

# Functional Anatomy of a dsRNA Trigger: Differential Requirement for the Two Trigger Strands in RNA Interference

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## Summary

In RNA-mediated interference (RNAi), externally provided mixtures of sense and antisense RNA trigger concerted degradation of homologous cellular RNAs. We show that RNAi requires duplex formation between the two trigger strands, that the duplex must include a region of identity between trigger and target RNAs, and that duplexes as short as 26 bp can trigger RNAi. Consistent with *in vitro* observations, a fraction of input dsRNA is converted *in vivo* to short segments of ~25 nt. Interference assays with modified dsRNAs indicate precise chemical requirements for both bases and backbone of the RNA trigger. Strikingly, certain modifications are well tolerated on the sense, but not the antisense, strand, indicating that the two trigger strands have distinct roles in the interference process.

## Introduction

Double-stranded RNA (dsRNA) induces potent cellular responses in diverse biological systems (Fire, 1999; Sharp, 1999; Williams, 1999). Models to explain dsRNA responses have centered on possible defense against deleterious RNAs, including viral transcripts and replication intermediates (Ratcliff et al., 1997; Williams, 1999) and transposons (Ketting et al., 1999; Tabara et al., 1999). In mammalian cells, dsRNA is associated with a sequence-nonspecific response that includes induction of interferon, phosphorylation of translation initiation factor eIF2 (which leads to a general block in translation), and induction of a 2'-5' oligoadenylate synthetase (which can stimulate the RNA degrading enzyme RNase L) (Williams, 1999).

A distinct type of response (termed RNAi) has been associated with dsRNA in numerous species of invertebrates, plants, and protozoa (for reviews, Fire, 1999; Sharp, 1999). In these cases, the response to dsRNA includes a dramatic and sequence-specific destabilization of cellular RNA transcripts that correspond to the RNA trigger.

A subset of the transgene-triggered processes referred to in plants as posttranscriptional gene silencing

(PTGS) appear to be related or identical to dsRNA-associated RNA destabilization, although it is not clear whether dsRNA is always the trigger (de Carvalho et al., 1992; Baulcombe, 1996; Vaucheret et al., 1998; Wassenegger and Pellissier, 1998; Jorgensen et al., 1999). As with the global (non-gene-specific) responses to dsRNA in mammalian cells, PTGS in plants has been identified as an antiviral mechanism (Baulcombe, 1999).

The dsRNA-associated gene-specific responses observed in plants and invertebrates are likely to involve (at some stage) the pairing of antisense RNA sequences derived from the trigger with the endogenous sense RNA. (This feature is held in common by all models proposed to date for PTGS.) Antisense nucleic acids have long been known to be involved in specific cases of physiological regulation and to be applicable in certain cases as tools for selective genetic disruption (Takayama and Inouye, 1990). The key (as yet unresolved) questions in analysis of dsRNA-associated PTGS are (1) Why are both strands required in the trigger RNA? and (2) How can dsRNA exert an effect at concentrations that are substantially lower than those of the endogenous target RNA? Several models have been proposed to explain the second observation, including the possibilities of multiround catalytic degradation of target RNAs using a denatured region of the double-stranded trigger RNA (Montgomery et al., 1998) or production of numerous short antisense RNA copies of the incoming trigger RNA (Wassenegger and Pellissier, 1998).

Genetic screens for components necessary for PTGS/RNAi have been one approach toward identifying molecular components of the mechanism (e.g., Cogoni and Macino, 1999; Tabara et al., 1999; Dalmay et al., 2000; Mourrain et al., 2000). While the cloned genes and mutant strains provide hints and useful tools for future studies, direct biochemical roles for the genetically identified PTGS factors have yet to be assigned.

A complementary approach toward understanding PTGS comes from attempts to understand the chemical character of the trigger RNA that is critical for inducing interference. The nematode system offers a several advantages for this analysis, since small quantities of synthetic trigger RNAs can be assayed for interference activity *in vivo* by a straightforward injection assay. This provides a sensitive means to test model substrates and to analyze the effects of various perturbations to the RNA trigger. In this paper, we investigate the chemical and sequence requirements for RNA-triggered gene silencing in *C. elegans*.

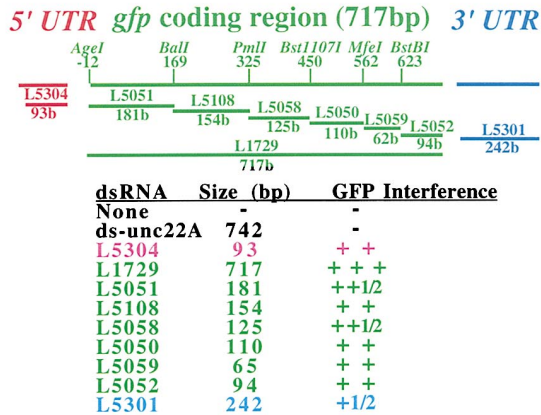
## Results

### Requirements for Length and Sequence Composition of the RNAi Trigger

From first principles, it was conceivable that RNA-mediated interference required a specific sequence in the interfering RNA or target RNA. The ability to target large numbers of different genes in *C. elegans* for interference suggests that any such sequence must be common in

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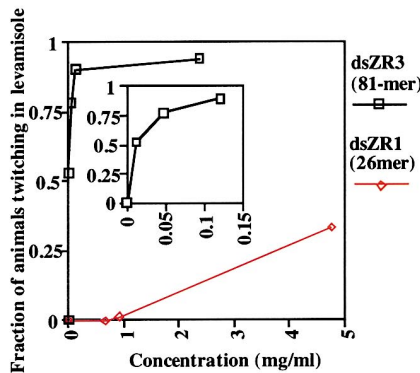
A



B

dsRNA/bp Start	Sequence of sense strand	Fraction F1 twitching	
		16°	25°
zr1 26	uccucugucucgucuccucucggcggg (No A)	26/124	0/200
zr9 27	aaccacucuccaccaauaacaau (No G)	0/600	0/80
zr11 32	aaaaagcaagccaacgaaccaaaggaccaa (No U)	16/174	0/80
zr7 37	aaguugaagguuaagaugauugggagaggaugaag (No C)	32/100	27/100
zr3 81*	gugcucggaaaaccacuagcccauugggaccuuuggaagugucg aaugucuacgaagaucgcgcagauuuggaguggaaa	96/100	94/100

C



the genome. Sequences commonly used for interference are sufficiently long (400–1000 bp) that a requirement for a short motif in the interfering segment might have been missed. As a more stringent test for the generality of RNAi, eight nonoverlapping dsRNA segments of 62–242 bp that span the 717 base *gfp* coding region (as well as the 5' and 3' nontranslated regions) were assayed for ability to interfere with GFP expression. Each of these segments produced an interference effect (albeit of slightly different magnitudes), indicating a lack of strong sequence specificity in interference (Figure 1A).

