Gene silencing in *Caenorhabditis elegans* by transitive RNA interference

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ABSTRACT

When a cell is exposed to double-stranded RNA (dsRNA), mRNA from the homologous gene is selectively degraded by a process called RNA interference (RNAi). Here, we provide evidence that dsRNA is amplified in *Caenorhabditis elegans* to ensure a robust RNAi response. Our data suggest a model in which mRNA targeted by RNAi functions as a template for 5' to 3' synthesis of new dsRNA (termed transitive RNAi). Strikingly, the effect is nonautonomous: dsRNA targeted to a gene expressed in one cell type can lead to transitive RNAi-mediated silencing of a second gene expressed in a distinct cell type. These data suggest dsRNA synthesized *in vivo* can mediate systemic RNAi.

Keywords: RNA interference; transitive RNAi; *C. elegans*; *pha-4*; *RdRP*

INTRODUCTION

Introduction of dsRNA into cells induces degradation of homologous mRNA by a process called RNA interference (RNAi) in animals or post-transcriptional gene silencing (PTGS) in plants (Bernstein et al. 2001b; Mango 2001; Sharp 2001; Zamore 2001). A two-step mechanism has been proposed for dsRNA-mediated degradation based on genetic and biochemical studies. In the first stage, dsRNA, bound by cellular factors such as Dicer/DCR-1 (Bernstein et al. 2001a; Grishok et al. 2001; Ketting et al. 2001; Knight and Bass 2001), is processed into 21–23 nucleotide pieces called siRNAs. In the second stage, siRNAs guide the degradation machinery to target mRNA, which is cleaved endonucleolytically (Zamore et al. 2000; Elbashir et al. 2001a). The machinery responsible for degradation is called RISC and includes *Argonaute2* in *Drosophila* (Bernstein et al. 2001a; Hammond et al. 2001). A similar complex probably exists in other organisms (Catalanotto et al. 2002; Hutvagner and Zamore 2002; Mourelatos et al. 2002; Tabara et al. 2002). Additional genes required for RNAi have been identified, although their precise functions are unclear. These include genes homologous to RNA helicases (Domeier et al. 2000; Wu-Scharf et al. 2000; Dalmay et al. 2001; Tijsterman

et al. 2002), DNA helicases (Cogoni and Macino 1999b; Ketting et al. 1999), RNA-directed RNA polymerases (Cogoni and Macino 1999a; Mourrain et al. 2000; Smardon et al. 2000), double-stranded RNA-binding proteins (Tabara et al. 2002), as well as additional *Argonaute* members (Tabara et al. 1999; Fagard et al. 2000). In several organisms, *Argonaute* homologs are not required to produce siRNAs, suggesting this family may function downstream of dsRNA processing (Parrish and Fire 2001; Catalanotto et al. 2002; Tijsterman et al. 2002). The loss-of-function phenotypes associated with some of these loci suggest that RNAi-related mechanisms are important for multicellular development and protection from transposons and viruses (Sharp 2001; Zamore 2001).

Previous experiments revealed that even small amounts of dsRNA are effective for silencing, suggesting the RNAi signal may be amplified. For example, an estimated ~2 dsRNA molecules per cell are sufficient to inactivate abundant *unc-22* RNAs (Fire et al. 1998). One explanation for this effect is that the initial RNAi signal is replicated to produce new dsRNA (discussed in Mango 2001). Recently, Fire and colleagues provided evidence that dsRNA is amplified in *Caenorhabditis elegans* (Sijen et al. 2001). Similarly, Paterson and colleagues observed *de novo* synthesis of dsRNA in *Drosophila* extracts using either dsRNA or target mRNA as template (Lipardi et al. 2001). However, questions concerning replication of dsRNA remain because other *Drosophila* studies failed to detect evidence of *de novo* synthesis *in vitro* (Zamore et al. 2000; Elbashir et al. 2001a;

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Hutvagner and Zamore 2002) or in vivo (Celotto and Graveley 2002). Similarly, Parrish et al. (2000) showed that exogenous dsRNA is not a template for replication in vivo in *C. elegans*, at least to any significant degree. Finally, the *Drosophila* genome lacks obvious candidate genes to mediate RNA-directed RNA synthesis. These conflicting observations raised the question of whether dsRNA is synthesized in vivo and, if so, the nature of the template. Our findings suggest that dsRNA is amplified in vivo in *C. elegans*, in agreement with Sijen et al. (2001). We also provide genetic evidence that amplified dsRNA can move between cells.

