLS-GFP-LACZ), while the secondary

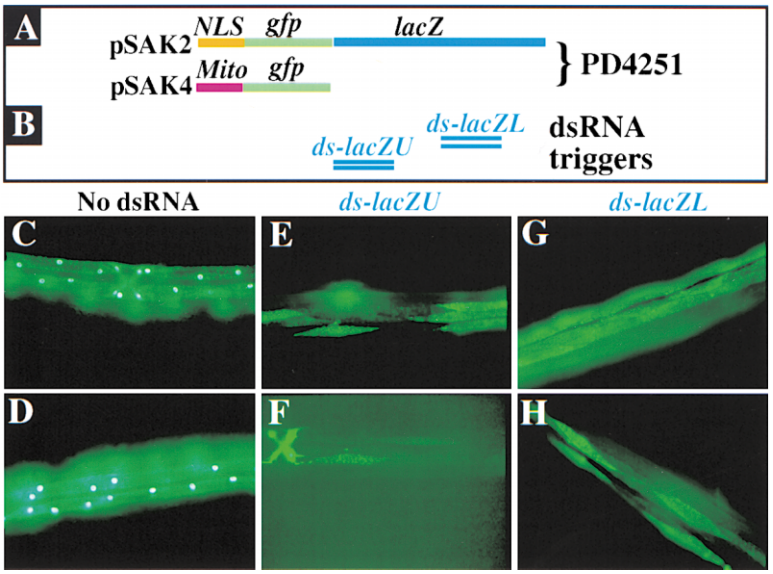



Figure 3. Assays for Transitive RNAi Using Distinct *gfp* Transgenes

The transgenic line used for this assay (PD4251) carries two different *gfp* reporter constructs (A). pSAK2 produces nuclear-localized GFP fused at the C terminus to additional sequences encoding *E. coli* β-galactosidase (*lacZ*). pSAK4 produces mitochondrially localized GFP with no additional sequences at the C terminus. PD4251 animals express both nuclear and mitochondrial GFP forms in all cells of the body musculature (Fire et al., 1998). Young adult progeny of adult animals injected with specific dsRNA segments (B) were examined to determine the level of interference with nuclear- and mitochondrial-targeted *gfp*s.

(C and D) Mock injected control animals with both GFP isoforms expressed in each muscle cell.

(E and F) Progeny of animals injected with ds-*lacZU.* This injection produced a strong transitive RNAi effect, interfering in a majority of cells not only with the nuclear targeted *gfp*::*lacZ* transgene, but also with the mitochondrial-targeted *gfp*. (A bright "X" shape in [F] shows vulval muscles fortuitously included in the photo; these cells are generally nonresponsive to parentally injected dsRNA; Fire et al., 1998)

(G and H) Progeny of animals injected with ds-*lacZL*. This segment had only a modest effect on the expression of mitochondrially targeted *gfp*, so that the majority of cells continue to produce GFP in mitochondria but not nuclei. (F) and (H) are representative of the strongest transitive RNAi response in each population, while (E) and (G) are representative of the weakest effect. As negative controls, PD4251 animals injected with a variety of unrelated dsRNA segments (*unc-22*A, *unc-22*B, *lin-26*IVS3) showed no evident decrease in either nuclear or mitochondrial GFP. Animals injected with *gfp* dsRNA show near-complete (98%) loss of both nuclear and mitochondrial GFP (Fire et al., 1998).

target encodes a mitochondrially targeted GFP (MtGFP) which has no sequences from *lacZ* (both transgene mRNAs are driven by the *myo-3* promoter). As a control, animals carrying only one of the two transgene constructs show the expected effects: both GFPs are dramatically reduced in progeny of animals injected with dsRNA corresponding to GFP, while only the NLS-GFP-LACZ construct is affected by dsRNAs corresponding to *lacZ* (data not shown). A line carrying both transgene constructs produces both nuclear LACZ-GFP and mitochondrial GFP (PD4251; Figures 3C and 3D). Injection of dsRNA segments from *lacZ* into the line carrying both transgenes produces a transitive effect: reduction of both nuclear GFP-*lacZ* and mitochondrial GFP. Of two different *lacZ* segments tested, a trigger that was located just 3′ to the *gfp*::*lacZ* junction (*ds-lacZU*) was most potent in the transitive RNAi assay, producing reduction of mitochondrial GFP to background in 60% of targeted cells, while a dsRNA trigger located further downstream (*ds-lacZL*) produced a more modest effect (reduction of GFP in 28% of cells) (Figure 3 and data not shown).

A second example of transitive RNAi is presented in Figure 4. In this case, the primary target is an *unc-22*::*gfp* fusion transgene (Figure 4C), while the secondary target is an endogenous gene (*unc-22*; Brenner, 1974; Moerman et al., 1988). Injection of dsRNA corresponding to *gfp* into wild-type animals (no transgene) produced no phenotype; injection of ds*gfp* RNA into animals carrying a transgene expressing GFP alone produced a decrease in GFP but no *unc-22* phenotype. Injection of ds*gfp* RNA into animals expressing the *unc-22*::*gfp* transgene produced the twitching phenotype that is characteristic of loss of *unc-22* expression (e.g., *ds-gfpA*; Figure 4C).