The *unc-22* gene provides a somewhat more sensitive assay for gene function than *gfp* (see Experimental Procedures). Chemical synthesis was used to prepare sense and antisense oligoribonucleotides corresponding to five nonoverlapping segments of 26, 27, 32, 37, and 81 nt from *unc-22* (Figure 1B). The four shorter segments were distinguished in nucleotide composition: these correspond to the longest segments of the *unc-22* mRNA that lack A, G, U, and C residues, respectively. When injected as dsRNA, four of the segments

Figure 1. Sequence and Size Requirements for the RNA Trigger

(A) A series of nonoverlapping fragments of the *gfp* coding region. dsRNAs corresponding to each fragment were prepared enzymatically and injected at concentrations in the range of 20–50  $\mu$ g/ml into transgenic animals carrying a *myo-3::gfp* transgene (PD4251, see Experimental Procedures). Interference levels (loss of GFP) were quantitated as described in Experimental Procedures. Slight differences in potency between the short RNAs could reflect some variation in concentration or purity between RNA preparations. (B) Five short dsRNA sequences from the *unc-22* coding region that were prepared synthetically. Fractions shown are numbers of F1 progeny following injection that twitched in 0.3 mM levamisole. Synthetic sense and antisense strands were annealed and injected into wild-type *C. elegans* (concentrations 9.5 mg/ml [zr7, zr9, zr11]; 4.8 mg/ml [zr1]; 2.3 mg/ml [zr3]. Additional injections of zr11 at 19 mg/ml showed no appearance of twitching progeny. As controls, single-stranded (sense and antisense) molecules of zr1 and zr3 were injected separately (4.8 mg/ml), with no twitching phenotypes seen in >100 progeny each. Additional injections of dsRNA for zr1 and zr3 into the RNAi-resistant strains *rde-1(ne219)*, *rde-2(ne221)*, *rde-3(ne298)*, and *rde-4(ne299)* likewise produced no twitching progeny. All duplexes were blunt-ended except for zr3, for which the antisense strand had a 4 base 5' extension.

(C) Concentration dependence for interference by dsRNA from zr1 and zr3.

(all except for the 27-mer lacking G) caused an *Unc-22* phenotype in a fraction of progeny. The short dsRNAs were not equal in their interference activities (Figure 1B); in particular, titrations of the 81 bp and 26 bp dsRNAs showed at least a 250-fold higher concentration requirement for the 26 bp RNA (Figure 1C). The high concentration of the short dsRNA required for interference raised the possibility that this sequence might be interfering with gene expression by an alternative mechanism. In particular, high doses of the 26-mer might conceivably obviate the need for an amplification or catalytic component of the interference. To address the relationship between interference induced by the 26-mer and interference induced by longer dsRNAs, we assessed the requirements for the two strands and for the products of four genes that are required for full susceptibility to RNAi (*rde-1*, *rde-2*, *rde-3*, and *rde-4*; Tabara et al., 1999). Interference by the 26-mer required the products of the four *rde* genes and required both strands (data not shown), indicating common (if not identical) elements in the action of this dsRNA and that of previously characterized larger dsRNAs.

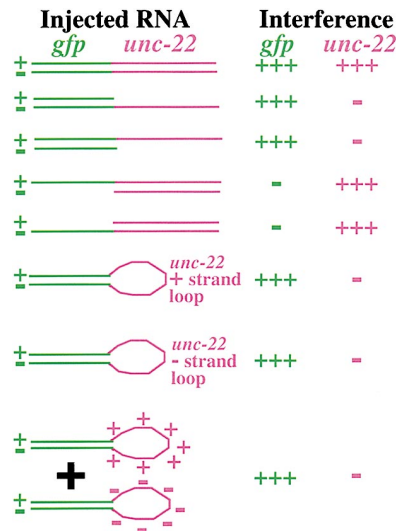


Figure 2. Requirements for Double-Stranded Character in the RNAi Trigger

The RNA molecules described in this figure are composed of segments corresponding to the entire 717 base *gfp* coding region (green) and a 1034 base coding segment of *unc-22* (*unc-22B*) [Fire et al., 1998]; red). RNAs were synthesized by standard enzymatic methods, annealed, and injected at approximately 40  $\mu$ g/ml.

Although no interference activity was observed for the 27-mer dsRNA lacking G, it is possible that a higher dose or lower growth temperature would have shown such an effect. Alternatively, this sequence might correspond to an inaccessible region of the target transcript (due to secondary structure or protein coat).

Taken together with the *gfp* data, the ability of four short double-stranded oligoribonucleotides with dramatically different sequence compositions to produce a specific *Unc-22* phenotype argues strongly against any sequence requirement for RNAi.

#### Requirements for Double-Stranded Character in the Interfering RNA

In earlier studies for which RNA was prepared enzymatically with T3 or T7 RNA polymerase, we had observed residual interference even with the most highly purified single-stranded RNA preparations [Fire et al., 1998]. This left open the question of whether ssRNAs were capable of inducing interference or whether interference seen with ssRNA preparations was due to contaminating dsRNA. With the ability to make highly active interfering RNAs using an oligonucleotide synthesizer, it became possible to test this in a more critical manner. For the 81 bp duplex (the most active of the dsRNA oligonucleotides), we observed no interference with sense or antisense alone, even when injected at concentrations (10 mg/ml) that were 1000-fold above the effective concentration for the equivalent dsRNA (Figure 1C and data not shown).

Observations that an effective RNAi trigger requires both sense and antisense strands leave several possibilities as to the contributions of the two strands to the triggering mechanism. Figure 2A shows experiments de-

signed to test whether interference by ssRNA could be stimulated by covalent linkage to an unrelated segment of dsRNA. Each molecule has an ssRNA region corresponding to one target gene linked to a dsRNA region corresponding to a second target. When injected into *C. elegans*, these molecules produce interference effects corresponding only to the double-stranded portion of the trigger. These experiments indicate that triggering of RNAi requires both strands in the region of identity to the target.

A second means to address the nature of the two-strand requirement comes from experiments in which both strands of RNA are provided to the animal under conditions that either favor or prevent formation of duplex. Although the two strands of *unc-22* RNA can produce an interference effect when injected separately into the same animal, a stimulation of activity is seen if the strands are first annealed before injection [Fire et al., 1998]. To further investigate the requirements for formation of dsRNA structure, we produced a pair of stem-loop molecules in which the two strands of *unc-22* were both present but would be prevented from rapid hybridization by topological constraints. No interference with *unc-22* function was observed when these two stem-loop molecules were coinjected (Figure 2B). These results indicate a requirement for sense/antisense duplex formation in the RNAi trigger.

