Identification and intracellular localization of the *unc-22* gene product of *Caenorhabditis elegans*

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The *unc-22* gene is one of a set of genes identified using classical genetics that affect muscle structure and function in the free-living nematode *Caenorhabditis elegans*. Since cloning the *unc-22* gene by transposon tagging, we have used conventional techniques combined with a set of Tc1 transposon insertion alleles to characterize the gene and its products. The gene extends over more than 20 kb of genomic sequence and produces a transcript of approximately 14 kb. A polyclonal antibody raised against an *Escherichia coli* β -galactosidase-*unc-22* fusion protein recognizes a polypeptide in nematode extracts that is between 500,000 and 600,000 daltons and labels the muscle A-band in indirect immunofluorescent microscopy. The Tc1-induced alleles have been used at every stage to verify these conclusions. The Tc1 insertions are spread over much of the region that contributes to the mature transcript; in most alleles, Tc1 sequences are incorporated into a composite *unc-22*-Tc1 transcript. The large protein is either absent or severely reduced in amounts in the mutants. In one case, a truncated polypeptide was also identified. The location of the protein in the A-band, along with earlier genetic data, suggests that the *unc-22* product may interact with myosin to regulate its function.

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The discovery of mutator strains of *Caenorhabditis elegans* in which the element Tc1 transposes at high frequencies has made possible the recovery of DNA sequences for genes without prior knowledge of the protein product (Moerman and Waterston 1984; Eide and Anderson 1985; Greenwald 1985; Moerman et al. 1986). Among the first genes isolated via Tc1 tagging was the *unc-22* locus (Moerman et al. 1986), which is a particularly frequent target of the element and has been used extensively in characterizing the mutator activities. The *unc-22* locus is also of interest as one of more than 25 genes implicated in normal muscle formation in *C. elegans* (Brenner 1974; Waterston et al. 1980; Zengel and Epstein 1980). This set of genes has been studied intensively, and the genes for many of the major components of the myofilament lattice have been identified, including myosin heavy-chain isoforms (Epstein et al. 1974; MacLeod et al. 1977b; Karn et al. 1983), actins (Landel et al. 1984), and paramyosin (Waterston et al.

1977; H. Kagawa and J. Karn, pers. comm.). The product of the *unc-22* gene, however, remained unknown.

Among the muscle-affecting genes, *unc-22* is particularly intriguing because of its unusual phenotype. Mutant alleles of the locus lead to varying degrees of impaired movement and muscle disorganization (Moerman 1980; Waterston et al. 1980). Weak alleles have little effect on movement or muscle structure. Strong alleles lead to almost complete paralysis of adult animals and to abnormal muscle structure in which thick and thin filaments, though present in normal quantities, are not in their normal register. All alleles, however, produce a nearly constant, subcellular twitch in all the body wall muscle cells. This phenotypic feature otherwise results only from rare alleles of the *unc-54* myosin heavy-chain gene (MacLeod et al. 1977a; Dibb et al. 1985) and from one allele of *lev-11* (Lewis et al. 1980). Whether the twitch represents an unregulated contraction or relaxation is unclear, but the twitch is exacerbated by choline agonists (Moerman and Baillie 1979).

Genetic analysis has yielded several insights into the nature of the gene and its function. The gene is an unusually large mutational target (Zengel and Epstein 1980), and intragenic recombination frequencies for alleles at opposite ends of the gene are similar to or higher

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than frequencies seen for the most distant allelic pairs of the *unc-54* gene (Moerman and Baillie 1979; Waterston et al. 1982), which encodes the 228,000-dalton myosin heavy chain (Karn et al. 1983). Thus, the gene is likely to be much larger than average. Most alleles are conditionally dominant, that is, heterozygous *unc-22*/+ animals move normally and have normal muscle structure but can be induced to twitch violently in solutions of choline agonists (Moerman and Baillie 1979). As this dominance is shown even by likely null alleles, including an amber allele *s32* and deletion alleles, the *unc-22* gene probably encodes a protein required stoichiometrically. Most intriguing of all, reversion analysis of *unc-22* alleles revealed that certain missense alleles of the myosin heavy-chain gene *unc-54* can suppress the *unc-22* phenotype (Moerman et al. 1982). Not only is the twitch suppressed and motility partially restored, but muscle structure is also improved. Sequence analysis of the *unc-54* suppressors showed that all of these mutations lie in the region coding for the myosin head; several are located near the nucleotide binding domain, and others are near the conserved thiol domain (Dibb et al. 1985; J. Kiff, D.G. Moerman, and R.H. Waterston, unpubl.).

In this study, we have used the recently obtained DNA clones from the *unc-22* region (Moerman et al. 1986) to investigate the molecular organization of the gene and the nature of its product. In addition, because *unc-22* is a frequent target of the *C. elegans* var. Bergerac mutator system, and because the mutator-induced alleles are generally intermediate in strength, we examined several mutator-induced alleles to determine the nature of the mutations and their effects on *unc-22* gene expression.

Our results show that the *unc-22* gene extends over more than 20 kb, that *Tc1* insertions probably account for all the mutator-induced alleles of this set, and that the insertions occur throughout much of the *unc-22* transcribed region. In addition, a 14-kb transcript has been identified, which is increased in size in the *Tc1* mutants by the inclusion of the *Tc1* sequence. The polypeptide identified with an antibody produced to a fusion protein is larger than rabbit nebulin, which has been reported to be approximately 500,000 daltons in size (Wang and Williamson 1980). This protein is absent in amber and deletion alleles and is altered in size in a *Tc1*-induced allele. The *unc-22* polypeptide in wild type is located in muscle cells and is apparently cell autonomous. Its colocalization with myosin at the light microscopic level in several strains, together with the previous genetic data, suggests that the *unc-22* protein interacts with components of the thick filament.

