

# Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*

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**Experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene<sup>1,2</sup>. Such effects have been proposed to result from a simple antisense mechanism that depends on hybridization between the injected RNA and endogenous messenger RNA transcripts. RNA interference has been used in the nematode *Caenorhabditis elegans* to manipulate gene expression<sup>3,4</sup>. Here we investigate the requirements for structure and delivery of the interfering RNA. To our surprise, we found that double-stranded RNA was substantially more effective at producing interference than was either strand individually. After injection into adult animals, purified single strands had at most a modest effect, whereas double-stranded mixtures caused potent and specific interference. The effects of this interference were evident in both the injected animals and their progeny. Only a few molecules of injected double-stranded RNA were required per affected cell, arguing against stoichiometric interference with endogenous mRNA and suggesting that there could be a catalytic or amplification component in the interference process.**

Despite the usefulness of RNA interference in *C. elegans*, two features of the process have been difficult to explain. First, sense and antisense RNA preparations are each sufficient to cause interference<sup>3,4</sup>. Second, interference effects can persist well into the next generation, even though many endogenous RNA transcripts are rapidly degraded in the early embryo<sup>5</sup>. These results indicate a fundamental difference in behaviour between native RNAs (for example, mRNAs) and the molecules responsible for interference. We sought to test the possibility that this contrast reflects an underlying difference in RNA structure. RNA populations to be injected are generally prepared using bacteriophage RNA polymerases<sup>6</sup>. These polymerases, although highly specific, produce some random or ectopic transcripts. DNA transgene arrays also produce a fraction of aberrant RNA products<sup>3</sup>. From these facts, we surmised that the interfering RNA populations might include some molecules with double-stranded character. To test whether double-stranded character might contribute to interference, we further purified single-stranded RNAs and compared interference activities of individual strands with the activity of a deliberately prepared double-stranded hybrid.

The *unc-22* gene was chosen for initial comparisons of activity. *unc22* encodes an abundant but nonessential myofilament protein<sup>7-9</sup>. Several thousand copies of *unc-22* mRNA are present in each striated muscle cell<sup>3</sup>. Semiquantitative correlations between *unc-22* activity and phenotype of the organism have been described<sup>8</sup>:

decreases in *unc-22* activity produce an increasingly severe twitching phenotype, whereas complete loss of function results in the additional appearance of muscle structural defects and impaired motility.

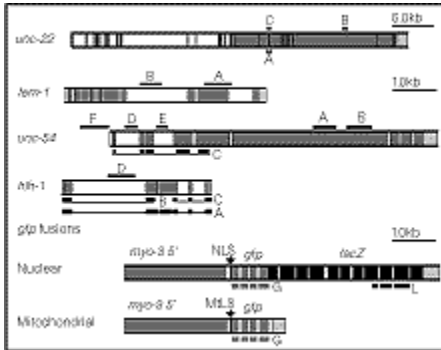
Purified antisense and sense RNAs covering a 742-nucleotide segment of *unc-22* had only marginal interference activity, requiring a very high dose of injected RNA to produce any observable effect (Table 1). In contrast, a sense–antisense mixture produced highly effective interference with endogenous gene activity. The mixture was at least two orders of magnitude more effective than either single strand alone in producing genetic interference. The lowest dose of the sense–antisense mixture that was tested, ~60,000 molecules of each strand per adult, led to twitching phenotypes in an average of 100 progeny. Expression of *unc-22* begins in embryos containing ~500 cells. At this point, the original injected material would be diluted to at most a few molecules per cell.

The potent interfering activity of the sense–antisense mixture could reflect the formation of double-stranded RNA (dsRNA) or, conceivably, some other synergy between the strands. Electrophoretic analysis indicated that the injected material was predominantly double-stranded. The dsRNA was gel-purified from the annealed mixture and found to retain potent interfering activity. Although annealing before injection was compatible with interference, it was not necessary. Mixing of sense and antisense RNAs in low-salt concentrations (under conditions of minimal dsRNA formation) or rapid sequential injection of sense and antisense strands were sufficient to allow complete interference. A long interval (>1 h) between sequential injections of sense and antisense RNA resulted in a dramatic decrease in interfering activity. This suggests that injected single strands may be degraded or otherwise rendered inaccessible in the absence of the opposite strand.