To test whether transitive RNAi could proceed with endogenous genes as targets, we carried out the two experiments shown in Figure 5. In-frame deletion alleles of *unc-22* and *unc-52* provide a useful genetic tool: these alleles each produce proteins that lose a fraction of the coding region (658 amino acids for *unc-22*(*st528*); 150 amino acids for *unc-52*(*ra511*)) but retain full wild-type function (Kiff et al., 1988; Fire et al., 1991; Rogalski et al., 1993; Mullen et al., 1999). As expected, dsRNAs corresponding to the deleted regions produced strong gene-specific RNAi effects in wild-type animals, but no effect in animals homozygous for the corresponding deletion alleles. The test for transitive RNAi in each case consists of introducing these trigger RNAs into heterozygous animals carrying both wild-type and mutant alleles. In each case, we found a strong transitive RNAi effect: heterozygotes exhibited interference with both deletion and wild-type alleles. These experiments demonstrate that transitive RNAi is not limited to transgene targets, but can also target physiological expression of cellular genes.

### Structural Requirements for Triggering of Transitive RNAi

Certain features of transitive RNAi are illuminated by the requirements for structure and dose of the primary trigger. A prediction of the model in Figure 1C is that the effect should exhibit a defined polarity, with interference depending on the order of the two segments in the primary target mRNA. This was the case, as shown by the lack of sensitivity to transitive RNA when the order of segments in the transgene construct was reversed (Figure 4E).

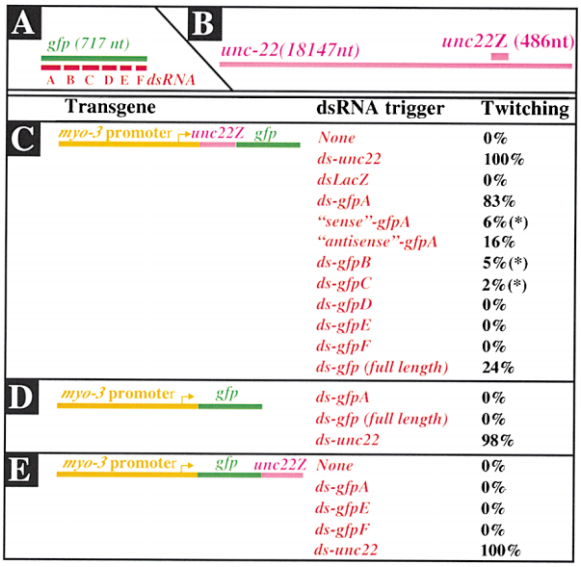


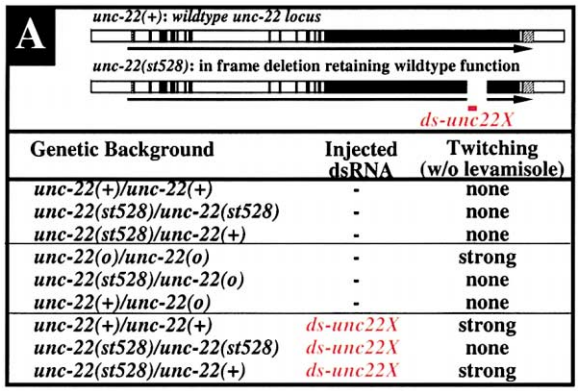

Figure 4. Assays for Transitive RNAi Using a Chimeric *unc-22*::*gfp* Transgene

Transgenic lines used for these assays carry the *C. elegans myo-3* promoter driving the indicated combinations of the *gfp* coding region (717 nt) and a segment within the *unc-22* gene (*unc-22z*; 486 nt). Following propagation of clonal transgenic lines for several generations, transitive RNAi was assayed by injecting adults with a variety of dsRNAs. After ~3.5 days, injected animals and postinjection progeny (>50 animals derived from 5–20 injected parents) were scored for twitching in levamisole. Assays marked with an "*" showed twitching predominantly in the injected adults; the remaining positive assays showed twitching in both injected adults and progeny, while negative assays showed twitching in neither injected adults nor progeny.

(A and B) Segments used in this analysis. mRNA structures are shown; the *gfp* coding region is interrupted in each DNA construct by three 51 nt introns. The *gfp*-derived dsRNAs (Parrish et al., 2000) were each functional in primary RNAi, as assayed by reduction of GFP in injected adults and progeny.

(C) A twitching phenotype was observed when the injected dsRNA corresponded to sequences from *gfp* downstream of the *unc-22*::*gfp* junction. Note that *ds-gfpA* produced the most effective twitching response, presumably by producing the highest molar concentration of siRNAs immediately downstream of the *unc-22*::*gfp* junction.

(D and E) Transitive RNA was specific to the structure and arrangement of the initial dsRNA trigger and transgene.



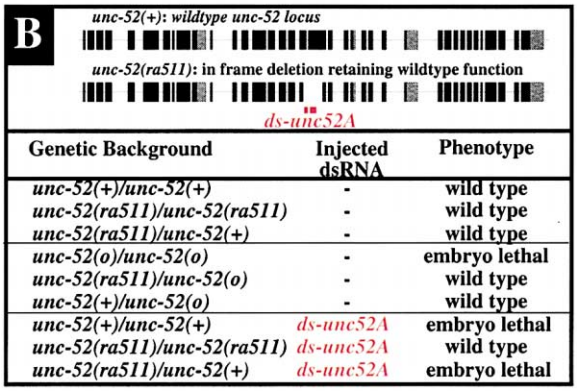




Figure 5. Transitive RNAi Can Operate on Native Chromosomal Genes

(A) Maps of wild-type *unc-22* and an in-frame deletion (*st528*) that retains wild-type function (Moerman et al., 1988; Benian et al., 1993; Kiff et al., 1988; black, exons; white, introns). *unc-22* null mutants exhibit a strong twitching behavior in the absence of levamisole (we used *unc-22*(*e66*) as a canonical null for this analysis; Brenner, 1974). The strong twitching phenotype is not seen with animals that have a single functional dose of the wild-type or *st528* allele. Following injection of *ds-unc22X* RNA, twitching without levamisole was observed in 100% of *unc-22*(+) animals, 0% of *unc-22*(*st528*)/*unc-22*(*st528*) animals, and 60% of *unc-22*(*st528*)/+ animals.

