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Journal Title: Journal of Cell Biology
Volume: Volume 132, Number 5
Publisher: Rockefeller University Press | 1996-03-01, Pages 835-848
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1083/jcb.132.5.835
Permanent URL: https://pid.emory.edu/ark:/25593/rqccv

Final published version: http://dx.doi.org/10.1083/jcb.132.5.835

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Accessed October 23, 2019 2:56 AM EDT
The Caenorhabditis elegans Gene unc-89, Required for Muscle M-line Assembly, Encodes a Giant Modular Protein Composed of Ig and Signal Transduction Domains

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Abstract. Mutations in the Caenorhabditis elegans gene unc-89 result in nematodes having disorganized muscle structure in which thick filaments are not organized into A-bands, and there are no M-lines (Waterston, R.H., J.N. Thomson, and S. Brenner. 1980. Dev. Biol. 77:271-302). Beginning with a partial cDNA from the C. elegans sequencing project, we have cloned and sequenced the unc-89 gene. An unc-89 allele, st515, was found to contain an 84-bp deletion and a 10-bp duplication, resulting in an in-frame stop codon within predicted unc-89 coding sequence. Analysis of the complete coding sequence for unc-89 predicts a novel 6,632-amino acid polypeptide consisting of sequence motifs which have been implicated in protein-protein interactions. UNC-89 begins with 67 residues of unique sequence, SH3, dbl/CDC24, and PH domains, 7 immunoglobulin (Ig) domains, a putative KSP-containing multiphosphorylation domain, and ends with 46 Ig domains. A polyclonal antiserum raised to a portion of unc-89 encoded sequence reacts to a twitchin-sized polypeptide from wild type, but truncated polypeptides from st515 and from the amber allele e2338. By immunofluorescent microscopy, this antiserum localizes to the middle of A-bands, consistent with UNC-89 being a structural component of the M-line. Previous studies indicate that myofilament lattice assembly begins with positional cues laid down in the basement membrane and muscle cell membrane (Williams, B.D., and R.H. Waterston. 1994. J. Cell Biol. 124:475-490; Hresko, M.C., B.D. Williams, and R.H. Waterston. 1994. J. Cell Biol. 124:491-506). We propose that the intracellular protein UNC-89 responds to these signals, localizes, and then participates in assembling an M-line.

In striated muscle, the bare zone is the central part of the A-band in which bipolar thick filaments are free of myosin heads. In the center of the bare zone lies the electron dense M-line, also known as the M-band or M-disc, which is 75–100 nm wide and is believed to anchor thick filaments and help maintain them in proper register. From electron microscopic examinations, models for the complex lattice-work that constitutes the M-line in vertebrates, especially the frog, have been developed (Knappeis and Carlsen, 1968; Luther and Squire, 1978), but the assignment of every known component to structures is not well understood.

Five non-myosin proteins are known to reside in the vertebrate M-line: (a) MM-creatine kinase (43 kD; Strehler et al., 1983), (b) the COOH-terminal portion of the giant 3 x 10^6-D polypeptide called titin, also known as connectin (Gaulet al., 1993; Maruyama, 1994; Labeit and Kolmerer, 1995b), (c) M-protein (165 kD; Noguchi et al., 1992), (d) myomesin (190 kD; Vinkemeier et al., 1993), and (e) skelemin (195 kD; Price and Gomer, 1993). Titin/connectin copurifies with M-protein and myomesin (Nave et al., 1989). The last four of these proteins are members of the intracellular muscle branch of the immunoglobulin (Ig) superfamily and consist primarily of multiple copies of Ig and fibronectin type III (FnIII) domains. The founding member of this branch of the Ig superfamily is Caenorhabditis elegans twitchin, a 753,494-D polypeptide (Benian et al., 1989, 1993) encoded by the gene unc-22, and localized to muscle A-bands (Moerman et al., 1988). Twitchin consists of a single protein kinase domain, 31 FnIII domains, and 30 Ig domains. Other members of this intracellular branch of the Ig superfamily include insect projectin (Ayme-Southgate et al., 1991, 1995; Fyrberg et al., 1992), and the vertebrate proteins smooth muscle (Olson et al., 1990) and non-muscle (Shoemaker et al., 1990) myosin light chain kinases, telokin (Gallagher and Herling, 1991), C-protein (Einheber and Fischman, 1990), 86-kD protein (Vaughan et al., 1993), kettin (Lakey et al., 1993), and the above-mentioned M-line proteins. The Ig and FnIII domains are likely to mediate interaction with myosin and other proteins, as has been shown for titin/con...
Materials and Methods

Nematode Strains

*Caenorhabditis elegans* strains were obtained from the *Caenorhabditis elegans* stock center, R.H. Waterston, H.F. Epstein, L. Avery, and D.L. Riddle. Wild-type worms were *Caenorhabditis elegans* variety Bristol, strain N2. The following *unc*-89 alleles were tested for polymorphisms with cosmid CS4E1 and K08D2: *unc*-539, *e1460*, *e2338*, *st79*, *st85*, *st515*, *su75*, *su78*, *su227*, and *su240*. *unc*-89(*st515*) was originally isolated from a mutator strain (and thus likely to be transposon-induced) and then out-crossed 8× to N2 by S. Rioux in R.H. Waterston’s laboratory (Washington University). *st85* and *e2338* are known to be amber (UAG) chain termination mutations because their phenotypes are suppressible by the amber suppressor mutation *sup-7*. *unc*-539, *e1460*, *e2338* are null for myoB (encoded by the *unc*-54 gene) but can produce thick filaments because it has a duplication of the myo-3 gene and overproduces myoA (Riddle and Brenner, 1978; Maruyama et al., 1989).

Northern and Southern Blots

Total RNA was isolated from wild type and *unc*-22(*es37*) by a slight modification of published procedures (Chirgwin et al., 1979). Northern blots were prepared and hybridized as described previously (Moerman et al., 1988). Genomic DNA was prepared from wild type and each of the above-mentioned *unc*-89 strains. Separate aliquots were digested with eight restriction enzymes, and probed with cosmid CS4E1 and K08D2 on Southern blots as described in Moerman et al. (1988).

