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Production of antisense RNA leads to effective and specific inhibition of gene expression in *C. elegans* muscle

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Summary

We have used an antisense strategy to effectively disrupt the expression of two genes encoding myofilament proteins present in *C. elegans* body wall muscles. DNA segments from the *unc-22* and *unc-54* genes have been placed in reverse orientation in vectors designed to produce RNA in body wall muscles. When the resulting plasmids are injected into oocytes, progeny with defects in muscle function are produced. These animals have phenotypes consistent with reduction and/or elimination of function of the gene to which antisense RNA has been produced: twitching and disorganization of muscle filaments for the *unc-22* antisense constructs and lack of muscle tone, slow movement, and egg laying defects for the *unc-54* antisense constructs. A fraction of the affected animals transmit the defective-muscle trait to subsequent generations. In these cases the transforming DNA is present at high copy number and cosegregates with the observed muscle defects. We have examined several of the *unc-22* antisense plasmid transformed lines to determine the mechanistic basis for the observed phenotypes. The RNA product of the endogenous *unc-22* locus is present at normal levels and this RNA is properly spliced in the region homologous to the antisense RNA. No evidence for modification of this RNA by deamination of adenosine to inosine was found. In affected animals the level of protein product from the endogenous *unc-22* locus is greatly reduced. Antisense RNA produced from the transforming DNA was detected and was much more abundant than 'sense' RNA from the endogenous locus. These data suggest that the observed phenotypes result from interference with a late step in gene expression, such as transport into the cytoplasm or translation.

Key words: antisense RNA, *C. elegans*, muscle.

Introduction

The ability to disrupt expression of specific genes can provide a valuable tool to relate cloned genes to their underlying functions. The most elegant methods for gene disruption utilize homologous recombination between injected DNA and the corresponding chromosomal locus; at present such methods are only available in a limited set of experimental systems. An alternative approach has been proposed whereby expression of specific genes could be effectively disrupted by the presence of excess quantities of negative-stranded nucleic acid (Izant and Weintraub, 1984). Antisense inhibition has been tried for several different systems and applications; varying degrees of success have been reported (for review see Takayama and Inouye, 1990). We have used a set of muscle expression vectors in *Caenorhabditis elegans* to produce antisense RNA directed to genes encoding abundant muscle filament proteins. We show that production of antisense RNA homologous to a specific gene leads to a decrease in expression of the endogenous chromosomal locus and to structural, functional and biochemical defects in muscle that parallel the effects of known mutations in the corresponding chromosomal locus.

Materials and methods

Selection for unc-22 *disruption in transformed lines*

Oocyte injections were performed as described (Fire, 1986). Following injection, animals were grown at 20°C or 25°C and progeny examined for any evident alterations from the wildtype. For detection of weak twitching phenotypes, we used the procedure of Moerman and Baillie (1979). The animals were washed into 1% nicotine (in H_2O) as late L4

larvae or young adults, and placed in an empty Petri plate. Wild-type animals hypercontract in this assay while the *unc-22* hypomorphs (animals with reduced *unc-22* activity) are evident by their inability to hypercontract and their characteristic twitching. Free nicotine is extremely toxic both as a vapor and if absorbed through skin, and should only be used in well-ventilated containment conditions.

The strong twitching phenotypes could be easily seen without the nicotine selection. We transferred the injected adults to fresh seeded Petri plates at 1 day intervals; this allows slower growing or odd progeny to be easily identified. Among the progeny are twitching animals, which are particularly evident after the animals have been adults for 1–2 days.

DNAs

Construction and growth of plasmid DNAs were as described (Fire *et al.* 1990). DNAs in TE [10 mM Tris–HCl pH 7.4, 1 mM EDTA] were diluted directly into injection buffer (Fire, 1986) and used for injection. Linear DNAs to be injected were cleaved with the appropriate restriction enzyme and then checked by gel electrophoresis to confirm complete digestion. Sequences and restriction maps have been compiled using the program DNA Strider (Marck, 1988).

RNA

Mixed stage (non-starved) populations of animals were floated in [1 M sucrose, 1 mM EDTA] (to remove bacteria and agarose) and washed repeatedly in [10 mM NaEDTA pH 7.5, 100 mM NaCl] before freezing at −80 °C in aliquots containing 100 μl of packed worms. To extract an aliquot, 800 μl of 4 M guanidine isothiocyanate (Chirgwin *et al.* 1979) and 600 μl of acid washed sand were added and the samples shaken vigorously for 5 min; SDS was added to 17 mM and the samples were extracted three times with phenol–$CHCl_3$ and once with $CHCl_3$, added to 3.5 ml of [4 M NH_4Ac, 4 mM EDTA], and precipitated by the addition of 10.5 ml of ethanol. Samples were resuspended in 300 μl of [40 mM Tris pH 7.5, 1 mM EDTA] and reprecipitated twice from 1 M NH_4Ac by addition of 2.5 volumes of ethanol. This yields a preparation of total *C. elegans* nucleic acid, which is then treated with DNAase I (RNAase free; Boehringer-Mannheim) to give a 'total RNA' preparation that has not been preselected for single-stranded character, size, or poly(A) content.

Primed cDNA was prepared by mixing 10 ng of oligonucleotide primer with RNA from 2.5 μl of packed worms in 8.0 μl of TE8.4 [10 mM Tris–HCl pH 8.4, 1 mM EDTA], heating to 72 °C for 5 min, mixing with 2.0 μl of EB1 [0.25 M Tris–HCl pH 8.4, 0.25 M KCl], cooling slowly (approx. 1 °C per min) to 48 °C, and mixing with 15 μl of EB2 [50 mM Tris–HCl pH 8.4, 50 mM KCl, 8 mM $MgCl_2$, 5 mM DTT]. AMV reverse transcriptase was then added (20 units to start reactions + 10 units added 30 min later; total incubation 70 min, 48 °C).

Results

Injection of some unc-22 *DNA segments causes a twitching phenotype*

Initial suggestions that antisense inhibition might be possible in *C. elegans* grew out of attempts to develop a homologous recombination system for gene knockout. The *unc-22* gene seemed particularly suitable for these experiments: mutants with reduced *unc-22* activity are unable to sustain muscle contraction and therefore twitch (Brenner, 1974; Moerman and Baillie, 1979). The twitching phenotype is a relatively specific indicator for disruption of *unc-22* function. Over 500 mutants exhibiting the distinctive 'twitcher' phenotype have been characterized; all except for three are *unc-22* mutations (the three exceptions are rare non-null alleles of the gene *lev-11*I; Moerman and Baillie, 1979; Lewis *et al.* 1980; Moerman and Waterston, 1984; R. Waterston; D. Clark and D. Baillie, personal communications). When animals are examined in the presence of nicotine, the twitching phenotype becomes a very sensitive assay for partial reduction in *unc-22* activity (Moerman and Baillie, 1979). Table 1 shows the relationship between dosage and phenotype for *unc-22* (from Moerman, 1980). Animals with a 30–50 % decrease in *unc-22* expression can reproducibly be identified in large populations ($>10^6$ animals) using the nicotine screen (Moerman and Waterston, 1984).