(B) Maps of wild-type *unc-52* and a deletion allele that removes nonessential sequences (*unc-52*(*ra511*); Mullen et al., 1999; black, exons; white, introns; hatched, alternatively spliced exons). The null phenotype for *unc-52* is a zygotic-effect embryonic lethality with paralysis (Williams and Waterston, 1994; Rogalski et al., 1993). A chromosomal deficiency (mnT11; Herman et al., 1982) was used to definitively determine *unc-52*(+)/*unc-52*(o) and *unc-52*(*ra511*)/*unc-52*(o) phenotypes. Animals that have a single functional dose of the wild-type or *ra511* allele show no lethal or visible phenotype. Following injection of *ds-unc52A* RNA, embryonic lethality with paralysis was observed in 100% of *unc-52*(+) animals, 0% of *unc-52*(*ra511*)/*unc-52*(*ra511*) animals, and 100% of *unc-52*(*ra511*)/+ animals.

Interference showed a dose response to the concentration of primary trigger, with a modest interference response observed at doses as low as $1.5 \times 10^6$ molecules per injected parent (data not shown). Given the expression levels of *unc-22* (Fire et al., 1991), and assuming equal dispersion of trigger RNA among the cells of the affected progeny, this corresponds to a stoichiometry on the order of ~100 molecules of trigger RNA for ~5000 molecules of target mRNA in each muscle cell of the affected animals. Triggering also appeared to be structure-specific: although some interference was observed with sense or antisense RNA preparations alone, there was a dramatic stimulation upon mixing the two preparations. As with previous studies (e.g., Fire et al., 1998), it was not straightforward to distinguish whether residual activity of our ssRNA preparations was due to low levels of dsRNA contamination even after purification. In any case, these data indicate that the initial triggering reaction is either fully dependent on, or greatly stimulated by, delivery of a trigger RNA with double-stranded character.

Not all potential trigger RNAs were capable of producing transitive interference. For each target RNA, we observed a graded effect as a function of distance between primary and secondary target sequences. The precise relationship between distance and effectiveness appeared to depend on the details of the experiment (compare positional dependence in Figures 3E–3H with that

in Figure 4C), but in each case, the effect decreased with increasing distance between the segments.

### The Cellular RdRP Homolog *rrf-1* Is Required in Somatic Cells for Production of Secondary siRNA Triggers and for Transitive RNAi

Genetic screens for factors responsible for RNA-triggered silencing phenomena in diverse organisms have identified (among many other components) factors with substantial homology to a cellular RdRP isolated from viroid-infected tomato leaves (Schiebel et al., 1998; Cogoni and Macino, 1999; Smardon et al., 2000; Dalmay et al., 2000; Mourrain et al., 2000). *C. elegans* has four members of this gene family (*ego-1*, *rrf-1*, *rrf-2*, and *rrf-3*) (Smardon et al., 2000). Two of these genes, *ego-1* and *rrf-1*, are closely linked (0.9 kb apart in tandem orientation), while *rrf-2* and *rrf-3* map to distinct loci. *ego-1* is an essential gene required for fertility: adult *ego-1* homozygotes can only be derived as progeny of heterozygous mothers, thus it is not possible to carry out RNAi assays in the complete absence of maternal and zygotic *ego-1* product (Smardon et al., 2000). Despite this limitation, Smardon et al. (2000) were able to demonstrate a requirement for *ego-1* in producing an efficient RNAi response in the adult germline; no role for *ego-1* during RNAi in somatic tissue has been detected.

To extend our understanding of the RdRP gene family in *C. elegans*, we produced deletion alleles of the *rrf-1*, *rrf-2*, and *rrf-3* genes through PCR-based screening of a chemical deletion library (Figure 6A; protocol from Jansen et al., 1997). We obtained single deletion alleles for each *rrf-* gene: *rrf-1*(*pk1417*) deletes 401 aa, including the majority of the residues conserved in the RdRP family; *rrf-2*(*pk2040*) deletes the presumed promoter region and the first five exons; *rrf-3*(*pk1426*) produces an out-of-frame truncation after the fourth exon, effectively removing most or all of the RdRP domain. These three deletions would be predicted to behave as genetic nulls. Each of the three *rrf* deletions was viable and fertile; none showed any obvious morphological or growth defects (the *rrf-3*(*pk1426*) strain produces a slightly higher incidence of male progeny than wild-type; the source of this effect has not been characterized). While this work was being carried out, an additional transposon (Tc1)-induced allele of *rrf-3* was obtained (F.S. and R.P., unpublished data; protocol from Zwaal et al., 1993). Although the majority of our analysis was carried out with the three deletion alleles, the transitive RNAi properties of *rrf-3* (see below) were confirmed with the Tc1 allele.

As shown in Figure 6B, the *rrf-2* and *rrf-3* deletion strains were sensitive to RNA interference in all tissues (soma and germline) and for all assays performed (both standard RNAi assays and transitive RNAi assays). For *rrf-2*(*pk2040*), we observed no differences from wild-type in any of the RNAi assays. These results indicate either a redundant role for RRF-2 in RNAi or (alternatively) a role in a distinct cellular process. Interestingly, the *rrf-3* deletion and Tc1 insertion strains both showed reproducible increases in sensitivity to RNAi when compared to wild-type animals. This increase in sensitivity is evident for several different target genes and for both standard and transitive RNAi assays (Figure 6B and data not shown). While it is interesting to speculate on possible negative roles for *rrf-3* in the RNAi response (e.g., loss of *rrf-3* function might release specific RdRP cofactors for use in RNAi), the nature of the effect will require further experimental analysis; the major conclusion that we can draw at this point is that RRF-3 is nonessential for the RNAi responses tested.

