The *Caenorhabditis elegans* UNC-87 Protein Is Essential for Maintenance, but Not Assembly, of Bodywall Muscle

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Abstract. Mutations in the *unc-87* gene of *Caenorhabditis elegans* cause disorganization of the myofilament lattice in adult bodywall muscle. In order to assess the organization of specific bodywall muscle components in the absence of the *unc-87* gene product, we examined the bodywall muscles of mutant animals using phalloidin and monoclonal antibodies to various muscle proteins. These studies indicated that the bodywall muscle of *unc-87* embryos is initially almost wild type in its organization, but at later stages, the muscle becomes severely disorganized. To address the possibility that this disorganization is due to deterioration of the muscle as a result of contraction, we introduced into the *unc-87* mutant background a mutation that decreases myosin heavy chain activity but does not substantially affect muscle structure. The improved muscle structure and motility of the double mutants are consistent with the hypothesis that at least part of the disorganization phenotype of *unc-87* mutants is a consequence of the wild-type levels of force generated during muscle contraction. These results imply that the role of the *unc-87* gene product is not in specifying organization but rather in serving as a structural component maintaining lattice integrity during and after contraction.

THE myofilament lattice of striated muscle is a dynamic, precisely ordered array containing many different proteins. The roles of individual proteins in the structure and function of muscle have been deduced from biochemical studies as well as immunolocalization of proteins to specific structures in the sarcomere. Genetic analysis of muscle offers an added advantage of being able to eliminate or alter individual components of the myofilament lattice and then assess the effects on muscle structure and function. Further, multiple components of the system can be manipulated either by creating double mutants with other muscle-affecting genes or through suppression analysis. Thus, it is possible to test whether a particular mutation can enhance or alleviate another mutant phenotype and also to identify mutations in genes involved in related processes by their suppression of the phenotype under examination.

The study of muscle in *C. elegans* has been facilitated by the availability of monoclonal antibodies to over 40 muscle proteins (Miller et al., 1983; Francis and Waterston, 1991) and genetic screens aimed at identifying genes that affect muscle structure and function (reviewed in Wood, 1988; also Waterston, 1989; Barstead and Waterston, 1991; Williams and Waterston, 1994). Over 50 muscle-affecting genes have been characterized to date. The organization of the myofilament lattice is known (Waterston et al., 1980), as is the distribution of specific components in both adult (Francis and Waterston, 1991) and embryonic (Hresko et al., 1994) muscle. This knowledge makes it possible to determine which, if any, antigens are affected in different mutant backgrounds (Hresko et al., 1994; Williams and Waterston, 1994). This approach has been useful in demonstrating that certain components of the sarcomere are required for a subset of others to assemble properly. Genetic suppression analysis has also provided clues to the role of muscle proteins by identifying interacting genes, or genes with related functions (Greenwald and Horvitz, 1982; Moerman et al., 1982).

We have employed such techniques to study the role of the *unc-87* gene product in muscle. Originally identified by mutations which result in severe paralysis and bodywall muscle disorganization, the *unc-87* gene has now been cloned (Goetinck and Waterston, 1994). Its predicted protein sequence is similar to those of several other proteins known to associate with thin filaments. Antibodies raised against the *unc-87* gene product show it resides in the region of muscle that contains thin filaments. Furthermore, the presence of seven evenly spaced sequence repeats in UNC-87 suggests that it may principally play a structural role in the I-band.

Here we examine the effect of a severe reduction-of-function mutation in the *unc-87* gene on the distribution of other components of the sarcomere. Our results indicate that, although larval and adult worms have severely disrupted bodywall muscle structure, the structure of late-stage

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embryonic muscle is almost wild type. We also show that the later disorganization can be suppressed by impairing bodywall muscle activity, consistent with the hypothesis that disorganization results, at least in part, from the breakup of the myofilament lattice under the force of contraction.

Materials and Methods

General Techniques

Handling and maintenance of stocks has been described (Brenner, 1974).

Immunohistochemistry

Adult worms were prepared and reacted with antibodies as described (Francis and Waterston, 1991). Embryos were prepared and stained as described (Barstead and Waterston, 1991). Monoclonal antibodies were used at the following dilutions: DM5.6, 1:500; MH24, 1:125; MH44, 1:100; anti-actin C4 (from ICN Biomedicals, Inc., Costa Mesa, CA), 1:100. Secondary antibodies were from Chemicon Intl. Inc. (Temcula, CA).

Phalloidin Staining

Phalloidin staining of adults and L1 larvae was performed as described (Waterston et al., 1984). Rhodamine-phalloidin was purchased from Molecular Probes, Inc., (Eugene, OR). To obtain populations of L1 larvae, gravid adults and eggs were rinsed off plates in M9 buffer (Brenner, 1974) and washed several times in the same to remove bacteria. After a 9-min treatment with alkaline hypochlorite (30% bleach, 250 mM potassium hydroxide), the remaining embryos were washed several times with M9 buffer and then allowed to hatch overnight on an NGM-agar plate without food. The resulting L1 larvae were then collected in M9 buffer for phalloidin staining. For embryos, eggs were isolated as described above, washed four times in M9 buffer, fixed in 3% formaldehyde in PBS for 10 min, washed twice with PBS, extracted with −20°C acetone for 10 min at −20°C, washed again, and stained with PBS (J. Waddle and P. Robbins, personal communication).