DNA Sequencing

The entire sequence of the cosmid CS4E1 was determined by sequencing clones from a shotgun library in mp10M13. Cosmid mini-prep DNA was sheared by sonication, separated on an agarose gel, and fragments larger than 1.1 kb were purified by GeneClean (BIO 101 Inc., La Jolla, CA). This DNA was end-repaired with sequential treatment with T4 DNA polymerase (+dNTPs) and Klenow fragment, phosphorylated with T4 polynucleotide kinase, ligated into Smal-cut and phosphatased mp10M13 (Amersham Corp., Arlington Heights, IL) and transformed into competent JM101 cells. Single-stranded DNA from over 800 independent clones was propagated and sequenced (~400 bp each) with the M13 universal primer using Taq DyeDeoxy Terminator Cycle Sequencing Kits (Applied Biosystems, Inc., Foster City, CA) on an Applied Biosystems 373A automated fluorescent DNA sequencer. Each ABI raw data file was transferred to a SUN computer; bases were called and M13 vector sequences were clipped-off with AUTOTED (Wilson et al., 1994), and assembled into contigs and edited with XBAP (Dear and Staden, 1991). To obtain one contig for the entire cosmid sequence on both strands, 31 oligonucleotides were used as sequencing primers on selected shotgun clones.

Exon Prediction

The GeneMark program (Borodovsky and McIninch, 1993) was used for predicting exons in the CS4E1 sequence. The key element of the GeneMark method is an inhomogeneous (three-periodical) Markov model that provides an accurate statistical description of a protein-coding sequence (Borodovsky et al., 1986; Tavare and Song, 1989; Kleffe and Borodovsky, 1992). A given DNA fragment is identified as residing in a region that is either (a) protein-coding, (b) non-coding but complementary to coding sequence (gene shadow), or (c) totally non-coding. Parameters of the Markov model for DNA sequence of a particular type are determined from training sets of experimentally identified sequences of the same type. A fragment S, of newly sequenced DNA, is associated with one of several possible sequence types (models, k = 1, . . . , K) using a probabilistic measure defined by Bayes theorem:

\[
P(\text{model } k | \text{ sequence } S) = \frac{P(\text{sequence } S | \text{ model } k) \cdot P(\text{model } k)}{\sum_{k} P(\text{sequence } S | \text{ model } k) \cdot P(\text{model } k)}
\]

The above expression is used to identify possible reading frames in coding sequence (k = 7). Then, seven probability values Pp, i = 1, . . . , 7 describe the likelihoods that the sequence S belongs to one of seven mutually exclusive states: six states of protein-coding in six different frames and one non-coding state. If one of Pp, i = 1, . . . , 6 is greater than threshold 0.5, then the DNA fragment S (or its complement) is identified as protein-coding in a proper reading frame. If Pp is larger than 0.5 or if none of Pp, i = 1, . . . , 6 is larger than 0.5, then S (and its complement) is identified as non-coding. Long sequences are analyzed using a sliding window technique and parsed into coding and non-coding regions. The GeneMark method

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The application of GeneMark to \textit{C. elegans} required some modifications. Since the non-homogeneity of the \textit{C. elegans} genome hampers the accuracy of gene identification, a large set of known \textit{C. elegans} DNA sequences was divided into two sets with "high" and "low" GC content using cutoff values of 46%, the average GC content for the \textit{C. elegans} genome. There were 153 spliced genes (227,010 bp) and 155 non-coding regions that fall into the high GC content range, the high GC models were used in the prediction of these sequences. The correct identification was done in 91% of cases for the low GC content range and 94% of cases for the high GC content range. Since the \textit{unc-89} sequence falls into the low GC content range, the low GC models were used in the GeneMark program. Predicted exons were translated and compared to protein sequences in the databases by the BLASTp method (Altschul et al., 1990).

\section*{Partial cDNA Clones, Reverse Transcriptase PCR, and 5' RACE}

The original cDNA, cm20d2, was used as a probe to screen an oligo-dT-primed cDNA library (kindly provided by R. Barstead, Oklahoma Medical Research Foundation) and two additional overlapping partial cDNAs were recovered, laA2 and 17b-2a. These clones were sequenced manually (Sanger et al., 1977) using Sequenase (Stratagene, Inc., La Jolla, CA). All three clones lack in-frame stop codons and have artificial polyA tails probably due to annealing of oligo-dT to A-rich coding sequences often found in the AT-rich \textit{C. elegans} genome. Comparison of these cDNA sequences to genomic sequence defined 8 exons. These encode sequence 45 bp upstream of the EcoRI cloning site in pBluescript SK (Stratagene, Inc.). The resulting clone was obtained and called 3'11A and found to extend further 3'. Upon sequencing, it was found to contain an in-frame stop codon, 3' untranslated region of 680 bp, a polyadenylation signal, and a polyA tail. Thus, the approximately 1,982 bp of the \textit{unc-89} mRNA was determined only by sequencing cDNAs and not genomic DNA.

\section*{Antisense Production and Western Blots}

A 364 residue polypeptide, corresponding to sequence including the end of Ig no. 2 through the middle of Ig no. 6, was expressed in \textit{E. coli} as a glutathione S-transferase fusion protein. This was done by PCR using Pfu polymerase (Stratagene) with primers corresponding to sequence within cDNA cm20d2, to which were added BamHI and EcoRI sites and cloned into pGEX-2T (Smith and Johnson, 1988). The cloned used for expression was verified, by sequencing, to not contain errors introduced by PCR. The GST fusion protein was expressed and purified with a glutathione-agarose batch method essentially as described by Smith and Johnson (1988) except that IPTG induction was conducted at room temperature. Milligram quantities of GST fusion protein were supplied to Spring Valley Labora-

tories (Sykesville, MD) for production of polyclonal rabbit antisera. From the preimmune sera of 10 rabbits, 2 rabbits were selected for immunization because they had the lowest level of antibodies cross-reacting to total protein extracts of nematodes on Western blots. Following the third series of immunizations, Western blots showed reaction to a polypeptide of \textsim 750 kD. Removal of most of the anti-GST antibodies and affinity purifi-
cation was performed as described in Benian et al. (1993). Immunoblot experiments were conducted as follows: wild-type and \textit{unc-89} mutant strains were grown on single 1-cm Petri plates seeded with \textit{E. coli} OP50 until the bacteria was nearly depleted. Worms were washed free from bacteria by several washes and pelleting through M9 buffer (86 mM NaCl, 22 mM KH2PO4, 43 mM Na2HPO4, 1 mM MgSO4) in a microfuge. To the ~0.1 ml of packed worms was added 1 ml of 95°C 2× Laemmli buffer (Laemmli, 1970), vortexed for 20 s, heated at 95°C for 5 min, and vortexed again for 20 s. This mixture was then spun in a microfuge for 10 min, after which the supernatant was decanted to a fresh Eppendorf tube and stored at -70°C until used. Samples were thawed briefly at 37°C, and 10 μl of each were run lanes of a 1-mm thick 5% polyacrylamide Sds Laemmli gel. Western blotting (Laemmli, 1970) with a 3% stacking gel run in a Bio-Rad, Inc.'s mini-
gel as follows: 50 volts for 40 min, 100 volts for 10 min, and 200 volts for 50 min. Immobilization of the gel in nitrocellulose membrane (Towbin et al., 1979) for 2 h at 100 constant volts with cooling in a Bio-