By contrast to the RNAi sensitivity observed in *rrf-2* and *rrf-3* mutants, we observed complete resistance of the *rrf-1* deletion allele to certain RNAi triggers. As shown in Figure 6B, there was a strong correlation between site (tissue) of function for the target gene and the efficacy of interference: interference for genes expressed in somatic tissue was lost in *rrf-1* deletion mutants, while interference was retained for genes expressed in the germline. Consistent with our analysis of *rrf-1(pk1426)*, D. Conte and C. Mello (personal communication) have observed loss of RNAi in soma but not germline tissue in an independently isolated set of *rrf-1* missense mutations.

We used two assays to address the production of secondary siRNAs in the RdRP mutants. These assays were carried out for somatic targets, since infertility of *ego-1* mutants (likely to affect germline RdRP; Smardon et al., 2000) would confound our biochemical and genetic assays. We first transformed each *rrf* deletion mutation with a DNA construct (*myo-3*::*unc-22Z*::*gfp*, as shown in Figure 4) that allows a functional test for transitive interference. In these assays, we observed no loss of transitive interference in *rrf-2*(*pk2040*) and *rrf-3*(*pk1426*), while *rrf-1*(*pk1417*) completely blocked the transitive interference. In parallel, we assayed directly for physical production of secondary trigger molecules (Figure 6C). By this assay, we failed to detect upstream (secondary) siRNAs in *rrf-1*(*pk1417*) animals. *rrf-2*(*pk2040*) and *rrf-3*(*pk1426*) retained the ability to produce the secondary triggers. Interestingly, *rrf-1*(*pk1417*) mutants retain the ability to produce a small population of siRNA molecules corresponding to the original trigger RNA. The siRNAs produced in *rrf-1*(*pk1417*) may represent the primary trigger RNAs. These results are consistent with an RdRP-independent cleavage of the initial dsRNA trigger, followed by RdRP- and target-dependent amplification of the trigger population.

A variety of genes have been shown to play essential or contributory roles in RNAi in *C. elegans*. To identify additional genetic requirements for transitive RNAi, we first assayed two genes for which the most straightforward genetic tools were available. *rde-1* and *rde-4* are the only *C. elegans* genes known to be essential for RNAi in all tissues. Since both genes are dispensable for organismal viability and fertility, the assays for transitive RNAi were straightforward. We found that both genes were required for the transitive RNAi assay (Figure 6B).

We note an ambiguity that is inherent in both siRNA and transitive RNAi assays: since both assays depend on early steps in the RNAi pathway, the results with *rrf-1*, *rde-1*, and *rde-4* mutants do not distinguish between (1) a specific loss of secondary siRNAs and (2) a decrease in secondary siRNAs as a result of inefficiency in earlier stages in the RNAi pathway (e.g., primary siRNA production). For *rde-1*, this ambiguity is addressed by previous results. Extracts of *rde-1* mutant animals are comparable to wild-type extracts in cleavage of labeled dsRNA into short siRNA fragments (Ketting et al., 2001). This initial cleavage process also proceeds in vivo: after injection of a $^{32}$P-labeled dsRNA trigger into the syncytial

germline, *rde-1*(*ne300*) null mutants are comparable to wild-type in the production of $^{32}$P-labeled siRNAs (Parrish and Fire, 2001). *rde-4* mutants have also been analyzed in the in vivo assay; *rde-4* shows a decreased primary siRNA production, suggesting a possible defect in primary siRNA production (Parrish and Fire, 2001). For the RdRP products, the straightforward assay for cleavage of labeled dsRNA after germline injection (or extract preparation) is not available: since *ego-1* is an essential gene, we have no source of healthy RdRP(−) animals for direct assays of siRNA production.

A second test that has been used to address mutational effects on the role of siRNAs in the interference reaction involves injection of a large population of synthetic siRNAs directed at a specific target sequence. The siRNAs are prepared with the characteristic duplex structure and 2-base 3′ overhang (Elbashir et al., 2001). For *C. elegans*, synthetic siRNAs of 24–25 bp yield robust interference in wild-type animals and partially bypass the RNAi defect in *rde-4* mutants (but not in *rde-1* mutants) (Caplen et al., 2001; Parrish and Fire, 2001). When tested in the *rrf-1* mutant backgrounds (point or deletion), we observed no interference by preformed siRNAs, even at concentrations 10-fold above those required for interference in a wild-type background (Figure 6B and data not shown).

### An Essential Role for Secondary siRNAs and RdRP Activity in the RNAi Mechanism

The insensitivity of *rrf-1* mutants to phenotypic interference in the soma suggested that the initial siRNA:target interaction might be insufficient to produce a phenotypically significant effect on gene expression. This was particularly surprising with an *unc-22* target, since a relatively modest decrease in gene expression (on the order of 30%–40%) is detectable using the assays employed. Additional experiments were carried out using quantitative RNase protection in attempts to detect small decreases in *unc-22* expression in the *rrf-1*(*pk1426*) mutant animals; no decrease in mRNA level was observed (Figure 6E).

## Discussion

### A Working Model for RNA Interference in the *C. elegans* Soma

We have demonstrated that RNA interference in *C. elegans* involves the production of at least two populations of siRNA molecules. One group of siRNA molecules had been previously described and is derived from the initial injected dsRNA. A second group of siRNAs has sequence, structural, and biological characteristics that indicate formation by an RdRP, potentially following priming of target RNAs by the antisense strand of primary siRNAs. Based on the results of this work and of the many studies of RNAi in diverse biological systems over the last several years, we present a working model for RNA interference and related pathways in the *C. elegans* soma (Figure 7).

