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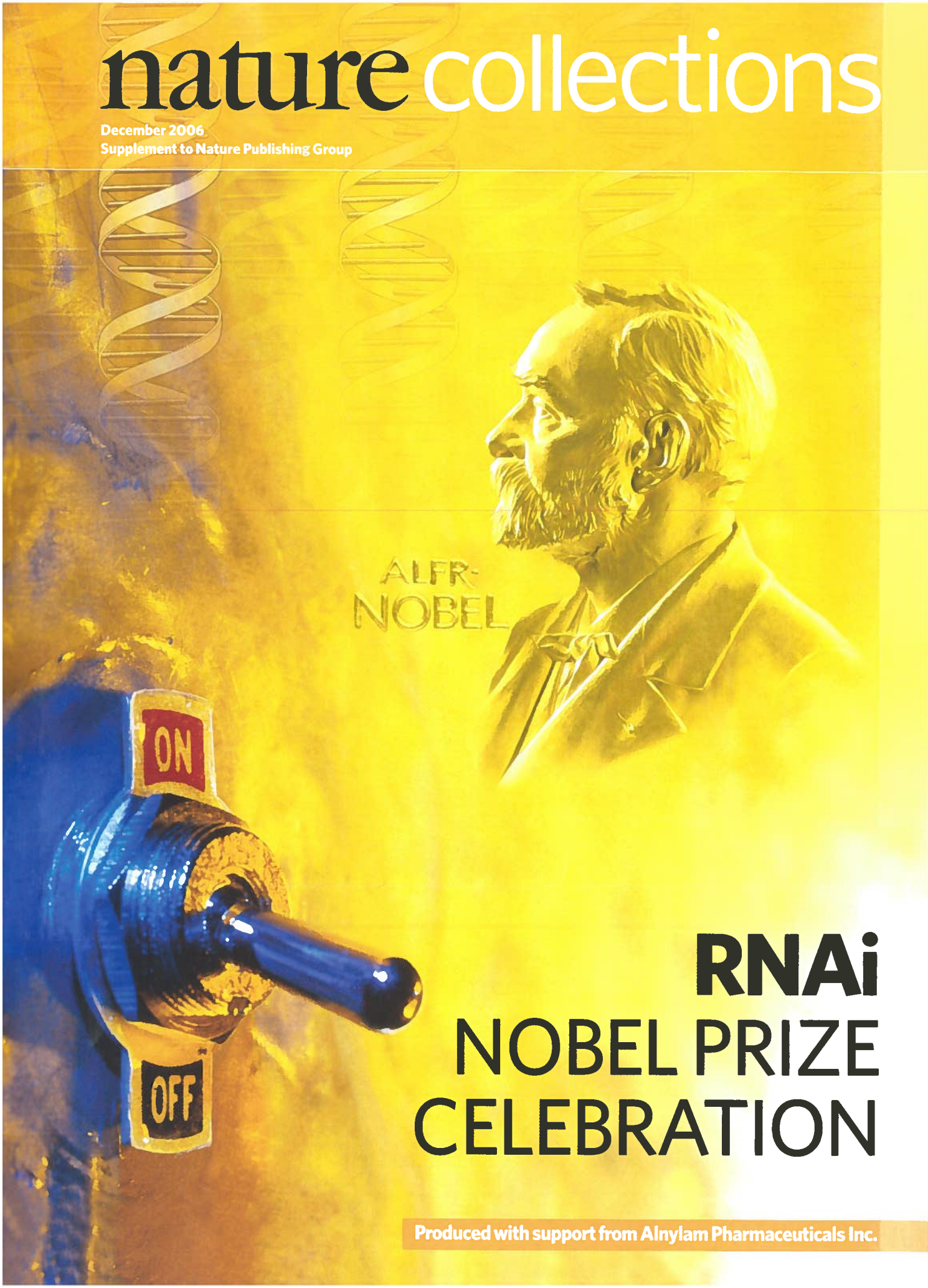


RNAi
NOBEL PRIZE CELEBRATION

Produced with support from Alnylam Pharmaceuticals Inc.

