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Caenorhabditis elegans UNC-45 defines a novel class of myosin-associated proteins

BY

Wanyuan Ao 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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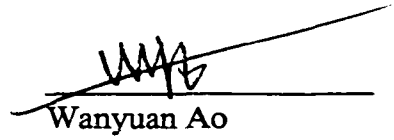

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "*Caenorhabditis elegans* UNC-45 Defines a Novel Class of Myosin-Associated Proteins" submitted by Wanyuan Ao in partial fulfillment for the degree of Doctor of Philosophy in Molecular Biology and Genetics

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Abstract

Caenorhabditis elegans unc-45 is an essential gene with a maternal effect. Animals homozygous for *unc-45* missense mutations show an uncoordinated phenotype and have disorganized body wall muscle structure. Homozygotes of *unc-45* null mutations are inviable and arrest at the two-fold stage of embryogenesis. UNC-45 contains three tetratricopeptide repeats and has limited similarity to fungal proteins, but its biochemical function is unknown.

unc-45 reporters are expressed exclusively in muscle cells, and a functional UNC-45::GFP fusion is localized in body wall muscles in a pattern similar to the A-bands. Immunofluorescence studies show that UNC-45 colocalizes to thick filaments with myosin heavy chain (MHC) B in wild-type worms, but not in a mutant in which MHC B is absent and in mutants in which MHC B is absent but the level of MHC A is increased. Thus, UNC-45 shows a localization pattern identical to and dependent on MHC B. Based on these observations, I propose that UNC-45 is a component of muscle thick filaments. The role of UNC-45 in muscles may be as a cofactor for assembly or stabilization of MHC B.

In addition to its co-localization with MHC B in muscles, UNC-45 is also detectable in the germline cells of the gonad and pre-morphogenesis embryos. Moreover, I have demonstrated that UNC-45 interacts specifically with a non-muscle myosin (NMY-2) in yeast two-hybrid assays and the two proteins are also co-localized at the embryonic cleavage furrow *in vivo* in *C. elegans*. The localization of UNC-45 at the cleavage furrow is also dependent on the presence of NMY-2. These results suggest that maternally contributed UNC-45 may have a function other than muscle development.

Furthermore, UNC-45 also interacts with an additional unconventional myosin in yeast. This has allowed me to define a region of 140 amino acids that seems to be sufficient for UNC-45 to bind to myosin. Database searches also indicate that UNC-45 may have sequence homologues in *Drosophila* and humans. With these results and the previous genetic evidence that UNC-45 may interact with both MHC A and MHC B in body wall muscles, I propose that UNC-45 defines a novel class of myosin-associated proteins.

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Abbreviations used in this thesis

UNC: Uncoordinated

MHC: Myosin heavy chain

NMY: Non-muscle myosin

UBS: UNC-45 binding site

GFP: Green fluorescent protein

UFD2: Ubiquitin fusion degradation protein-2

U box: UFD2-homology region

CeUFD2: *C. elegans* UFD2 homologue

DIC: Differential interference contrast

TPR: Tetratricopeptide repeats

UTR: Untranslated region

PCR: Polymerase chain reaction

RNAi: RNA interference

bHLH: Basic helix-loop-helix

MADS box: Homology among yeast (MCM1, Agamous), plant (Deficiens) and vertebrate (SRF) proteins

SH3: Src homology type 3

PH: Pleckstrin-homology

KSL: Lysine-serine-proline

IQ: Isoleucine-glutamine

CDC: Cell division cycle

Ub: Ubiquitin

kbp: Kilobase pairs

FERM domain: (4.1, ezrin, radixin, moesin)-like domain

She: Suppressor of *HO* expression

Pat: Paralyzed and arrested at two-fold stage

Par: Partitioning-defective

(ts): Temperature sensitive mutation

(lf): Loss of function mutation

(0): Null mutation

TRITC: Tetramethyrhodamine B isothiocyanate

FITC: Fluorescein isothiocyanate

DAPI: Diamidophenylindole

DTT: Dithiothreitol

PIPES: 1,4 piperazinediethanesulfonic acid

1. Introduction

1.1 Goals of this thesis

The aim of this thesis was to determine the expression pattern of the *C. elegans unc-45* gene, to characterize the function of the UNC-45 protein and to gain an understanding of its role in the development of *C. elegans*. The phenotype of *unc-45* (*ts*) alleles has suggested that UNC-45 may play a regulatory role during muscle development, but how it plays such a role was unclear. Is UNC-45 a cytoplasmic regulator required for muscle lattice assembly? Or is it a component of muscle filaments with a catalytic activity? Is UNC-45 specifically required for muscle development? Or does it have a role other than in muscle? To test these hypotheses, the following approaches were used.

1). Northern analysis and reporter genes to determine the expression pattern of *unc-45*.

2). Yeast two-hybrid screens to identify proteins that may interact with UNC-45.

3). Immunofluorescence to determine the sub-cellular localization of UNC-45.

4). Database searches to identify putative homologues of *unc-45* in other species.

1.2. *C. elegans* as a model system

How does muscle develop? The answer to this question may help to improve the performance of a nation's athletes on the Olympic stage. Whereas some people are born to win gold medals, some are born with hereditary muscle diseases (Mastaglia and Laing, 1996). Thus, investigations of muscle development should also shed light on the improvement of human health. The use of an animal model will be useful for such investigations since humans are not directly suitable for laboratory experiments (Nonaka, 1998).

C. elegans has proven to be an excellent model system for genetic and developmental analysis since its introduction into the scientific community by Brenner about 27 years ago (Brenner, 1974). This is largely due to its simplicity, fixed cell lineage, transparent body, facility of genetic analysis and the development of technology for reverse genetics (Waterston, 1988; Moerman and Fire, 1997). However, in the area of muscle development, there is another unique advantage to the use of *C. elegans* as a model, which is its male/hermaphrodite sexual system. Hermaphrodites are self-fertile and any viable mutants can be easily maintained as homozygous stocks. Moreover, the fertilization of hermaphrodite worms is internal and they do not need to move to feed and mate (Waterston, 1988). Thus, even completely paralyzed worms are still viable and fertile. This has led to the isolation of many mutations in *C. elegans* which affect movement (uncoordinated or *unc* mutants) (Brenner, 1973 and 1974; Epstein and Thomson, 1974; Epstein, et al., 1974; Waterston, et al., 1974, 1977 and 1980; Zengel and Epstein, 1980) which would normally be lethal in other metazoans such as *Drosophila*.

The mechanisms governing muscle development and the regulation of muscle contraction are highly conserved across the metazoans (Waterston, 1988; Moerman and Fire, 1997; Geeves and Holmes, 1999; Perry and Rudnicki, 2000; Sabourin and Rudnicki, 2000; Gordon, et al., 2000). Most of the basic transcription factors governing myogenesis such as MyoD and Twist have been shown to play a similar role in both vertebrates and invertebrates (Harfe, et al., 1998b; Zhang, et al., 1999; Corsi, et al., 2000), although the *C. elegans* MEF2 homologue seems to have evolved a divergent role in development (Naya and Olson, 1999; Dichoso et al., 2000). Not only are most of the major proteins of the contractile apparatus conserved between *C. elegans* and vertebrates (Waterston, 1988; Moerman and Fire, 1997; Gordon, et al., 2000), but also the processes of myofibril assembly are very similar (Barral and Epstein, 1999; Cripps, et al., 1999; Gregorio, et al., 1999; Gregorio and Antin, 2000). It has been expected that any discoveries regarding muscle development, structure and function using a model system will be applicable to medical research and benefit humans as well.

The precisely organized sarcomeres in the muscle myofibrils arise partially by self-assembly of the actin and myosin filaments (Holtzer, et al., 1997). However, there must also be unidentified proteins participating in the assembly process or regulating muscle development in general. The availability of genetic tools and the functional conservation make *C. elegans* one of the good model organisms to search for such new molecules as mentioned above. It was in *C. elegans* that the first complete sequence of a myosin heavy chain (Epstein et al., 1974; MacLeod et al., 1981; Karn et al., 1983; Dibb et al.,1985 and 1989) and the best evidence that thick and thin filaments show independent assembly (Epstein and Fischman, 1991) were provided. *C. elegans* has also

allowed the discovery of new muscle proteins or proteins involved in the regulation of muscle development (Goetinck and Waterston, 1994; Benian et al., 1996; Venolia et al. 1999; Hresko, et al., 1999; Ding and Candido, 2000; Rogalski, et al., 2000; Plenefisch, et al., 2000).

1.3 *C. elegans* muscle

1.3.1 *Muscle development*

In vertebrates, several families of transcription factor have been shown to be involved in the regulation of myogenesis (Sabourin and Rudnicki, 2000; Perry and Rudnicki, 2000), including the MyoD family of myogenic regulatory factors, the MEF2 family of conserved myocyte enhancer factors and a subfamily of Twist transcription factors. The MyoD family is a group of basic helix-loop-helix (bHLH) transcription factors, with four identified members: MyoD, Myf-5, myogenin and MRF4 (Davis, et al., 1987; Wright et al., 1989; Edmonson and Olson, 1989; Braun, et al., 1989a, 1989b and 1990; Rhodes and Konieczny, 1989; Miner and Wold, 1989), each of which has distinct roles during myogenesis. MyoD and Myf-5 are required at an early stage of skeletal muscle differentiation (Rudnicki, et al., 1992 and 1993; Rudnicki and Jaenisch, 1995), whereas myogenin plays a role in a late stage in the differentiation process (Hasty, et al., 1993; Nabeshima, et al., 1993). The MEF2 family is a group of MADS-box-containing transcription factors and is required for full transcription of muscle-specific genes (Black, et al., 1996; Black and Olson, 1998; Naya and Olson, 1999). Twist homologues are also bHLH transcription factors required for muscle differentiation in vertebrates (Hopwood, et al., 1989; Wang, et al., 1997; Anant et al., 1998).

In *C. elegans,* there are a total of 156 muscle cells in adult males and 135 in adult hermaphrodites (Waterston, 1988). Although the generation of these cells has been well described in cell lineages (Sulston and Horvitz, 1977; Sulston et al., 1983), the molecular mechanisms governing muscle development and its function are unclear. *C. elegans* has only one member of the MyoD family, which is encoded by the *hlh-1* gene. The expression of *hlh-1* can convert a mouse fibroblast cell line into muscle and the protein product (CeMyoD) of *hlh-1* is expressed in myoblasts (Krause et al., 1990 and 1992), indicating that *hlh-1* may be a functional homologue of MyoD. CeMyoD is expressed exclusively in the body wall muscles and their precursor cells but not in the pharyngeal muscles or their precursor cells (Krause et al., 1990) Genetic evidence also suggests that CeMyoD expression during embryogenesis is essential for proper muscle function and for overall morphogenesis of *C. elegans* (Chen et al., 1994; Krause, et al., 1994). An animal homozygous for an *hlh-1* null mutation has body wall muscles, but displays only partial body wall muscle contractile function, and arrests as an embryo (Chen et al., 1992). The role of *hlh-1* in development is still unclear since it has also been shown that *hlh-1* is involved in the specification of non-muscle cell fate during postembryonic development of the mesoderm (Harfe, et al., 1998a). However, it has been recently demonstrated that the function of MyoD homologues is evolutionarily conserved since the *Drosophila* and chicken MyoD factors can rescue the CeMyoD loss-of-function phenotype in *C. elegans* (Zhang, et al., 1999).

A *C. elegans* Twist homologue (CeTwist) has also been recently identified and analyzed (Harfe, et al., 1998b; Corsi, et al., 2000). CeTwist is encoded by *hlh-8* and plays an essential role in non-striated muscle development. It is required for embryonic

formation of the non-striated enteric muscle cells, the later formation of the non-striated sex muscles and patterning in the post-embryonic M mesodermal lineage (Corsi, et al., 2000). However, it seems that the *C. elegans* MEF2 homologue (CeMEF2) has a divergent role in development in the nematode compared with *Drosophila* and vertebrates. CeMEF2 is not essential for myogenesis or development and a null *mef-2* allele can not enhance or suppress the phenotype of CeMyoD or CeTwist mutants (Dichoso, et al., 2000).

1.3.2 Muscle structure

Anatomically, the muscle system of *C. elegans* is very simple. As mentioned above, there are a total of only 156 muscle cells in adult males and 135 in adult hermaphrodites (Waterston, 1988; Moerman and Fire, 1997). These muscle cells can be subdivided according to their functions. For locomotion, there are 95 body wall muscles which are the only striated muscles (multiple sarcomeres) in the worm and are arranged in four quadrants with 23 in the left and 24 each in the other three (right, ventral and dorsal. Figure 1.1). 81 of these cells are generated during embryogenesis and the other 14 muscles are generated during larval development. For grinding food, there are 20 pharyngeal muscles that are organized into eight distinct muscle layers, each of which has three fold radial symmetry. There are also two intestinal muscles used for intestinal contraction and two anal muscles (one sphincter and one depressor) used for defecation. In males, there are 41 additional specialized muscles that are added to the tail during post-embryonic development and are used for mating. In hermaphrodites, 16 (eight vulval and eight uterine muscles and the contractile gonad sheath) muscles are added that

are used to control fertilization and egg-laying (Waterston, 1988; Moerman and Fire, 1997).

The basic organization and structure of *C. elegans* muscles are similar to those of vertebrates, but there are also some differences (Waterston, 1988; Moerman and Fire, 1997). A body wall muscle cell can be divided into three main parts: (i) the cell body, which contains the nucleus and cytoplasmic organelles, (ii) the muscle arm, which extends from the cell body to the nerve cord for receiving synaptic input from the motor neurons, and (iii) the contractile myofilament lattice, which is spindle-shaped and lies beneath the cell surface and is anchored to the membrane adjacent to the hypodermis (Waterston, 1988; Moerman and Fire, 1997). The basic unit for muscle contraction is similar to the sarcomere in vertebrates which is composed of mainly thick filaments, thin filaments, M-lines and dense bodies (corresponding to the Z-lines in vertebrate muscle. Figure 1.2). The myosin-containing thick filaments are stacked in the center and aligned by the M-lines which are anchored to the membrane and function to maintain the alignment (Francis and Waterston, 1985). The thick filaments are not directly attached to any peripheral cellular structures but interact with two sets of actin-containing thin filaments which in turn are attached to the dense bodies at both ends (Waterston, et al., 1980). The dense bodies are finger-shaped structures originating from the cell membrane that is adjacent to the hypodermis and play a role in the maintenance of the alignment of thin filaments. The dense bodies are connected to the basement membrane by a transmembrane complex which allows the transmission of contractile force from the hypodermis to the overlying cuticle (Moerman and Fire, 1997). As they appear under the electron microscope, the A-bands are the thick-filament-containing regions whereas the I-

bands are the thin-filament-containing regions (without the overlapping regions). The H-zones are those regions that contain only thick filaments (Moerman and Fire, 1997).

There are three major aspects that make *C. elegans* muscle structure different from that of vertebrates (Waterston, 1988; Moerman and Fire, 1997). The nematode muscle is organized obliquely compared with the cross-striated muscle in vertebrates (Rosenbluth, 1965; Francis and Waterston, 1985). The filaments are longitudinally oriented with an angle of about 6° to the axes of both the A-band and the body of the animal (Mackenzie and Epstein, 1980). The size and length of filaments in the *C. elegans* adult are also different from those in vertebrates. In *C. elegans*, the thick filaments are about 10 μm in length and taper in diameter from about 33.4 nm in the center to 14 nm in the distal (Mackenzie and Epstein, 1980; Epstein, et al., 1985), whereas vertebrate thick filaments in striated muscle are 1.6 μm in length and 12.0-14.0 nm in diameter (Harrington, 1979). In addition to myosin, *C. elegans* thick filaments contain paramyosin as a major component which is not seen in vertebrates. The thin filaments are about 6 μm long in *C. elegans* and 1 μm in vertebrates, but they are similar in diameter. Another difference is the attachment of muscles and, in turn, the transmission of tension. In vertebrates, the ends of muscle cells contain attachment plaques which can transfer the tension. However, the tension is transferred by the lateral attachments directly to the cuticle mediated by structures of the dense bodies and M-lines in the nematode (Waterston, 1988; Moerman and Fire, 1997).

1.3.3 Muscle genes

Besides the mutations originally reported by Brenner (1973 and 1974) as affecting muscle structure in *C. elegans*, several other screens have also been carried out to identify

additional genes that may affect muscle structure, function or development in *C. elegans* (Waterston, et al., 1980 and 1984; Williams and Waterston, 1994; Ahnn and Fire, 1994; Plenefisch, et al., 2000). In particular, Williams and Waterston have isolated a class of genes with a "Pat" phenotype (paralyzed and arrested at two-fold stage) which is a typical severe phenotype of muscle defects (Williams and Waterston, 1994). To date, more than 70 genes that are involved in the regulation of muscle development and structure have been identified. Mutations in most of these genes affect the structure of muscle, whereas some others seem to be involved in the regulation of muscle contraction and attachments (Waterston, 1988; Rogalski, et al., 1993 and 1995; Williams and Waterston, 1994; Mullen et al., 1999; Plenefisch et al., 2000). Table 1-1 is a list of the genes and their products that affect muscle development or structure. There are also a few proteins that have been characterized biochemically and proposed to be components of muscle thick filaments (α- and γ- filagenin), but their gene loci have not yet been reported (Liu et al., 1998; Liu and Epstein, per. comm.).

myo-1, myo-2, myo-3 (Albertson, 1985; Miller et al., 1986; Waterston, 1989) and *unc-54* (Mackenzie, et al., 1978; Bejsovec and Anderson, 1988) are the four structural genes that encode MHC C, D, A and B, respectively. MHC C and D are found in the pharyngeal muscles and MHC A and B in other muscles (Ardizzi and Epstein, 1987). *unc-15* encodes paramyosin (Mackenzie and Epstein, 1980; Kagawa, et al., 1989) which is also a major component of thick filaments in all muscles (Ardizzi and Epstein, 1987). β-filagenin has also been shown to be a core protein of thick filaments (Liu et al., 1998). *mlc-1* and *mlc-2* are two genes encoding the regulatory myosin light chains (Rushforth, et al., 1998). *mlc-1* and *mlc-2* have some overlapping function in some muscle types and

mlc-2 is essential (Rushforth, et al., 1998). Actin isoforms of the major components of thin filaments are encoded by *act-1* to *act-4* (Files, et al.,1983; Krause and Hirsh, 1987; Stone and Shaw, 1993), whereas tropomyosin and troponin C and T which are also components of thin filaments are encoded by *lev-11*, *pat-10* and *mup-2*, respectively (Williams and Waterston, 1994; Kagawa, et al., 1989 and 1995; Myers, et al., 1996; Terami, et al., 1999). *unc-60* and *unc-87* encode two actin-associating proteins (McKim et al., 1994; Goetinck and Waterston, 1994a and 1994b). The UNC-60 protein shows similarity to the ADF/cofilin family of actin-binding proteins and is required for proper assembly of actin into myofibrils (Ono, et al, 1999), while the UNC-87 protein has some sequence similarity to vertebrate calponin and is a structural component for maintaining the integrity of myofibril lattice of body wall muscles during and after contraction (Goetinck and Waterston, 1994a).

Genes encoding components of other parts of muscle structure or regulatory factors required for muscle development have also been characterized. *deb-1, pat-2, pat-3* and *unc-52* encode vinculin, α-integrin, β-integrin and perlecan, respectively (Barstead and Waterston, 1989 and 1991; Barstead, et al., 1991; Williams and Waterston, 1994; Gettner, et al., 1995; Rogalski, et al., 1993), which are components of the extracellular matrix or basement membrane and are involved in the attachment of muscle cells. *unc-22* and *unc-89* encode two large proteins belonging to the twitchin/titin family (Moerman, et al., 1986 and 1988; Benian, et la., 1989, 1993 and 1996). The UNC-22 protein possesses a kinase domain at its C-terminus and is localized to the A-band region of the sarcomere, suggesting a role in the regulation of muscle contraction (Benian, et al., 1989). The UNC-89 protein is probably associated with the M-line and may be involved in the G-protein-

mediated signal transduction pathway regulating the alignment of thick filaments (Benian, et al., 1996). The *emb-9* and *let-2* gene products are type IV collagen molecules which are also components of muscle-associated basement membranes (Sibley, et al., 1993 and 1994). *atn-1* encodes α-actinin, the major component of dense bodies (Barstead, et al., 1991). The *unc-93* gene product is a putative trans-membrane protein that regulates muscle contraction (Levin and Horvitz, 1992), *unc-97* encodes a LIM protein that functions in adherens junction assembly of muscles (Hobert, et al., 1999) and *unc-112* encodes a protein of the FERM family that is required to organize β-integrin into the basal cell membrane (Rogalski, et al., 2000). *hlh-1* and *hlh-8* encode two transcription factors: MyoD and Twist, respectively (Krause et al., 1994 and Corsi, et la., 2000) as discussed earlier. *etr-1*, encoding a RNA-binding protein, is essential for muscle development (Milne and Hodgkin, 1999). UNC-68, a ryanodine receptor, is involved in the regulation of calcium ions in the striated muscles (Maryon et al., 1998). HSP-25, a heat shock protein, is associated with the dense bodies and M-lines of body wall muscle (Ding and Candido, 2000). Some *mua* genes have also been shown to be required for muscle attachments (Plenefisch, et al., 2000). Finally, the subject of this thesis, *unc-45*, one of the first mutants reported in *C. elegans,* is involved in the regulation of muscle thick filament assembly (Epstein and Thomson, 1974) and is also essential for muscle development (Venolia and Waterston, 1990), but its molecular function is unclear.

1.3.4 Muscle assembly

As mentioned above, *C. elegans* muscle thick filaments are mainly composed of paramyosin, myosin and filagenins, whereas thin filaments consist of actin, tropomyosin

and troponin (Figure 1.3). However, how these individual components are assembled into the precisely organized myofilament lattice is still not well understood.

Based on immuno-electron microscopy studies and computer analyses, a three-dimensional model for *C. elegans* muscle thick filaments has been proposed (Epstein, et al., 1995; Liu, et al., 1997; Schmid and Epstein, 1998; Barral and Epstein, 1999). In this model, muscle thick filaments are rigid coupled tubules and their core structures are composed of an outer layer of seven paramyosin-containing subfilaments which are cross-linked by other proteins, such as filagenins and additional internal proteins (Figure 1.4 A). Each subfilament consists of two strands of paramyosin molecules which are staggered by 72 nm with respect to one another with a 22 nm gap between consecutive paramyosin molecules in each strand (Epstein et al., 1995. Figure 1.4 B). The non-helical ends of paramyosin and the unpaired regions contain sites for interaction with other molecules which may be critical for the assembly of thick filaments and their regulation. Upon this backbone, additional paramyosin and myosin isoforms and other additional proteins are assembled.

The members of the twitchin/titin family may also be involved in the regulation of muscle assembly or contraction (Johnson and Quiocho, 1996; Gregorio, et al., 1999; Gregorio and Antin, 2000). In *C. elegans*, twitchin is encoded by *unc-22* and is a 750 kD giant protein which contains a single protein kinase domain at its C-terminus, 31 copies of a fibronectin type-III-like domain, and 30 copies of an immunoglobulin superfamily C2-like domain (Benian et al., 1989 and 1993). Twitchin co-localizes with MHC B in muscle A-bands and may play a role in the regulation of muscle contraction, since *unc-22* mutant animals have near-normal muscle structure at young stages but progressively get

disorganized muscle structure with age, which is probably caused by the constant twitching of muscle cells (Moerman et al., 1988; Benian, et al., 1989 and 1993). *unc-89* also encodes a 732 kD member of the twitchin/titin family (Benian et al., 1996) which contains a complex series of SH3, CDC24 and PH (Pleckstrin-Homology) domains, seven immuno-globulin domains, a KSP (Lysine-Serine-Proline)-containing multiphosphorylation domain, and 46 immuno-globulin domains in tandem. UNC-89 is localized to the middle of muscle A-bands and may be associated with the M-line (Benian, et al., 1996). It may function in regulation of the alignment of thick filaments since *unc-89* mutant animals have a normal thick filament number but improper alignment (Waterston, et al., 1980). UNC-45 is another protein which has been suggested to be involved in the regulation of thick filament assembly (Epstein and Thomson, 1974; Venolia and Waterston, 1990). UNC-45 contains three tetratricopeptide repeats (TPR) at its N-terminus and much of its C-terminus shows similarity to the yeast She4 protein (Barral and Epstein, 1998; Venolia, et al., 1999), but its function is unknown.

1.3.5 Muscle myosin

Myosins are a large superfamily of actin-dependent molecular motors characterized by their interaction with actin, hydrolysis of ATP and production of movement using the generated energy (Titus, 1998; Sellers, et al., 2000; Rodriguez and Cheney, 2000). Typically, a myosin molecule has three functional domains: (i) the head domain (also called the motor domain) which interacts with actin and binds ATP, generating energy; (ii) the neck domain which binds to myosin light chains or calmodulin, functioning in regulation; and (iii) the rod domain (or the tail domain) which serves to anchor and position the motor domain to interact with actin (Figure 1.5). The

head domains are highly conserved, while the tail domain can vary widely in length and sequence. The neck domains usually contain a few IQ motifs that bind with light chains or calmodulin, whereas the tail domains may contain one or more functional domains, such as SH3 and FERM domains (Sellers, 2000). In addition, the tail of many myosins are coiled-coil sequences which function in dimerizing and producing two-headed myosins (Figure 1.5).

Phylogenetically, the myosin family is divided into 15 classes. The largest and best-characterized group includes the class II myosins which are the conventional filament-forming myosins found in both muscle and non-muscle cells. Little is known about the cellular functions of other unconventional myosins (Mooseker and Cheney, 1995; Baker and Titus, 1997) except that class I myosins are involved in organelle translocation, ion channel gating and cytoskeletal reorganization (Barylko, et al., 2000); class V myosins function in mRNA transport, cell polarity and membrane trafficking (Reck-Peterson, et al., 2000); and class III & IX myosins appear to play a role in phototransduction and other signaling pathways (Bahler, 2000). Interestingly, there is no conventional class II myosin found in plants, but some myosin classes (VII, XI and XIII) are found exclusively in plants. However, all animals examined contain at least one myosin II gene and usually multiple myosin I genes (Seller, 2000). While most characterized myosins move only towards the barbed (+) end of the actin filament, an unconventional myosin (class VI) has recently been shown to move "backwards" to the pointed (-) end of the actin filament (Schliwa, 1999; Wells, et al, 1999). This raises intriguing questions about the molecular mechanisms of "backward" movement and biological roles of this myosin (Cramer, 2000; Rodriguez and Cheney, 2000; Titus,

2000). There is evidence suggesting that myosin VI may play a role in organelle or particle transport and membrane-cytoskeleton interaction (Mermall, et al., 1994; Hasson, et al., 1997; Kelleher, et al., 1999), but this transportation may bring materials or membrane into the cell (Titus, 2000) and this interaction could be involved in generation of a cell expansion force (Cramer, 2000).

In *C. elegans*, there are four genes for muscle myosin II (*myo-1, myo-2, myo-3* and *unc-54*), two for non-muscle myosin II (*nmy-1* and *nmy-2*), two for myosin I (*hum-1* and *hum-5*), and one each for myosin V (*hum-2*), myosin VI (*hum-3*), myosin VII (*hum-6*) and myosin XII (*hum-4*) as reported so far (Baker and Titus, 1997). The four muscle myosin II genes encode the **m**yosin **h**eavy **c**hain isoforms (MHC A, B, C and D), respectively, which are major components of thick filaments as mentioned earlier. The non-muscle myosin II gene, *nmy-2*, encodes NMY-2 which is detected in the germ-line cells of the gonad and premorphogenesis embryos, and is involved in regulating embryonic polarity and cytokinesis (Guo and Kemphues, 1996; Shelton, et al., 1999). *nmy-1* and all other unconventional myosin genes have not yet been characterized.

MHC C and D are expressed exclusively in the pharyngeal muscles, whereas MHC A and B are expressed in body wall muscles and other single-sarcomere muscles (Ardizzi and Epstein, 1987), but how these myosin isoforms are assembled into muscle thick filaments is not yet well understood. As discussed earlier, the core structure of thick filaments is composed of seven paramyosin subfilaments and associated proteins, which may be a template for later myosin assembly. Although there are two myosin isoforms in an individual muscle cell, these myosins primarily exist as homodimers rather than heterodimers (Schachat et al., 1978). In the body wall muscles, it has been demonstrated

that MHC A and B are differentially localized in the thick filaments (Miller, et al., 1983). MHC A is localized in the central area (1.8 μm long) of the thick filament and MHC B in the two polar regions (about 4.4 μm in length) with about 0.45 μm overlap region on each side with MHC A. MHC A is essential for the initiation of assembly since animals lacking MHC A are arrested at the two-fold stage and no normal thick filaments are formed in muscles (Waterston, 1989). However, MHC B null animals are viable but have disorganized thick filaments, some of which are wild-type in length and entirely composed of MHC A (Epstein, et al., 1974 and 1986). This indicates that MHC A is capable of at least partially replacing MHC B function. This is further supported by *sup-3* mutants in which MHC A is overexpressed, thick filaments are nearly normal and movement is largely restored even if MHC B is absent (Riddle and Brenner, 1978; Maruyama, et al., 1989). In contrast, overexpression of MHC B cannot rescue mutants that lack MHC A (Waterston, 1989). Interestingly, it has also been shown that the differential localization of MHC A and B in thick filaments is disrupted in *unc-45* (*ts*) mutants (Barral and Epstein, 1998), but how the *unc-45* gene product (UNC-45) is involved in the assembly or localization of MHC A and B is unclear.