Results

Sequences important for unc-22 expression extend over more than 20 kb

The region of genomic DNA cloned in previous work extends over 70 kb (Coulson et al. 1986; A. Coulson and J. Sulston, pers. comm.), of which more than 30 kb has

been recovered in phage clones (Fig. 1; Moerman et al. 1986). Various *unc-22* alleles were screened for rearrangements within this region to help define the extent of the *unc-22* gene. The positions of the deficiency *sDf19* (Rogalski and Baillie 1985), which by genetic criteria breaks within the genes, and two *Tc1* insertions, *st136::Tc1* and *st137::Tc1*, were determined previously (Moerman et al. 1986). Several diepoxybutane-induced alleles were found to have small deficiencies to the right of the *st136::Tc1* position (C. Trent, pers. comm.). In addition, we examined 12 more mutator-induced *unc-22* alleles for changes in restriction fragments in the *unc-22* region. All 12 alleles showed alterations in their hybridization patterns; in each mutant, a change consistent with a single insertion of 1.6 kb, the size of *Tc1* (Rosenzweig et al. 1983), was found.

The 12 different *Tc1* insertions were placed into at least 10 different sites by analyzing the patterns produced on Southern transfers of DNA from each allele digested with four different restriction enzymes (Fig. 1). For example, in DNA from the *st140::Tc1* allele, the 3.5-kb *SacI* fragment of the wild type has been replaced by a 5.1-kb fragment (Fig. 2). A weak signal is present in this DNA at 3.5 kb, but this is presumed to be the result of somatic excision of *Tc1* (Emmons and Yesner 1984). The *st181::Tc1* allele affects the same *SacI* fragment, but *st140::Tc1* and *st181::Tc1* are clearly at different sites as in *st140* DNA the 0.4-kb *BglII* fragment is replaced with a 2.0-kb fragment whereas in the *st181* DNA the adjacent 3.4-kb *BglII* fragment is altered (data not shown). Data for two additional alleles, *st185::Tc1* and *st139::Tc1*, are also shown (Fig. 2). Eleven of the insertions are distributed over 17 kb. The twelfth insertion, the *st139::Tc1* allele, lies about 12 kb outside this group. To verify that this insertion indeed represents the relevant sequence change producing the *Unc-22* phenotype, we recovered a mutator-induced revertant of *st139::Tc1*; its restriction pattern is indistinguishable from that of wild type (Fig. 2). The *st139::Tc1* allele is unusual in that it results in such a weak phenotype that it is necessary to use the choline agonist nicotine to score the homozygote reliably. The leftmost-located *Tc1* insertion *st185::Tc1*, which lies under the *sDf19* deficiency, results in a weak phenotype, intermediate between *st139::Tc1* and the other *Tc1* alleles.

unc-22 produces a 14-kb mRNA

To determine what transcripts might arise from the region defined by the rearrangements, we probed Northern transfers of size-fractionated total RNA with various radiolabeled fragments from the genomic clones. A 1.6-kb RNA species was recognized only by the 4.6-kb *SacI* fragment that extends to the left of the *sDf19* deficiency break point (data not shown). No probes from other regions recognized this RNA, so we considered that it was likely to be the product of another gene. A much larger species, estimated to be 14 kb using single-stranded DNA markers (see Fig. 3), also hybridized with the 4.6-kb *SacI* fragment, as well as with probes throughout the

Figure 1. The *unc-22* region and sites of Tc1 insertion. The genomic segment containing the *unc-22* gene is shown, along with several DNA rearrangements that define its extent. Three λ clones, which together span most of the region, are also shown. The minimum region contributing to the transcript is indicated, and the direction of transcription, as determined using single-stranded probes, is from right to left. The positions of the large deficiency *sDf19* (Rogalski and Baillie 1985; Moerman et al. 1986) and three smaller deficiencies, *ct36*, *ct37*, and *ct38* (C. Trent, pers. comm.), are shown as open boxes. The break point of *sDf19* was used to show that the orientation of the physical map to the genetic map is as depicted (Moerman et al. 1986). The approximate position of 14 Tc1 insertions that result in the *Unc-22* phenotype are indicated by triangles. The asterisks above two of the triangles indicate that two independent insertions have been localized within the interval. The order and approximate sizes of the *SacI* fragments, as well as the order of the *unc-22::Tc1* alleles, are from right to left: 7.2 kb (*st139*), 1.0 kb, 0.9 kb, 4.3 kb, 1.1 kb, 3.7 kb, 0.9 kb (*st187*), 3.9 kb (*st188*), 1.7 kb (*st137*, *st136*), 0.3 kb, 1.0 kb (*st184*), 0.2 kb, 1.5 kb ([*st141*, *st191*]), 3.5 kb [*st181*, *st140*, (*st186*, *st182*)], 4.6 kb (*st183* and *st185*). The orientation of the Tc1 insertion, where known, is shown by an arrow. The striped box denotes the 1.9-kb *SacI* fragment that was subcloned into the expression vector λ RB1 (R.J. Barstead and R.H. Waterston, unpubl.).

region containing the majority of the insertion sites. The rightmost fragment tested that gave a positive signal was the 1.1-kb *SacI* fragment. The adjacent 4.3-kb *SacI* fragment yielded no hybridization signal. Fragments farther to the right were not tested. The region covered by these fragments and thus contributing to the 14-kb transcript extends over about 20 kb. Single-stranded DNA probes showed that the orientation of the transcription unit is from right to left on the conventional genetic map.

To confirm that the 14-kb transcript is the product of the *unc-22* locus, we also examined RNA from several *unc-22* mutants that might be expected to have altered mRNA levels or sizes. Amber alleles are known in other genes to reduce the steady-state mRNA levels (Losson and LaCroute 1979; Brown et al. 1982), and one *unc-22* amber allele, *s32*, has been identified (Moerman 1980). In RNA from *s32* homozygotes, the signal from the 14-kb band was reduced markedly compared to wild type when hybridized with an *unc-22* region probe; in control experiments using an *unc-54*-specific probe against the same filter, the signal from the myosin message was similar between the wild-type and *s32* lanes (Fig. 3).