Table 1. *Phenotypes of genetically isolated strains with reduced* unc-22 *activity*

unc-22 activity fraction of wild-type	Behavior	
	Normal Growth Medium	In 1 % Nicotine
0–25 %	twitches	twitches
33–67 %	normal	twitches
75–100 %	normal	hypercontracted

Animals with null mutations at the *unc-22* locus exhibit a characteristic twitching phenotype. The tendency to twitch is enhanced in 1 % nicotine so that animals with only one functional *unc-22* copy can readily be distinguished from wild-type adult hermaphrodites. Heterozygotes, hemizygotes, and polyploids have been used to determine the effects of intermediate *unc-22* levels as shown above (from Moerman, 1980.)

In an attempt to disrupt the endogenous *unc-22* gene by homologous recombination, we injected a variety of *unc-22* plasmid and lambda DNA clones into wild-type animals and selected in the next generation for the effective disruption of one of the copies of *unc-22*. Each of the *unc-22* clones injected contained only part (3.6–15 kb) of the *unc-22* gene (Fig. 1). From the viewpoint of achieving homologous gene disruption, these experiments were unsuccessful: there were no cases of homologous recombination. There was, however, an unexpectedly high frequency of twitching animals derived from the *unc-22* DNA injections. In most cases, the twitching trait could be transmitted to progeny, so that lines could be established. These lines fell into two classes. (1) Three *bona fide unc-22* alleles which appear to be small deletions in the gene. The deleted segments did not correlate with the injected DNAs, and we assume that the relatively high frequency of 'spontaneous' *unc-22* alleles that was observed in these experiments results from generally mutagenic effects of the injected DNA and/or the microinjection process. (2) A large number of lines (37 total) in which the endogenous *unc-22* loci were apparently unaltered, but that contained several hundred copies of the injected DNA in a large tandem array

The *unc-22* locus

Twitching lines derived after injecting *unc-22* DNA segments

Injected DNA	Number of Animals Injected	Unstable Twitcher Lines	Strong	Weak	Ni	Stable *unc-22* alleles
λDm22	33	14	14	0	≥4	1
λDm24	33	0	0	0	0	1
λDm20	40	5	1	4	≥3	0
λDm23	33	0	0	0	0	0
pGB3.5 cut *Bgl* II	33	3	3	0	3	0
λDm20 cut *Xho*I+*Sac*I	40	4	0	4	≥3	0
λDm20 cut *Xho*I	159	11	4	7	≥9	0

Fig. 1. Results of injecting cloned fragments of the *unc-22* gene. Top: map of the *unc-22* locus (derived from DNA sequence; Benian *et al.* 1989; Benian, G., Morris, M., L'Hernault, S., and Tobin, K., personal communication). Transcription proceeds to the right; exons are filled black, non-translated 5′ leader and 3′ tail are hatched. Fragments and clones used in this work are indicated beneath the map (Moerman *et al.* 1986; Benian *et al.* 1989). Bottom: high copy number twitching lines derived. Injected animals were either wild-type (N2) or *rec-1*(*s180*; Rose and Baillie, 1979). No reproducible difference in these experiments was observed between recipient strains. Animals in the F_1 were screened as fourth stage larvae or young adults for twitching in 1% nicotine. 'Strongly' twitching lines are those that can be observed to twitch in the absence of nicotine. 'Weakly' twitching lines twitch only in the presence of nicotine. 'Ni' indicates the minimum number injected parents that yielded twitching lines. Because the lines were originally established in the first generation after injection, each line must represent transformation of a different oocyte following injection. It is possible, however, that in some cases more than one transformed line was obtained from a single injected parent. The injected parents were maintained in groups of four, and Ni is the number of these groups that yielded transformed lines. The two stable *unc-22 alleles* obtained from these experiments were short deletions in the *unc-22* coding region. Approximately 1.4×10^5 chromosomes were screened. The spontaneous mutation frequency at *unc-22* is normally less than 10^{-6} (Moerman and Waterston, 1984). Cut DNAs were tested in a similar way to the uncut lambda phage. The *Bgl*II end was filled-in partially with Klenow fragment of DNA polymerase +ATP +GTP in an (unsuccessful) attempt to prevent religation.

(Fig. 2). The inheritance of the twitching phenotype in these lines indicates that the twitching is due to extrachromosomal elements. Different twitching lines have transmission frequencies of 5% to 95% per generation. No homozygous lines could be obtained; some progeny in each generation are completely wild-type (i.e. no extrachromosomal *unc-22* DNA, no alterations in the *unc-22* locus and no twitching). Animals were separated into twitching and non-twitching classes and stained with dye (DAPI or Hoechst 33258) to label the DNA. The twitching phenotype is correlated with the presence of extra-chromosomal 'spots' of staining material present in metaphase nuclei (data not shown). Similar spots are observed with other extrachromosomal arrays and in cases of genetically isolated free duplications (Herman *et al.* 1976; Stinchcomb *et al.* 1985). The twitching lines can apparently be maintained indefinitely (at least 20 generations) by continually choosing twitching animals.

Twitching lines can be derived from a variety of *unc-22* clones with no common region of overlap (Fig. 1). Lines have been divided into categories based on the severity of the twitching phenotype. The 'weakly' twitching lines can only be scored in nicotine, while the 'strongly' twitching strains exhibit clear twitching behavior even in the absence of nicotine. Not every *unc-22* clone has the ability to cause twitching when injected to generate the long tandem arrays. Indeed,