#### Requirements for Homology between Trigger and Target RNAs

To assess homology requirements for RNAi, we used a series of altered *gfp* coding regions with different degrees of identity to the transgene target (Figure 3A; Cramer et al., 1996; Cormack et al., 1997; Fukumura et al., 1998; Cohen and Fox, unpublished data). We found effective interference with dsRNAs that were 96% identical to the target sequence (193 nt maximum uninterrupted identity), less effective interference with a trigger that was 88% identical (41 nt maximum uninterrupted identity), and no interference with dsRNA triggers that were 78% or 72% identical to the target (23 and 14 nt maximum uninterrupted identity; Figure 3B). By comparison with dilutions of the wild-type *gfp* dsRNA, we found that the 78% identity and 72% identity RNAs reduced the effectiveness of interference by at least 100-fold (data not shown).

#### Requirements for Quality of the Interfering RNA Duplex

The availability of altered *gfp* isoforms allowed us to test the triggering capacity of heteroduplexes in which mismatches between the two interfering strands were present. As shown in Figure 3C, such heteroduplexes can be triggers for RNAi, but with reduced effectiveness when compared to perfectly matched duplexes. No significant difference was observed as a function of which strand was more closely related to the RNAi target.

#### Saturation of the RNAi Machinery in *C. elegans*

To measure the saturability of the RNAi machinery in *C. elegans*, we carried out experiments in which a defined concentration of a single dsRNA species (an 81 bp segment of *unc-22*) was mixed with increasing concentra-

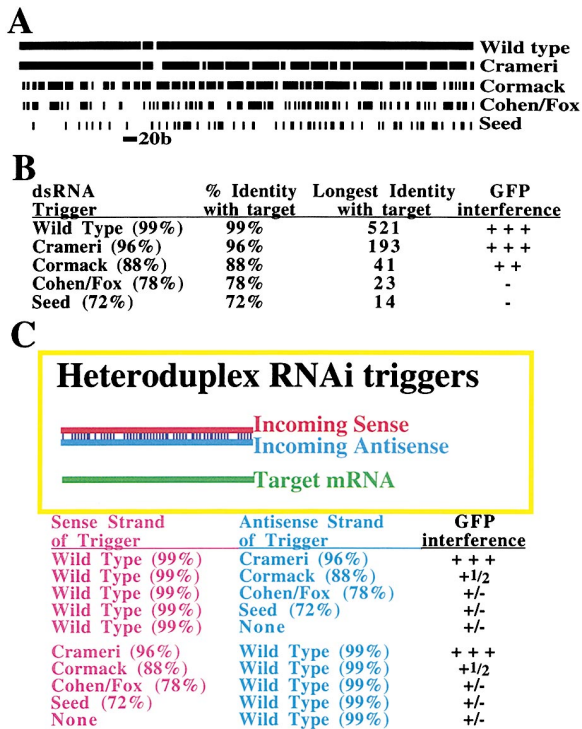


Figure 3. Requirements for Homology between Trigger and Target RNAs

Wild-type *gfp* and four multiply mutant versions were inserted into standard vectors for synthesis of RNA using T3 and T7 RNA polymerases. "Crameri" (GFP-uv; Crameri et al., 1996) is a result of combinatorial mutagenesis, "Cormack" (Cormack et al., 1997), "Cohen/Fox" (Cohen and Fox, unpublished data), and "Seed" (Fukumura et al., 1998) are codon-optimized synthetic forms of the gene that were originally produced for use in yeast nuclei, yeast mitochondria, and human cells, respectively.

(A) Regions of identity between the five versions of GFP and the target gene over the 717 bp GFP coding sequence. (Note that the *gfp* variant used in the transgene target differs at five positions from wild-type *gfp*.) Darkened bars represent regions of identity of  $\geq 5$  bp.

(B and C) Interference activities of homoduplex and heteroduplex dsRNAs. Single-stranded RNAs were prepared from each gene and annealed as described in Experimental Procedures and injected at concentrations of approximately 40  $\mu$ g/ml into GFP reporter strain PD4251. GFP levels in progeny animals were scored by counting GFP-positive cells in L4 larvae and young adults (see Experimental Procedures). Although the wild-type *gfp* sense and antisense RNAs in these experiments were purified through two rounds of electrophoresis, we were unable to completely remove low level interference activity of each ssRNA preparation alone ("+/-" signals, presumably due to trace levels of dsRNA contamination). For this reason, a low level of interference by Cohen/Fox::WildType or Seed::WildType heteroduplexes would have been missed.

tions of an unrelated competitor dsRNA. The competitor dsRNA (codon-humanized *gfp*) was unrelated to any gene in *C. elegans*. We found in these experiments that the RNAi effect was indeed saturable (Figure 4A). Saturation of the response required double-stranded character in that the individual single strands of the competitor RNAs had less than 2% of the competitor activity seen with dsRNAs (data not shown). Competition by the long *gfp* dsRNA was surprisingly effective, with complete competition observed even with a 5-fold lower mass of

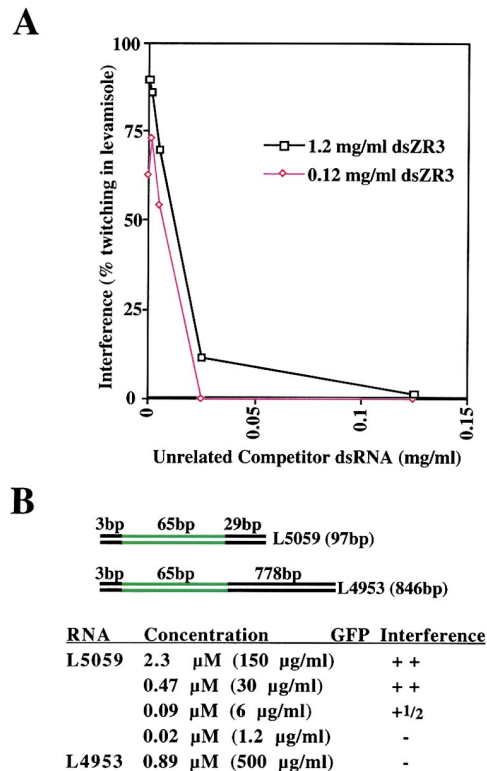


Figure 4. *cis* and *trans* Saturability of the RNAi Machinery in *C. elegans*

(A) Response following injection of wild-type animals with mixtures of a synthetic dsRNA oligonucleotide (zr3, 81 bp) with increasing concentrations of an unrelated dsRNA (enzymatically prepared dsRNA corresponding to the humanized "Seed" version of *gfp* (724 bp). Percentage of twitching animals (in 0.3 mM levamisole) amongst F1 progeny is shown.