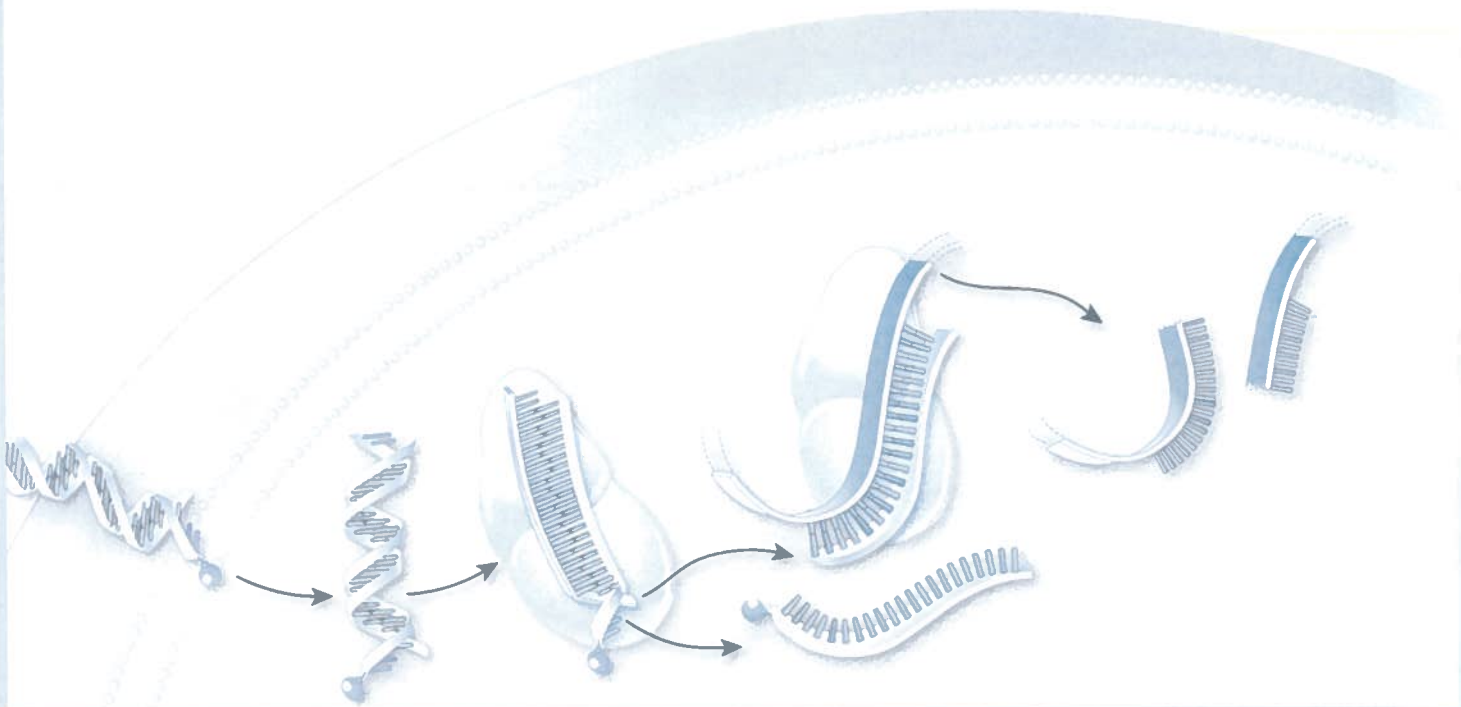
Alnylam Pharmaceuticals,
a leading RNAi therapeutics company,
is pleased to congratulate the

2006 Nobel Prize recipients in Physiology or Medicine

Dr. Andrew Z. Fire and Dr. Craig C. Mello

for their revolutionary discovery of RNA interference

The discovery of RNA interference (RNAi), a natural biological pathway for control of gene expression, is widely recognized as one of the most important scientific discoveries in decades. RNAi has revolutionized post-genomic research for target validation and has enabled the opportunity to create a whole new class of drugs to harness the RNAi pathway to silence disease-causing genes.



Alnylam Pharmaceuticals

HARNESSING A REVOLUTION IN BIOLOGY FOR HUMAN HEALTH®

I am pleased and honored to have this opportunity to congratulate Dr. Andrew Z. Fire and Dr. Craig C. Mello for their receipt of the 2006 Nobel Prize in Physiology or Medicine for their discovery of RNA interference, or RNAi. Fire and Mello's groundbreaking findings have sparked new excitement in the field of drug discovery and development. Indeed, RNAi represents one of the most promising and rapidly progressing frontiers in biology and drug discovery today, with many scientists worldwide working to advance Fire and Mello's discovery to develop novel products and technologies that benefit human health.

Less than a decade after the publication of their breakthrough research in *Nature*, the creation of a major new class of medicines, known as RNAi therapeutics, is visible on the horizon. By harnessing the natural biological process of RNAi occurring in our cells, RNAi therapeutics target the genetic cause of diseases by silencing specific messenger RNA, thereby preventing disease-causing proteins from being made. While there's more work to do to realize RNAi's full potential, this important discovery may be able to treat disease and help patients in an entirely new way.

Fueled by the potential of RNAi therapeutics, the progress in translating Fire and Mello's scientific discoveries to creation of innovative therapeutics has been remarkable. Researchers across the globe are using RNAi to investigate and develop drugs for a wide variety of important diseases, including respiratory diseases, cancer, neurological diseases, and ocular diseases. Already, three RNAi therapeutic candidates have entered human clinical trials, with dozens more likely to come over the next few years.

At Alnylam, we are committed to being at the forefront of the discovery and advancement of RNAi therapeutics. Having assembled a foundation of pioneering RNAi scientists and the industry's leading intellectual property estate, we are uniquely and singularly focused on playing a leading role in establishing RNAi therapeutics as a fundamentally important new class of medicines.