A question of specificity arises when considering known cellular responses to dsRNA. Some organisms have a dsRNA-dependent protein kinase that activates a panic-response mechanism<sup>10</sup>. Conceivably, our sense–antisense synergy might have reflected a nonspecific potentiation of antisense effects by such a panic mechanism. This is not the case: co-injection of dsRNA segments unrelated to *unc-22* did not potentiate the ability of single *unc-22*-RNA strands to mediate inhibition (data not shown). We also investigated whether double-stranded structure could potentiate interference activity when placed in *cis* to a single-stranded segment. No such potentiation was seen: unrelated double-stranded sequences located 5' or 3' of a single-stranded *unc-22* segment did not stimulate interference. Thus, we have only observed potentiation of interference when dsRNA sequences exist within the region of homology with the target gene.

The phenotype produced by interference using *unc-22* dsRNA was extremely specific. Progeny of injected animals exhibited behaviour that precisely mimics loss-of-function mutations in *unc-22*. We assessed target specificity of dsRNA effects using three additional genes with well characterized phenotypes (Fig. 1, Table 1). *unc-54* encodes a body-wall-muscle heavy-chain isoform of myosin that is required for full muscle contraction<sup>7,11,12</sup>; *fem-1* encodes an ankyrin-repeat-containing protein that is required in hermaphrodites for sperm production<sup>13,14</sup>; and *hlh-1* encodes a *C. elegans* homologue of myoD-family proteins that is required for proper body shape and motility<sup>15,16</sup>. For each of these genes, injection of related dsRNA produced progeny broods exhibiting the known null-mutant phenotype, whereas the purified single RNA strands produced no significant interference. With one exception, all of the

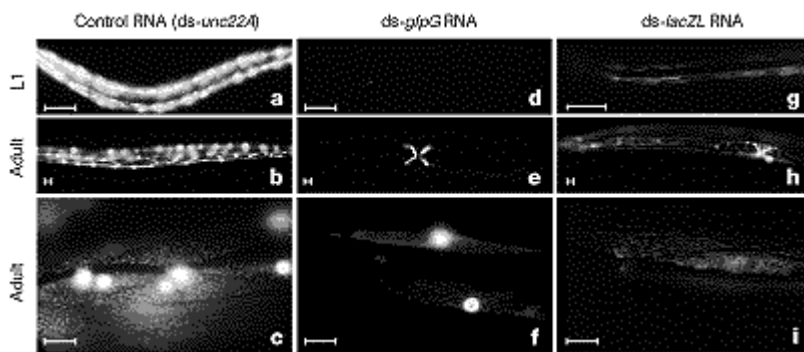
phenotypic consequences of dsRNA injection were those expected from interference with the corresponding gene. The exception (segment *unc54C* which led to an embryonic- and larval-arrest phenotype not seen with *unc-54*-null mutants) was illustrative. This segment covers the highly conserved myosin-motor domain, and might have been expected to interfere with activity of other highly related myosin heavy-chain genes<sup>17</sup>. The *unc54C* segment has been unique in our overall experience to date: effects of 18 other dsRNA segments (Table 1; and our unpublished observations) have all been limited to those expected from previously characterized null mutants.



**Figure 1** Genes used to study RNA-mediated genetic interference in *C.elegans*. [Full legend](#)

[High resolution image and legend](#) (13k)

The pronounced phenotypes seen following dsRNA injection indicate that interference effects are occurring in a high fraction of cells. The phenotypes seen in *unc-54* and *hnh-1* null mutants, in particular, are known to result from many defective muscle cells<sup>11,16</sup>. To examine interference effects of dsRNA at a cellular level, we used a transgenic line expressing two different green fluorescent protein (GFP)-derived fluorescent-reporter proteins in body muscle. Injection of dsRNA directed to *gfp* produced marked decreases in the fraction of fluorescent cells (Fig. 2). Both reporter proteins were absent from the affected cells, whereas the few cells that were fluorescent generally expressed both GFP proteins.



