The *Caenorhabditis elegans* Muscle-affecting Gene *unc-87* Encodes a Novel Thin Filament-associated Protein

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Abstract. Mutations in the *unc-87* gene of *Caenorhabditis elegans* affect the structure and function of bodywall muscle, resulting in variable paralysis. We cloned the *unc-87* gene by taking advantage of a transposon-induced allele of *unc-87* and the correspondence of the genetic and physical maps in *C. elegans*. A genomic clone was isolated that alleviates the mutant phenotype when introduced into *unc-87* mutants. Sequence analysis of a corresponding cDNA clone predicts a 357-amino acid, 40-kD protein that is similar to portions of the vertebrate smooth muscle proteins calponin and SM22alpha, the Drosophila muscle protein mp20, the deduced product of the *C. elegans* cDNA cm7g3, and the rat neuronal protein np25. Analysis of the genomic sequence and of various transcripts represented in a cDNA library suggest that *unc-87* mRNAs are subject to alternative splicing. Immunohistochemistry of wildtype and mutant animals with antibodies to an *unc-87* fusion protein indicates that the gene product is localized to the I-band of bodywall muscle. Studies of the UNC-87 protein in other muscle mutants suggest that the *unc-87* gene product associates with thin filaments, in a manner that does not depend on the presence of the thin filament protein tropomyosin.

THE myofibrillar lattice of striated muscle is a precisely ordered structure whose assembly and function requires the proper localization of many different proteins. In order to understand muscle assembly, the protein constituents must be identified and their interactions with each other characterized. The nematode *Caenorhabditis elegans* offers a combined genetic, molecular and cell biological approach to this problem. *C. elegans* has several types of striated muscles, the most prominent of which are the bodywall muscles. Muscle function can be assessed by the motility of animals, and the structure of the myofilament lattice can be readily evaluated using polarized light and electron microscopy and immunohistochemistry. Mutations in genes that affect muscle structure and function have been isolated in *C. elegans*, and many tools for cloning genes are available. This allows the genetic analysis of known muscle-affecting proteins, as well as the discovery of previously unknown proteins. New proteins have also been identified with monoclonal antibodies to biochemical preparations enriched in muscle components (Francis and Waterston, 1991).

The genes for many muscle proteins—the myosin heavy (Schachat et al., 1977; Waterston, 1989) and light (Cummins and Anderson, 1988) chains, actin (Waterston et al., 1984), alpha-actinin (Barstead et al., 1991), tropomyosin (H. Kagawa, personal communication), paramyosin (Waterston

et al., 1977; Kagawa et al., 1989) and the troponins TnT (E. Bucher, personal communication; Goh, 1991), TnI, and TnC (H. Kagawa, personal communication)—have been identified in *C. elegans*, and several have mutations associated with them. However, the existence of additional muscle-affecting mutants that do not correspond to the genes for these proteins indicates that not all the proteins important for normal muscle structure are known. *unc-87* is an example of such a gene.

The *unc-87* gene was identified by its paralyzed mutant phenotype in a screen for homozygous viable muscle mutants (Waterston et al., 1980). All *unc-87* alleles are fully recessive, but some are more severe than others. Homozygous animals exhibit variable degrees of paralysis (from slow moving to completely paralyzed) throughout their larval stages. This impaired motility is correlated with abnormal bodywall muscle organization: polarized light microscopy reveals greatly reduced birefringence in mutant animals, indicating severe disorganization of the myofilament lattice. Electron microscopy of mutant bodywall muscle shows patches of thick and thin filaments, instead of the normal interdigitation of the two sets of filaments. Interestingly, almost all animals move better as adults. *unc-87* mutants also have an egg-laying defect and a reduction in brood size from the wildtype ~300 (Wood et al., 1988) to ~70 (unpublished results), suggesting the *unc-87* gene is also important in egg laying muscles.

To understand the role of *unc-87* in muscle structure and function, we set out to clone the gene and localize its prod-

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uct. In this study, we provide evidence that we have cloned the *unc-87* gene, present sequence analysis of corresponding genomic and cDNA clones, investigate the role of an alternatively spliced mRNA, and describe the localization of the product in nematode muscle. Our results suggest that the gene product is similar to several thin filament-associated proteins, is localized to the I-band of bodywall muscle, and probably interacts with thin filaments. In addition, we tested the interdependence of the *unc-87* gene product and tropomyosin on their ability to associate with thin filaments.

Materials and Methods

General Methods

General techniques for DNA and RNA manipulation and characterization are as described (Sambrook et al., 1989) unless noted. Enzymes and other reagents for molecular techniques were purchased from New England Biolabs (Beverly, MA), Boehringer Mannheim Corp. (Indianapolis, IN), Perkin-Elmer Cetus Instrs. (Norwalk, CT), GIBCO BRL (Gaithersburg, MD), or Pharmacia LKB Nuclear (Gaithersburg, MD). With one exception noted below, all sequencing reactions were performed using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochem. Corp., Cleveland, OH). Gene-specific primers were made on an Applied Biosystems, Inc. (Foster City, CA), DNA synthesizer. Polymerase chain reactions were performed in a Perkin-Elmer Cetus DNA Thermocycler. Bacterial plasmids pBluescript and pBluescript II SK+ were from Stratagene Inc. (La Jolla, CA). Plasmids for routine cloning were propagated in the bacterial hosts XL-1 Blue (Stratagene Inc.) or DH5alpha. Nematode genomic DNA was purified on two successive CsCl gradients following Proteinase K/Sarkosyl treatment as described (Kondo et al., 1988). Nematode RNA was prepared as follows (D. Pilgrim, personal communication). Standard precautions were taken to make all materials free from RNAse (Sambrook et al., 1989). Mixed-stage populations of worms were washed off plates in 0.1 M NaCl, rinsed to remove bacteria and either processed immediately or frozen in liquid nitrogen for subsequent storage at −70°C. Up to 500 μl of packed worms were brought to 0.5 ml with 0.1 M NaCl, as necessary. To this mixture, 2.0 ml GUest buffer (4.0 M guanidine isothiocyanate, 50 mM Tris-Cl, pH 7.4, 10 mM EDTA, 0.1% sarkosyl, 1.0% beta-mercaptoethanol), 2.0 ml PCI (phenol/chloroform/isoamyl alcohol, 25:24:1), and 6 g of glass beads (425–600 μm, Sigma Chem. Co., St. Louis, MO) were added. Prior to use, the glass beads were washed in 55% nitric acid, rinsed with sterile water and baked. Tubes were vortexed for 4 min on high speed at room temperature. The liquid was removed and dispensed into 1.5-ml microfuge tubes. The pelleted glass beads were rinsed with 0.5 ml PCI; this was combined with the first wash. Tubes were centrifuged at high speed for 2 min in a microfuge, and the aqueous phase was removed. One-tenth volume of 3.0 M Na acetate, pH 6.0, was added to the aqueous phase; this was reextracted with PCI and precipitated with 2 vol of 100% ethanol at −20°C for 20 min. The pellet was resuspended in 200 μl of water and quantitated using a spectrophotometer. Approximately 10 μg of the nucleic acids were loaded on a formaldehyde gel for subsequent Northern analysis. No attempt was made to remove genomic DNA. The control probe used in Fig. 2 is a portion of a *C. elegans cap-1* cDNA clone and was provided by J. A. Waddle (Washington University, St. Louis, MO).

Nematode Strains and Maintenance

General methods of culture and handling were used (Brenner, 1974). The following strains were used: N2, RW7227: *unc-87(st1005)*; RW7158: *unc-87(st1005)*; RW7164: *unc-87(st1005)*; RW7165: *unc-87(st1005)*; RW7-162: *unc-87(st1005)*; RW2749: *unc-87(st443)*; RW2755: *unc-87(st444)*; RW2-759: *unc-87(st445)*; RW2763: *unc-87(st446)*; CB843: *unc-87(e843)*; CB1216: *unc-87(e1216)*; CB1458: *unc-87(e1458)*; CB1459: *unc-87(e1459)*; RW55: *unc-87(st55)*; RW253: *unc-87(st253)*; RW257: *unc-87(st257)*; RW22: *act-3(st22)*; RW15: *act-3(st15)*; RW3566: *lev-11(st557)*.

Isolation and Sequencing of the unc-87 Genomic Clone

A 6-kb *BglII* fragment from K01B11 was subcloned into the *BamHI* polylinker site of pBluescript to generate pSG2. Plasmid pSG7 was made by

filling in the ends of the 6-kb *NcoI* fragment from K01B11 and ligating it to pBS that had been cut with *XbaI* and then filled in to generate blunt ends. The *NcoI* and the *XbaI* sites were destroyed in the construction of this clone. Clone pSG8 was constructed by joining pSG2 and pSG7 at their common internal *ScaI* sites. With one exception (noted below), the sequence of the region of pSG8 encompassed by the cDNA was determined by using insert-specific primers or by using vector primers to sequence subcloned restriction fragments or subclones representing nested deletions of larger fragments. Nested deletions were generated as described (Ausubel et al., 1987a) with minor modifications. The *ClaI* fragment (positions 5003–5771, Fig. 3) that contains the internal 750 basepairs of an inverted repeat was subcloned into pBluescript SK II (+) to generate plasmid pSG12. Its sequence was determined using the TN1000 system (Gold Biotechnology, St. Louis, MO), except that random insertions of a bacterial transposon were isolated directly in pSG12. Clones with transposons in the insert DNA were identified and sequenced using primers specific for each end of the transposon. Sequencing reactions were performed using the Sequenase kit and, in one instance, the cycle sequencing protocol (Craxton, 1991). The inverted repeat was oriented relative to the rest of the genomic clone using the polymerase chain reaction. A primer complementary to the unique central portion of the inverted repeat was tested for its ability to generate a product from the genomic clone pSG2 with each of two primers that flank the inverted repeat. An amplification product was detected only with the downstream primer, thus determining the orientation of the unique region shown in Fig. 3. The sequence of the genomic clone was determined for both strands, except in some regions in which it corresponded to the cDNA clone.

cDNA Cloning and Sequencing

Insert DNA from clone pSG1 was isolated, radiolabeled using the Prime-it Kit (Stratagene Inc.) and used to screen 1 × 10^6 pfus of a Lambda ZAP (Stratagene Inc.) cDNA library (courtesy of R. Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK) plated on the bacterial host BB4. 259 positive plaques were identified. Automatic excision (Stratagene Inc.) of the insert from one of the positive plaques generated the plasmid pSG3, which contains the cDNA insert at the *EcoRI* site of pBluescript. Initial DNA sequence was obtained using the vector primers T3 and T7; all other sequencing primers were designed based on derived sequence. The sequence of the cDNA clone was determined for both strands.