Microscopy and Photography

Worms were mounted for fluorescence microscopy in 90% glycerol, 20 mM Tris-Cl, pH 8.0, containing 0.2 M DABCO (Sigma Chem. Co., St. Louis, MO). T-MAX 400 film (Kodak) was used to record fluorescent images.

Construction of unc-87 unc-54 Double Mutants

The same procedure was used to construct *unc-87(e843) unc-54(s95)* and *unc-87(e1459) unc-54(s95)*. These strains have been designated RW1523 and RW1524, respectively. *dpy-5* is 1.5 map units to the left of *unc-87*; *unc-87* is 25 map units to the left of *unc-54*. *unc-87/+* males were mated to *dpy-5(e61)unc-54(s95)* hermaphrodites. Wild-type hermaphrodites (cross progeny) were cloned onto individual plates, and from plates that segregated one quarter Unc animals, UncNonDpy animals were cloned. From those clones which segregated DpyUnc animals, lines were established which no longer segregated Dpys. That these strains were homozygous for both *unc-87* and *unc-54(s95)* was confirmed by individual complementation tests for both alleles (Brenner, 1974).

Results

Bodywall Muscle Organization of unc-87(e843) Adults

In the accompanying manuscript, we showed that *unc-87(e843)* adults fail to accumulate detectable UNC-87 antigen (Goetinck and Waterston, 1994). Here we examine the effect of the *unc-87(e843)* mutation on the organization of other muscle antigens using phalloidin and three different monoclonal antibodies. The distribution in *unc-87(e843)* of thin filaments, an I-band component (p440) (Francis and Waterston, 1991), thick filaments and dense bodies was evaluated. Our results demonstrate that these structures are disrupted in the bodywall muscle of *unc-87(e843)* adults.

Phalloidin binds to filamentous actin and marks the organization of thin filaments when it is used to stain muscle. A comparison of wildtype and *unc-87* muscle structure is shown in Fig. 1. In the wild type, regular striations are apparent, reflecting the highly ordered myofilament lattice. In the mutant, the striations are irregular, and accumulations of thin filaments are present in the ends of the bodywall muscle cells. This is in agreement with previous electron microscopic observations (Waterston et al., 1980).

MH44 recognizes a pair of high molecular mass (440 and 400 kD) polypeptides on Western blots and reacts with the I-band of bodywall muscle (Francis and Waterston, 1991). In wild-type animals, regular parallel striations are interrupted along their length by periodic negatively stained ovals which correspond to dense bodies (Fig. 2 *a*). This staining pattern is in agreement with that first observed by Francis and Waterston (1991). In *unc-87(e843)* the pattern is disorganized: striations are irregular and uneven in width (Fig. 2 *b*).

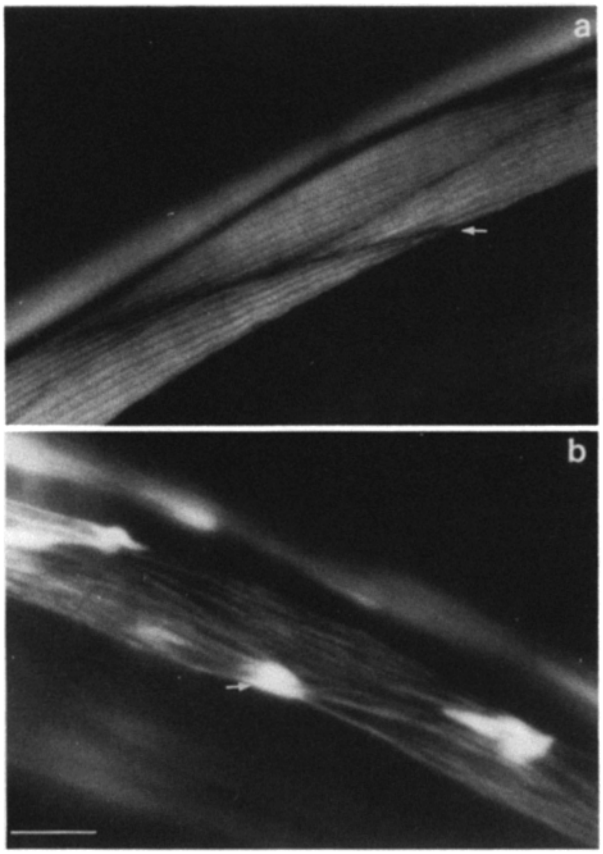

Figure 1. Fluorescence micrographs of adult bodywall muscle stained with rhodamine-phalloidin. One muscle quadrant is in focus in each panel. Arrows indicate boundaries between the spindle shaped bodywall muscle cells. (*a*) Wild type. A striated pattern is observed, reflecting the highly ordered structure of the myofilament lattice. (*b*) *unc-87(e843)*. The myofilament lattice is disorganized; regular striations are not observed. In addition, concentrations of positively stained material are present in the ends of the cells. Previous electron microscopic analysis of another *unc-87* allele, *e1216*, suggests these are aggregates of thin filaments. Bar, 10 μm.

