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Distinct roles for RDE-1 and RDE-4 during RNA interference in *Caenorhabditis elegans*

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ABSTRACT

RNA interference (RNAi) is a cellular defense mechanism that uses double-stranded RNA (dsRNA) as a sequence-specific trigger to guide the degradation of homologous single-stranded RNAs. RNAi is a multistep process involving several proteins and at least one type of RNA intermediate, a population of small 21–25 nt RNAs (called siRNAs) that are initially derived from cleavage of the dsRNA trigger. Genetic screens in *Caenorhabditis elegans* have identified numerous mutations that cause partial or complete loss of RNAi. In this work, we analyzed cleavage of injected dsRNA to produce the initial siRNA population in animals mutant for *rde-1* and *rde-4*, two genes that are essential for RNAi but that are not required for organismal viability or fertility. Our results suggest distinct roles for RDE-1 and RDE-4 in the interference process. Although null mutants lacking *rde-1* show no phenotypic response to dsRNA, the amount of siRNAs generated from an injected dsRNA trigger was comparable to that of wild-type. By contrast, mutations in *rde-4* substantially reduced the population of siRNAs derived from an injected dsRNA trigger. Injection of chemically synthesized 24- or 25-nt siRNAs could circumvent RNAi resistance in *rde-4* mutants, whereas no bypass was observed in *rde-1* mutants. These results support a model in which RDE-4 is involved before or during production of siRNAs, whereas RDE-1 acts after the siRNAs have been formed.

Keywords: dsRNA; Piwi; PTGS; RNAi; silencing; siRNA

INTRODUCTION

Defense mechanisms that protect against deleterious gene expression are essential for long-term survival of a species. These genome surveillance mechanisms allow selective silencing or destruction of aberrant nucleic acids, including molecules of both endogenous and parasitic origin. RNA interference (RNAi), an example of such a mechanism, uses double-stranded RNA (dsRNA) to trigger the sequence-specific degradation of homologous transcripts (Fire et al., 1998; reviewed in Sharp, 2001). dsRNAs are frequently a byproduct of viral or transposon gene expression. One proposed function of RNAi is to recognize these dsRNAs as indicators of unwanted gene expression, thus providing a means to silence parasitic genetic elements (reviewed in Baulcombe, 1996, 1999; Ketting et al., 1999; Tabara et al., 1999). Aspects of RNAi and related post-transcriptional silencing (PTGS) mechanisms have been conserved across diverse phyla from all four eukaryotic kingdoms (reviewed in Cogoni & Macino, 2000; Bosher & Labouesse, 2000). The ancient origin of RNAi and other PTGS processes suggests these mechanisms may represent a primitive nucleic-acid-based immune response.

Although the precise molecular mechanisms of RNAi remain to be elucidated, several recent observations have provided the beginnings of a working model. A particularly significant observation was made by Hamilton & Baulcombe (1999), who identified a population of small (~25-nt) RNAs (now referred to as siRNAs), whose presence correlates with ongoing silencing. siRNAs are also observed in *Drosophila* extracts carrying out an RNAi-like reaction (Hammond et al., 2000; Zamore et al., 2000). siRNA formation in vitro and in vivo has shown to result at least in part from cleavage of the dsRNA trigger (Parrish et al., 2000; Yang et al., 2000; Zamore et al., 2000). Intriguingly, Hamilton & Baulcombe (1999) reported an example in which the appearance of a strong siRNA signal was dependent on the presence of an RNA target. This suggests that a population of siRNAs formed during initial cleavage of the trigger RNA may be subjected to subsequent (a) amplification by a target-dependent copying process, and/or (b) stabilization by the presence of an mRNA target.

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The siRNAs possess a characteristic structure, with a 5′ phosphate, 3′ hydroxyl, and a two- or three-base 3′ overhang on each strand of the duplex (Elbashir et al., 2001b). This siRNA structure bears a striking resemblance to products formed by RNase III cleavage of long dsRNAs. Consistent with the hypothesis that an RNase III-like activity is involved in RNAi, Bernstein and colleagues (2001) have demonstrated that a protein with homology to RNase III (DICER) is present in *Drosophila* extracts and is capable of producing short RNA products from a longer dsRNA trigger. Importantly, synthetic 21- or 22-nt RNA duplexes that mimic the structure of siRNAs are capable of effectively triggering RNAi in vitro and in vivo, implicating siRNAs as direct participants in the RNAi process (Caplen et al., 2001; Elbashir et al. 2001a, 2001b). The siRNAs, probably assembled in a ribonucleoprotein complex, have been shown to direct cleavage of the cognate mRNA within the region of homology, with the single cleavage site located in the center of the protected area (Zamore et al., 2000; Elbashir et al., 2001b).