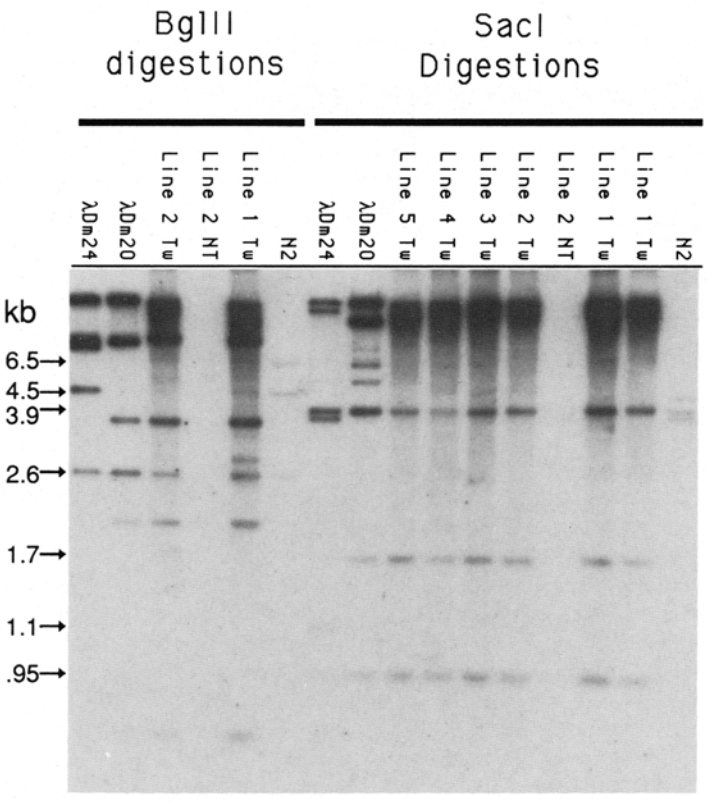


Fig. 2. Southern blot of high copy number transformed lines. DNA preparations derived from five lines transformed with *Xho*I cut λDm20 have been analyzed. Each of the lines was a weak twitching strain, exhibiting the twitching phenotype only in nicotine. Individual animals from each strain were tested for twitching in nicotine and picked individually onto 6 cm Petri plates. After 6 days (2 generations) the plates were harvested and DNA prepared. At this stage, 25–60 % of the animals in the populations exhibited twitching behavior in nicotine. The DNAs were digested with restriction enzymes as indicated, resolved on an agarose gel, transferred to nitrocellulose (Southern, 1975) and hybridized with nick translated λDm24. The high relative molecular mass bands presumably correspond to the phage arms. Wild-type *C. elegans* DNA (strain N2; Brenner, 1974) and phage DNAs have been run as markers. The N2 DNAs were loaded in excess of the DNAs from the transformed lines (5–10-fold more) in order to be able to visualize the single copy bands. For Line 1, results from two twitching subclones from the original line are shown. For Line 2, a non-twitching animal ('Line 2 NT') was picked; the derived population lacks the exogenous DNA from the injected clones. Bands from the wild-type *unc-22* locus are visible in these lanes upon further exposure. The 3.9 kb *Sac*I fragment and 2.6 kb *Bgl*II fragments observed on the blot are evidence for religation of the *Xho*I cut ends of the incoming DNA.

the cloned DNAs that were used in these experiments apparently generate different spectra of twitching levels: some constructs give rise primarily to weakly twitching lines and some to strongly twitching lines. The ability to induce twitching behavior is specific to *unc-22* DNA: strains with high copy numbers of recombinant DNAs with other *C. elegans* genes have not exhibited twitching behavior (Stinchcomb *et al.* 1985; Jefferson *et al.* 1987; Way and Chalfie, 1988).

We considered several possible ways in which these arrays could cause a twitching phenotype.

(1) Aberrant protein products might be produced from array transcripts which interfere with muscle function to cause twitching.

(2) Some level of indiscriminate transcription from the arrays might produce antisense RNA molecules that would specifically interfere with expression of the endogenous *unc-22* gene.

(3) Regulatory sites contained within the injected DNA might compete with the endogenous *unc-22* gene for specific factors required for expression.

(4) Chromosomal pairing between the endogenous loci and the large number of extrachromosomal copies of *unc-22* might disrupt normal *unc-22* gene expression.

The latter two possibilities seemed less likely, given the observed patterns of active and inactive constructs. Protein or antisense RNA effects would both require transcription of the injected DNA segments in muscle. Note that the 5′ end of *unc-22* is not included in any of the injected segments. Any expression of the arrays must therefore be promoted from ectopic sites in vector DNA or from within the *unc-22* gene. The pattern of active and inactive constructs could reflect differences in orientation and placement of the inserted *unc-22* sequences relative to the corresponding bacterial vectors. Since we could not detect any abundantly produced protein product from the arrays (data not shown), we suspected that the above phenomena could be due to antisense interference.

Deliberate disruption of gene activity by an 'antisense' strategy

In order to test directly for the ability of antisense RNA to disrupt gene expression in *C. elegans* muscle, we designed a series of expression constructs predicted to produce RNA complementary to the *unc-22* message. For these 'antisense' constructions, we used an expression vector (pPD12.01: Fig. 3 and Fire and Harrison, unpublished) containing the promoter, enhancer and 3′ nontranslated elements from the gene encoding the major myosin heavy chain isoform expressed in body wall muscle (Epstein *et al.* 1974). Since *unc-22* is also expressed in body wall muscle, this vector with an appropriate *unc-22* insert should produce an *unc-22* antisense transcript.

When the expression vector pPD12.01 (without insert) was injected into animals, no unusual phenotypes were observed among the progeny (Table 2). Injection of pPD12.01 derivatives carrying either of two different *unc-22* segments in antisense orientation leads to the production of F_1 animals with strong twitching behavior (Table 2). Approximately half of these F_1 twitchers give some twitching F_2 progeny, and in each of these cases, the twitching phenotype segregates in further generations as a dominant genetic element. The twitching phenotype varies somewhat between strains, but tends to be a much more severe phenotype than

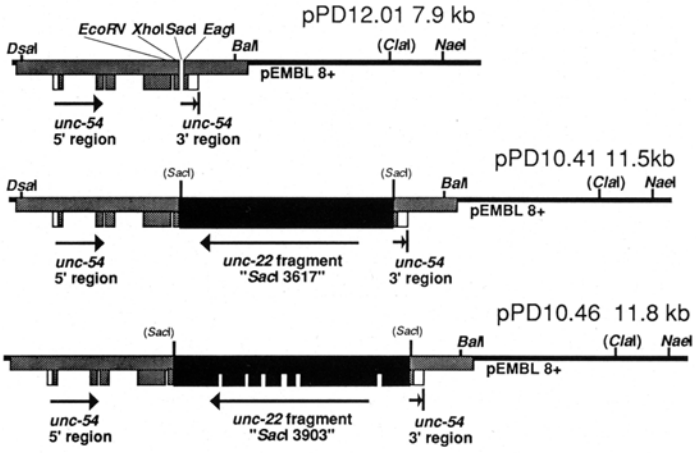


Fig. 3. Maps of muscle expression vector pPD12.01 and *unc-22* antisense expression constructs pPD10.41 and pPD10.46. Plasmid pPD12.01 was constructed by cutting plasmid pUNK54 (from I. Maruyama, described in Fire and Waterston, 1989) with *Sac*I and religating. Constructs pPD10.41 and pPD10.46 result from insertion of *Sac*I fragments from the *unc-22* gene into pPD12.01 in an antisense orientation. Unique restriction sites in nonessential regions of the plasmids are shown. (The *Cla*I site shown in parenthesis in pPD10.46 is unique only if the plasmid is grown in Dam+ bacteria).

with the lines derived after injection of *unc-22* DNA fragments cloned into vectors without muscle expression signals (e.g. those described in Fig. 1). In some of the lines made with the pPD12.01 driven antisense constructs, very severely affected animals are observed. These have phenotypes similar to the null phenotype for the *unc-22* locus (Moerman, 1980): highly disorganized muscle tissue with weak twitching and very little movement or egg laying in adults.

For most of these lines, the dominant twitching behaviour segregates as an extrachromosomal genetic element similar to lines described in Fig. 1 (i.e. a dominant, heritable twitch inducing 'locus' with transmission between 5% and 95% per generation). In all lines examined (10), the injected DNA is present in a high copy number array (>100 copies per nucleus). In one case the dominant twitching phenotype is caused by a long array which has integrated into one of the chromosomes, generating a semi-dominant twitch-inducing locus that can be maintained in a homozygous stock.