As discussed earlier, MHC A and B are conventional myosin II molecules. Their tail domains exhibit the typical features of coiled-coil structure and form homodimers. Whereas MHC B dimer molecules are packed in a parallel manner in the two polar regions of thick filaments, MHC A must be packed in an antiparallel manner in order to generate the central bare zone where no myosin heads are present (Figure 1.6). MHC A is present in this central region and it must have some unique features that allow it to assemble at the M-line and be capable of antiparallel packing. To map the regions unique

to MHC A that are sufficient for the initiation of thick filament assembly, DNA constructs that can encode MHC A/MHC B chimerical proteins *in vivo* after transformation were produced (Hoppe and Waterston, 1996. Figure 1.7). After analysis of their ability to rescue an MHC A null mutant and produce thick filaments, two MHC A-function specific regions have been determined. One is a 263-residue segment localized in the middle of the rod domain of MHC A and the other one is 169-residues near the end of the C-terminus of MHC A (Figure 1.7). Either is sufficient to confer MHC B the ability to rescue a MHC A null mutant (Hoppe and Waterston, 1996). In these two critical regions, MHC A is more hydrophobic on the rod outer surface which is thought to mediate dimer-dimer interactions for filament assembly. Moreover, this feature makes MHC A more similar to paramyosin and has led to the suggestion that close contacts between MHC A and paramyosin may be critical for the antiparallel packing of MHC A in the central region (Hoppe and Waterston, 1996). There is evidence that ionic interactions play an important role in myosin rod assembly (Arrizubeita and Bandman, 1998) and that even a single charge change within the rod domain can inhibit filament assembly (Moores and Spudich, 1998).

Whereas the rod domains of myosins are involved in the assembly of thick filaments, the head domains are mainly required for other functions, such as ATP hydrolysis for converting chemical energy into mechanical energy and actin-binding for generating movement. There is a class of missense mutations within the MHC B head domain, which largely affect the contraction-relaxation cycle with a long duration of tight binding between myosin and actin but have no or little effect on muscle structure and motility (Moerman, et al., 1982; Dibb et al., 1985). There is also evidence from

Drosophila that myosins without the head domain can assemble into thick filaments (Cripps, et al., 1999). However, there is another class of MHC B missense alleles that suggest a role for the myosin head domain in filament assembly (Bejsovec and Anderson, 1988 and 1990). These alleles were identified as dominant-negative mutations. Heterozygous *unc-54* (*d*) worms are paralyzed and have disorganized thick filaments, whereas homozygous animals are very sick or inviable. It has been proposed that the *unc-54* (*d*)-induced lethality is caused by the toxicity of the remaining altered MHC B in the muscles since *unc-54*(*0*) mutants are viable although paralyzed (Bejsovec and Anderson, 1990). How the myosin head domain is involved in the assembly or stability of thick filaments is not understood.

1.4 *C. elegans* embryogenesis

1.4.1 Overview of embryogenesis

C. elegans embryogenesis has been described in detail (Sulston, et al., 1983; Wood, 1988; Strome, 1989). At 20°C, it takes about 14 hours for a newly fertilized zygote to complete embryogenesis and hatch into a larva. After fertilization, the embryo undergoes a series of unequal cell divisions to produce somatic founder cells (AB, E, MS, C and D) and the primodial germ cell (P4) in the first few hours (Figure 1.8). At the 28-cell stage, gastrulation begins when the two daughters of E move to the interior of the embryo, followed later by P4 and some of the descendants of other somatic founder cells. Continued proliferation generates a triploblastic embryo of 300 cells, with an internal cylinder of pharynx and gut primordia, an outer layer of hypodermal and neuronal precursors, and four quadrants of body wall myoblasts. Most of the cell divisions are complete by the time the embryo has reached about 550 cells. During the later half of

embryogenesis, it undergoes morphogenesis, organogenesis and elongation of the body until hatching. Figure 1.9 shows some of the stages of early embyrogenesis which will be referred to in the text.

1.4.2 Embryonic polarity

The anterior-posterior polarity in the *C. elegans* zygote is initiated shortly after sperm entry into a previously symmetrical oocyte (Goldstein and Hird, 1996). Based on the position of the sperm pronucleus and associated centrosomes, a polarized cytoplasmic flow occurs within the zygote. Internal cytoplasm flows toward the paternal pronucleus-centrasomal complex whereas cytoplasm near the cortex flows away, resulting in an arrangement where the pronucleus is at one end of the oblong zygote defining this position as the posterior pole (Goldstein and Hird, 1996). The first cleavage produces two daughter cells with different sizes and developmental potentials (Cowan and Macintosh, 1985; Priess and Thomson, 1987. Figure 1.10). Further development results in the dorsal-ventral asymmetry in addition to the anterior-posterior polarity. The microfilament-mediated cytoplasmic flow has been suggested to play an important role in this process (Strome and Wood, 1983; Hill and Strome, 1988; Hird et al., 1996; Golden, 2000).

P granules, which are ribonucleoprotein particles and distributed evenly in the oocyte, become localized to the posterior end of the zygote after fertilization and are partitioned asymmetrically at each of the unequal divisions (Strome and Wood, 1982 and 1983. Figure 1.10). In the embryo, they are associated with the germ-line cell fate during development, and remain associated with the germ lineage throughout the life of the

worm. Because of their asymmetrical distribution, P granules are useful markers of early embryonic polarity.

1.4.3 Genes required for embryonic polarity

Genetic screens for early regulators of pattern formation have led to the identification of six *par* genes (for partitioning-defective), which are required for some aspects of anterior-posterior polarity in the zygote, such as P-granule localization, pseudocleavage, cytoplasmic streaming and asymmetric placement of the first cleavage spindle (Kemphues, et al., 1988; Kemphues, 1989; Kirby et al., 1990). The PAR proteins have been shown to co-localize with actin microfilaments in the cortical regions of the cytoplasm and PAR-1, a serine/threonine kinase, interacts with a non-muscle myosin II heavy chain (NMY-2) which is also present in the cortical region of early embryos (Guo and Kemphues, 1996). This interaction is consistent with the role of NMY-2 in embryonic polarity and cytokinesis (Guo and Kemphues, 1996). Recently, a non-muscle myosin II regulatory light chain (MLC-4) has also been shown to be required for cytokinesis and anterior-posterior polarity (Shelton, et al., 1999). It may be involved in the *let-502/mel-11* regulatory pathway (Shelton, et al., 1999). LET-502 (a Rho-dependent kinase) and MEL-11 (a myosin phosphatase), which were originally identified as regulators for the hypodermal microfilament cytoskeleton during embryonic elongation (Wissmann, et al., 1997 and 1999), may also have a role in cytokinesis in early embryos (Piekny and Mains, pers. comm.). The coronin-like protein POD-1(actin-binding protein) has also recently been shown to be required for anterior-posterior axis formation and cellular architecture (Rappleye, et al., 1999). It may play a role in intracellular trafficking and organizing specific aspects of the actin cytoskeleton.

1.5 Genetic analysis of *unc-45*

A huge collection of *C. elegans* mutant strains was generated using ethyl methanesulphonate by Dr. Sydney Brenner and co-workers in the middle 1960's after he had chosen this little soil nematode as a model system for genetic and developmental analysis (Brenner, 1973 and 1974). *unc-45* (*e286*), which is temperature-sensitive and recessive, is one of the first mutations reported from this collection (Epstein and Thomson, 1974). Wild-type nematodes (N2) are highly motile between 15°C and 25°C. In contrast, when grown at temperature of 20°C or above, homozygous *e286* mutant animals are paralyzed with very slow body movements and a smaller brood size. However, if grown at 15°C, the movement and brood size of *e286* mutants are indistinguishable from those of wild-type worms (Epstein and Thomson, 1974). Heterozygous worms (+/e286) are normal at either temperature regarding both movement and structure, indicating that one dose of normal UNC-45 is sufficient for its function. There is an interesting observation pertaining to the temperature-sensitive phenotype of this allele. If the homozygous mutants are grown at 15°C in their early stages (before L4), shifting the temperature to 25°C will result in paralyzed adults. Conversely, mutant worms growing at 25°C will lead to normal motile animals if switched to 15°C before the L4 stage (Epstein and Thomson, 1974). Under the polarized light microscope, motile *e286* mutant adults raised at 15°C exhibit the typical wild-type muscle structures with distinguishable A-bands, I-bands, H zones and dense bodies, but paralyzed adult worms raised at 25°C do not exhibit these regular muscle structures. Moreover, when examined by electron microscopy, the paralyzed worms have a reduced number of recognizable thick filaments. Moreover, they are disorganized with thin filaments presenting at various

angles to the thick filament arrays, compared with wildtype worms which have parallel thin filament arrays around thick filaments (Epstein and Thomson, 1974. Figure 1.11). Also, it seems that there is no apparent change in the sizes and amounts of some major myofilament proteins in the mutant worms grown at either temperature. These observations have led to the suggestion that the *unc-45* (*e286*) mutation may affect the formation of thick filaments during the period of muscle development. One possible explanation is that UNC-45 has a catalytic function required for proper assembly of thick filaments (Epstein and Thomson, 1974). Other *unc-45* (*ts*) alleles were subsequently isolated from different laboratories (Venolia and Waterston, 1990): *m94* (D. L. Riddle), *r450* (R. P. Anderson), *b131*(D. Hirsh) and *su2002* (H. E. Epstein). All these alleles show the paralyzed phenotype at 25°C with differing severity.

The isolation of three recessive lethal alleles of *unc-45* by a non-complementation screen has demonstrated that *unc-45* is an essential gene (Venolia and Waterston, 1990). The two more severe alleles *st601* and *st603* show identical phenotypes, whereas the *st604* allele has a maternal effect. Grown at either 15°C or 20°C, *st601* and *st603* homozygous embryos from an *unc-45* (*ts*)/(*st601* or *st603*) mother show a "Pat" phenotype (paralyzed and arrested at two-fold stage). These embryos fail to show pharyngeal pumping and hatching is much delayed or blocked, but other aspects of development seem normal at the two-fold stage. *st601* and *st603* homozygous embryos from an *unc-45* (+)/(*st601* or *st603*) mother show a milder phenotype: most can develop until the three-fold stage and slight muscle movements can be seen; some even show pharyngeal pumping and can hatch but arrest at the L1 stage without unfolding (Venolia and Waterston, 1990). *st604/st604* embryos from an *st604/unc-45* (*ts*) mother arrest at

various embryonic or L1 stages but before any larval molting occurs. In these animals, some body wall muscle contraction can be seen, but the hatched larvae generally fail to unfold. However, *st604* homozygotes from an *st604/unc-45* (+) mother are viable and fertile, although they show an "*unc*" phenotype, especially at higher temperatures, which is not as severe as *unc-45* (*ts*) mutants at the restrictive temperatures. The progeny of such *st604* homozygotes arrest mostly as embryos at the two-fold stage with a few (1-2%) that can survive to adulthood and show a similar phenotype as the maternally rescued animals. The function of this maternally provided *unc-45* product in embryogenesis is unclear, but the rescue of *st604* (probably not null) and failure to rescue *st601* or *st603* (probably null) indicate that the maternal product may be necessary for normal early development.

In *unc-45* (*lf*) animals, the expression of *unc-54* and *myo*-3 is normal and the assembly of MHC A and B can even be initiated but no A-bands can be formed (Venolia and Waterston, 1990). There is evidence that UNC-45 may interact with myosin heavy chain isoforms in muscles. Previous genetic analyses show that *unc-54* is epistatic to *unc-45* (Waterston, et al., 1980; Waterston and Curry, cited in Waterston, 1988). This can be explained if MHC B molecules are abnormal or non-functional in *unc-45* animals. The toxic MHC B proteins can not account for the lethality of *unc-45* (*lf*) animals, since the double mutant *unc-54* (*0*); *unc-45* (*lf*) in which MHC B has been removed has the same phenotype as *unc-45* (*lf*) single mutant. UNC-45 must interact with other proteins in addition to MHC B, since the *unc-45* (*lf*) phenotype (lethal) is much more severe than the *unc-54* (*0*) phenotype (paralyzed but viable). The similar "Pat" phenotype of *myo-3* (*lf*) and *unc-45* (*lf*) mutants suggest that MHC A may be another target of UNC-45. In *unc-*

45 (*lf*) allele animals, MHC A molecules may also be in an inactive form as MHC B. This is consistent with the abolition of maternal rescue of *st604* when MHC A is overexpressed in a *sup-3* background. A double mutant *unc-54* (*0*); *myo*-3 (*0*) in which both MHC A and MHC B have been removed has normal pharyngeal pumping function, but pharyngeal pumping is not observed in *unc-45* (*lf*) animals though MHC A and B are not expressed in the pharynx (Venolia and Waterston, 1990). This indicates that UNC-45 function is required in the pharynx, presumably through its interaction with MHC C and/or D.

1.6 Molecular analysis of *unc-45*

When I joined this lab in 1995, the *unc-45* gene had been cloned and sequencing had begun. This gene is located on the left end of linkage group III between *vab-6* and *fem-2* (Pilgrim, 1993), and encodes a novel protein of 961 amino acids. This protein contains three tandem tetratricopeptide repeats (TPR) at its amino terminus and a region with similarity to fungal CRO1/She4 proteins in the carboxyl terminal half (Venolia et al., 1999. Figure 1.12). The central region (about 350 amino acids) does not show similarity to any proteins of known functions. TPR are loosely conserved 34-amino acid sequence motifs that have been shown to function as scaffolding structures to mediate protein-protein interactions with apparently divergent cellular functions (Sikorski, et al., 1991; Goebel and Yanagida, 1991; Das, et al., 1998; Blatch and Lassle, 1999). Most TPR-containing proteins are associated with multi-protein complexes and important to the function of chaperone, cell-cycle, transcription and protein transport complexes (Blatch and Lassle, 1999; Melville et al., 2000). A TPR-containing cyclophilin has been shown to be required for larval muscle development in parasitic and free-living nematode

species (Page and Winter, 1998) and another TPR-containing protein (P581PK) has also recently been shown to have co-chaperone and oncogenic properties (Melville, et al., 2000). This suggests that UNC-45 may function as a chaperone involved in myosin assembly in *C. elegans*.

The C-terminal domain of UNC-45 is similar to part of the *Podospora* CRO1 protein and the *S. cerevisiae* She4 protein. The CRO1 protein is required for the transition between the syncytial and cellular states of the filamentous fungus *P. anserina* (Berteaux-Lecellier et al., 1998). The She4 protein is involved in the mating type switching of the budding yeast (Jansen et al., 1996; Wendland et al., 1996). In yeast, an asymmetrical cell growth/division occurs in some cases, such as pseudohyphal growth, mating type response and cell fate determination (Madden and Snyder, 1998). Two mating types exist in yeast: **a** and α. In nature, the haploid yeast can switch their mating type: **a** cells convert to α and α cells change to **a**. In this process, mother cells can switch but daughter cells do not. This is due to mother cell-specific expression of the *HO* protein, which is an endonuclease that initiates gene conversion of the mating type locus (Strathern and Herskowitz, 1979; Nasmyth, 1993). The expression of the *HO* gene is repressed in the daughter cell by the Ash1 protein (Bobola et al., 1996; Sil and Herskowitz, 1996), which is a zinc finger protein related to GATA transcription factors and has also been shown to be involved in pseudohyphal growth of diploid yeast cells (Chandarlapaty and Errede, 1998). Ash1 mRNA has been shown to be asymmetrically localized in the daughter cell and this localization requires five *She* genes (Jansen et al,, 1996; Takizawa et al., 1997; Long et al., 1997. Figure 1.13). *She1* encodes a type V myosin, Myo4p, which has been shown to bind to the 3' UTR of *Ash1* mRNA and is required for the transport of this

mRNA into the daughter cell (Munchow et al., 1999; Takizawa and Vale, 2000; Fischer, 2000). She2p and She3p are also involved in this process (Bertrand, et al., 1998; Munchow et al., 1999; Takizawa and Vale, 2000) and it has also been suggested that She4p may be required for the assembly of the transport particle although its specific function is unclear (Bertrand, et al., 1998). Based on the sequence similarity of UNC-45 and She4p, UNC-45 may be involved in thick filament assembly by interactions with one or both myosin heavy chains isoforms in body wall muscles directly or indirectly.

Moreover, sequence analysis of *unc-45* mutant alleles has revealed that the CRO1/She4 domain is critical for thick filament assembly (Barral and Epstein, 1998). Three of the four *ts* mutations sequenced (*e286*, *su2002*, and *m94=r450*) are missense substitutions in codons that encode conserved residues located in the CRO1/She4 domain and the other *ts* mutation (*b131*) is also a missense substitution found just upstream of the conserved domain (Figure 1.12). Two lethal alleles (*st601* and *st603*) are found to be amber mutations in the central region and they are predicted to produce truncated proteins lacking the CRO1/She4 domain. Thus, the change of one amino acid in the CRO1/She4 domain can cause an uncoordinated phenotype and the absence of the CRO1/She4 domain is correlated with the lethal phenotype.

1.7 Summary of this thesis

As mentioned earlier, this *unc-45* project was started when this gene had been cloned and its protein sequence had been analyzed as above. However, the function of UNC-45 and it's involvement in thick filament assembly was unclear, as was whether UNC-45 is a component of the thick filaments, or a cytoplasmic regulatory factor required for thick filament assembly. To address these questions, I mainly applied the

following approaches: the use of reporter genes and Northern analysis to determine the expression of *unc-45*; yeast two-hybrid screens to identify proteins that may interact with UNC-45; immunofluorescence studies to determine UNC-45 subcellular localization; and analysis of UNC-45 sequence homologues in other species. A summary of my major findings follows.

I showed that *unc-45*::GFP reporter fusions are expressed in all muscle cells and a functional UNC-45:GFP fusion is localized to the A-bands of thick filaments in body wall muscles. Immunofluorescence experiments demonstrated that UNC-45 co-localizes with MHC B in the thick filaments of wild-type worms and is also associated with the abnormal thick filaments in *unc-45* (*ts*) mutants grown at the restrictive temperature. In *unc-54* null animals, in which MHC B is absent, and in *unc-54*; *sup-3* mutants, in which increased MHC A partially compensates for lack of MHC B, UNC-45 cannot be detected in association with thick filaments. Therefore, I concluded that UNC-45 is a component of muscle thick filaments due to its co-localization with MHC B in the body wall muscles. Furthermore, I showed that UNC-45 may be added to thick filaments after MHC isoforms have been assembled.

The UNC-45 protein is also maternally contributed and is present in all cells of the early embryo. Zygotic UNC-45 expression is only detected in the developing muscle cells. Yeast two-hybrid screens demonstrated that UNC-45 interacts with NMY-2, a non-muscle type II myosin. These two proteins also co-localize at cell boundaries in early embryos. Localization of UNC-45 at these boundaries is dependent on the presence of NMY-2. These results suggest that UNC-45 may interact with more than one type of myosin and have a function in the embryo other than in thick filament assembly and

stability during muscle development. Moreover, UNC-45 may also define a novel class of myosin-associated proteins.

2. Materials and Methods

2.1 Strains and genetics

N2 (wild-type) and strains carrying *unc-45* mutant alleles *e286*, *r450*, and *m94* were obtained from the stock collection of the MRC Laboratory of Molecular Biology, Cambridge, UK; CB190 [*unc-54* (*e190*)] from the *Caenorhabditis* Genetics Center, University of Minnesota, USA and double mutant RW2329 [*unc-54* (*e190*); *sup-3* (*e1407st90st92*)] and triple mutant RW2665 [*unc-54* (*e190*); *unc-45* (*m94*); *sup-3* (*e1407st90st92*)] from the laboratory of R. H. Waterston (Washington University, St. Louis, USA). The strains were maintained as described (Wood, 1988).

The yeast strain PJ69-4A (along with the series of expression vectors pGAD-1, -2, -3 and pGBDU-1, -2, -3) for the two-hybrid screen was kindly provided by Dr. Philip James (University of Wisconsin, Madison, WI, USA) and maintained as described (James, et al., 1996). All yeast transformations were done using the high efficiency lithium acetate method (Gietz and Schiestl, 1995).

Drosophila strain 11602 [P(ry$^{+t7.2}$=PZ)Tom34^{03692}ry506/TM3, ry^{RK}5b^{1}Ser^{1}] was obtained from the Bloomington *Drosophila* stock center (USA) and this strain was originally generated in Dr. Allen Spradling's laboratory (University of California at Berkeley).

2.2 Northern analysis

Total RNA was prepared from mixed-stage cultures of wild-type animals using TRIzol reagents (GIBCO BRL) and poly A$^+$ RNA was purified from the total RNA using oligo(dT) cellulose columns (GIBCO BRL) according to the manufacturers' instructions. Northern analysis was performed using 1% agarose gels containing formaldehyde as

described (Sambrook et al., 1989). Radiolabelled antisense RNA probes were synthesized using T7 or T3 RNA polymerases as directed by the manufacturer (Promega), using a linearized *unc-45* cDNA clone on a pBluescript vector as a template.

2.3 Sequencing of the *C. briggsae unc-45* homologue

Using low stringency hybridization, the genomic DNA corresponding to a putative *C. briggsae unc-45* homologue was previously cloned in this lab, and part of one subclone (pDP#Cb004, about 1.8 kbp) had been sequenced. The sequence of the remainder of pCb004 was determined during this thesis, which corresponds to exons 6 to 10 of the *C. elegans unc-45* gene with two introns. Another subclone pDP#Cb002 was also sequenced and it connects with pCb004 within exon 6 and extends to the 5' end of exon 1 of the *C. briggsae unc-45* gene. To finish the whole sequence, two more fragments were subcloned and sequenced: one corresponds to exon 11 and the 3'-untranslated region of the *C. briggsae unc-45* gene, the other overlaps from exon 10 to exon 11 of the *C. briggsae unc-45* gene.

2.4 Transgenic lines

An approximately 4.4 kbp promoter region upstream of the *unc-45* gene was amplified by PCR from genomic DNA using the primers DPU1 (5' GCGTCTAGAAAAGCTGGCCC 3') and DPU2 (5' CAATACTGCAGAGAGTCACG 3'). The PCR product was digested with *Pst*I, and the resulting 4.4 kbp fragment was cloned into pBluescript KS^- to yield pDP#WA002. This should contain the upstream region and the first 18 codons of the predicted *unc-45* coding region, as verified by restriction mapping. The 4.4 kbp *Pst*I fragment from pDP#WA002 was subcloned into the promoterless Green Florescent Protein (GFP) vector pPD95.81 (Ahn et al., personal

communication) to yield pDP#WA009 (*unc-45*:GFP), and into the promoterless nuclear-localization-signal containing (lacZ:SV40NLS) vector pPD95.10 (Fire et al., 1990) to yield pDP#WA004 (*unc-45*:lacZ:NLS). Both vectors should result in fusion proteins containing the first 18 amino acids of UNC-45.

A "minigene" cDNA version of the *unc-45* gene was also amplified by RT-PCR as described below. First-strand cDNA synthesis was primed with WA08 (5' ATCCACGTGGATGAAATATAGCAG 3') from total RNA (mixed stages). A fragment containing the open reading frame was amplified using the primers LV+7 (5' ATGGTTGCTCGAGTACAGAC 3') at the 5' end and WA05 (5' CAACCCGGGTTCCTGAATGGTGCTCATTTG 3') at the 3' end. The 2.9 kbp product was purified, digested by *Pst*I (at the 5' end) and *Sma*I (introduced at the 3' end by the primer) and then cloned into vector pPD95.79 (Ahn et al., pers. comm.) at these two sites. The 4.4 kbp-*Pst*I fragment containing the *unc-45* upstream region (which also contains the sequence of exon 1 before the *Pst*I site) from pDP#WA002 was inserted into the *Pst*I site of the above construct to make a plasmid pDP#WA036 that should contain the full length *unc-45* cDNA and driven by the *unc-45* promoter. pDP#WA036 was used to inject *unc-45* (*r450*) mutant animals together with the pRF4 plasmid containing the dominant marker *rol-6* (*su1006*). The mutant worms were grown at 15°C before injection and at 25°C after injection. The resulting transgenic lines were examined for rescue under the dissecting microscope by examining their ability to move. A transgenic line (DP193 edEx74) generated as described (Mello, et al., 1991) contains this construct as part of an extrachromosomal array along with pRF4 (*rol6* [*su1006dm*]). An isolate of this line

showing transmission of the array to more than 95% of the progeny was used in this study.

A 4.9 kbp promoter region of *CeUFD2* was amplified using primers WA32 (5' CTCAGCTGCAGTGATTTGCC 3') at about 4.9 kbp upstream of the start codon and WA33 (5' ACCACTGCAGCATCACTTATGTC 3') at the end of first exon (about 18 amino acids downstream of the start codon). A *Pst*I restriction site was introduced on both ends. The amplified PCR product was digested with *Pst*I and cloned into the *Pst*I site of the GFP vector pPD95.79. The orientation of the construct was confirmed by restriction analysis. This construct was injected into wild-type worms (L4 or young adults) with dominant co-injection marker pRF4 to generate transmitting lines.

The putative *Drosophila unc-45* homologue cDNA was cloned by RT-PCR. The first strand of cDNA was synthesized using primer WA27 (5' CGCGATGACACTGTGAGTAAAGTAAA 3') at the 3' UTR from total RNA (kindly provided by Zhigang Jin and Graham Banting) purified from wild-type *Drosophila* adult females. A segment containing the open reading frame (about 2.9 kbp) was amplified using primer WA28 (5' TAACGTCGACAAACACCATCAACAGC 3') at the 5' end of this homologue and WA29 (5' TTTCCCGGGATCATCGATAATCTCAG 3') at the 3' end. The 2.9 kbp PCR product was purified, digested by *Sal*I (introduced at the 5' end by the primer) and *Sma*I (introduced the 3' end by the primer), and then cloned into the GFP vector pPD95.79 (Ahn, et al., pers. comm.) at these two sites.

Animals transgenic for the lacZ fusions were fixed and stained for β-galactosidase activity as described (Fire et al., 1990). GFP expression was examined under

fluorescence with a Zeiss Axioskop (Carl Zeiss) or a confocal microscope (Molecular Dynamics 2001).

2.5 RNA interference

A 1.6 kbp fragment of *nmy-2* cDNA, which corresponds to a segment of the NMY-2 protein from amino acids 469 to 999 on the plasmid pACT (Elledge et al., 1991; Durfee et al., 1993) isolated from the yeast two-hybrid screen, was re-cloned into the pBluescript KS^- vector. A double-stranded linear fragment which contains the 1.6 kbp *nmy-2* cDNA and the T7 promoter region at both ends was amplified by PCR using the primers 5' AGCTCGGTAATACGACTCACTATAGGGAAC 3' and 5' CCAGTGAATTGTAATACGACTCACTAT 3'. Double-stranded RNA was amplified using a MEGAscript T7 *in vitro* transcription kit (Ambion) as described by the manufactory's guide. After microinjection of the RNA into the gonad of adult hermaphrodites, the same phenotype was observed in the progeny as previously described (Guo and Kemphues, 1996). To generate RNA interference (RNAi) worms for immuno-staining, a soaking method (Tabara, et al., 1998) was used. Briefly, 1 μl of Lipofectin (Gibco-BRL) and 4 μl of dsRNA prepared as above were mixed and incubated at room temperature for 15- 30 min in the bottom of a 1.5 ml microfuge tube. 15-20 L4-stage hermaphrodites were picked from agar plates and added to the RNA-lipsome mixture. The tube was capped and incubated for 10-24 hours at room temperature. After incubation, the worms were transferred to an agar plate with *E. coli* (OP50) and grown up to mid-adulthood for immuno-staining of their embryos.

2. 6 Yeast two-hybrid screen

The full length *unc-45* cDNA was amplified from pDP#WA036 which contains the *unc-45* "mini gene" (Venolia et al., 1999) by PCR using primer WA06 (5' TTTCCCGGGATGGTTGCTCGAGTACAGACT 3', at the 5' end of *unc-45*) and WA05 (5' CAACCCGGGTTCCTGAATGGTGCTCATTTG 3', at the 3' end of *unc-45*). The PCR product was digested at the *Sma*I site that was introduced by PCR at both ends and cloned into the *Sma*I site of the yeast two-hybrid expression vector pGBDU-C1 which contains a uracil selection marker (James et al., 1996), resulting in plasmid pDP#WA039. This expression plasmid was used as a bait to screen a *C. elegans* cDNA expression library (λACT-RB1) generated from mixed stages of wild-type worms (kindly provided by Dr. Robert Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA). This library represents 10^7 independent clones and the vector λACT contains a leucine selection marker (Elledge, et al., 1991).

Plasmid pDP#WA039 was transformed into the yeast strain PJ69-4A and maintained on synthetic complete (SC) media minus uracil as described (James. et al., 1996). To screen the library, 2 μg of cDNA was used to transform PJ69-4A containing plasmid pDP#WA039 using a high efficiency method (Gietz and Schiestl, 1995) and positive transformants were identified from the strictest selective media (SC without uracil, leucine, histidine and adenine) as described (James, et al., 1996). Uracil and leucine markers were used to select the bait plasmid and the prey plasmid, respectively. Histidine and adenine reporter genes, which are driven by the *GAL1* and *GAL2* promoters, respectively, were used to select for clones which show the interaction between the bait and the prey. The interactions of the positive clones with the UNC-45

bait were confirmed by a third reporter gene, *LacZ*, which is driven by the *GAL7* promoter (James, et al., 1996), since those clones were blue in the presence of X-gal which indicates expression of ß-galactosidase. About 10^6 transformants (estimated from the efficiency of transformation selected from the media of SC lacking uracil and leucine) were screened and three strong positive clones were identified in the screen. Their interactions in the yeast assays were initially tested by re-purifying the positive plasmid DNAs and re-transforming them into the yeast. As a control, the bait plasmid pSE1112 (Durfee, et al., 1993), which encodes the SNF1 fusion protein, was tested. SNF1 did interact with SNF4 protein in the yeast assays as expected, but did not interact with those positives isolated in this screen judged by lack of growth on appropriate selective media. After sequencing the cDNA fragments and re-cloning into pGAD vectors, their interactions with UNC-45 were further tested in the yeast assays. The three isolated positive cDNAs are a 1. 6 kbp fragment of *nmy-2*, a 420 bp fragment of *hum-2* and a 2.9 kbp fragment of *CeUFD2*, respectively.