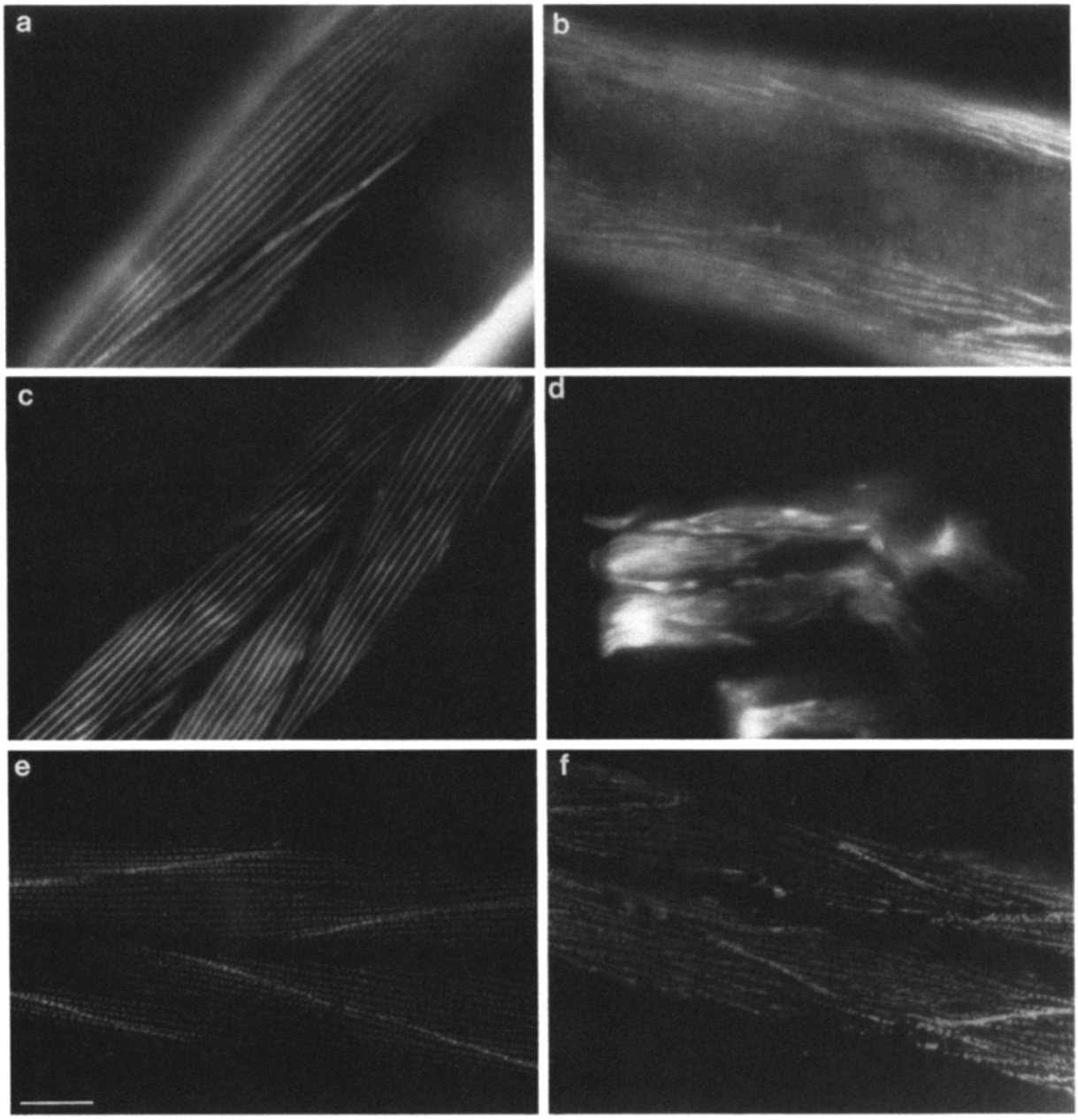

Figure 2. Fluorescence micrographs of adult bodywall muscle. Wild type (*a*, *c*, and *e*) and *unc-87(e843)* (*b*, *d*, and *f*) adult worms reacted with the monoclonal antibodies MH44 (*a* and *b*), DM5.6 (*c* and *d*) and MH24 (*e* and *f*). The bodywall muscles of the *unc-87(e843)* adults are disorganized. Bar, 10 μm.