As insertion of transposable elements into the transcribed portion of a gene has been shown to affect the transcript in any of a variety of ways (Doring et al. 1984; Levis et al. 1984; Pirrotta and Brockl 1984; Rowland and Strommer 1985; Zachar et al. 1985), we also examined RNA from several of the Tc1-induced alleles. These include *st139::Tc1*, *st137::Tc1*, *st136::Tc1*, *st141::Tc1*, *st140::Tc1*, and *st185::Tc1*, which span the region of interest (Fig. 1). When hybridized with probes from *unc-22*

(Fig. 4a), in five of the six cases, the hybridizing transcript appeared slightly larger than that in wild type, but of similar abundance. Because an increased size might result from incorporation of Tc1 sequences into the transcript, similar Northern transfers were hybridized with a Tc1 sequence. In the wild type, no RNA in this region of the transfer hybridized with Tc1.

Both orientations of Tc1 insertion are represented in these five alleles. The *st136::Tc1* and *st137::Tc1* alleles have Tc1 inserted in opposite orientations (Moerman et al. 1986). Hybridization of Northern transfers with single-stranded Tc1 probes showed that *st140::Tc1* has the same orientation as *st137::Tc1* and that *st141::Tc1* has the same orientation as *st136::Tc1*. The orientation of the *st185::Tc1* allele was not determined.

The only allele of the six that tested positive with *unc-22* probes but negative with the Tc1 probe was *st139::Tc1*. This is the very weak allele whose site of Tc1 insertion is well to the right of the position of the other DNA rearrangements associated with *unc-22* phenotypes.

The *unc-22* protein is larger than 500,000 daltons

To determine the size of the *unc-22* protein product and its location in muscle, we raised antibodies against a polypeptide produced by the fusion of *unc-22* sequences to the β -galactosidase gene of *Escherichia coli*. Limited sequencing of a region near the *st136::Tc1* insertion site revealed an open reading frame extending through a *SacI* site that would remain in frame when ligated to the *SacI* site within the β -galactosidase gene (data not shown). We inserted the corresponding 1.9-kb *unc-22* *SacI* frag-

Figure 2. Spontaneous *unc-22* alleles isolated in mutator background have Tc1 insertions in the *unc-22* region. DNA from each of the 12 mutator-induced *unc-22* alleles was isolated and compared to wild-type DNA by Southern blot analysis. By using the λ clones in Fig. 1 as probes, each mutant strain was shown to contain an insert of 1.6 kb in single *SacI* and *BglII* fragment. Once a mutation was localized to a small region, the appropriate *SacI* or *BglII* fragment spanning the insertion site was used as a probe to confirm this assignment. The simpler pattern obtained allowed unambiguous interpretation of the results. Shown are two examples, *st185* and *st140*, where we have probed digested DNA from strains containing these mutations with a *SacI* fragment spanning the Tc1 insert, and one example, *st139*, where we have used a λ clone. The *st185* mutation was probed with the left-most 4.6-kb *SacI* fragment, *st140* was probed with the 3.5-kb *SacI* fragment adjacent to the 4.6-kb fragment, and *st139* was probed with λ DM22 containing the right-most region of the gene. In all three cases, a band 1.6 kb larger than the corresponding wild-type band was detected. Weaker hybridization at the position of the wild-type band was also seen, which was probably a result of somatic excision, a characteristic of all Tc1 insertions so far identified. Similar results were obtained for the other 11 mutants described in Fig. 1. A revertant of *st139::Tc1* was recovered and included in the analysis. The revertant, RW2418, has lost the 8.8-kb *SacI* hybridizing band and regained the wild-type 7.2-kb hybridizing band.

ment in the desired orientation into a modified λ gt11 vector that has a unique *SacI* site within the β -galactosidase gene (Young and Davis 1983; R.J. Barstead and R.H. Waterston, unpubl.). When β -galactosidase expression was induced, a fusion peptide of about 155 kD appeared in the λ lysogens, the size expected if the open reading frame was maintained across the entire 1.9-kb *SacI* fragment, with a subsequent out-of-frame fusion to the remainder of the β -galactosidase sequence (data not shown).

To obtain antiserum, the polypeptide was partially purified and injected into rabbits. The resultant serum,

called R11-3, was adsorbed extensively against full-length β -galactosidase and used against Western transfers. The antiserum reacted strongly against the 155-kD protein in λ lysogens (data not shown) and with a very large protein or proteins in either 2-aminoethanol or SDS-soluble proteins from *C. elegans* wild-type animals (Fig. 5). The signal, depending on the preparation, was either a single band or a closely spaced doublet. Extrapolating from the mobilities of myosin heavy-chain (225,000 daltons; $R_f = 0.66$) and rabbit nebulin (500,000 daltons; Wang and Williamson 1980; $R_f = 0.22$) markers, the wild-type protein, or proteins, was esti-

Figure 3. *unc-22* encodes a 14-kb message. Total RNA isolated from the indicated strains was examined by Northern analysis, as described in Materials and methods. The filter on the left shows a comparison of N2 to an *unc-22* amber allele, *s32*, after hybridization with λ DM17. In N2, a prominent band near the top of the filter is observed, which has a dark smear below it. In *s32*, this band and smear are greatly reduced. Hybridization of this filter with an *unc-54*-specific probe shows that the 6.1-kb myosin heavy chain message is not degraded and that the RNA amounts in each lane are comparable. The filter on the right has RNA from N2 and the mutant strain *st136::Tc1*, as well as single-stranded DNA size markers from λ phage. The probe in this case was again λ DM22. By comparison of the high-molecular-weight band in N2 to the λ size markers, the steady-state *unc-22* message is approximately 14 kb in size. The *unc-22::Tc1* strain produces an even larger message (see text and Fig. 4).

mated to have a molecular weight of 500,000 to 600,000 ($R_f = 0.16$).