To further test the interactions of different domains of UNC-45 with these positive clones, two more *unc-45* subclones were constructed as below. A 2.6 kbp partial *unc*-45 cDNA fragment was amplified by PCR from pDP#WA039 using primer WA05 (5' CAACCCGGGTTCCTGAATGGTGCTCATTTG 3') at the 3' end of *unc-45* and WA07 (5' GAT CCCGGGATTGTTGAAGTTCTTCAG 3') at the 5' end of *unc-45* exon 4, and cloned into vector pGBDU-C1, resulting in plasmid pDP#WA040 which encodes a truncated UNC-45 protein lacking the TPR domain (the first 112 amino acids at the N-terminus). The plasmid pDP#WA061 was generated by cutting pDP#WA039 with *Nhe*I which recognizes a site about 441 bp downstream of the start codon and *Bgl*II at the 3'

end, removing a 2.5 kbp cDNA fragment, filled in with Klenow enzyme and ligated again. This construct encodes only the TPR domain (the first 127 amino acids of UNC-45 at the N-terminus). These two baits were transformed into yeast and their interactions with the positive clones were further tested.

2.7 Motility Assay

The N2 [wild-type], *unc-45* (*m94*), double mutant RW2329 [*unc-54* (*e190*); *sup-3* (*e1407st90st92*)] and triple mutant RW2665 [*unc-54* (*e190*); *unc-45* (*m94*); *sup-3* (*e1407st90st92*)] strains were grown at 25°C. Five young adult worms of each strain were put in the center of five plates which were completely seeded with a lawn of *E. coli* (OP50). The worms were allowed to crawl for 1 hour at 25°C and the traces were marked with a pen and photographed. All five worms of the same strain showed similar motility.

2.8 Antibody production

A fragment of the *unc-45* cDNA, which corresponds to a 58-residue region from amino acid 18 to 76 of the predicted UNC-45 protein, was fused in frame to the glutathione S-transferase coding region in the expression vector pGEX-2T (Amersham Pharmacia Biotech). This glutathione S-transferase::UNC-45 fusion was expressed in *E. coli* strain BL21 (DE3) under standard conditions and purified on glutathione agarose (Amersham Pharmacia Biotech) as described (Smith and Johnson, 1988). Rabbit antiserum (7N5) to this fusion protein was raised and purified as described (Ausubel, et al., 1991). The preimmune serum did not detect the putative UNC-45 protein on Western blots and did not show any significant staining in immunofluorescence. The purification of antiserum and Western blots was performed by Heather Lemon and Shawna Maguire, technicians in this laboratory.

2.9 Microscopy

Polarized light microscopy was performed as described (Waterston et al., 1980; Hobert et al., 1999). For immunofluorescence, embryos from either wild-type or *nmy-2* RNAi-treated hermaphrodites and young larvae were fixed as described (Miller and Shakes, 1995) using the methanol/acetone fixation method and air-dried after fixation. The fixed embryos were incubated in diluted primary antibodies for 1 hour at room temperature, followed by three 5 minutes washes in PBS buffer (150 mM NaCl, 10 mM sodium PO_4, pH 7.2). The primary antibodies were detected using either fuorescein or rhodamine conjugated anti-rabbit secondary antibodies. For whole-mount fixation of older larvae and adults, the method used was originally developed by Finney and Ruvkun (1990) and modified for the study of muscle as described (Miller and Shakes, 1995). Briefly, the worms were fixed in an eppendorf tube with in 1X Ruvkun buffer (160 mM KCl, 40 mM NaCl, 20 mM Na_2 EGTA, 10 mM spermidine-HCl, 30 mM Pipes, pH 7.4, and 50% methanol) and 1-4% formaldehyde (made freshly from dry paraformaldehyde, J. T. Baker Inc.) with 30 minutes to overnight incubation on ice, followed by 15 minutes treatment with 1X BO_3 buffer (50 mM H_3BO_3, 25 mM NaOH), 10 mM DTT in 1X BO_3 buffer and 0.3% H_2O_2 in 1X BO_3 buffer, respectively. The fixed worms were stained as described above. To stain the gonads of hermaphrodites, the worms were placed on a slide in M9 buffer and cut beneath the pharynx to release the intact gonads from the worm bodies. The released gonads were stained using the same protocol as embryos.

Monoclonal antibodies against MHC isoforms (kindly provided by Dr. David Miller) were used at 1:100 dilution for DM 5-6 growth media and 1:1,000 dilution for DM 5-8 ascites, respectively and MH27 (provided by Dr. Joel Rothman, University of

California, Santa Barbara, CA, USA, but originally from the Waterston lab, University of Washington, St. Louis, MI, USA), which stains the boundaries of hypodermal cells (Francis and Waterston, 1985), was used at 1:1000 dilution. Affinity-purified polyclonal antibody against NMY-2 (a gift from Dr. Ken Kemphues, Cornell University, Ithaca, NY, USA) was used at 1:200 dilution and antibody 7N5 against UNC-45 described above was used at 1:500-1:1,000 dilution. The secondary antisera (Sigma) used were FITC-labeled anti-rabbit (at 1:1,000 dilution) or TRITC-labeled anti-mouse (at 1:1,000 dilution) immunoglobulins. DAPI (diamidophenylindole) was used at 1μg/ml in the mounting media. The immunofluorescence images were taken using a Zeiss Axioskop (Carl Zeiss) or a confocal microscope (Molecular Dynamics 2001) and processed using Adobe Photoshop 5.0.

3. Results

3.1 *unc-45* is a muscle-specific gene.

3.1.1 unc-45 reporter genes are expressed specifically in muscles.

To determine whether the *unc-45* gene is expressed in muscle cells, as expected from the mutant phenotype, a 4.4 kbp fragment containing the presumed promoter region and the first 18 amino acids of exon 1 of the *unc-45* gene was fused in-frame to both the *E. coli lacZ* gene (Fire et al., 1990) and the *Aequorea victoria* GFP gene (Chalfie et al., 1994). Transgenic animals containing these constructs were examined for enzymatic activity (*lacZ* fusion) or fluorescence (GFP fusion). The results from both fusions are consistent. In the adult, the reporter genes are detectable in the body wall muscle cells, in the pharyngeal muscle cells, in the sex-specific muscle cells (e.g., vulval muscles in hermaphrodites and diagonal muscles of the male tail) and in the anal muscles (Figure 3.1). Expression is also found in the embryos at the comma stage to two-fold stage, when muscle lattices are being assembled (Figure 3.2). β-galactosidase staining from the lacZ fusion is detected in muscle cells and early embryos (Figure 3.3). This expression pattern indicates that UNC-45 may be expressed in all muscle cells early in their development and continue through adult life, and sequences sufficient for muscle-specific expression lie in the upstream 4.4 kbp.

3.1.2 Functional UNC-45::GFP is expressed in all muscle cell types.

The GFP transgenic arrays examined above were unstable as they existed as extra-chromosomal elements. Only a fraction of the progeny inherited the arrays and only a fraction of the muscle cells in any transgenic animal expressed the GFP reporter. In addition, subcellular localization of the fusion protein which contains only a small

fragment of UNC-45 would not be informative for the native protein. Following UV treatment which increases the recombination frequency and can integrate extra-chromosomal genes into chromosomes, a stable transgenic *unc-45* cDNA::GFP line was obtained, in which GFP is fused in frame to the entire UNC-45 coding region. More than 95% of the progeny inherit the array, and the array is mitotically stable. This *unc-45* cDNA::GFP construct can rescue the *unc-45* (*ts*) mutant phenotype at the restrictive temperature of 25^{0}C assayed by both improved motility and muscle structure (Figure 3.4 and 3.5), indicating that the fusion protein retains UNC-45 function. In this transgenic line, GFP expression is detected in all muscle cells examined, including body wall muscle cells, pharyngeal muscle cells, anal-intestinal muscle cells, gonad sheath muscle cells, and sex-specific muscle cells in both males and hermaphrodites (Table 3.1). This supports a general role of UNC-45 in development and function of all muscles.

3.1.3 UNC-45 protein is detected in the germline cells of the gonad and pre-morphogenesis embryos

Although genetic evidence suggests that *unc-45* mRNA or protein is contributed maternally (Venolia and Waterston, 1990), transgenic arrays are generally silent in the germline (Kelly et al., 1997) and any maternal germline expression would not have been detected by the reporter fusions as described in the previous section. Using immunofluorescence, UNC-45 is present in germline cells (Figure 3.6) including mature oocytes (data not shown), as well as pre-morphogenesis embryos (Figure 3.7). Furthermore, *in situ* hybridization has shown that *unc-45* mRNA is also enriched in the gonad of adult worms as well as embryos (Figure 3.8, Dr. Y. Kohara, database at http://watson.genes.nig.ac.jp/db/, EST yk44f2). These results indicate that the maternal

effect of *unc-45* is consistent with the presence of the UNC-45 protein or mRNA in the oocytes.

3.1.4 UNC-45 begins to be enriched in the muscle cells at about the 420-cell stage

UNC-45 staining is generally detectable throughout the pre-morphogenesis embryos, rather than being restricted to myogenic lineages (Figure 3.7). However, *unc-45* reporter genes (expressed from extra-chromosomal transgenic arrays) are expressed only in muscles (Figure 3.2), and UNC-45 immunostaining is only detectable (besides the germline staining described above) in the somatic muscle cells in the adult. Therefore, there must be a transition in expression pattern. In examining older embryos, we found that UNC-45 begins to be enriched in the muscle cells at about 300 min after fertilization (Figure 3.9). At 270 min, UNC-45 is evenly distributed in all cells; but at 300 min (about 420 cells), UNC-45 is more concentrated in muscle cells (the two lateral bands of muscle cells can be easily distinguished). At 320 to 350 min, UNC-45 is mainly detectable in muscle cells. Other muscle structural proteins are first detectable at about this time (Epstein et al., 1993; Hresko et al., 1994; Moerman and Fire, 1997). This is consistent with a zygotic function for *unc-45* in muscle development.

3.1.5 Zygotic UNC-45 is only expressed in muscles whereas maternal UNC-45 is only present in the early embryos

Many *C. elegans* genes show poor maternal expression when present on extra-chromosomal transgenic arrays, since they exist as repetitive sequences on which genes are transcriptionally repressed in germ cells (Seydoux, et al., 1996; Kelly et al., 1997). Thus, reporter genes on an extra-chromosomal transgenic array may only indicate the zygotic expression of the gene. Figure 3.2 shows the expression of an extra-chromosomal

transgenic UNC-45::GFP fusion at the 420-cell stage and two-fold stage of embryogenesis. Expression mirrors the detection of other muscle-specific proteins (Epstein et al., 1993; Hresko et al., 1994; Moerman and Fire, 1997). Moreover, the expression of UNC-45 is co-localized with MHC B in muscle cells (Figure 3.10).

Taking these results together, maternal UNC-45 protein persists in all cells of the embryo up until gastrulation, at which time it fades. At the same time, zygotic UNC-45 is synthesized in a muscle specific manner.

3.1.6 Northern analysis indicates that unc-45 gene has only one major product.

The sequence of the genomic *unc-45* gene predicts a messenger RNA of approximately 3.5 kb. Northern analysis was used to confirm this. When total mRNA from wild type worms was probed with antisense RNA generated from an *unc-45* cDNA, a ~3.5 kb band was detected. However, since it was almost the same size as the larger ribosomal band (26S: about 3.5 kb) it was formally possible that the band was artifactual, representing non-specific hybridization of the probe to an abundant transcript. To distinguish the *unc-45* transcript from the ribosomal RNA, poly A^+ enriched RNA and poly A^+ depleted RNA along with total RNA were examined. The same amount (2 μg) of the enriched poly A^+, the enriched rRNA and total RNA were used in the blot, but the intensity of the band from the enriched poly A^+ is stronger than the other two (Figure 3.11, lane b). This indicates that the 3.5 kb message RNA is the major transcript of the *unc-45* gene, as expected. As a control, the *act-1* gene was also examined and the 2.0 kb *act-1* mRNA was detected in all samples (Figure 3.11, lane d).

These data are consistent with the results of 5' RACE and the sequences of the cDNA clones. As well, the *unc-45* cDNA fused to the genomic upstream region also

rescues the "unc" phenotype of *unc-45* mutants. Together, these results indicate that the 3.5 kb mRNA is the predominant transcript of the *unc-45* gene, at least in mixed stage cultures.

3.1.7 unc-45 C. briggsae homologue can functionally rescue unc-45 (ts) mutants.

For the identification of putative functional domains of the protein, likely to be conserved through evolution, a genomic DNA fragment from the related species *C. briggsae* was cloned and sequenced. In previously identified *C. briggsae* homologues of *C. elegans* genes, sequence identities range from 44 to 100% (de Bono and Hodgkin, 1996), and have allowed the prediction of structure-function relationships. A strongly hybridizing 1.8 kbp *Xba*I fragment of *C. briggsae* genomic DNA from a cosmid library was sequenced and showed significant identity to the central portion of the *C. elegans unc-45* coding region. Two more *Xba*I fragments flanking this were also sequenced. When conceptually spliced and translated, these three fragments were found to account for a predicted *C. briggsae* protein similar to the entire *C. elegans unc-45* coding region, as well as 150 bp of putative upstream region.

The *C. briggsae unc-45* homologue covers only 3.1 kbp of genomic DNA, compared to nearly 12 kbp in *C. elegans* (Figure 3.12). The major difference between the two is in the size of the introns. *C. briggsae unc-45* contains five introns, all with 56 bp or less, compared to 10 in *C. elegans*. In contrast to the difference in the genomic organization, the predicted *C. briggsae* UNC-45 protein sequence is very similar to the *C. elegans* sequence (Figure 3.13). The two proteins are 88% identical over their entire length, similar to what has been seen for other *C. briggsae* and *C. elegans* homologues (de Bono and Hodgkin, 1996). The three TPR domains in the *C. briggsae* homologue are

95% identical to those in *C. elegans*. Moreover, the *C. briggsae* gene is able to restore partial movement to *unc-45(r450)* mutants at both 20°C and 25°C, and significantly increase the viability at 25°C when present as a transgene (Venolia, et al, 1999). This confirms that the conservation seen at the sequence level is a reflection of functional conservation as well.

3.1.8 Putative Drosophila unc-45 homologue.

A search of the *Drosophila* genomic DNA sequence database using the carboxyl-terminal half of UNC-45 sequence was done, and a putative *Drosophila unc-45* homologue (*Dm-unc-45*) has recently been found (D. Pilgrim, pers. comm.). Although similar TPR repeats are found in many proteins, the predicted *Drosophila* gene product also has a domain similar to the CRO1 and She4 proteins, but most significantly, shows similarity to UNC-45 between these sequence motifs as well. The *Dm-unc-45* gene has only one intron (59 bp) and its predicted protein shows 32% identity and 51% similarity to the *C. elegans* UNC-45 protein (Figure 3.14). The sizes of these two proteins are also similar (*C. elegans* UNC-45 is 961 amino acids in length and *Drosophila* UNC-45 is 948 amino acids), suggesting that they may have the same cellular function. Three of the missense mutations found in *unc-45* (*ts*) alleles are residues conserved between *C. elegans* and *Drosophila*. A *Drosophila* strain with a P element insertion mutation at the 5' end of the *Dm-unc-45* gene (within 500 bp of the initiation ATG) has been obtained from the *Drosophila* stock center. This insertion strain is homozygous lethal, but the terminal phenotype has not been reported. Using RT-PCR, the entire cDNA of *Dm-unc-45* has been cloned and fused to a GFP vector. Whether this *Dm-unc-45* cDNA could

functionally complement *C. elegans unc-45* mutants is currently being tested by another graduate in this lab.

3.1.9 Putative human unc-45 homologue.

A BLAST search of the human genomic sequences suggests that there is a human *unc-45* homologue as well (D. Pilgrim, pers. comm.). Sequences that match the TPR, CRO1/She4 and linking region of UNC-45 exist on linked contigs at 15q26.1 (Figure 3.15). However, further analysis of this putative homologue must await the completion of the genomic sequence in this region by the sequencing consortium.

3.2. UNC-45 is a component of muscle thick filaments and co-localizes with myosin heavy chain B, but not myosin heavy chain A.

3.2.1 Functional UNC-45::GFP is localized to the A-bands of body wall muscles.

It has been suggested that UNC-45 protein may be involved in muscle thick filament assembly (Epstein and Thomson, 1974). UNC-45 could act catalytically in the cytoplasm to modify the thick filament components for assembly, or it could act as a component of the thick filament itself. To distinguish between these alternatives, we examined the expression pattern of this functional UNC-45::GFP in the body wall muscle cells in adult worms. The GFP expression pattern resembles the pattern of A-bands of thick filaments (Figure 3.5 A). To confirm this, the same field was examined under polarized light microscopy and an identical pattern was seen (Figure 3.5 B). This indicates that functional UNC-45::GFP is associated with thick filaments in body wall muscles.

3.2.2 UNC-45 is localized to the polar regions of thick filaments as MHC B in the body wall muscles of wild-type worms.

Since the functional UNC-45 ::GFP fusion protein is localized to the A-bands of body wall muscles, we examined these cells in wild-type adult worms by immunofluorescence. In body wall muscles, there are two myosin isoforms, MHC A and B. MHC A is localized in the central part of thick filaments while MHC B is present in the two polar regions (Miller et al, 1983). Double-staining with 7N5 and either anti-MHC B antibody or anti-MHC A antibodies showed that UNC-45 expression pattern overlaps the MHC B expression pattern, leaving an un-stained central gap (Figure 3.10 C), whereas MHC A expression is localized in the central part of the A-bands and overlaps only slightly bilaterally with UNC-45 (Figure 3.10 F). Figure 3.10 I shows the MHC A and B double-staining pattern which is similar to that of UNC-45 and MHC A double-staining. This indicates that UNC-45 may co-localize with MHC B, but not MHC A, in the body wall muscles. Figure 3.16 is a schematic diagram of muscle thick filaments.

3.2.3 Mutant UNC-45 is still associated with thick filaments in unc-45 (ts) mutant worms grown at the restrictive temperature.

unc-45 (ts) mutants grown at the restrictive temperature (20°C or above) are paralyzed, and show a reduced number of thick filaments which are present in a disorganized arrangement (Epstein and Thomson, 1974). To test if mutant UNC-45 protein is still associated with thick filaments in the *unc-45* (*ts*) mutants grown under restrictive conditions, we examined worms doubly stained with UNC-45 and MHC A or MHC B antisera. We chose the conditional mutants *unc-45 (r450*) and *unc-45 (e286)* which are both missense alleles resulting in different amino acid substitutions in the CRO1/She4 domain (Barral et al., 1998) and should therefore still react with the polyclonal antisera. For the worms grown at both 22°C and 25°C, the mutant UNC-45

protein is still associated with the disorganized thick filaments. The UNC-45 staining pattern (Figure 3.17 A) is similar to the MHC A staining pattern (Figure 3.17 B) as well as the MHC B staining pattern (data not shown). These results suggest that the association of UNC-45 with the thick filaments may not be affected in these mutants, but that this assembly or association is not sufficient to provide wild-type UNC-45 activity. In this case, it cannot be determined whether UNC-45 still differentially localizes with MHC B or MHC A, since the two myosins are no longer differentially localized on thick filaments in the *unc-45* (*ts*) mutant worms grown at the restrictive temperature (Barral et al, 1998).

3.2.4 UNC-45 is not localized to thick filaments in unc-54 null mutants in which MHC B is absent.

If UNC-45 co-localizes only with MHC B, but not MHC A, in thick filaments in wild-type worms, it is expected that UNC-45 will not be localized to thick filaments in mutants homozygous for the *unc-54* null allele in which MHC B protein is absent. *unc-54* (*e190*) is a null mutant which produces viable but paralyzed adults with disorganized thick filaments in which no MHC B is detectable (Epstein, et al., 1974; Dibb, et al. 1985; Bejsovec and Anderson, 1988). Immuno-fluorescence shows that UNC-45 is indeed not localized to these disorganized thick filaments (Figure 3.17 C) while MHC A is still present (Figure 3.17 D). Therefore, we conclude that UNC-45 does not associate with MHC A in the body wall muscles of worms lacking MHC B.

3.2.5 UNC-45 is not localized to thick filaments in unc-54 (0); sup-3 mutants in which MHC B is absent, but the amount of MHC A is increased.

sup-3 is an unusual allele of *myo-3* (encoding MHC A) which is a strong suppressor of *unc-54* null alleles, as well as *unc-15* missense alleles (Riddle and Brenner, 1978; Waterston, 1988; Maruyama, et al., 1989). The double mutant RW2329 [*unc-54* (*e190*);*sup-3* (*e1407st90st92*)] has improved structure of muscle thick filaments and much better movement than *unc-54* (*e190*) (Waterston, 1988). This improvement is due to the increased expression of MHC A encoded by the *myo-3/sup-3* locus (Maruyama, et al., 1989). To determine if UNC-45 activity is still required in this *sup-3* background, the motility of animals from strains RW2329 [*unc-54* (*e190*); *sup-3* (*e1407st90st92*)] and RW2665 [*unc-54* (*e190*); *unc-45* (*m94*); *sup-3* (*e1407st90st92*)] was assayed at the restrictive temperature (25°C). As shown in Figure 3.4, there is no apparent difference between the motility of the double and triple mutants as judged by the distances they crawled for 1 hour at 25°C. This indicates that full UNC-45 activity is not required in an *unc-54*; *sup-3* background for assembly of functional thick filaments. In addition, immuno-fluorescence also shows that UNC-45 is not localized to thick filaments in these mutants (Figure 3.17, E and G). MHC A is still present in thick filaments (Figure 3.17, F and H) which have much better organization than those seen in the *unc-54* (*e190*) null allele. These results suggest that the assembly of thick filaments containing only MHC A is UNC-45-independent, at least in adult animals.

3.2.6 UNC-45 may be added to thick filaments after MHC isoforms are assembled.

The specific mechanism by which *C. elegans* muscle thick filaments are assembled remains unclear. In the nematode, it has been proposed that body wall muscle thick filaments are composed of a core structure and an outer layer (Epstein et al, 1985 and 1988; Deitiker and Epstein, 1993). The core structure contains paramyosin as its major

protein and at least three other proteins, α, β and γ-filagenins (Deitiker and Epstein, 1993; Epstein et al, 1995; Liu et al, 1998). The outer layer is composed of two differentially localized myosin isoforms , MHC A and B, and associated myosin light chains in body wall muscles. MHC A and B may be involved in the initiation and termination of the assembly, respectively, suggested by their differential localization on thick filament in the wild-type worms (Miller, et al., 1983). In early larval stages, we have observed that MHC B has been assembled into the thick filaments near the cell membrane along the face of the muscle cell that is adjacent to the hypodermis while UNC-45 is still mainly diffuse in the cytoplasm (Figure 3.18). This indicates that UNC-45 may be added to thick filaments after the assembly of MHC A and B. While the stoichiometry of UNC-45 and MHC B in these cells at this stage can not be accurately measured, this temporal difference in localization to the thick filament suggests that while UNC-45 is a thick filament component in adult muscles, it does not form a scaffold in the embryo upon which MHC A and B are assembled.

3.3 UNC-45 interacts with myosins in yeast two-hybrid assays.

3.3.1 Yeast two-hybrid screens

To search for proteins that may interact with UNC-45 *in vivo*, a yeast two-hybrid screen was performed. An *unc-45* full length cDNA clone (pDP#WA039) was used as a bait to screen a *C. elegans* cDNA expression library using the yeast two-hybrid method (Fields and Sternglanz, 1994; Phizicky and Fields, 1995; Gietz and Schiestl, 1995; James et al., 1996). In the screens, three strong and one weak positive clones were identified (Figure 3. 19). The weak positive clone was not further characterized. Of the three strong positives, two encode myosin proteins: one is a 1.6 kbp cDNA fragment of a non-muscle

class II gene (*nmy-2*) (Guo and Kemphues, 1996) and the other one is a 420 bp fragment of *hum-2*, an unconventional class V myosin (Baker and Titus, 1997). The third is a 2.9 kbp cDNA of a gene (T05H10.5) which encodes a predicted protein with significant similarity to the yeast UFD2 protein and will be discussed in next section.

The fragment of *hum-2* corresponds to amino acids 540 to 680 in the head domain of HUM-2, which is located about 20 amino acids upstream of the actin binding site and downstream of the ATP binding site (Figure 3.20). The identified NMY-2 fragment also contains that portion. Although the function of HUM-2 has not yet been characterized, these observations do suggest that this small portion of myosin may represent a conserved binding site for UNC-45. This information will be useful in the future for mapping possible UNC-45 binding sites in myosin.

3.3.2 UNC-45 interacts with a non-muscle myosin

As mentioned earlier, UNC-45 also interacts with the non-muscle myosin, NMY-2, in the yeast assays. A 530 amino acid fragment of the NMY-2 head domain (amino acid 469 to 999) is sufficient for that interaction, but both the TPR domain and the CRO1/She4p similarity domain of UNC-45 are required for that interaction. NMY-2 was initially identified as a non-muscle myosin II heavy chain that interacts with the C-terminus of PAR-1, a putative kinase that is necessary for embryonic polarity (Guo and Kemphues, 1996). NMY-2 is present in the oocytes and the germline cells of the gonad and is necessary for normal asymmetric cell divisions in early embryos (Guo and Kemphues, 1996). Furthermore, it has also been shown that the NMY-2 protein is localized at the embryonic cleavage furrow and required for polarized cytoplasmic flow at cytokinesis (Shelton et al., 1999).

3.3.3 UNC-45 co-localizes with NMY-2 at the cell boundaries of the early embryo

Both NMY-2 and maternal UNC-45 proteins are present in the oocyte. The staining patterns of both UNC-45 and NMY-2 show concentration at the cortex of the cell in early embryos (Figure 3.7; Schumacher, et al., 1998; Shelton et al., 1999). Embryos labeled with both NMY-2 and UNC-45 antibodies were examined by confocal microscopy. Figure 3.21 shows reconstructed 3-dimensional images of 2-cell, 4-cell and >8-cell stages of wild-type embryos, demonstrating that both NMY-2 and UNC-45 are indeed concentrated at the cell cortex (first and second columns), and the staining patterns are coincident (third column). In addition, the UNC-45 concentration at the cortex is disrupted if NMY-2 is removed by RNA interference (fourth column), indicating that the localization of UNC-45 at the cleavage furrow is dependent on NMY-2. Thus, UNC-45 is similar to PAR proteins in having an embryonic asymmetric localization that is NMY-2 dependent.

I also attempted to deplete UNC-45 by RNA interference and examine the staining pattern of NMY-2. Although *unc-45* RNAi into wild-type worms did result in a "Pat" phenotype, maternal UNC-45 could not be removed completely as judged by immunofluorescence. In these RNAi embryos, both UNC-45 and NMY-2 were still localized at the cell boundaries (data not shown).

3.4 Preliminary characterization of CeUFD2, a homologue of yeast ubiquitin fusion degradation protein-2 (UFD2)

3.4.1 CeUFD2 interacts with the TPR domain of UNC-45 in the yeast two-hybrid system.

As mentioned earlier, another positive clone identified in the yeast two-hybrid assays is the cDNA from the *C. elegans* T05H10.5 gene, which is predicted to encode a

homologue of yeast UFD2 (ubiquitin fusion degradation protein-2). CeUFD2 contains 6 exons encoding a protein of 980 amino acids. If most of the C-terminal part of UNC-45 is removed and only the TPR domain at the N-terminal is tested, these two proteins still interact with each other in the yeast two-hybrid assays, indicating that the N-terminal TPR domain of UNC-45 is sufficient for that interaction (Figure 3.22).

3.4.2 CeUFD2::GFP fusion is expressed in nerve cells and the intestine, but not muscles.

The *CeUFD2* gene has not yet been characterized. If CeUFD2 interacts with UNC-45 in *C. elegans*, its expression pattern should overlap with at least part of UNC-45. To test this, the upstream region (~4.9 kbp) of CeUFD2 was cloned into the vector pPD95.79 and fused to the GFP coding region. Transgenic worms containing extra-chromosomal arrays of this construct showed GFP expression in some nerve cells, the ventral cord and the intestine (Figure 3.23 and 3.24) but not muscle cells. This indicates that the interaction of UNC-45 and CeUFD2 may not be biologically relevant.

4. Discussion

4.1 UNC-45 has distinct maternal and zygotic expression.

The evidence that UNC-45 or its mRNA is contributed maternally was previously indirect, as the lethal alleles of *unc-45* show maternal effects (Venolia and Waterston, 1990). All reported phenotypes of *unc-45* mutants could be rescued by zygotic expression of the gene, although the precise nature of the defect that leads to the lethality has not been adequately explained (Venolia and Waterston, 1990). Although UNC-45 localizes to thick filaments in an MHC B dependent fashion, and the need for wild type UNC-45 activity is eliminated if MHC B protein is removed by mutation and MHC A expression is increased, UNC-45 must have other roles in development, as the lethal phenotype of the strongest *unc-45* alleles is still stronger than the phenotype of MHC B (*unc-54*) null alleles (Venolia and Waterston, 1990). Since *unc-45* reporter gene expression is detected in all types of muscle, the lethal phenotype may derive from defects outside the body wall muscles, possibly in the pharynx (Venolia and Waterston, 1990). Reporter transgenes in *C. elegans* are poorly expressed in the germline (Seydoux et al., 1996; Seydoux and Dunn, 1997; Kelly et al., 1997), and any germline expression or maternal contribution of *unc-45* product would have been overlooked by this technique. *In situ* hybridization to whole animals using *unc-45* cDNA as a probe suggests that *unc-45* transcripts are widespread in the early embryo as well as being strongly expressed in the adult hermaphrodite germline (Figure 3.8), but since components of the body wall muscle and pharynx are normally zygotically transcribed, the implications of this finding were unclear.

Not only is *unc-45* mRNA maternally contributed, but using antisera we show that the protein is provided maternally as well. The GFP reporter transgenes likely represent only the zygotic, and not maternal, expression of *unc-45* (Seydoux et al., 1996; Seydoux and Dunn, 1997; Kelly et al., 1997), while the antisera detect both maternal and zygotic UNC-45. The simplest explanation for the pattern that we see is that maternal UNC-45 is segregated to all cells in the embryo, and persists until the early comma stage, at which point zygotic UNC-45 is synthesized only in muscle cells. The ubiquitous maternal UNC-45 is then degraded. In this way zygotic UNC-45 synthesis mirrors the pattern of other muscle thick filament components (Moerman and Fire, 1997).