Several properties of the transformed lines are consistent with an antisense mechanism as the cause for the twitching phenotypes. One prediction would be that disrupting expression of a specific gene by antisense should result in a specific reduction of the corresponding protein product. To assay levels of *unc-22* and *unc-54* protein products we stained animals with antisera specific for either of the two proteins (Miller *et al.* 1983; Moerman *et al.* 1988), comparing twitching animals from the transgenic strains with their nontwitching siblings and wild-type animals. Body wall muscle in the control animals stains very strongly with both antisera. The strongly twitching animals show dramatically reduced unc-22 staining in body wall muscle, but show no such reduction in the *unc-54* product (Fig. 4 and data not shown). The reduction in *unc-22* staining represents at least a ten-fold difference. Less dramatic reductions in unc-22 staining were seen upon staining of the less severe twitching lines (data not shown). The reductions in unc-22 staining are not due simply to disorganization of the muscle cells: *unc-54* null mutant animals with very disorganized muscle structure stain intensely with antibodies to unc-22 protein (Moerman *et al.* 1988; A.F. unpublished results).

Faint residual staining with the unc-22 antibody is seen in muscle from the strongly twitching transgenic lines. This staining could be due to a residual level of *unc-22* product in these lines, although residual staining with this antiserum has also been observed upon

Table 2. *Disruption of muscle gene function following injection of antisense constructs. Results obtained with following injection of 'antisense' and 'sense' plasmids carrying* unc-22 *and* unc-54 *gene sequences*

Insert in 12.01	Orientation	Plasmid	Odd phenotypes	Injected adults	Affected F_1	Lines	Peptide coding potential
None	–	pPD 12.01	none	7	0	0	30K of myosin head
*unc-22 Sac*I-3.6	antisense	pPD 10.41	strong twitchers	15	13	5	30K of myosin head+11K nonsense
*unc-22 Sac*I-3.9	antisense	pPD 10.46	strong twitchers	9	9	4	30K of myosin head+0.5K nonsense
*unc-22 Sac*I-3.6	sense	pPD 10.69	strong twitchers	7	10	3	30K of myosin head+0.5K nonsense
*unc-22 Sac*I-3.9	sense	pPD 10.73	strong twitchers	7	4	4	30K of myosin head+5K nonsense
*unc-54 Sac*I-B	antisense	pPD 10.35	slow or paralyzed (flaccid)	15	38	4	30K of myosin head+33K nonsense
*unc-54 Sac*I-B	sense	pPD 10.34	none	14	0	0	All 87K of myosin head

'12.01' is the muscle expression vector pPD12.01 (Fig. 3). Plasmids were injected into wild-type adults and progeny screened under standard growth conditions for abnormal phenotypes. The phenotypes observed are described under 'Odd Phenotypes': the 'strong twitchers' behave similarly to *unc-22* mutants while the 'slow or paralyzed' animals (from pPD10.35) have a similar flaccid phenotype to that caused by *unc-54* mutations. The numbers represent the total number of injected adults, the number of their progeny showing muscle defects, and the number of F_1 progeny that gave rise to transformed lines. The predicted product from translation of the fusion messages in each construct are described under 'Peptide coding potential.' For each of the *unc-22* constructions, the fusion is out of frame, and translation would terminate soon after the fusion point. The two *unc-54* constructions pPD 10.34 and pPD10.35 can encode considerably larger proteins as shown. Another *unc-54* antisense construction with much less coding potential (4K mhcB+28K nonsense) yields results similar to pPD10.35 following injection (data not shown).

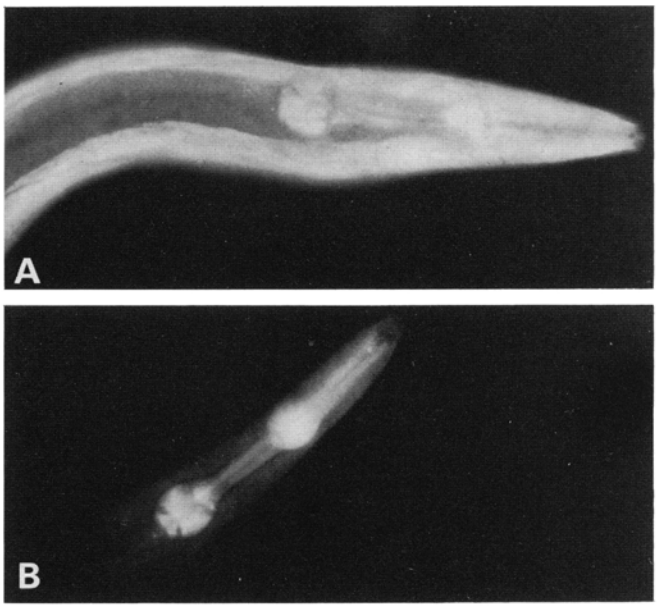


Fig. 4. Staining with antibody to the *unc-22* protein. Animals were fixed and stained as described (Albertson, 1984). Strain PD60 carries an extrachromosomal tandem array of the plasmid pPD10.41. A single brood was separated into twitching and non-twitching animals and the two populations fixed and stained with a polyclonal antibody to *unc-22* protein (R11–3; Moerman *et al.* 1988). A secondary antibody labelled with rhodamine was used as a second layer to visualize the stain. The samples were also counterstained with the DNA enhanced fluorochrome DAPI. A small extrachromosomal fragment of DAPI staining material was present in oocyte nuclei of the twitching animals but absent in oocytes of the non-twitching animals. (A) A non-twitching animal derived from strain PD60. This animal does not carry the extrachromosomal tandem array of pPD10.41 (as determined by DAPI staining). Wild-type animals (N2) were stained in parallel and give an identical staining pattern to that shown here. (B) A twitching animal from PD60 which has the extrachromosomal array of pPD10.41 as seen by DAPI staining. The observed staining is similar to that seen in null mutants in the *unc-22* gene. The pharyngeal staining is due to a protein that is antigenically related to the *unc-22* protein but which is probably encoded by a different gene (Moerman *et al.* 1988). Animals from two other strains carrying tandem arrays of the *unc-22* antisense constructions pPD10.41 and pPD10.46 show essentially the pattern shown here. In some cases, individual muscle cells or groups of cells show some *unc-22* staining, consistent with the stained animals being mosaic. Each of the above strains has also been stained with antibodies to mhcB. The mhcB isoform is present in body wall muscle in all of the twitching lines (data not shown).

staining of deletions of the endogenous *unc-22* locus (Moerman *et al.* 1988).

The antibody used to localize *unc-22* product also stains a pharyngeal protein, possibly an *unc-22* homologue encoded by a separate gene (Moerman *et al.* 1988). The pharyngeal staining is not affected in the transgenic twitching lines. This would be expected from the tissue specificity (non-pharyngeal muscle) of both *unc-22* and the expression vector pPD12.01.