Again, to ensure that the reactive polypeptide was indeed the product of the *unc-22* locus, we examined mutant alleles in which we could anticipate that the amount or size of the product would be altered. No signal was detected from homozygotes of the deficiency allele, *ct37* (Fig. 5; see also Fig. 1 for the location of *ct37*). In *st136::Tc1* extracts, a closely spaced doublet of lower molecular weight and reduced intensity was observed (Fig. 5). After an exposure of the blot approximately five times longer than that shown in Fig. 5, a weak signal

was observed in the *st136::Tc1* extracts at the same mobility as the wild-type signal. Even after this longer exposure, no signal was detected from the *ct37* extracts.

The unc-22 protein is located in the A-bands of striated muscle

After adsorption, the R11-3 serum was also used to determine the in vivo location of the *unc-22* protein. The antiserum reacts specifically, by indirect immunofluorescence, with A bands of wild-type muscles, not only including the body wall muscles (Fig. 6) but also the anal, vulval, and pharyngeal muscles (data not shown).

Figure 4. Hybrid *unc-22*-*Tc1* transcripts from *unc-22::Tc1* insertion alleles. Duplicate Northern filters of total RNA were prepared from the wild-type N2 strain and six *unc-22::Tc1* strains and hybridized with either λ DM22 or *Tc1*. The *unc-22* probe detected a single high-molecular-weight band, but in five of six mutant strains the hybridizing band was even larger than in N2. However, the *st139::Tc1* strain, which has the most 5' *Tc1* insert, showed a band at the same level as in N2. The higher-molecular-weight band observed in the five mutant strains using an *unc-22* probe was also observed with a *Tc1* probe. In contrast, the N2 and *st139::Tc1* strains did not have any signal in this region of the filter. Less RNA was loaded for the *st185::Tc1* strain and presumably accounts for the reduced signal. Additional bands and smears observed in the *st140*, *st141*, *st139*, and *st183* lanes relative to the N2, *st136*, and *st137* lanes on the *Tc1*-probed filter correlate with strain background. The first four alleles mentioned are in strains with active mutators, and these *unc-22* alleles are all unstable. N2 is a nonmutator strain, whereas *st136* and *st137* were moved from mutator-active to mutator-inactive backgrounds [see Moerman and Waterston (1984) for details on the genetics of mutator strains]. Whether these extra hybridizing bands observed with a *Tc1* probe are due to RNA or contaminating DNA has not been determined.

Figure 5. Antiserum to an *unc-22*- β -galactosidase fusion polypeptide identifies a polypeptide in *C. elegans* that is larger than 500,000 daltons. Rabbit antibodies were generated to an *unc-22*- β -galactosidase fusion polypeptide (see text for details), and the resulting serum, R11-3, was absorbed against β -galactosidase before use. Total *C. elegans* protein extracts, prepared as described in Materials and methods, were separated on a polyacrylamide gel and either stained with Coomassie blue or transferred to nitrocellulose, reacted with the R11-3 antiserum and 125 I-labeled protein A, and visualized after autoradiography. The molecular weight markers shown here are paramyosin (pm) at 105 kD and myosin heavy chain (mhc) at 220 kD. The left pair of panels shows that in wild type animals the antisera react with a large protein or proteins of ~ 500 kD. Although not visible here, in some extracts a faint band, corresponding to the band detected with the antiserum, can also be observed after Coomassie blue staining. The panels on the right demonstrate that the antibody reactive band is either absent or altered in *unc-22* mutants. Mutants bearing the *unc-22* internal deficiency *ct37* lack this large protein; no signal can be detected even after prolonged exposure. From a *st136::Tc1*-bearing strain, a shorter polypeptide that runs as a doublet is detected with the R11-3 antiserum. In some preparations, the reactive species in N2 can sometimes be a doublet.

No signal was observed in any of these muscle cells in animals treated with the preimmune serum. In the body wall muscles, the R11-3 reactive region appears to be nearly coextensive with the region stained by an *unc-54*-specific monoclonal antibody. This colocalization is observed even in a mutant where the *unc-54* myosin is present but is not part of a normal A-band (Fig. 7).

Mutant animals were again used to determine the contribution of the *unc-22* gene to the observed signal (Fig. 6). In *ct37* animals, the antibody staining is drastically reduced in the body wall, vulval, and anal muscles. In contrast, the pharyngeal signal is identical to wild type. The slight residual staining of the body wall muscles in wild type is confined to narrow strips between each row of dense bodies. The pattern obtained

with the anti-*unc-54* antibody is abnormal, but the signal remains strong. Similar results were obtained with the antibody when used on most *st136::Tc1* animals.

unc-22 protein expression is cell autonomous

Because *Tc1* is known to excise somatically (Emmons and Yesner 1984; Eide and Anderson 1985), we elected to see if we could identify animals in which one or a few muscle cells contained normal or nearly normal levels of the antibody reactive protein. In previous work we had obtained a strain containing *st136::Tc1* in which germ line excision is exceedingly rare ($<10^{-7}$), yet somatic excision, as judged by Southern analysis of exponentially

Figure 6. Subcellular localization of the *unc-22* protein relative to the *unc-54* myosin heavy chain protein. Wild-type (a,b) or *unc-22(ct37)* mutant animals (c,d) were prepared for immunofluorescence, as described in Francis and Waterston (1985) and Materials and methods. They were then double stained with the monoclonal antibodies DM28.2 (Miller et al. 1983) and DM5-8, which are specific for the *unc-54* myosin heavy chain protein (a,c) and with the R11-3 serum (b,d). The R11-3 staining in wild type (b) is localized to the same region as the *unc-54* myosin (a). Overall, the A-bands are severely disorganized in the mutant, as shown by the myosin distribution (c), and the R11-3 staining is greatly reduced compared to wild type (d). The residual signal in the mutant is midway between the rows of dense bodies (not stained). The large arrows in a and b point to the center of the same A-band. Smaller arrows indicate the cell boundaries. The longitudinal axis of the nematode is horizontal. Bar, 20 μ m.