RESULTS AND DISCUSSION

To determine whether target mRNA is a likely template for dsRNA synthesis in vivo, we designed an assay to detect spreading of the RNAi signal with respect to mRNA target sequences ("transitive RNAi"; Fig. 1A). We generated transgenic worm carrying a translational fusion of green fluorescent protein (GFP) inserted into the *pha-4* gene (Labouesse and Mango 1999; see Materials and Methods). We initiated RNAi with this strain by microinjecting full-length GFP dsRNA (GFP-FL, the trigger dsRNA). If *pha-4::GFP* target mRNA is used as a template to synthesize new dsRNA, some of this dsRNA should be homologous to *pha-4* and able to silence both the chimeric mRNA and endogenous *pha-4*. Silencing of endogenous *pha-4* is predicted to kill progeny worms because *pha-4* is essential (Mango et al. 1994), whereas silencing of the *pha-4::GFP* chimera alone should not affect viability.

Our data demonstrate that transitive RNAi can lead to silencing of new mRNA targets, presumably by de novo synthesis of dsRNA. GFP dsRNA introduced into *pha-4::GFP* transgenic worms resulted in striking lethality in which >80% of the progeny of most injected worms died (Fig. 1B). These worms arrested at the first larval stage, similar to animals with reduced but not null *pha-4* activity

FIGURE 1. Genetic evidence for dsRNA amplification in vivo. (A) If target mRNA functions as a template for synthesis of new dsRNA, then dsRNA directed against a translational fusion between GFP (green) and a *C. elegans* gene (red) will lead to the production of new dsRNA that can target the endogenous *C. elegans* gene (red lines). This phenomenon is called *transitive RNAi*. Here and throughout, target mRNAs are depicted as boxes and dsRNA as lines. (B) Injection of full-length GFP dsRNA (GFP-FL) leads to killing of *pha-4::GFP* and *tlf-1::GFP* worms but not *pGFP* or wild-type animals (top four rows). Injection of full-length *unc-60B* dsRNA kills wild-type worms but not *unc-60B* mutants (bottom two rows). (n') The number of mothers whose progeny were scored except for *unc-60B* where mothers were typically scored. A total of 83% of wild-type animals injected with *unc-60B* dsRNA died and, for the surviving mothers, none of their progeny survived. (C) *unc-60* produces two mRNAs, A and B, which share a common first exon (hatched box). RNAi is initiated using *unc-60B* dsRNA and progeny animals scored for lethal (*unc-60A+B*) or uncoordinated (*unc-60B*) phenotypes. (FL) Full-length 453-bp dsRNA for either *unc-60A* or *unc-60B*. (5') 358-bp dsRNA located 2 nucleotides from the exon 1/2 boundary. (3') 360-bp dsRNA located 96 nucleotides from the exon 1/2 boundary.

(Gaudet and Mango 2002). Lethality depended on *pha-4* exonic sequences as GFP dsRNA injected into either wild-type worms or worms expressing a transcriptional fusion between *pha-4* and GFP had no effect (*pGFP*; Fig. 1B). Importantly, GFP dsRNA injected into *pGFP* transgenic

animals abolished GFP expression, indicating that RNAi-mediated silencing had occurred (data not shown; Fig. 5B, below).

We tested two additional genes to demonstrate the generality of transitive RNAi. The first was a translational fusion between the essential gene *tlf-1* (Kaltenbach et al. 2000) and GFP. GFP dsRNA was used to target the *tlf-1::GFP* fusion by microinjection and consequently endogenous *tlf-1* (Fig. 1B). These embryos arrested with Tlf-1 phenotype (Fig. 2). The second gene examined was the endogenous *unc-60* locus (Fig. 1B,C). Normally, *unc-60* produces two mRNAs, A and B, which share a common first exon but are otherwise different (McKim et al. 1994). Mutations in *unc-60A* and *unc-60B* are associated with different phenotypes, lethal and uncoordinated, respectively, enabling us to determine which mRNAs were degraded during RNAi. Introduction of dsRNA specific for *unc-60B* by microinjection resulted in a lethal phenotype for either injected mothers or >50% of their progeny (Fig. 1B). This result suggests that *unc-60B* dsRNA targeted both *unc-60A* and *unc-60B* transcripts.