**Figure 2** Analysis of RNA-interference effects in individual cells.

[Full legend](#)

[High resolution image and legend](#) (23k)

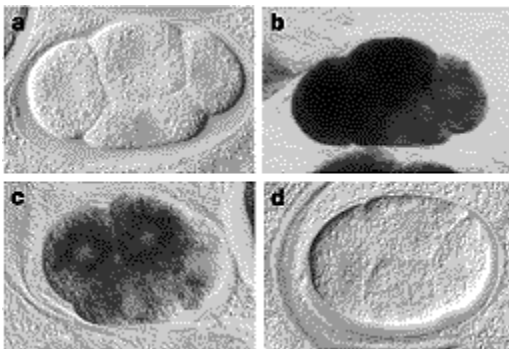
The mosaic pattern observed in the *gfp*-interference experiments was nonrandom. At low doses of dsRNA, we saw frequent interference in the embryonically derived muscle cells that are present when the animal hatches. The interference effect in these differentiated cells persisted throughout larval growth: these cells produced little or no additional GFP as the affected animals grew. The 14 postembryonically derived striated muscles are born during early larval stages and these were more

resistant to interference. These cells have come through additional divisions (13–14 divisions versus 8–9 divisions for embryonic muscles<sup>18,19</sup>). At high concentrations of *gfp* dsRNA, we saw interference in virtually all striated body-wall muscles, with occasional lone escaping cells, including cells born during both embryonic and postembryonic development. The non-striated vulval muscles, which are born during late larval development, appeared to be resistant to interference at all tested concentrations of injected dsRNA.

We do not yet know the mechanism of RNA-mediated interference in *C. elegans*. Some observations, however, add to the debate about possible targets and mechanisms.

First, dsRNA segments corresponding to various intron and promoter sequences did not produce detectable interference ([Table 1](#)). Although consistent with interference at a post-transcriptional level, these experiments do not rule out interference at the level of the gene.

Second, we found that injection of dsRNA produces a pronounced decrease or elimination of the endogenous mRNA transcript ([Fig. 3](#)). For this experiment, we used a target transcript (*mex-3*) that is abundant in the gonad and early embryos<sup>20</sup>, in which straightforward *in situ* hybridization can be performed<sup>5</sup>. No endogenous *mex-3* mRNA was observed in animals injected with a dsRNA segment derived from *mex-3*. In contrast, animals into which purified *mex-3* antisense RNA was injected retained substantial endogenous mRNA levels ([Fig. 3d](#)).



**Figure 3** Effects of *mex-3* RNA interference on levels of the endogenous mRNA. [Full legend](#)

[High resolution image and legend](#) (41k)

Third, dsRNA-mediated interference showed a surprising ability to cross cellular boundaries. Injection of dsRNA (for *unc-22*, *gfp* or *lacZ*) into the body cavity of the head or tail produced a specific and robust interference with gene expression in the progeny brood ([Table 2](#)). Interference was seen in the progeny of both gonad arms, ruling out the occurrence of a transient 'nicking' of the gonad in these injections. dsRNA injected into the body cavity or gonad of young adults also produced gene-specific interference in somatic tissues of the injected animal ([Table 2](#)).

The use of dsRNA injection adds to the tools available for studying gene function in *C. elegans*. In particular, it should now be possible functionally to analyse many interesting coding regions<sup>21</sup> for which no specific function has been defined. Although the effects of dsRNA-mediated interference are potent and specific we have observed several limitations that should be taken into account when designing RNA-interference-based experiments. First, a sequence shared between several closely

related genes may interfere with several members of the gene family. Second, it is likely that a low level of expression will resist RNA-mediated interference for some or all genes, and that a small number of cells will likewise escape these effects.

Genetic tools are available for only a few organisms. Double-stranded RNA could conceivably mediate interference more generally in other nematodes, in other invertebrates, and, potentially, in vertebrates. RNA interference might also operate in plants: several studies have suggested that inverted-repeat structures or characteristics of dsRNA viruses are involved in transgene-dependent co-suppression in plants<sup>22,23</sup>.

There are several possible mechanisms for RNA interference in *C. elegans*. A simple antisense model is not likely: annealing between a few injected RNA molecules and excess endogenous transcripts would not be expected to yield observable phenotypes. RNA-targeted processes cannot, however, be ruled out, as they could include a catalytic component. Alternatively, direct RNA-mediated interference at the level of chromatin structure or transcription could be involved. Interactions between RNA and the genome, combined with propagation of changes along chromatin, have been proposed in mammalian X-chromosome inactivation and plant-gene co-suppression<sup>22,24</sup>. If RNA interference in *C. elegans* works by such a mechanism, it would be new in targeting regions of the template that are present in the final mRNA (as we observed no phenotypic interference using intron or promoter sequences). Whatever their target, the mechanisms underlying RNA interference probably exist for a biological purpose. Genetic interference by dsRNA could be used by the organism for physiological gene silencing. Likewise, the ability of dsRNA to work at a distance from the site of injection, and particularly to move into both germline and muscle cells, suggests that there is an effective RNA-transport mechanism in *C. elegans*.