Effects of injecting unc-54 *antisense constructs*

The phenotype caused by the expression driven antisense constructs is dependent on the source of the inverted segment. If a segment from the *unc-54* gene is inserted in reverse orientation into pPD12.01 and the resulting plasmid (pPD10.35) is injected into wild-type animals, uncoordinated animals are produced (Table 2). These animals do not twitch but have a lack of muscle tone, consistent with a decrease in *unc-54* gene function. The most severely affected animals have a paralyzed phenotype virtually identical to the *unc-54* null mutant phenotype (Brenner, 1974; Epstein *et al.* 1974). The majority of the uncoordinated animals derived from pPD10.35 injection are less severely affected. Levels of gene expression in individual cells have been assayed by staining of whole animals with antisera (Miller *et al.* 1983; Moerman *et al.* 1988) to the *unc-22*, *unc-54*, and *myo-3* protein products [*myo-3* encodes the minor myosin heavy chain isoform (mhcA) present in body wall muscle; Miller *et al.* 1986]. The disorganized cells stain strongly with antibodies to *myo-3* and *unc-22* products but exhibit very little staining with antibodies to *unc-54* product, while the well-organized cells yield essentially a wild-type pattern of staining with all three antibodies (data not shown).

Orientation dependence

In order to test the orientation dependence of the putative 'antisense' phenomenon, we have used the 'sense' equivalents of each of the plasmids as shown in Table 2. In the case of the *unc-54* insertion, the uncoordinated phenotype is apparently dependent on the orientation of the inserted segment. This strongly argues against a direct titration of DNA binding factors as the cause for the uncoordinated phenotype, since the two plasmids pPD10.34 and pPD10.35 have the same sequences in different orientations. For the *unc-22* constructs, no strong dependence on the orientation of the inserted segment was observed. Immunofluorescent staining (not shown; performed as described in Fig. 4) revealed dramatically reduced *unc-22* protein levels in lines transformed with the two *unc-22* 'sense' constructs. For this reason, as well as the results described in Fig. 1, we believe that the effects of *unc-22* 'sense' constructs actually result from antisense RNA. Indiscriminate transcription from the extrachromosomal arrays apparently occurs (Jefferson *et al.* 1987; also see legend to Fig. 6C). In addition, the expression vector pPD12.01 carries a muscle specific enhancer element that can act in either orientation and can enhance expression from both correct and ectopic initiation sites (Fire and Harrison, unpublished data). Thus it seems conceivable even with a 'sense' construct that sufficient antisense RNA could be made from the large arrays to interfere with expression of the endogenous *unc-22* gene.

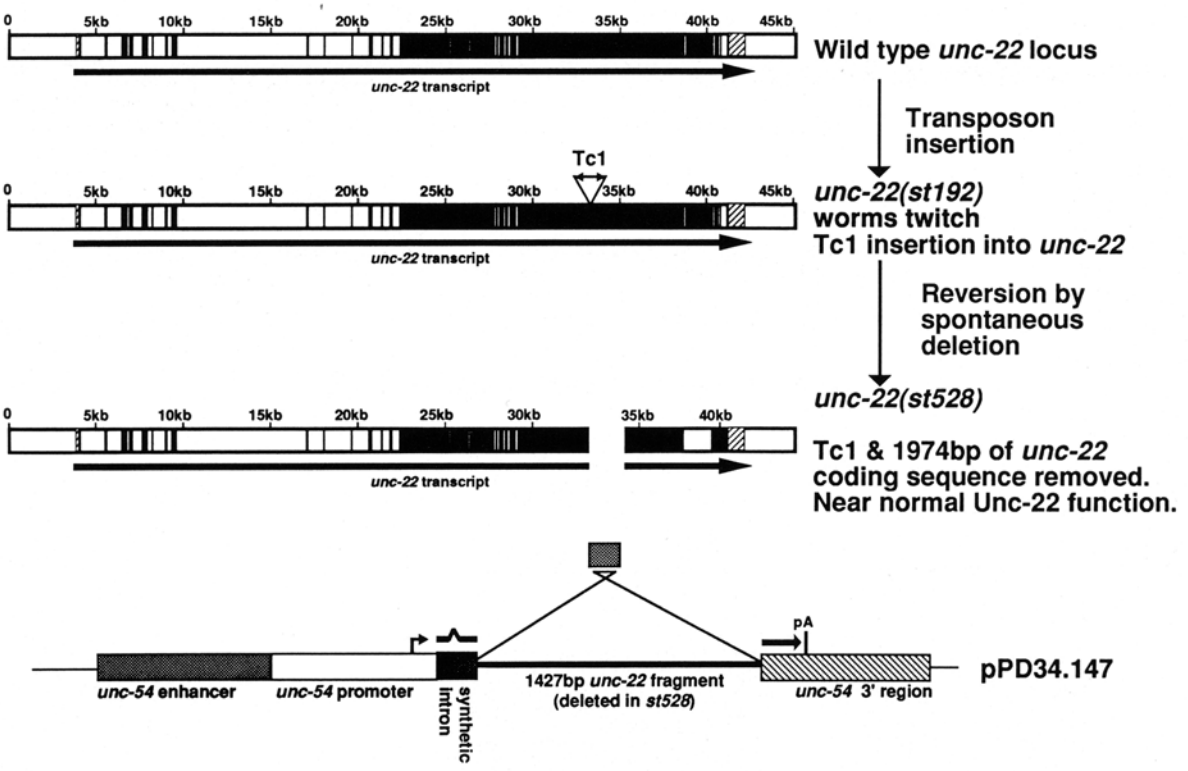


Fig. 5. Derivation and structure of a functional *unc-22* allele (*st528*) carrying an in-phase deletion (from Kiff *et al.* 1988). Below, structure of pPD34.147, a plasmid designed to express *unc-22* sequences deleted in *st528* as antisense RNA.

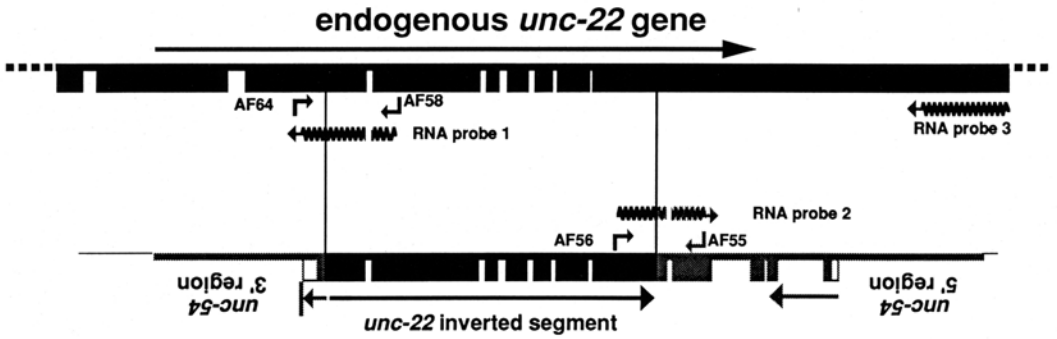
Genetic evidence for an antisense mechanism