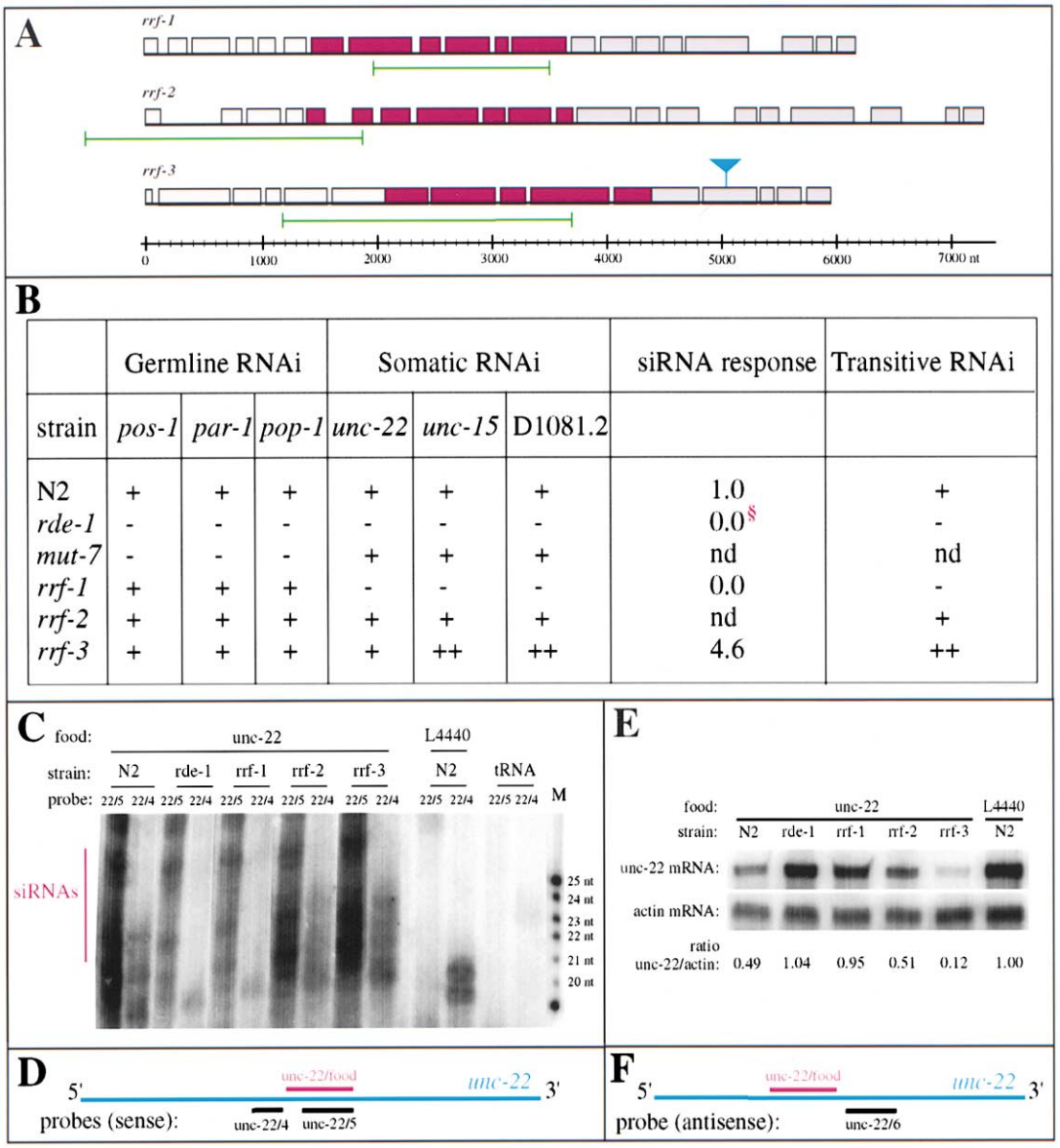
The first steps in the RNAi pathway involve uptake of dsRNA by cells and an inefficient cleavage of the original trigger RNA into short fragments. The cleavage reaction has been studied in detail in extracts of *Drosophila* and *C. elegans* (Bernstein et al., 2001; Zamore et al., 2000; Elbashir et al., 2001; Ketting et al., 2001), and has been shown to be mediated by the RNaseIII-like factor DICER; genetic experiments in *C. elegans* suggest, in addition, the involvement of RDE-4 (Tabara et al., 1999; Parrish and Fire, 2001). These initial siRNAs are apparently not numerous enough (or not of the proper structure) to effect an efficient interference response in vivo. They must, however, have an appropriate structure to allow interaction in vivo with complementary sequences on the target mRNA. Two possible consequences could follow this initial interaction: the siRNA might prime synthesis of longer antisense RNA; alternatively, cleavage of the target mRNA in the region of siRNA homology might produce an end structure which signals RdRP to initiate de novo synthesis of antisense RNA on the cleaved mRNA template. Interestingly, the biochemical analysis of plant RdRP is consistent with either model: the tomato RdRP activity is capable of both primed and unprimed synthesis (Schiebel et al., 1993a, 1993b). Whatever the mechanism by which synthesis of new antisense RNA is primed, the subsequent activity of DICER or another dsRNA-specific nuclease could function both (1) to destroy the mRNA and (2) to amplify the population of siRNA triggers.