We performed two controls to demonstrate that lethality induced by *unc-60B* dsRNA was specific. First, we used a dsRNA positioned 96 bp from the exon 1–2 boundary to induce silencing (Fig. 1C, 3' construct). Because transitive RNAi can travel only short distances (see below), we predicted this dsRNA would inactivate *unc-60B* but not *unc-60A*. As expected, we observed Unc animals but no lethality when this dsRNA was used to induce silencing by microinjection (7/10 Unc, 0/10 Let). A dsRNA of a similar size but located 2 base pairs from the exon 1–2 boundary was

able to induce lethality similar to full-length dsRNA indicating that location of the dsRNA, not its size, was important for killing (0/8 Unc, 7/8 Let; Fig. 1C, 5' construct). Second, the allele *unc-60(su158)* carries a deletion within *unc-60B* that is predicted to destabilize the mature *unc-60B* mRNA while leaving *unc-60A* intact (Fig. 1C; Mango 2001; S. Ono, pers. comm.). We reasoned that if transitive RNAi depends on mature *unc-60B* sequences, this allele should suppress the lethality associated with *unc-60B*-mediated RNAi because mature *unc-60B(su158)* mRNA could not function as a template. We injected *unc-60B* dsRNA into *unc-60(su158)* homozygotes and found, as expected, that all mothers and offspring survived (Fig. 1B). Our data suggest *unc-60A* mRNA is a target for transitive RNAi from *unc-60B* sequences.

One intriguing possibility is that antisense RNAs from the trigger dsRNA function as primers during transitive RNAi. If so, the process should be directional. Synthesis of an antisense strand of RNA from 5' to 3' using the 3' hydroxyl of the antisense RNA primer (Elbashir et al. 2001a) is predicted to produce dsRNA complementary to mRNA sequences 5' of the domain targeted by the trigger dsRNA, but not 3'. Three lines of evidence support this model. First, we showed directionality for the mRNA target. We constructed a transgene carrying *pha-4* sequences 3' of *GFP* (*GPTxg*) and initiated RNAi with *GFP-FL* dsRNA (Fig. 3). Whereas *GFP-FL* dsRNA could silence GFP expression as effectively for this transgene as for *pha-4::GFP*, we observed no transitive RNAi-induced killing (Fig. 3). These findings indicate that transitive RNAi targets mRNA sequences located 5' of the mRNA sequences homologous to incoming dsRNA.

As a second approach, we tested directionality of exogenously introduced dsRNA. We used different fragments of GFP dsRNA to initiate RNAi by microinjection (Fig. 3). *GFP-5'* and *GFP-3'* are dsRNAs homologous to the 5' and 3' halves of full-length GFP, respectively. *GFP-mid* is an equivalently sized dsRNA from the middle of GFP. We reasoned that all three GFP dsRNAs should be competent to interfere with expression of GFP transgenes. However, GFP dsRNA homologous to sequences close to the appropriate *pha-4*–*GFP* junction of the target mRNA should be more effective for transitive RNAi-mediated killing than GFP dsRNA homologous to sequences far from the junction. This prediction rests on the idea that killing requires synthesis of new dsRNAs that extend from the priming antisense RNA to sequences homologous to *pha-4* and that spreading is likely to be sensitive to distance. *GFP-5'* and *GFP-3'*

FIGURE 2. Phenotype of *tlf-1::GFP* transgenic animals treated with GFP dsRNA. (Top) Wild-type worms treated with no dsRNA (WT) or *tlf-1* dsRNA (*tlf-1* RNAi). (Bottom) Two examples of arrested embryos from *tlf-1::GFP* transgenic worms treated with GFP dsRNA (GFP RNAi).