One approach to understand the silencing mechanism has relied on genetic screens for genes that can mutate to a silencing-deficient phenotype (Cogoni & Macino, 1997; Elmayan et al., 1998; Tabara et al., 1999). One class of mutations in *Caenorhabditis elegans*, consisting of alleles of the genes *rde-1* and *rde-4* (*rde*: RNAi defective), shows global RNAi resistance without any evidence of additional phenotypic consequences (Tabara et al., 1999). The *rde-1* gene has been cloned and encodes a member of the large PIWI/STING/Argonaute/Zwille/eIF2c protein family (e.g., with 24 representatives in *C. elegans*; Tabara et al., 1999). Analyses of PIWI/STING/Argonaute/Zwille/eIF2c homologs in other systems have suggested that some are similar to *rde-1* in exhibiting a specific role in RNAi/PTGS, whereas others are involved in developmental regulation of gene expression (Cox et al., 1998; Catalanotto et al., 2000; Fagard et al., 2000; Grishok et al., 2001). Certain members of this family may have dual function in development and genome surveillance (Fagard et al., 2000).

Consistent with the hypothesis of mechanistic commonality between RNAi and developmental control processes, a large class of mutations has been shown to affect both RNAi and developmental progression. In *C. elegans*, mutations in the genes *rde-2*, *rde-3*, *mut-2*, *mut-7*, *mut-8*, *mut-9*, and *ego-1*, exhibit variable degrees of RNAi resistance accompanied by a host of other phenotypes, such as defective chromosome segregation (all), increased transposon mobilization (*rde-2*, *rde-3*, and *mut*-7, -8, -9), and chromatin condensation defects (*mut-7*) (Ketting et al., 1999; Tabara et al., 1999; Dernburg et al., 2000; Ketting & Plasterk, 2000; Smardon et al., 2000). All of these mutations produce sterility, as does loss of function for the *C. elegans* DICER homolog *dcr-1* (Grishok et al., 2001).

The description of molecular intermediates in a reaction, combined with the availability of numerous mutants affecting the mechanism provides an excellent starting point for the construction of a functional pathway (Edgar & Wood, 1966). The most straightforward starting point for such analysis are genes that are required for RNAi but not essential for viability. Such cases allow a clear analysis of the reaction in a living system completely lacking a specific gene product. Mutations with partial loss of RNAi and those that have accompanying fertility defects are somewhat more of a challenge for analysis in a whole organism. For each case, rescue by maternal dowry, overlapping gene function, and other considerations prevent complete loss of RNAi activity from being achieved in a living system.

In this work, we have utilized assays for production of siRNAs and interference by siRNAs in *rde-1* and *rde-4* mutant animals to begin constructing a pathway for the RNAi reaction.

RESULTS AND DISCUSSION

Genetic requirements for the production of siRNAs during RNA interference

We have previously described an in vivo assay to follow the fate of the dsRNA trigger in *C. elegans* (Parrish et al., 2000). Briefly, *C. elegans* adults are injected with ^{32}P-labeled dsRNA and harvested for RNA extraction after 12 h of growth at 16 °C. RNA molecules from the injected animals are resolved on denaturing polyacrylamide gels and visualized by phosphorimager analysis. In wild-type recipient animals, this assay detects a small proportion (approximately 1%) of the dsRNA trigger that is processed into small RNAs (Parrish et al., 2000). These small RNAs are approximately 25 nt in length, similar to siRNAs observed during RNAi in diverse species (Hamilton & Baulcombe, 1999; Hammond et al., 2000; Parrish et al., 2000; Yang et al., 2000; Zamore et al., 2000). To investigate the genetic requirements for production of siRNAs, we utilized this in vivo assay to assess siRNA formation in *rde-1* and *rde-4* mutants.

In mutant strains carrying either of two different *rde-1* alleles (*rde-1*(*ne219*) or *rde-1*(*ne300*); Tabara et al., 1999), levels of ~25 nt siRNAs produced from the dsRNA trigger were comparable to that of wild-type N2 worms (*ne300*: Fig. 1; *ne219*: data not shown). This indicates that RDE-1 is not required for the initial processing of the trigger RNA into siRNAs. The two *rde-1* alleles used have distinct molecular lesions: *rde-1*(*ne300*) is a predicted null that contains a premature stop codon, whereas *rde-1*(*ne219*) harbors a missense mutation in the conserved PIWI domain (Tabara et al., 1999).