DNA Sequence Analysis

Sequence assembly was done using the XDAP program (Dear and Staden, 1991) on a Sun Workstation. Analysis of open reading frames and intron/exon boundaries was performed using GENEFINDER (L. Hillier and P. Green, unpublished observation). Similarities to other proteins were determined with a BLASTP (Altschul et al., 1990) search of the nonredundant protein database (on June 24, 1993) at the NCBI. A score of 70 or greater was considered significant. DNA similarities were determined using a BLASTN (Altschul et al., 1990) search of Genbank release 77.0. A score of 200 or greater was considered significant. Protein and DNA sequences were retrieved from the Entrez database (National Center for Biotechnology Information) for subsequent analysis. DNAstar (DNAstar, Madison, WI) was used to format sequences for subsequent alignment with Megalign (DNAstar).

Transformation Rescue

DNA for transformation rescue of *C. elegans* was prepared by alkaline lysis, polyethylene glycol precipitation and a final phenol/chloroform extraction. Clones pSG2, pSG7, and pSG8 were each coinjected with plasmid pRF4, which carries the dominant allele *rol-6(su1006)* (Mello et al., 1991), at a 1:10 ratio of test plasmid to pRF4. The total DNA concentration was 200–250 ng/μl. Microinjection of DNA into the gonads of young adult hermaphrodites was performed as described (Fire, 1986), with minor modifications. Needles for microinjection were pulled on a Model P-80C Brown-Flaming Micropipette Puller, and injections were done using a Narashige IM-200 Microinjector to control delivery of the DNA solution, a Zeiss IM-35 inverted microscope and a Leitz Micromanipulator.

Polymerase Chain Reactions

Polymerase chain reactions were performed using standard reaction conditions (Saiki et al., 1988). 0.25 ng of *C. elegans* N2 genomic DNA, or one

microliter of the cDNA library described above, were used as template for the reactions shown in Fig. 6. Thirty cycles (30 s at 94°C, 30 s at either 58°C for reactions with the primer to exon A or 56°C for reactions with the forward primer to exon B, 3 min at 72°C) were performed for each reaction.

In Vitro Mutagenesis

Two termination codons were introduced using in vitro mutagenesis (Kunkel, 1985) into the clone pSG8 following each of the two translation start sites predicted by GENEFINDER. The SpeI–BamHI fragment (positions 1784–3727) from pSG2 was subcloned into pBS SK II (+) to generate the clone pSG10, which was used for production of single-stranded DNA. VCSM13 helper phage was from Stratagene Inc. The two mutagenic primers were 5'GCAGCAGAAGCTTATCACGTCATTTCTGGAT3' and 5'GTT-GTGTTGTTGAATCATTACATTCTTTAAACCAA3' (positions 2818–2788, and 2386–2351, respectively; see Fig. 3). The altered nucleotides are underlined. A plasmid with the desired changes was identified by sequencing and named pSG10term. The SpeI–BamHI fragment was reintroduced into pSG2 to generate pSG2term, and this clone was spliced to pSG7 at the ScaI site to generate pSG8term. The sequence containing these changes was analyzed using the GENEFINDER program, and no novel splicing patterns were predicted.

Production and Purification of Fusion Proteins

The portion of pSG3 containing the COOH-terminal 241 codons, the 3' untranslated region, and the polylinker region up to the EcoRI site of pBluescript was ligated in frame to the glutathione S-transferase coding region in the expression vector pGEX-3X (Smith and Johnson, 1988) to form pSG5 as follows: pSG3 was linearized with BamHI, partially filled in with dGTP and dATP, and treated with mung bean nuclease to generate blunt ends. This DNA was then cut with EcoRI, and the insert fragment was ligated to pGEX-3X that had been cut with EcoRI and SmaI. This GST–unc-87 fusion was expressed in the protease-deficient strain UT5600 (from J. Cooper, Washington University, St. Louis, MO) for large scale preparations as described (Ausubel et al., 1987b).

For affinity purification of antibodies to UNC-87 epitopes, a fusion of unc-87 coding sequence to the trpE coding sequence was made in the vector pATH3 (Koerner et al., 1991). An oligonucleotide primer corresponding to positions 3267–3288 in Fig. 3 was used in the PCR with the primer 5'TAGAGAAACCTAGCAGAGATTTCGGGCGGATTACTCGCCCG-AAAAAAAATTCGAAGGG 3'. This second primer contains the last 21 nucleotides of the unc-87 coding region, the trpA transcription terminator (Christie et al., 1981), and a HindIII recognition site. The PCR product was digested with BamHI and HindIII, gel purified and ligated to BamHI/HindIII-digested pATH3 to generate clone pSG6. This fusion protein contains the same portion of UNC-87 as the GST–UNC-87 fusion. Inductions of fusion protein were carried out in the strain RR1 (Koerner et al., 1991). Protein preparations were performed as described (Koerner et al., 1991).

Antibody Production, Affinity Purification, and Immunohistochemistry

UNC-87-GST fusion protein was purified on glutathione-S-agarose (Sigma Chem. Co.), subjected to SDS-PAGE, visualized using negative staining with 4 M sodium acetate and excised from the gel with a razor blade. The gel slice was passed through successively smaller hypodermic needles, brought to 1 ml with phosphate-buffered saline (per liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4, pH 7.2) and emulsified with adjuvant by sonication. Freund's complete adjuvant was used for the initial immunization; Freund's incomplete adjuvant was used for subsequent boosts. Approximately 150 μg of fusion protein were injected at a total of four sites into two New Zealand White rabbits (obtained from Boswell Bunny Farm, Pacific, MO). The first boost was performed one month following the initial immunization. Subsequent boosts were given at least one month apart. Bleeds were performed 10 to 14 d following immunizations. Following collection, blood was incubated at 37°C for 1 h and at 4°C overnight to encourage clotting. Serum was collected, cleared by centrifugation, brought to 0.02% sodium azide and stored at −20°C.

For affinity purification of antibodies to UNC-87 epitopes, inclusion bodies containing the trpE–UNC-87 fusion were subjected to SDS-PAGE and blotted to Immobilon P (Millipore Corp., Bedford, MA) using a Genie blotting apparatus (Idea Scientific Company, Minneapolis, MN) for 1.5 h at 12 volts with a battery charger (MC-1; Schumacher, Chicago, IL). The

fusion protein was visualized by staining the blot with Ponceau S (Sigma Chem. Co.), and the corresponding strip was excised with a razor blade. Protein was fixed to the filter in 25% isopropanol, 7% acetic acid for 2 min and then mock eluted for 3 min with 100 mM glycine, pH 2.5, to remove loosely bound antigen. The strip containing the fusion protein was incubated overnight at 4°C in TBS-T (Tris-buffered saline-0.5% Tween-20; per liter: 8 g NaCl, 0.2 g KCl, 3 g Tris base, pH adjusted to 8.0 with HCl, 5 ml Tween-20), 10% normal goat serum (GIBCO BRL), 1% bovine serum albumin (Stratagene Inc.), 0.2% sodium azide and 1% total immune serum. The membrane was washed five times for 5 min each in TBS-T to remove nonspecifically bound antibodies. Specifically bound antibodies were eluted in 250 μl 100 mM glycine, pH 2.5, for 3 min at room temperature. The eluant was neutralized with 37 μl 1 M Tris-Cl, pH 8, 0.1 N NaOH, and supplemented with 10 μl 30% bovine serum albumin, 32 μl normal goat serum, 10 μl 10% sodium azide and stored at 4°C for up to several days. Following elution of antibody, the strip was neutralized in TBS-0.5% Tween-20 and stored in a seal-a-meal bag at −20°C. The strip has been used successfully over 15 times.

Western Blot Analysis of Wildtype and unc-87 Mutants

To prepare total SDS-soluble nematode protein, worms were washed off NGM (nematode growth media) agar plates with M9 buffer (Brenner, 1974), centrifuged to remove bacteria and resuspended in an equal volume of 50 mM ethanolamine, 5 mM dithiothreitol, 2 mM EDTA, 1 mM PMSF (Moerman et al., 1988), containing 10 μl of protease inhibitors (10 mg/ml leupeptin, 1 mg/ml pepstatin in methanol, 1 mg/ml N-alpha-p-Tosyl-L-Arginine Methyl Ester [TAME]). Tubes were microwaved on the highest setting for 25 s and placed immediately in a boiling water bath. One-third volume boiling 4× Laemmli sample buffer containing proteinase inhibitors was added, and the sample was boiled for 5–10 min. The sample was passed through a 26-gauge needle to shear the DNA and centrifuged for 1 min at high speed in a microcentrifuge to pellet insoluble components. The denatured, SDS-soluble components were transferred to a fresh tube and either frozen at −70°C or used immediately. Protein samples were electrophoresed through an 8% gel and blotted to Immobilon P as above. A parallel gel was stained with Coomassie Blue to ensure comparable loading of the samples. The blot was stained with Ponceau S, and the positions of the molecular weight standards (Promega Corp., Madison, WI) were marked on the blot with a soft lead pencil. Ponceau S was removed by washing with TBS-T. The blot was blocked for 10 min at room temperature in TBS-T containing 10% normal goat serum and 2% BSA (Sigma Chem. Co.). Affinity-purified antibody (see above) was added at a 1:100 dilution and incubated for 1 h at room temperature with shaking. The filter was washed four times, 5 min each wash, with TBS-T and then blocked again as above. The secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit Ig (TAGO), was added at a dilution of 1:2,500 and incubated and washed as above. The filter was rinsed once in prewarmed (37°C) alkaline phosphatase substrate buffer (APSB, 12.1 g Tris base, 4.84 ml diethanolamine, 20 ml 5 M NaCl, 4 ml 1 M MgCl2, 4 μl 0.5 M ZnCl2 per liter, 0.02% sodium azide, pH 9.55). 30 ml fresh APSB containing the color development reagents (all from Sigma Chem. Co.) phenazine methosulfate (200 μl at 2 mg/ml), 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 100 μl at 41 mg/ml) and Nitro Blue Tetrazolium (NBT, 100 μl at 99 mg/ml in ethanol) was added, and the alkaline phosphatase reaction was allowed to proceed at room temperature until the desired intensity of bands was reached. Reactions were stopped by removal of APSB and addition of 25 mM Tris, pH 2.9.