drad mini trans-blot apparatus. After staining with Ponceau S solution (Sigma Chem Co., St. Louis, MO), the membrane was blocked at 37°C for 4 h in 5% non-fat dry milk, 0.1% Tween-20, tris-buffereed saline; this solution was used also for dilution of primary and secondary antibodies. Monoclonal MH42 (kindly provided by M. Costello) was used at a 1:400 dilution, and the rabbit polyclonal anti-twitchin (developed to central Fn III Fn III Ig motifs of twitchin by M. Valenzuela and G.M. Benian) at 1:10,000 dilution. The secondary antisera were peroxidase-conjugated donkey anti–mouse or anti-rabbit immunoglobulin, and detection was by enhanced chemiluminescence (Amersham).

\section*{Indirect Immunofluorescence}

Most of our observations were made on whole nematodes using a method originally designed by Ruvkun and Giusto (1989) and Finney and Ruvkun (1990) and modified for the study of muscle by D.M. Miller (1995). For localizing UNC-89 in the pharynx, we performed fluorescence microscopy on frozen sections, as described later in this section.

For the whole worm procedure, most spines were carried out at 764 g. 430 g sections were used for fragile mutant strains. Disposable plastic conical centrifuge tubes, with screw-on lids were used for most steps. Glass coni-

cal centrifuge tubes were necessary for the β-mercaptoethanol (BME),
dithiothreitol (DTT), and H$_2$O$_2$ incubations to prevent worms from sticking to the sides of the tube. All reactions were carried out at room temperature, unless otherwise noted. One volume is equivalent to 5 ml.

Worms were washed off plates with 2 vol M9 buffer and spun for 2 min, and then washed several times, leaving behind a worm pellet of 0.1–0.5 ml. The worms were resuspended in 1 vol M9 and left on ice until ready to use. The fixative, containing 1% formaldehyde and 71% methanol was made as follows: 0.25 ml 16% paraformaldehyde (obtained from E M Sciences), 2.85 ml 100% methanol, and 0.9 ml 4x Modified Ruvkun’s wash buffer (M9 buffer without methanol (320 mM KCl, 80 mM NaCl, 40 mM EGTA [pH 7.4], 20 mM spermidine, 60 mM Pipes [pH 7.4]). The worms were spun again, and excess M9 was aspirated off. The 4 ml of fixative were quickly added. The tube was inverted to mix and plunged into liquid nitrogen until frozen. The tube was then placed on ice to thaw. In some cases, the freeze-thaw cycle was repeated two more times. The worms were then incubated for 1 h on ice, and mixed by gentle inversion at 5-min intervals. The worms were again pelleted and resuspended in 1 vol 1× TTB (Tris Triton buffer: 100 mM Tris-HCl [pH 7.4], 1% Triton X-100, 1 mM EDTA). After gently inverting three times, the worms were spun and washed again twice. The worms were then resuspended in 1 vol 1× TTB and transferred to a glass tube. Then, 1% BSA was added and the worms were incubated at 37°C for 2 h on a rotating wheel at low speed. The worms were then washed in 1 vol 1× BO$_3$ buffer (50× BO$_3$ buffer: 1 M H$_2$BO$_3$, 0.5 M NaOH, pH 9.2). They were then resuspended in 10% 0.1 M DTT in 1 1/2 vol 1× BO$_3$ and gently rotated on the wheel for 15 min. The worms were then washed in 1 1/2 vol 1× BO$_3$ twice. The worms were then resuspended in 1 1/2 vol 1× BO$_3$ and 0.075 ml 30% H$_2$O$_2$ was added. The tube was allowed to set, uncovered, for 15 min, with gentle inversion every 5 min. The worms were then washed in 1 vol of 1× BO$_3$ three times with 1 vol of 1× BO$_3$ buffer by buffer B: 1× PBS, 0.1% BSA, 0.5% Triton X-100, 1 mM NaN$_3$, 1 mM EDTA), and left on the wheel for 20 min. The worms were then resuspended in 1 vol AbA (antibody buffer A: same as AbB, except 10% BSA). The solution was divided among the number of tubes necessary and then pelleted. Primary antibodies were diluted at 1:50 in AbA to a volume of 0.5 ml, and added to worm pellets. The lids were placed on the tubes, and wrapped in parafilm, then incubated on the wheel overnight. The worms were then washed four times in 1 vol of AbB for 30 min each on the wheel. The worms were then resuspended in 1 vol AbA and pellets. Rhodamine- or fluorescein-conjugated secondary antibodies (Cappel, Durham, NC) were diluted 1:250 or 1:500 in AbA and added to the pellets. The tubes were capped, covered in foil, and incubated for 2 h on the wheel. Samples were handled individually as follows. Worms were pelleted and the solution was aspirated, leaving a small amount of supernatant above the worms. 30-µl samples were placed on slides and 50 µl Aquamount (Lerner Labs., Pittsburgh, PA) was then added per slide. Coverslips were applied and then the slides were viewed with a Zeiss Axioplan microscope, equipped with an HBO 100 watt mercury illuminator. Photographs were taken with an MC100 Spot camera, using Kodak Ektachrome P1600, EPH 135-36 or Fuji Fujichrome 1600, EPH 135-36 film. Fluorescein samples were photographed at 1600 ASA and rhodamine samples at 800 ASA. Film was pushed two process steps two.