When *rde-4*(*ne299*) animals (Tabara et al., 1999) were examined by this assay, a reduction in the amount of

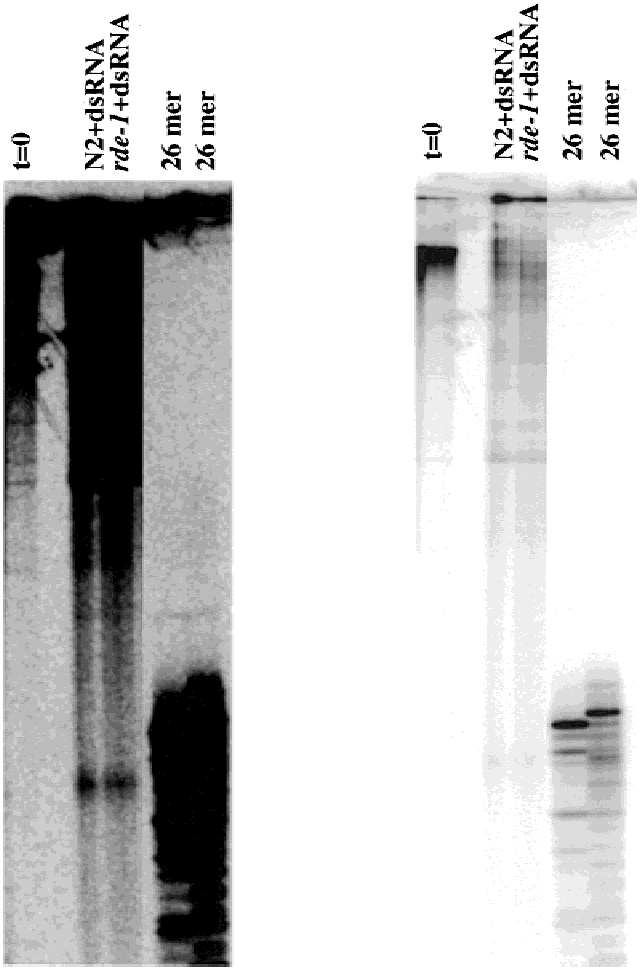


FIGURE 1. Processing of injected dsRNA into ~25-nt siRNAs is not impaired in *rde-1* mutants. ^{32}P-labeled dsRNA was injected into N2 (wild-type) and *rde-1(ne300) C. elegans* adults. RNAs were uniformly labeled by in vitro synthesis in the presence of ^{32}P-UTP (to a specific activity of 15 Ci/mMol UTP) and injected at a concentration of ~2.5 mg/mL. Animals were allowed to recover for 12 h at 16 °C and subsequently harvested for RNA extraction. Samples were resolved by electrophoresis on 12% denaturing polyacrylamide gels and visualized by phosphorimager analysis. Two different images of the gel are shown; the high gain image is located on the left and low gain image is on the right. The $t = 0$ sample contained a mixture of *C. elegans* and labeled dsRNA that was then subjected to the standard RNA extraction. End-labeled ssRNA oligos (zr1 and zr2, each 26 nt in length; Parrish et al., 2000) were utilized as markers.

