Genetic Analysis of Myosin Assembly in *Caenorhabditis elegans*

Henry F. Epstein

Department of Neurology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030

Contents

Molecular Neurobiology Ed.: N. G. Bazan

Abstract

The established observations and unresolved questions in the assembly of myosin are outlined in this article. Much of the background information has been obtained in classical experiments using the myosin and thick filaments from vertebrate skeletal muscle. Current research is concerned with problems of myosin assembly and structure in smooth muscle, a broad spectrum of invertebrate muscles, and eukaryotic cells in general.

Many of the general questions concerning myosin assembly have been addressed by a combination of genetic, molecular, and structural approaches in the nematode *Caenorhabditis elegans*. Detailed analysis of multiple myosin isoforms has been a prominent aspect of the nematode work. The molecular cloning and determination of the complete sequences of the genes encoding the four isoforms of myosin heavy chain and of the myosin-associated protein paramyosin have been a major landmark.

The sequences have permitted a theoretical analysis of myosin rod structure and the interactions of myosin in thick filaments. The development of specific monoclonal antibodies to the individual myosins has led to the delineation of the different locations of the myosins and to their special roles in thick filament structure and assembly.

In nematode body-wall muscles, two isoforms, myosins A and B, are located in different regions of each thick filament. Myosin A is located in the central biopolar zones, whereas myosin B is restricted to the flanking polar regions. This specific localization directly implies differential behavior of the two myosins during assembly. Genetic and structural experiments demonstrate that paramyosin and the levels of expression of the two forms are required for the differential assembly. Additional genetic experiments indicate that several other gene products are involved in the assembly of myosin. Structural studies of mutants have uncovered two new structures. A core structure separate from myosin and paramyosin appears to be an integral part of thick filaments. Multifilament assemblages exhibit multiple nascent thick filament-like structures extending from central paramyosin regions. Dominant mutants of myosin that disrupt thick filament assembly are located in the ATP and actin binding sites of the heavy chain.

A model for a cycle of reactions in the assembly of myosin into thick filaments is presented. Specific reactions of the two myosin isoforms, paramyosin, and core proteins with multifilament assemblages as possible intermediates in assembly are proposed.

Index Entries: Myosin; assembly; genetics; *Caenorhabditis elegans;* thick filaments; muscle; myosin assembly.

Introduction

Myosin may be defined as an enzyme that catalyzes the translational movement of bound actin filaments (Warrick and Spudich, 1987). There are two general classes of myosin: a single-headed form that does not assemble into filaments (Pollard and Korn, 1973) and the two-headed form that assembles into some form of filament (Huxley, 1963; Lowey et al., 1969). The single-headed myosin has been localized to the ruffling membranes of *Acanthameba* (Gadasi and Korn, 1980) and to the microvilli of intestinal mucosa (Conzelman and Mooseker, 1987; Coluccio and Bretscher, 1987). Two-headed myosin is required for cell division in the slime mold *Dictyostelium* (Knecht and Loomis, 1987; De Lozanne and Spudich, 1987) and is the classical motor and structural protein of contractile assemblies in muscle (Huxley, 1969). This review will deal with the problem of the assembly of two-headed myosin into thick filaments of striated muscles (Fig. 1A).

The model structure for myosin assembly has been the thick filament of vertebrate skeletal muscle (Huxley, 1963; Kensler and Stewart, 1983; Davis, 1988). The myosin-containing length of these filaments is 1.53 μm (Morimoto and Harrington, 1973). The structures are bipolar. The central bare zone contains myosin rods interacting in antiparallel, whereas in the flanking polar regions, the assembled myosin molecules

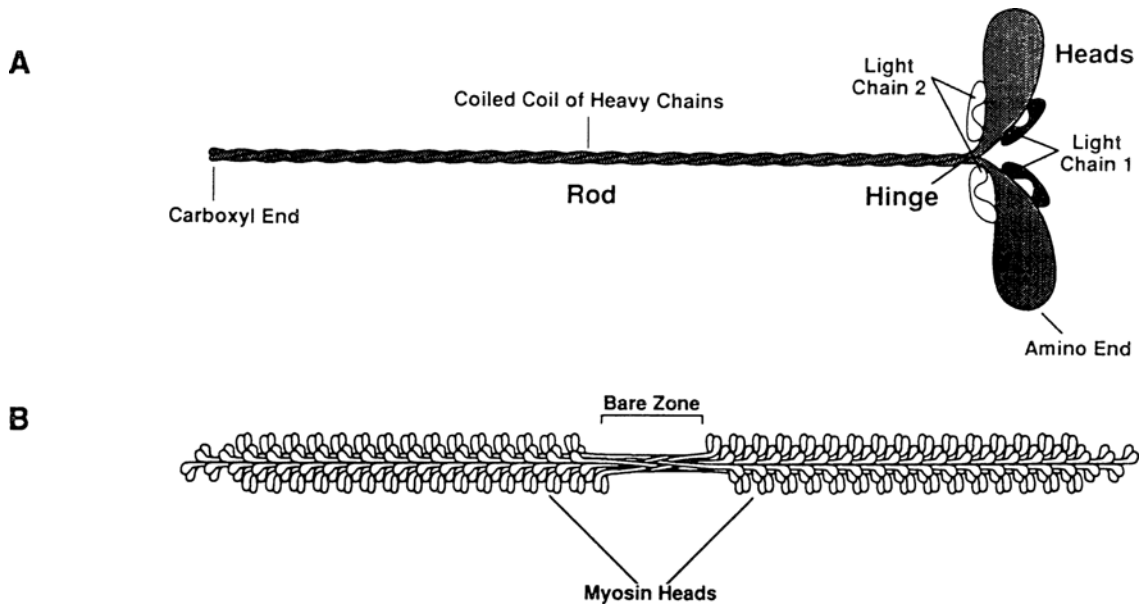

Fig. 1. A. Model of the myosin molecule. B. Model of the thick filament. Both the myosin and thick filament models are based primarily on the structures of the vertebrate striated muscle species.

are in parallel (Fig. 1B). This configuration permits contraction or movement of actin filaments by the action of myosin heads towards the center. The myosin-containing thick filaments and the actin-containing thin filaments are organized into regular alternating arrays that appear as the characteristic striations or bands.