4.2 UNC-45 is a component of muscle thick filaments.

Our results show that UNC-45 co-localizes with MHC B in the two polar regions of the thick filament in adult body wall muscles. In *unc-45 (ts)* mutant animals grown at the restrictive temperature, UNC-45 staining is still associated with disorganized thick filaments. However, this localization to thick filament remnants is lost in *unc-54* null mutants, suggesting that UNC-45 localization is dependent on the presence of MHC B. Based on these results, we propose that UNC-45 is a component of thick filaments and that UNC-45 is associated with MHC B in the outer layer of thick filaments in wild-type worms. Immunoblotting experiments demonstrated that the accumulation of MHC B, but not MHC A, is decreased in *unc-45* (*ts*) mutant worms grown at the restrictive temperature (Barral et al., 1998). This indicates that the accumulation of MHC B, but not MHC A, requires wild-type UNC-45 activity. The overlap of UNC-45 immunolocalization with MHC B, but not MHC A, in the thick filaments is consistent with this.

4.3 Is the assembly of MHC A independent of UNC-45?

Several lines of evidence suggest that the stable incorporation of MHC A into thick filaments appears to be independent of UNC-45. First, MHC A is localized in the central part of the thick filament, where polarized myosin assembly is thought to initiate (Waterston, 1989) and embryos homozygous for *unc-45* lethal alleles begin MHC A assembly into thick filaments (Venolia and Waterston, 1990). Second, unlike MHC B, the steady-state accumulation of MHC A in adult muscle is not dependent on UNC-45 (Barral et al., 1998). Third, *unc-45* (*ts*); *sup-3* mutants show muscle structure that is no better than in *unc-45* (*ts*) mutants, but much worse structure than in *unc-54* (*0*);*unc-45* (*ts*);*sup-3* mutants, indicating that MHC B, but not MHC A, is deleterious in an *unc-45* background (Waterston, 1988). Moreover, our immuno-fluorescence studies have shown that UNC-45 does not co-localize with MHC A either in wild-type worms, or in the *sup-3* background where MHC A is the only myosin in the body wall muscle thick filaments.

However, there is also genetic evidence that UNC-45 may have some role in MHC A assembly or stability earlier in the embryo. Indirectly, *unc-45* lethal alleles (*st601* and *st603*) and an MHC A null mutant allele [*myo-3* (*st378*)] have a similar embryonic lethal phenotype but *unc-54* null alleles are viable (Dibb et al. 1985; Waterston, 1989; Venolia and Waterston, 1990). Also, increasing the level of MHC A (as in some *sup-3* alleles) can antagonize the maternal rescue seen in some of the *unc-45* lethal alleles (Venolia and Waterston, 1990). It is possible that UNC-45 may interact with MHC A for the initiation of thick filament assembly, but this interaction may only

be required at an early stage in development and the *unc-45* (*ts*) alleles still maintain sufficient UNC-45 activity for that interaction.

4.4 A model for UNC-45 function in muscles.

At least one of the roles of UNC-45 seems to be to ensure the stability of MHC B in the thick filament of the adult worm. Since MHC B is the major myosin in the nematode and it covers most of the thick filament of body wall muscles (except a narrow central region which is occupied by MHC A (Miller et al., 1983)), the assembly and stability of MHC B would be expected to play a major role in the stability of the thick filament as a whole. In wild-type worms, UNC-45 co-localizes with MHC B and the thick filaments are normal and stable, while in *unc-45* (*ts*) mutants grown at the restrictive temperature, even though mutant UNC-45 still co-localizes with MHC B, the thick filaments are short and disorganized. MHC B levels are reduced in these worms, suggesting that unassembled or mis-assembled MHC B is degraded (Barral et al., 1998). UNC-45 shows no thick filament localization in MHC B null mutants. The simplest explanation for these results is that *unc-45* (*ts*) mutant animals are defective for an activity which stabilizes MHC B in long, polarized thick filaments. The lack of this activity leads (i) to the instability of the thick filament and (ii) directly or indirectly to the lack of a polarized arrangement of MHC A and B. *unc-45* (*ts*) mutants are caused by missense mutations within the CRO1/She4 domain of UNC-45 (Barral et al., 1998). This suggests that full activity of the CRO1/She4 domain is critical for the MHC B stabilizing function of UNC-45. To test this hypothesis, one would need to be able to remove all UNC-45 function from a muscle cell once the filaments have been assembled normally, and examine the stability of the thick filament with time.

While we have detected co-localization of UNC-45 and MHC B staining, and the disorganized muscle structure and function resulting from *unc-45* (*ts*) alleles can be suppressed by removing all MHC B and increasing MHC A levels, UNC-45 must be playing a larger role in muscle patterning. As discussed earlier, UNC-45 may have some role with MHC A for the initiation of thick filament assembly since the *sup-3* alleles of MHC A cannot suppress or ameliorate the phenotype of the lethal alleles of *unc-45* (Venolia and Waterston, 1990). Also, in embryos homozygous for lethal alleles of *unc-45*, MHC A assembly begins, but MHC B assembly into thick filaments (even disorganized ones) is not seen (Venolia and Waterston, 1990). This lack of MHC B assembly is similar to that seen in strong *myo-3* alleles, where MHC B remains dispersed until late in embryogenesis (Waterston, 1989). Thus, UNC-45 appears to be necessary for the early stages of myosin assembly, even though it does not appear to be concentrated in or around the thick filament at that time. Later, in the adult, it is also necessary for filament stability, and thick filament localization is seen.

UNC-45 is also required outside the body wall muscles. Strong UNC-45 reporter gene expression and immunofluorescence is seen in the pharyngeal muscles, where thick filaments are composed of MHC C and D. No disorganization of pharyngeal muscle structure has yet been reported in *unc-45* mutants, but *unc-45* lethal alleles result in embryos where no pharyngeal pumping is seen, while embryos homozygous for null alleles of MHC A and B do not have apparent pharyngeal defects (Venolia and Waterston 1990). Thus, UNC-45 may play a role in assembly or stability of thick filaments in pharyngeal muscles (and other muscles) as well as in body wall muscles. Examination of

pharyngeal muscle structure using electron microscopy may be necessary to address this hypothesis.

A model for the function of UNC-45 in body wall muscles is shown in Figure 4.1. In the early stages of development, UNC-45 is required for the initiation of assembly of MHC A and B onto the thick filament core structure. Although it is not associated with thick filaments in these stages, it may function as a catalyst to modify MHC B (and/or MHC A) before assembly or directly participate in the assembly itself. For *unc-45* lethal alleles *st601* and *st603* (which encode mutant UNC-45 containing stop codons between the TPR domain and the CRO1/She4 domain (Barral et al., 1998)), loss of UNC-45 function results in embryonic lethality. In the larval stages, UNC-45 becomes localized, perhaps onto an intermediate in the assembly of thick filaments and associated with MHC B. In the adult, UNC-45 is a component of thick filaments and functions as a stabilizer for MHC B. In the case of *unc-45* missense mutations *e286* and *r450* (which encode mutant UNC-45 carrying amino acid changes within the CRO1/She4 domain), MHC B may not be modified properly in the early stages, so that it cannot be distinguished from MHC A and their localization on the thick filament is no longer differential. Moreover, MHC B is unstable on the filament and shows reduced accumulation, resulting in disorganized muscle structure. UNC-45 may function in a similar fashion with MHC C and D in the pharyngeal muscles. This model makes some testable predictions: (i) MHC A assembly into thick filaments in early embryos should initiate, but not proceed far in *unc-45* (null) embryos; (ii) UNC-45 should physically interact with some form of MHC B, but not MHC A *in vitro* and (iii) UNC-45 should purify with thick filaments isolated from adult worms, and should be stoichiometrically related to the level of MHC B in the

preparation. To fully understand UNC-45 function, especially its role in the initiation of assembly in the early stages, we will need a better understanding of the nature of the MHC A and B molecules in the earliest stages of thick filament assembly in the embryo.

As discussed above, UNC-45 may function as a stabilizer, an assemblase or a chaperone. As a chaperone, it would be required for the proper folding of a protein (presumably in the cytoplasm) but does not stably associate with the protein. My results do not support this, since UNC-45 does physically associate with thick filaments in adult worms although UNC-45 staining is cytoplasmic at the time thick filaments are being assembled (L1 stage). An assemblase would be required during the process of myosin assembly but should disassociate with myosin once the process completes. This is partially supported by the reversible phenotypes during larval stages when the temperature is shifted between permissive and restrictive temperatures and that UNC-45 does not associate with MHC B in early larval stages. In contrast, however UNC-45 associates with MHC B in adult worms. It seems that UNC-45 most likely functions as a stabilizer. This is evidenced by its physical association with MHC B on muscle thick filaments and the unstability of MHC B in *unc-45* (*ts*) mutants grown at restrictive temperatures.

4.5 Maternal UNC-45 interacts with a non-muscle myosin NMY-2.

Why is UNC-45 mRNA and protein provided to the embryo, if it is not required until later in zygotic development? We have been unable to address this question genetically, since the strongest alleles of *unc-45* are zygotic embryonic lethals, and it has not been possible to examine embryos which lack any maternal contribution. Likewise, we have attempted to use RNA-mediated interference (RNAi) to deplete any maternal

contributions, but have been unable to produce embryos which are devoid of UNC-45 cross reacting material. The localization of UNC-45 staining to the cell cortex, most obviously to cell boundaries in early embryos, suggested that UNC-45 may have an early-embryo specific role that has remained obscure until now. The staining pattern is not unique, as several maternally contributed components show asymmetrical localization in the two-cell embryo (Rose and Kemphues, 1998), including the non-muscle myosin NMY-2 (Guo and Kemphues, 1996) which is also enriched in the germline cells of the adult and early embryos.

The observations that UNC-45 and NMY-2 co-localize in the embryo, that UNC-45 interacts with NMY-2 in the yeast two hybrid assay, and that UNC-45 localization in the embryo is disrupted if NMY-2 is removed using RNAi compel us to suggest a specific function for UNC-45 at this stage, even if there is not yet genetic evidence for such a role. NMY-2 has been shown to be required for embryonic polarity (Guo and Kemphues, 1996). It interacts through it's tail domain with PAR-1 and participates in the asymmetric localization of PAR proteins (Guo and Kemphues, 1996). Moreover, NMY-2 is also required for cytokinesis and body morphology during embryogenesis (Guo and Kemphues, 1996; Shelton et al., 1999) and a non-muscle myosin regulatory light chain, MLC-4, is also involved in this process (Shelton et al., 1999). Thus, this is the second instance of UNC-45 subcellular localization being dependent on a type II myosin molecule, the first being MHC B in the body wall muscle.

4.6 The possible role of maternal UNC-45 in early embryos.

As mentioned above, both UNC-45 and NMY-2 are expressed in the germline cells of the gonad and the pre-morphogenesis embryos, and the localization of maternal

UNC-45 at the cell boundaries is also dependent on the presence of NMY-2. It is tempting to assume that the biochemical role which UNC-45 has in its interaction with MHC B in the body wall muscle is related to its interaction with NMY-2. As mentioned above, the nature of this interaction is not yet clear, but UNC-45 may act as an assemblase, necessary for the proper polarized assembly of the myosin molecules, or as a stabilizer, preventing disassembly and degradation of myosin (I have discussed the difference between these, and how they may be ascertained, in the end of section 4.4).

Since the role of nonmuscle myosin II, particularly in cytokinesis, is a dynamic one where there is constant remodeling of the cytoskeleton, there is not an obvious need for a myosin stabilizer. However, this dynamic process may certainly involve factors necessary for targeting, assembly and disassembly of myosin filaments (Bresnick, 1999). Overexpression of Myo4p in yeast leads to defects in cytokinesis and polarized growth and the Ash1 protein may be helping to arrange or stabilize the myosin (Haarer et al., 1994). Given the similarity between UNC-45 and She4p, it is tempting to think the role may be evolutionarily conserved.

Since UNC-45 clearly plays a role in myosin assembly or stability in muscles, it seems most likely that its role in the embryo is mediated through NMY-2. Therefore, unless there is evidence for a completely independent role for UNC-45, it is simplest to assume that any defects in embryonic patterning that arise in embryos lacking all maternal UNC-45 may be due to failure of NMY-2 to properly assemble, or a failure of assembled myosin filaments to remain stable. Indeed, such defects may not be limited to embryos, as cytokinesis is necessary for proper oogenesis, and the F1 progeny of *nmy-2* RNAi develop to sterile adults. The UNC-45 homologue CRO1 is necessary for transition

from syncytial to cellular growth in *Podospora anserina* (Berteaux-Lecellier et al., 1998) and UNC-45 may play a similar role in the hermaphrodite gonad.

It is also interesting to note that both *C. elegans* HUM-2 and *S. cerevisiae* Myo4 belong to the same myosin subgroup, myosin V. Although there is no evidence that She4 interacts directly with Myo4 for regulating the asymmetrical localization of *Ash1* mRNA in yeast as discussed earlier, the interaction of UNC-45 and HUM-2 in yeast assays supports this possibility. This in turn supports the possibility of interaction of UNC-45 and NMY-2 in the regulation of asymmetric division in *C. elegans*. Moreover, this shows that UNC-45 is capable of interacting with multiple myosins *in vivo* and is involved in different cellular processes (muscle thick filament assembly and embryonic polarity).

4.7 UNC-45 defines a novel class of myosin-associated proteins

In muscles, we have shown that zygotic UNC-45 co-localizes with MHC B in the thick filament and this localization is dependent on the presence of MHC B and required for the assembly and stability of muscle thick filaments. Although we have not been able to show that they interact directly *in vivo*, genetic evidence does support such a conclusion. We have also shown that maternal UNC-45 co-localizes with a non-muscle myosin, NMY-2, at the cleavage furrow of early embryos and this localization requires the presence of NMY-2. Finally, we have shown that UNC-45 interacts with an unconventional myosin HUM-2 in yeast two-hybrid assays. Therefore, UNC-45 may associate with multiple tissue-specific myosins during development in *C. elegans*. Moreover, it has been shown that She4 may interact with an unconventional myosin Myo4p (She1) in the process that affects the mating type switching ability of the yeast cell (Jansen et al., 1996; Bertrand, et al., 1998) and both Myo4 and HUM-2 are class type

V myosins. We have shown that UNC-45 may have homologues in *Drosophila* and humans, although their functions remain to be characterized and it has not been tested whether they interact with myosin. Based on these observations, I propose that UNC-45 defines a novel class of myosin-associated proteins.

In yeast two-hybrid screens, a 140 amino acid region of HUM-2 was identified and the identified NMY-2 fragment also contains the corresponding region (Figure 3.20). I will refer to this region as a potential UNC-45 binding site (UBS). The first 30 amino acids at the N-terminal end of this region is highly conserved among HUM-2, NMY-2, MHC B and MHC A (Figure 4.2). If this smaller region is an actual UNC-45 binding site, UNC-45 may be expected to interact with all four of these myosins. However, MHC A is different from MHC B, NMY-2 and HUM-2, since there is no evidence that it associates with UNC-45 in muscle cells and its interaction with UNC-45 in yeast has not yet been able to be detected. At the C-terminal end of this region, MHC A does have two amino acids (Thr631 and Gln657) which are different from the conserved amino acids (lysine and glycine) in the other three myosins (Figure 4.2). This difference may affect the affinity of MHC A to UNC-45.

4.8 Ubiquitination system and CeUFD2

As mentioned earlier, one strong positive identified from the yeast two-hybrid screen is CeUFD2, which is very similar in sequence to the yeast ubiquitination system protein UFD2. In eukaryotic cells, protein activities and levels are regulated by differential gene expression, translation regulation and post-translational regulation. One of the post-translational regulations is the ubiquitin-mediated selective protein degradation. This ubiquitin-mediated proteolysis system plays an important role in a

broad range of cellular processes. In addition to the degradation of cyclins in cell cycle regulation, it is also involved in removal of abnormal proteins, generation of free amino acids, cell growth and cellular differentiation (Jentsch, 1992; Hochestrasser, 1996; Ciechanover, et al., 2000). In most cases, the ubiquitin-targeted substrates are degraded by the 26S proteasome, the major protease in eukaryotes. In some cases, ubiquitination can also target proteins to lysosomes for degradation or mediate other regulatory functions (Hochestrasser, 1996; Ciechanover, et al., 2000). Recently, the discovery of other ubiquitin-like proteins suggests that there are other proteolytic pathways which are similar but distinct from the ubiquitination pathway (Tanaka, et al., 1998; Jentsch and Pyrowolakis, 2000; Yeh, et al., 2000).

Ubiquitin is a highly conserved 76-residue protein (~ 8kD) in all eukaryotic cells (Hochestrasser, 1996). It exists in a free form or as part of a branched covalently linked protein-protein complex in which its C-terminus is attached to a lysine side chain of acceptor proteins during the process of substrate protein degradation. The degradation process requires the ubiquitin-activating enzyme (E1) and the ubiquitin-conjugating enzymes (E2). In some cases, E3s (auxiliary substrate recognition proteins) are also required (Hochestrasser, 1996). More recently, it has been shown that a novel additional conjugation factor, E4 (UFD2), is required for efficient multiubiquitin chain assembly in yeast (Johnson, et al., 1995; Koegl, et al., 1999. Figure 4.3). The CeUFD2 protein identified from my yeast two-hybrid screens and encoded by the *C elegans* T05H10.5 gene (named after the cosmid sequence on which it was found) shows high sequence similarity to this yeast UFD2 and therefore is a proposed member of E4 family.

The UFD2-like proteins are highly conserved from yeast to humans (Pukatzke, et al., 1998; Koegl et al., 1999). Database searches indicate one fission yeast (*S. pombe*) homologue, one *D. discoideum* homologue (NOSA), one putative *Drosophila* homologue and two putative human homologues in addition to CeUFD2. *NOSA* is essential for the normal development of *D. discoideum* (Pukatzki, et al., 1998). Although CeUFD2 is expressed in the intestine and some nerve cells, its role in *C. elegans* development remains unclear. There is evidence that the ubiquitination pathway plays an important role in the nervous system of *C. elegans*. *let-70 (ubc-2),* a member of the ubiquitin-conjugating enzymes (E2s), has been shown to be an essential gene required for larval development and it is expressed in the nervous system in the adult (Zhen, et al., 1993 and 1996).

The interaction between UNC-45 and CeUFD2 in yeast assays suggests that CeUFD2 may interact *in vivo* with a protein that contains TPR repeats similar to those in UNC-45. Also, the expression pattern of such a protein should overlap with that of CeUFD2. The best candidate in the *C. elegans* genome is a predicted gene (T09B4.10), which encodes a predicted protein of 272 amino acids with a TPR domain at its N-terminus and a UFD2-homology domain at its C-terminus (Figure 3.22). However, the expression pattern of the T09B4.10 gene has not been examined.

4.9 Sequence comparison of *C. elegans* and *C. briggsae unc-45* homologues

As expected, *C. elegans* UNC-45 and its *C. briggsae* homologue show high identity in their protein sequences (Figure 3.13) because they are evolutionarily closely-related species (de Bono and Hodgkin, 1996). The two proteins are the same size (961 amino acids) and 88% identical overall increasing to 95% in the TPR domain. The

overall level of similarity made it difficult to predict functional domains based only on the local level of conservation.

Despite the high conservation at the protein level, the organization of their exons differs considerably (Figure 3.12). From initiation to termination codons, the *C. elegans unc-45* gene (~ 12.0 kbp) is almost four-fold larger than its *C. briggsae* homologue (~3.3 kbp). This difference can be accounted for entirely by the difference in the number and size of the introns. *C. briggsae* has five introns and all are about 50 bp long, whereas the *C. elegans* homologue contains ten introns, four of them larger than 1.0 kbp (the largest intron is almost 1.6 kbp). This high identity of homologue protein sequences with strong divergence in non-coding regions has also been reported in other *C. briggsae* homologues of *C. elegans* genes (Hansen and Pilgrim, 1998; Maduro and Pilgrim, 1996; Blumenthal, per. comm.).

4.10 Speculations for putative *Drosophila* and human *unc-45* homologues

Based on the similarity in size and amino acid sequence between the putative *Drosophila* UNC-45 homologue and *C. elegans* UNC-45, it is expected that the two homologues may have a similar cellular function. The fly homologue may also function in the development of muscles and probably interacts with myosin isoforms. It should be noted that the expression of MHC isoforms in *Drosophila* is different from what is seen in *C. elegans*. There is only a single MHC gene in *Drosophila,* which is spliced in a developmentally and muscle-type specific manner to express different isoforms (Berstein et al., 1983; George, et al., 1989; Morgan, 1995). The *Drosophila* gene is 36 kbp in length and contains 19 exons, of which some are common and expressed in all the isoforms while five exist as alternatively spliced exon groups (Standiford, et al.,1997;

Davis, et al., 1998; Hordge et al., 1999). The potential UNC-45 binding site (UBS) in the head domain of myosin (Figure 4.2), judged from its interaction with HUM-2 and NMY-2, is probably located in the common exon 10 or 12 of the fly gene (Davis, et al., 1998; Hordge et al., 1999). If there is indeed only a single UNC-45 homologue in *Drosophila,* that will mean that it may interact with all the isoforms. There is a *Drosophila* strain which has a P-element insertion mutation in the 5' end of the predicted *unc-45* homologue and it is a homozygous lethal, but the terminal phenotype of this strain has not yet been characterized.

Although the sequence of the putative human *unc-45* homologue is still not complete, an examination of the available sequence suggests that the human and worm homologues may also be highly conserved. In humans, there are eight sarcomeric MHC genes identified so far (Weiss, et al., 1999) and some of them have been correlated with specific MHC isoforms, such as MHCs-α, and -β(I), MHC-Emb, MHC-Neo, MHC-IIa, MHC-IIx, MHC-IIb, and MHC-EOM (Weiss and Leinwand, 1996; Talmadge, 2000). These isoforms are expressed in different developmental stages or muscle types, while some of them also co-exist in the same muscle type. MHC-EOM is only found in the muscles that control eye movement, whereas MHC-Emb and MHC-Neo are limited to pre- and early postnatal muscle development. In the adult limb skeletal muscles, only MHC-I, MHC-IIa and MHC-IIx are predominantly expressed and found singularly in type I, IIA and IIX fibers, respectively (Smerdu et al., 1994; Talmadge, et al., 1996).

4.11 Future directions

The role of UNC-45 in muscle development and thick filament assembly is apparent, but whether the maternal UNC-45 has a role in embryogenesis is still not clear.

Whether UNC-45 is required for the assembly of MHC A or how it is involved in its assembly is also not understood. How the interaction of UNC-45 with myosins is related to its function is also a question. Following are some suggestions that may be helpful to address these questions in the future.

(i). To Determine the role of maternal UNC-45

I have been unable to directly address the role of UNC-45 in embryogenesis, although NMY-2 co-localizes with UNC-45 in the cleavage furrow and is required for the localization of UNC-45. This is because I have been unable to completely remove the maternal contribution of UNC-45 in embryos either through mutations or by the regular RNA interference approach.

A potential solution is to try feeding worms *E. coli* which expresses a dsRNA construct (Timmons and Fire, 1998). In this way, the worms are continuously exposed to the dsRNA. This may improve the efficiency of RNA interference and lead to a depletion of the maternal UNC-45 in the progeny. Also, this approach can be applied to *unc-45* (*ts*) and *unc-45* (*let*) as well, in addition to wild-type worms.

Another approach is mosaic analysis. In the well established extra-chromosomal transgenic lines, the transgene is poorly expressed in germ-line cells but well expressed in somatic cells (Kelly, et al., 1997). This is actually a mosaic expression for the transgene whose product is not present in the germ-line cells. We already have a functional UNC-45::GFP strain which carries an extra-chromosomal array of the transgene. Crossing this strain to *unc-45* (*st601*) heterozygous males can lead to a strain which will produce *unc-45* (*st601*)homozygotes that carry the UNC-45::GFP transgene. If this transgene can rescue the *st601* allele, these *st601* homozygous worms will grow to adults which will not

be able to produce any maternally rescued F2 progeny since the transgene (UNC-45::GFP) is not well expressed in the germline. However, an examination of the F2 embryos for any defects in early embryogenesis (such as cytokinesis or embryonic polarity) may reveal the role of maternal UNC-45.

(ii). To examine the role of UNC-45 in the assembly of MHC A

If UNC-45 does not have a role in the embryo outside of muscle cells, the lethal phenotype of *unc-45* mutant alleles is most likely caused by the failure of UNC-45 in a putative interaction with MHC A, since both *unc-45* and *myo-3* null mutants have a similar "Pat" phenotype and genetic evidence does suggest that UNC-45 interacts with MHC A.

In *unc-45* lethal mutants, MHC A assembly is observed to initiate (Venolia and Waterston, 1990). This could be due to the function of maternal UNC-45. If maternal UNC-45 is completely removed, can the assembly of MHC A still be initiated? The approaches mentioned above can be used to produce embryos that are devoid of maternal UNC-45. An examination of these embryos by immuno-fluorescence should be able to answer this question. If these embryos can develop to the two-fold stage (still "Pat") but the initiation of MHC A assembly can no longer be observed, this will tell us that UNC-45 is indeed required to initiate the assembly of MHC A.

Immunoprecipitation is another approach to examine the interaction of UNC-45 with MHC A *in vitro*. It will not be surprising to see MHC B in the immunoprecipitates using UNC-45 antibodies, but how about MHC A (especially from the protein extracts from early embryos)? If MHC A can be pulled out in the assays, it will support the idea that UNC-45 plays a role in the initiation of MHC A assembly.

(iii). To map the UNC-45 binding site in myosin

Based on the co-localization of UNC-45 with MHC B and NMY-2 *in vivo* and its interaction with NMY-2 and HUM-2 in yeast two-hybrid assays, I propose that there is an UNC-45-binding site (UBS) in the myosin type II molecules. In particular, the 140 amino acid small segment from the HUM-2 head domain is sufficient for that interaction (Figure 3.19). Although I failed to show an interaction of UNC-45 with MHC B in the yeast assay system, it is possibly due to the poor expression of MHC B in yeast, since the whole large molecule was used for the assays. To further test MHC B in yeast, it may be helpful to use small fragments of MHC B, particularly those that include the region identified in HUM-2.

Another strategy to determine the UBS in MHC B is to use MHC A and MHC B molecular chimeras that can confer MHC A-specific function and rescue *myo-3* null mutations (Hoppe and Waterston, 1996). Using the UNC-45 antibody to stain the thick filaments of these transgenic worms, if UNC-45 is still localized to thick filaments, it will mean that the chimeric molecule contains the UBS of MHC B since I have shown UNC-45 is only associated with MHC B but not MHC A *in vivo*. Conversely, if UNC-45 is not localized to thick filaments, it will indicate that the UBS of MHC B is no longer present in that chimera. Since many different chimeras are available, these may help to determine the specific UBS of MHC B. I have already tested two strains which are rescued by two different chimeras (chimera 7 and 8 in Figure 1.7. Hoppe and Waterston, 1996). In these two strains, UNC-45 is still localized to thick filaments. This is what I expected, since these two chimeras contain the potential UBS region of MHC B in the head domain. I

would not expect UNC-45 to be localized to thick filaments in the strains that are rescued by chimeras that do not contain that region (such as chimera 11 and 13 in Figure 1.7).

(iv). To screen for suppressors of unc-45 mutations

UNC-45 is associated with MHC B in the thick filament in the adult, but in the larval stages UNC-45 is not physically associated with nascent thick filaments. UNC-45 may play a regulatory role in early stages for the initiation of thick filament assembly and a structural role in the adult for the stability of thick filaments. It will be very interesting (at least to me) to know how UNC-45 is involved in these processes. Are there any other proteins involved in these processes with UNC-45? Genetic screens for suppressors of *unc-45* mutations may be a way to answer these interesting questions. However, it should been noticed that *sup-3* may be one of these suppressors since high expression of *myo-3* gene can result in near normal muscle structure without UNC-45 activity.

5. Acknowledgement

We are grateful to Dr. Ken Kemphues for providing antibody against NMY-2, Dr. David Miller II for antibodies against MHC isoforms (5-6 and 5-8), Dr. Joel Rothman for MH27 and Dr. Andrew Fire for GFP vectors. We are also in debt to Dr. Robert Barstead for providing *C. elegans* cDNA libraries and Dr. Philip James for the yeast two-hybrid strain (PJ69-4A) and expression vectors. We thank Dr. Yuji Kohara for sharing the database of *in situ* hybridization patterns. We also thank Drs. Pam Hoppe, Don Moerman, Lee Venolia, Paul Mains, and Morris Maduro for comments, and anonymous reviewers for helpful suggestions for the manuscripts, Rakesh Bhatnagar for helping with confocal microscopy, Heather Lemon, Shawna Maguire and Janette Berg for help in generating the UNC-45 antibody and immunoblotting. Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). This work was supported by Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research.

6. Figures

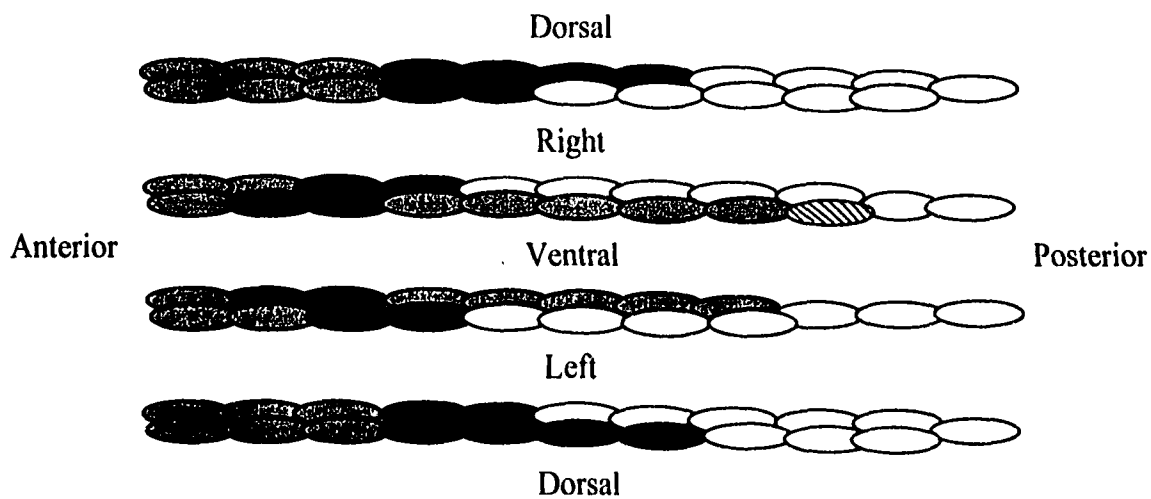

Figure 1.1 A diagram showing the four quadrants of body wall muscle cells in cylindrical projection. The 28 gray cells originated from the MS founder cell; the 32 unshaded cells from the C founder cell; the 20 dark cells from the D blastomere and the striped cell from the AB founder cell (Modified from Krause and Weintraub, 1992).