The Nobel Prize is a crowning achievement in the short and sparkling history of RNAi. All of us who are now involved in moving RNAi forward have a both a great opportunity and an important responsibility to work to ensure that RNAi has a significant and lasting impact on human health. At Alnylam, we are steadfastly dedicated to making RNAi vital to the future of drug development – that is why we are working to bring the first RNAi products to market, and why we have sponsored this *Nature* publication.

John M. Maraganore, Ph.D.
President and CEO
Alnylam Pharmaceuticals, Inc.

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A big award for a small RNA

By general agreement, it was only a matter of time before the work that initiated the RNA interference (RNAi) revolution would be honoured by the Nobel Prize committee. The only surprise was the speed with which the committee recognized this discovery, the effects of which have permeated so many areas of biological research.

Since the early twentieth century, biologists had noted mysterious phenomena that were triggered by virus infection of plants. Less than 20 years ago, experiments with transgenes suggested that there were both transcriptional and post-transcriptional mechanisms of gene silencing, but the mechanisms were unclear. Nevertheless, researchers began to exploit the observation that the introduction of RNA into a cell could interfere with endogenous gene expression; it was believed that this resulted from an antisense interaction between the exogenous RNA and the messenger RNA (mRNA).

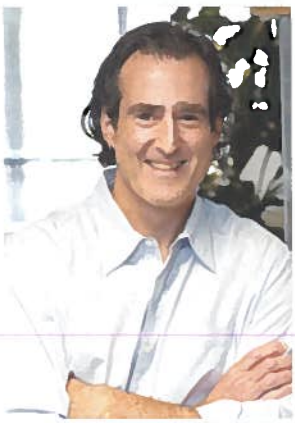
The breakthrough came when Andrew Fire, Craig Mello, and their colleagues tried to understand the RNA requirements for this reaction. Their work, published in *Nature* in 1998, showed that injection of double-stranded RNA, rather than single-stranded RNA, elicited a much more potent silencing response. This finding was contrary to what was expected if a simple antisense mechanism operated. The second surprise was that the reaction seemed catalytic. Finally, silencing was observed not only in the injected worm, but also in its progeny. These provocative results left no doubt that a previously unrecognized but powerful system of gene regulation existed.

In this collection *Nature* presents the original paper by Fire, Mello and colleagues, the News and Views commentary piece by Richard Wagner and Lin Sun that accompanied the original paper, and the recent *Nature* News story that accompanied the announcement of the prize.

Nature Publishing Group is grateful to Alnylam Pharmaceuticals, a leading developer of RNAi-based therapeutics, for sponsorship of this collection.

Angela K. Eggleston

Youthful duo snags a swift Nobel for RNA control of genes



Silence is golden: Craig Mello (top) and Andrew Fire.

STANFORD UNIV., UNIV OF MASSACHUSETTS

The call from Stockholm was received by two unusually young scientists, an impressively short time after they demonstrated a fundamental control of gene expression.

The 2006 Nobel Prize in Physiology or Medicine has gone to Andrew Fire, now at Stanford University, California, and Craig Mello, now at the University of Massachusetts Medical Center in Worcester. Both are in their forties. It honours a discovery that has transformed biological research and may, in the future, prove useful in treating human disease.

The discovery is called RNA interference, or RNAi. When a gene is to be expressed, it sends instructions to the cell's protein synthesis machinery. The intermediary is messenger RNA (mRNA), which has a structure complementary to that of the gene. In their breakthrough paper, published in *Nature* in 1998, Fire and Mello, and colleagues, demonstrated that these mRNAs can be targeted for destruction by specific double-stranded forms of RNA (A. Fire *et al. Nature* **391,** 806–811; 1998).

Researchers already knew that 'antisense' RNA — an artificial molecule whose sequence complements the mRNA — could silence specific genes when taken up by a cell. But the effect was modest and inconsistent. And, confusingly, the same effect was seen with 'sense' RNA.

In a series of simple and elegant experiments on a muscle gene in the nematode worm *Caenorhabditis elegans*, Fire and Mello showed that a powerful and consistent effect required the sense and antisense RNAs to be stuck together, as double-stranded RNA. When injected with the double-stranded RNA, the worms twitched awkwardly, just like mutant worms lacking the muscle gene. The researchers also showed that mRNA was destroyed by the treatment, rather than being masked as others had believed. And they showed that the double-stranded RNA can cause more copies of itself to be made, can spread between cells and can even be inherited by progeny.

In the *Nature* paper the researchers speculated that organisms might use this mechanism to control expression of their genes — a fact soon shown to be true. RNAi turned out to be an important way of controlling 'jumping genes', which can insert themselves throughout the genome and disrupt gene function. It is also thought to help protect against viral infections, at least in simple organisms.

Fire and Mello's subsequent research, and that of the many researchers who flooded the field after them, have since filled in the details. The RNAi pathway has been fully worked out and shown to apply to all genes — not just in worms but in nearly all other species.