Immunohistochemical Techniques and Phalloidin Staining

Fluorescently labeled secondary antibodies were from Chemicon and used at a dilution of 1:100. Specimens were mounted in 90% glycerol, 20 mM Tris-Cl, pH 8.0, and 0.2 M 1,4-diazabicyclo[2.2.2]octane (DABCO, from Sigma Chem. Co.). Photographs were taken using Kodak TMAX 400 film. Adult worms were prepared and stained as described (Francis and Waterston, 1991). Affinity purified antibodies were used at a 1:5 dilution. To insure that the affinity-purified antibodies were generated in response to immunization with the unc-87-GST fusion protein, preimmune serum was subjected to the affinity-purified protocol, and the resulting eluant was used to stain wildtype worms. No specific staining was observed. The anti-actin antibody, C4 (Chemicon Intl. Inc., Temecula, CA), and MH44 (Francis and Waterston, 1991) ascites were used at 1:100. Anti-tropomyosin antibodies, a gift from L. Schriefer and M. Hresko, were used at 1:5. Phalloidin (Mo-

lecular Probes) staining of nematodes was performed as described (Waterston et al., 1984).

Antibody Staining of Embryos

Embryos were stained as described (Barstead and Waterston, 1991). Briefly, an alkaline hypochlorite treatment of gravid adults and laid eggs caused release of the eggs from the parents and permeabilization of the eggshell. Embryos were fixed in 3% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 30 min at room temperature and then with −20°C methanol for 10 min. In some cases, embryos were stored in methanol at −20°C for up to 3 wk before proceeding with antibody staining.

Results

Cloning and Sequencing of unc-87 and an unc-87 cDNA

The unc-87 allele st1005 was isolated in a strain with a high frequency of transposition of the transposable element Tc1 (T. Sanford and D. Moerman, personal communication). The st1005 mutation readily reverts to wildtype, suggesting that this mutation is caused by insertion of a transposable element into the unc-87 gene. With an appropriate probe, such an insertion should be detectable as a restriction fragment length polymorphism on genomic Southern blots containing DNA from st1005 and a revertant. Comparison of st1005 with coisogenic revertants on Southern blots using Tc1 as a probe showed no Tc1 elements associated with the st1005 allele (J. Kiff, personal communication). For the present study, we chose instead an approach that did not make any assumptions about the nature of the inserted element.

Genetically, unc-87 maps to linkage group I, 0.05 map units to the left of the dpy-14 gene. In this region of the genome, 0.05 map units corresponds to a physical distance of ~50 kbp (Starr et al., 1989). Cosmid clones known to contain DNA present in the region of the chromosome to the left of the dpy-14 gene (Coulson et al., 1991) were selected for hybridization to a Southern blot containing genomic DNA prepared from the original mutant, st1005 and a coisogenic revertant (Fig. 1). The probe K01B11 detects an 8.5-kb BglII band in the mutant and a 6.0-kb BglII band in the revertant; all other mutant and revertant bands comigrate. Genomic DNA from two additional independent revertants was tested; both contained the 6.0-kb BglII band (data not shown.) These results are consistent with a 2.5-kb insertion into the wildtype gene causing the unc-87(st1005) phenotype. Because most Tc1 elements are 1.6 kb, the st1005 mutation is probably not due to the insertion of a typical Tc1 element, consistent with earlier findings (J. Kiff and R. Waterston, personal communication). The 2.5-kb transposable element Tc3 (Collins et al., 1989) does not hybridize to the polymorphism (data not shown). Thus, the element responsible for the polymorphism remains unidentified.

The wildtype 6-kb BglII fragment was subcloned from K01B11 to create pSG2. To determine whether the BglII fragment contained coding sequence, total RNA prepared from mixed-stage populations of st1005, revertant and wildtype animals was subjected to Northern analysis and hybridization to pSG2. The genomic clone detected a 1.3-kb transcript in RNA derived from wildtype and the revertant animals, but failed to hybridize to RNA prepared for st1005 (Fig. 2). A control hybridization with an unrelated probe, a portion of a C. elegans cap-1 cDNA, indicated that intact st1005 RNA
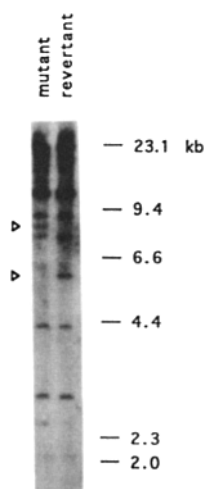

Figure 1. Southern blot of BglII-digested genomic DNA prepared from unc-87-(st1005) and a coisogenic revertant. The blot was hybridized with the radiolabeled cosmid clone K01B11. The polymorphic fragments are indicated at left with arrowheads. Positions of molecular size standards are at right.

was present on the blot. These results suggest that the 1.3-kb RNA represents a transcript from the unc-87 gene; the inability to detect this transcript in st1005 RNA also suggests that st1005 is a severe reduction of function allele of unc-87. In turn, the genomic clone pSG2 was used to screen a cDNA library, and a 1.3-kb cDNA was identified that gave the same hybridization pattern on Northern blots as the genomic clone.

The cDNA insert was sequenced in its entirety. A poly(A) tail at one end of the cDNA clone and nine nucleotides of the trans-spliced leader sequence SL1 (Krause and Hirsh, 1987) present at the other end indicate that the cDNA is full length. The longest open reading frame contained in the cDNA clone is indicated in boldface in Fig. 3. There are two potential translation initiation codons, the second of which most closely fits the C. elegans consensus and initiates a 357-amino acid, 40-kD polypeptide. A diagonal plot comparison of this polypeptide to itself revealed the presence of seven similar, evenly spaced, 23-amino acid stretches. As shown in Fig. 4 a, the repeats are 39 to 87% identical to one another. The number of amino acids between successive repeats is indicated to the right of each repeat; the 9-amino acid stretches following the third and fourth repeats are identical.

Comparison of the entire UNC-87 protein sequence to the nonredundant protein database revealed significant (see Ma-
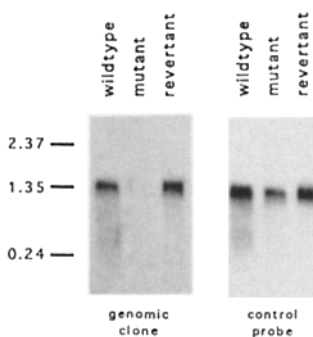
Figure 2. Northern blot containing total RNA prepared from wildtype, unc-87-(st1005) and a revertant. (a) The blot was hybridized with the radiolabeled genomic clone pSG2, which contains the 6-kb BglII fragment from cosmid K01B11. A band is present at 1.3 kb in the wildtype and revertant, but not in the mutant. (b) The same blot, stripped and hybridized with an unrelated probe, a portion of a C. elegans cap-1 cDNA clone, to show that intact RNA was prepared from unc-87(st1005). Positions of molecular size standards are indicated at left.

terials and Methods) similarity to the vertebrate smooth muscle proteins calponin (Vancompernolle et al., 1990; Takahashi and Nadal-Ginard, 1991) and SM22 (Pearlstone et al., 1987; Nishida et al., 1991), the rat neuronal protein (GenBank Accession number M84725), and the predicted polypeptide encoded by the *C. elegans* cDNA cm7g3 (Waterston et al., 1992). The cm7g3 clone does not derive from the *unc-87* gene: it is distinct in sequence and has been positioned on chromosome V (C. Huynh and R. Waterston, personal communication). Weak similarity (see Materials and Methods) also exists to the Drosophila muscle protein mp20 (Ayme-Southgate et al., 1989). Except for the cm7g3 polypeptide, the overall identity of UNC-87 to these proteins ranges from 21-34%; the identities to the repeated regions in UNC-87 range from 39-70%. The overall percent identity to the cm7g3 polypeptide is 60%, reflecting the fact that most of its primary structure consists of the sequence repeats. An alignment of a consensus (Fig. 4 *a*) of the UNC-87 repeats to the similar regions from the other proteins and the corresponding pairwise identities is presented in Fig. 4 *b*.

To determine the organization of the *unc-87* gene, the region of pSG2 containing the exons present in the cDNA clone was sequenced. The exon containing the first 38 nucleotides of the cDNA was not present in pSG2, so an overlapping clone, pSG7, was subcloned from K01B11 and sequenced in the direction opposite existing *unc-87* sequence (Fig. 3). The new sequence contains the potential inhibitor methionine residue and 5' untranslated sequences in the cDNA. The genomic sequence encompassing all of the exons present in the cDNA was further analyzed using the GENEFINDER program. GENEFINDER predicts open reading frames and intron donor and acceptor splice sites, using available information on nematode codon bias and splice site consensus sequences. All the splice sites determined by alignment of the cDNA and genomic sequences were also predicted by GENEFINDER. In addition, GENEFINDER predicted a 605-bp exon that is not contained in the cDNA (see Figs. 3 and 6). This exon (referred to as exon B) is predicted to splice in frame to the second exon present in the cDNA. The encoded amino acids are boxed in Fig. 3, and the two potential translation start sites are each indicated by a #. Comparison of this exon to the non-redundant database did not reveal any significant similarities.