At some point in the RNAi process, there is an absolute requirement for a member of the Argonaute superfamily. Although there are 24 Argonaute homologs in *C. elegans*, RDE-1 is completely required for specific interference responses to exogenous dsRNA (Tabara et al., 1999). Potential roles for RDE-1 would be to stabilize the primary siRNAs (M. Tijsterman et al., submitted) and/or to facilitate scanning of potential target RNAs for regions of homology. Consistent with these proposals are recent studies by Hammond et al. (2001) showing that *Drosophila* Argonaute-2 forms a tight complex with siRNAs during RNAi in *Drosophila* cultured cells.

Certain biochemical features of RdRP-derived amplification are suggested from our in vivo observations. In particular, our analyses of positional dependence showed a loss of the transitivity and secondary siRNA signals at distances greater than several hundred base pairs from the original trigger. Given that this distance may reflect multiple rounds of elongation and reduction to siRNAs, these data suggest that only relatively short transcripts are produced by RdRP in our assays. Several different aspects of the reaction might limit the extent of dsRNA formed: (1) the processivity of the enzyme in vivo may be very limited; (2) the enzyme may be blocked from producing large dsRNAs by secondary structure or protein factors bound to target RNA, or (3) templates available for RdRP may be of limited length (perhaps short segments of sense RNA that are derived through partial degradation of the target mRNA). Given the ability of the RdRP enzyme to initiate RNA synthesis at the end of a short RNA segment (Schiebel et al., 1993a, 1993b), it is certainly possible that the RdRP would carry out an additional reaction of copying sense segments of the input siRNA.

### A Diversity of Roles RdRP and Amplification Processes in Gene Silencing?

One surprising aspect of our data was the lack of measurable RNAi response in *rrf-1* mutant soma. Given that some siRNAs are produced in the mutant, and given




| strain | Germline RNAi | | | Somatic RNAi | | | siRNA response | Transitive RNAi |
|---|---|---|---|---|---|---|---|---|
| | *pos-1* | *par-1* | *pop-1* | *unc-22* | *unc-15* | D1081.2 | | |
| N2 | + | + | + | + | + | + | 1.0 | + |
| *rde-1* | - | - | - | - | - | - | 0.0§ | - |
| *mut-7* | - | - | - | + | + | + | nd | nd |
| *rrf-1* | + | + | + | - | - | - | 0.0 | - |
| *rrf-2* | + | + | + | + | + | + | nd | + |
| *rrf-3* | + | + | + | + | ++ | ++ | 4.6 | ++ |

Figure 6. Contributions of the RdRP-Homologous Genes *rrf-1*, *rrf-2*, and *rrf-3* to RNAi and Secondary siRNA Production

(A) Predicted structures of *rrf* genes and mutant alleles. Boxes represent exons. Red boxes: RdRP-related segments (*rrf-1*: nt 1413–3837/aa 471–1279; *rrf-2*: nt 1362–3771/aa 454–1257; *rrf-3*: nt 2049–4383/aa 683–1461). Green lines: *rrf-1*(*pk1417*), *rrf-2*(*pk2040*), and *rrf-3*(*pk1426*) remove nt 1991–3407, 572–1878, and 1190–4205, respectively. Blue triangle: *rrf-3*(*pk2042*) has a Tc1 transposon inserted between nt 5016 and 5017.

(B) RNAi sensitivity assays. Animals were fed *E. coli* producing different dsRNAs and progeny scored for survival (germline-expressed genes) or uncoordinated or paralyzed phenotype (somatically expressed genes). "−": resistance to RNAi (full survival or normal movement). "+": sensitivity to RNAi (no survival or uncoordinated movement; effects comparable to those in wild-type animals). "++": hypersensitivity to RNAi (greater sensitivity to RNAi than observed in wild-type; this was only testable for the *unc-15* and *D1081.2* genes for which the dsRNA-producing bacteria yielded a partially penetrant phenotype in wild-type animals). "siRNA response": twitching behavior for progeny of animals injected with 5 mg/ml of a synthetic 25 nt siRNA from *unc-22* (23 bp duplex with 2 base 3′ overhangs; Caplen et al., 2001). Percentages of animals twitching in levamisole are normalized to fractions observed in wild-type. "§": data from Parrish and Fire (2001). "Transitive RNAi" refers to the assay in Figure 4C: mutant strains were transformed with the *myo-3*::*unc-22*::*gfp* fusion construct to generate several independent transgenic lines, and animals from these lines injected with dsRNA for segment *gfpA*. No twitching in levamisole (i.e., no transitive RNAi) was observed in *rrf-1*(*pk1417*) (two lines), *rde-1*(*ne300*) (two lines), or *rde-4*(*ne299*) (one line). For *rde-1* and *rde-4* (where fewer lines were derived), efficacy of each transgene as a substrate for transitive RNAi was confirmed by crossing out of the *rde* background and assaying in a wild-type background. For *rrf-3*(*pk1426*) (two lines) and *rrf-3*(*pk2042*) (two lines), we observed apparent increases in transitive RNAi, as evidenced by an increase of 10- to 15-fold in twitching response to a dsRNA segment located further downstream of the *unc-22::gfp* junction (*gfpB*).