"There is hardly a lab not using the tools of RNAi to turn off the genes that they are studying," says Tom Tuschl of Rockerfeller University, New York, who joined the RNAi élite soon after the Fire and Mello discovery. RNAi reagents have been used to find genes involved in everything from ageing to cancer. Clinical hopes are pinned on finding ways to use RNAi to reduce the activity of genes that cause disease.

Most Nobel prizes are given many years after the relevant discovery. The Fire and Mello award, given just eight years after publication of their paper, is reminiscent of Kary Mullis's 1993 chemistry Nobel. That prize was awarded for his 1985 invention of the polymerase chain reaction — a method of gene amplification that invaded research labs just as fast and comprehensively as the RNAi technique has.

Ronald Plasterk of the University of Utrecht in the Netherlands, who worked with Fire when they were young researchers at the Laboratory of Molecular Biology in Cambridge, UK, says he felt that a Nobel was around the corner. But he didn't think it would be so soon. For a laugh, on the morning of the announcement Plasterk asked his postdoc to call Fire in a pronounced Swedish accent. "But then we heard he had really won, so the joke had to be dropped."

Mello seemed stunned when interviewed on the Nobel webcast, not long after he had heard the news. "I seem too young," he said. "And isn't the gap unusually short?"

Alison Abbott

Functional genomics

Double-stranded RNA poses puzzle

Richard W. Wagner and Lin Sun

The human genome is predicted to contain between 50,000 and 100,000 genes[1]. To work out what these genes do, an array of techniques is needed to evaluate the protein–protein interactions and biochemical pathways of any gene product. The nematode worm *Caenorhabditis elegans* is an excellent system for such studies because of its well-understood genetics and development, evolutionary conservation to human genes, small genome size and relatively short life cycle. The 100-megabase-pair genome will be completely sequenced this year, and a total of 17,000 genes have been predicted, many with human counterparts. Approaches used to manipulate gene expression in *C. elegans* include transposon-mediated deletion[2], antisense inhibition[3] and direct isolation of deletions after mutagenesis[4,5]. Although these methods have proved useful, limitations still exist.

On page 806 of this issue, Fire and colleagues[6] describe a remarkable and surprising technique for inhibiting gene function in *C. elegans*. They turned off a specific gene in progeny worms by microinjecting double-stranded RNA (dsRNA) complementary to the coding region of the gene into the gonads of adult animals. Using a well-characterized gene, *unc-22*, which encodes a non-essential myofilament protein, they showed that injection of dsRNA produced a phenotype characteristic of *unc-22* inhibition — twitching.

In a series of well-controlled studies, the authors also found that injection of dsRNA targeted to a reporter gene for green fluorescent protein resulted in a dramatic — and specific — decrease in protein production. Furthermore, when they injected dsRNA targeted to another gene, *mex-3*, the result was a loss of *mex-3* RNA in early-stage embryos. In other words, at the levels of phenotype, RNA and protein, the interference with gene expression was specific and reproducible.

Perhaps most astounding is the phenomenon that the dsRNA causes gene inhibition. Previously[3], Fire and co-workers had been puzzled by the fact that antisense RNA alone — which is often used to inactivate sense messenger RNA — was only marginally effective. Furthermore, results using the antisense RNA were mimicked by injection of sense RNA, a control in their studies. They later found out that these data could be largely explained by an artefact of the transcription process that was used to generate the antisense and sense RNAs; namely, dsRNA fragments.

Additional experiments by Fire *et al.*, designed to shed light on the possible mechanism of the dsRNA-mediated inhibition, painted an even more mystifying picture. For example, even when only a few copies of the dsRNAs are present in each cell, they are active against highly abundant RNAs. This indicates that the interference occurs either by a catalytic mechanism or at the chromosomal level — and not by a conventional antisense mechanism. The authors also found that only dsRNAs that are complementary to coding regions of the gene are active, and not, for example, those targeted to introns or promoter regions. This argues against a generalized mechanism involving chromosomal inactivation, such as chromosomal deletion. Moreover, dsRNA interference seems to cross cellular boundaries with ease. Gene inhibition was observed in progeny when dsRNA was injected into the body cavity of the adult (gonadal injections had been thought to be necessary), and in somatic tissues of young adults after injection into their body cavity.