To prepare frozen sections, ~200 µl of a 50:50 mixture of nematodes in M9 buffer were injected into a mold filled approximately halfway with OCT compound (Miles, Inc., Elkhart, IN). Additional OCT compound was added gently to encircle the area containing the worms and to fill up the mold. Freezing was accomplished by immersing the mold in 2-methylbutane at ~72°C. Frozen sections of ~3 µm thickness were mounted on Superfrost/Plus (charged) slides (Fisher Scientific, Pittsburgh, PA). After staining a representative section by hematoxylin and eosin, we observed that ~10% of the sections were cross sections through the pharynx. We obtained best results, in terms of numbers of sections retained on slides and intensity of staining, when the entire procedure was carried to completion on the same day that the sections were cut. The following washes were performed in Copland jars at room temperature: PBS for 5 min, 95% ethanol for 10 min, and PBS for 5 min (x2). After blotting away excess liquid, 200 µl of 10% goat serum in PBS was applied and incubated at room temperature for 15 min to block nonspecific binding of secondary antibodies. After blotting off excess goat serum, 200 µl of primary antibody diluted 1:200 in PBS was added and the slide was placed in a humidified chamber at 37°C for 1 h. The slide was then washed at room temperature in PBS for 5 min, and then in 1% BSA, PBS for 5 min. After removing excess liquid, 200 µl of fluor-conjugated goat secondary antibodies diluted 1:200 or 1:400 in 1% BSA, PBS was added and the slide placed in a humidified chamber at 37°C for 30 min. This was followed by two washes at room temperature in the dark: 1% BSA, PBS for 5 min, and then PBS for 5 min. After removing most of the liquid, the sections were covered with 30 µl of Aquamount and a coverslip, and photographed as noted above.

**Results**

cDNA cm20d2 Encodes Six Ig Domains and Hybridizes to an ~22-kb mRNA

Recently, Waterston et al. (1992) determined ~400 bp of sequence from the 5’ ends of each of 1517 clones from a sorted cDNA library, then translated and compared these sequences to the databases. One cDNA, cm20d2, had highest similarity to myosin light chain kinases and twitchin. We were alerted and kindly given the clone. We finished sequencing the 1978-bp insert. It has no similarity to the 55-kb genomic sequence that contains unc-22 (Benian et al., 1989, 1993). cm20d2 encodes part of one and five complete Ig domains (No. 2–7 in Fig. 3 a). These six domains give the best BLAST scores to Ig domains from members of the intracellular muscle branch of the Ig superfamily (data not shown).

When cm20d2 was used as a probe against Northern blots, a very large mRNA, approximately the size of the twitchin mRNA (21.6 kb; Benian et al., 1993) was detected (Fig. 1). To eliminate the possibility that cm20d2 merely cross-hybridized to the twitchin mRNA, we also probed RNA prepared from the unc-22 allele ct37. ct37 has a several-kilobase deletion of unc-22 coding sequence, and lacks the twitchin polypeptide on an immunoblot (Moerman et al., 1988). As shown in Fig. 1, although no mRNA was detected from ct37 with an unc-22 probe, an mRNA was detected from ct37 with cm20d2 as probe.

cm20d2 Maps to a Genomic Cosmid Which, as a Probe, Detects a Rearrangement in the unc-89 Allele sl515

Upon hybridization to a filter of 960 physically-mapped YAC clones (kindly provided by R. Waterston), the cm20d2 cDNA detects 2 YACs, Y56D12 and Y74A11. We obtained cosmids covering the overlapping region from J. Sulston and A. Coulson. cm20d2 hybridized to the cosmids as indicated in Fig. 2. This portion of the physical map corresponds to the central portion of chromosome I. The closest gene that had been cloned was unc-73. unc-73 is required for axon guidance and segregation of developmental potential. unc-73 has been cloned via transposon tagging (Steven, R., and J. Culotti; Mt. Sinai Hospital Research Institute, Toronto, personal communication). cm20d2 is not encoded by unc-73 for the following reasons: (a) just to the left and slightly overlapping cosmids that hybridize to cm20d2 is cosmids C11B5, which as a transgene rescues unc-73; (b) there is no DNA sequence homology between cm20d2 and the unc-73 sequence; (c) unc-73 mRNAs are 6.3 and 7.7 kb; and (d) unc-73 encodes a protein homologous to yeast CDC24, and lacks Ig domains. Inspection of the genetic map revealed that one of the closest genes to unc-73 is unc-89, only 0.21 map units away. Mutations in unc-89, as described above, specifically affect muscle. Eleven unc-89 alleles were examined by Southern blots for RFLPs with cosmids clones C34E1 and K08D2 as probes. For the allele sl515, isolated from a mutator background (Rioux, S., and R. Waterston, personal communication), and thus
Figure 1. cDNA cm20d2 hybridizes to an ~22-kb mRNA. C. elegans total RNA (~20 μg per lane) was fractionated on a 0.7% agarose-formaldehyde gel, transferred to nitrocellulose membrane and hybridized with the indicated probes. unc-22(ct37) is a several kilobase deletion within the unc-22 (twitchin) gene which produces no detectable twitchin (Moerman et al., 1988). As shown in A, a probe specific for unc-22 detects the mRNA known from sequence analysis to be 21.6 kb from wild type, but no mRNA from ct37. This blot was stripped of probe and rehybridized with a probe specific for unc-54 (myosin heavy chain B) to show that the RNA was largely undegraded and that RNA was present in the ct37 lane (B). A portion of the same blot was hybridized with cDNA cm20d2. As shown in C, cm20d2 hybridizes to an mRNA of approximately the same size as the mRNA for unc-22 (twitchin). Since this message can be detected in RNA from ct37, it rules out the possibility that cm20d2 is merely cross-hybridizing to the unc-22 message. D shows the result of rehybridizing the membrane used in C with an unc-54 probe. likely to be transposon-induced, a polymorphism was seen with C34E1 as probe, consistent with a deletion of ~100 bp within a 2.0-kb Hind III fragment. This 2.0-kb fragment was subcloned into Bluescript, and several hundred bp of sequence were determined from each end. Primers were designed from the sequence and used to produce, by PCR, the corresponding segment from both wild type and st515. After sequencing each segment, st515 was found to contain an 84-bp deletion and 10-bp duplication, resulting in an in-phase TGA stop codon (Fig. 2 b).

unc-89 Encodes a 731,897-D Polypeptide Consisting of SH3, dbl/CDC24, PH, Multiphosphorylation, and Ig Domains