(C) RNase protection assays of total RNA from animals raised on *E. coli* containing the *unc-22* dsRNA expression construct pTS302, or the empty vector (L4440); lanes labeled "tRNA" show RNase protection assays carried out on yeast tRNA. Probes (all of sense polarity) are indicated above the lanes. The putative siRNA region of the gel (22–26 nt RNAs) is noted; lower bands in the gel (in the 20–21 nt region,

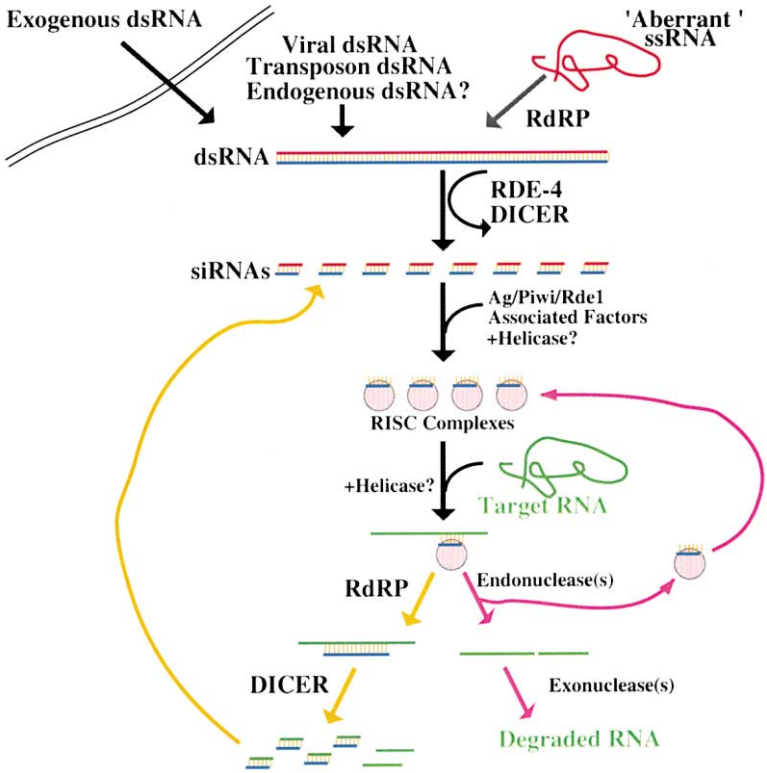



Figure 7. A Working Model for RNA Interference

Two different aspects of the model enhance the potency of the RNAi reaction. Reuse of RNA-loaded RISC complexes (magenta arrows) should provide the reaction with a catalytic component, while physical amplification by RdRP (orange arrows) provides a physical amplification of the initial trigger RNA.

that siRNAs can be injected at high concentrations, we might have expected at least a modest interference response. The lack of such an effect suggests one of three possibilities. The first would be a quantitative insufficiency: it is conceivable that the levels of primary siRNAs (even following the injection of preformed siRNA at high concentration) are insufficient for a measurable response (perhaps incorporation of injected siRNAs into RISC complexes [Hammond et al., 2001] is much less efficient than the incorporation of secondary siRNAs formed in vivo). Alternatively, the initial siRNA::mRNA interaction may be relatively transient or unstable in vivo and may require stabilization through the polymerization of additional bases on the end of the duplex. Under such circumstances, the formation of a region of duplex by RdRP may be sufficient to block gene expression even before (or in the absence) or further cleavage by DICER/RNaseIII. A third possibility is perhaps mechanistically most intriguing: RRF-1 and other RdRP-like factors could have an additional biochemical role in the RNAi reaction. Since these factors must be capable of interacting with dsRNA, their binding could promote or stabilize interactions between siRNAs and target RNA. More speculatively, RdRP-like factors might catalyze phosphorolysis reactions in addition to template-dependent nucleotide polymerization, perhaps breaking the target mRNA or tagging it for destruction.

Genetic analysis in plants of RdRP function during silencing and pathogen defense has suggested both commonality and diversity of roles. One of the *Arabidopsis* RdRP homologs, SDE-1/SGS-2, is required for RNA-triggered silencing by a variety of sense transgenes and for RNA-triggered defense against some but not all viral genomes (Mourrain et al., 2000; Dalmay et al., 2000). Dalmay and colleagues proposed that silencing by sense transgenes might require RdRP to produce a dsRNA trigger, which then enters a (potentially RdRP-independent) RNAi pathway. Alternatively, a central role for RdRP in RNAi might be obviated during certain viral infections by unwitting amplification of specific trigger RNAs by viral replicase. Xie et al. (2001) describe the involvement of a distinct RdRP homolog in tobacco viral resistance; it is not clear whether this factor has a role in RNAi.

Given the complexity of RNAi and other gene silencing responses, it seems likely that multiple amplification processes cooperate to provide a highly sensitive and selective response. The absence of an identified RdRP homolog in *Drosophila* and mammals suggests either (1) that other RNA copying enzymes are used in these

particularly with the 22/4 probe) represent background hybridization that is observed in the absence of ongoing RNAi (e.g., L4440 lanes).
(D) *unc-22* mRNA with positions of *E. coli* produced dsRNA and probes.
(E) RNase protection assay on total RNA isolated from animals fed with *E. coli* producing *unc-22* dsRNA. *unc-22* and an actin (*act-1*) probes, both of antisense polarity, were both added during hybridization. *act-1* and *unc-22* steady-state mRNA levels were quantitated and the ratio *unc-22*/*act-1* mRNA determined.
(F) Relative positions of probes and bacterially produced dsRNAs for (E).

systems for amplification or (2) that the primary siRNAs may be sufficient to produce a detectable interference response (as is observed in *Drosophila* extracts). With or without an RNA copying process, a variety of additional amplification mechanisms may contribute to silencing. In this regard, it is of interest to note two previous examples of transitive silencing: Pal-Bhadra et al. (1999) observed examples of transitive silencing in *Drosophila*, while Voinnet et al. (1998) reported transitive silencing with a GFP transgene target in plants. These examples may reflect different underlying processes than we have reported; in particular, neither study noted a specific polarity in the transitivity, and the biological systems that were used are known to enforce silencing both at a posttranscriptional level and at the level of chromosome modification (methylation in plants [Wassenegger, 2000]; polycomb-group binding in animals [Pal-Bhadra et al., 1997]). A number of extant models for gene silencing in plants propose an amplification step relying on such chromosome-targeted effects (e.g., Bender, 2001). It will be of interest in the future to understand the breadth of different amplification events operating in gene silencing and their biological roles.