(B) Two dsRNAs that each have 65 bp of identity to a *gfp* reporter transgene (PD4251, see Experimental Procedures). The shorter RNA (L5059, see Figure 1A) has only short segments on each end (derived from the vector multiple cloning site), while the L4953 RNA is linked in *cis* to an extended region of dsRNA corresponding to *unc-22*. The progeny of animals injected with L4953 RNA show a strong twitching phenotype but no evident reduction in the number of cells with GFP fluorescence. Note that a longer region of *gfp* (717 base) dsRNA in *cis* to *unc-22* dsRNA produced effective *gfp* interference (line 1 of Figure 2).

the competitor (24  $\mu$ g/ml of competitor dsRNA versus 120  $\mu$ g/ml of interfering RNA). This could reflect a general preference for longer dsRNA in triggering RNAi, greater stability of the longer dsRNA, or specific properties of the individual sequences used in the competition assay.

It was conceivable that the ability to saturate the RNAi response represented a limitation in the number of dsRNA molecules that could be recognized. This could be the case, for instance, if the limiting factor were responsible for the initial recruitment of individual dsRNA molecules to a multicomponent complex. To address the nature of the saturation, we produced molecules in which competitor RNA was linked in *cis* to the interfering RNA. These experiments demonstrated that triggering activity of a dsRNA could be effectively competed by excess RNA provided in *cis* (Figure 4B).

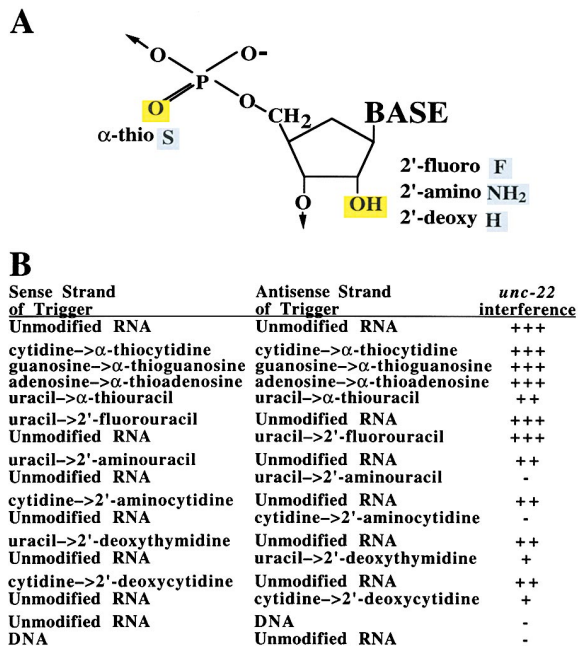


Figure 5. Effects of Backbone Modifications on Activity of the RNAi Trigger

(A) A schematic RNA backbone with a description of the modifications used in this work. Note that the Uracil→deoxyThymidine substitution also entails a change in the base moiety (see Figure 6A). (B) Activities of backbone modified RNAs. RNAs were prepared as described in Experimental Procedures, annealed, and injected into wild-type *C. elegans*. The degree of interference was assessed by examination of phenotypes of progeny animals with and without levamisole treatment. Although assays were in general qualitative, we have included an indicator of interference strength based on severity of phenotypes for the majority class of progeny animals scored. Unmodified RNA and modified RNAs described as “+++” produced a strong twitching phenotype in the absence of levamisole (Fire et al., 1998). Modified RNAs described as “++” produce a weak twitching phenotype that is evident without levamisole treatment as a twitch during movement; these animals twitch strongly following levamisole treatment. Interference described as “+” was observed only in levamisole and only in a fraction of progeny. Interference described as “+/-” was at a level that was indistinguishable from the preparations of unmodified sense or antisense RNA used to make the modified duplex. Titrations of unmodified RNA (Fire et al., 1998) indicate that levels of twitching described as ++ correspond to activities in a range of 3%–10% of unmodified RNA, while those described as “+” correspond to 1%–3% of unmodified RNA. 2'-fluorouracil, 2'-aminouracil, 2'-deoxythymidine, and 2'-deoxycytidine were incorporated into individual strands of the 742 nt *unc-22A* segment using T3 and T7 RNA polymerases (Experimental Procedures). Incorporation of  $\alpha$ -thio nucleotides was carried out with an RNA hairpin carrying *unc-22* sequences in the stem. This allows rapid formation in the polymerase reaction mix of duplex structure, which appeared to stabilize the resulting modified RNA. RNA concentrations in injection mixes were in the range of 30–60  $\mu$ g/ml. RNA:DNA hybrids were constructed with synthetic RNA and DNA corresponding to the 81 nt *nr3* segment (Figure 2) and were injected at 5 mg/ml.

### Chemical Requirements in the RNA Trigger: Phosphate–Sugar Backbone

We tested several modifications in the phosphate–sugar backbone for their effects on the ability of dsRNA to trigger interference (Figure 5). Modification of phosphate residues to thiophosphate could be effectively carried

out by incorporating thiophosphate nucleotide analogs with T7 and T3 RNA polymerase. Although the  $\alpha$ -thiophosphate modifications caused some chemical instability in the RNA, we were able to demonstrate interference activity following incorporation of any single modified residue. Modifications of A, C, or G residues were compatible with full interference activity, while modified U caused some decrease in interference activity (Figure 5B). Interestingly, Zamore et al. (2000) have noted a preference for U residues in RNA-associated cleavage in vitro. RNAs with two modified bases also had substantial decreases in effectiveness as RNAi triggers (data not shown); modification of more than two residues greatly destabilized the RNAs in vitro and we were not able to assay interference activities.

A second position at which modifications were tested was the 2' position of the nucleotide sugar. Modification of cytidine to deoxycytidine (or uracil to thymidine) on either the sense or the antisense strand of the trigger was sufficient to produce a substantial decrease in interference activity (Figure 5B). In the case of cytidine to deoxycytidine substitution, this effect must be a consequence of a change at the 2' position, while the effects of uracil to thymidine substitution could reflect effects of the additional methyl group on the thymidine base. Modification of uracil with 2'-fluorouracil was compatible with RNAi activity, while modification with 2'-aminouracil or 2'-aminocytidine produced a decrease in activity comparable to that seen with the deoxynucleotide modification.

A second means to assess requirements at the 2' position involves the question of whether RNA:DNA hybrids can trigger RNAi. Such hybrids were prepared synthetically and enzymatically and found to lack interference activity (Figure 5B and data not shown).

Interestingly we observed a preferential effect on interference activity for several modifications (uracil→2'-aminouracil, cytidine→2'-aminocytidine, uracil→thymidine, and cytidine→2'-deoxycytidine) depending on whether the sense or antisense strand was modified. In each case, trigger activity was more sensitive to modification of the antisense strand than of the sense strand.