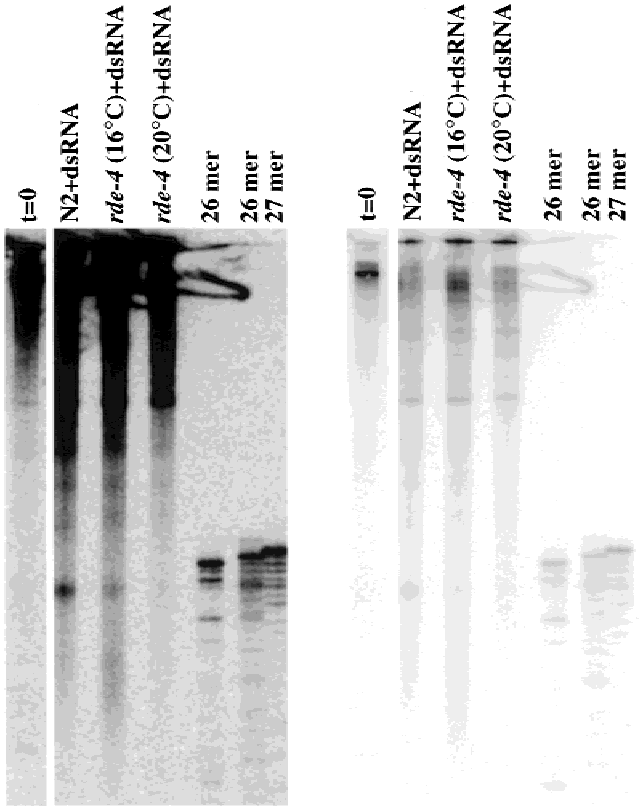


FIGURE 2. Reduced levels of ~25-nt siRNA products from a dsRNA trigger in *rde-4* mutants. Labeled dsRNA was injected into N2 and *rde-4(ne299)* adults (grown at 16 °C or 20 °C) and analyzed as described in Figure 1. End-labeled ssRNA oligos (zr1 and zr2, each 26 nt in length and zr10, 27 nt in length; Parrish et al., 2000) were utilized as markers.

siRNA generated from the trigger was observed, with a more dramatic decrease seen at elevated temperatures (Fig. 2). The RNAi process in *rde-4*(*ne299*) appears to be somewhat temperature sensitive, allowing no interference at 20 °C or 23 °C, while permitting weak interference when grown at 16 °C (data not shown). At present we have no data to distinguish whether temperature effects with the *ne299* allele reflect a temperature-sensitive protein (i.e., residual RDE-4 protein activity at 16 °C) or a temperature-sensitive process (ability of the RNAi reaction to proceed at a low level in the absence of RDE-4 at 16 °C). Assays for initial siRNA formation in the *rde-4* mutant at different temperatures paralleled overall RNAi sensitivity. At 16 °C, siRNA products from the injected RNA were detected in *rde-4*(*ne299*) animals, but were present at a level that was substantially below levels in wild-type (Fig. 2). When animals were grown at 20 °C (Fig. 2) or 23 °C (data not shown), the levels of small RNA generated in *rde-4*(*ne299*) were consistently below the detection threshold of our assay. These results suggest an involvement (direct or indirect) of RDE-4 in the initial processing the dsRNA trigger.

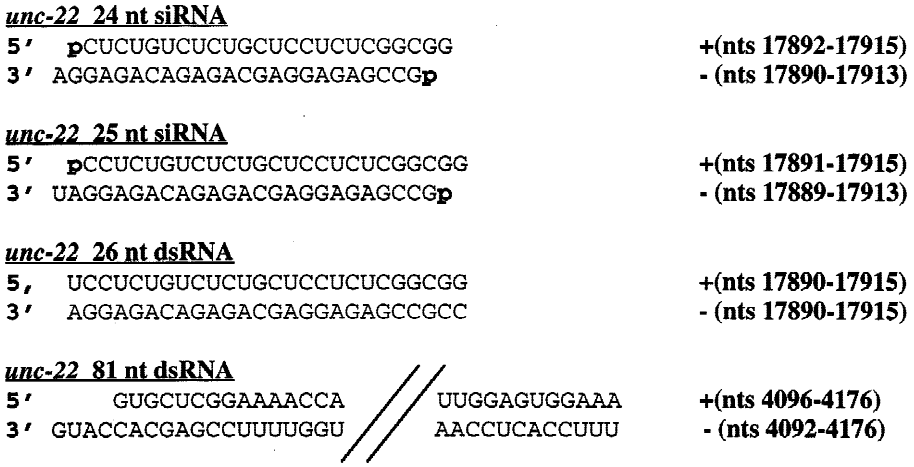
Assays for interference by siRNAs distinguish the roles for RDE-1 and RDE-4 in RNAi

In experiments performed in *Drosophila* embryo extracts, siRNAs derived from the dsRNA trigger have been shown to possess a characteristic structure with a 5′ phosphate, a 3′ hydroxyl, and a 3′ two- or three-base overhang on each strand of the duplex (Elbashir et al., 2001b). Synthetic dsRNA oligos with a siRNA structure have been shown to be capable of triggering RNAi upon injection into *C. elegans* (Caplen et al., 2001).

To determine if the roles of RDE-1 and/or RDE-4 could be bypassed by precleavage of the trigger RNA, we examined whether chemically synthesized siRNAs could produce a phenotypic effect in *rde-1* or *rde-4* mutants.