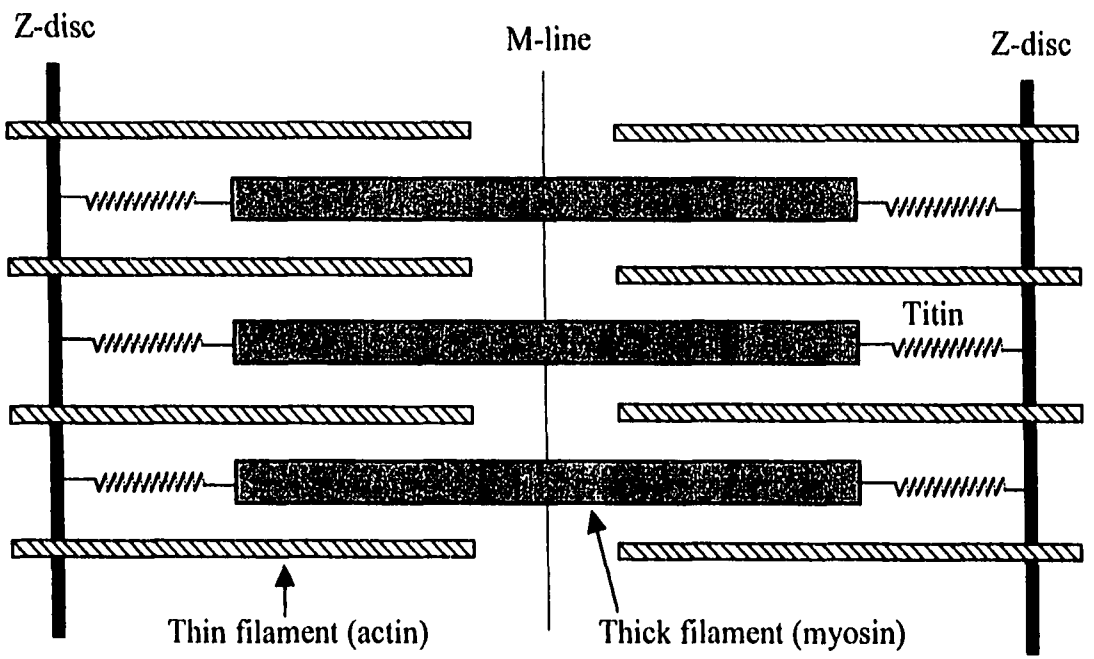

Figure 1.2 A diagram of the sarcomere in vertebrates, the basic unit of muscle contraction. M-line, Z-disc, thick and thin filaments are indicated. Titin, a giant protein kinase, is also shown.

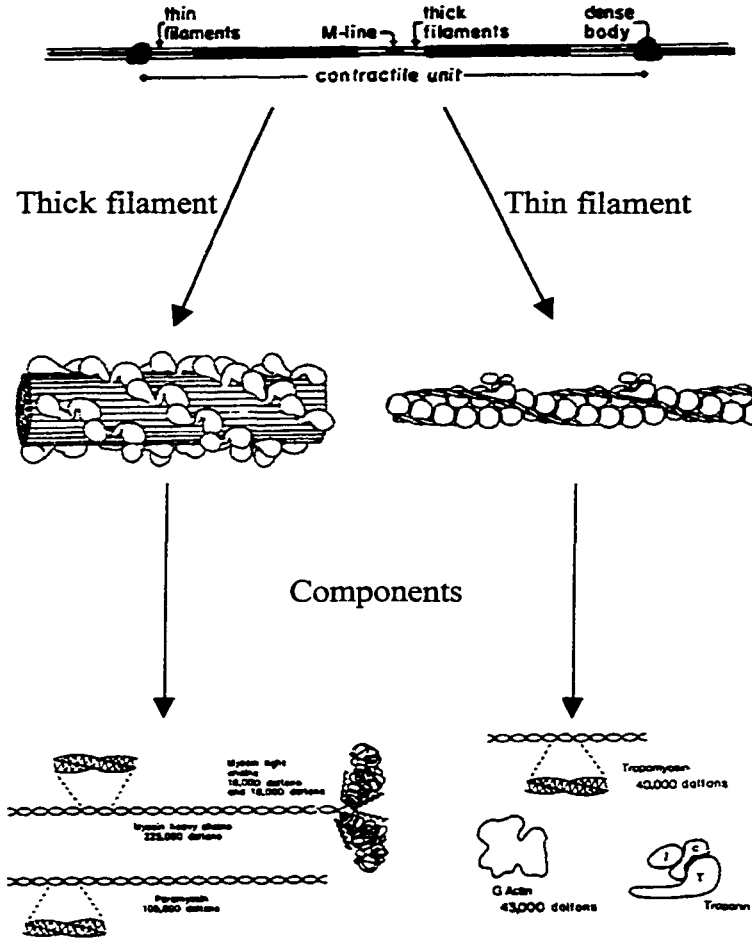

Figure 1.3 Components of *C. elegans* thick and thin filaments. Top, the contractile unit of muscle; Middle, thick and thin filaments; Bottom, the known protein components of thick and thin filaments. (modified from Waterston, 1988)

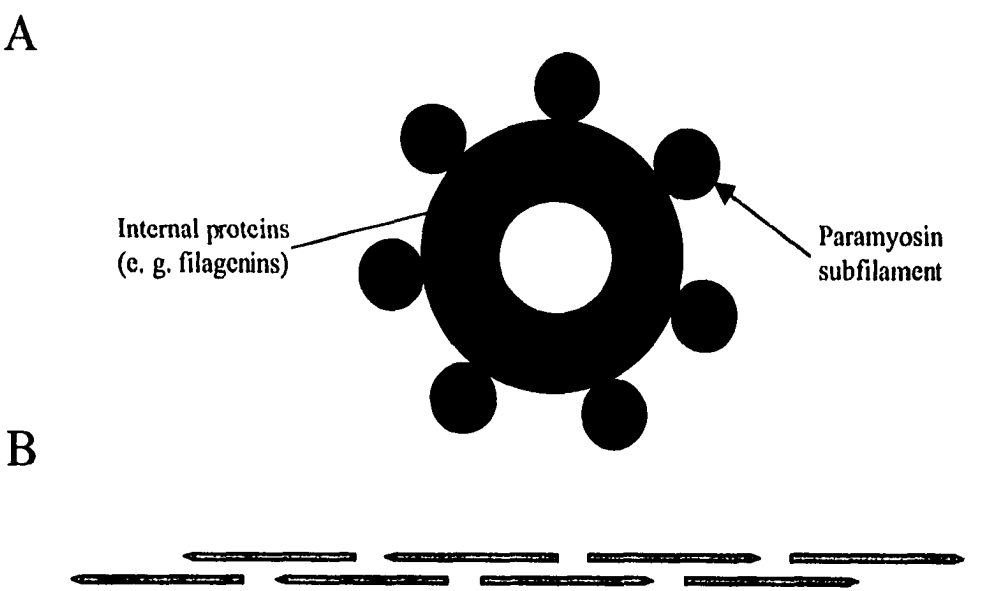

Figure 1.4 (A) A diagram of the cross section of the rigid tubule model of the core structure of *C. elegans* muscle thick filaments (Esptein et al., 1995). The outer layer is composed of seven paramyosin subfilaments (dark dots). These subfilaments are cross-linked with internal proteins (the gray ring). **(B) Each subfilament consists of two strands of paramyosin molecules.** Paramyosin dimers are packed in an anti-parallel manner in the center and in a parallel manner in the polar regions.

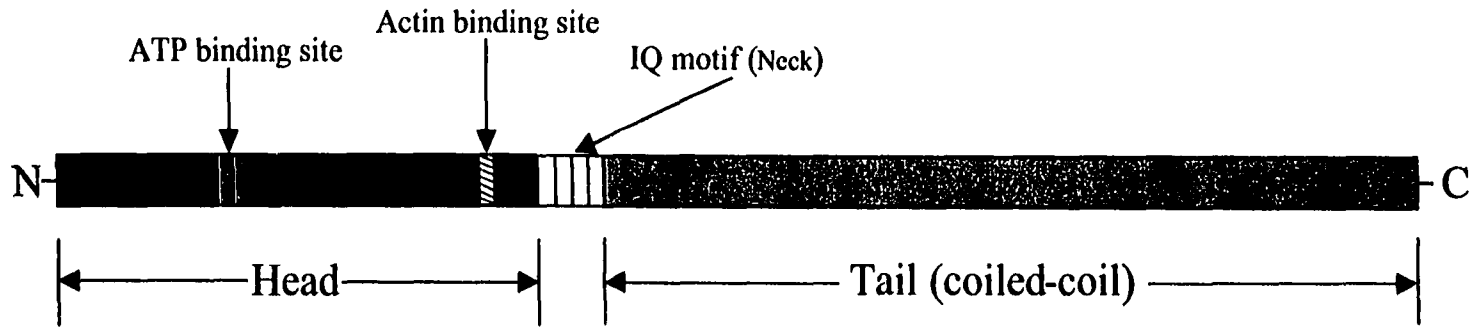

Figure 1.5. A diagram of a conventional myosin II molecule, showing the ATP and actin binding sites in the head domain, IQ motif (light chain and calmodulin binding sequence) in the neck domain and the coiled-coil tail domain.

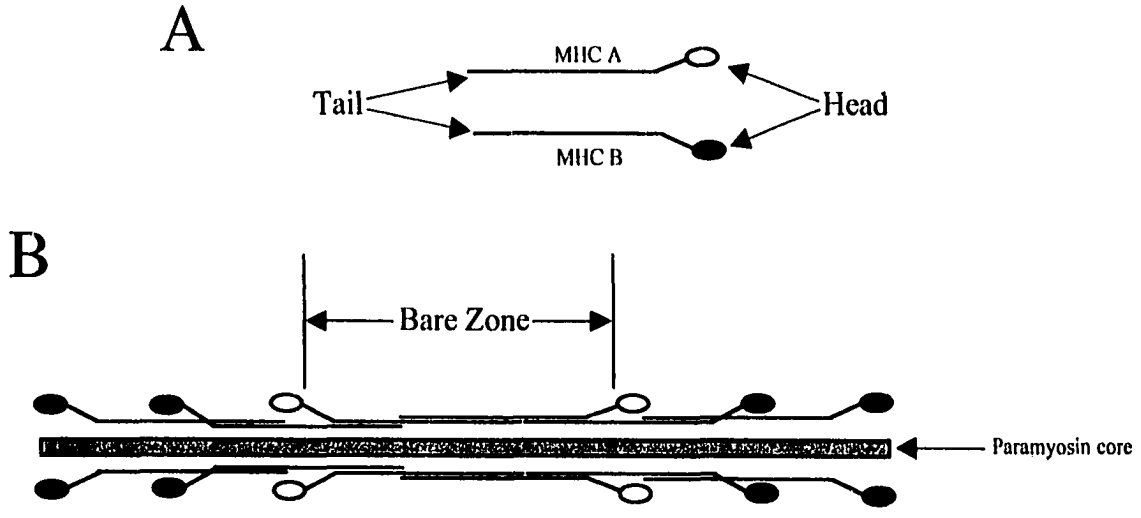

Figure 1.6 The two myosin isoforms of *C. elegans* body wall muscle. (A), MHC A and MHC B (homodimer molecules), head and tail domains are indicated. (B), MHC B dimers are packed in a parallel manner in the two polar regions of thick filaments, whereas MHC A molecules are packed in an anti-parallel manner, resulting in a central bare region where no myosin heads are present. The core structure of thick filaments consists of paramyosin subfilaments (Figure 1.4).

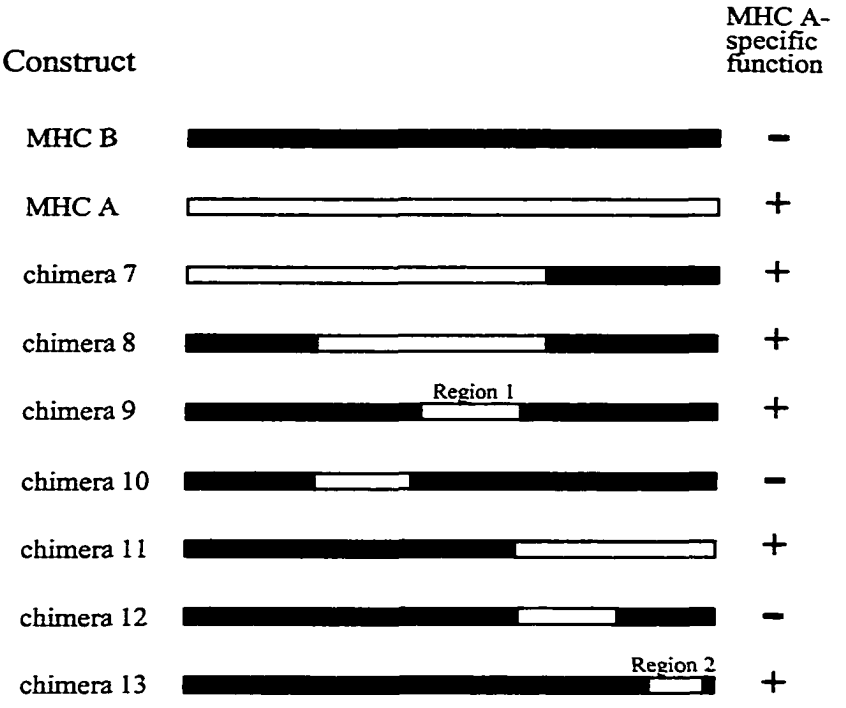

Figure 1.7 MHC A/MHC B chimeras used to test MHC A-specific function (Hoppe and Waterston, 1996). Two regions have been determined to be able to confer upon the chimera the ability to rescue a *myo-3* null mutant and initiate filament assembly. Region 1 is 263 amino acids in length and region 2 is a segment of 169 residues. Black boxes are regions from MHC B and white boxes are regions from MHC A.

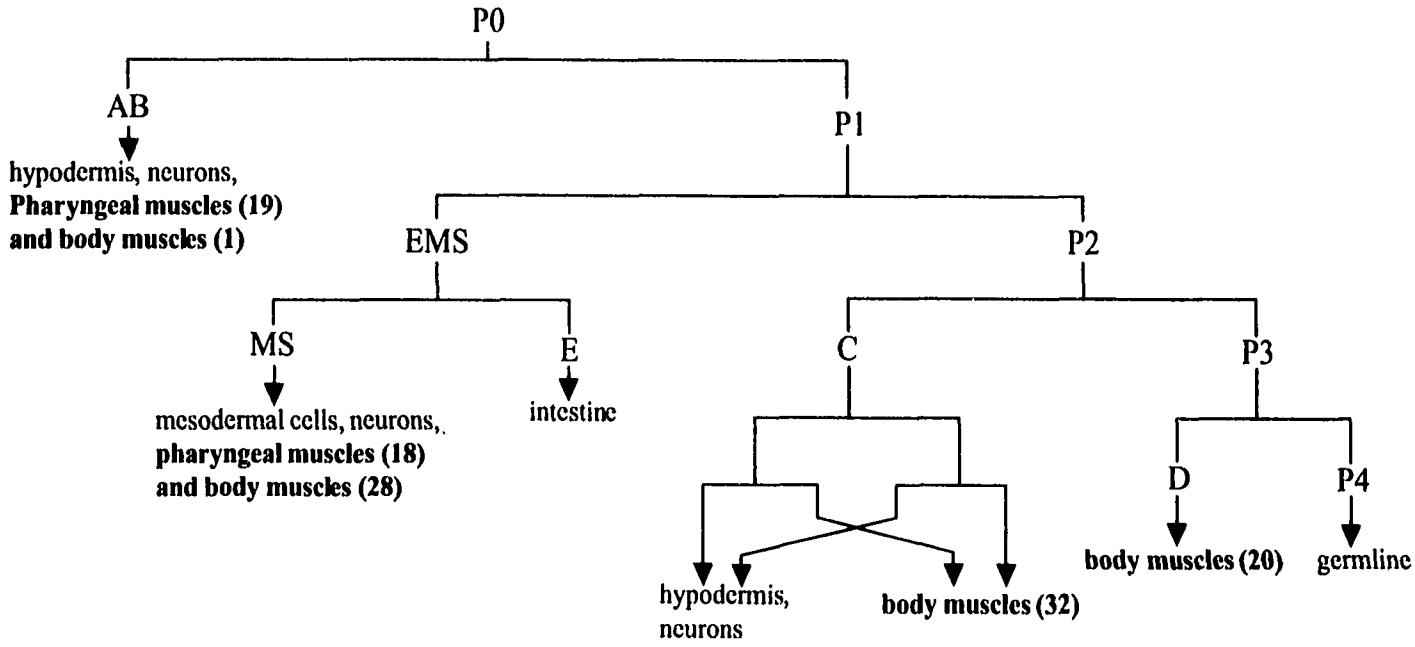

Figure 1.8 Embryonic cell lineage of a *C. elegans* hermaphrodite, showing the origin of muscle cells and other cell types from the founder cells (the number of muscle cells is shown parenthetically. Modified from Krause and Weintraub, 1992).

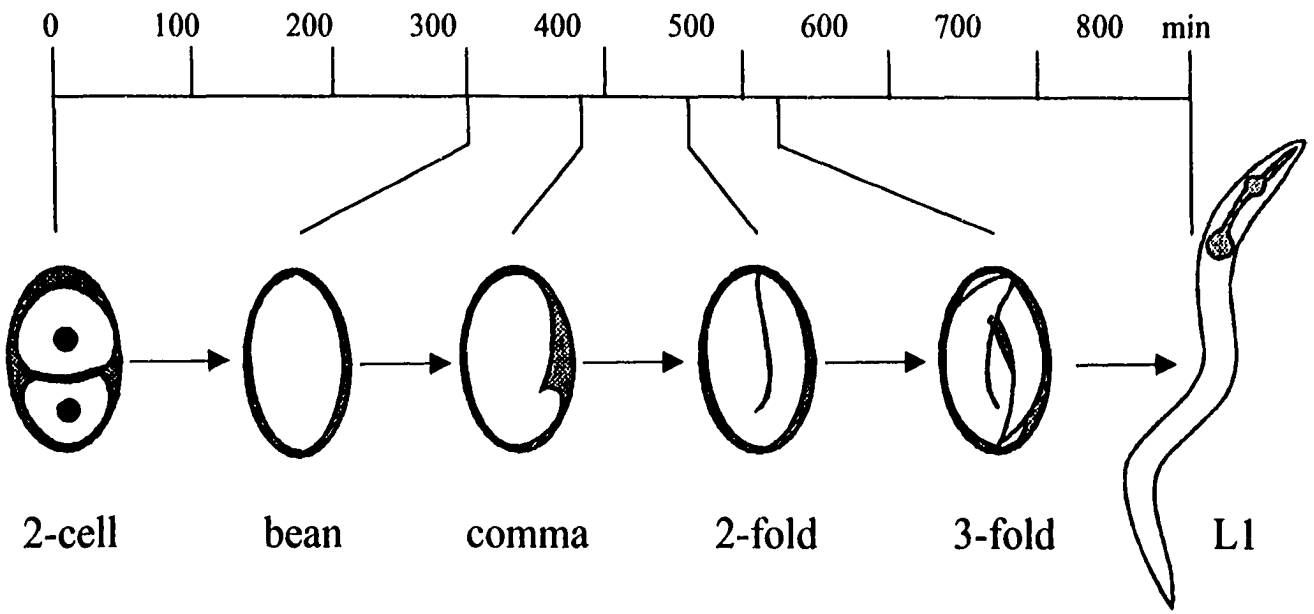

Figure 1.9 A diagram showing some of the stages of *C. elegans* embryogenesis. The developmental time of each stage is indicated in minutes after fertilization (Modified from Williams and Waterston, 1994).

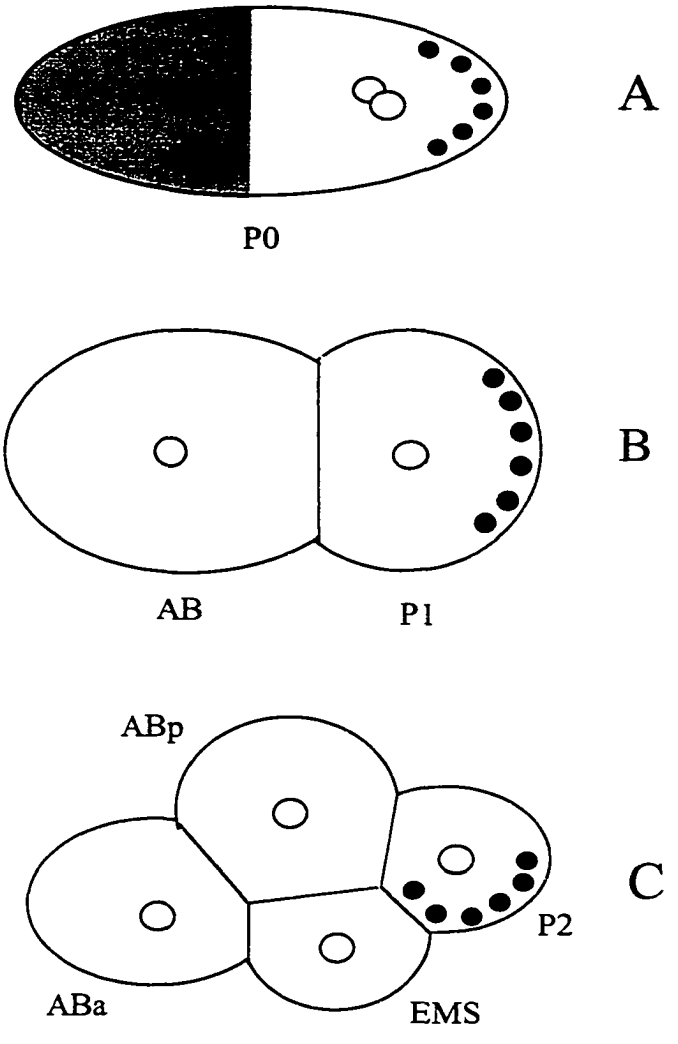

Figure 1.10 Polarity of the early embryo. (Left is anterior and right is posterior) A, late one-cell stage; B, two-cell embryo; C, four-cell embryo. Dark dots, P granules; small circles, nuclei (Modified from Kemphues and Strome, 1997).

25°C

15°C

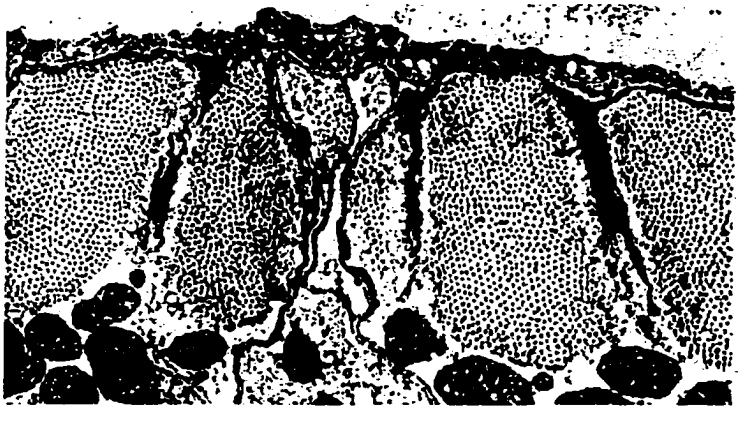

Figure 1.11 Electron microscopy images of *unc-45* (*e286*) at different temperatures, showing the defects in body wall muscles at the restrictive temperature of 25°C. (adapted from Epstein and Thomson, 1974)

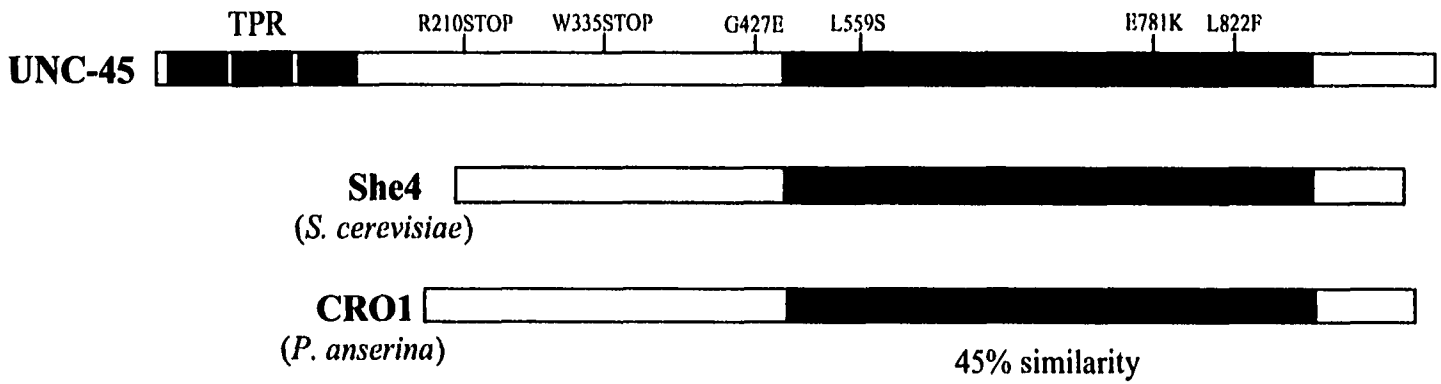

Figure 1.12 UNC-45 sequence alignment. *unc-45* encodes a protein of 961 amino acids, which contains three tetratricopeptide repeats (TPR) at its N-terminus. The C-terminus end of UNC-45 shows high similarity with the *S. cerevisiae* She4 protein and *Podospora* CRO1 protein. UNC-45 mutations are indicated as R210STOP (*st603*), W335STOP(*st601*), G427E(*b131*), L559S(*su2002*), E781K(*m94/r450*) and L822F(*e286*) (Barral et al., 1998).

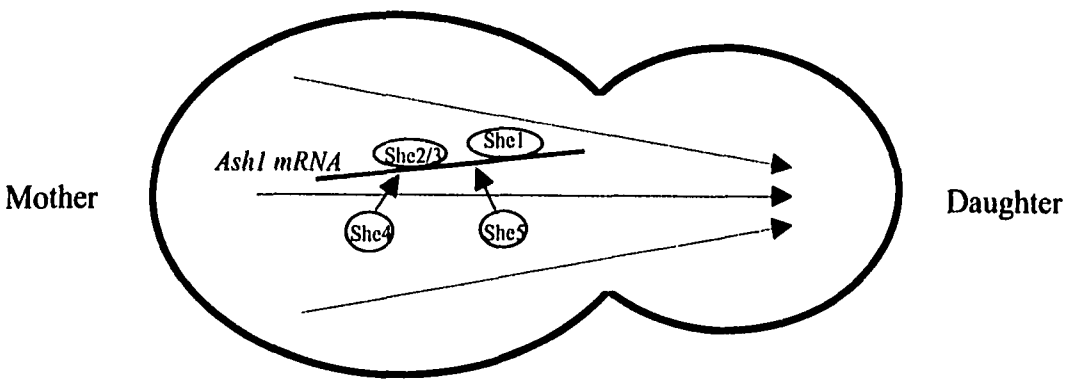

Figure 1.13 Transportation of *Ash1* mRNA into the daughter cell in the budding yeast. Ash1 is required to repress the *HO* expression in the daughter cell. Five She proteins are involved in this process and She1 is the mini-myosin Myo4 (Jansen et al., 1996).

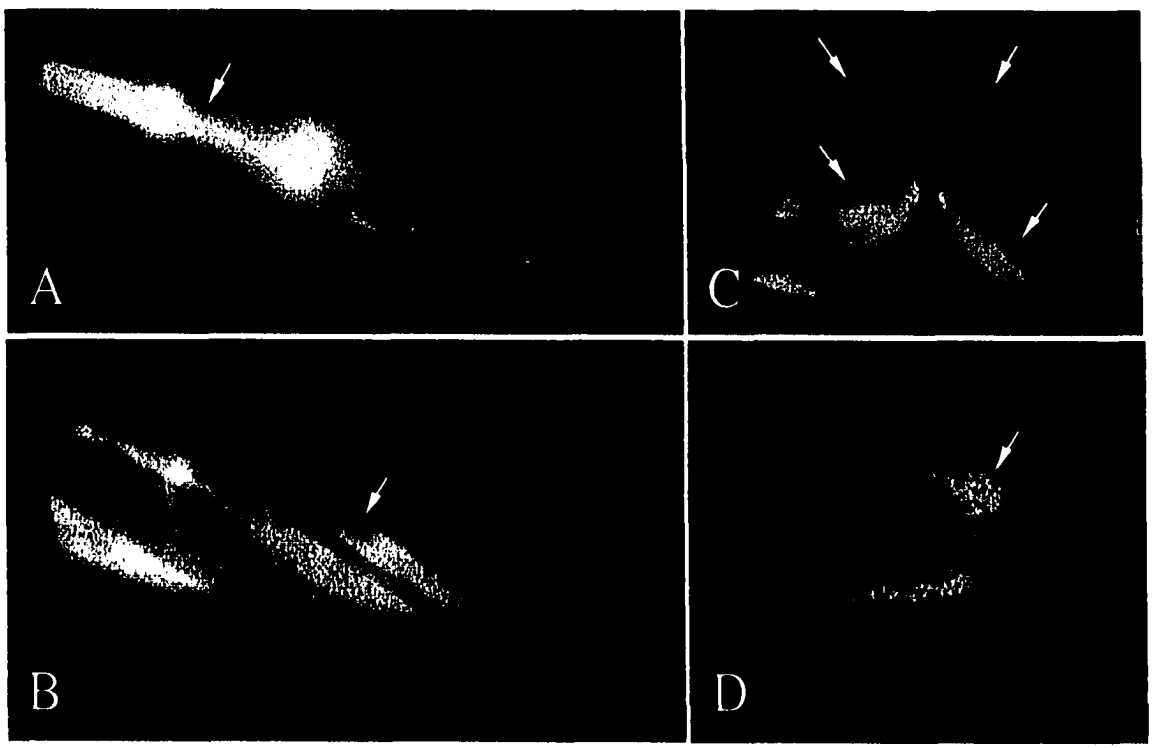

Figure 3.1 *unc-45*::GFP expression. A GFP reporter driven by the *unc-45* promoter is expressed in pharyngeal muscles (A), body wall muscles (B), vulval muscles (C) and anal muscles (D).