growing populations, is as high as that in mutator strains (Moerman and Waterston 1984; Moerman et al. 1986). In fragments prepared from random populations of this *st136::Tc1* strain, we observed rare specimens with muscle cells that had strong R11-3 reactivity distributed within the cells in a striated pattern (Fig. 8). Within the same fragments, cells were also present that did not stain with the antibody, and the limits of the staining clearly corresponded to muscle cell boundaries. As a control, the same animals were also stained with the *unc-22*-specific antibody, and all cells stained. In addition, no cell-limited staining was ever observed in wild-type animals prepared the same way. In cells that reacted with both antibodies, the *unc-54* and *unc-22*

proteins appear to be colocalized even in instances where the A-bands are somewhat disorganized (Fig. 8).

Discussion

Genetic studies indicated that *unc-22* might be an unusually large gene, both from the frequency of mutation and the recombinational distances between *unc-22* alleles. Our molecular studies confirm these earlier inferences. The genomic region comprising the *unc-22* gene must be at least 20 kb, based on the rearrangements analyzed. The major cluster of Tc1 insertion sites is distributed over 17 kb, and the diepoxybutane-induced deletions extend the region a minimum of another 3 kb. DNA throughout the 20-kb region contributes to the es-

Figure 7. Colocalization of *unc-22* and *unc-54* proteins in a thick filament mutant. Shown is an animal homozygous for the dominant missense allele *unc-54(st273)* stained with DM5-8 (a), a monoclonal antibody specific to the *unc-54* protein and R11-3 (b), a polyclonal antibody that reacts with the *unc-22*-encoded protein (see Dibb et al. 1985 for a fuller description of this class of *unc-54* mutation). Homozygotes of *st273* have severely disorganized thick filaments, as shown in a using the *unc-54*-specific monoclonal antibody. The *unc-22* R11-3 serum reveals a similar subcellular staining pattern (b) to that observed in a, which indicates that even in cells with disorganized A-bands the *unc-22* protein and myosin are colocalized. Arrows in a and b point to similar position. Bar, 20 μ m.

Figure 8. The *unc-22* gene product is cell autonomous. Mosaic animals were detected when R11-3, the polyclonal antibody to the *unc-22* protein (b,d) and DM28.2, a monoclonal antibody to the major myosin heavy chain (a,c), were used against *unc-22(st136::Tc1)* animals. (a) Three body wall muscle cells stained with the myosin antibody; (b) the same three cells as in a treated with the *unc-22* antibody; (c) the ends of two body wall muscle cells reacted with the myosin antibody; (d) the same muscle cells as in c treated with the *unc-22* antibody. (b,d) Arrows indicate the edge of a nonrevertant muscle cell. Note that the *unc-22*-expressing cell in c has better organized A-bands than the nonrevertant cell (see also Fig. 6c). Bar, 10 μ m.

timated 14-kb transcript. The gene may be larger still, as the *st139::Tc1* insertion site lies several kilobase pairs to the right of the small deficiencies. However, this allele is unusual in that the resultant phenotype is very weak and *Tc1* sequences are not incorporated into the transcript. Until the 5' end of the gene and its normal regulatory sequences are defined, we cannot rule out the possibility that several large introns or an unusually large regulatory region could place even the *st139::Tc1* site within the boundaries of the gene. The large stable transcript detected indicates that most of the 20-kb region is comprised of exons, which is not atypical of *C. elegans* genes (Kramer et al. 1982; Karn et al. 1983; Blumenthal et al. 1984; Spieth and Blumenthal 1985). Most of this mRNA must be coding sequence to yield the protein product or products of the gene greater than 500,000 daltons, as defined by the antibody and the analysis of the mutants.

The genetic analysis had also suggested that the *unc-22* protein might be associated with the myosin heavy-chain product of the *unc-54* locus. Only rare alleles of the *unc-54* locus are able to suppress *unc-22* mutants; no other loci were identified despite exhaustive screening (Moerman et al. 1982; D.G. Moerman, unpubl.). In addition, other rare alleles of the *unc-54* locus partially mimic the *unc-22* twitching phenotype. The two analyzed alleles of this class have small in-frame deletions that remove a section of the myosin rod (Dibb et al. 1985). The location of the *unc-22* protein in the A-band, as determined by the antibody R11-3, supports the notion that the *unc-54* and *unc-22* proteins are associated, and studies on *unc-54* missense mutants reveal that in mutants where the *unc-54* protein is present but displaced from its normal A-band location, the *unc-22* protein colocalizes with the *unc-54* myosin (Fig. 7). This colocalization of the protein with *unc-54* myosin in wild-type and mutant animals suggests that the *unc-22* protein might bind directly to myosin or another component associated with it. Additional biochemical and morphological studies will be necessary to determine whether the *unc-22* protein is indeed a component of the thick filament.

The R11-3 antibody not only detects reactive material

in the body wall muscles but also in the anal, vulval, and pharyngeal muscles. All of these muscles, including the pharyngeal, also react with the R11-3 antibody after it has been affinity purified against the fusion polypeptide (D.G. Moerman et al., unpubl.). In *unc-22* mutants the antibody staining is significantly reduced in all the muscles except the pharyngeal muscles, where the signal is identical to wild type. These observations on mutants, including genetic mosaics (Fig. 8), clearly indicate that staining of the body wall, anal, and vulval muscles in wild-type animals is due to the presence of the *unc-22* protein in these cells. That *unc-22* mutants have no effect on pharyngeal staining is in agreement with earlier studies showing that pharyngeal pumping and pharyngeal muscle structure were normal in multiple mutant isolates of *unc-22* (Waterston et al. 1980). Apparently, an immunologically related protein is expressed in the pharynx, which is not the product of the *unc-22* locus. Our inability to detect this second protein on Western blots is perhaps not surprising considering that pharyngeal muscles account for less than 5% of the total muscle mass. The weak residual staining of the body wall muscles observed in *unc-22* mutants may be due to this second protein, or possibly even a third. Probing low-stringency genomic Southern blots with *unc-22* fragments has not, as yet, revealed any strongly cross-hybridizing bands (D.G. Moerman et al., unpubl.). However, there is precedent for differential expression among gene family members in nematode muscle: Myosin heavy chains are encoded by four genes, two of which are expressed in the pharyngeal muscle and two of which are expressed in the body wall muscle (Epstein et al. 1974; Waterston et al. 1982; Karn et al. 1984; Miller et al. 1984).