DM 5.6 reacts specifically with MHC-A, the isoform of the myosin heavy chain located in the central region of the bodywall muscle thick filaments (Miller et al., 1983). A fluorescence micrograph of a wild-type animal stained with this antibody shows a striated pattern, reflecting the regularity of the A-band (Fig. 2 *c*). In *unc-87(e843)* animals, the central portions of the A-bands have a wavy appearance and are no longer parallel to each other (Fig. 2 *d*).

MH24 recognizes vinculin, a component of nematode dense bodies (Barstead and Waterston, 1989). In wild-type animals, the staining pattern is one of rows of periodic spots (Fig. 2 *e*) (Francis and Waterston, 1991). In the mutant, however, the spots appear as diffuse patches (Fig. 2 *f*).

Although the patterns we observed were abnormal, they were nevertheless reminiscent of the highly organized wild-type pattern. Observations of other muscle-affecting mutants have shown that a primary disruption of one structure can secondarily affect others. The primary defect of *unc-54* null mutants, which lack the most abundant myosin heavy chain isoform (MacLeod et al., 1977), is a severe reduction in the number of thick filaments. However, as adults these mutants also have poorly organized thin filaments and irregular dense bodies (Wood, 1988). Mutant alleles of the actin genes primarily affect thin filaments but also result in poorly organized thick filaments in adults (Waterston et al., 1984). In order to avoid possible secondarily induced effects on muscle

structure, we examined embryos just as the striated pattern in the bodywall muscle becomes apparent.

Organization of Bodywall Muscle in unc-87(e843) Embryos

Previous work has shown that the threefold *C. elegans* embryo is the youngest stage that possesses the same organization of the myofilament lattice as that of larval and adult muscle, although the sarcomeres are not as deep and are fewer in number (MacKenzie et al., 1978; Hresko et al., 1994). (The term threefold refers to the length of the embryo, which at this stage is three times the length of the egg. Animals hatch when they are over three times the length of the egg.) At this timepoint, the bodywall muscles have been functioning for only ~100 min (Sulston et al., 1983). In earlier embryos, thick filament-containing A-bands are well delineated, but actin staining is not clearly resolved into striations. Thus we compared threefold *unc-87* embryo muscle structure to wild-type of an equivalent stage. Wild type, but not *unc-87*, threefold embryos contain detectable UNC-87 antigen (Goetinck and Waterston, 1994).

Fig. 3 *a* shows rhodamine-phalloidin staining of a wild-type embryo, which at this stage contains two sarcomeres in each bodywall muscle cell and stains positively for Unc-87 (Goetinck and Waterston, 1994). The *unc-87(e843)* embryo in 3 *b* looks almost identical to the wild type, although the thin filament bands are slightly variable in width. Thus thin filaments in the mutant are capable of achieving nearly wild-type organization despite the much more severe disorganization seen in mutant adults and L1 stage larvae (see Fig. 5 *c*). We have detected occasional late stage embryos whose phalloidin staining pattern resembles that seen in first stage (L1) larvae and adults. These are possibly older embryos that would have soon hatched into an L1 larva; alternatively, these might represent variability in assembly or in the onset of disorganization (see also Discussion).

We also examined threefold *unc-87(e843)* embryos stained with the same three monoclonal antibodies used for analysis of the UNC-87 adults. As was the case for the thin filaments, the organization of the antigens detected by MH44, DM5.6, and MH24 in *unc-87(e843)* embryos was nearly identical to that seen in wild type (Fig. 4). This is in contrast to the severely disrupted organization seen in *unc-87(e843)* adults (see Fig. 1).

Because most threefold *unc-87(e843)* embryos have bodywall musculature nearly identical to that of wild type, we conclude that normal levels of the *unc-87* gene product are not essential for the initial assembly of the myofilament lattice. However, because the myofilament lattice of all L1 larvae and some late stage embryos is disrupted, it appears that UNC-87 has a role either in subsequent growth of the lattice or in maintaining its integrity.

Disorganization Is Influenced by Contractile Activity

We were intrigued by the later disruption of the initially organized myofilament lattice in *unc-87* mutants. It seemed possible that the disorganization was a result of failure of the structure of the myofilament lattice under the force of contraction. To test this hypothesis, we decreased contractile activity in the *unc-87* single mutants, *e843* and *e1459*, by introducing a second mutation, *unc-54(s95)*, that decreases