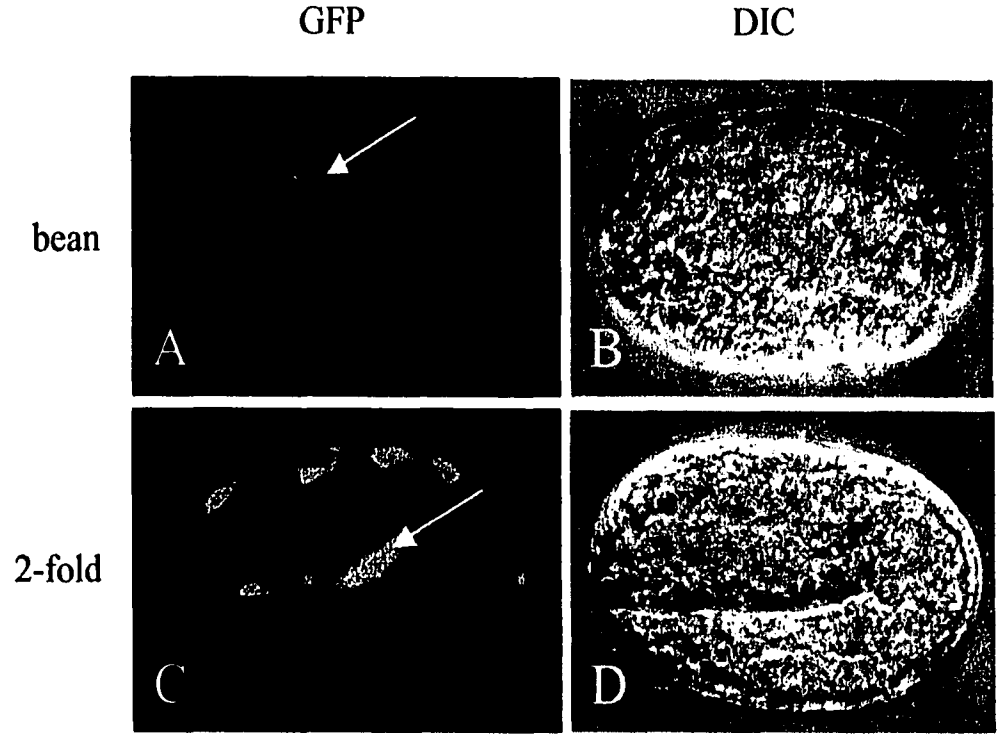

Figure 3.2 *unc-45*::GFP from an extra-chromosomal transgenic array is expressed only in the muscle cells, representing zygotic expression (anterior is to the left and dorsal to the top in A and B). A and C show the GFP expression in muscle cells at bean and two-fold stages of development, respectively. B to D are DIC images of the same embryos as A and C. Arrows indicate the GFP signal in the muscle cells. Bar, 10 μm.

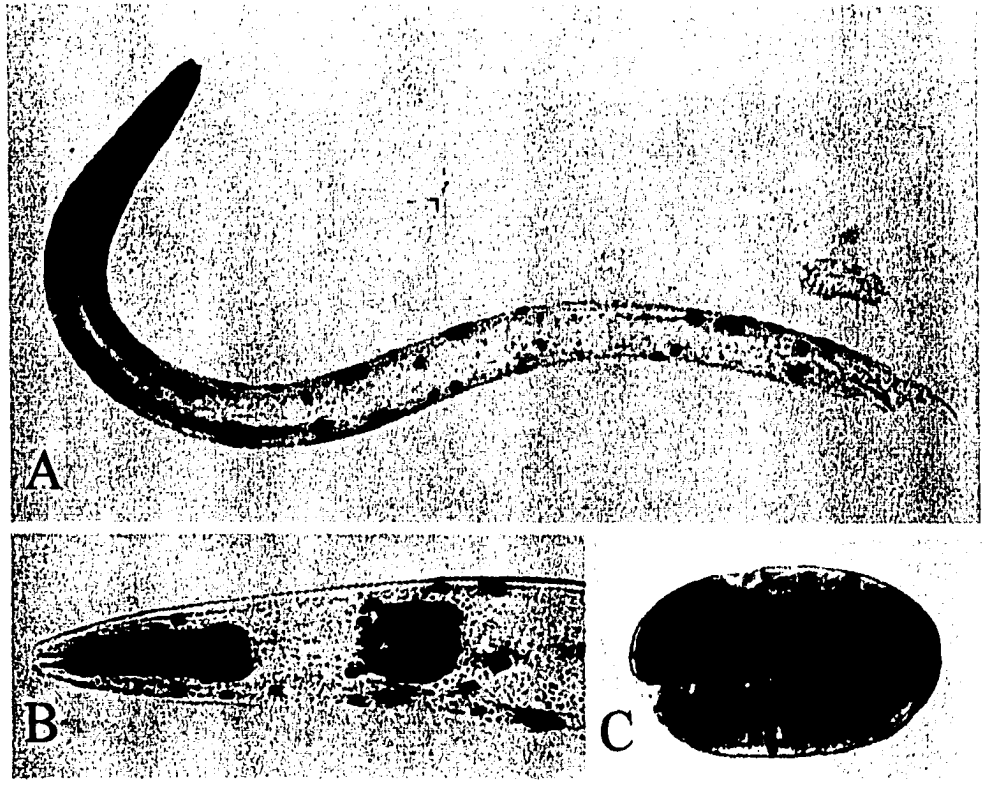

Figure 3.3 ***unc-45*::LacZ expression**. The ß-galactosidase signal is localized in the nucleus of the muscle cells since this fusion protein contains a nuclear localization signal. A, a young male adult worm, showing signal in body wall muscles and strong signal in the head. B, high magnification of the head of an adult worm, showing signal in pharyngeal muscles. C, ß-galactosidase signal in a two-fold embryo.

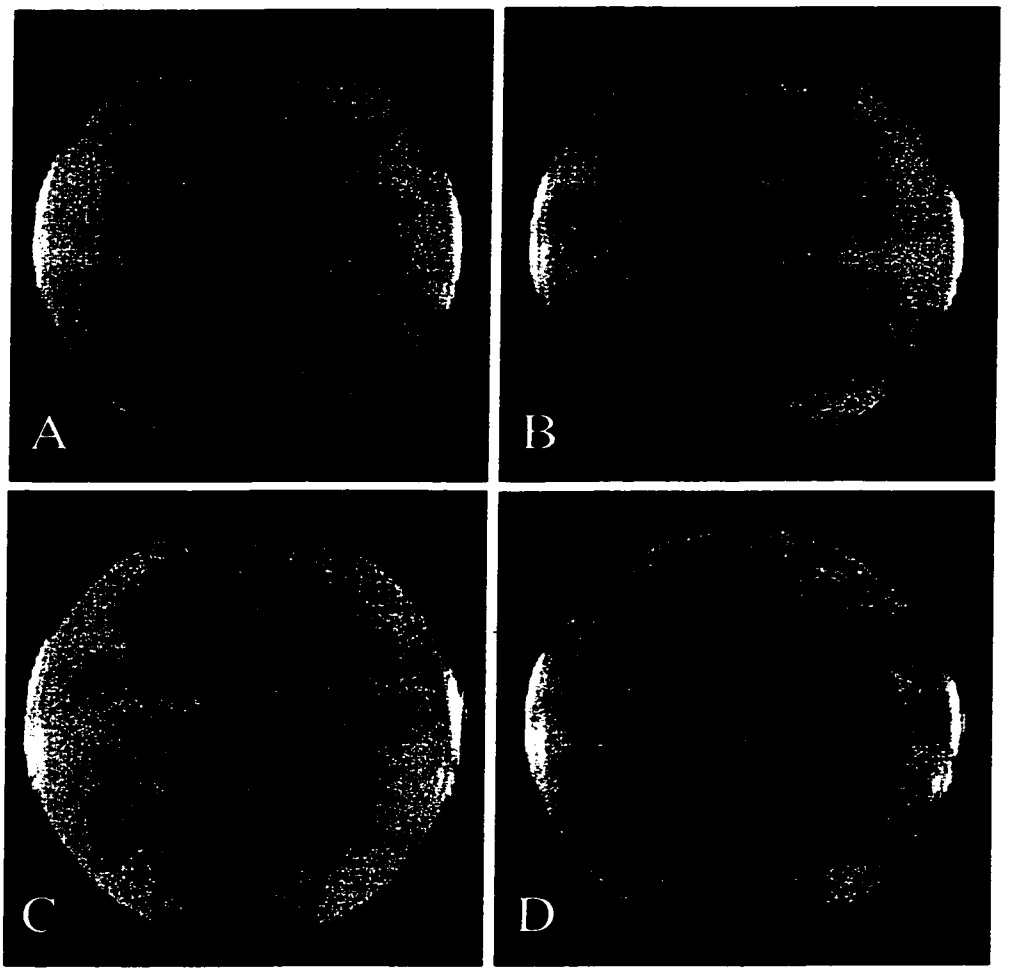

Figure 3.4 Motility assay for RW2329 [*unc-54* (*e190*) ; *sup-3* (*e1407st90st92*)] and RW2665 [*unc-54* (*e190*) ; *unc-45* (*m94*); *sup-3* (*e1407st90st92*)] mutants. A, B, C and D are plates showing the traces of worms after crawling for 1 hour at 25°C for RW2665[*unc-54* (*e190*) ; *unc-45* (*m94*); *sup-3* (*e1407st90st92*)], RW2329 [*unc-54* (*e190*) ; *sup-3* (*e1407st90st92*)], N2 and *unc-45* (*m94*), respectively. The dots in the centers of the plates are the start points and the others are the end points. N2 and *unc-45 (m94)* were used as controls. Five young adults were assayed for each strain and all showed similar motility. There is no apparent difference for the motility between RW2329 and RW2665.

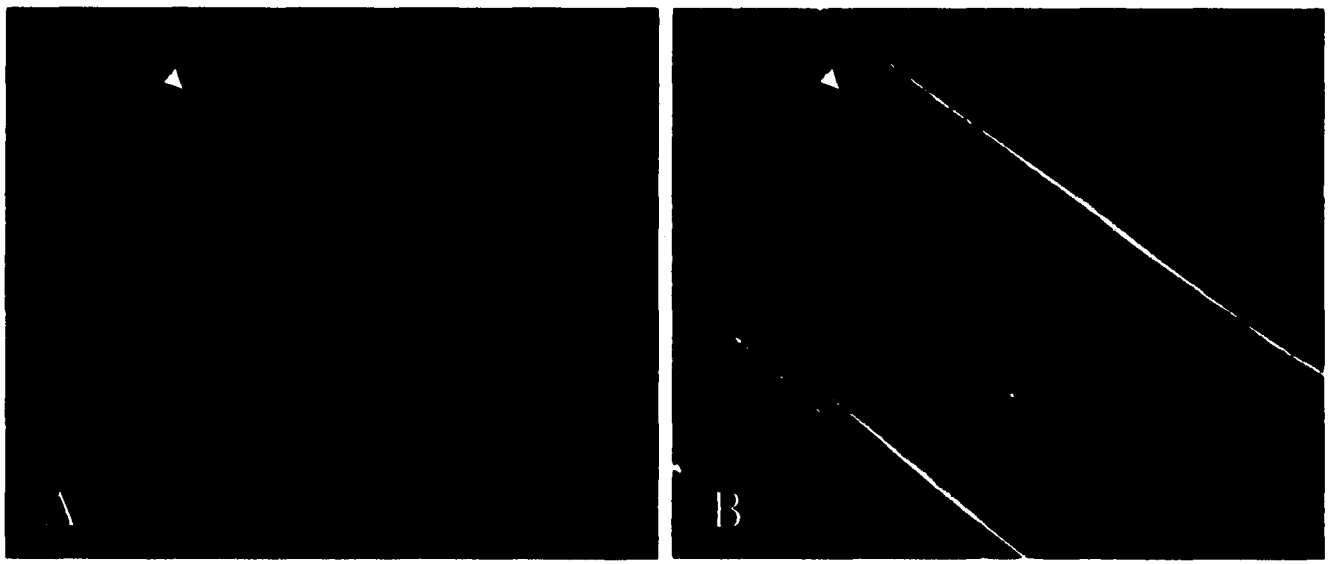

Figure 3.5 Comparison of the functional UNC-45::GFP protein distribution and the muscle filament pattern under polarized light microscopy in the body wall muscles of the rescued *unc-45* (*r450*). (A) is the GFP signal associated with the UNC-45::GFP fusion protein. (B) is the same field showing the muscle filament pattern as visualized by polarized light microscopy. The arrows indicate the thick filaments-containing A-bands. The bar is 10 μm.

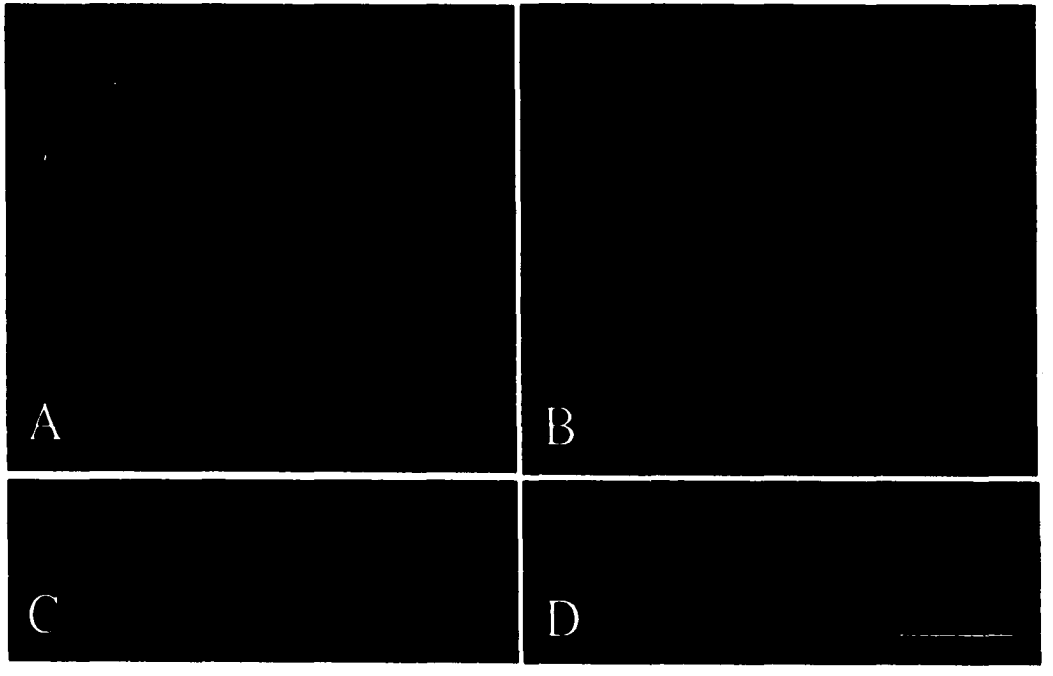

Figure 3.6 UNC-45 protein is detectable in the germline cells of the hermaphrodite gonad. A and B are the same distal part of a hermaphrodite gonad stained with anti-UNC-45 antibody (7N5) and DAPI, which stains the cell nuclei, respectively. C and D are the distal part of another hermaphrodite gonad stained with 7N5 pre-immune serum and DAPI, respectively as controls. Bar, 10 μm.

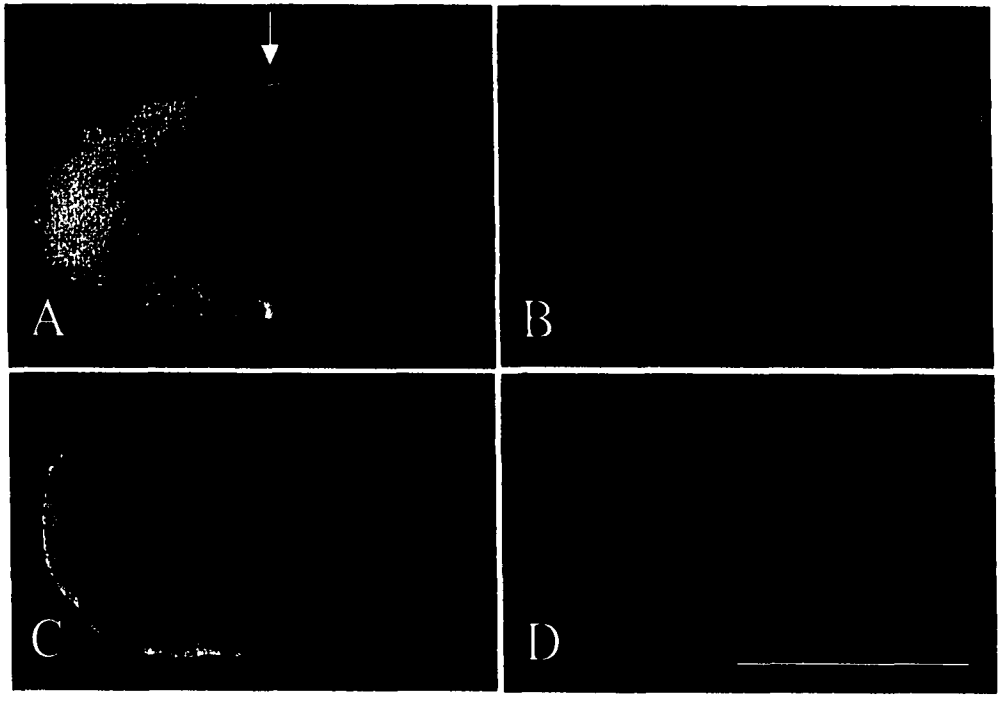

Figure 3.7 UNC-45 is expressed in the pre-morphogenesis embryos (anterior is to the left in all embryos). A and C are stained with anti-UNC-45 antibody (7N5). B and D are the same embryos as A and C, respectively stained with DAPI to show the cell nuclei. The developmental stages of these embryos are approximately (A) 40 min (two-cell)and (C) 100 min, respectively. The arrow in A indicates the UNC-45 concentration at the cleavage furrow. Bar, 10 μm.

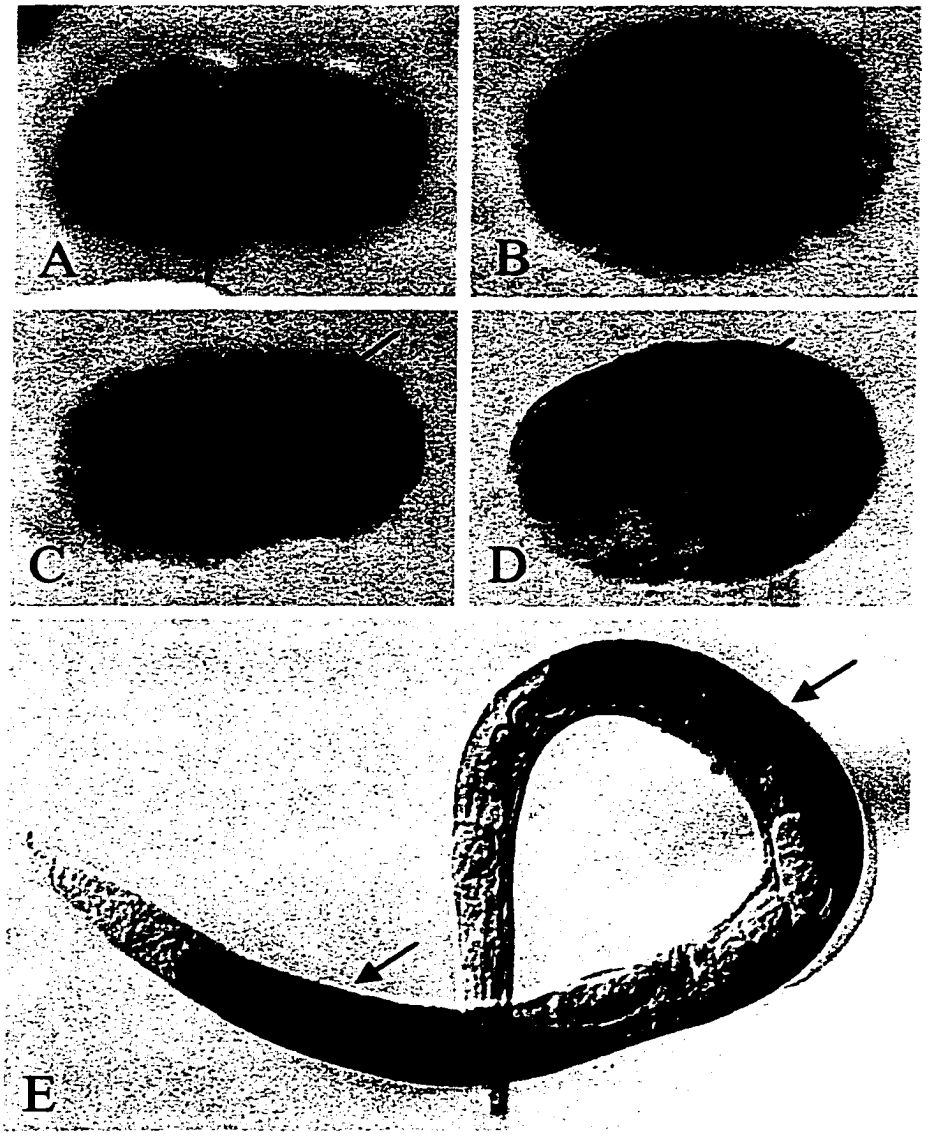

Figure 3.8 *In situ* hybridization with *unc-45* cDNA probe. A and B are embryos at the developmental time about 40 min (two-cell stage) and 100 min, respectively after fertilization, showing that *unc-45* mRNA is distributed in all cells. C and D are embryos at comma and two-fold stages, respectively. Arrows indicate muscle cells in which *unc-45* mRNA is enriched. E is a hermaphrodite adult, showing *unc-45* mRNA in the germline cells of the gonad. Data from Dr. Yuji Kohara at http://watson.genes.nig.ac.jp/db/keysrch.html.

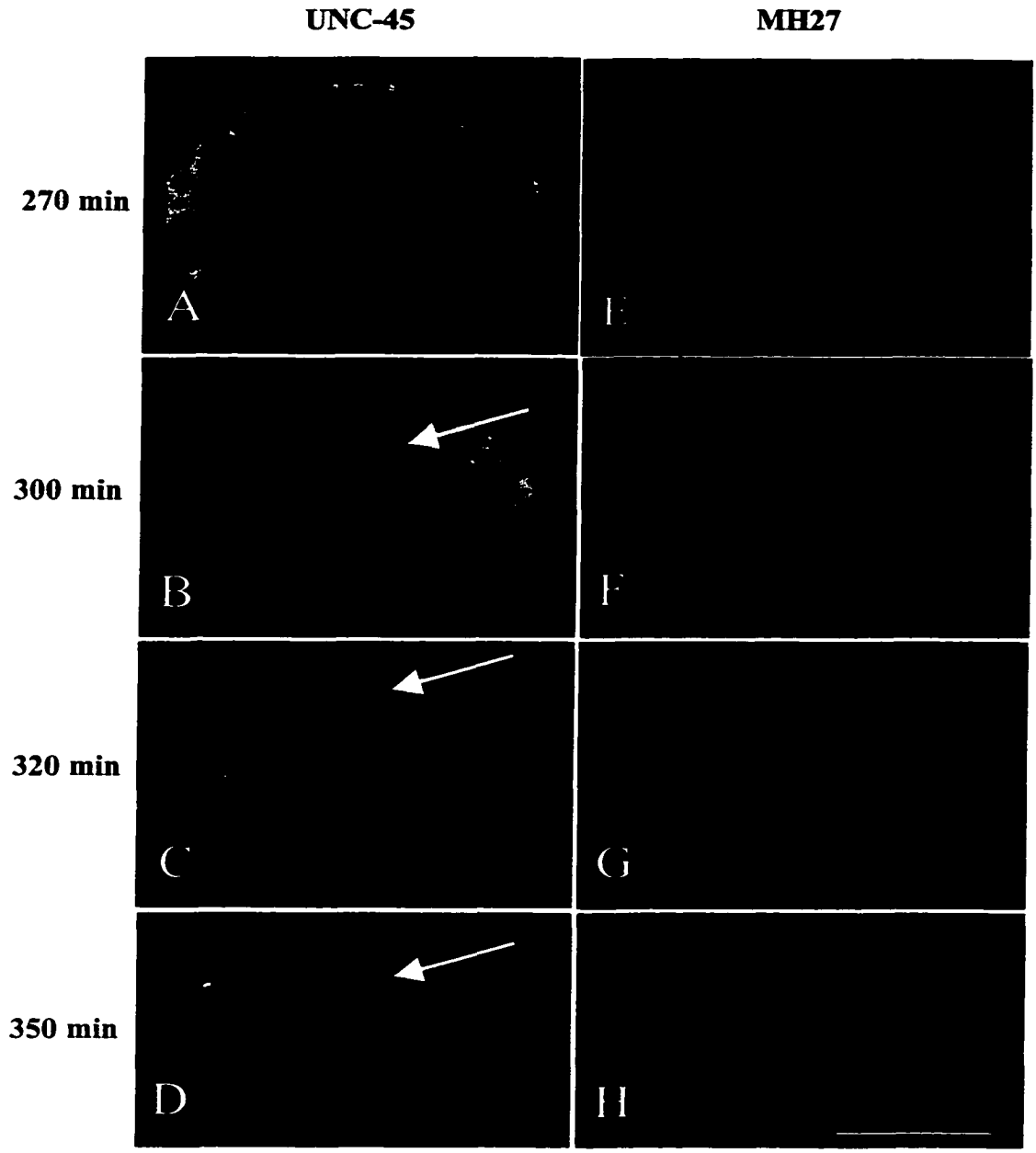

Figure 3.9 UNC-45 protein begins to be enriched in the muscle cells at about 420 cell stage of embryogenesis (See the legend on next page).

Figure 3.9 UNC-45 protein begins to be enriched in the muscle cells at about the 420 cell stage of embryogenesis (anterior is to the left and dorsal to the top in all embryos). A to D are stained with anti-UNC-45 antibody (7N5). E to H are the same embryos as A to D, respectively, stained with MH27 monoclonal antibody. The developmental stages of these embryos are (A) 270 min, (B) 300 min, (C) 320 min and(D) 350 min after fertilization, respectively. Arrows in B, C and D indicate the staining in the muscle cells. UNC-45 distribution is still even in all the cells at 270 min (A) but begins to be concentrated in the muscle cells by 300 min (B). Bar, 10 μm.

Figure 3.10 UNC-45 co-localizes with MHC B (Please see the legend on next page)

Figure 3.10 UNC-45 co-localizes with MHC B in the thick filaments of body wall muscles of wild-type adult worms. A, B and C are the same field of body wall muscle labeled with anti-UNC-45 (7N5), anti-MHC B (DM 5-8) or double-labeled with 7N5 and DM 5-8, respectively. D, E and F are the same field of body wall muscle labeled with 7N5, anti-MHC A (DM 5-6) or double-labeled with 7N5 and DM 5-6, respectively. G, H and I are the same field of body wall muscle labeled with DM 5-8, DM 5-6 or double-labeled with DM 5-8 and DM 5-6, respectively. Note that DM 5-8 reacts with MHC B which is localized on the two polar regions of the A-bands and DM 5-6 reacts with MHC A which is localized in the middle of the A-bands. C shows the overlapped pattern of UNC-45 and MHC B at the two polar regions of the thick filaments. F and I show a similar pattern with a central labeled strip when double-labeled with UNC-45 and DM-5-6 or DM 5-8 and DM 5-6, respectively. The arrows indicate the central unlabeled strip for UNC-45 (A and D) and for MHC B (B and G), the central unlabeled strip for double-staining(C) as shown in Figure 3.9 E and the central labeled strip for double-staining (F and I) in Figure 3.9 F. The bars are 10 μm.