Because cosmid C34E1 is the most centrally placed of the five cosmids to which cm20d2 hybridizes, and C34E1 contains the site of deletion for st515, we determined the complete 44,848-bp sequence of the C34E1 insert. Exons were predicted by the computer program, GeneMark, and then, exon-intron boundaries were confirmed by sequencing 200–900-bp reverse transcriptase PCR products. These PCR products revealed several small (less than 100 bp) exons not predicted by the program. Alignment of cm20d2 and two other partial cDNAs (each ~2.2 kb) with this genomic sequence confirmed several other exons. The confirmed exons predicted one transcriptional unit, beginning at position 5,870 and continuing until the end of the cosmid insert. Because an in-frame stop codon was not found, screens of oligo-dT-generated cDNA libraries were performed, and yielded two cDNA clones that extended the sequence an additional 1,982 bp. The sequence of the furthest 3'-clone revealed an in-frame stop codon, a 680 bp 3'-untranslated sequence, and an AATAAA consensus polyadenylation signal 19 bp upstream of a poly(A) tail. After finding coding sequence that could account for a protein ~99% the size of twitchin, a primer designed from the furthest 5' exon was used in 5' RACE to determine the 5' end of the unc-89 message. An in-frame initiator methionine beginning at position 4,920 and a 145-bp 5' untranslated sequence starting at position 4,774 were found. The unc-89 gene extends over at least 42,041 bp of the genome and contains at least 30 introns. These sequence data are available from GenBank under accession number U33058. The 5' end of the unc-73 gene lies at position 773 in our sequence and is transcribed in opposite orientation to unc-89 (Steven, R., and J. Culotti, personal communication), giving a distance of 4,001 bp between unc-73 and unc-89.

The entire UNC-89 polypeptide consists of 6,632 amino acids with a calculated molecular weight of 731,897. As shown in Fig. 3 a, the UNC-89 sequence begins with 67 residues having no homologies to sequences in the databases, followed by an SH3 domain, a dbl/CDC24 domain, a PH domain, 7 Ig domains, 645 amino acids consisting of several repeats including 44 copies of the amino acid triplet lysine serine proline (KSP), and then 46 Ig domains. The region with homology to dbl/CDC24 was recognized by a BLASTP search which gave the highest scores to products of the dbl (Eva et al., 1988; Ron et al., 1991) and Ost oncogenes (Horii et al., 1994), which are members of the CDC24 family of guanine-nucleotide release factors that link rho and rac signaling pathways (Fig. 3 b). Because these domains are often followed by PH domains (Gibson et al., 1994), such a sequence was searched for, by inspection. As shown in Fig. 3 c, there is indeed a PH domain which matches a consensus for PH domains and a prediction of secondary structure indicates that the key el-
Antisera Raised to an unc-89 Encoded Peptide and Truncated Polypeptides in Two unc-89 Alleles

Steven's analysis of unc-73 indicates that the transcription start sites of the two genes are separated by 4001 bp, and they are oppositely oriented (directions indicated by arrows). On the genetic map, unc-73 and unc-89 are separated by 0.21 map units. lin-44 lies in the 20 kb between C24G7 and K05E6, within the 4.1 kb just left of K05E6 (Herman et al., 1995; indicated by light shading). unc-89 and lin-44 have not been separated by recombination. The 3' end of unc-89 has not been placed on this physical map. (B) Sequence alteration in the unc-89 allele st515. The mutator-induced st515 mutation consists of an 84-bp deletion and 10-bp duplication, resulting in an in-frame TGA stop codon within coding sequence for Ig no. 5 of the predicted UNC-89 protein (see Fig. 3). This is predicted to result in a truncated polypeptide of 135 kD that can indeed be detected by Western blot (see Fig. 4).

Antisera Raised to an unc-89 Encoded Peptide React with a Twitchin-sized Polypeptide in Wild Type and Truncated Polypeptides in Two unc-89 Alleles

To confirm the presence of the very large polypeptide predicted from sequence analysis, we raised antisera to a portion of the polypeptide. A 364-amino acid peptide, coded by the majority of cDNA cm20d2 and corresponding to sequence including the end of Ig domain 2 through the middle of Ig domain 6, was expressed as a glutathione-S-transferase fusion protein. An affinity-purified rabbit antiserum to this fusion protein (EU30) reacts to a polypeptide of about the same size as twitchin (~750 kD) on immunoblots containing total Laemmli-soluble nematode proteins (Fig. 4 a). This antiserum reacts with smaller, probably truncated polypeptides from e2338 (an unc-89 amber allele; Fig. 4 b). The 135,000-D size of the truncated polypeptide from st515 is consistent with the position of the stop codon introduced in the coding sequence.

R. Francis had previously generated ~40 monoclonal antibodies to muscle cells, and their underlying basement membranes and hypodermis (Francis and Waterston, 1985, 1991). One of these monoclonals, MH42, localized to the center of A-bands by immunofluorescence (Waterston, 1988). Previously, R. Francis failed to detect a polypeptide on a Western blot with MH42. Using the more recently developed and highly sensitive enhanced chemiluminescent method, we detect reaction of MH42 with a polypeptide of the same size as with EU30 (Fig. 4). MH42 fails to react to a polypeptide in st515, but detects approximately the same-sized truncated polypeptides in e2338 as EU30. That the polyclonal antiserum EU30 and monoclonal MH42 react to the same protein, the product of the unc-89 gene, is suggested by the results from both these Western experiments and immunofluorescent localization in both wild type and in unc-89 mutants (see below).
Figure 3. The deduced UNC-89 polypeptide. (A) Schematic representation. Ig domains are denoted as shaded boxes (1-53), the 645 amino acid residues containing 44 KSps are indicated with dots, the PH domain with a thin diagonal striping, the dbl/CDC24 domain with thick diagonal striping, and the SH3 domain with a "wood grain pattern." Clear boxes denote sequences with no homologies to proteins in the databases. The short vertical lines projecting above represent the positions of introns. The intron-exon structure is not known from the middle of Ig no. 50 until the COOH terminus. (Indicated by dotted line) a represents 22 amino acids encoded by an alternative exon. (B) The dbl/CDC24 domain of UNC-89 (residues 151-329) aligned with comparable domains from human dbl, rat ost, and yeast cdc24, using prettybox with the similarity option. (C) The PH domain of UNC-89 (residues 343-451) aligned with comparable domains from human dbl and rat ost, using prettybox with the similarity option. (D) A pair-wise comparison of UNC-89 residues 68-122 with a profile made from an SH3 alignment (and Gonnet PAM160 matrix). The SH3 alignment is a revised and expanded version of one made by Musacchio et al. (1992). (E) The 645 amino acids lying between Ig no. 7 and Ig no. 8 are highly repetitive and highly ordered. This sequence is presented consecutively, NH2-terminal to COOH-terminal, but arranged to emphasize the repeating linear sequence motifs. The KSps(44) and related sequences ASPs(7), GTP(1), PSP(1), SSP(5), RTS(1), TSP(1), KTP(1) are mostly arranged in a repeat of 10 residues (typically KSPTKKEKSP) and present in 26 copies. Most of these are separated by a third repeating sequence varying between three and eight residues. In one region, there is a regular alternation of eight and seven residues. Except for the alternative exon sequence (limited by parentheses) this region is encoded by a single exon. (F) A consensus sequence derived by the PILEUP program for all 53 Ig domains which consists of any residue present at a given position in at least 20 of the 53 Ig domains. This UNC-89 consensus is aligned to a consensus for the 30 Ig domains of twitchin (Benian et al., 1989) and a consensus for Ig domains found in six of the myosin-associated intracellular Ig proteins (MAPC) calculated by Price and Gomer (1993). Dots are gaps introduced to align the three consensus sequences.
Antisera Localize the UNC-89 Protein to the Center of Muscle A-Bands