What kind of mechanism have Fire and colleagues uncovered? This is not the first puzzle posed by dsRNA. Almost ten years ago, Bass and Weintraub[7] and Wagner *et al.*[8] discovered an enzyme that binds dsRNA and deaminates adenosines in the duplex to inosines. After a feverish hunt for the cellular function of the dsRNA adenosine deaminase, it was found to be involved in the post-transcriptional editing of messages. Inosines are read by the cellular machinery

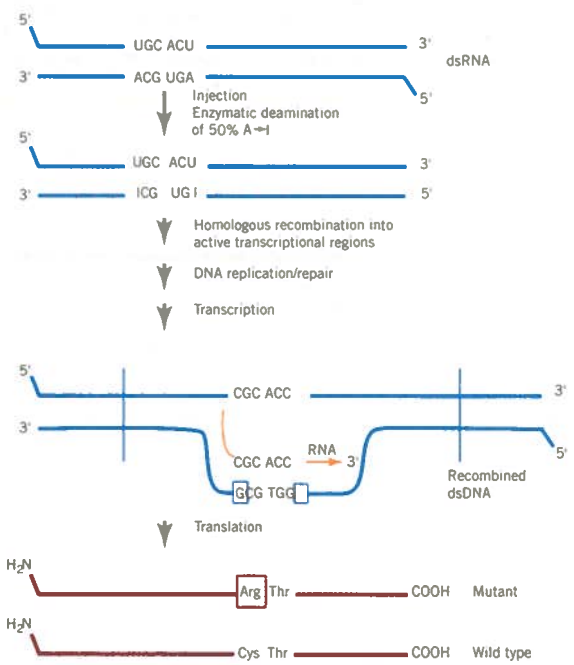


Figure 1 Possible mechanism for inhibition of gene expression in *C. elegans* by double-stranded RNA. Fire *et al.*[6] have convincingly shown that, at the phenotype, RNA and protein levels, dsRNA-mediated interference with gene expression is specific and reproducible. Perhaps, on injection into worms, dsRNA is modified by dsRNA adenosine deaminase. Transfer of this information back into the chromosome may occur by a recombination event. After replication and mismatch repair, transcription and translation result in mutant proteins that have impaired function.

as guanosine, so the enzyme could alter the genetic make-up of mRNA (reviewed in refs 9, 10).

Could this dsRNA adenosine deaminase be involved in a complicated pathway that results in gene inhibition in *C. elegans*? Quite possibly. The enzymatic activity has been found in *C. elegans*, and would probably treat the injected dsRNA as a substrate. A specialized homologous recombination system would be needed, which would use the modified dsRNA to transfer the genetic alterations into the chromosome (Fig. 1).

This model fits some of the data: modification of adenosines to inosines alters the genetic make-up of the injected dsRNA; transfer of this information into the genome by recombination would affect coding (but not intronic) regions; and mutations introduced by the inosine substitutions would affect the ability to detect mRNA and, at least partially, the function of the protein. These mutations could account for the surprising result that only a few copies of dsRNA are required per cell, because they would have an effect at the level of the chromosome. Of course, such a model is a stretch of the imagination and is not supported by all of the data. For example, attempts to use homologous recombination with dsDNA in *C. elegans* have largely failed[3].

Fire and colleagues[6] have uncovered a complex and intriguing mode of regulation in *C. elegans*. Does dsRNA perform a biological function in *C. elegans* (and is this function titrated out by the microinjected dsRNA)? Does a similar phenomenon exist in other organisms? What would happen if transgenic animals or plants were generated expressing both the sense and antisense strands of a transgene? A similar mode of action would not be suspected to occur in mammals, because injection of dsRNA is often used as a control for antisense experiments, albeit at the individual cell (and not organism) level. Nevertheless, perhaps specific 'knockouts' can be generated this way, for organisms in which genetic material cannot be delivered by microinjection. Whatever the mechanism might be, dsRNA-mediated inhibition of gene expression will provide a useful alternative for working out gene function in *C. elegans* and, maybe, in other animals and plants. □

Richard W. Wagner and Lin Sun are at Phylos Inc., 300 Putnam Avenue, Cambridge, Massachusetts 02139, USA.
e-mail: rwagner@phylos.com

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

Andrew Fire*, SiQun Xu*, Mary K. Montgomery*, Steven A. Kostas*†, Samuel E. Driver‡ & Craig C. Mello‡

* Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, Maryland 21210, USA
† Biology Graduate Program, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218, USA
‡ Program in Molecular Medicine, Department of Cell Biology, University of Massachusetts Cancer Center, Two Biotech Suite 213, 373 Plantation Street, Worcester, Massachusetts 01605, USA

Experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene[1,2]. 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[3,4]. 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 stochiometric 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[3,4]. Second, interference effects can persist well into the next generation, even though many endogenous RNA transcripts are rapidly degraded in the early embryo[5]. 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[6]. These polymerases, although highly specific, produce some random or ectopic transcripts. DNA transgene arrays also produce a fraction of aberrant RNA products[3]. 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. *unc-22* encodes an abundant but nonessential myofilament protein[7–9]. Several thousand copies of *unc-22* mRNA are present in each striated muscle cell[3]. Semiquantitative correlations between *unc-22* activity and phenotype of the organism have been described[8]: 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[10]. 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[7,11,12]; *fem-1* encodes an ankyrin-repeat-containing

protein that is required in hermaphrodites for sperm production[13,14]; and *hlh-1* encodes a *C. elegans* homologue of myoD-family proteins that is required for proper body shape and motility[15,16]. 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[17]. 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.