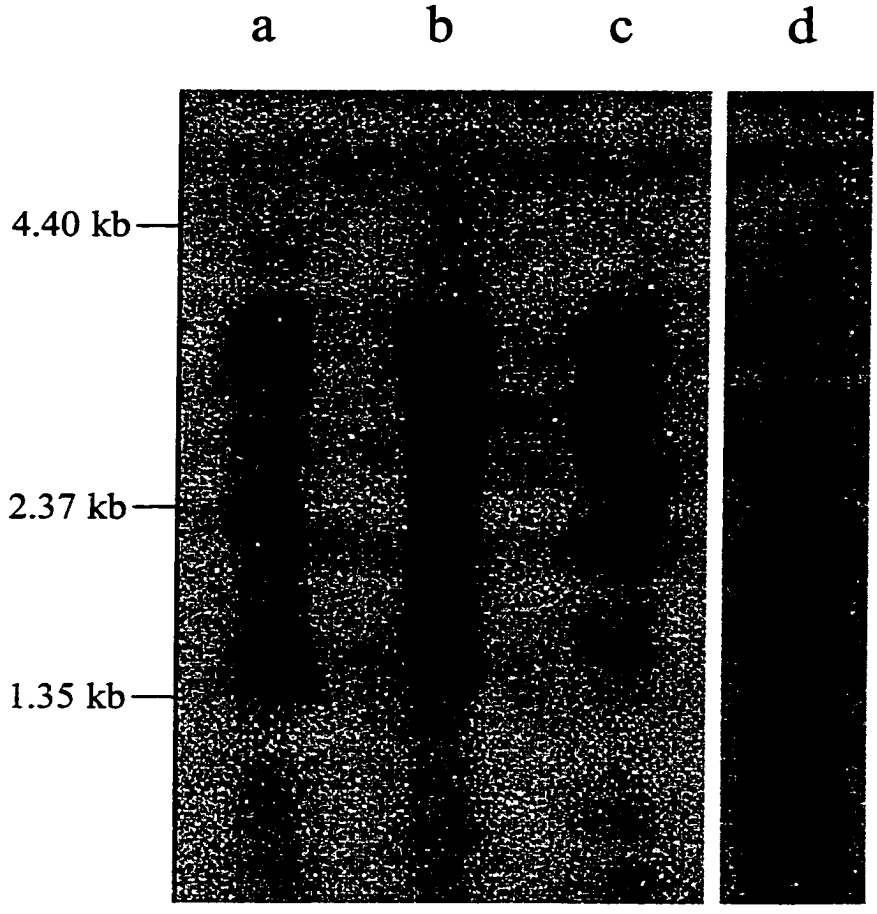

Figure 3.11 Northern analysis. Lanes a-c, the 3.5 kb *unc-45* mRNA was detected using an anti-sense probe (a. 2 μg of total RNA; b. 2 μg of poly A^+ enriched RNA; c. 2 μg of poly A^+ depleted RNA); d, as a control, the 2.0 kb *act-1* mRNA was detected with the *act-1* cDNA probe (2 μg of total RNA). The positions of RNA molecular size standards are indicated.

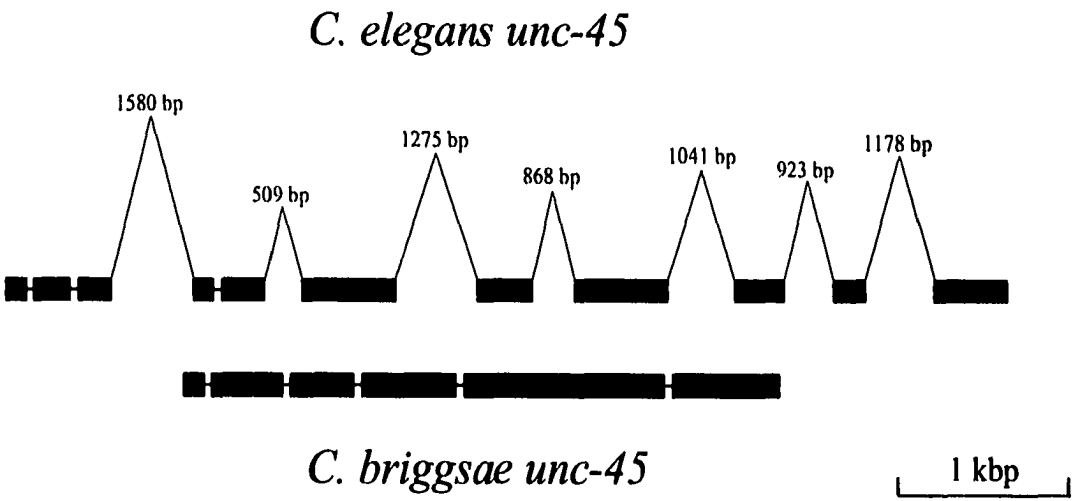

Figure 3.12 Comparison of genomic organization of *C. elegans unc-45* gene with its *C. briggsae* homologue. *C. elegans unc-45* has 11 exons and covers about 12 kbp of genomic DNA, whereas its *C. briggsae* homologue has only 6 exons and covers about only 3.1 kbp of genomic DNA. These two genes are drawn to scale. Black bars are exons (Large introns are drawn as peaks and their sizes are indicated).

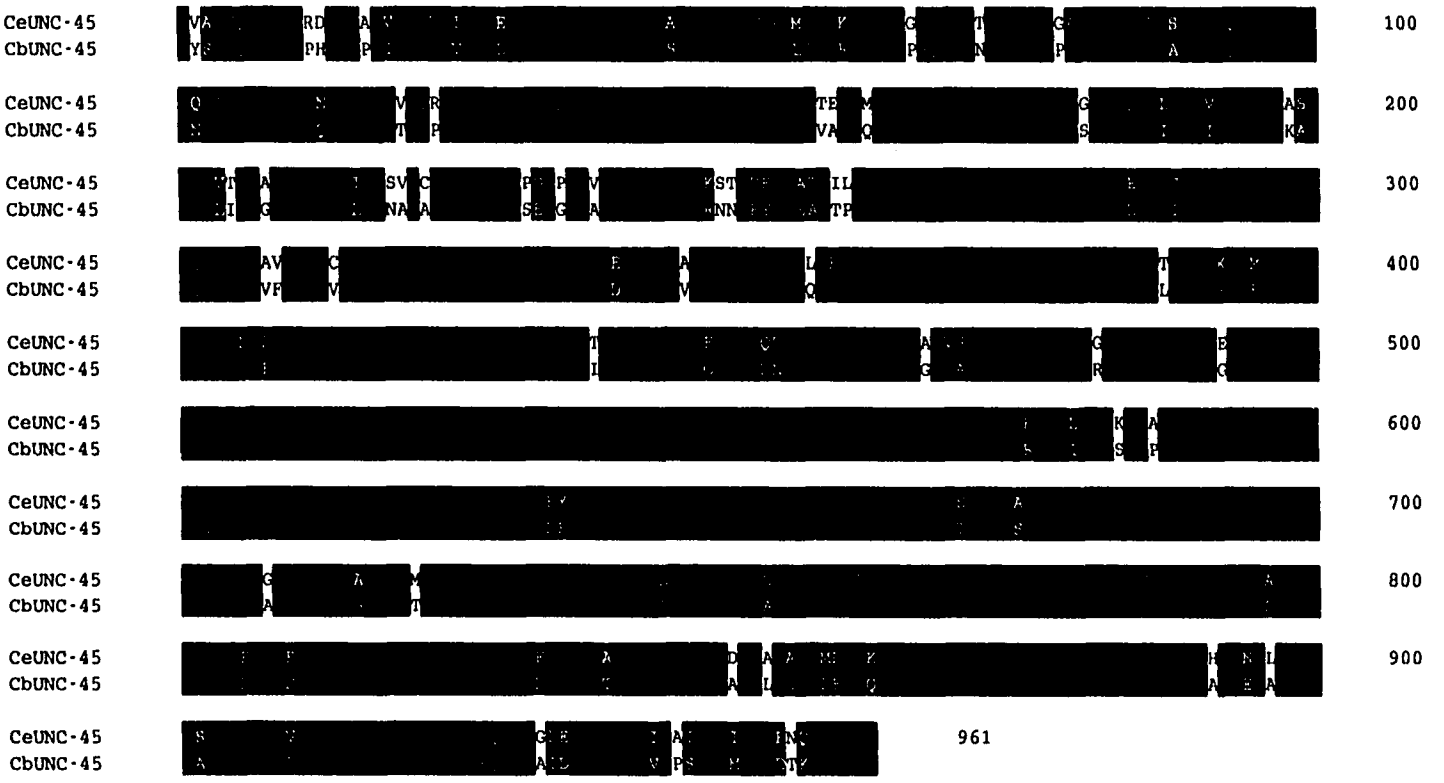

Figure 3.13 Alignment of *C. elegans* and *C. briggsae* UNC-45 homologue sequences. Identical amino acids are darkly shaded and similar amino acids are lightly shaded.

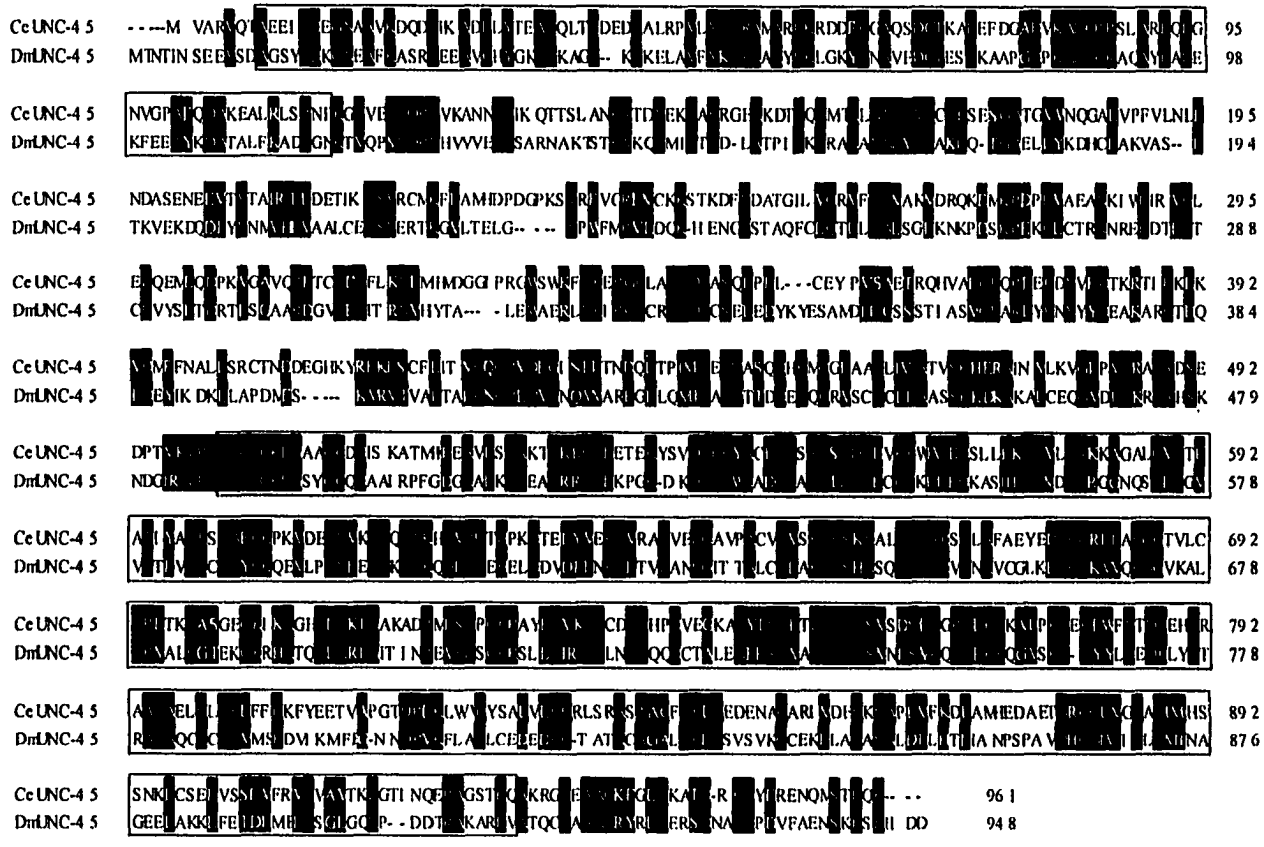

Figure 3.14 Alignment of UNC-45 with its *Drosophila* homologue. Identical amino acids are darkly shaded and similar amino acids are lightly shaded. The TPR domain at the N-terminus and the CRO1/She4 domain at the C-terminus are boxed (*Drosophila* homologue accession #AE003677).

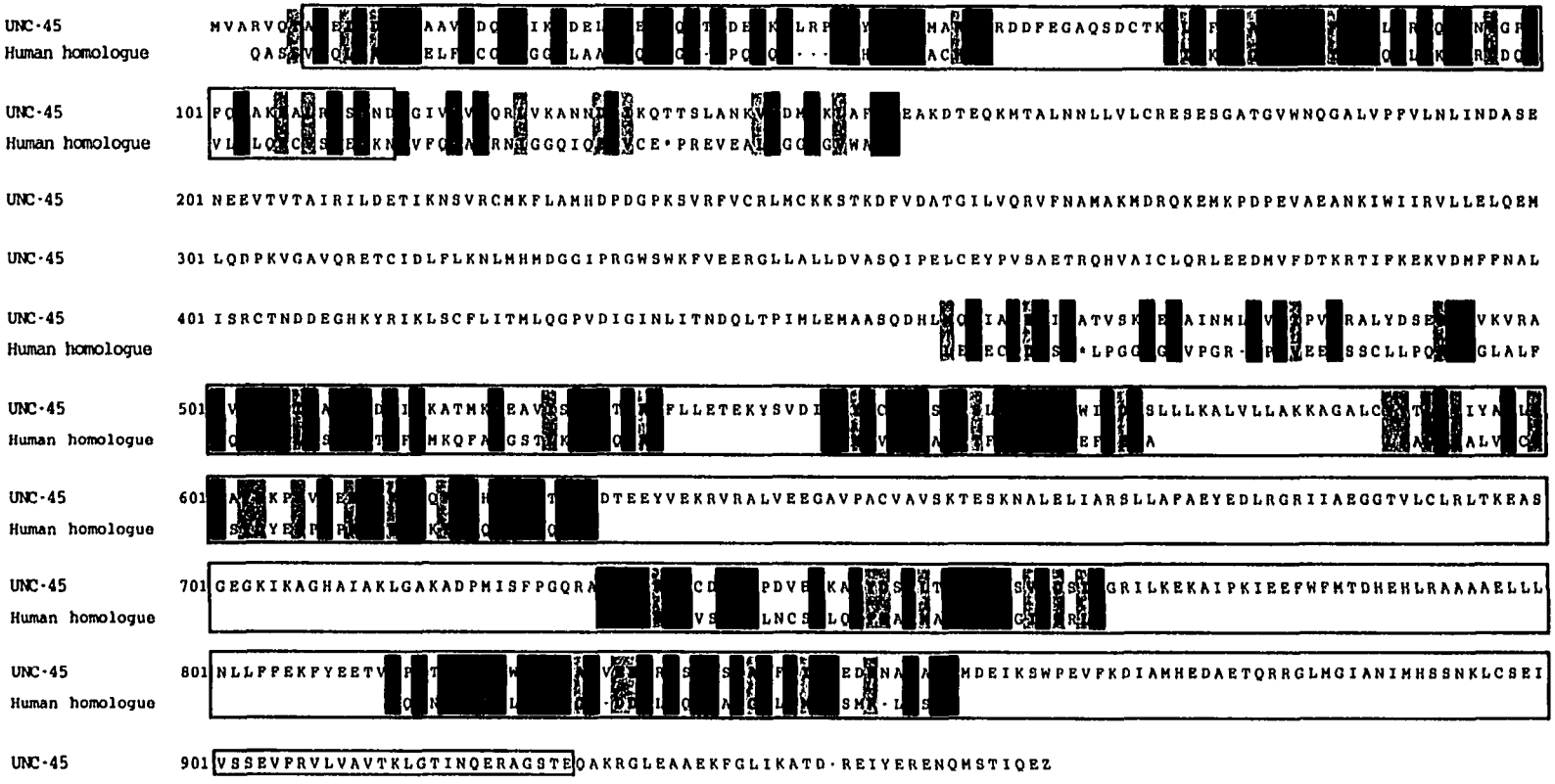

Figure 3.15 Alignment of UNC-45 with partial sequences of its putative human homologue. These sequences are 7 unordered pieces from human chromosome 15q6.1 (Accession #AC004586.1). The TPR domain at the N-terminus and the CRO1/She4 domain at the C-terminus are boxed.

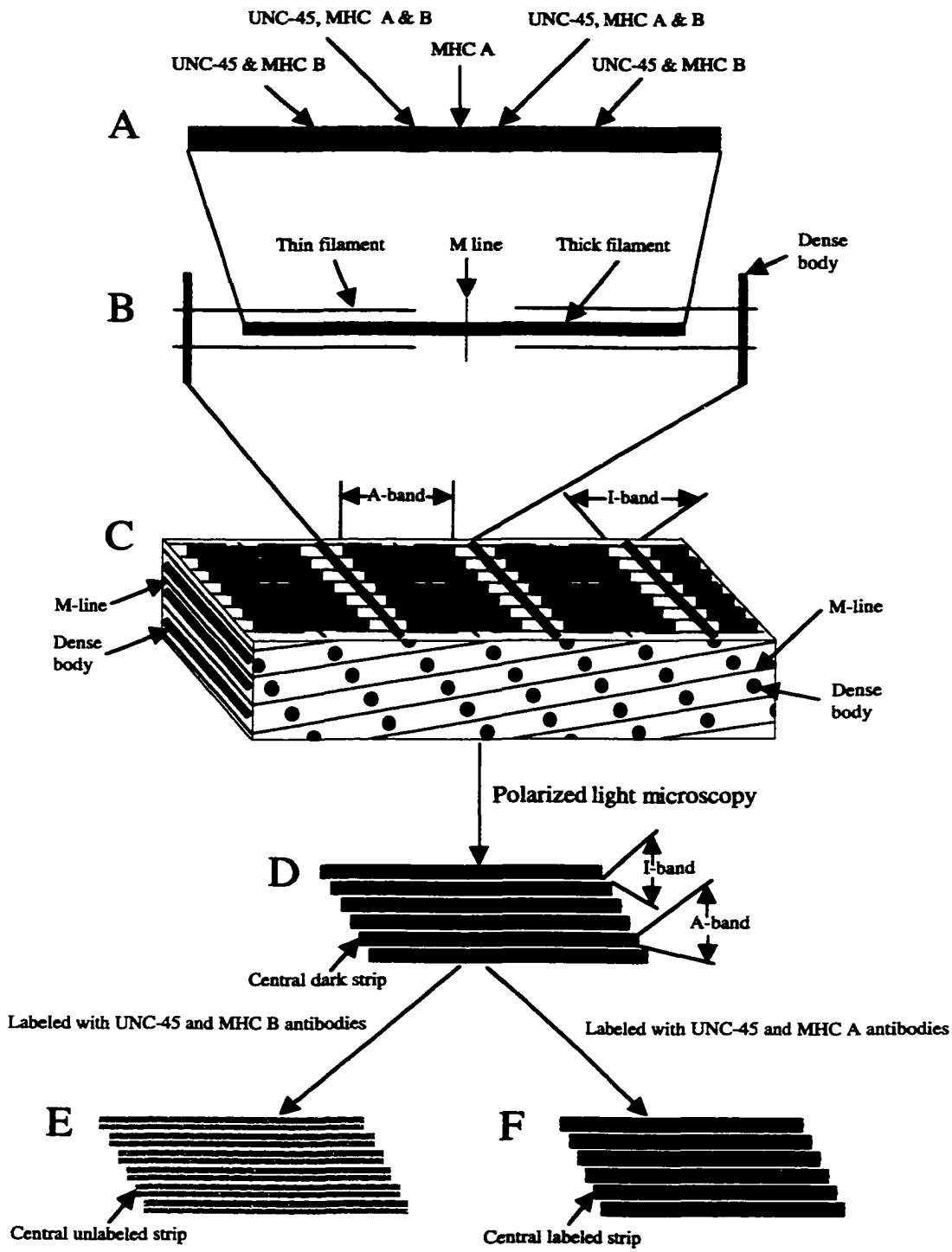

Figure 3.16 A schematic diagram of muscle thick filaments (please see the legend on next page).

Figure 3.16 A schematic diagram of muscle thick filaments. (A) Thick filament, depicting the localization of UNC-45, MHC A and B. (B) Part of a sarcomere, showing the structure of thick filament, thin filament, M-line and dense body. (C) Part of a body wall muscle, showing A-band, I-band, M-line and dense body. (D) Schematic view of thick filaments as visualized by polarized light microscopy, showing A-band and the central dark strip in the A-band which corresponds to the region containing only the thick filaments but not thin filaments. (E) Schematic view of thick filaments when labeled with both UNC-45 (green) and MHC B (red) antibodies as shown in Figure 3.8 C. The color appears as light yellow, since the UNC-45 and MHC B are completely overlapped. The central unlabeled strip corresponds to the region containing only MHC A, but not MHC B or UNC-45. (F) Schematic view of thick filaments when labeled with both UNC-45 (green) and MHC A (red) antibodies as shown in Figure 3.8 F. The central labeled strip corresponds to the whole region containing MHC A and appears as dark yellow due to the overlapping parts of MHC A and UNC-45 and the central part containing only MHC A.

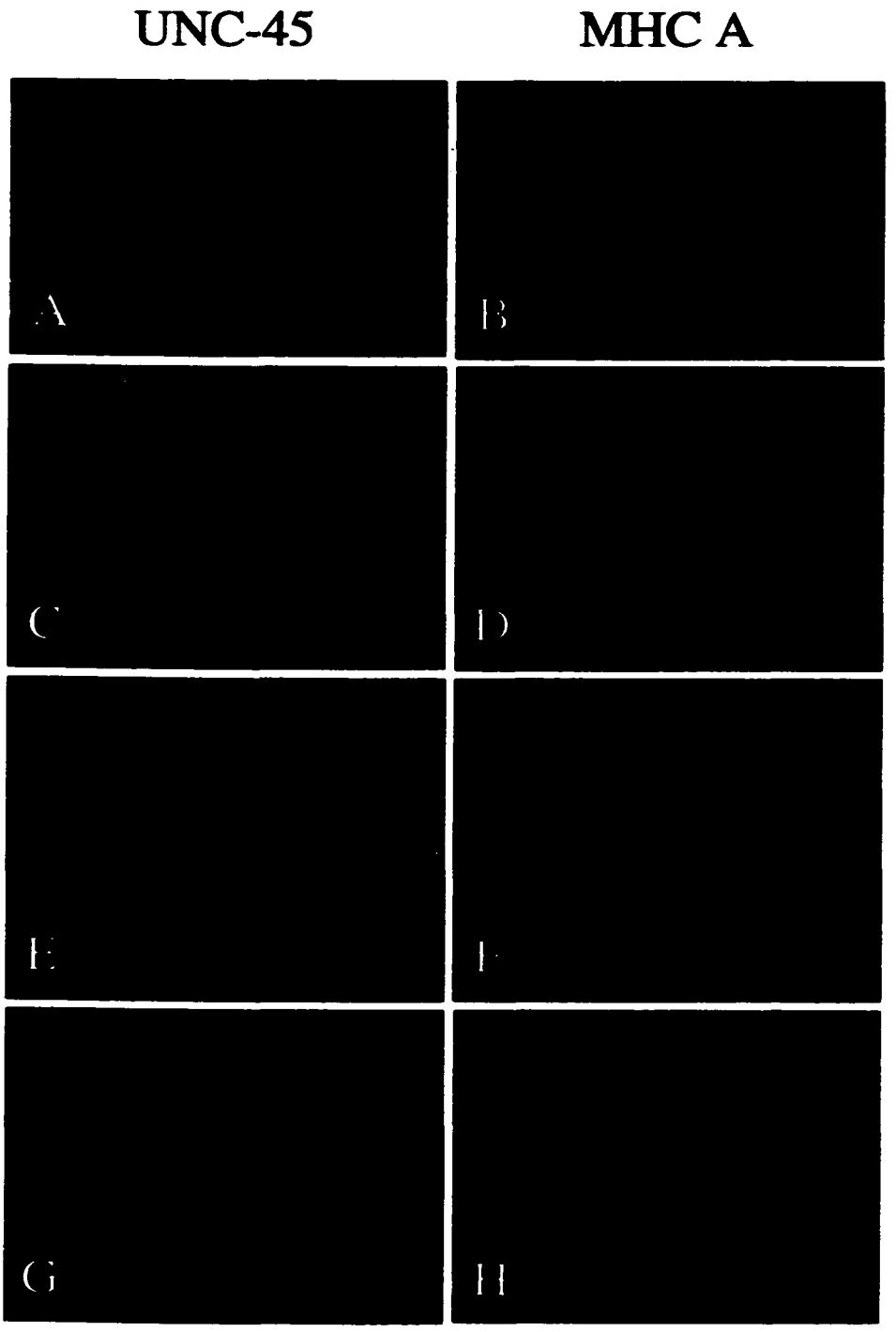

Figure 3.17 UNC-45 and MHC A staining in mutant worms
(Please see the legend on next page).

Figure 3.17 UNC-45 and MHC A staining in mutant worms. A and B are the same field of body wall muscle from *unc-45* (*r450*) mutant animals grown at the restrictive temperature (22°C) labeled with 7N5 and DM 5-6, respectively. C and D are the same field of body wall muscle from *unc-54* (*e190*) mutants labeled with 7N5 and DM 5-6, respectively. E and F are the same field of body wall muscle from *unc-54* (*e190*) ; *sup-3* (*e1407st90st92*) mutants labeled with 7N5 and DM 5-6, respectively. G and H are the same field of body muscle from *unc-54* (*e190*) ; *unc-45* (*m94*); *sup-3* (*e1407st90st92*) mutants labeled with 7N5 and DM 5-6, respectively. Note that UNC-45 is localized to thick filaments in *unc-45* (*ts*) mutants (A) , but not in *unc-54* (*0*) mutants (C) in which MHC B is absent and *unc-54* (*0*); *sup-3* mutants (E and G) in which MHC B is absent, but the level of MHC A is increased. Bars, 10 μm.

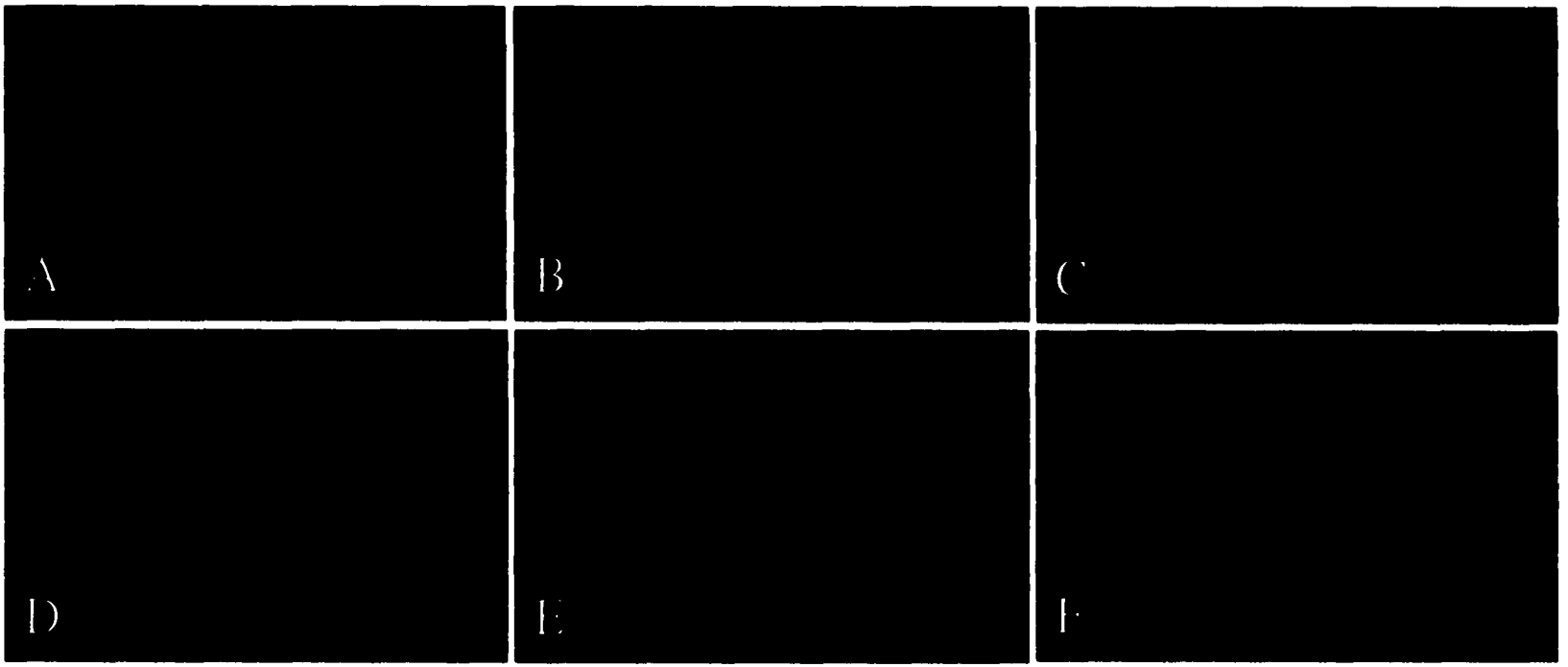

Figure 3.18 UNC-45 may be added into thick filaments after MHC isoforms. A, B and C are the same part of a wild-type worm at the L1 larval stage labeled with 7N5, DM 5-8 or double-labeled with 7N5 and DM 5-8, respectively. D, E and F are enlarged for the boxed areas of A, B and C, respectively, showing one muscle cell. Note that UNC-45 is diffuse in the cytoplasm (D) whereas MHC B has been assembled into thick filaments near the cell membrane along the face of the muscle cell that is adjacent to the hypodermis (E). The bars are 10 μm.