The siRNAs were not able to bypass the RNAi defects in *rde-1*(*ne300*) animals (Fig. 3). siRNAs containing 24 or 25 nt of *unc-22* sequence, which produced the characteristic and specific twitching phenotype indicative of *unc-22* interference in wild-type animals (Fire et al., 1998; Caplen et al., 2001), produced no interference in *rde-1*(*ne300*) animals. Similarly, a blunt-ended dsRNA oligo containing 26 nt could not mediate interference in *rde-1*(*ne300*), nor could a dsRNA oligo 81 nt in length. The inability of the siRNAs to bypass RNAi resistance in *rde-1* mutants suggests the defect in this mutant lies downstream of trigger processing. This hypothesis is also consistent with the observation (Fig. 1) that ~25-nt siRNAs are present after injection of labeled dsRNA into *rde-1* mutants.

We found that synthetic siRNAs were able to partially bypass the requirement for RDE-4 in the interference response (Fig. 3). For two alleles of *rde-4* (*ne299* and *ne301*), interference was induced at 20 °C after injection of the 24-nt or the 25-nt siRNA. Although injection of siRNAs produced interference in an *rde-4* mutant background, bypass of the block to interference appeared incomplete. Evidence for an incomplete restoration of interference comes from a comparison of the fraction of progeny affected in wild-type versus *rde-4* mutant animals injected with the 25-nt siRNA (approximately a threefold increase in wild-type animals). Interestingly, a blunt-ended 26-nt dsRNA did not trigger significant interference in *rde-4* mutant animals, despite triggering modest interference in wild-type worms. This result implies that the structural modifications imparted by an RNase III cleavage event are important for generating efficient RNAi. For *rde-4*(*ne299*), there was a slight background level of interference with the 26-nt and 81-nt dsRNAs; this may have resulted from degradation in the RNA preparations, perhaps resulting in a small population of RNAs resembling the structure of the siRNAs. The ability of siRNAs to bypass the RNAi defect in the *rde-4* mutant, in addition to the reduced amount of ~25-nt siRNAs generated during RNAi in this mutant (Fig. 2), suggests that RDE-4 is involved in generating siRNAs from dsRNA trigger molecules.



FRACTION AFFECTED (NUMBER SCORED)

RNA Injection	N2	*rde-1* (*ne300*)	*rde-4* (*ne 299*)	*rde-4* (*ne301*)
unc-22 siRNA **24 nts**	3.6% (279)	0% (400)	2.6% (1443)	5.5% (1252)
unc-22 siRNA **25 nts**	16.3% (768)	0% (1275)	3.2% (1375)	5.8% (289)
unc-22 dsRNA **26 nts**	3.1% (609)	0% (275)	0.2% (2371)	0.0% (740)
unc-22 dsRNA **81 nts**	88.9% (180)	0% (305)	0.4% (2023)	0.0% (593)

FIGURE 3. siRNAs can bypass RNAi resistance in *rde-4* but not in *rde-1* mutants. Shown at the top are structures of synthetic RNAs injected into *C. elegans* (Parrish et al., 2000, Caplen et al., 2001). Shown at the bottom are interference activities of synthetic RNAs in wild-type and mutant *C. elegans* (wild-type data as described, Caplen et al., 2001). Double-stranded RNAs (5 mg/mL) were injected into the gonad of *C. elegans* adults grown at 20 °C. Percentages represent the fraction of F1 progeny exhibiting the *unc-22* twitching phenotype in 0.33 mM levamisole. Numbers in parentheses indicate the total number of animals scored.

Sequential activity of RDE-4 and RDE-1 in RNAi

Our data are consistent with a sequential model in which RDE-4 is involved in a step prior to or during the initial cleavage of the dsRNA trigger to produce siRNA-type molecules, while RDE-1 is involved in a subsequent step in interference (Fig. 4). As with other complex multiprotein/multi-RNA assemblies, the events in RNAi might occur within a short time range and in a large complex (e.g., Bernstein et al., 2001). There is thus likely to be some degree of freedom in the formation and action of protein:RNA complexes that execute RNAi. It should be pointed out that our experiments do not rule out an association of RDE-1 with the precleavage RNAi complex, nor do they rule out the continued association of RDE-4 with the complex after cleavage.