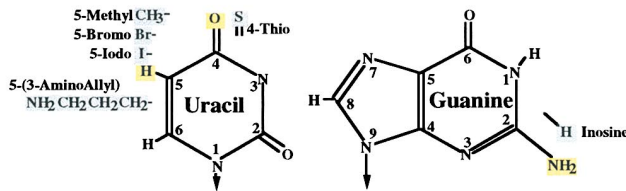
### Chemical Requirements for the RNA

#### Trigger: RNA Bases

We tested a small number of RNA base modifications for their effects on the efficacy of RNA interference reaction. The modifications were chosen based on the commercial availability of nucleoside triphosphates that could be incorporated using T3 or T7 RNA polymerases. Five such base analogs (Figure 6A) were tested; the uracil analogs 4-thiouracil, 5-bromouracil, 5-iodouracil, and 5-(3-aminoallyl)-uracil could be readily incorporated in place of uracil, while inosine was incorporated in place of guanosine.

As with the backbone modifications, we were particularly interested to learn whether there were distinct base requirements for the two strands of the RNAi trigger. As shown in Figure 6, 4-thio-uracil and 5-bromo-uracil (which were compatible with interference) and inosine (which was compatible but produced a substantial decrease in interference activity) showed no detectable difference in effect between the two strands. By con-

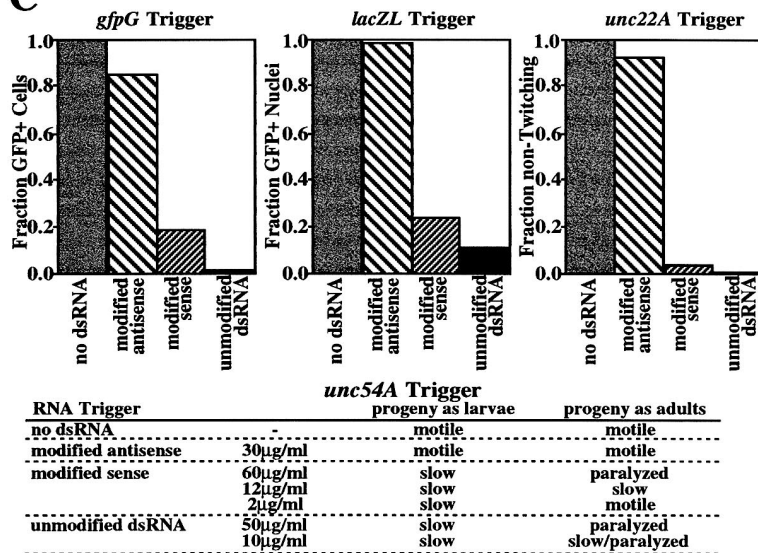
A



B

Sense Strand of Trigger	Antisense Strand of Trigger	<i>unc-22</i> interference
Unmodified RNA	Unmodified RNA	+++
uracil->4-thiouracil	Unmodified RNA	+++
Unmodified RNA	uracil->4-thiouracil	+++
uracil->5-bromouracil	Unmodified RNA	+++
Unmodified RNA	uracil->5-bromouracil	+++
uracil->5-iodouracil	Unmodified RNA	++
Unmodified RNA	uracil->5-iodouracil	+
uracil->5-(3-aminoallyl)uracil	Unmodified RNA	++
Unmodified RNA	uracil->5-(3-aminoallyl)uracil	+/-
guanosine->inosine	Unmodified RNA	+
Unmodified RNA	guanosine->inosine	+

C



trast, 5-iodouracil and 5-(3-aminoallyl)-uracil showed remarkably strand-specific effects, with substantially greater effects on the antisense strand.

It was important to test whether greater sensitivity to modification for the antisense strand was a general phenomenon or a unique property of the *unc-22A* segment used as a trigger. Although the sense and antisense strands of the *unc-22* had comparable base composition, it was conceivable that some specific feature of the sequence might account for the difference in susceptibility between the two strands. We therefore tested three other RNAi trigger segments for sensitivity to modification by 5-(3-aminoallyl)-uracil (Figure 6C). For each trigger, we observed (a) full or near full activity with a modified sense strand and (b) greatly reduced activity of the modified antisense strand. We obtained a rough quantification of the effect by carrying out titrations of the active sense-modified RNAs. These experiments indicated minimal differences of 10- to 25-fold between the antisense and sense strands in susceptibility of trig-

Figure 6. Effects of Base Modification on Activity of the RNAi Trigger

(A) Formulas for uracil and guanosine with a description of the modifications used in this work.

(B) Activities of backbone modified RNAs. RNAs were prepared as described in Experimental Procedures, annealed, and injected into wild-type *C. elegans*. The degree of interference was assessed as in Figure 5.

(C) Activities of 5-(3-aminoallyl)-uracil substituted RNAs. The triggering segments *unc22A*, *gfpG*, *lacZL*, and *unc54A* were as described in Fire et al. (1998). RNA concentrations were 30 µg/ml for *lacZL* and *gfpG*, and 40 µg/ml for *unc-22A*. *gfpG* and *lacZL* injections were carried out in strain PD4251 (Fire et al., 1998). *unc-22A* and *unc-54A* injections were carried out in wild type (N2) animals. For *unc-22A* injections, twitching fractions shown are in the absence of levamisole (L4 larvae and young adults). Some animals with weak twitching in the presence of levamisole were observed in antisense-modified *unc-22A* injections (57/200 animals); this was only marginally above the background level of weak levamisole-induced twitching following injection of the preparation of sense RNA alone (30/200 animals); this limited signal could represent residual contamination of dsRNA in the individual ssRNA preparations. For *unc-54A* injections, movement was scored in L1 larvae and again in young adults. Titrations of 5-(3-aminoallyl)uracil sense-modified RNAs for *unc-54A* indicate that this RNA is at least 15-fold more active than the equivalent antisense modified *unc-54A* trigger. Similar experiments with *lacZL*, *gfpG*, and *unc-22A* indicate in each case at least a 10-fold differential strand effect for 5-(3-aminoallyl)uracil substitution.

ger activity to 5-(3-aminoallyl)-uracil modification (Figure 6C and data not shown).

There are two situations in which a given base modification might block activity of the trigger. The first would involve a specific need for the unmodified base in the function of the trigger RNA, while the second would involve an ability of the modified base to block normal function of the dsRNA. The ability of a 26-mer dsRNA lacking uracil in the antisense strand (Figure 1B) to function as an interference trigger argues against any specific requirement for chemical groups on uracil. Instead, it seems likely that the large substituents on the 5-position of uracil act by sterically blocking recognition or catalysis at a key step in RNAi.