To confirm that the disruption of expression requires homology between 'sense' RNAs from the endogenous gene and antisense RNA products of the arrays, we made use of an in-frame deletion mutation *unc-22*(*st528*), which lacks a 1964 bp region of the *unc-22* gene but has *unc-22* function close to normal (Kiff *et al.* 1988). A 1427 bp fragment contained entirely within this 1964 base deletion fragment was cloned in reverse orientation into the muscle expression vector pPD21.36 to make plasmid pPD34.147 (pPD21.36 is similar to pPD12.01, but devoid of *unc-54* coding sequences; Fig. 5). When injected into N2, this plasmid yields many twitchers (41 twitching progeny from 16 injected adults), while no twitchers have been derived after many injections into *st528* (zero from 19 injected adults). When arrays of pPD34.147 from two strongly twitching lines derived from N2 injections were moved genetically into an *st528* background, the twitching phenotype was lost. These experiments confirm that homology between the inverted region and the wild-type chromosome is necessary for the observed disruption of gene function. This is consistent either with an antisense mechanism or with a requirement for pairing between the array and the endogenous gene at the DNA level. A simple pairing model is incompatible both with the observed pattern of active and inactive constructs (Fig. 1 and Table 2) and with the lack of a change in endogenous mRNA level (see below) in strains with disrupted gene function.

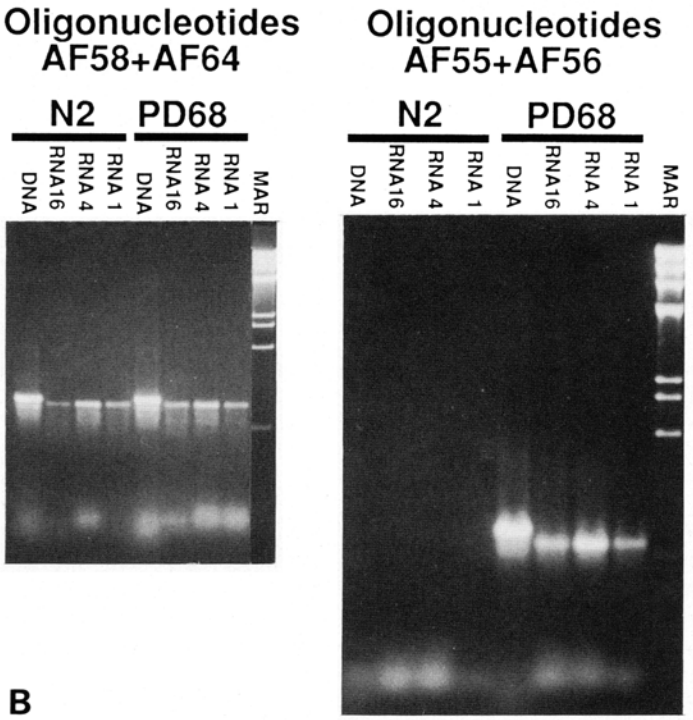
Analysis of sense and antisense RNA

To analyze RNA structures and levels in a uniform population of animals, we have taken advantage of a strain (PD68) in which a long tandem array containing the *unc-22* antisense plasmid pPD10.46 has integrated, leading to a stable, strong, twitcher phenotype. Two techniques for RNA analysis have been used: polymerase chain reaction (PCR) of reverse transcriptase products (Saiki *et al.* 1988; Frohman *et al.* 1988) and quantitative RNAase protection (Melton *et al.* 1984). In these experiments, care was taken to avoid any selection step that would distort the true ratios of different RNA species that might be present. Thus total RNA was prepared without selection for polyadenylation or single stranded character (see Materials and methods).

Results of the PCR confirm the presence of both sense and antisense RNAs (Fig. 6). The PCR products were cloned and sequenced to confirm their identity. Plasmid pPD10.46 contains the first four introns of the *unc-54* gene upstream of the *unc-22* insertion point. The fourth intron lies within the region amplified by the primers AF55 and AF56. From gel analysis of PCR products and sequencing of clones, this intron appears to be quantitatively spliced out in the transcript derived from pPD10.46. Likewise an intron from *unc-22* is present in the segment of sense RNA amplified from the endogenous *unc-22* locus. This intron is also precisely removed in PCR products from sense RNA in both N2 and PD68 animals. The spliced nature of the



A antisense construction pPD10.46



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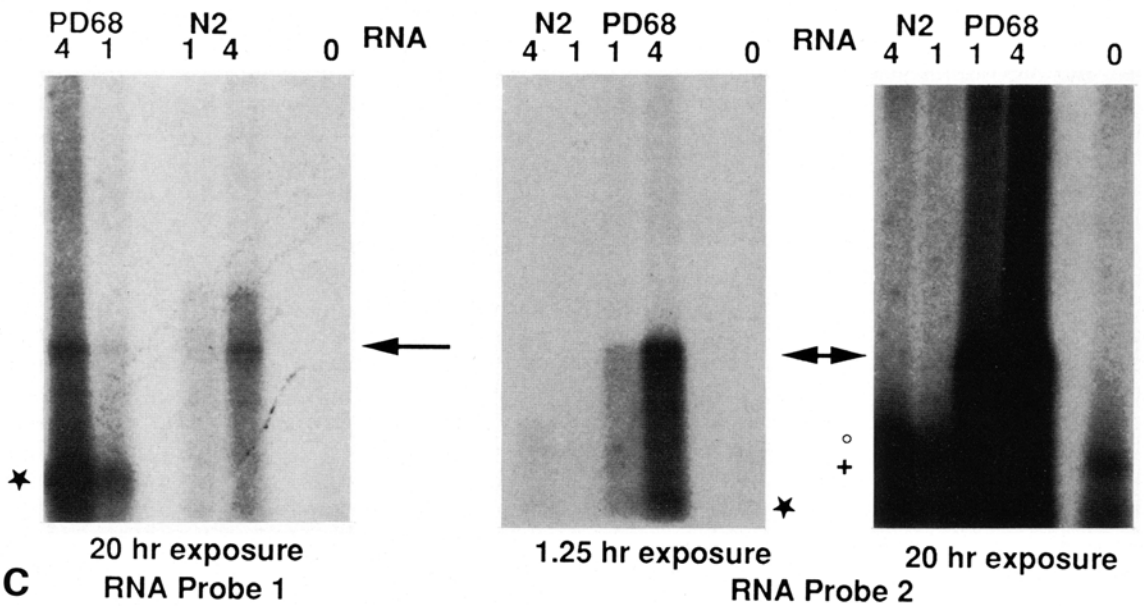