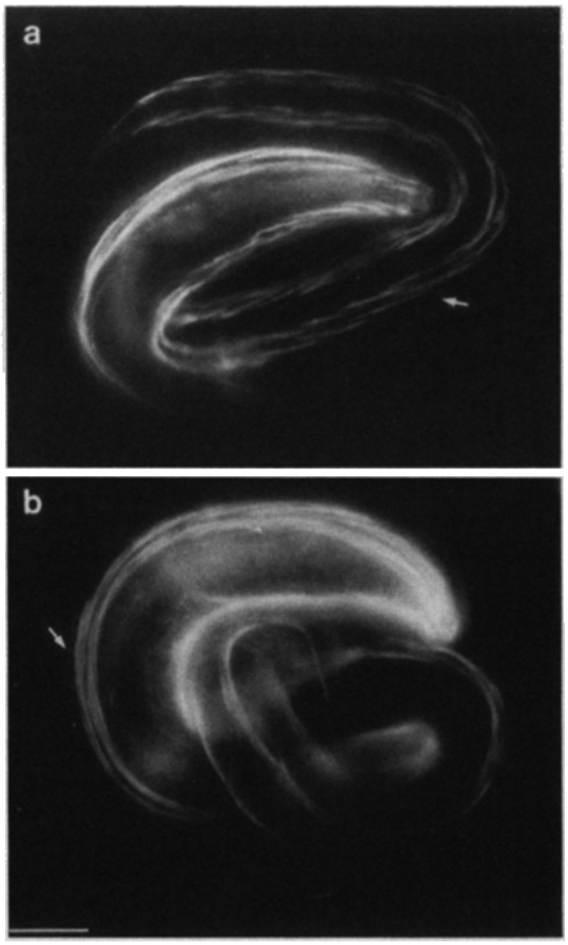

Figure 3. Fluorescence micrographs of threefold stage embryos stained with rhodamine-phalloidin. Bodywall muscle of wild type (*a*) and unc-87(e843) (*b*) threefold embryos. Arrows indicate muscle quadrants. Bar, 10 μm.

myosin heavy chain activity but does not substantially affect muscle assembly or structure (Moerman et al., 1982). Neither *unc-87(e843)* nor *unc-87(e1459)* accumulates detectable UNC-87 antigen as embryos and adults (Goetinck and Waterston, 1994, and data not shown). We then compared thin filament organization in the doubly mutant L1 larvae to that of wild type or *unc-54(s95)* and *unc-87* single mutants (Fig. 5).

The bodywall muscles of *unc-54(s95)* are mildly disorganized relative to wild-type animals (compare Fig. 5, *b* to *a*), while the thin filaments in the *unc-87* single mutants are severely disorganized (Fig. 5, *c* and *d*). Specifically, the normal myofilament periodicity is disrupted in *unc-87* larvae, and thin filament aggregates accumulate at the ends of muscle cells. Strikingly, the muscle structure of both *unc-87 unc-54(s95)* double mutants (Fig. 5, *e* and *f*) is less disrupted than that of the corresponding *unc-87* single mutants. The abnormal collections of thin filaments in the ends of the bodywall muscle cells are no longer present, and the striations appear more even. Concordant with this structural observation, the motility of the double mutants is better than