To determine if exon B is transcribed in vivo, along with other sequences present in the original cDNA clone, we performed the polymerase chain reaction on a nematode cDNA library, using various exon-specific primers. Amplification products were obtained using a forward primer specific to exon B in combination with primers from each of the downstream exons (Fig. 6, C, D, E, F, and G). In contrast, no products amplified with a forward primer from the upstream exon (Fig. 6, A) and a reverse primer in exon B (see Fig. 5). As expected from the structure of the original cDNA clone, we were able to amplify products using primers specific to exon A and exons C, D, E, F, and G. The sizes of the PCR products are consistent with the splicing pattern predicted by GENEFINDER and exclude the possibility that genomic DNA was the starting material for the amplified product. Exon B has a 5' splice acceptor site; it may be *trans*-spliced to a leader sequence or spliced to an exon farther upstream.

In addition to the coding sequence, the *unc-87* gene contains an 877-bp element present in the final intron (see Fig.

3). It consists of inverted repeats separated by a 112-bp segment. The two copies of the repeat, 382 and 383 base pairs, respectively, are identical, with the exception of an additional nucleotide in the left portion of the inverted repeat. Inverted repeats are common in the *C. elegans* genome, with an estimated frequency of one every six kilobases. These sequences most frequently reside in introns and intergenic regions (R. Durbin, personal communication). Similarities exist among some of these elements, and the inverted repeat reported here is 84% similar to a 60-bp region in the *C. elegans* cosmid clone ZK370 (Sulston et al., 1992).

Rescue of the unc-87 Phenotype by Exogenous Copies of unc-87

In order to determine if the sequence that was covered by the cDNA was able to rescue *unc-87* mutants in transformation experiments (Fire, 1986), we tested it along with clones pSG7 and pSG2 by microinjection into *unc-87* mutants. A diagram of these clones and the results of the rescue experiments are shown in Fig. 6. As indicated, pSG7 and pSG2 overlap, and each contains only part of the sequence contained in the cDNA; pSG8 contains all of this sequence. Each clone was coinjected into *unc-87*(e843) hermaphrodites along with pRF4, a plasmid that encodes the dominant *sul006* allele of the *rol-6* gene (Mello et al., 1991). When expressed, pRF4 DNA causes the worms to roll, thus providing a visual marker for transformed animals. Rolling animals whose parents had been injected with the clones pSG7 or pSG2 rolled poorly and did not show improved organization of bodywall muscle. In contrast, rolling animals whose parent had also been injected with pSG8 rolled vigorously. These transformants had organized bodywall muscle, as observed by polarized light microscopy, and had near wildtype brood sizes. These results show that pSG8 has all the sequences necessary for function of the *unc-87* gene in bodywall muscle.

Our results thus far suggested that the *unc-87* gene encodes two alternative mRNAs. In order to address which of the two transcripts was providing the rescuing activity, we performed the rescue experiments with a clone that would eliminate production of protein from transcripts containing exon B. We used in vitro mutagenesis to introduce two translation termination codons immediately following each of the two predicted translation start sites in exon B (Fig. 3). The resulting clone, pSG8term, was tested for its ability to rescue the *unc-87* mutant phenotype. Transformed progeny from hermaphrodites injected with pRF4 and pSG8term rolled vigorously, had wildtype bodywall muscle structure and had an average brood size of 187. We were unable to find any difference between worms rescued by pSG8 and pSG8term. These results suggest that translation of exon B is not necessary to correct the bodywall muscle defect of the *unc-87(e843)* animals.

Preparation and Characterization of Antibodies to UNC-87

To determine the subcellular location of the *unc-87* gene product, a polyclonal antibody was raised against a bacterially expressed fusion protein containing the COOH-terminal 241 amino acids of UNC-87 fixed to the carrier protein glutathione S-transferase. Antibodies were affinity-purified

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caactctcgccctcctgactatatacatcagaaaaaaaaactattgaaaaggttttccctcgttgttttatattgcattgtctacgtttattacatgaacatttctattcgttttttttc      120
                                                      *      #
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                                                          M  P  P  T  E  D  Q  V  V  A  E

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tacaatgaaatgataattttttaaactggttcaatccagtgagcatttaccaatctacaaaagcctcgaagcattatgcattctaaaatttacctttttaaagaatttttatttcgatacga   960

aatttgaaaataaatataatctatagtaattcttctctctaattcccttgttcgatgctcccactaatcttttcgggtatggtgctgcgacctctgtagtaaccgaaaacttaaaccgaa   1080

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                                              #·   +  .+
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                                                                                   M  L  S  F  N  N  T  T  S  A  S  S  F

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E  A  L  E  R  L  R  P  N  T  A  S  R  E  R  N  I  P  I  Q  F  T  G  K  N  P  T  T  N  S  A  L  E  E  Y  K  P  V  P  H

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V  Q  V  T  S  P  K  S  S  I  V  P  N  F  V  S  E  Q  R  G  L  Q  P  Q  P  T  S  Q  A  P  T  N  Y  R  Q  F  V  A  A  P
               #·   +  +
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GGATTCGGATCCGGACGTGATGTGTGCCGTGAGGGAGTCCGTGTCGCTCAGAACCCAGCTGACTTGGCTgtaagttacttttacatagtagctctgtaacctttcatcacttcagtaaaa   3840
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Figure 3. Sequence of the *unc-87* gene and predicted amino acid sequence. Introns and untranscribed sequences are shown in lowercase; exons are shown in uppercase. Exons present in the cDNA are shown in boldface. Amino acids predicted by the alternatively spliced product are boxed. The inverted repeats in the final intron are underscored. The first nucleotide in the cDNA which is downstream of the *trans*-spliced leader SL1 (omitted because it is not colinear) is indicated by an asterisk (*) above the sequence. The first and last nucleotide positions of exon B (see text) are indicated by a caret (^) below the sequence. Potential initiator methionines, as predicted by GENEFINDER, are indicated by a #. The nucleotides changed by in vitro mutagenesis to create the plasmid pSG8term are marked with a + above the sequence. The sequence data for the cDNA and genomic clones are available from GenBank under the accession numbers U04711 and U04712, respectively.

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P S I S W Q N R K S Q G M V R L Q S G T N R F A S Q A   277

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A G W N K G D S Q K   322

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                                                                               K M T S F G A   328

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aatttaacaaaaaattaaaaaattagttcgtgttcaggg 6639

Figure 3.
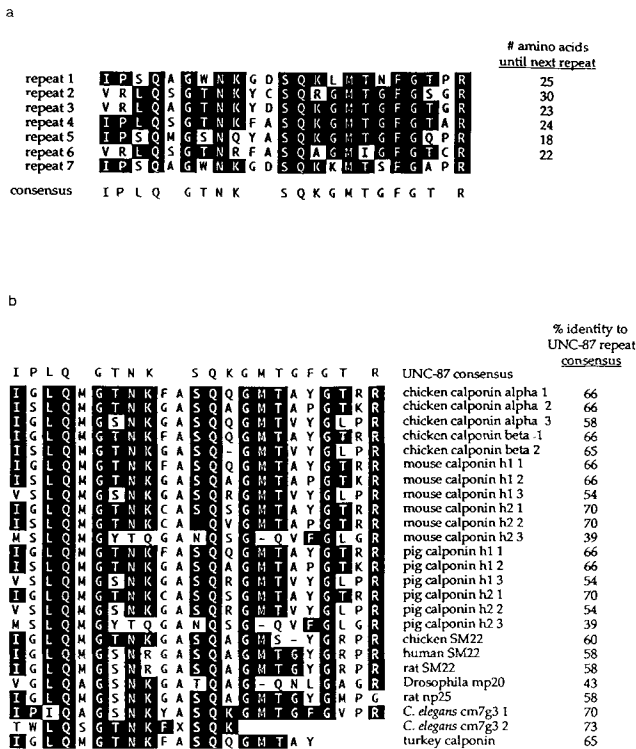
Figure 4. Alignments of the sequence repeats in UNC-87 and related proteins. (*a*) The seven sequence repeats in UNC-87 are aligned. Amino acid residues are included in the consensus if they are present in four or more of the repeats. The number of amino acids separating a repeat from the next one is shown at the right.

from immune serum by adsorption to a filter containing the UNC-87–derived peptide fused to the trpE protein. To determine the specificity of the affinity-purified antibody, Western blot analysis was performed on total SDS-soluble nematode protein prepared from wildtype and *unc-87* mutant worms (Fig. 7). Because both fusion proteins were derived from exons common to the alternatively spliced products, the antibodies were expected to recognize both isoforms. The principal reactive band detected in wildtype extracts is 42 kD; a weaker band at 65 kD and other minor bands are also detected. The size of the major isoform is consistent with that predicted by the *unc-87* cDNA clones. The size of the larger isoform conforms with that predicted if the translation begins in exon B.

Specific alterations in the 42- and 65-kD bands in several of the mutants confirmed that both are products of the *unc-87* gene. Both proteins are absent in strains carrying the *unc-87* alleles *e843*, *e1469*, *st55*, and *st257*, while the 42-kD protein, but not the 65-kD band, is detectable in *unc-87(e1216)* and *unc-87(e1458)*. *unc-87(st253)* is the only allele in which both UNC-87 isoforms are detectable. Fig. 7, lane 2 contains protein prepared from the *st1005* strain originally used for RNA isolation (see above). In this strain, both proteins are

(*b*) The consensus derived in *a* above aligned with the similar regions of other proteins. The numbers following each repeat name refer to the order of the repeat in the protein. For pig and mouse calponin, two genes exist and are designated h1 and h2 in both cases. The percent identity of these regions and the UNC-87 consensus is shown to the right.
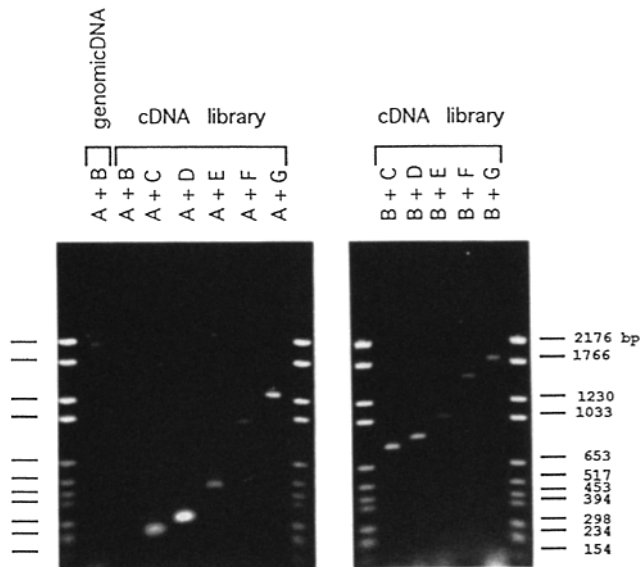
Figure 5. Polymerase chain reaction amplification products from a *C. elegans* cDNA library and *C. elegans* genomic DNA using exon-specific primers. The exons (see Fig. 6) represented by the primers used and the substrate for amplification are shown above the lanes. Positions of molecular weight standards are shown to the left. Amplification products were electrophoresed through a 1.2% agarose gel and visualized by staining with ethidium bromide.

smaller. However, no hybridization between RNA from this strain and an *unc-87* cDNA was detected on Northern blots. It is possible that mRNA corresponding to the *unc-87* gene is present in this strain, but not detectable under the conditions we used. If this is the case, the transposable element apparently excises from the mRNA (Rushforth, 1993). Alternatively, the element has excised from the genome during maintenance of the strain after the Southern blot was performed. In either case, the alteration affects both proteins detected here, suggesting both are products of the *unc-87* gene. The minor bands may represent cross reactive antigens or could result from nonspecific antibodies. These minor bands are presumed to be products of other genes because they are unaltered on the mutants.