Table 1 Effects of sense, antisense and mixed RNAs on progeny of injected animals

Gene	segment	Size (kilobases)	Injected RNA	F_1 phenotype
unc-22				*unc-22*-null mutants: strong twitchers[18]
*unc22A**	Exon 21–22	742	Sense	Wild type
			Antisense	Wild type
			Sense + antisense	Strong twitchers (100%)
unc22B	Exon 27	1,033	Sense	Wild type
			Antisense	Wild type
			Sense + antisense	Strong twitchers (100%)
unc22C	Exon 21–22†	785	Sense + antisense	Strong twitchers (100%)
fem-1				*fem-1*-null mutants: femal (no sperm)[13]
fem1A	Exon 10‡	531	Sense	Hermaphrodite (98%)
			Antisense	Hermaphrodite (>98%)
			Sense + antisense	Female (72%)
fem1B	Intron 8	556	Sense + antisense	Hermaphrodite (>98%)
unc-54				*unc-54*-null mutants: paralysed[19]
unc54A	Exon 6	576	Sense	Wild type (100%)
			Antisense	Wild type (100%)
			Sense + antisense	Paralysed (100%)§
unc54B	Exon 6	651	Sense	Wild type (100%)
			Antisense	Wild type (100%)
			Sense + antisense	Paralysed (100%)§
unc54C	Exon 1–5	1,015	Sense + antisense	Arrested embryos and larvae (100%)
unc54D	Promoter	567	Sense + antisense	Wild type (100%)
unc54E	Intron 1	369	Sense + antisense	Wild type (100%)
unc54F	Intron 3	386	Sense + antisense	Wild type (100%)
hlh-1				*hlh-1*-null mutants: lumpy-dumpy larvae[16]
hlh1A	Exons 1–6	1,033	Sense	Wild type (<2% lpy-dpy)
			Antisense	Wild type (<2% lpy-dpy)
			Sense + antisense	Lpy-dpy larvae (>90%)‖
hlh1B	Exons 1–2	438	Sense + antisense	Lpy-dpy larvae (>80%)‖
hlh1C	Exons 4–6	299	Sense + antisense	Lpy-dpy larvae (>80%)‖
hlh1D	Intron 1	697	Sense + antisense	Wild type (<2% lpy-dpy)
myo-3-driven GFP transgenes¶				
myo-3::NLS::gfp::lacZ				Makes nuclear GFP in body muscle
gfpG	Exons 2–5	730	Sense	Nuclear GFP–LacZ pattern of parent strain
			Antisense	Nuclear GFP–LacZ pattern of parent strain
			Sense + antisense	Nuclear GFP–LacZ absent in 98% of cells
lacZL	Exon 12–14	830	Sense + antisense	Nuclear GFP–LacZ absent in >95% of cells
myo-3::MtLS::gfp				Makes mitochondrial GFP in body muscle
gfpG	Exons 2–5	730	Sense	Mitochondrial-GFP pattern of parent strain
			Antisense	Mitochondrial-GFP pattern of parent strain
			Sense + antisense	Mitochondrial-GFP absent in 98% of cells
lacZL	Exon 12–14	830	Sense + antisense	Mitochondrial-GFP pattern of parent strain

Each RNA was injected into 6–10 adult hermaphrodites (0.5×10^6–1×10^6 molecules into each gonad arm). After 4–6 h (to clear prefertilized eggs from the uterus), injected animals were transferred and eggs collected for 20–22 h. Progeny phenotypes were scored upon hatching and subsequently at 12-24-h intervals.

* to obtain a semiquantitative assessment of the relationship between RNA dose and phenotypic response, we injected each *unc22A* RNA preparation at a series of different concentrations (see figure in Supplementary information for details). At the highest dose tested (3.6×10^6 molecules per gonad), the individual sense and antisense *unc22A* preparations produced some visible twitching (1% and 11% of progeny, respectively). Comparable doses of double-stranded *unc22A* RNA produced visible twitching in all progeny, whereas a 120-fold lower dose of double-stranded *unc22A* RNA produced visible twitching in 30% of progeny. † *unc22C* also carries the 43-nucleotide intron between exons 21 and 22. ‡ *fem1A* carries a portion (131 nucleotides) of intron 10. § Animals in the first affected broods (layed 4–24 h after injection) showed movement defects indistinguishable from those of *unc-54*-null mutants. A variable fraction of these animals (25%–75%) failed to lay eggs (another phenotype of *unc-54*-null mutants), whereas the remainder of the paralysed animals did lay eggs. This may indicate incomplete interference with *unc-54* activity in vulval muscles. Animals from later broods frequently show a distinct partial loss-of-function phenotype, with contractility in a subset of body-wall muscles. ‖ Phenotypes produced by RNA-mediated interference with *hlh-1* included arrested embryos and partially elongated L1 larvae (the *hlh-1*-null phenotype). These phenotypes were seen in virtually all progeny after injection of double-stranded *hlh1A* and in about half of the affected animals produced after injection of double-stranded *hlh1B* and double-stranded *hlhlC*. A set of less severe defects was seen in the remainder of the animals produced after injection of double-stranded *hlh1B* and double-stranded *hlh1C*). The less severe phenotypes are characteristic of partial loss of function of *hlh-1* (B. Harfe and A.F., unpublished observations). ¶ the host for these injections, strain PD4251, expresses both mitochondrial GFP and nuclear GFP–LacZ (see Methods). This allows simultaneous assay for interference with *gfp* (seen as loss of all fluorescence) and with *lacZ* (loss of nuclear fluorescence). The table describes scoring of animals as L1 larvae. Double-stranded *gfpG* caused a loss of GFP in all but 0–3 of the 85 body muscles in these larvae. As these animals mature to adults, GFP activity was seen in 0–5 additional body-wall muscles and in the 8 vulval muscles. Lpy-dpy, lumpy-dumpy.