The twitching aspect of the phenotype of *unc-22* mutants has been used to infer that the *unc-22* gene product is involved in the regulation of muscle contraction. The location of the protein in the A bands seems to rule out the possibility that the *unc-22* protein is part of the muscle membrane or the sarcoplasmic reticulum. Its size also eliminates the possibility that it is one of the known thin-filament regulatory proteins or one of the myosin light chains. One intriguing possibility consis-

tent with our findings is that the *unc-22* protein may modulate myosin activity directly.

The intracellular location of the *unc-22* protein is different from any of the few very large proteins identified in muscles of other animals. Z-nin (Suzuki et al. 1981) and zeugmatin (Mahler et al. 1985) are found in the Z-line region of vertebrate muscle. Another large protein, nebulin, is only slightly smaller than the *unc-22* protein, but its location in the N-2 line of the I band (Wang and Williamson 1980) makes it an unlikely analog. Titin is much larger in size, with a molecular weight of more than 1,000,000 (Wang et al. 1979; Trinick et al. 1984). Its apparent position within the sarcomere seems to depend on sarcomere length, but at resting length the immunoreactive groups are restricted to the A- to I-band junction. Another potentially large protein discovered recently is the product of the Duchenne's muscular dystrophy locus, which encodes an mRNA of about 16 kb (Monaco et al. 1986). However, no hybridization of the Duchenne's cDNA probes to the *unc-22* genomic clones was detected even under low stringency (L. Kunkel, pers. comm.). In addition, partial sequences for both are known and they are not similar (unpubl.; L. Kunkel, pers. comm.). Whether any of these proteins or others are analogs of the *unc-22* protein must await either the development of functional tests for these large proteins or more complete sequence comparisons.

The Tc1-induced alleles of *unc-22* have been crucial at each step in our investigations and, in turn, our investigations of these alleles has revealed new aspects of Tc1 biology. The wide distribution of insertion sites helped to demonstrate the size of the gene. Although the disperse distribution of these insertions might suggest that Tc1 has little or no insertion site specificity, a much more limited set of insertion sites has been found for *unc-54* (Eide and Anderson 1985), and our own sequence analysis of Tc1 insertion sites demonstrates that Tc1 has a preferred target sequence (Mori et al. 1987). Apparently the *unc-22* gene is unusually rich in such sites.

The incorporation of Tc1 sequences in whole or in part into a full-length stable message aided in defining the region of the gene contributing to mature mRNA and is an unusual finding for transposable elements. The incorporation is independent of Tc1 orientation and largely independent of Tc1 position within the gene. In five of the six cases we studied, the insertions are in either orientation and are spread over a region of about 12 kb. The only exception is the *st139::Tc1* allele, where a normal-sized message is made.

The incorporation of a transposable element into a full-length RNA has been reported for a *Ds* insertion element into the alcohol dehydrogenase (ADH) gene of maize (Doring et al. 1984), but, in general, insertion of a transposon into a gene results in a truncated message (Levis et al. 1984; Pirrotta and Brockl 1984). The intensity of signal from the full-length composite *unc-22::Tc1* transcript in the examples here suggests that very little, if any, termination is occurring within the Tc1 sequence, but we have not ruled out that termination occurs either at a variety of sites or results in an un-

stable RNA that is not readily detected on Northern analysis. In fact, a few faint bands can be detected on long exposures of Northern transfers that are of a size consistent with their production via truncation within Tc1 (G.M. Benian and D.G. Moerman, unpubl.). Nevertheless, our results suggest that Tc1 contains either no, or only very weak, transcription-termination signals.

The incorporation of Tc1 sequences into the messages of genes with Tc1 insertions has several practical implications. First, it may serve to verify that a particular mRNA is the transcript of the gene under study, as was done here. Second, hybridization with a Tc1 probe of a Northern transfer of total RNA from a mutant strain might be useful in verifying that a mutation represents a Tc1 insertion. Such a composite RNA might even be used as an aid in cloning the gene in unusual circumstances. At present, we do not know how often Tc1 insertions will result in a composite mRNA. Results from an analysis of three *unc-15::Tc1* alleles reveal that none of these mutations generates a hybrid message (R.P. Anderson, S. Rioux, and R.H. Waterston, unpubl.). Recent sequence analysis of these mutations (S. Rioux and R.H. Waterston, unpubl.) and of the paramyosin gene (H. Kagawa, pers. comm.) suggests that these insertions lie near the 5' end of the gene. These alleles are non-null alleles and may be analogous to the *st139* allele. On the other hand, Tc1 insertions into *tra-2* result in a transcript larger than wild type (P. Okkema and J. Kimble, pers. comm.).