Localization in Bodywall Muscle

C. elegans bodywall muscle cells are located in four strips along the longitudinal axis of the body, immediately below the hypodermal cells, which in turn are adjacent to the outer
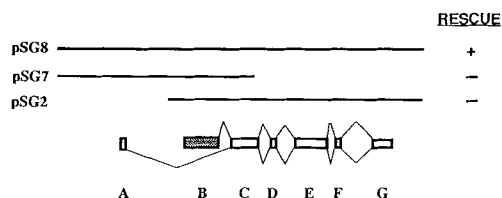
Figure 6. Summary of transformation rescue results and exon map of the *unc-87* gene. The genomic clones tested in transformation rescue experiments are shown at the left (pSG2, pSG7, and pSG8). The ability of each clone to rescue the *unc-87(e843)* phenotype is indicated to the right by the symbols + (rescue) and − (no rescue). The positions of the exons present in the cDNA (*open boxes*) and exon B (*shaded box*, see text) are shown at the bottom.
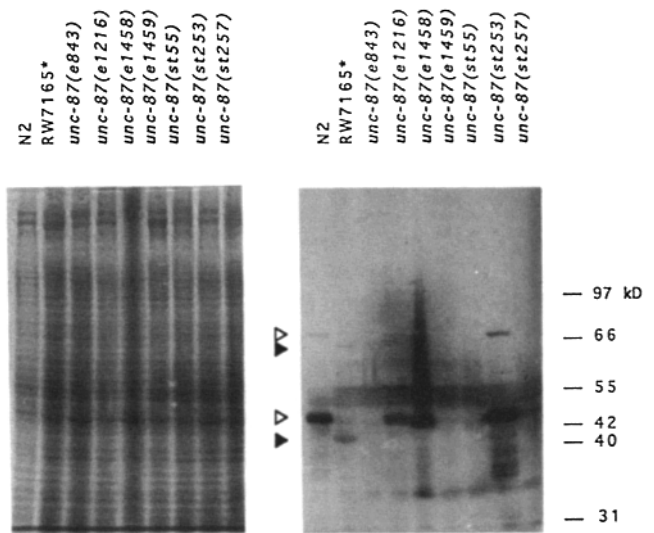
Figure 7. Western blot analysis of total nematode SDS-soluble protein prepared from wildtype and *unc-87* mutant animals using affinity-purified antibodies to an UNC-87-trpE fusion protein. (*a*) Parallel gel stained with Coomassie Blue to show that roughly comparable levels of protein were loaded in each lane. (*b*) Western analysis of a similar gel, blotted and reacted with anti-UNC-87 antibodies. The *unc-87* allele used is indicated above each lane, and N2 was used as the wildtype strain. See Results for explanation of strain RW7165*. Positions and molecular mass of standards are indicated to the right.

cuticle, or skin, of the worm. The myosin-containing thick filaments and the actin-containing thin filaments are located in the region of the muscle cells closest to the hypodermis. They run parallel to the long axis of the worm and slide past each other during muscle contraction. The force generated during contraction causes the worm to move because these filaments are anchored in membrane-bound structures that are linked to the outer cuticle via the hypodermis. The thin filaments are anchored in dense bodies, structures that are functionally equivalent to the Z-disc of vertebrate cross-striated muscle. The thick filaments are anchored in a structure analogous to the M-line of cross-striated muscle. In vertebrate cross-striated muscle, laterally adjacent sarcomeres are in register. In contrast, adjacent sarcomeres are offset longitudinally with respect to each other in the obliquely-striated muscle of *C. elegans*. As in cross-striated muscle, the regular arrangement of the contractile filaments in bodywall muscle of *C. elegans* provides easy localization of component proteins using immunohistochemical techniques.

Immunohistochemical staining of wildtype adult bodywall muscle with affinity-purified anti-UNC-87 antibodies revealed a striated pattern (Fig. 8 *a*). The striations are interrupted periodically along their length by unstained regions. Double labeling experiments showed that the anti-UNC-87 staining pattern corresponded with that of the monoclonal antibody MH44 (Fig. 8 *b*), which recognizes polypeptides of 400 and 440 kD (Francis and Waterston, 1991). This pattern has been previously shown to represent the I-band, the region of the sarcomere containing the thin filaments where they do not overlap with thick filaments. At the center of the I-band are the dense bodies; these correspond to the unstained regions within the striations.
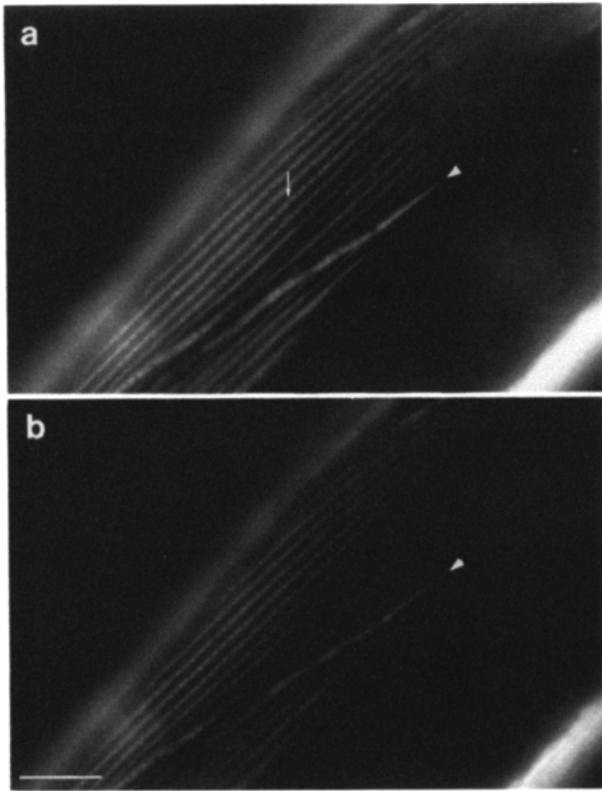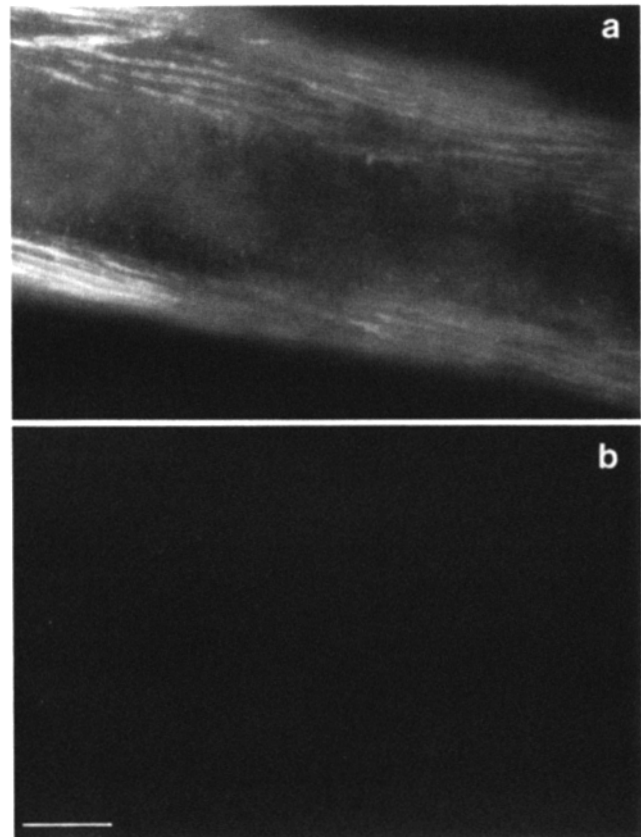
86

Figure 8. Fluorescence micrographs of wildtype bodywall muscle double-labeled with MH44 and antibodies to UNC-87. (*a*) Body-wall muscle fragment reacted with affinity-purified antibodies to an *unc-87* fusion protein. (*b*) The same field as in *a*, stained with the monoclonal antibody MH44. The staining patterns of the antibodies are identical. The gaps in the linear pattern (*arrow*) correspond to the dense bodies. Arrowheads indicate the longitudinal muscle cell boundaries. Bar, 10 μm.

Figure 9. Fluorescence micrographs of *unc-87(e843)* bodywall muscle double-labeled with MH44 and antibodies to UNC-87. (*a*) Bodywall muscle fragment reacted with MH44. (*b*) The same field as in *a*, stained with anti–UNC-87 antibodies. The pattern seen with MH44 is irregular, reflecting the disorganization of the bodywall muscle. The staining with UNC-87 antibodies is greatly reduced; where it is visible, it is coincident with the MH44 staining. Bar, 10 μm.