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 *hlh-1* null mutants, in particular, are known to result from many defective muscle cells[11,16]. 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.

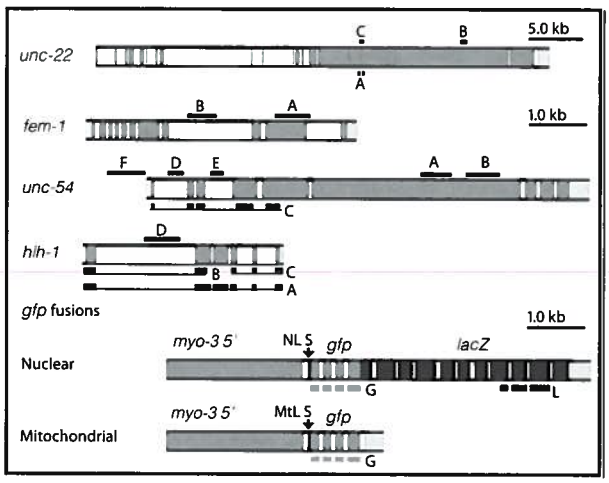

Figure 1 Genes used to study RNA-mediated genetic interference in *C. elegans*. Intron–exon structure for genes used to test RNA-mediated inhibition are shown (grey and filled boxes, exons; open boxes, introns; patterned and striped boxes, 5′ and 3′ untranslated regions. *unc-22*. ref. 9, *unc-54*, ref. 12, *fem-1*, ref. 14, and *hlh-1*, ref. 15). Each segment of a gene tested forRNA interference is designated with the name of the gene followed by a single letter (for example, *unc22C*). These segments are indicated by bars and upper-case letters above and below each gene. Segments derived from genomic DNA are shown above the gene; segments derived from cDNA are shown below the gene. NLS, nuclear-localization sequence; MtLS, mitochondrial localization sequence.

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[18,19]). 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[20], in which straightforward *in situ* hybridization can be performed[5]. 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).

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).

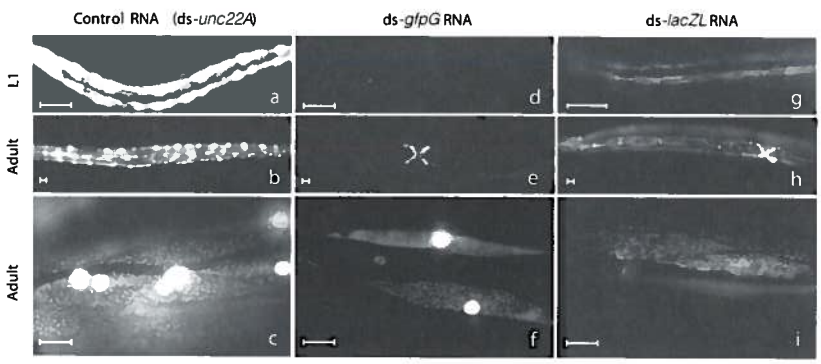

Figure 2 Analysis of RNA-interference effects in individual cells. Fluorescence micrographs show progeny of injected animals from GFP-reporter strain PD4251. **a–c**, Progeny of animals injected with a control RNA (double-stranded (ds)-*unc22A*). **a**, Young larva, **b**, adult, **c**, adult body wall at high magnification. These GFP patterns appear identical to patterns in the parent strain, with prominent fluorescence in nuclei (nuclear-localized GFP–LacZ) and mitochondria (mitochondrially targeted GFP). **d–f**, Progeny of animals injected with ds-*gfpG*. Only a single active cell is seen in the larva in **d**, whereas the entire vulval musculature expresses active GFP in the adult animal in **e**. **f**, Two rare GFP-positive cells in an adult: both cells express both nuclear-targeted GFP–LacZ and mitochondrial GFP. **g–i**, Progeny of animals injected with ds-*lacZL* RNA: mitochondrial-targeted GFP seems unaffected, while the nuclear-targeted GFP–LacZ is absent from almost all cells (for example, see larva in **g**). **h**, A typical adult, with nuclear GFP–LacZ lacking in almost all body-wall muscles but retained in vulval muscles. Scale bars represent 20 μm.