A puzzling aspect of the *unc-22::Tc1* alleles is that they are all phenotypically milder in their effects than are amber alleles or other likely null alleles. This is particularly noticeable at the level of muscle structure (see Figs. 6 and 7; Moerman and Waterston 1984). Previously, we postulated that the insertions might be in introns where they could be processed out (Moerman and Waterston 1984). Instead, the Tc1 sequences are incorporated into the stable transcript, and in the case of *st136::Tc1*, a likely truncated peptide has been found. Furthermore, the integration sites of the *st136::Tc1* and *st137::Tc1* alleles lie within long open reading frames, not introns (I. Mori, G.M. Benian, and D.G. Moerman, unpubl.). Another possible explanation is that somatic excision of Tc1, which results in the appearance of several percent of the signal at the size of the wild-type band, could result in the production of sufficient protein to yield a weak phenotype. However, the *unc-22* product appears to be cell autonomous, and only very rare cells show restoration of near normal levels of antibody-reactive material. Apparently, many of the somatic excision events, as judged by Southern analysis, must produce out-of-frame deletions or unstable, nonfunctional polypeptides. Another explanation is suggested by our present data: perhaps, the truncated polypeptide detected in *st136::Tc1* animals (Fig. 5) is sufficient to produce discernible lattice organization but is unable to inhibit twitching. In severe alleles, lattice structure is disrupted more severely (Moerman and Waterston 1984). This possibility of different functional domains within the *unc-22* locus is supported by partial sequence anal-

ysis currently under way (J. Kiff, I. Mori, R.H. Waterston, and G.M. Benian, unpubl.).

The tentative assignment of *unc-22* to the set of genes affecting the thick filament is significant. In addition to *unc-22*, the genes for both myosin heavy-chain isoforms and paramyosin have been discovered and subjected to a combined molecular and genetic analysis. The genes for the myosin light chains have been cloned (C. Cummins and P. Anderson, pers. comm) and, once localized to the genetic map, may also be subjected to genetic analysis. In addition, several genes have tentatively been assigned to the thick filament by genetic criteria, including *unc-87*, *unc-82*, and *unc-89* (Waterston et al. 1980). Mutator-induced alleles of both *unc-87* and *unc-89* are available, and efforts are under way to recover DNA for these genes (D.G. Moerman et al., unpubl.). A thorough study of these and other genes yet to be discovered may provide us with a complete picture of thick filament assembly and function.

Materials and methods

Growth and description of nematode strains

Stock maintenance and strain growth for protein and nucleic acid preparations were as described in Brenner (1974) and Sulston and Brenner (1974). All *unc-22::Tc1* mutants were isolated in either the BO strain of *C. elegans* var. Bergerac (Nigon 1949; Liao et al. 1983; Moerman and Waterston 1984) or in strains derived from crosses between BO and the N2 strain of *C. elegans* var. Bristol (Brenner 1974).

Nucleic acid procedures

High-molecular-weight nematode genomic DNA was isolated following the protocol of Wills et al. (1983). Standard methods were used for restriction digestion, gel electrophoresis, and Southern blotting (Southern 1975; Maniatis et al. 1982). DNA probes were labeled by nick translation (Rigby et al. 1977), and hybridization conditions were as described by Goodman et al. (1977). DNA clones used for localizing the *unc-22::Tc1* inserts included λ DM17, λ DM20, and λ DM22 (Moerman et al. 1986), which span the 30-kb *unc-22* region. In addition to these clones, several single *SacI* and *BglII* fragments from *unc-22*, cloned into puc18 or puc19 (Yanish-Perron et al. 1985), were used as hybridization probes. Methods for growth and for DNA isolation from these phage and plasmid clones have been described (Karn et al. 1980; Maniatis et al. 1982).

Total RNA was prepared by a modification of the guanidine hydrochloride method of Chirgwin et al. (1979). A mixed population of nematodes was harvested from five 100-mm petri dishes, washed to remove *E. coli*, and frozen at -70°C . Worms were ground in liquid nitrogen for 10 min, using a mortar and pestle, and lyophilized for 5 hr. This powder was added to 4 ml of a solution containing 6 M guanidine hydrochloride, 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol, and 0.1% antifoam A emulsion (Sigma, St. Louis, MO), pH 7.0, and vortexed for 80 sec. Debris was removed by centrifugation at 8000 rpm in a Sorvall SS-34 rotor for 10 min. The supernatant was then layered onto a 1.25-ml CsCl pad [5.7 M CsCl, 0.1 M EDTA, pH 7.0] and centrifuged in a Sorvall AH-650 rotor at 36,000 rpm for 13 hr. The supernatant was discarded, and the pellet was resuspended in 0.3 ml of guanidine hydrochloride solution (described above) by pipetting and heating at 65°C for 5 min. The RNA was ethanol-

precipitated, washed with absolute ethanol, and dissolved in 0.1–0.3 ml of a solution containing 10 mM Tris and 0.1 mM EDTA at pH 8.

Fifteen micrograms of total RNA was electrophoresed through 0.7% agarose-formaldehyde gels (Maniatis et al. 1982) and transferred to nitrocellulose filters (Thomas 1980; Schleicher and Schuell, Inc., Keene, NH). Filters were pre-hybridized for several hours at 42°C in 50% formamide, $5\times$ SSC, 0.1% SDS, and 100–200 mg/ml denatured calf thymus DNA. This solution was discarded, and the filters were hybridized for 16 hr at 42°C to ^{32}P -labeled DNA or RNA probes in the same buffer, with the addition of dextran sulfate to 10%. Extensive washings were done, initially in $2\times$ SSC and 0.1% SDS at room temperature, and then in $0.1\times$ SSC and 0.1% SDS at $55\text{--}60^{\circ}\text{C}$. Nitrocellulose filters were blotted dry and then exposed to Kodak XAR-2 film with an intensifying screen (DuPont Cronex Lightning-Plus, Dupont Co., Wilmington, DE) for 1–5 days at -70°C .