Indirect immunofluorescence was used to determine the location of this protein in the sarcomere. Wild-type animals were simultaneously reacted with an antiserum to twitchin and to MH42 (Fig. 5, A and B). This reaction was visualized by the use of secondary antibodies conjugated to rhodamine and fluorescein. Moerman et al. (1988) observed that twitchin is localized to the A-bands with a central gap of staining. In Fig. 5, A and B, the arrows point to the middle of the same A-band, and indicate that this gap is filled with the protein recognized by MH42. In Fig. 5, C and D are shown results of staining the same animals with EU30 and MH42 staining occurs in the middle (Fig. 5, F and G) of the radial muscle fibers of the pharynx, as compared with the broad A-band distribution (Fig. 5 E) of the pharyngeal muscle-specific myosin heavy chain, myoC (Miller et al., 1986).

In unc-89 Mutants, UNC-89 Protein Is Missing, or Is Still Located in the Middle of A-Bands

To obtain further evidence that the antibody reagents are directed against the unc-89 product, we examined unc-89 mutants by immunofluorescence microscopy. One group of nematodes was stained at the same time with antitwitchin and MH42 and viewed with different filters. A separate set of animals was reacted with EU30. Reaction to twitchin antibodies was used to check the success of the immunofluorescence procedure and as an independent measure of A-band organization. Two nonsense (amber) alleles of unc-89, st85, and e2338, (Waterston, R.H., personal communication) show very much reduced staining as compared to wild type. st85 still shows some localization to the center of A-bands (data not shown), but the staining in e2338 is confined to dots or clumps (Fig. 5, B and C). unc-89(e1460) yields an abundant normal-sized polypeptide on
immunoblots with either EU30 or MH42 (data not shown). Consistent with this, immunofluorescence microscopy shows heavy but discontinuous reaction to the center of A-bands (Fig. 6, D and E). Both MH42 and EU30 show the same intensity of staining and localization for wild type and these three unc-89 mutants (e1460, st85, and e2338).

Fig. 6, F and G display the results of staining one st515 animal with MH42, and another st515 animal with EU30. Consistent with the Western data, no staining is seen with MH42, but weak staining, surprisingly much of it localized to the center of A-bands, is seen with EU30. This suggests that most of the determinants for localization of the UNC-89 protein to the middle of A-bands reside in the first 135 kD of the UNC-89 polypeptide.
Figure 6. EU30 and MH42 staining of body wall muscle in mutant animals. (A and B) Photographs of the same portion of muscle from an e2338 animal simultaneously reacted with α-twitchin (in A) and MH42 (in B). (C) Photograph of a separate e2338 animal reacted with EU30. (D) Photograph of body wall muscle of e1460 stained with MH42 (simultaneous staining with α-twitchin showed a nearly normal staining pattern). (E) A separate e1460 animal stained with EU30. (F) A st515 animal stained with MH42. (G) A st515 animal stained with EU30. This mutant that produces a fairly abundant truncated 135-kD polypeptide shows reduced staining but much of it is still restricted to the middle of A-bands. (H and I) Localization of UNC-89 protein in body wall muscle with thick filaments having myosin heavy chains of only the myoA isoform. Nematodes of genotype eDp23;unc-54(e190) were stained with both α-myoA and EU30. The arrows point to the center of the same A-band. In H it can be seen that MyoA is distributed throughout the A-band, whereas in I it can be seen that UNC-89 (EU30 staining) is confined to the center of A-bands, as it is in wild type. Twitchin is also distributed as it is in wild type, with a lack of staining in the middle (data not shown). Bars, 5 μm.

MyoA Is Not the Determinant for UNC-89 Localization

Given that twitchin colocalizes with myoB (Moerman et al., 1988), and UNC-89 seems to colocalize with myoA, it is tempting to hypothesize that in wild-type, twitchin associates with myoB and UNC-89 protein associates with myoA. This was tested by performing immunofluorescence microscopy on the strain eDp23; unc-54(e190) which produces no myoB (encoded by the unc-54 gene) but forms thick filaments because of overexpression of myoA due to a duplication of the myoA gene. As shown in Fig. 6 H, anti-myoA stains the entire, broad A-band. Nevertheless, UNC-89 (EU30) is still narrowly restricted to the center of A-bands (Fig. 6 I). Arrows point to the center of the same A band in both Fig. 6, H and I. Similar results have been obtained with MH42 staining of unc-54(e190) animals transformed with extrachromosomal arrays of the myoA gene (Coutu Hresko, M., P. Hoppe, and R. Waterston, personal communication). Thus, the most important determinants for UNC-89 localization appear to reside in proteins/structures different from myoA. In addition, twitchin is seen to have the same distribution as in wild type, with the same gap in the middle of A-bands (data not shown). Thus, in this strain, it would seem that twitchin, in the absence of myoB can instead associate with myoA and/or other proteins.
**Discussion**

We have characterized a >42-kb gene that encodes a 20.7-kb mRNA and 732-kD polypeptide consisting primarily of 53 Ig domains, a possible multiphosphorylation sequence, and regions with homology to SH3, CDC24, and PH domains. That this gene corresponds to *unc-89* is based on the following evidence: (a) genomic and cDNA clones localize to a region of the physical map that corresponds to a region of the genetic map which contains *unc-89*. (b) An antiserum raised to a peptide encoded by a portion of the sequence reacts specifically to muscle, localizing to the middle of the A-band, a region that contains the M-line. The M-line is missing in one *unc-89* allele examined by EM. On Western blots, this antiserum reacts with an ~750-kD polypeptide from wild type, and with smaller, truncated polypeptides from an *unc-89* deletion (st515) and an *unc-89* amber nonsense mutation (e2338). Indirect immunofluorescence microscopy results are consistent with the immunoblot data in that staining is reduced or greatly reduced in four *unc-89* alleles. (c) *unc-89*(st515) was shown to contain an 84-bp deletion and 10-bp duplication resulting in an in-frame stop codon in the coding sequence of the gene, consistent with the 153-kD polypeptide detected by Western blot.