Bodywall muscle from *unc-87(e843)* was stained with anti–UNC-87 antibodies and the monoclonal antibody MH44 (Fig. 9). Staining was greatly reduced with the anti–UNC-87 antibodies, consistent with the Western blot results. *unc-87(e843)* animals reacted with MH44, although the pattern was abnormal, reflecting the disorganization of the mutant muscle. Together, these results suggest that the antibodies recognize the *unc-87* gene product, that the protein is a component of the myofilament lattice and that it is located in the same region as the actin-containing thin filaments.

Localization in Other Muscles

In addition to the bodywall muscles, *C. elegans* also possesses a muscular pharynx for feeding, an anal depressor and sphincter for defecation, intestinal muscles, and, in the case of the hermaphrodite, vulval and uterine muscles. Examination of wildtype animals indicated that antibodies to UNC-87 react with all of these muscles (data not shown). In all cases, UNC-87 was contained within the pattern observed with phalloidin or anti-actin antibodies, suggesting that, as in bodywall muscle, the antigen recognized in these tissues colocalizes with thin filaments.

It is interesting that both wildtype bodywall and pharyn-

geal muscles react with antibodies to UNC-87, yet only the bodywall, and not the pharyngeal, muscles appear affected in *unc-87* mutants examined by polarized light microscopy. Likewise, the pharynges of *unc-87* mutants pump as well as those of wildtype. There are several possible explanations for this observation. One is that the pharyngeal antigen is the product of the *unc-87* gene, but that its absence in mutant pharynges does not have an adverse effect. Alternatively, the *unc-87* mutations might not affect expression of the gene product in the pharynx. A third possibility is that the pharyngeal antigen is the product of another gene. To determine if the pharyngeal staining was affected by mutations in the *unc-87* gene, we examined pharynges from two different *unc-87* alleles, *e843* and *e1459*, using antibodies to UNC-87. The results for *e1459* are shown in Fig. 10; identical results were obtained for *e843* (data not shown). Pharyngeal muscle, but not the bodywall muscle in the head region, reacted with antibodies to UNC-87, consistent with the idea that either a cross reactive antigen, presumably the product of a distinct gene, or an alternately spliced form of the UNC-87 protein, is present in the pharynx.

The vulval muscles in *unc-87(e843)* hermaphrodites do not react with UNC-87 antibodies. Therefore, the antigen recognized there must be a product of the *unc-87* gene. We
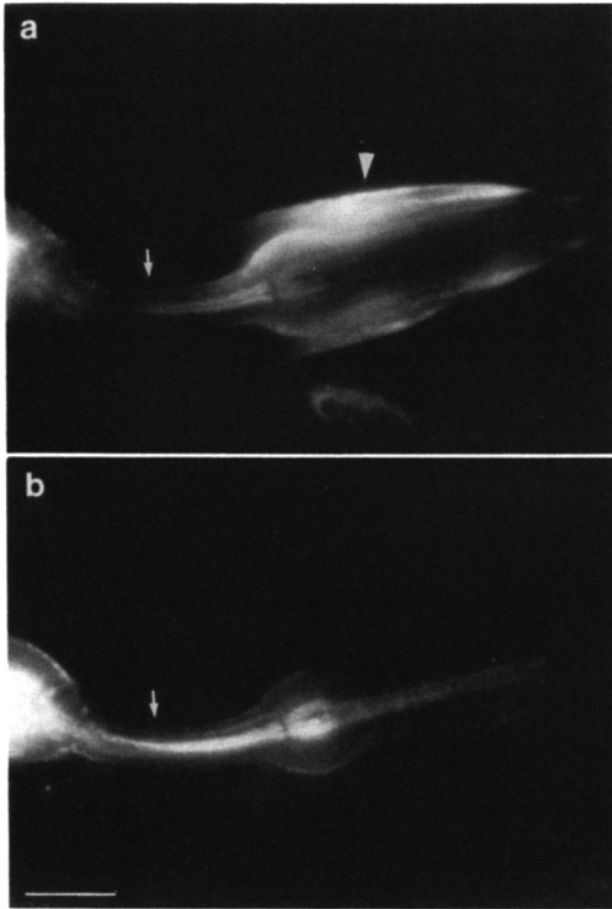
Figure 10. Fluorescence micrographs of *unc-87(e1459)* pharynx and bodywall muscle double-labeled with antibodies to actin and UNC-87. Fragments of *unc-87(e1459)* animals were reacted with antibodies to actin and UNC-87. The pharynx (*arrow*) and the bodywall muscle (*arrowhead*) in the head region shown have been severed from the rest of the animal. (*a*) Anti-actin staining. (*b*) The same field as in *a*, stained with antibodies to UNC-87. The pattern in the pharynx is similar for both antibodies. The bodywall muscles are out of focus and react strongly with the anti-actin antibodies, but only very weakly with the UNC-87 antibodies. Bar, 10 μm.

have not determined how the antigen(s) recognized in the anal depressor, the intestinal muscles and the uterine muscles are affected in *unc-87* mutants.

Thin Filament Association of UNC-87

To learn whether the UNC-87 protein product was associated directly with thin filaments or merely located in the region of the I-band, we tested whether UNC-87 can colocalize with thin filaments displaced from their normal location. *st22* is a semi-dominant allele of the actin gene *act-3* (L. Schriefer and R. Waterston, personal communication), which causes thin filaments to aggregate at ectopic locations within bodywall muscle cells (Waterston et al., 1984). Immunofluorescence studies of *act-3(st22)* embryos with anti-UNC-87 antibodies and an anti-actin antibody (Fig. 11, *c* and *d*) indicated that UNC-87 was also located in these aggregates. Positive staining of these structures with antibodies

to tropomyosin suggests that these resemble muscle thin filaments and are not just accumulations of filamentous actin (L. Schriefer and M. Hresko, personal communication). A wildtype embryo stained with anti-UNC-87 and anti-actin antibodies is shown for comparison (Fig. 11, *a* and *b*). The same results were obtained with the *act-3* allele *st15*, which has a phenotype similar to *act-3(st22)* (data not shown). These results suggest that the *unc-87* gene product is a component of thin filaments in *C. elegans*.

Because UNC-87 and the MH44 antigen colocalize in wildtype animals, we were curious whether their localization is coordinately disrupted in *st22* animals. Double staining of *act-3(st22)* embryos with MH44 and antibodies to UNC-87 indicated that, although the localization of UNC-87 is severely disrupted, the localization of the MH44 antigen is not (Fig. 12, *a* and *b*). Instead, the organization of the MH44 antigen appears almost wildtype (see also Goetinck and Waterston, 1994), although slight concentrations of antigen are sometimes visible in the location of the thin filament aggregates.

Because the *unc-87* gene product appears to associate with thin filaments, we tested whether this interaction is dependent on the presence of other known thin filament components. *st557* is a lethal allele of *lev-11* (Williams and Waterston, 1994), the candidate gene for tropomyosin. The genetic map position of *lev-11* corresponds to the physical map position of the cloned tropomyosin gene (A. Coulson and H. Kagawa, personal communications), and *st557* homozygotes fail to stain with polyclonal antibodies to tropomyosin (Williams and Waterston, 1994). To test whether the *unc-87* gene product colocalizes with thin filaments in the absence of detectable tropomyosin, we stained homozygous *st557* embryos with anti-*unc-87* and anti-actin antibodies (Fig. 13). The colocalization of the *unc-87* gene product with actin in these embryos suggests that it associates with thin filaments in the absence of tropomyosin.

To determine if the interaction of tropomyosin with thin filaments is dependent on the presence of the *unc-87* gene product, we stained *unc-87(e843)* embryos with antibodies to tropomyosin and actin. The *unc-87(e843)* embryos do not react with anti-UNC-87 antibodies (data not shown) but do stain with the tropomyosin antibody. The results of these experiments (Fig. 14) show that tropomyosin colocalizes with actin in an *unc-87* mutant, suggesting that it associates with thin filaments in the absence of detectable *unc-87* gene product.

Discussion

UNC-87 Is a Muscle Protein with Similarities to Proteins in the Muscles of Other Animals

We have cloned the *unc-87* gene and its cDNA by taking advantage of a transposon tagged allele and the correspondence of the genetic and physical maps in the region near *unc-87*. Complementation of the mutant phenotype with a wildtype copy of the gene has defined a region of DNA which restores wildtype muscle to *unc-87* mutants. Analysis of the predicted protein product shows that it contains seven copies of a repeated sequence motif that is similar to regions of other muscle proteins. The best characterized of these is
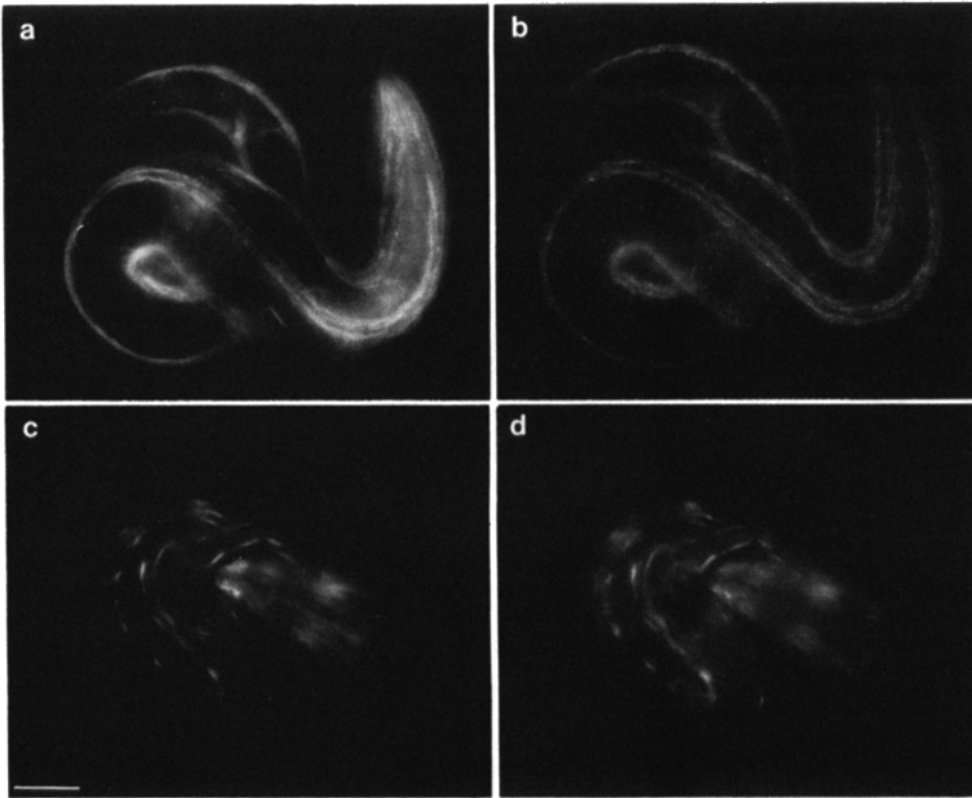
Figure 11. Fluorescence micrographs of wildtype and *act-3(st22)* embryos double labeled with antibodies to actin and UNC-87. (*a* and *b*) Wildtype embryos doubly stained with anti-actin (*a*) and anti-UNC-87 (*b*) antibodies. The staining pattern of both antibodies in the bodywall muscles is similar. (*c* and *d*) *act-3(st22)* embryos doubly stained with anti-actin (*c*) and anti-*unc-87* (*d*) antibodies. Actin filaments and UNC-87 colocalize even in ectopic patches (see text) in *act-3(st22)*. In both experiments, the pharynx stains with antibodies to actin and is out of focus. In contrast to the adult (Fig. 10), we have not observed pharyngeal staining of embryos with antibodies to UNC-87. Bar, 10 µm.