## Methods

**RNA synthesis and microinjection.** RNA was synthesized from phagemid clones by using T3 and T7 polymerase<sup>6</sup>. Templates were then removed with two sequential DNase treatments. When sense-, antisense-, and mixed-RNA populations were to be compared, RNAs were further purified by electrophoresis on low-gelling-temperature agarose. Gel-purified products appeared to lack many of the minor bands seen in the original 'sense' and 'antisense' preparations. Nonetheless, RNA species comprising <10% of purified RNA preparations would not have been observed. Without gel purification, the 'sense' and 'antisense' preparations produced notable interference. This interference activity was reduced or eliminated upon gel purification. In contrast, sense-plus-antisense mixtures of gel-purified and non-gel-purified RNA preparations produced identical effects.

Sense/antisense annealing was carried out in injection buffer ([ref. 27](#)) at 37 °C for 10–30 min. Formation of predominantly double-stranded material was confirmed by testing migration on a standard (nondenaturing) agarose gel: for each RNA pair, gel mobility was shifted to that expected for dsRNA of the appropriate length. Co-incubation of the two strands in a lower-salt buffer (5 mM Tris-Cl, pH 7.5, 0.5 mM EDTA) was insufficient for visible formation of dsRNA *in vitro*. Non-annealed sense-plus-antisense RNAs for *unc22B* and *gfpG* were tested for RNA interference and found to be much more active than the individual single strands, but twofold to fourfold less active than equivalent preannealed preparations.

After preannealing of the single strands for *unc22A*, the single electrophoretic species, corresponding in size to that expected for the dsRNA, was purified using two rounds of gel electrophoresis. This material retained a high degree of interference activity.

Except where noted, injection mixes were constructed so that animals would receive an average of  $0.5 \times 10^6$  to  $1.0 \times 10^6$  RNA molecules. For comparisons of sense, antisense, and double-stranded RNA activity, equal masses of RNA were injected (that is, dsRNA was used at half the molar concentration of the single strands). Numbers of molecules injected per adult are approximate and based on the concentration of RNA in the injected material (estimated from ethidium bromide staining) and the volume of injected material (estimated from visible displacement at the site of injection). It is likely that this volume will vary several-fold between individual animals; this variability would not affect any of the conclusions drawn from this work.

**Analysis of phenotypes.** Interference with endogenous genes was generally assayed in a wild-type genetic background (N2). Features analysed included movement, feeding, hatching, body shape, sexual identity, and fertility. Interference with *gfp* ([ref. 25](#)) and *lacZ* activity was assessed using *C. elegans* strain PD4251. This strain is a stable transgenic strain containing an integrated array (ccIs4251) made up of three plasmids: pSAK4 (*myo-3* promoter driving mitochondrially targeted GFP); pSAK2 (*myo-3* promoter driving a nuclear-targeted GFP-LacZ fusion); and a *dpy-20* subclone<sup>26</sup> as a selectable marker. This strain produces GFP in all body muscles, with a combination of mitochondrial and nuclear localization. The two distinct compartments are easily distinguished in these cells, allowing easy distinction between cells expressing both, either, or neither of the original GFP constructs.

Gonadal injection was done as described<sup>27</sup>. Body-cavity injections followed a similar procedure, with needle insertion into regions of the head and tail beyond the positions of the two gonad arms. Injection into the cytoplasm of intestinal cells is also effective, and may be the least disruptive to the animal. After recovery and transfer to standard solid media, injected animals were transferred to fresh culture plates at 16-h intervals. This yields a series of semisynchronous cohorts in which it was straightforward to identify phenotypic differences. A characteristic temporal pattern of phenotypic severity is observed among progeny. First, there is a short 'clearance' interval in which unaffected progeny are produced. These include impermeable fertilized eggs present at the time of injection. Second, after the clearance period, individuals that show the interference phenotype are produced. Third, after injected animals have produced eggs for several days, gonads can in some cases 'revert' to produce incompletely affected or phenotypically normal progeny.

[Supplementary information](#) accompanies this paper.

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