In nematode obliquely-striated body wall muscle, we have localized the UNC-89 protein to the middle of the A-band, where the M-line resides, but it may extend into a broader central region of the A-band. Although at the resolution of immunofluorescence microscopy, the UNC-89 protein is located in the same central region of the A-band as the minor myosin heavy chain myoA, we have shown that the localization of UNC-89 is independent of myoA. This conclusion is based on finding UNC-89 restricted to the center of A-bands even when the entire A-band contains myoA, as in the mutant *dpy-23;unc-54(e190)*. Moreover, this is consistent with UNC-89 being a component of the M-line itself. The pharynx of the nematode is a neuromuscular pump used for feeding. The pharynx has radially oriented myofilaments (Albertson and Thomson, 1976). We found that UNC-89 is also localized to the center of the A-bands in pharyngeal muscle, which suggests that this muscle also has an M-line. Thus, in two different muscle types, UNC-89 has a similar location, and quite probably a similar function. Possibly different isoforms of UNC-89, generated by alternative splicing, are expressed in pharyngeal vs. body wall muscle. At least one example of alternative splicing in *unc-89* is known, and is indicated in Fig. 3. Also, there is at least one allele of *unc-89, ad539*, which has abnormal pharyngeal, but normal body wall muscle structure (Avery, 1993; T.L. Tinley and G.M. Benian, unpublished data).

The UNC-89 polypeptide is unusual in being composed primarily of Ig domains, and having no FnIII domains. Except for telokin, which is essentially one Ig domain, all other members of the intracellular branch of the Ig superfamily contain both Ig and FnIII domains. Although having no linear sequence homology, Ig and FnIII domains form similar structures (Leahy et al., 1992). Whereas both Ig and FnIII domains have been implicated in protein-protein interactions, functional differences, such as in binding affinities, preferences for target domains etc., are not known. The observation of 46 Ig domains in tandem in UNC-89 is surpassed only by the 90 tandem Ig domains in the I-band region of some skeletal muscle isoforms of vertebrate titin/connectin (Labeit and Kolmerer, 1995). Given the immunolocalization of UNC-89 and the phenotype of *unc-89* mutants, it is likely that the function of these tandem Ig domains in UNC-89 is to interact with other proteins in or near the M-line, especially myosin in the shaft of the thick filament. The postulated function of the very large arrays of Ig domains in 1-band titin/connectin is different, probably providing length to the titin/connectin filament and resistance to stretching, rather than interacting with other proteins (Labeit and Kolmerer, 1995).

Large numbers of KSPs have previously been found in two of three subunits of human neurofilament proteins (NF-M has 12 copies [Myers et al., 1987]; and NF-H has 41 copies [Lees et al., 1988]). Neurofilaments comprise the major cytoskeleton in axons and consist of parallel arrays of 10-nm filaments linked to each other by cross-bridges. Antibody decoration experiments have shown that the COOH-terminal tail of NF-H forms cross-bridges (Hirokawa et al., 1984). In addition, by transfection experiments with NF-L and various deletion mutants of NF-M, it appears that the COOH-terminal tail of NF-M also forms cross-bridges (Nakagawa et al., 1995). The KSPs reside in the COOH-terminal tails of NF-M and NF-H and become phosphorylated at the serines by a neuronal cdc2-like kinase (Lew and Wang, 1995). It is hypothesized that phosphorylation of the KSPs causes the COOH-terminal tails of NF-M and NF-H to project out perpendicular to the filament core, thus forming cross-bridges (Nixon and Sihag, 1991). Interestingly, 1 KSP has been found in the first FnIII domain of myomesin (Vinkemeier et al., 1993), and 4 KSPs have been found in the COOH-terminal, M-line portion of human cardiac titin/connectin (Gautel et al., 1993). The KSPs in titin/connectin are phosphorylated by a cdc2-like kinase activity in developing, but not differentiated muscle (Gautel et al., 1993). These authors suggest that early in development, the COOH-terminal portion of titin/connectin, phosphorylated at the KSPs might be inhibited from attaching to M-line proteins. By analogy, the KSP-containing region of the UNC-89 protein might be a major binding site of the UNC-89 protein to itself or to other M-line proteins. The fact that the truncated polypeptide produced in *unc-89*(st515), which lacks all of the KSPs, can still localize to the center of A-bands (perhaps to the M-line) suggests that, for UNC-89, the KSPs are not necessary for this localization. In addition, whether the serines of the KSPs in UNC-89 are phosphorylated, and whether this phosphorylation is developmentally regulated is not yet known. It is also interesting to note that the organization of the KSPs is roughly similar in four proteins: the separation of KSPs alternates between four and primarily seven or eight residues in UNC-89, between three and five in NF-H, between two and five in NF-M, and in the pattern of four, three, and three residues separating the four KSPs in titin/connectin. These intervening sequences are very similar within one protein (e.g., in UNC-89, TKKE is the predominant sequence that separates two closely-spaced KSPs, whereas for NF-H it is predominantly EKA and for NF-M it is most frequently, VP). Presumably, this reflects how the region evolved by multi-
ple rounds of duplication. Moreover, these "intervening sequences" are very similar between UNC-89 and NF-H; in fact, there is a strong bias for K occurring as the second residue past each KSP (i.e., KSP_K) in both proteins. It is interesting to note that the consensus phosphorylation site motif for p34cdc2-cyclin is (S/T)P(K/R) (Moreno and Nurse, 1990). Thus, it is likely that in UNC-89, the KSP_K sequences serve as multiple sites for serine phosphorylation by a nematode muscle cd2-like kinase.

The most surprising domains encountered in the UNC-89 sequence are the SH3, dbl/CDC24 and PH domains, which are well-known in signal transduction molecules (Cohen et al., 1995). The SH3 domain is an ~60-amino acid residue domain first identified as a conserved sequence in the NH2 termini of src tyrosine protein kinases and subsequently found in a large number of other proteins. The functions of SH3 domains are not certain, but because many SH3-containing proteins are localized to the plasma membrane or the cytoskeleton, it suggests that SH3 domains mediate localization to these regions (Cohen et al., 1995). A number of ligands for SH3 have been identified and the binding sites all contain proline-rich sequences, especially a P-X_P motif (Musacchio et al., 1994).