#### Fate of Labeled RNA Molecules Injected into *C. elegans*

To follow the fate of RNA injected into *C. elegans*, we injected uniformly labeled (<sup>32</sup>P) dsRNA into *C. elegans*. The ability of the labeled RNA to produce interference

was confirmed by the appearance of an Unc-22 null phenotype. RNAs from injected animals were then extracted and analyzed by polyacrylamide gel electrophoresis followed by autoradiography. Two conclusions can be drawn from the patterns of labeled dsRNAs that are observed. First, a substantial fraction of the injected dsRNA persists as high molecular weight material. Second, we see evidence for cleavage of the injected dsRNA into a population of short RNA segments. These segments migrate in a band of approximately 25 nt and are thus similar in size to small RNA populations that have been seen to associate with PTGS effects in plants and in extracts of *Drosophila* cells (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000). In our experiments, we found a relatively small fraction of cleaved RNAs, accounting for approximately 1% of the recovered radioactivity.

### Discussion

In characterizing the molecular requirements for dsRNA-triggered genetic interference in *C. elegans*, we have observed the outlines of an effective, ancient, and highly specific cellular defense mechanism. Although the parameters of the RNAi-triggered silencing mechanism might be expected to vary between biological systems, it seems likely that the central aspects of the mechanism will be conserved. These key features are the double-stranded character of the trigger, the ability to target native or "foreign" mRNAs for degradation, and the molecular nature of at least a subset of protein components involved in the machinery.

### Requirements for the RNAi Trigger in *C. elegans*

In *C. elegans*, we find that a broad range of dsRNA sequences are capable of triggering the interference process. Interference was induced with duplexes as short as 26 nt and with a variety of nucleotide compositions. Using short dsRNA triggers, we were able to rule out a specific requirement for any sequence motif in the trigger or target RNA and were able to rule out any requirement for A, U, or C residues in the targeted sequence.

The lack of a requirement for adenine in the target sequence is interesting in light of models in which modification by ADAR (adenosine deaminase acting on RNA; Bass, 1997) was proposed as a potential component of RNAi (Wagner and Sun, 1998; Sharp, 1999). Such models would generally predict a requirement for adenine in the target or in one of the two trigger strands. Zamore et al. (2000) observed limited ADAR activity in *Drosophila* extracts carrying out RNAi, although they found no evidence for a functional connection. Our results argue strongly against models in which RNAi requires ADAR modification of the target RNA. (Note that ADAR-based models in which either of the two strands can be modified, although somewhat less plausible, are not ruled out by our data).

**A Limited Capacity for Utilization of the dsRNA Trigger** Encouraged by preliminary data from colleagues optimizing RNAi as a tool for functional genomic screens (P. G6nczy, C. Echeverri, and A. Hyman, personal com-

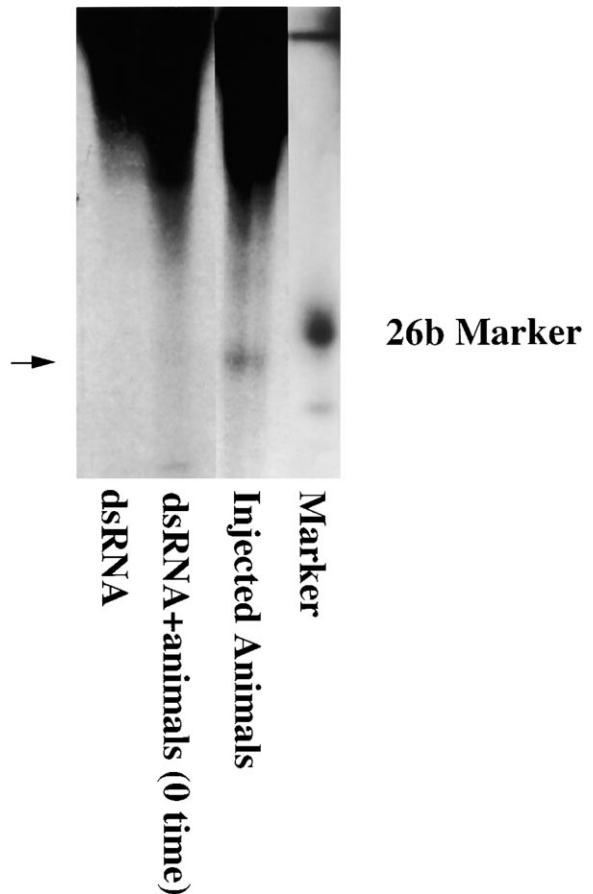


Figure 7. Fate of Radioactive dsRNA Injected into the *C. elegans* Germline

A radioactively labeled dsRNA segment was prepared enzymatically by incorporation of <sup>32</sup>P UTP during synthesis with T3 and T7 RNA polymerase. The two strands were extracted, hybridized (without further purification), and injected into 70 wild-type *C. elegans* adults. An 1172 nt segment covering sequences from *gfp* and *unc-22* was used and was labeled to a specific activity of 15 Ci/mMol UTP. Injection mixes contained approximately 2.5 mg/ml labeled RNA. After 12 hr at 16°C, RNAs were extracted and resolved by electrophoresis on a denaturing polyacrylamide gel (20% acrylamide, 8.3 M urea, 10 mM EDTA, 100 mM Tris-Borate [pH 8.4]), and exposed to X-Ray film for 7 days. Also shown are the input dsRNA ("dsRNA"), an aliquot of the RNA mixed with *C. elegans* and taken through a mock extraction ["dsRNA+animals (0 time)"] and a 26 base ssRNA marker (5'-end-labeled *zr1*; see Figure 1B). A set of parallel injections with <sup>32</sup>P UTP (injected with or without unlabelled dsRNA) were carried out to address the possibility that the ~25 nt material observed in these experiments might represent degradation of the input RNA and reincorporation of label into short cellular RNA species; no evidence for such reincorporation was observed (data not shown).

munication), we carried out competition experiments that document a limited capacity of the RNAi system in *C. elegans* to utilize injected dsRNA. Combined with similar competition experiments with *Drosophila* embryo extracts (Tuschl et al., 1999), these results should be considered in interpreting genetic requirements for RNA interference. In particular, any mutation or treatment that (directly or indirectly) causes an increase in the native concentration of target-unrelated dsRNA could potentially block the ability of exogenous dsRNA to produce interference.

### Does Triggering of RNAi Require an A Form Helical Structure?

We found a requirement not only for providing the two interfering strands for an RNAi trigger, but also for allowing the strands the opportunity to hybridize to form an RNA duplex. Although this process can occur in vivo if the strands are provided in the same area in rapid succession, the instability of ssRNA in vivo seems to ensure that the process will be most effective when the introduced RNA is double stranded in character. Consistent with this conclusion, we found that topological manipulations that would inhibit rapid duplex formation prevented interference by mixtures of sense and antisense RNA.

A limited set of chemical modification experiments begin to address the structural features required in the trigger. Modifications with a tendency to reduce helical A form character (2'-deoxy and 2'-amino substitutions for one of the four bases on one of the two trigger strands) decreased the effectiveness of the trigger, while modification of at least 1/4 of bases to a 2' fluoro group (which preserves A form structure; e.g., Cummins et al., 1995; Lesnik and Freier, 1995) were compatible with function as an RNAi trigger.