C

Fig. 6. Analysis of sense and antisense RNA in wild-type (N2) and in PD68 (transformed line with an integrated array of pPD10.46). (A) PCR primers and RNA probes. Primer design: to avoid a bias against RNAs with A to I transitions, primers within the region of antisense were chosen to contain only 1–2 thymidines. These primers were synthesized as degenerate oligonucleotides with both pyrimidines at each thymidine position. 5′ ends of the oligonucleotides were designed to facilitate eventual cloning of PCR products (treatment with T4 DNA polymerase+dGTP+dCTP leaves one *Eco*RI and one blunt end).
AF55=aattcCTTCGCCGCTGTCGGTGCCTCCC;
AF56=GCGGAGAGAGCAAGGAYACCAAC;
AF58=CCGAGCACAAYGACCYCAACGGAA;
AF64=aattcGGAAGATGGGTTCCATGCGCC.
(Y=50%C+50%T). PCR products were cloned between *Eco*RI and *Hinc*II sites in Bluescript KS+ (Stratagene). Two cDNA clones from PD68 were used to make RNA probes: pPD34.20 is bordered by AF55 and AF56; pPD34.15 is bordered by AF64 and AF58. RNA probe 3 was made from pPD35.38, a genomic clone (*Hind*III 1030 fragment; Fig. 1). To synthesize probes, templates were cut with *BsaA*I or *Xba*I and transcribed with T3 RNA polymerase, using [^{32}P]UTP. The *BsaA*I cut probes extend 400 nt beyond the end of the insert, into Bluescript vector sequences. (B) PCR reactions resolved on agarose gels and stained with Ethidium bromide. DNA amplifications were carried out with 0.2 μg of genomic DNA from the indicated strain. 'RNA' amplifications contained cDNA primed with the appropriate primer (AF56 or AF58). Amplifications containing three different amounts of input cDNA are shown ('RNA16' corresponds to total RNA approximately equivalent to 60 ng wet weight of whole worms). The amplified products from genomic DNA are slightly larger due to the presence of introns in both amplified regions. Marker lanes (MAR) contain *Hind*III+*Kpn*I digested λDNA. The PCR fragments migrate with the expected mobilities: for AF58+AF64, 795 (genomic product) and 748 (cDNA product); for AF55+AF56, 714 (genomic) and 635 (cDNA).
(C) Ribonuclease protection assays: Total RNA from N2 and PD68 were hybridized to probe RNAs (2 h at 50°C in 50 mM Tris–HCl pH7.4, 0.45 M NaCl, 10 mM EDTA), treated with RNAaseA (35 min, 30°C in 40 mM Tris–HCl pH7.4, 0.3 M NaCl, 7 mM EDTA) and separated (as dsRNA) on a 2% agarose gel. Two quantities of each RNA were used: '1' corresponds to 0.6 μg wet weight of whole worms, '4' to 2.4 μg. The preparations of N2 and PD68 RNA were equivalent as judged by ribosomal RNA content and by RNAase protection using a cDNA clone of ribosomal protein rp21C (data not shown). The large arrows point to the expected protection products from the processed *unc-22* and pPD10.46 transcripts. Two smaller bands result from other RNAs in the samples. Band * (probes 1 and 2; PD68 only) results from transcription of pEMBL vector sequences in pPD10.46; these hybridize to bluescript sequences present in all of the *BsaA*I cut probes. Band * does not appear when RNA probes lacking bluescript sequences are used (data not shown). Band ° (probe 2; N2 and PD68) has been analyzed on sequencing gels, and corresponds precisely in size (315 bp) to the expected partial protection of probe 2 by native *unc-54* mRNA. Band + (probe 2) is independent of input RNA and may represent trace amounts of dsRNA in the probe. After drying, gel was exposed to preflashed film using an intensifying screen. The two probes had identical specific activities (800 Ci mmol^{-1} UTP); the endogenous *unc-22* RNA product should protect 251 U residues in probe 1 while the pPD10.46 product should protect 180 U residues in probe 2. To avoid decay due the presence of multiple labelled U residues in each probe molecule, hybridization and electrophoresis were completed within 10 h of probe synthesis. Protection assays with RNA probe 3 show similar results to those seen with probe 1 (equivalent amounts of *unc-22* transcript in wild-type and PD68.)

PCR products rules out the possibility that the PCR signals are due to contaminating DNA.

A dsRNA unwinding enzyme has been detected in *Xenopus* embryos (Bass and Weintraub, 1987; Rebigliati and Melton, 1987). This enzyme acts by catalyzing conversion of adenines to inosines in double stranded regions of RNA (Bass and Weintraub, 1988; Wagner and Nishikura, 1988). Similar activities have been found in a variety of mammalian cell types (Wagner *et al.* 1989) and in mixed stage embryos from *C. elegans* (M. Krause, personal communication). The adenine to inosine transitions in the RNA could be detected as complementary T to C transitions in cDNA clones (Kimelman and Kirschner, 1989). In order to test for such an activity in *C. elegans* muscle, we have sequenced segments of five different cDNA clones derived from the sense transcript of the endogenous *unc-22* locus in the region homologous to the antisense RNA. Our PCR oligonucleotides were designed so that adenine to inosine transitions in the regions of homology would not affect the ability of the primers to bind RNA. This was done by choosing priming segments with few (0, 1 or 2) adenines in the coding strand, and making the primers 2–4-fold degenerate to account for possible mismatches at these positions caused by covalent modification of the RNA. Approximately 300 bases of each clone were examined, with no evident point changes, either by PCR errors or by covalent transitions in the original RNA sample.

We used cDNA clones derived from the PCR reactions to quantify sense and antisense RNA. Radioactive RNA probes were synthesized *in vitro* from these clones, hybridized to the total RNA population following denaturation and treated with RNAase A (Melton *et al.* 1984). The protected products were analyzed on both native (agarose) and denaturing (acrylamide) gels. The abundance of the sense RNA is similar in wild-type and PD68 animals (Fig. 6C). The antisense RNA is seen in PD68 but not in N2. The antisense RNA in PD68 is approximately 16-fold more abundant than the sense RNA. Two other bands appear in examining the hybridizations. A short protection product (316 nt), seen with RNA probe 2 and either input RNA, is due to partial overlap of the endogenous *unc-54* mRNA with this probe (Fig. 6A). A second band appears in PD68 hybridized with any of the probes used and has subsequently been shown to result from transcription of plasmid vector (pEMBL) sequences in PD68. The abundance of transcripts from plasmid vector sequences is not surprising in the light of suggestions that high copy number arrays may be subject to some degree of spurious transcription.

Mosaicism

Stinchcomb *et al.* (1985) showed that certain extrachromosomal tandem arrays could be lost mitotically, yielding mosaic animals. This phenomenon is evident in some but not all of the extrachromosomal transformed lines described in this work, and is manifest in two classes of animals. Some animals from transformed strains show phenotypic mosaicism in muscle function:

parts of the animal move normally while other parts show an uncoordinated phenotype. Mosaicism at the single cell level is evident when the mitotically unstable lines are examined by immunofluorescence or polarized light microscopy (some cells are normal while others show disorganized filament structure; data not shown). Mitotic loss of the extrachromosomal arrays is also evidenced by a class of animals that exhibit twitching behavior but do not pass the trait on to any of their progeny (loss of the array in the germline). The degree of mosaicism seems to be a property of the particular transformed line: some extrachromosomal arrays exhibit no detectable mitotic loss (these also tend to be the more stable arrays meiotically) while other lines exhibit high frequency loss (with a majority of affected animals being visibly mosaic).