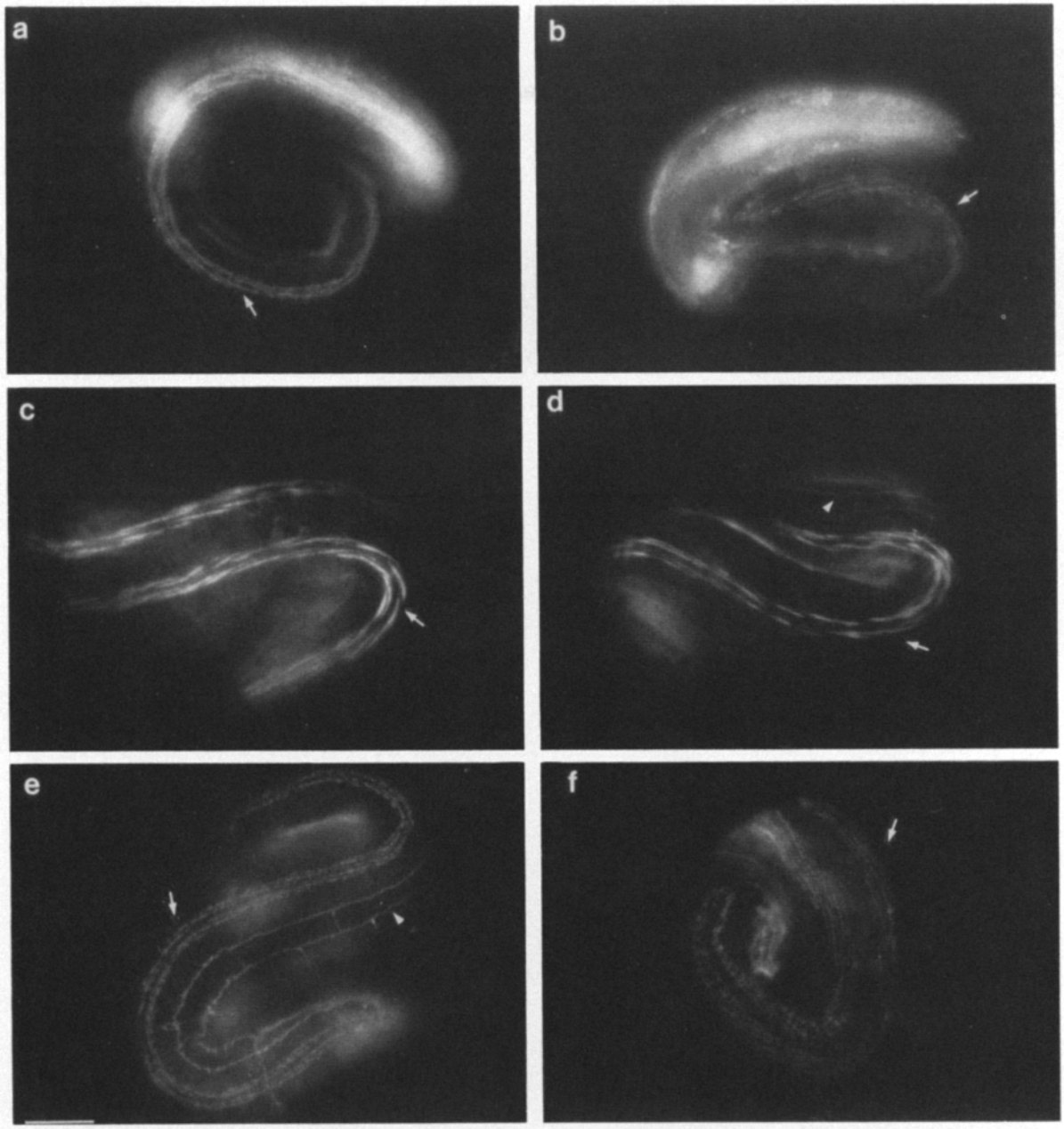

Figure 4. Fluorescence micrographs of threefold stage embryos. Wild type (*a*, *c*, and *e*) and *unc-87(e843)* (*b*, *d*, and *f*) embryos reacted with the monoclonal antibodies MH44 (*a* and *b*), DM5.6 (*c* and *d*) and MH24 (*e* and *f*). The bodywall muscle quadrants are indicated by arrows. Unlike the adults, the bodywall muscle organization of *unc-87(e843)* embryos is very similar to wild type. Embryos in panels (*d*) and (*e*) are also stained with the monoclonal antibody MH27, which reacts with hypodermal cell boundaries (*arrow heads*). Bar, 10 μm.

that of the *unc-87* single mutants, but still worse than that of *unc-54(s95)* and wildtype.

Discussion

The bodywall muscle of *unc-87* mutants was examined as a step in understanding the role *unc-87* plays in muscle assembly. Phalloidin staining and immunofluorescence studies indicated that the distribution of thin filaments, dense bodies, thick filaments and the epitope recognized by the monoclonal antibody MH44 was close to that of wild type in threefold-stage *unc-87* embryos but was severely disrupted in adults. Wild type, but not *unc-87* threefold embryos, stain positively with antibodies to UNC-87 (Goetinck and Waterston, 1994). Staining of first stage *unc-87* larvae with rhodamine–phalloidin indicated that the distribution of thin filaments was also severely disrupted at this time. Thus, normal levels of the *unc-87* gene product are not necessary for initial assembly of the myofilament lattice, but organization becomes abnormal shortly after the onset of forceful contractions. One interpretation of these results is that UNC-87 does not function in *unc-87* embryos, but is only important