Figure 4 shows our working model for the roles of RDE-1, RDE-4, and siRNAs in the interference process: RDE-4 is invoked in the recognition of long duplex RNA molecules as aberrant RNAs (allowing cleavage to form the characteristic siRNA guide), whereas RDE-1 (and homologous factors) are invoked in subsequent steps that utilize a short segment of guide RNA as a means to recognize and/or act on a target transcript.

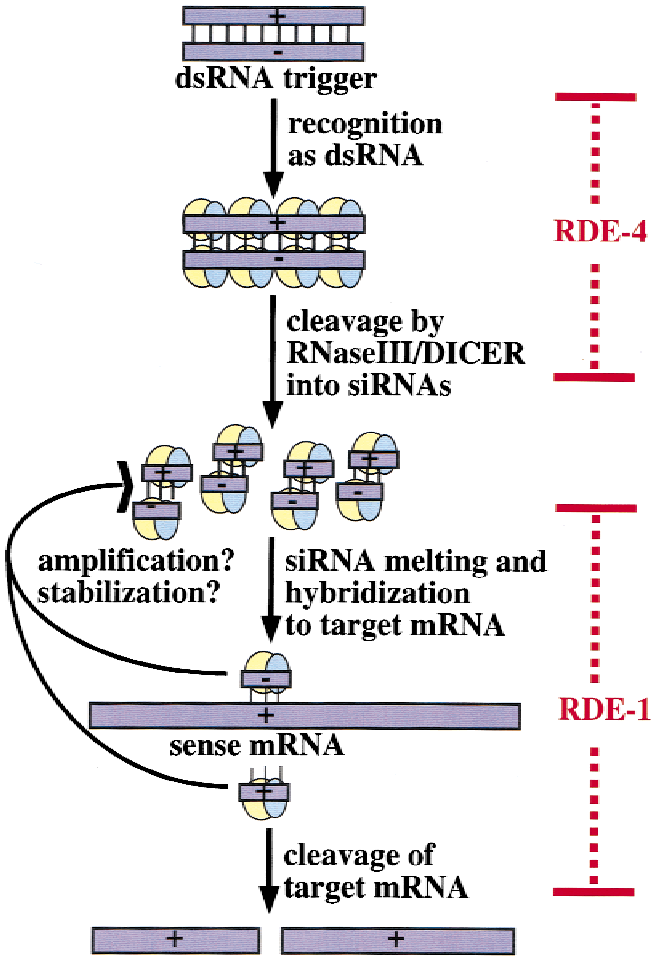


FIGURE 4. A working model showing roles of RDE-1, RDE-4, and siRNAs in the interference reaction.

MATERIALS AND METHODS

Strains

The *C. elegans* stock N2 (Brenner, 1974) was used as the representative wild-type strain. Strains carrying the RNAi-defective mutations *rde-1*(*ne219*), *rde-1*(*ne300*), *rde-4*(*ne299*), and *rde-4*(*ne301*) (Tabara et al., 1999) were each extensively backcrossed to wild-type (and were gifts from H. Tabara and C. Mello). Each strain contained no other genetic markers.

Assays for fate of the dsRNA trigger

The in vivo fate of radioactively labeled trigger molecules was analyzed as described previously (Parrish et al., 2000). Radioactive RNA was synthesized through incorporation of ^{32}P-UTP (specific activity, 15 Ci/mMol UTP) in standard T3/T7 polymerase reactions followed by RNA extraction, sense/antisense hybridization, and injection into *C. elegans* at a concentration of ~2.5 mg/mL. The injected dsRNA molecules contained sequence from *gfp* (326 nt), *unc-22* (743 nt), and from restriction enzyme linkers (103 nt). Following growth for 12 h at 16 °C, injected animals were harvested for RNA extraction. RNAs were resolved on 12% denaturing polyacrylamide gels and visualized using a Molecular Dynamics Storm Phosphorimager.

Assays for interference by small RNAs

RNA oligonucleotides containing sequences of the *unc-22* gene (Fig. 3) were chemically synthesized using 2′-*O*-(triisopropyl)silyloxy-methyl chemistry by Xeragon AG (Zurich, Switzerland; Parrish et al., 2000; Caplen et al., 2001). The 26-nt and 81-nt RNA oligomers were further purified by anion exchange HPLC. RNA oligos were annealed and injected into *C. elegans* strains (grown at 20 °C) at a concentration of ~5 mg/mL. The degree of interference was measured as the fraction of progeny exhibiting the Unc-22 twitching phenotype in 0.33 mM levamisole.

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