FIGURE 3. Transitive RNAi moves 5' to 3'. dsRNA homologous to full-length, 5', 3', or middle of *GFP* was used to initiate RNAi in worms carrying one of five transgenes: *PG txg*, *GPltxg*, *pha-4::GFP*, *tlf-1::GFP*, or *pGFP*. (*) Effects of *GFP-FL* for *pha-4::GFP*, *tlf-1::GFP*, and *pGFP* worms are from Figure 1.

dsRNA each flank a *pha-4-GFP* junction, but at different positions. The junction is 5' relative to the mRNA sequences homologous to trigger dsRNA for *GFP-5'* and 3' for *GFP-3'* (Fig. 3). We found that *GFP-5'* dsRNA was as competent to induce a lethal phenotype as *GFP-FL* for both *pha-4::GFP* and *tlf-1::GFP* transgenes. This result suggests that dsRNA synthesis targets mRNA sequences located 5' of those homologous to the exogenous dsRNA. By contrast, neither *GFP-3'* nor *GFP-mid* dsRNA induced killing even though they were as effective for silencing *GFP* as *GFP-5'*. These experiments suggest that dsRNA is synthesized from 5' to 3' and is sensitive to distance.

Our third line of evidence for directionality of transitive RNAi is based on the *unc-60A/B* experiment described above. The only sequences shared between these mRNAs are those of the common first exon, suggesting that synthesis of dsRNA is 5'–3' (Fig. 1C). Together, these experiments demonstrate that transitive RNAi-induced killing involves mRNA sequences located 5' of those homologous to the trigger dsRNA. We conclude that transitive RNAi moves from the initiating siRNA (or other antisense RNA) toward the 5' end of the mRNA (i.e., 5'–3' synthesis), which agrees with the findings of Sijen et al. (2001) and Lipardi et al. (2001). What determines directionality? One possibility is that the siRNA functions as a primer to initiate elongation. Previous analyses are consistent with a primer function during RNAi in *C. elegans* (Tijsterman et al. 2002). Alternatively, directionality may reflect differential stability of potential templates for RNA synthesis derived from a cleaved target mRNA. The 5' fragment may be more stable than the 3' fragment and therefore used more frequently as a template. Finally, the machinery required for RNA synthesis may be associated with RISC and positioned by this complex for unidirectional RNA synthesis.

We observe only short-range effects, fewer than 100 or 180 bases. Neither *GFP-3'* nor *GFP-mid* dsRNA induced lethality even though these dsRNAs were only 358 bases and 180 bases, respectively, from the 5' *pha-4-GFP* junction. Similarly, the *unc-60B* 3' construct was only 96 bases from

the exon 1–2 boundary and yet failed to induce transitive RNAi. This result is similar to Sijen et al. (2001), but different from Lipardi et al. (2001), who observed synthesis of full-length RNAs.

We next examined transitive RNAi from the perspective of the trigger dsRNA. We used a dsRNA hairpin that carried *GFP* sequences in the stem and *unc-22* sequences in the loop (Fig. 4A; Winston et al. 2002). We predicted these molecules would be processed into siRNAs starting from the free end of the stem and ending with the last fragment of dsRNA carrying the *unc-22* loop at one end. This prediction stems from in

vitro data that revealed single-stranded RNA can block dsRNA processing in *Drosophila* (Elbashir et al. 2001a). We asked whether we could observe *Unc-22* mutant phenotypes as a consequence of *unc-22* dsRNA synthesized de novo.