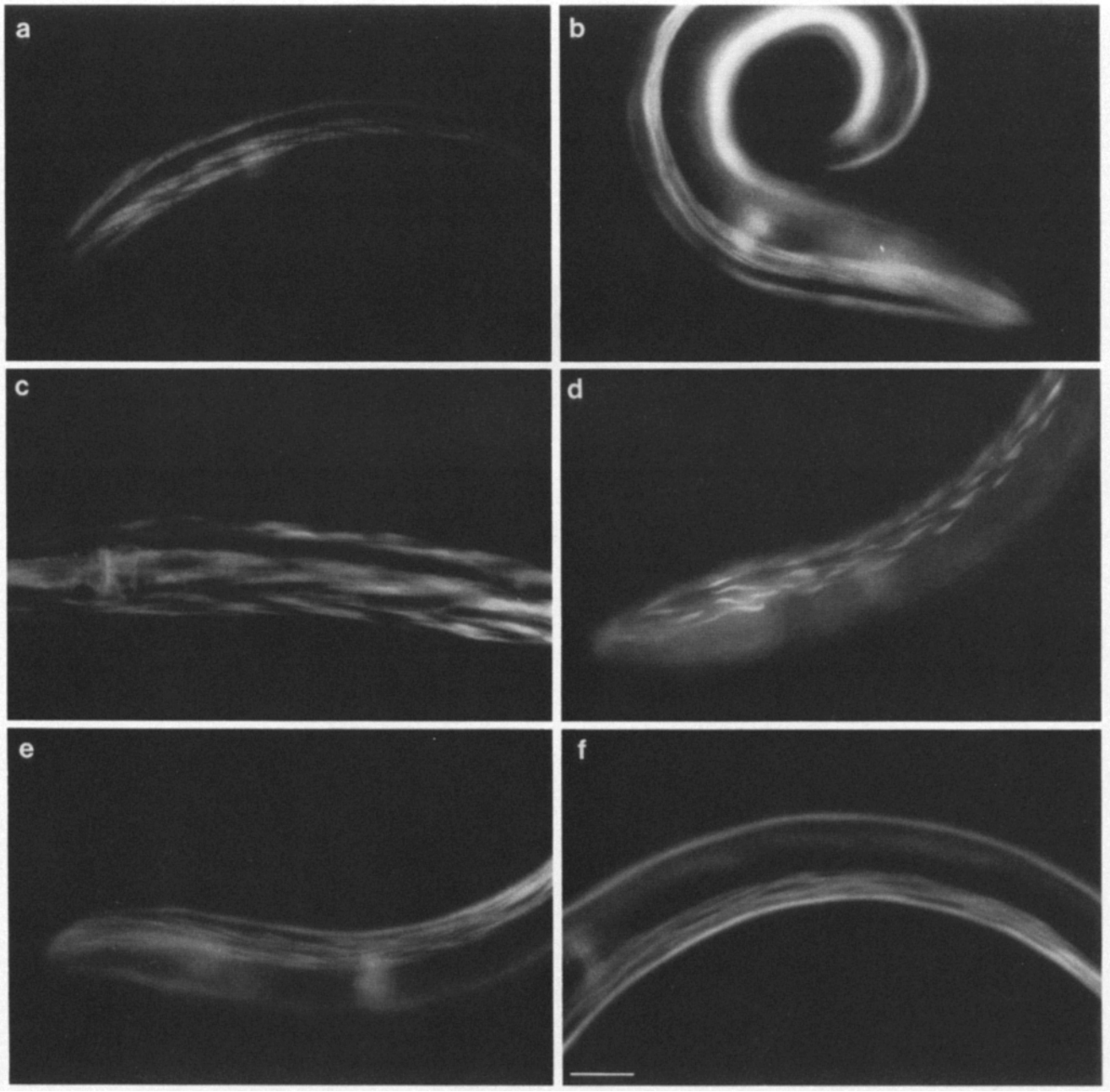

Figure 5. Fluorescence micrographs of L1 larvae stained with rhodamine-phalloidin. (*a*) N2, (*b*) *unc-54(s95)*, (*c*) *unc-87(e843)*, (*d*) *unc-87(el459)*, (*e*) *unc-87(e843) unc-54(s95)* and (*f*) *unc-87(el459)unc-54(s95)* L1 larvae were stained with rhodamine phalloidin. A portion of one bodywall muscle quadrant is in focus in each panel. *unc-54(s95)* has slightly abnormal musculature. The brightly staining patches of phalloidin-positive material present in *unc-87(e843)* and *unc-87(el459)* are not present in the double mutants *unc-87(e843)unc-54(s95)*, and *unc-87(el459)unc-54(s95)*. Bar, 10 μm.

in the further growth of the myofilament lattice that takes place in larvae and adult worms. However, we feel this is unlikely, because the configuration of late-stage embryonic muscle is the same as that of larvae and adults (MacKenzie et al., 1978; Hresko et al., 1994), and *unc-87* antibodies react strongly with embryonic bodywall muscle (Goetinck and Waterston, 1994). Rather, we suggest that the myofilament lattice has a limited capacity to withstand contractions in the absence of UNC-87. In this model, UNC-87 is incorporated into the lattice as it is formed and helps to stabilize the lattice when contractions occur. It should be noted that *unc-87* adults move better, in general, than *unc-87* larvae. The reason for this is unclear, and possible explanations are considered in the Discussion of the accompanying manuscript (Goetinck and Waterston, 1994).

To test the hypothesis that UNC-87 provides stability to the myofilament lattice, we decreased myosin heavy chain activity in *unc-87* mutants by constructing double mutants with the *unc-54* allele, *s95*. Both motility and muscle organization were improved in the double mutants, consistent with

the hypothesis that at least some of the disorganization results from a degeneration of the myofilament lattice under the force of contraction.