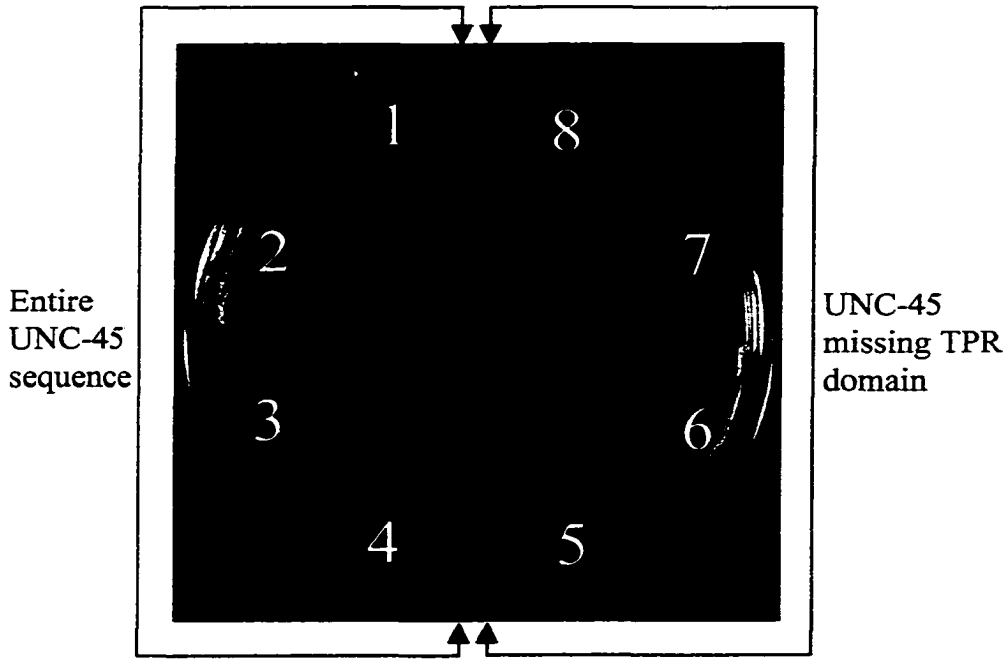

Figure 3. 19. Four positive clones identified from yeast two-hybrid screens. 1-4 are their interactions with the *unc-45* whole cDNA bait (pDP#WA039) on the selective media. 5-8 are their interactions with a 2.6 kbp *unc-45* cDNA bait (pDP#WA040) in which the region encoding the TPR domain has been removed. 1 and 5 are CeUFD2. 2 and 6 are HUM-2. 3 and 7 are NMY-2. 4 and 8 are from a weak positive, which was not further characterized.

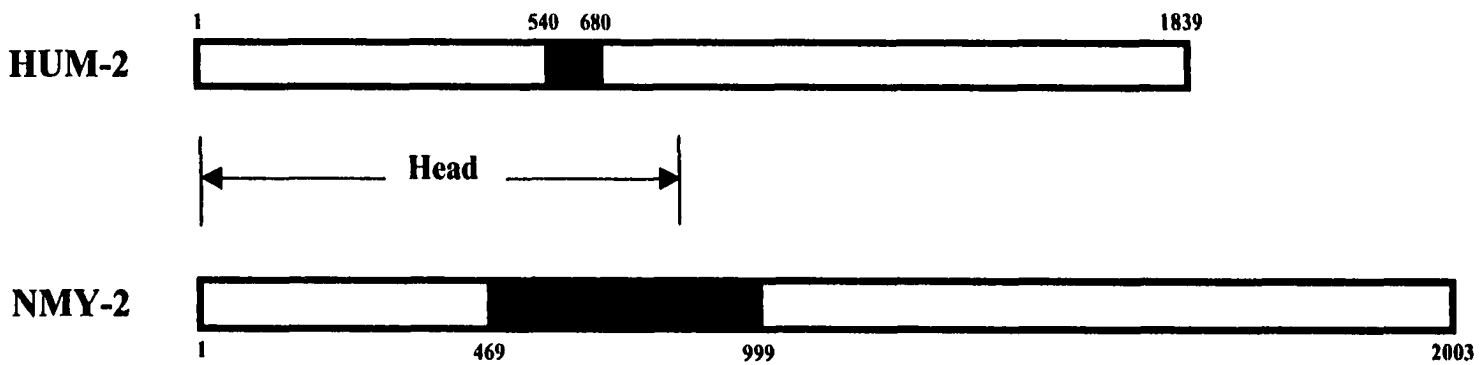

Figure 3.20. Two myosins identified from the yeast two-hybrid screens that interact with UNC-45. HUM-2 is an unconventional type V myosin and the identified cDNA encodes a 140 amino acid segment located in the head domain (gray part). NMY-2 is a non-muscle type II myosin and a region of 530 amino acids (gray part) located mostly in the head domain interacts with UNC-45. Note that these two identified regions overlap in the highly conserved head domain as indicated.

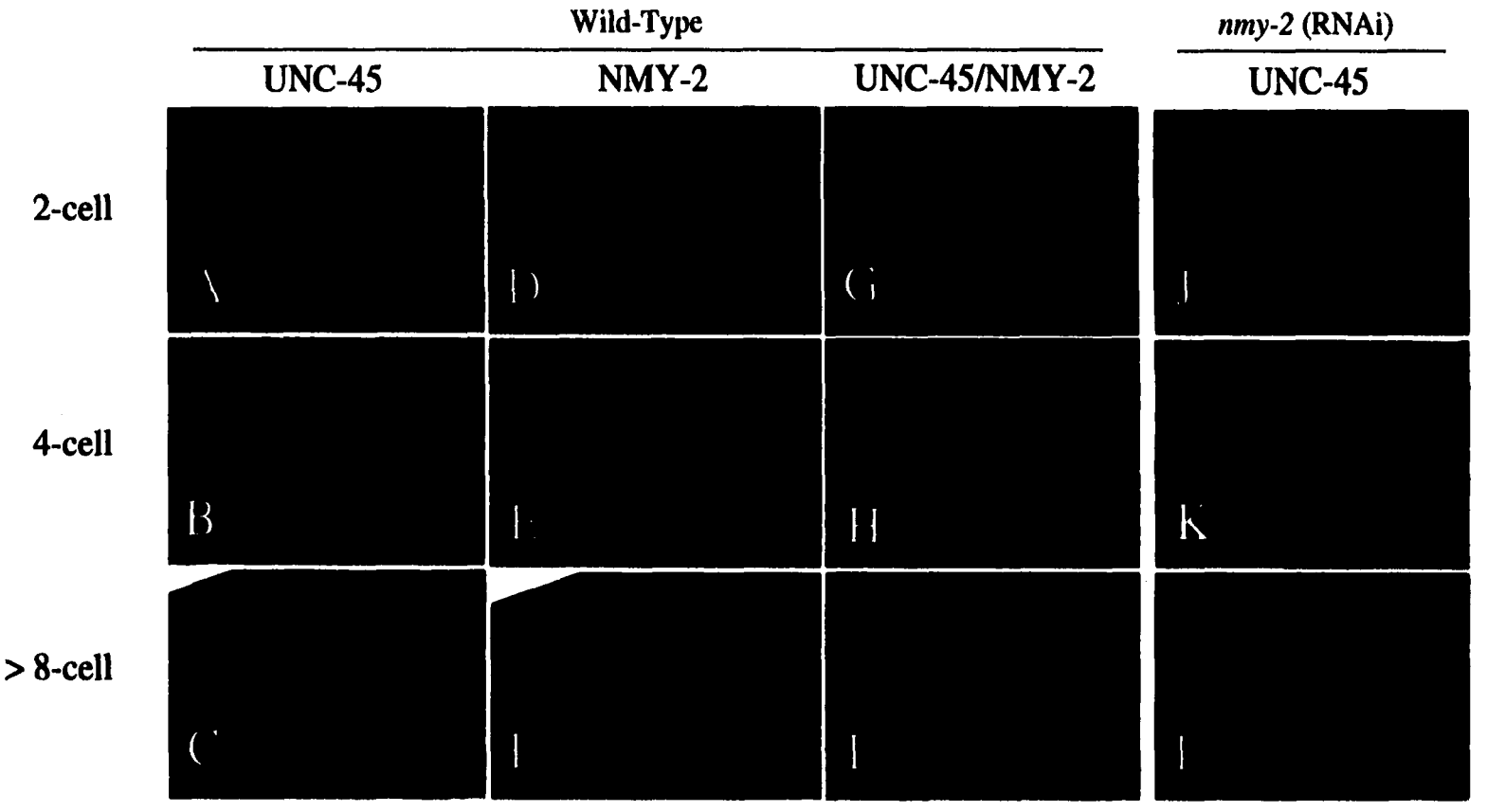

Figure 3.21. Confocal images of embryos, showing the localization of UNC-45 and NMY-2 in wild-type and the localization of UNC-45 in the *nmy-2* (RNAi) embryos (See the legend on next page).

Figure 3.21. Confocal images of embryos, showing the localization of UNC-45 and NMY-2 in wild-type embryos and the localization of UNC-45 in *nmy-2* (RNAi) embryos. A to C are wild-type embryos stained with anti-UNC-45 antibody (7N5) at 2-cell, 4-cell and >8-cell stages, showing its concentration at the cell boundaries. D to F are the same embryos as A to C but stained with anti-NMY-2 antibody, showing NMY-2 is also concentrated at the cell boundaries. G to I are the same embryos as A to C but double-stained with anti-UNC-45 and anti-NMY-2 antibodies. J to L are NMY-2-depleted embryos by RNAi stained with anti-UNC-45 antibody (7N5) at 2-cell, 4-cell and >8-cell stages, showing that the concentration of UNC-45 at the cell boundaries is disrupted. Bar, 10 μm.

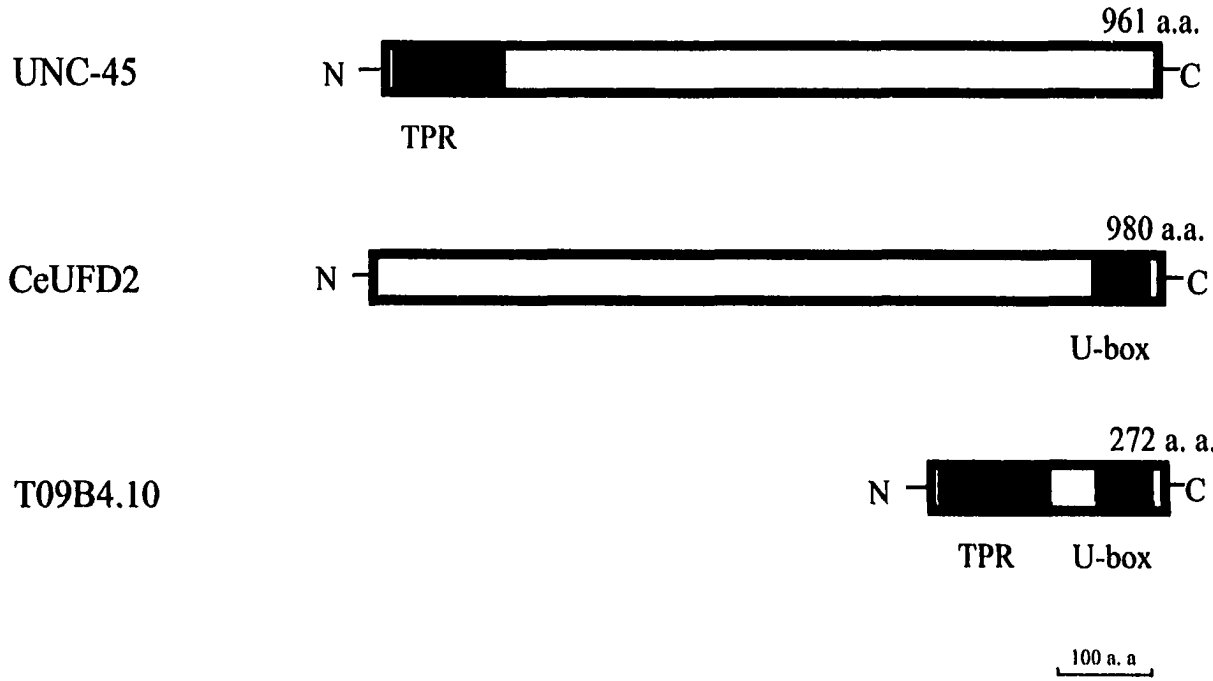

Figure 3.22 Comparison of UNC-45, CeUFD2 and T09B4 Black boxes indicate the UFD2 homology region and the gray boxes indicate the tetratricopeptide repeats.

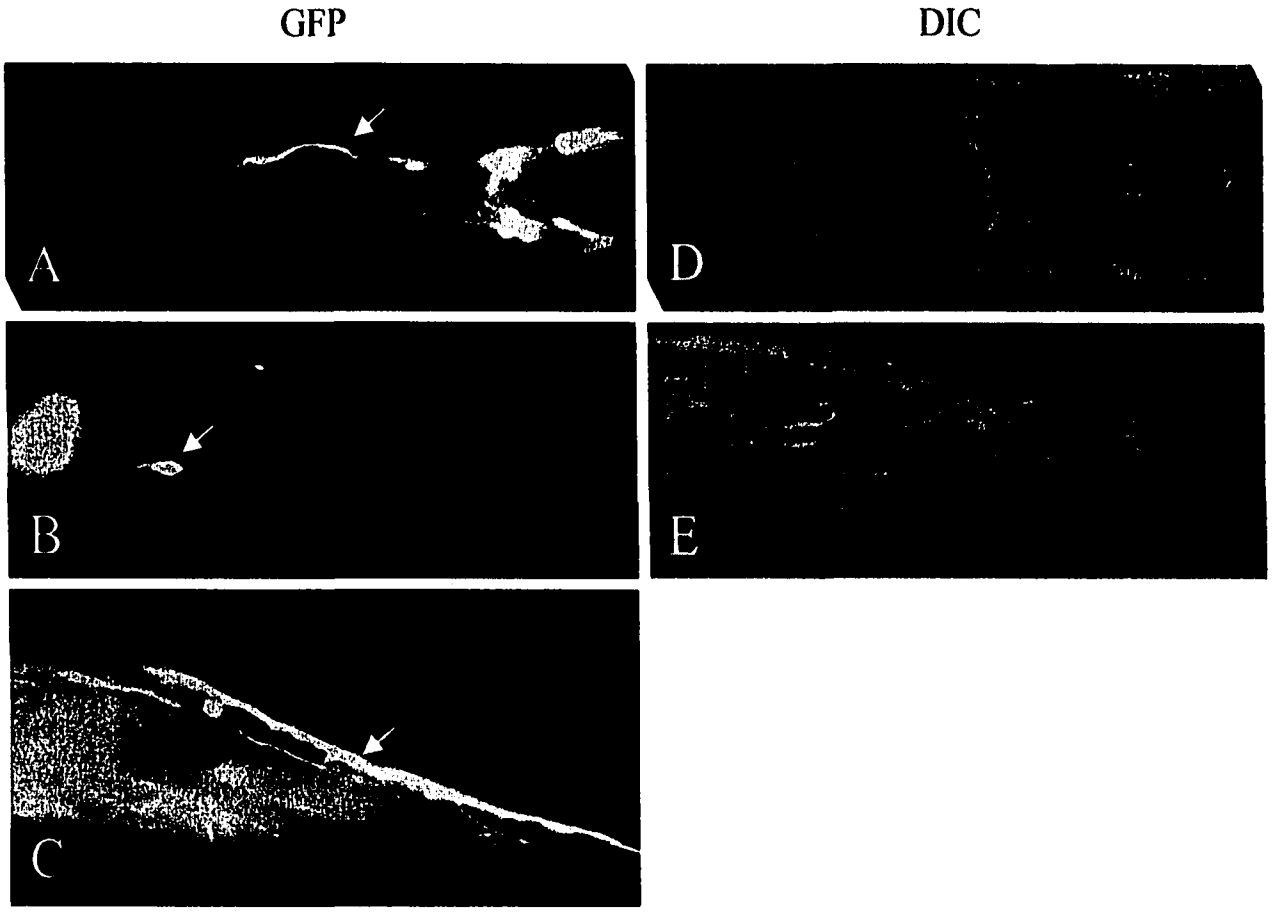

Figure 3.23 CeUFD2::GFP is expressed in nerve cells. A, B and C, GFP expression in nerve cells. D and E, DIC images of the same part as A and B (DIC image is not available for C). Arrows indicate amphid process bundles in the head (A), probably PLM or PHB in the tail (B) and the ventral cord (C).

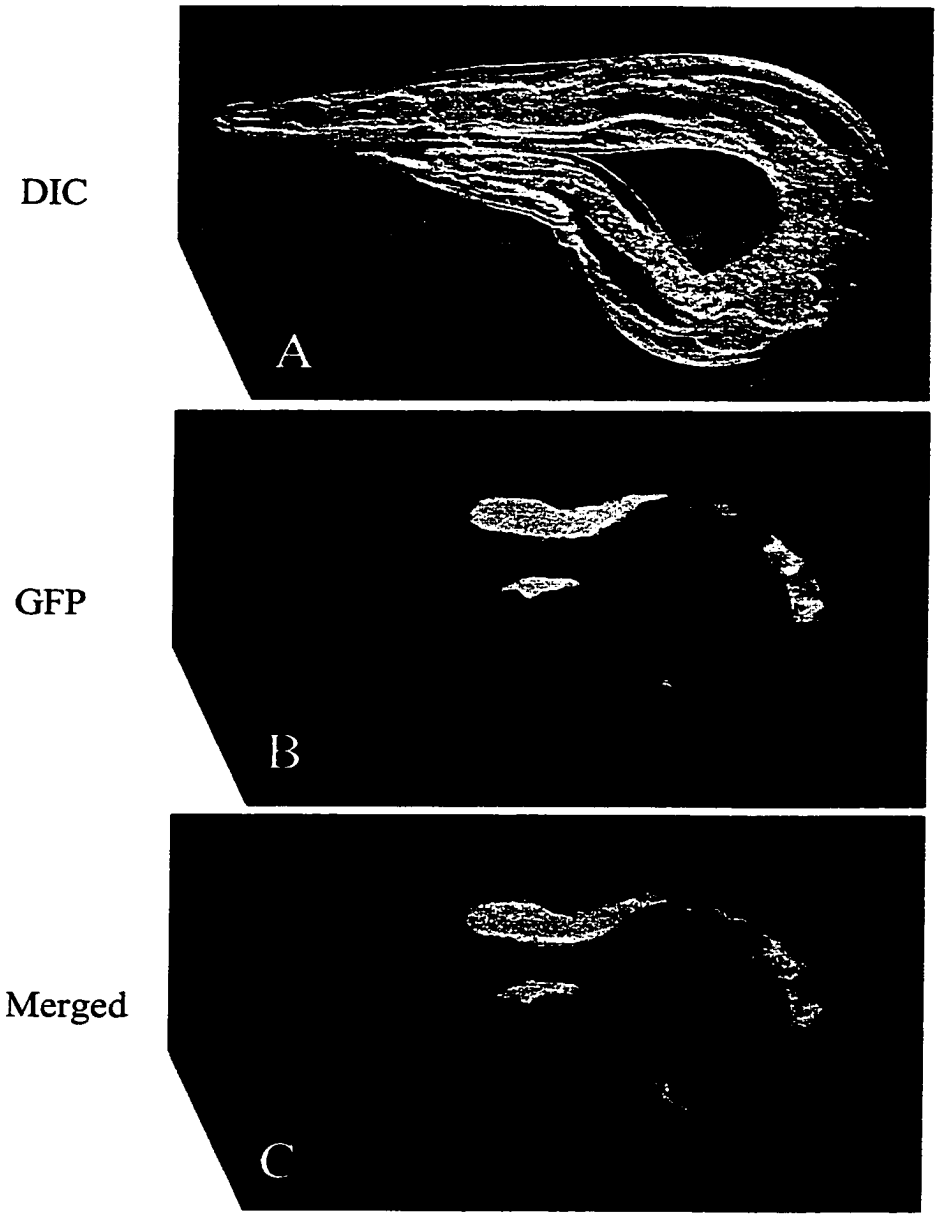

Figure 3.24 CeUFD2::GFP is expressed in the intestine. A, DIC image of an adult worm. B, CeUFD2::GFP expression in the gut of the same worm. C, merged image of A and B.

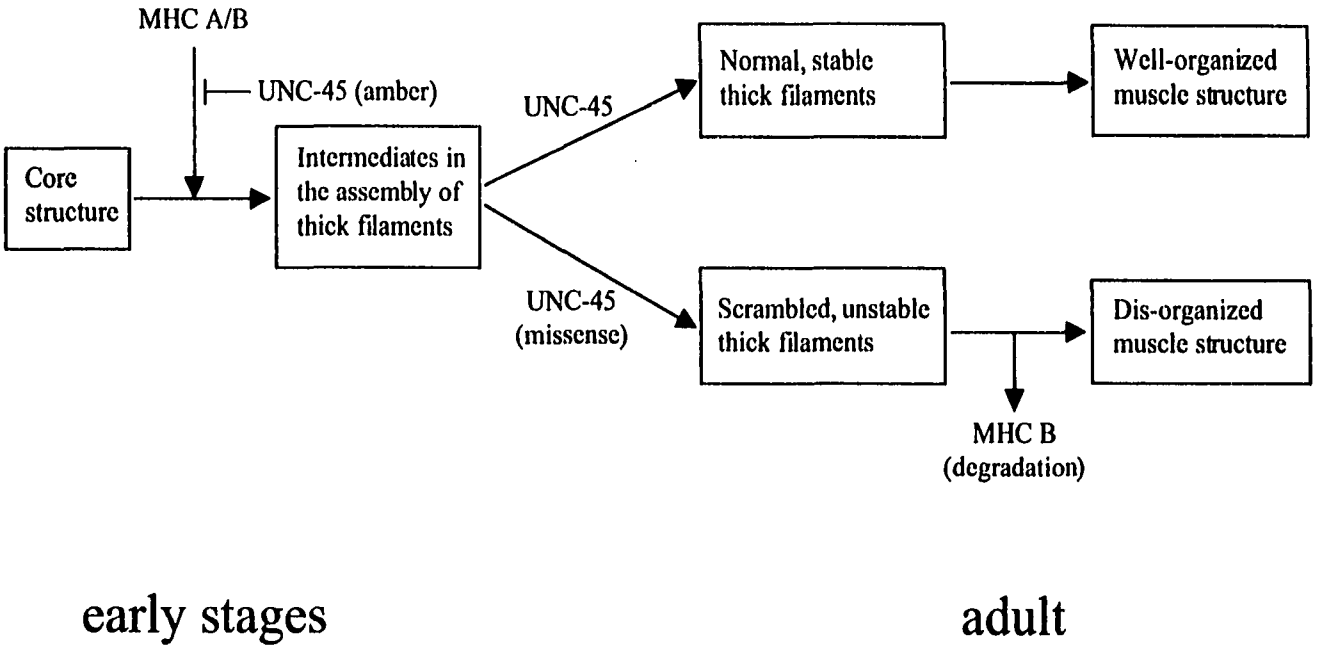

Figure 4.1. A model for UNC-45 function (please see the legend on next page).

Figure 4.1. A model for UNC-45 function. UNC-45 is required for the initiation of thick filament assembly in the early stages of development. It may function as a catalyst to modify MHC B (and/or MHC A) before assembly or directly participate in the assembly itself. In the case of UNC-45 amber mutations, UNC-45 cannot function properly and causes embryonic lethality. Later, UNC-45 is localized to thick filaments in the same patterns as MHC B. In the adult, UNC-45 normally functions as a stabilizer for MHC B. In animals carrying UNC-45 missense mutations in the CRO1/SHE4 domain at the restrictive temperature, MHC B turnover is increased, either due to inherent MHC B instability on the thick filament or an inability to properly assemble MHC B into the thick filament, followed by increased turnover of unassembled MHC B.

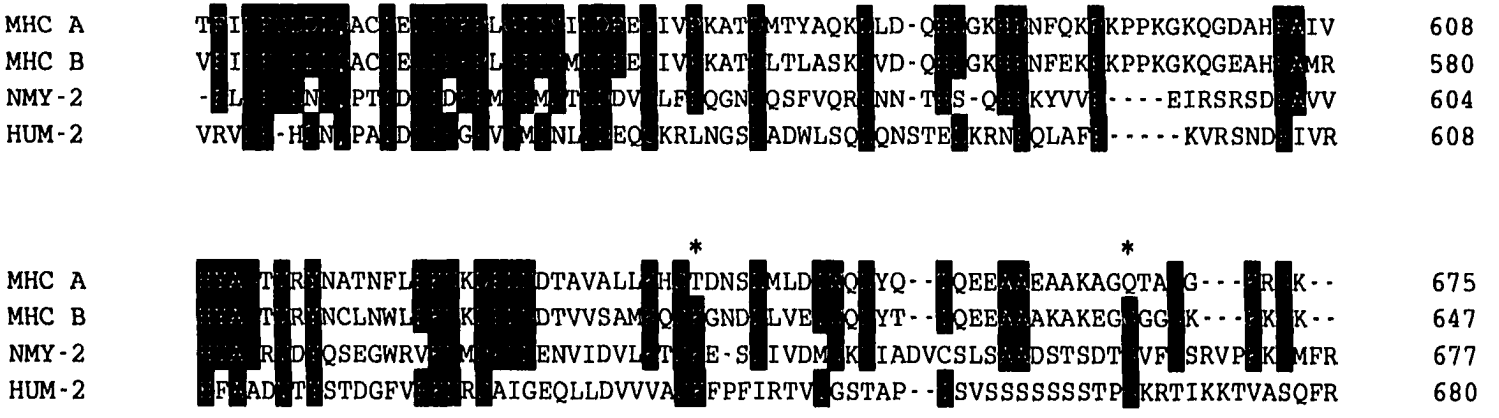

Figure 4.2 Sequence alignment of potential UBS sites in myosins. The shaded amino acids are identical in three or four of the four proteins. Broken lines indicate gaps and asterisks indicate the amino acids in MHC A that are different from those in the three other myosins.

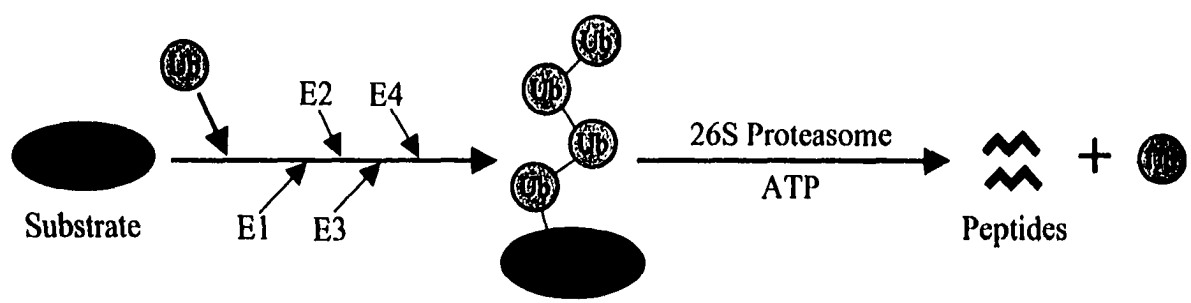

Figure 4.3 The ubiquitination pathway. Ubiquitin (Ub) is activated by the ubiquitin-activating enzyme (E1) and then transferred to the substrate by a ubiquitin-conjugating enzyme (E2). E3 is required for some substrate recognition and E4 is a newly identified protein required for efficient multiubiquitin chain assembly. The conjugated substrate is degraded into peptides by the 26S proteasome.

7. Tables

Table 1.1 *C. elegans* genes affecting muscle development for which protein products are known

Locus	Protein encoded (*C. elegans* homologues)	References
act-1 to act-5	actin	Files et al., 1983; Krause and Hirsh, 1987
atn-1	alpha-actinin	Barstead et al., 1991
deb-1	vinculin	Barstead and Waterston, 1989 and 1991
emb-9	collagen Type IV	Guo and Kramer, 1989; Guo et al., 1991; Sibley, et al., 1994
etr-1	related to ELAV	Milne and Hodgkin, 1999
hlh-1	myoD	Chen et al., 1994; Krause et al., 1994
hlh-8	Twist	Corsi, et al., 2000
hsp-25	heat shock protein	Ding and Candido, 2000
lev-11	tropomyosin	Kagawa et al., 1995 and 1997
let-2	collagen Type IV	Guo and Kramer, 1989; Sibley, et al., 1994
mlc-1	regulatory myosin light chain	Rushforth, et al., 1993 and 1998
mlc-2	regulatory myosin light chain	Rushforth, et al., 1993 and 1998
myo-1	myosin heavy chain C	Miller et al., 1986
myo-2	mycoin heavy chain D	Miller et al., 1986
myo-3	myosin heavy chain A	Waterston, 1989; Miller et al., 1986; Albertson, 1985
mup-2	Troponin T	Myers, et al., 1996
pat-2	alpha-integrin	Williams and Waterston, 1994
pat-3	beta-integrin	Gettner et al., 1995
pat-10	troponin C	Williams and Waterston, 1994; Terami et al., 1999
unc-15	paramyosin	Mackenzie, and Epstein, 1980; Kagawa et al., 1989
unc-22	twitchin (titin)	Moerman et al., 1988; Benian et al., 1989
unc-45	TPR and CRO-1/She4 domains	Venolia et al., 1999
unc-52	perlecan	Rogalski, et al., 1993
unc-54	myosin heavy chain B	Mackenzie, et al., 1978; Bejsovec and Anderson, 1988
unc-60	cofilin similarity	McKim, et al., 1994; Ono et al., 1999
unc-68	ryanodine receptor	Maryon, et al., 1998
unc-87	calponin	Goetinck and Waterston, 1994a and 1994b
unc-89	related to UNC-22	Benian, et al., 1989 and 1996
unc-93	putative trans-membrane protein	Levin and Horvitz, 1992
unc-97	LIM protein	Hobert, et al., 1999
unc-112	FERM protein	Rogalski, et al., 2000

Table 3.1: Muscle cell types and UNC-45 and MHC isoform expression

Muscle cell types	Proteins	References
Body wall muscles	UNC-45	This work
	myosin heavy chain A	Miller, et al; 1983
	myosin heavy chain B	Miller, et al; 1983
Pharyngeal muscles	UNC-45	This work
	myosin heavy chain C	Epstein et al 1982; Ardizzi and Epstein, 1987
	myosin heavy chain D	Ardizzi and Epstein, 1987
Anal-intestinal muscles	UNC-45	This work
	myosin heavy chain A	Ardizzi and Epstein, 1987
	myosin heavy chain B	Ardizzi and Epstein, 1987
Sex-specific muscles	UNC-45	This work
	myosin heavy chain A	Ardizzi and Epstein, 1987
	myosin heavy chain B	Ardizzi and Epstein, 1987
Gonadal contractile muscles	UNC-45	This work
	myosin heavy chain A	Ardizzi and Epstein, 1987
	myosin heavy chain B	Ardizzi and Epstein, 1987

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