Unc-22 animals were generated when *GFP-unc-22* hairpin dsRNA produced in bacteria was fed to animals carrying *pha-4::GFP*, *pGFP*, or *tlf-1::GFP* transgenes (Figs. 4B and 5A). The severity of the *Unc-22* phenotype differed between the three strains, suggesting that the *Unc-22* phenotype depended on the abundance or cellular expression of the mRNA target (Fig. 4B; data not shown). The *pha-4::GFP* transgenic strain had the strongest phenotype, with 100% living worms becoming severely uncoordinated. *tlf-1::GFP* gave the weakest phenotype, with only mild effects on movement. By contrast, *GFP-unc-22* hairpin dsRNA never induced an *Unc-22* phenotype in wild-type animals that lacked a *GFP* transgene, indicating that an mRNA target was essential (Figs. 4B and 5A). To ensure that all three strains were susceptible to *unc-22* RNAi, we also performed a titration of *unc-22* dsRNA alone. We observed some variability in responsiveness to *unc-22* dsRNA, but it was not sufficient to explain the dramatic differences in *Unc-22* phenotypes induced by the hairpin dsRNA. For example, in some experiments *pha-4::GFP* behaved identically to wild-type worms, whereas in others we saw a mild enhancement of RNAi (data not shown). We suggest that *unc-22* dsRNA is produced via transitive RNAi from hairpin constructs. One speculative but exciting model is that cleaved mRNA can function as a primer for dsRNA synthesis (Fig. 4A, left). Alternatively, siRNAs homologous to *GFP* may function as primers with a hairpin dsRNA template (Fig. 4A, right). In the latter scenario, the role of the *GFP* transgene might be to produce large numbers of siRNAs or structurally modified siRNAs that could function as primers.

The difference in phenotypes with various mRNA targets and *GFP-unc-22* hairpin dsRNA provides a possible explanation why transitive RNAi using dsRNA hairpins was not detected previously (Parrish et al. 2000). The mRNA targets used in the previous study may not have been as effective as

FIGURE 4. Two models for RNAi with loop sequences of a dsRNA hairpin. (A) *GFP_{hp}* is a dsRNA hairpin with *GFP* sense RNA(s) on the 5' end of the stem and *GFP* antisense RNA (as) on the 3' end of the stem; *unc-22* sequences are located in the loop (Winston et al. 2002). The hairpin is predicted to undergo processing to form siRNAs, with the last fragment of dsRNA bearing the *unc-22* loop. Association of siRNAs with the *GFP* mRNA will induce endonucleolytic cleavage of the target. Cleaved *GFP* mRNA may function as a primer to synthesize a complementary strand of *unc-22* RNA using the loop-containing siRNA as template (1, left). Alternatively, siRNAs generated during *GFP* RNAi may prime dsRNA synthesis with a new *GFP_{hp}* (2, right). (B) Silencing of *GFP* and *unc-22*. (*) Percentage of animals with *GFP* completely off. (†) Percentage of uncoordinated animals. (+++) Animals that were paralyzed on the plate; (+) animals that were sluggish or egg-laying defective. *pGFP* is not silenced effectively by *GFP_{hp}* feeding, but presumably some RNAi is occurring since this strain was tested concurrently with *pha-4::GFP*.

pha-4::GFP. Moreover, we observed *Unc-22* phenotypes when hairpin dsRNA was administered continuously but not when a single dose of dsRNA was applied by microinjection (see Materials and Methods). Parrish et al. (2000) relied on microinjection in their studies. One possibility is that a stable supply of hairpin dsRNA is important for a sustained reduction of *unc-22* mRNA and generation of an *Unc-22* phenotype.

The *GFP-unc-22* hairpin experiment revealed that a molecule produced during transitive RNAi, presumably dsRNA,

can move between cells. Previous work showed that RNAi can spread from cell to cell (for review, see Sharp 2001; see also Winston 2002). The systemic nature of RNAi is functional in *rde-4* mutants even though these animals cannot produce siRNAs efficiently (Grishok et al. 2000; Parrish and Fire 2001). These data suggest that siRNAs are not required for systemic RNAi but leave open the nature of the dsRNA molecule involved. One possibility is that a larger dsRNA could be involved. Tabara et al. (2002) detected a larger 100-bp RNA associated with RDE-4 in worms undergoing RNAi and Boutla et al. (2002) have implicated an 85-bp species from plants that can induce RNAi in worms. These RNAs could represent cleaved trigger dsRNA or replicated dsRNA. In our experiments, we observed transitive RNAi when the primary mRNA target was expressed in one cell type and the secondary mRNA target in another. *GFP-unc-22* hairpin dsRNA introduced into *pha-4::GFP* or *pGFP* transgenic strains produced a strong *Unc-22* phenotype even though expression of these transgenes was restricted to the digestive tract whereas *unc-22* is limited to body wall muscles (Fig. 5B; Moerman and Fire 1997). Conversely, *tlf-1::GFP* transgenic worms gave the weakest *Unc-22* phenotype in side by side comparisons (Fig. 4B) even though *tlf-1::GFP* is expressed ubiquitously, including body wall muscles (Kaltenbach et al. 2000; data not shown). Although we cannot rule out that some small amount of *pha-4::GFP* is expressed in body wall muscles, we clearly do not observe a concordance between the level of *GFP* in body wall muscles and the *Unc-22* phenotype. We propose this effect may reflect the level of *GFP* in individual cells, especially those of the digestive tract that presumably receive most of the ingested dsRNA. Because transitive RNAi depends critically on a transgene, our data also suggest that a product of transitive RNAi, presumably dsRNA, can move between cells. This product may not be siRNA, based on the *rde-4* data described above.