calponin. Originally purified from thin filaments of chicken gizzard smooth muscle, calponin has been shown to bind F-actin, tropomyosin and Ca⁺²/calmodulin (Takahashi et al., 1986; Vancompernolle et al., 1990). Antibodies raised against calponin react with proteins present in smooth muscle tissues (Takeuchi et al., 1991; Frid et al., 1992; Gimona et al., 1992) and in certain non-muscle tissues (Takeuchi et al., 1991). It has been proposed that calponin is a thin filament-associated regulator of smooth muscle contraction, because in vitro addition of calponin inhibits the actin-activated myosin MgATPase (Abe et al., 1990; Nishida et al., 1990; Winder and Walsh, 1990). Other in vitro studies have shown that calponin's inhibitory and actin binding properties are both decreased when calponin is phosphorylated, thus providing a model for the mechanism of action of calponin (Winder and Walsh, 1990). However, phosphorylation of calponin in vivo has not been observed (Barany et al., 1991; Gimona et al., 1992).

Studies with various proteolytic fragments of calponin have defined regions of the molecule that are capable of binding to F-actin, Ca⁺²/calmodulin (Mezgueldi et al., 1992) and tropomyosin (Vancompernolle et al., 1990). The regions of calponin capable of binding to tropomyosin and Ca⁺²/calmodulin show no similarity to either of the predicted UNC-87 isoforms, suggesting that the calponin and the *unc-87* gene product(s) do not serve fully equivalent functions. Of the three sequence repeats present in the alpha isoform of calponin, almost an entire (19/23 amino acids) repeat is contained in a proteolytic fragment known to bind F-actin. However, a proteolytic fragment containing the other two repeats failed to bind to F-actin (Mezgueldi et al., 1992). In light of the colocalization of UNC-87 and actin in *C. elegans* muscle and the similarity of UNC-87 to calponin, a known

actin-binding protein, it is possible that the sequence repeats, which are present in both proteins, mediate actin binding. Further support for this hypothesis might be obtained through biochemical analysis of the ability of native, or bacterially expressed, UNC-87 to associate with F-actin.

SM22, rat neuronal protein, Drosophila muscle protein mp20 and the predicted gene product from the *C. elegans* cDNA cm7g3 are also similar to the predicted *unc-87* gene products. These similarities are all confined to the sequence repeat present in UNC-87, suggesting that these regions share some functional properties. These proteins have not been shown to associate with F-actin or with thin filaments.

unc-87 Is Alternatively Spliced

Analysis of the *unc-87* genomic sequence with GENE-FINDER and comparison to the cDNA suggested the possibility that *unc-87* is alternatively spliced. Polymerase chain reactions from a *C. elegans* cDNA library indicated that alternative splicing does, in fact, exist for this gene. We did not detect a message corresponding to the predicted size of the alternatively spliced product on Northern blots containing total RNA prepared from mixed-stage populations, although the probe we used should hybridize to both transcripts. However, subsequent Western blot experiments using antibodies to an UNC-87 fusion protein against various *unc-87* alleles suggest that this larger mRNA is translated, although the protein is present at lower levels than the smaller isoform. It is possible that this message is present at lower levels than the 1.3-kb message, perhaps because it exists in small organs or in a tissue that was underrepresented in the population used to isolate the RNA.

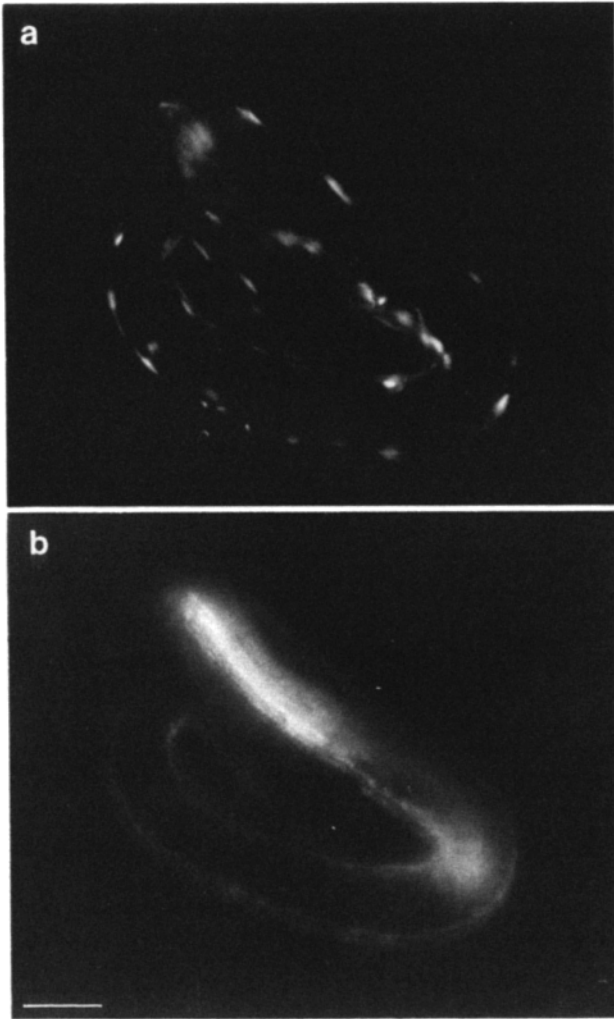We attempted to elucidate the function of the product en-

Figure 12. Fluorescence micrographs of *act-3(st22)* embryos double-labeled with MH44 and antibodies to UNC-87. (*a*) Anti-UNC-87 staining. (*b*) The same field as in *a*, stained with MH44. The colocalization of MH44p and UNC-87 observed in wildtype is disrupted in this mutant (Fig. 11). Bar, 10 μm.
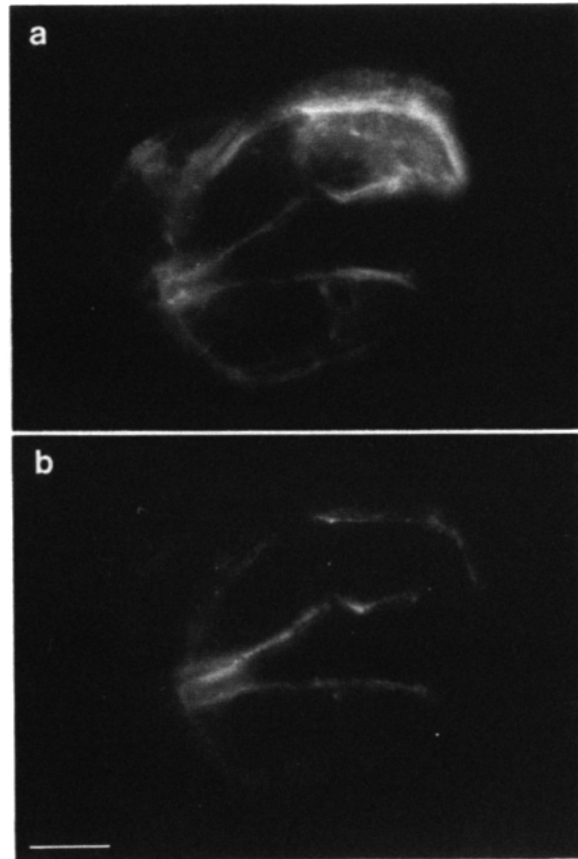

Figure 13. Fluorescence micrographs of *lev-11(st557)* embryos double-labeled with antibodies to actin and UNC-87. Homozygous *st557* embryos arrest elongation as twofold embryos and are misshapen (Williams and Waterston, 1994). (*a*) Anti-actin staining. (*b*) The same field as in *a*, reacted with antibodies to UNC-87. The staining pattern with both antibodies is similar; UNC-87 colocalizes with thin filaments in these embryos. Bar, 10 μm.

coded by the alternatively spliced mRNA by performing transformation rescue experiments with a genomic clone into which we had introduced translation termination codons that should eliminate the full-length product. The only protein encoded by the injected DNA should be that produced by initiation at exon A of the original cDNA clone. When introduced into *unc-87(e843)* animals, the altered construct rescued the mutant phenotype as well as the wildtype construct did, indicating that the 1.3-kb message represented by the cDNA is sufficient to rescue the *unc-87* mutant phenotype. The function of the alternative transcript remains unknown. Determination of the expression pattern of exon B using in situ hybridization techniques might provide clues to its function.