Precedence for an SH3 domain in a myofilament lattice protein is provided by the giant I-band protein called nebulin, where an SH3 domain at its COOH terminus is probably partly responsible for its anchorage at the Z-disc (Wright et al., 1993; Labet and Kolmerer, 1995a).

The human oncogene dbl, the yeast cell division cycle protein CDC24, and an expanding family of growth regulatory proteins share a homologous 238-amino acid sequence, generally termed a CDC24 domain. These domains from CDC24 (Zheng et al., 1994) and dbl (Hart et al., 1991) have been shown to stimulate the exchange of GDP for GTP on Rho-like GTPases, thereby activating Rho-like GTPase activity. Activation of Rho has been shown to cause a reorganization of actin filaments via an unknown mechanism. This triggers a diverse set of cell processes including bud formation in yeast, cytokinesis in zygotes, maintenance of cell shape, formation of stress fibers and focal adhesions, cell aggregation, smooth muscle contraction, etc. (Takai et al., 1995). Similar to UNC-89, some known guanine nucleotide exchange factors (e.g., Vav and CDC25) also contain an SH3 domain (Mayer and Baltimore, 1993). The presence of a possible Rho-like stimulator as a domain of the giant UNC-89 polypeptide, suggests that Rho-like molecules might also trigger the assembly of M-lines in striated muscle.

The PH domain, first defined in the platelet protein called plekstrin, is a 100-residue sequence that has been found in many different proteins involved in intracellular signalling or the cytoskeleton (Gibson et al., 1994). PH domains from several proteins have been shown to bind to inositol phosphates (Harlan et al., 1994; Hyvonen et al., 1995). Hyvonen et al. (1995) have proposed that this binding has two functions: (a) The anchoring of some proteins to membranes would occur through the interaction of a PH domain with membrane phosphoinositides. (b) In the case of CDC24 and its homologs, PH domains reside just COOH-terminal to the dbl/CDC24 domains, as is true for UNC-89. The binding of an inositol phosphate compound, such as Ins(1,4,5)P3, to the PH domain, might regulate the nucleotide exchange activity of the neighboring CDC24 domain.

In the obliquely-striated muscle of *C. elegans*, M-lines and dense bodies are attached to the muscle cell membrane. At these positions are the locations of β-integrin (Gettner et al., 1995) in the plasma membrane, and the UNC-52 protein, which is homologous to vertebrate perlecans (Rogalski et al., 1993), in the overlying basement membrane (Francis and Waterston, 1985, 1991). Certain mutations in *unc-52* and *pat-3* (which encodes β-integrin) result in a "pat" embryonic lethal phenotype which is characteristic of mutations in at least 13 genes essential for muscle development in *C. elegans* (Williams and Waterston, 1994). Failure of these *unc-52* and *pat-3* mutants to assemble actin and myosin (although both are synthesized) into thin and thick filaments suggests that myofilament lattice assembly begins at the extracellular matrix and the cell membrane. Further support for this model comes from antibody staining of embryos at different stages of development (Hresko et al., 1994). The data on UNC-89 can be incorporated into this model. Where an M-line is formed is dictated by the accumulation of UNC-52, β-integrin, and M-line–specific basement membrane and muscle cell membrane molecules, which in some way send a signal. This signal might be an inositol phosphate compound that binds to the PH domain of UNC-89 and increases the nucleotide exchange activity of the adjacent CDC24 domain. Regardless of the nature of the signal and how it is received, the signal causes the CDC24 domain of UNC-89 to activate a Rho-like GTPase. This results in a reorganization of the actin cytoskeleton near the muscle cell membrane. Anchorage of UNC-89, and hence the M-line, at the cell membrane might be provided by the SH3 domain interacting with this reorganized actin cytoskeleton. Alternatively, this anchorage might occur through a direct interaction of the PH domain of UNC-89 with the muscle cell membrane. The M-line structure would be built by interactions of UNC-89 with itself and other, as yet uncharacterized proteins. Some examples include the 200-kD H-zone polypeptide detected with monoclonal MH9 (Waterston, 1988), and nematode homologues of vertebrate M-line proteins (creatine kinase, M-protein and myomesin).

These interactions could occur through some of the Ig domains of UNC-89, and the KSP region (by analogy to the role of the four KSPs in vertebrate titin/connexin). The long tandem array of 46 Ig domains might be designed to interact with myosin rods in the shaft of the thick filament, as is postulated for the many copies of Ig and FnIII domains in the A-band portion of vertebrate titin/connexin (Labet et al., 1992; Labet and Kolmerer, 1995b). This idea, together with the role of the KSP-containing tails of NF-M and NF-H in neurofilament cross-bridge formation, suggests an attractive topology for UNC-89. From the NH2 terminus to some position within the KSP domain, UNC-89 is part of the M-line per se, at which point UNC-89 makes a bend which permits the 46 tandem Ig domains to bind to a thick filament.

The authors wish to thank the following colleagues: R. Waterston for suggesting the project to us and generously providing the clone em204d2; T. Gibson for recognizing the SH3 domain, and his alignments of regions of UNC-89 to dbl/CDC24 and PH domains; R. Steven for pointing us towards the PH domain; S. Sammons for installing and trouble-shooting the
Staden programs on a Sun computer and generous help with sequence analysis using the GCC programs; R. Santaniemi for devising and helping us with the frozen section immunofluorescence method; S. Warren and members of his laboratory (especially H. Liener and J. Iber) for instruction and generous access to their ABI sequencing machine; J. McNinch for invaluable programming assistance in obtaining the GeneMark program parameters for C. elegans; H. Joshi, D. Moerman, and J. Heierhorst for critically reading the manuscript; R. Francis and M. Coutu Hresko for generating and then suggesting and providing the monocular MH42; D. Miller for providing the monocular antibodies to myoA and myoC G. White, J. Waddell, and M. Gilbert for suggestions on fluorescence microscopy; D. DeLa Garza for technical assistance; N. Boguslavsky for oligonucleotide synthesis; D. Maurer and N. Larsen for running some sequencing reactions on their ABI machine; and R. Waterston, B. Williams, L. Avery, H. Epstein, and D. Riddle for providing nematode strains.

This work was supported in part by the National Institutes of Health (NIH) (to G.M. Benian and M. Borodovsky). Some nematode strains were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources. G.M. Benian is an Established Investigator of the American Heart Association.

Received for publication 11 October 1995 and in revised form 8 January 1996.

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