To look for *unc-22*-encoded RNA, nick-translated λ and plasmid clones (described above) were used as hybridization probes. For determining the direction of *unc-22* transcription, we used strand-specific M13 probes (Brown et al. 1982), which were derived from a 1.1-kb *EcoRV* fragment near the *st136::Tc1* insert (Moerman et al. 1986). The vector used to clone these fragments was mWB3295 (Barnes and Bevan 1983). A 1.6-kb *EcoRV* *Tc1* fragment from pDM4 (Moerman et al. 1986) was nick-translated and used to probe the Northern blot shown in Figure 4. To determine the orientation of *Tc1* within the composite *unc-22*–*Tc1* transcripts, labeled RNA was prepared from two SP6 recombinants that contain *Tc1* in opposite orientation (Zinn et al. 1983). The myosin heavy-chain gene used for probing Northern blots was the 2.8-kb *BamHI* fragment of *unc-54* (Karn et al. 1980).

Generation of the *unc-22* fusion protein and antibody production

The λ vector λ gt11 (Young and Davis 1983) was modified by oligonucleotide mutagenesis in order to eliminate the *SacI* restriction site outside of the β -galactosidase gene, thereby making the *SacI* site in the gene unique (R.J. Barstead and R.H. Waterston, unpubl.). Partial sequencing of a portion of the *unc-22* gene, as depicted in Figure 1, allowed us to identify a *SacI* fragment that would produce an in-frame fusion between the β -galactosidase of the modified vector, λ RB1, and this portion of *unc-22*.

The clone containing the *unc-22* insert, λ SL1, was used to establish a lysogen in the *E. coli* strain Y1089 (Young and Davis 1983). The phage can be induced by a heat pulse at 42°C to initiate a lytic cycle in this strain; however, the amber mutation S100 in the phage S gene prevents lysis of the cells. The lysogenic strain was grown at 30°C to an OD_{600} of 0.25 in L broth. The culture was then transferred to a $42\text{--}44^{\circ}\text{C}$ water bath and grown for 15 min after its temperature reached the temperature of the bath. Isopropal- β -D-thiogalactopyranoside (IPTG) was added to 1 mM, and growth was continued at 38°C for 1 hr. The cells were collected by centrifugation, resuspended in breaking buffer [50 mM NaH_2PO_4 , pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 M β -mercaptoethanol, and a mixture of protease inhibitors], and sonicated. The solution was spun at 18K for 15 min, at 4°C in a Sorvall SS-34 rotor. NH_4SO_4 was added to 35%.

The NH_4SO_4 precipitate containing the *unc-22* fusion protein was collected by centrifugation. The 155-kD fusion protein, first identified by its reaction with an anti- β -galactosidase serum (not shown; Cappel Laboratories), was further purified

by preparative SDS-PAGE (Laemmli 1970). Samples were run on 5% polyacrylamide gels, which were fixed and stained using standard procedures. The band containing the fusion protein was excised, neutralized, and passed through a French Press in 0.15 M NaH_2PO_4 (pH 7.4). The disrupted gel was then emulsified with two volumes of adjuvant by sonication. A New Zealand white rabbit was immunized with three subcutaneous injections over the course of a month with ~ 50 μg of fusion protein/injection. Immune serum was collected 7 days after the last injection. Anti- β -galactosidase activity was eliminated by adsorption to *E. coli* β -galactosidase (Sigma), coupled to cyanogen bromide (CNBr)-activated Sepharose (Sigma).

Western transfer procedures

To prepare protein samples, 0.1 g of freshly grown nematodes was suspended in 1 ml of a solution containing 50 mM 2-aminooethanol (pH 11), 5 mM dithiothreitol (DTT), 2 mM EDTA, and 1 mM PMSF. The samples were then heated in a microwave oven for 25 sec and transferred to a boiling water bath for 3 min. Laemmli sample buffer (1 ml) was added to each, and the samples were immediately loaded onto a 5% acrylamide-SDS gel (Laemmli 1970). The gels were either stained with 0.05% Coomassie Brilliant Blue R-250 using standard conditions or transferred electrophoretically to a nitrocellulose filter (Towbin et al. 1979). Transfer conditions were as described by Francis and Waterston (1985), except the electrode buffer contained 0.1% SDS. After electroblotting, the nitrocellulose filters were preincubated for 15 min in Tris-buffered saline (TBS: 50 mM Tris-HCl, pH 7, 150 mM NaCl) with 0.1% Tween-20 (Batteiger et al. 1982) and 5% nonfat dried milk (Johnson et al. 1984) as blocking agents. Filters were then incubated with gentle shaking for 1 hr in a sealed plastic bag containing this buffer and a 170-fold dilution of the R11-3 serum. After washing away unbound antibody with the same buffer, the filters were incubated with 1 μCi of affinity-purified ^{125}I -labeled protein A (Amersham, Arlington Heights, IL) for 1 hr. Once again, the filters were washed, as described above, and exposed at -70°C to Kodak XAR-2 film with an intensifying screen.

Immunofluorescence techniques

Nematodes were partially sheared in a French Press, according to the procedure of Francis and Waterston (1985), in the following rigor buffer: 100 mM KCl, 10 mM KH_2PO_4 (pH 7.0), 2 mM MgCl_2 , 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. They were washed twice in rigor buffer containing 1% NP-40 and fixed at room temperature for 3 hr to overnight in 3% formaldehyde made up by dilution of formalin in phosphate-buffered saline (PBS) (pH 7), with the addition of 5 mM EDTA. The monoclonal antibody DM28.2 is specific for the *unc-54* myosin heavy-chain peptide and is described in Miller et al. (1983). The monoclonal antibody DM5-8 is similar to DM28.2 (D.M. Miller, pers. comm.). Tetramethylrhodamine isothiocyanate-conjugated goat-anti-mouse and fluorescein isothiocyanate-conjugated goat-anti-rabbit secondary antibodies were obtained from Jackson ImmunoResearch Labs, Inc. (Avondale, Pennsylvania). Antibody incubations were done as in Francis and Waterston (1985). Stained specimens were viewed with a Zeiss Universal microscope (Carl Zeiss, Inc., Thornwood, New York), equipped with epifluorescent illumination. Photomicrographs were prepared as in Francis and Waterston (1985).

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