What molecules mediate transitive RNAi? One attractive candidate is RNA-directed RNA polymerase (RdRP), which is required for RNAi in worms (Smardon et al. 2000; Sijen et al. 2001), *Neurospora* (Cogoni and Macino 1999a), *Arabidopsis* (Dalmay et al. 2000; Fagard et al. 2000; Mourrain et al. 2000), and *Dictyostelium* (Martens et al. 2002). RdRPs have been studied in tomato, where they synthesize an RNA complement from a single-stranded RNA or DNA template in vitro (Schiebel et al. 1993a,b, 1998). In plants and *Dictyostelium*, the amount of siRNA decreases when RdRP is inactivated, consistent with the existence of a replicative dsRNA amplification step (Dalmay et al. 2000; Martens et al. 2002). However, because RdRPs are required for all RNAi, it is not possible to determine whether they play a specific role in transitive RNAi. The loss of RNAi in RdRP mutants could reflect the absolute requirement for an amplification step to produce enough siRNAs to reach a critical threshold for silencing. Alternatively, RdRPs could perform some other function that remains to be discovered. Con-

FIGURE 5. Phenotypes of worms exposed to dsRNA hairpins. (A) Motility of wild-type (WT), *pha-4::GFP*, or *pGFP* transgenic worms after RNAi induced with *GFP-unc-22* hairpin dsRNA (GFPhp). Three F1 animals were allowed to move freely for 11 min and photographed in the absence (no treat) or presence of *GFP-unc-22* hairpin dsRNA. (B) GFP fluorescence for *pha-4::GFP* transgenic worms treated with no dsRNA (no treat), or *GFP-unc-22* hairpin dsRNA (GFPhp).

sistent with this idea, loss of *SDE1/RdRP* activity in *Arabidopsis* blocks mRNA degradation even though siRNAs accumulate (Vaistij et al. 2002). In *C. elegans*, a biochemical activity for RdRP homologs has not yet been documented.

Interestingly, *Drosophila* and mammals do not carry an RdRP homolog, suggesting transitive RNAi may not occur in these organisms. Several studies are consistent with this idea, both in vivo (Celotto and Graveley 2002; Holen et al. 2002) and in vitro (Zamore et al. 2000; Elbashir et al. 2001b; Nykanen et al. 2001; Hutvagner and Zamore 2002). However, other studies have suggested amplification can occur in *Drosophila* (Lipardi et al. 2001). The discrepancy between the different studies may reflect differences in the balance between dsRNA synthesis versus mRNA degradation activities in vitro versus in vivo. Alternatively, perhaps some cells carry viruses that produce RdRP enzymes, providing a pos-

sible source for the potent replication activity seen in some studies but not others.

In closing, we note that the *GFP-unc-22* hairpin used in these studies may have a natural counterpart in vivo. RNAi suppresses transposition of Tc1/mariner type transposons, which carry inverted repeats at each end (Plasterk and van Luenen 1997; Sharp 2001; Zamore 2001). The configuration of transposon RNA is unknown, but at least some transcripts may be read-through products initiated at adjacent cellular promoters. If so, these RNAs would form hairpins. The *GFPhp* hairpin used in this study resembles the likely structure of hairpin transposon RNA, with the inverted repeats forming the stem and the body of the transposon in the loop (Plasterk and van Luenen 1997). The small size of the inverted repeat within some transposons suggests that few siRNAs would be made during initial RNAi and therefore transposon RNAs would be poor RNAi targets. Transitive RNAi provides the cell with an elegant solution to this problem. Synthesis of new dsRNA complementary to the transposon loop, analogous to the *unc-22* loop of *GFPhp*, would enable the cell to silence transposon RNAs more effectively.