Antibodies to UNC-87 Recognize Other Muscle Groups in C. elegans and a Related Gene Product

The antibodies to UNC-87 were tested for their reactivity with other muscle groups in *C. elegans*. Reactivity was ob-

served in all muscles examined, and, as in the bodywall muscles, the patterns were consistent with an I-band location. *unc-87(e843)* and *unc-87(e1459)* animals, which have greatly reduced levels of UNC-87 staining in their bodywall muscles, show wildtype reactivity in the pharynx. These results are consistent with the possibility that the pharyngeal antigen is the product of a distinct, but related, gene. The gene product predicted by the *C. elegans* cDNA cm7g3 is a candidate for the pharyngeal antigen. Pharyngeal-restricted expression exists for other muscle components. The myosin heavy chains present in the pharynx and the bodywall muscles are encoded by two distinct sets of genes (Miller et al., 1986; Ardizzi and Epstein, 1987). A protein that is immunologically related to the *unc-22* gene product, another component of bodywall muscle thick filaments, is present in the pharynx, but the *unc-22* gene product is not (Moerman et al., 1988). As we do not know the nature of the molecular lesions in the *unc-87* mutants, we cannot rule out the possibility that the pharyngeal antigen is the product of the *unc-87* gene, and that its expression in the pharynx is not affected in the mutants.

Interestingly, *unc-87* mutant animals move better as adults than they do as larvae. We have not determined the mechanisms underlying this component of the phenotype, but
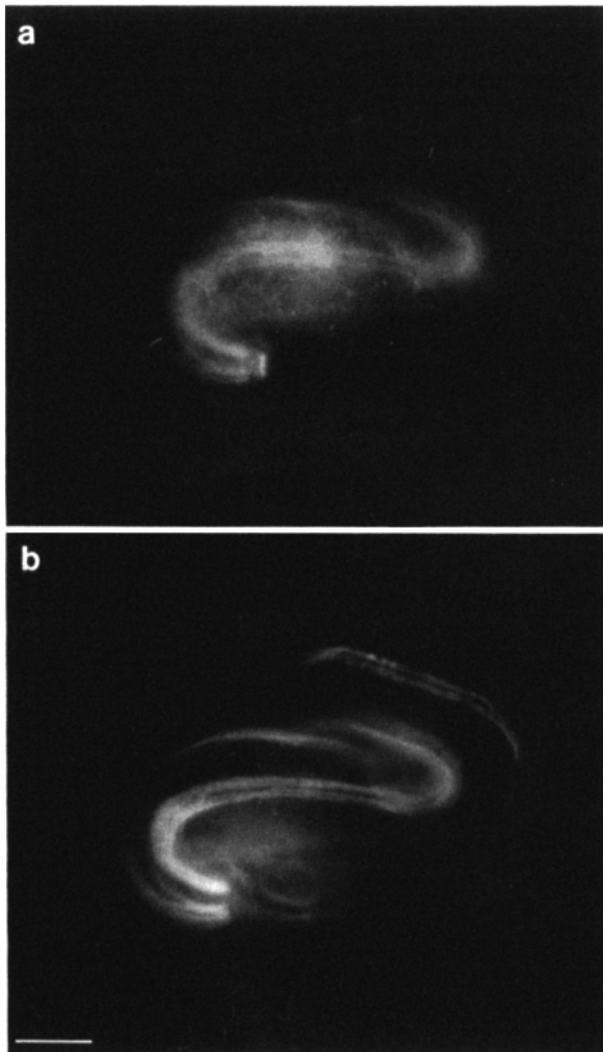
Figure 14. Fluorescence micrographs of *unc-87(e843)* embryos double-labeled with antibodies to actin and tropomyosin. (*a*) Anti-actin staining. (*b*) The same field as in *a*, stained with antibodies to tropomyosin. The staining pattern of both antibodies is identical in the bodywall muscle. The pharyngeal muscles, which are out of focus in these panels, react with antibodies to actin, but not to tropomyosin. Pharyngeal muscles in wildtype embryos also failed to react with anti-tropomyosin antibodies under the conditions we used. The reason for this is unclear. Bar, 10 μm.

based on our current understanding of the *unc-87* gene and its product, we offer several possible explanations. The *unc-87* mutants show faint reactivity with anti–UNC-87 antibodies in adults and in embryos under certain fixation conditions (unpublished results). Whether this is due to partial activity of the *unc-87* gene or whether the antigen recognized is the product of a related gene is unclear. Nevertheless, we failed to detect an increase in levels of the antigen over the lifetime of the worm. Therefore it seems unlikely that increased accumulation of the UNC-87 protein is responsible for the improved motility. Likewise, the organization of adult bodywall muscle does not improve relative to that observed in larval muscle (see Goetinck and Waterston, 1994). It is possible that adult bodywall muscle has a decreased requirement for the *unc-87* gene product, even though anti–*unc 87* antibodies

react strongly with these muscles in wildtype, suggesting the protein is normally present in high amounts. Alternatively, enough normal interdigitations between thick and thin filaments may accumulate by adulthood to allow the animals to move better.

UNC-87 Is Associated with Thin Filaments

Affinity-purified antibodies to UNC-87 react with the I-band of wildtype *C. elegans* bodywall muscle; the reactivity is specifically reduced in *unc-87(e843)* animals. These results indicate that the *unc-87* gene product is a component of muscle, consistent with the uncoordinated phenotype of *unc-87* mutants. Furthermore, the localization of UNC-87 to the same region as the actin-containing thin filaments is concordant with its similarity to thin filament-associated proteins and suggests that it may be a novel component of thin filaments in *C. elegans*.

The staining pattern of MH44 in the bodywall muscle is identical to that generated with antibodies to *unc-87*. MH44 recognizes a pair of high molecular mass proteins (440 and 400 kD). Based on its location and molecular weight, it was proposed (Francis and Waterston, 1991) that the MH44 protein might be analogous to nebulin. Wang and Wright (1988) have postulated that nebulin is an extended thin filament-associated protein that is anchored at the Z-line of vertebrate cross striated muscle and that serves as a ruler to govern the length of thin filaments. The localization of p440/400 in nematodes is consistent with a role in *C. elegans* analogous to that of nebulin in vertebrate muscle. However, the relationship between the molecular weight (and presumably protein length) of nebulins from different vertebrate muscles and the lengths of their thin filaments (Kruger et al., 1991) does not extend to p440/440 and *C. elegans* thin filaments, which are estimated to be 6 μm in length. It is possible that p440/400 may serve a related function but act by a different mechanism.

Based on the colocalization of UNC-87 and p440/400, it is reasonable to hypothesize that these two proteins interact. However, the proper organization of the MH44 protein does not appear to depend on wildtype levels of UNC-87: in contrast to adults, *unc-87(e843)* embryos have an MH44 staining pattern that closely resembles wildtype (see Goetinck and Waterston, 1994), but they do not react with anti-UNC-87 antibodies (data not shown).

To further test the hypothesis that UNC-87 is a component of thin filaments, we examined its distribution in *act-3(st22)* mutants. *st22* is a semi-dominant allele of *act-3* (L. Schriefer and R. Waterston, personal communication), one of four identified muscle actin genes in *C. elegans*. *act-3(st22)* animals are characterized by sluggish paralysis and aggregates of thin filaments that are ectopically located in the bodywall muscle cells (Waterston et al., 1984). Antibodies to UNC-87 react with these aggregates, as do antibodies to another known thin filament component, tropomyosin. This provides additional support for the idea that UNC-87 is a novel component of thin filaments in *C. elegans* and further suggests that the principal association of UNC-87 is not with p440/400. It should be possible to test this idea directly by purifying native thin filaments from *C. elegans* and assaying them for the presence of the *unc-87* gene product using anti–*unc-87* antibodies.

The existence of seven evenly spaced sequence repeats in UNC-87, a possible thin filament component, is interesting in light of the proposed periodicity of tropomyosin and nebulin along the thin filament. Tropomyosin is thought to lie in the groove of the actin filament and span the length of seven actin monomers (Ebashi et al., 1969). Partial amino acid sequence of nebulin indicates the molecule contains a tandem series of seven 31–38-amino acid repeats (Wang, K., M. Knipfer, Q. Q. Huang, L. Hsu, A. van Heerden, K. Browning, E. Quain, and H. Stedman. 1990. *J. Cell Biol.* 111:428a; Labeit et al., 1991). It has been proposed that these super repeats also coincide with the periodicity imposed by the tropomyosin molecules. The length of a sequence repeat and the following spacer (unrelated to the nebulin sequence) region in UNC-87, at 40–55 amino acids, is also long enough to adopt a similar arrangement.

Analysis of the effect of mutations in many thin filament-affecting genes on other thin filament components is now possible. For example, here we examined the effect of eliminating tropomyosin on UNC-87 distribution and, conversely, the effect of eliminating UNC-87 on the distribution of tropomyosin. In homozygous *lev-11(st557)* mutants, which do not react with anti-tropomyosin antibodies (Hresko et al., 1994), *unc-87* still colocalized with actin. These results suggest that the *unc-87* gene product does not depend on tropomyosin to associate with thin filaments. Likewise, absence of detectable UNC-87 did not disrupt the colocalization of tropomyosin with actin, indicating that wildtype levels of UNC-87 are not necessary for tropomyosin to assemble into thin filaments.

The genes for many known thin filament proteins are identified, and mutations exist for some of these genes. The tropomyosin gene has been cloned (H. Kagawa, personal communication), and a putative null allele exists (Williams and Waterston, 1994). Genes for troponin-T, -I, and -C have been identified, and mutations probably exist for TnT (E. Bucher, personal communication; Goh, 1991) and TnC (B. Williams and H. Kagawa, personal communications). We tentatively assign the *unc-87* gene product to the group of thin filament proteins that have mutations associated with them. In addition to mutant phenotypes associated with a loss of function of these gene products, antibodies are available for tropomyosin and *unc-87* and are being developed for the troponins. The monoclonal antibody MH44 may also prove useful in examining thin filament organization. With these tools in hand, it should be possible to analyze the effect of a mutation in a thin filament-affecting gene on the distribution of other thin filament antigens and thereby determine the requirements for those components to associate with thin filaments. This approach has been used to develop a model for the overall assembly of the myofilament lattice (Hresko et al., 1994; Williams and Waterston, 1994).

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