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[21] 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.

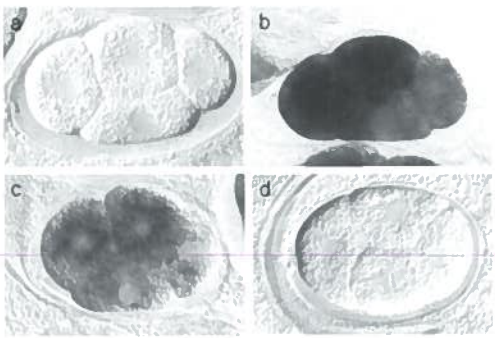

Figure 3 Effects of *mex-3* RNA interference on levels of the endogenous mRNA. Interference contrast micrographs show *in situ* hybridization in embryos. The 1,262-nt *mex-3* cDNA clone[20] was divided into two segments, *mex-3A* and *mex-3B*, with a short (325-nt) overlap (similar results were obtained in experiments with no overlap between interfering and probe segments). *mex-3B* antisense or dsRNA was injected into the gonads of adult animals, which were fed for 24 h before fixation and *in situ* hybridization (ref. 5; B. Harfe and A.F., unpublished observations). The *mex-3B* dsRNA produced 100% embryonic arrest, whereas >90% of embryos produced after the antisense injections hatched. Antisense probes for the *mex-3A* portion of *mex-3* were used to assay distribution of the endogenous *mex-3* mRNA (dark stain). Four-cell-stage embryos are shown; similar results were observed from the one to eight cell stage and in the germ line of injected adults. **a**, Negative control showing lack of staining in the absence of the hybridization probe. **b**, Embryo from uninjected parent (showing normal pattern of endogenous *mex-3* RNA[20]). **c**, Embryo from a parent injected with purified *mex-3B* antisense RNA. These embryos (and the parent animals) retain the *mex-3* mRNA, although levels may be somewhat less than wild type. **d**, Embryo from a parent injected with dsRNA corresponding to *mex-3B*; no *mex-3* RNA is detected. Each embryo is approximately 50 μm in length.

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[22,23].

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[22,24]. 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[6]. 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

Table 2 Effect of site of injection on interference in injected animals and their progeny

dsRNA	Site of injection	Injected-animal phenotype	Progeny phenotype
None	Gonad or body cavity	No twitching	No twitching
None	Gonad or body cavity	Strong nuclear and mitochondrial GFP expression	Strong nuclear and mitochondrial GFP expression
unc22B	Gonad	Weak twitchers	Strong twitchers
unc22B	Body-cavity head	Weak twitchers	Strong twitchers
unc22B	Body-cavity tail	Weak twitchers	Strong twitchers
gfpG	Gonad	Lower nuclear and mitochondiral GFP expression	Rare or absent nuclear and mitochondiral GFP expression
gfpG	Body-cavity tail	Lower nuclear and mitochondrial GFP expression	Rare or absent nuclear and mitochondrial GFP expression
lacZL	Gonad	Lower nuclear GFP expression	Rare or absent nuclear-GFP expression
lacZL	Body-cavity tail	Lower nuclear GFP expresison	Rare or absent nuclear-GFP expression

The GFP-reporter strain PD4251, which expresses both mitochondrial GFP and nuclear GFP–LacZ, was used for injections. The use of this strain allowed simultaneous assay for interference with *gfp* (fainter overall fluorescence), *lacZ* (loss of nuclear fluorescence) and *unc-22* (twitching). Body-cavity injections into the tail region were carried out to minimize accidental injection of the gonad; equivalent results have been observed with injections into the anterior body cavity. An equivalent set of injections was also performed into a single gonad arm. The entire progeny broods showed phenotypes identical to those described in Table 1. This included progeny of both injected and uninjected gonad arms. Injected animals were scored three days after recovery and showed somewhat less dramatic phenotypes than their progeny. This could be partly due to the persistence of products already present in the injected adult. After injection of double-stranded *unc22B*, a fraction of the injected animals twitch weakly under standard growth conditions (10 out of 21 animals). Levamisole treatment led to twitching of 100% (21 out of 21) of these animals. Similar effects (not shown) were seen with double-stranded *unc22A*. Injections of double-stranded *gfpG* or double-stranded *lacZL* produced a dramatic decrease (but not elimination) of the corresponding GFP reporters. In some cases, isolated cells or parts of animals retained strong GFP activity. These were most frequently seen in the anterior region and around the vulva. Injections of double-stranded *gfpG* and double-stranded *lacZL* produced no twitching, whereas injections of double-stranded *unc22A* produced no change in the GFP-fluorescence pattern.

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 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 0.5×10^6 to 1.0×10^6 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[26] 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[27]. 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.

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Correspondence and requests for materials should be addressed to A.F. (e-mail: fire@mail1.ciwemb.edu).

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