### Differential Function of the Two Trigger Strands

We found that large chemical substitutions on the 5-position of the uracil base were compatible with effective RNAi when present on the sense trigger strand, but not on the antisense strand. Several backbone modifications also appeared to preferentially block RNAi when carried out on the antisense strand. These results argue that the two strands have distinct roles at some stage in the interference process; the simplest interpretation would be to propose that the antisense strand of the trigger could interact directly with the target RNA, with the sense strand participating less directly in the RNAi process (perhaps protecting the antisense strand from degradation and allowing recognition of the trigger as an aberrant [ds] molecule).

Two modifications (substitution of inosine for guanine [Figure 6B] and sequence divergence [Figure 6C]) decreased trigger activity in a manner that appeared independent of which trigger strand was modified. These modifications presumably inhibit RNAi at a stage when the two trigger strands are still associated (e.g., by increasing nuclease attacks on the trigger or by partially blocking trigger recognition and melting by the RNAi apparatus).

### Evidence against Exponential Amplification of Trigger RNA

Sequence similarity between a purified RNA-dependent RNA polymerase (RdRP) from Tomato (Schiebel et al., 1998) and the coding regions of several genes that can mutate to produce altered PTGS/RNAi responses (Cogoni and Macino, 1999; Dalmay et al., 2000; Mourrain et al., 2000; Smardon et al., 2000) have supported suggestions that a physical amplification of trigger sequences might contribute to the remarkable efficacy of PTGS/RNAi. *C. elegans* and *Arabidopsis* each have multiple RdRP-like coding regions; in each case, the identified mutations affect only one RdRP and only block

a subset of RNAi/PTGS effects. This leaves the possibilities (A) that some RNAi/PTGS processes are RdRP-independent or (B) that different RdRP factors are responsible for distinct RNAi/PTGS processes.

Three types of models to account for an RdRP contribution to RNAi/PTGS (not mutually exclusive) could be envisioned: (1) RdRP might convert an aberrant ssRNA population to a duplex RNAi trigger; (2) RdRP might repeatedly copy the dsRNA trigger to produce a population of ssRNAs that could then interact with target RNA; and (3) RdRP might produce an exponential amplification in which copies of the trigger are subject to further copying to generate a "self-replicating" trigger population. Our data are consistent with models such as the first, in which RdRP might play no role in interference by an RNA that is already double stranded. Our data would place a significant constraint on models of the second class. The differential requirements that we observed (always a more stringent requirement on the antisense trigger strand) would imply a critical role of the nontemplate strand in utilization by RdRP (e.g., recognition by a helicase or ssRNA-binding protein to allow copying of the template strand). The third set of models would not be consistent with our data, since exponential amplification would produce a situation in which the differential contributions of the original trigger strands would be lost during replication. It should be noted that the experiments in this paper were all carried out using assays for genetic interference in somatic tissues of the animal in the first generation after injection. It is conceivable that distinct mechanisms might operate in longer term RNAi (Tabara et al., 1999; Grishok et al., 2000) or in specific tissues, such as the germline.

### Does RNAi Involve Short dsRNAs Derived from the Original Trigger?

Hamilton and Baulcombe (1999) observed a population of small (25 nt) RNAs associated with PTGS in plants. These short RNAs could represent RdRP products or products resulting from cleavage of the target and/or trigger RNAs. Subsequent experiments in *Drosophila* cell extracts showed copurification of a specific RNA degrading activity with ~25 bp RNAs (Hammond et al., 2000). A family of enzymes designated RNaseIII can degrade dsRNA duplexes to produce segments of defined size (Nicholson, 1996; Rotondo and Frendewey, 1996). Although the ubiquity of such enzymes is consistent with a critical role in RNAi, an alternative hypothesis (that the short RNAs have no role in RNAi but simply reflect exposure of the dsRNA trigger to abundant RNaseIII) must also be considered (Bass, 2000).

We found evidence for a population of ~25 nt RNAs following injection of labeled dsRNA into *C. elegans*. A key aspect of our results, consistent with recent results from *Drosophila* embryonic extracts (Zamore et al., 2000), has been to show that the short RNA products are at least in part derived directly from the trigger RNA. The fraction of dsRNA converted to small fragments (approximately 1% of the total input material) was lower in *C. elegans* than had been observed in the *Drosophila* extracts. This could reflect a low fraction of injected dsRNA participating in RNAi at a given time in *C. elegans*; alternatively, it remains conceivable that the ~25 nt

RNAs are a byproduct of RNAi rather than a direct participant.

Why cleave the trigger RNA to short fragments? We envision three benefits to an organism fighting viral infection. First, the fragmentation could increase the molarity of antiviral complexes within the cell and allow more efficient dissemination through the organism. Second, cleavage of a viral dsRNA into small fragments would irreversibly break up the viral genome, thus eliminating any possibility that the RNA interference machinery would contribute unwittingly to viral spread. Third, cleavage to segments of 21–25 nt might provide optimal specificity for a homology-based searching mechanism. Given the size of eukaryotic genomes, substantially shorter segments (e.g., 10–15 bp) would leave insufficient specificity to ensure against unwanted targeting of cellular genes, while a much longer segment than 25 nt might allow unwanted attacks on cellular genes with partial but extended identity to the trigger.

#### Experimental Procedures

##### Enzymatic RNA Preparation

Standard reaction conditions were used for initial RNA synthesis with T3 and T7 RNA polymerases (Fire et al., 1998). As previously reported, the unpurified “sense” and “antisense” reaction products show a substantial level of interference that can be greatly decreased if the RNAs are first purified by gel electrophoresis. With three exceptions, all enzymatically prepared RNAs described in this work were gel purified. The exceptions were (1) competitor RNA (Figure 4) and (2) <sup>32</sup>P RNA (Figure 7), for which single-strand purity was not critical, and (3)  $\alpha$ -thio RNAs (see Figure 5). As a consequence of restriction enzymes used to digest vector sequences, each dsRNA that was prepared enzymatically had 3–30 nt of dsRNA derived from polylinker sequences on each end, and polylinker-derived single stranded tails of 10–30 nt.

Incorporation of modified bases was carried out by replacing one of the four nucleoside triphosphates completely with a modified version of the base (each nucleotide was present at 0.5 mM). Omission of any of the four nucleotide triphosphates (with no substitution) resulted in a absence of any observed RNA product. This suggests that the synthetic RNAs have complete or nearly complete replacement with the modified base. Several modified nucleotides required modified reaction conditions for incorporation. Inosine was incorporated in place of guanine (T3 RNA polymerase only) by including a dinucleotide (GpG; 0.25mM) to promote initiation. Deoxycytosine and deoxythymidine were incorporated by including 20mM MnCl<sub>2</sub> in the synthesis reaction (similar to the procedure of Conrad et al., 1995) or using modified versions of the T3 and T7 RNA polymerases described by Sousa and Padilla (1995).