MATERIALS AND METHODS

Worm growth

Strains were maintained at room temperature (22°–23°) following Brenner (1974). For all but the hairpin constructs, dsRNA was synthesized and microinjected as described in Kaltenbach et al. (2000). Adults were injected except for the *unc-60B* experiments (360 and 358 bp dsRNA) where we obtained stronger phenotypes when we injected fourth-stage larvae. Progeny laid 40–48 h post-injection were scored for GFP fluorescence and lethality over the following two days. For the hairpin constructs, RNAi was initiated by bacterial feeding (Timmons and Fire 1998; Timmons et al. 2001). First- and second-stage larvae were placed on plates seeded with RNase III-deficient bacteria expressing dsRNA and induced with 1 mM IPTG (Timmons et al. 2001). Worms were transferred daily to freshly seeded plates. Progeny laid 48 h after Po larvae had been placed on the plates were scored for GFP fluorescence. *Unc-22* phenotypes were scored mostly in Po animals after 48 h although progeny were also *Unc* whereas wild-type animals were not. Four independent experiments were performed for the dsRNA hairpin experiments. The structures of the hairpin construct was confirmed by restriction enzyme digest before and after each experiment.

DNA constructs

pha-4::GFP carries GFP from pPD95.85 (<http://www.ciwemb.edu>; Fire et al. 1990) inserted into a unique *Mlu*I site at position 5612 relative to ATG1 of *pha-4*. The genomic *pha-4* sequences extend from -7110 to +7683. *GPTxg* carries the pan-pharyngeal promoter region from -589 to -1 of T05E11.3 (Gaudet and Mango 2002) fused to GFP from pAP10 (A. Paulson and S.E. Mango, unpubl.; G. Seydoux, pers. comm.). The 3' end of GFP is fused in-frame to *pha-4* cDNA from +4 to +507 relative to the first ATG. *pGFP* is described in Portereiko and Mango (2001) and *tlf-1::GFP* in Kaltenbach et al. (2000). *pha-4::GFP*, *tlf-1::GFP*, and *pGFP* are simple arrays integrated into the genome. *GPTxg* is a complex extrachromosomal array. *GFP_{hp}* is derived from L4271 (<http://www.ciwemb.edu>; 1999 Fire vector kit; Winston et al. 2002) with the lacZ promoter inactivated.

Site-directed mutagenesis

We inactivated the lacZ promoter from L4271 and pBluescript KS- by replacing six bases at the -10 LacZ with a *Sca*-I restriction site. Primers were designed for the pBluescript backbone (For lacZ *sca*-I, CACTTTATGCTTCCGGCTCGAGTACTTGTGGAATTGT GAGCGG; rev lacZ *sca*-I, CCGCTCACAAATTCACAAGTACTC GAGCCGGAAGCATAAAGTG) and amplified using *Pfu*TURBO and digested with *Dpn*I prior to transformation (QuickChange Site-Directed Mutagenesis Kit, Stratagene). Hairpins constructed in pCRII2.1 TOPO were inserted into pBluescript KS- by sequentially digesting with *Kpn*I and *Not*I and ligating with T4 DNA ligase following standard procedures.

dsRNA production

Amplicons were transcribed using T7 or SP6 transcription kits (Ambion Megascript). All cloned fragments were linearized prior to transcription. *GFP*: The full-length (1–715 bp), 5' (1–357 bp), middle (181–535 bp), and 3' (358–715 bp) GFP sequences were amplified from *GFP::unc-22::PFG* plasmid L4271 (<http://www.ciwemb.edu>; Fire et al. 1990). *unc-60A* and *unc-60B*: Primers containing T7 and SP6 promoters were designed to *unc-60A* (1–498 bp) and *unc-60B* (499–882 bp). Amplicons of the desired size were obtained from the Okkema embryonic cDNA library, gel purified, and transcribed. Most forward and reverse primers were designed with T7 or SP6 promoter sites for in vitro transcription. Primers with no promoter sites were cloned into pCRII TOPO (Invitrogen).

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