Discussion

Mechanism of antisense disruption of gene function in C. elegans muscle

Several different mechanisms have been proposed or demonstrated for antisense inhibition of gene expression *in vivo* or *in vitro* (Fig. 7). From our experiments, several of these models do not appear to be relevant to *C. elegans* muscle. The presence of the sense RNA in the disrupted lines at normal levels suggests that double-strand-specific RNAase mediated degradation and inhibition of transcription do not play a role in the observed disruption of expression. The ability of the RNA to properly splice in the region of presumed heteroduplex formation suggests that inhibition of splicing is likewise not a major factor, and in addition suggests that heteroduplexes between antisense and sense RNA take some time to form following transcription. Finally, the lack of observed transitions characteristic of the covalent unwinding activity described for *Xenopus* oocytes (Bass and Weintraub, 1988; Wagner *et al.* 1989) strongly argues against a cognate enzyme being effective in unwinding duplexes that form in *C. elegans* muscle. The latter result was somewhat surprising given that dsRNA unwindase activity has been observed in extracts of *C. elegans* embryos (M. Krause, personal communication). Perhaps the dsRNA unwindase activity is lost (or sequestered to the nucleus; see Bass and Weintraub, 1988) in mature muscles. In any case, the observations leave us with the suggestion that antisense RNA disrupts expression in *C. elegans* muscle by hybridization to the sense transcript followed by steric hindrance, blocking either a late processing step, RNA transport, or translation.

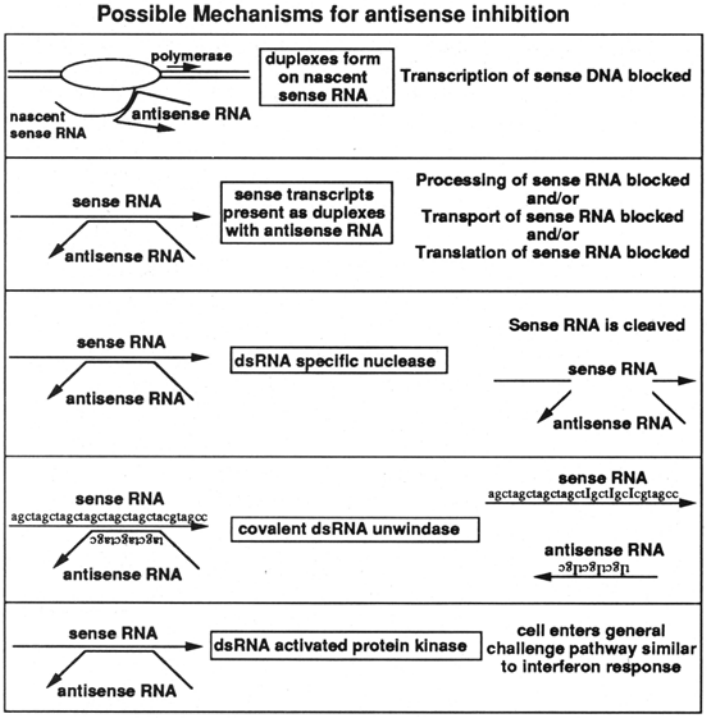

Fig. 7. Possible mechanisms for antisense inhibition. All of these mechanisms have been demonstrated in biological systems (Takayama and Inouye, 1990; Novick *et al.* 1989; Kimelman and Kirschner, 1989; Ma and Simons, 1990; Farrel *et al.* 1977).

A rough estimate of the efficiency of antisense RNA action *in vivo* can be obtained from the phenotype and relative RNA abundances in line PD68(*cc68*). When homozygous, the *cc68* locus leads to strong twitching, while *cc68*/+ heterozygotes have a weak but still visible twitching defect. From comparisons similar to those in Table 1, this places the activity in the heterozygote at approximately 25–33% of wild-type. In the *cc68* homozygote the ratio of antisense to sense RNA is approximately 16, which suggests that this ratio in the heterozygote would be at least 8. These data suggest that efficient interference with gene function requires a high concentration of the antisense RNA as well as a molar excess of antisense over sense RNA.

Prospects for general use of antisense in C. elegans

In certain cases one is presented with a cloned gene that has been obtained through non-genetic criteria: by homology to another gene of interest or by the biochemical properties of the protein or its expression pattern. In these cases it is usually of great interest to determine the phenotype of null mutations in the gene. Protocols for mutating endogenous genes using homologous recombination with incoming DNA are an obvious technique of choice, but this technology is not yet available for *C. elegans*. A second strategy involves precise localization of the gene on the chromosome, followed by extensive analysis of mutations induced in the region; this strategy relies on some ability to predict the phenotype of such a mutation (Waterston, 1989).

The antisense strategy could provide an alternative to these for determining null phenotypes (Izant and Weintraub, 1984). In order to do this, a set of segments internal to the gene would be reversed and inserted into either a genomic copy or into a universal expression vector active in all tissue types (such a universal vector has not yet been described for *C. elegans*). The resulting

constructs could then be injected into wild-type animals (with or without coselection for a second plasmid) and the phenotypes of the progeny carefully examined. As a guide, the frequencies of transformation observed with the antisense constructs directed to *unc-22* and *unc-54* are sufficiently high that any visible defects should readily be found.

Any phenotypes observed should be interpreted carefully. If long tandem arrays are obtained (and this is likely unless selected against, given their prevalence among *C. elegans* transformed lines) the possibilities of titration of DNA binding factors and aberrant peptide production must be considered. The production of antisense RNA could also have effects on homologous genes, and dsRNA could itself have physiological effects on certain cells (Farrel *et al.* 1977).

A second set of problems comes with the question of whether a gene has been completely knocked out. Obviously, biochemical criteria such as immunofluorescent staining are useful in this regard, but it is never possible to confirm biochemically complete loss of function. If the gene under study was required for embryonic viability, then any initial transformants with complete knockout due to antisense RNA would die as embryos, and these dead embryos could easily be missed among inviable progeny that are victims of the injection process itself. We noted with the putative *unc-22* and *unc-54* disruption experiments that a variety of severities were observed among the different affected animals ranging from a slightly hypomorphic phenotype to phenotypes close to those of well characterized deletions of the corresponding chromosomal loci. In addition, the transformed animals tend to be mosaics. We should thus expect to find some abnormal progeny in attempting to knock out any essential gene. These would in general be animals that are either mosaic for the transforming DNA or that are the result of only partial decrease in gene function. In initial experiments using constructs that should produce antisense RNA to the essential gene *myo-3* (Waterston, 1989) we generated a number of strains that segregate animals with varying degrees of muscle disorganization and some inviable animals. Encouragingly, the phenotypes of the most severely affected animals from these strains are close to the null phenotype for *myo-3* (data not shown).

Our current view of the use of antisense to study cloned *C. elegans* genes is that the strategy can best be used to yield hypotheses about null and/or hypomorphic phenotypes for a gene of interest. These hypotheses should then be useful for designing genetic screens for mutations, allowing the gene's normal role to be addressed independently.

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