We used the *unc-54(s95)* mutation because it is a member of a class of *unc-54* alleles identified by their ability to suppress the muscle phenotype of *unc-22* mutants (Moerman et al., 1982). The molecular lesion in *unc-54(s95)* and one other member of its class, *unc-54(s74)*, have been determined. Both are missense changes in the head of the myosin heavy chain (Dibb et al., 1985) near the ATP binding site and thus could affect ATPase activity and the contraction/relaxation cycle. Mutations in the *unc-22* gene cause the bodywall muscle to twitch; this presumably represents a defect in the regulation of muscle contraction. The bodywall muscle organization is also perturbed. Homozygous *s95* animals move slowly, but in contrast to null alleles of *unc-54*, their muscle structure is almost wild type. Because *s95*, as well as other members of its class, can suppress many different alleles of *unc-22*, Moerman et al. (1982) proposed that the suppression does not reflect a direct interaction between the two gene products (Moerman et al., 1982). Rather, the suppression of the twitching and disorganization might be due to a decrease in wild-type activity of the *unc-54* gene. Thus, if the muscle cannot contract with wild-type force levels, twitching cannot take place. The suppression of the disorganization could result from the reduced force of contraction in the double mutants. This suggests further that UNC-22 may have a structural role in stabilizing the lattice in the presence of the force generated during contraction.

Further evidence that this class of *unc-54* alleles reduces myosin heavy chain activity is their suppression of *unc-90* (G. R. Francis, personal communication). *unc-90* is a muscle-affecting gene represented by a single dominant allele; homozygous animals are severely hypercontracted and have disorganized bodywall muscle (Waterston et al., 1980). *unc-54(s95) unc-90* double mutants are less hypercontracted, and their muscle structure is more organized than that of *unc-90* single mutants. A paradox exists in the degree of suppression of *unc-90* by *unc-54(s95)* and the other members of its class: the alleles with the weakest phenotype (i.e., the best motility) are better suppressors of *unc-90* than those with the strongest phenotype. The basis for this is not understood, but nevertheless, the results indicate that the *unc-90* phenotype is dependent on wild-type activity of the *unc-54* gene.

The bodywall muscle of both *unc-87 unc-54(s95)* double mutants is better organized than that of the *unc-87* single mutants, indicating that *unc-54(s95)* suppresses the *unc-87* mutant phenotype. The improved motility of the double mutant compared to *unc-87* mutants alone presumably reflects this structural improvement. Thus, *unc-87* is a new member of the group of muscle-affecting mutants that can be suppressed by mutations which decrease myosin heavy chain function. It is possible the *unc-54(s95)* allele decreases the need for UNC-87 only for growth of the myofilament lattice in the larval and adult stages. However, based on the ability of *unc-54(s95)* to suppress a variety of muscle-affinity genes, we propose that suppression of the *unc-87* phenotype occurs because the force generated during contraction is decreased by the *unc-54(s95)* allele, and disruption is reduced.

Mutations in the genes encoding the thin filament components troponin T and troponin I in Drosophila also alter myofilament lattice integrity. In strains bearing the *heldup*^{2} allele of troponin I (Beall and Fyrberg, 1991) and the *upheld*^{101} and *indented thorax*^{3} alleles of troponin T (Fyrberg et al., 1990), the indirect flight muscles initially assemble relatively normally but begin to degenerate when the muscle is used. This is apparently not due to an instability of the thin filaments themselves because, in double mutants with a mutation that eliminates the myosin heavy chain from the indirect flight muscles, the myofilament lattice appears no worse than in the myosin heavy chain single mutant. Perhaps the altered regulation of contraction resulting from these mutations produces abnormal contraction forces which disrupt the lattice structure; some instability of the thin filaments due to the abnormal troponins could be exacerbated by the abnormal contractions.

Like the troponins, UNC-87 might associate with thin filaments. However, based on sequence comparisons it is probably not part of a troponin regulatory complex. The nematode does have genes encoding the troponin subunits (E. Bucher, personal communication; Goh, 1991) and tropomyosin (A. Coulson and J. Sulston, H. Kagawa, personal communications), and their protein products presumably account for thin filament regulation (Harris et al., 1977). The similarity of UNC-87 to calponin involves a region outside the portion of calponin known to interact with the regulatory proteins tropomyosin and calmodulin; instead, the region is implicated in actin bonding. In the absence of any evidence suggesting a role in contraction, and in light of the evenly-spaced sequence repeats in UNC-87, we believe the protein is likely to serve a structural role.

We propose that UNC-87 stabilizes the myofilament lattice by virtue of its association with thin filaments and perhaps other proteins; the disorganization of additional sarcomeric components likely represents secondary effects. Specifically, we suggest that UNC-87 may provide thin filament attachment points in addition to those located at the dense body. The protein's repeated structure and its distribution over the width of the I band are compatible with such a role. In vertebrate muscle the protein nebulin is proposed to play such a structural role by aligning along the thin filament and binding to it through 35-amino acid sequence modules (Jin and Wang, 1991*a*,*b*). Obviously the small size of UNC-87 rules out a role directly comparable to the much larger nebulin. Further, the predicted UNC-87 sequence bears no direct sequence similarity to the available nebulin sequence. Perhaps the UNC-87 protein itself can polymerize to form long chains lying along the thin filament. Alternatively, it might link the thin filament to another component, possibly p440, which has the same distribution as UNC-87 in wild-type bodywall muscle, to provide lineal integrity (see accompanying manuscript).

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