Following gel purification, RNAs were annealed in 25 mM KAC, 10 mM Tris, 1 mM EDTA (pH 7.0) by briefly heating to 70°C, then incubating for 20 min each at 37°C and 23°C. Formation of RNA duplexes was confirmed by a shift in mobility of duplex RNAs relative to the individual (nonhybridized) strands analyzed by agarose gel electrophoresis. With the exception of inosine-modified RNAs (which are distinctly retarded in gel mobility), none of the modifications affected gel mobility. All chemically modified RNAs readily formed duplex material under the conditions used. Sequence-heteroduplex RNAs (Figure 3) likewise formed readily under these conditions, with the exception of the most distantly related heteroduplex (Seed/Wild Type), which could not be confirmed by gel electrophoresis. (For this case, therefore, the injected RNAs may have been nonhybridized.) Concentrations of dsRNA were estimated (within 2-fold) from the intensity of ethidium bromide staining of gels. Substantial differences in ethidium bromide staining in RNAs resulting from one modified strand seem unlikely for the following reason: in experiments in which a constant concentration of an unmodified ssRNA was annealed to several different complementary RNAs, we observed efficient conversion to dsRNA mobility with only modest differences in total ethidium bromide staining.

Nucleotides were obtained from Pharmacia (ATP, GTP, CTP, UTP, dCTP, dTTP, 2'-amino-UTP, 2'-amino-ATP, 2'-amino-CTP, 2'-fluoro-UTP, 2'-fluoro-CTP, 4-thio-UTP), Amersham ( $\alpha$ -thio ATP,  $\alpha$ -thio CTP,  $\alpha$ -thio GTP), Dupont ( $\alpha$ -thio UTP), and Sigma (GpG, inosine triphosphate, 5-[3-aminoallyl]UTP, 5-iodo-UTP, 5-bromo-UTP).

##### Chemically Synthesized RNAs

Synthetic RNAs were prepared using 2'-O-(triisopropyl)silyloxy-methyl chemistry by Xeragon AG (Zurich Switzerland) and further purified by anion exchange HPLC. These RNAs appeared highly pure by electrophoretic analysis and formed stable dsRNA duplexes in vitro under standard hybridization conditions described above. Two independent preparations were tested for each strand of zr1, giving equivalent results. For each other oligo pair, only one preparation of each oligonucleotide was tested. Although the 2'-O-(triisopropyl)silyloxymethyl chemistry provides generally high quality RNA preparations (P. Weiss, personal communication; Caplen et al., 2000), we cannot rule out the possibility that some or all of the synthetic RNA populations contained altered chemical forms.

##### Assays for RNA-Mediated Interference

Assays for genetic interference were carried out by injecting the germline of adult animals with modified and unmodified RNAs and analyzing F1 progeny for functional consequences; phenotypic assays were carried out on progeny animals that were produced from eggs fertilized in the first day after injection, since this cohort of progeny have in general been found to respond most efficiently to RNA interference (Fire et al., 1998).

*unc-22* encodes an abundant striated muscle component named “twitchin” as a result of the characteristic twitching phenotype seen in *unc-22* null mutations (Moerman et al., 1988). Conventional genetic analysis of scores of independently isolated mutants with this twitching phenotype (Brenner, 1974; Moerman and Baillie, 1979; Moerman and Fire, 1996), suggest that *unc-22* is the only gene for which loss-of-function produces such a phenotype (although twitching phenotypes are produced by rare non-null alleles of at least two other genes). In some cases as noted, animals were soaked in 0.3 mM levamisole before assaying twitching behavior; this allows partial loss-of function for *unc-22* to be readily assessed (Brenner, 1974; Fire et al., 1998).

*unc-54* encodes the major myosin heavy chain in striated muscles (Epstein et al., 1974). At room temperature, wild-type adults on semisolid media execute a half-sine wave approximately 60 times per minute ( $m = 60$ ), while *unc-54* null mutant adults execute less than one half-wave per minute ( $m < 1$ ). Populations described as paralyzed had locomotory rates of less than 10 half-waves per minute; populations described as slow had locomotory rates in the range of 10–40 half-waves per minute.

Interference with *gfp* activity is assayed by injecting a stably integrated transgenic strain (PD4251; Fire et al., 1998) that has been engineered to express GFP in every cell of the striated body wall musculature. The PD4251 transgene incorporates two different GFP fusions. The first (pSAK2) contains *gfp* and *lacZ* coding sequence and a nuclear localization signal and produces GFP in the nucleus only, while the second (pSAK4) contains *gfp* with a mitochondrial targeting signal (without *lacZ* sequences). Interference with the activity of the PD4251 transgene is described as follows: “+++” (most potent interference) <1 strongly fluorescent cell per animal; “++<sup>1/2</sup>” = 1–5 strongly fluorescent cells per animal; “++” = 5–20 strongly fluorescent cells per animal; “+<sup>1/2</sup>” = 20–50 strongly fluorescent cells per animal; “+” = 50–75 strongly fluorescent cells per animal; “+/-” >75 strongly fluorescent cells per animal with some clearly negative cells; “-” = no difference seen from original PD4251 line (all animals have >90 positive cells).

The PD4251 strain provides a second specific assay for homology-dependent interference. Because the nuclear localized reporter construct consists of a *lacZ::gfp* fusion protein, specific interference with the *lacZ* gene can be assayed by examining animals for loss of the nuclear fluorescence signal (with retention of the mitochondrial signal, which is due to a *gfp* reporter that is not fused to *lacZ*).

None of the RNAi targets for this study are essential for *C. elegans*; hence, an indication of specificity comes from the observation that none of the modified RNAs in this paper produced extensive F1

lethality or other unexpected phenotypes. Irrespective of the identity of the injected material, a low level of nonspecific lethality (totaling 1%–5%) is present in the broods of most injections; this lethality may be due to damage during the injection process.

Negative results in RNAi assays (absence or low level of phenotypic effect) carry experimental uncertainties, including amount, purity, and stability of the material delivered. To minimize uncertainty, potential triggers were injected at a concentration (20–50  $\mu\text{g}/\text{ml}$  unless noted) for which strong loss-of-function phenotypes were invariably obtained after injection of a corresponding unmodified dsRNA at up to a 5-fold dilution. Each RNA preparation was injected into at least six recipient animals, and (with the exception of synthetic RNA zr9) at least two preparations of each noninterfering or weakly interfering RNA duplex were tested. It should be noted that we assayed interference in progeny animals several days after injection into an adult germline, and that none of the target transcripts are present in the *C. elegans* germline. A positive result in our assay thus requires an extended period of stability for the RNAi trigger (or a derivative thereof) in the absence of target RNA.

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