## Experimental Procedures

### dsRNAs

Previously described plasmids were used to produce dsRNA segments for *gfp* (*gfp*A–*gfp*F: L5051, L5108, L5058, L5050, L5059, L5052; Parrish et al., 2000), full-length *gfp* (*gfp*G; Fire et al., 1998), *unc-22* (*unc-22A*, *unc-22B*; Fire et al., 1998), and *lacZ* (*lacZ*L; Fire et al., 1998). Additional dsRNAs were from pRP1245 and *unc22X* (nt 16219–17207 and 10687–10861 of the spliced *unc-22* coding sequence), *ds-lacZU* (nt 158–1957 from the *lacZ* coding region), and *ds-unc52A* (nt 12002–12349 from *unc-52* (GenBank: CELUNC52X; Rogalski et al., 1993). *ds-lin26ivs3* (used for some negative controls) was identical in sequence to that described by Bosher et al. (1999); in our hands, injection of a highly purified and concentrated preparation of *ds-lin26ivs3* dsRNA produced no lethality or other phenotypes.

Plasmids for dsRNA production in *E. coli* were derivatives of vector L4440 (Timmons and Fire, 1998): pTS302 contained nt 11139–11728 of the spliced unc-22 coding region; pTS301 contained nt 183–620 of the spliced pos-1 coding region; pRP1251 contained the full pos-1 cDNA from pCCM114 (provided by H. Tabara; Tabara et al., 1999). dsRNA expression plasmids for *unc-15*, *D1081.2*, *pop-1*, and *par-1* (Fraser et al., 2000) were from J. Ahringer. Bacterial feeding was performed as described (Fraser et al., 2000); L4 hermaphrodites fed on dsRNA-producing bacteria (20–22°C) for approximately 24 hr were transferred to a second dsRNA-bacteria plate for >48 hr and progeny scored for behavior (both plates) or survival (second plate).

### RNase Protection Assays

RNase protection assays were chosen to provide maximally sensitive detection of small RNAs. By comparison with Northern blot procedures (e.g., Hamilton and Baulcombe, 1999), the avoidance of a filter transfer allows a substantial increase in fraction of siRNAs detected. A disadvantage of RNase protection assays is that the measured size (length of protected probe) is subject to possible imprecision of a few nucleotides due to frayed or overhanging ends produced by RNase. The siRNA size heterogeneity of several nucleotides seen in Figures 2 and 6 is likely to reflect (at least in part) this aspect of the assay.

To isolate RNA, animals or eggs lysed in protease K were extracted with phenol/chloroform, precipitated with isopropanol (1:1), and resuspended in 1 ml 1.5× STE (1× STE = 0.1 M Tris [pH 7.5], 0.1 M NaCl, and 10 mM EDTA). 100 mg cellulose powder (MN 301, Macherey-Nagel) was added, samples were mixed 10 min at room temperature, two aliquots of 290 μl 96% ethanol were added (mixing each time), and RNA was allowed to bind for one hour. After brief centrifugation, pellets were washed 3 times with 1× STE/35% ethanol, and RNA was eluted with 2 ml 1× STE, precipitated with ethanol, treated with RNase-free DNaseI, extracted with phenol/chloroform, and precipitated with ethanol.

$^{32}$P-labeled RNA probes were generated from cloned fragments by in vitro transcription with T3 or T7 polymerase followed by gel purification. Probes used were: *unc-22*/1 (10452–10562), *unc-22*/2 (10557–10798), *unc-22*/3 (10807–11004), *unc-22*/4 (10999–11138), *unc-22*/5 (11206–11728), *unc-22*/6 (11729–12075), *unc-22*/7 (12228–12564), *pos-1*/1 (1–188), *pos-1*/2 (183–620), *pos-1*/3 (615–795), *act-1* (199–390) (numbers from spliced coding sequences).

RNase protection assays were performed essentially as described (Sijen et al., 2001) with minor modifications: after hybridization, samples were treated with 20 μg/ml RNaseA, 10 U/ml RNaseT1, and 10 U/ml RNaseOne (45 min at 30°C followed by 45 min at 37°C). For each sample, 20 μg of total RNA was used.

### Derivation of Transgenic Lines

Derivation of transgenic lines using the markers *pha-1*(+) or pRF4 was as described (Granato et al., 1994; Mello and Fire, 1995). Some transgenic lines exhibit cosuppression in the absence of injected RNA (e.g., Fire et al., 1991), possibly reflecting unintended antisense products of the transgene that would complicate the subsequent analysis of polarity for transitive RNAi. We sought to minimize this problem in two ways: (1) to improve transport and stability of sense transcripts (thereby maximizing steady-state ratios of sense/antisense), our transgene structures were similar to native *C. elegans* genes in having short 5′ and 3′ UTR sequences and internal punctuation by introns; and (2) we screened lines to eliminate those with detectable cosuppression: the *gfp* transgenic line in Figure 3 (PD4251) was chosen from several similar lines based on uniformity of expression and lack of sporadic silencing. The *unc-22*::*gfp* and *gfp*::*unc-22* constructs (Figure 4) were used to make numerous independent lines; 10%–20% of these lines showed cosuppression and were eliminated. Of the remainder, 5–10 lines were tested for each construct and yielded essentially identical results.

### Rescue of *rrf-1*(*pk1417*)

A PCR product containing the wild-type *rrf-1* gene (1226 bp of upstream sequence, 568 bp downstream sequence) was injected (20 ng/ml, with 100 ng/ml pRF4) into *pk1417*. Transgenic animals showed a normal RNAi response to bacterially produced *unc-22* dsRNA.

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