Unresolved Problems in Myosin Assembly

The first problem in myosin assembly concerns the formation of the native myosin molecule, a hexameric complex. Two polypeptides, each of greater than 200 kilodaltons (kDa) mass, associate and fold into two domains, the complex globular head and the primarily α-helical tail. The tails intertwine into a coiled-coil rod at least 120 nm long (Lowey et al., 1969). Four additional polypeptides of 17–23 kDa associate with the two heads. Myosin molecules in various mammalian muscles appear to be posttranslationally modified. The amino terminals are acetylated (Starr and Offer, 1973), and specific lysines and histidines may be methylated (Huszar and Elzinga, 1972; Huszar, 1972). Myosins in various smooth muscle and nonmuscle cells are reversibly phosphorylated (Kuczmarski and Spudich, 1980; Scholey et al., 1980). The exact order of these events and their physiological significance are still unknown. Smooth muscle myosin appears to assemble into "side-polar" filaments by antiparallel packing along the entire length in contrast to the behavior of striated muscle myosin (Craig and Megerman, 1977; Trybus and Lowey, 1987).

The myosin molecule interacts with a variety of distinct proteins within the thick filament (Table 1). In the thick filaments of vertebrate skeletal muscle, the C, H, and X proteins are localized to specific sites along the surface (Craig and Offer, 1976) (Fig. 2). Indeed, particular muscle fibers show any one of a number of combinations of these associated proteins (Bennett et al., 1986). The interactions of these three proteins with the underlying myosin molecules clearly indicates complex non-

Table 1
Proteins of Thick Filaments

Polypeptide	Where found	Molecular mass
Myosin heavy chain II	All cells	220–230 kDa
Myosin light chain 1	All cells	20–25 kDa
Myosin light chain 2	All cells	16–18 kDa
Paramyosin	Nonvertebrate muscle	98 kDa
C protein	Vertebrate striated muscle	140 kDa
H protein	Vertebrate skeletal muscle	69 kDa
X Protein	Vertebrate skeletal muscle	152 kDa
M band protein	Vertebrate striated muscle (probable nonvertebrate analogs)	165 kDa
Myomesin	Vertebrate skeletal muscle	185 kDa
M-creatine kinase	Vertebrate striated muscle	42 kDa
Adenylate deaminase	Vertebrate skeletal muscle	kDa
Twitchin	Nematode body-wall muscle	665 kDa
Putative core proteins	Nematode body-wall muscle	Not known

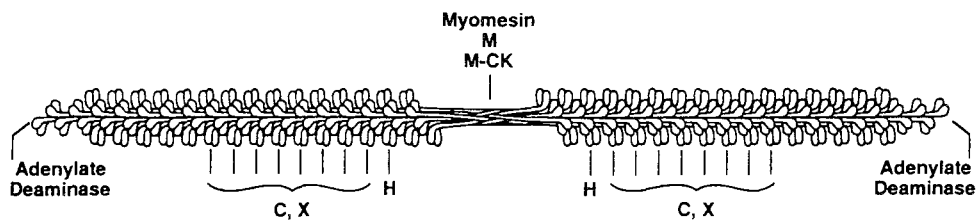

Fig. 2. Additional proteins of the vertebrate striated muscle thick filament.

equivalence of binding sites. M-creatine kinase (Turner et al., 1973), M-band protein (Masaki and Takaiti, 1974), and myomesin (Strehler et al., 1980; Grove et al., 1985) bind to the central region of the thick filament, whereas adenylate deaminase binds to the filament ends (Cooper and Trinick, 1984). The thick filaments of invertebrate muscles from a broad phylogenetic spectrum including nematodes (Waterston et al., 1974), mollusks (Szent-Györgyi et al., 1971), and insects (Bullard et al., 1973) contain paramyosin, a coiled-coil dimeric protein homologous to the myosin rod (Cohen et al., 1987). Paramyosin assembles into the backbone of the filament and serves as a substrate for the more peripherally placed myosin (Szent-Györgyi et al., 1971; Epstein et al., 1985) (Fig. 3). In *Dipteran* insect flight muscles, the paramyosin is a minor component, and the thick filaments appear hollow. In the larger molluscan thick filaments, paramyosin becomes the predominant protein component. The mechanisms by which myosin coassembles with these very different proteins are not understood. By definition, the formation of thick filaments in muscle cannot be the result of simple self-assembly of myosin; other proteins are specifically interacting with the myosin.

Myosin and its associated proteins not only assemble into thick filaments, but form muscle cell-specific filaments of characteristic length and diameter (Levine et al., 1976). The lengths of thick filaments in a given muscle can be measured precisely. In skeletal muscle, thick filaments contain exactly 294 or 300 myosin molecules (49 or 50 repeats of 3 myosins in 2 directions) (Kensler and Stewart, 1983). With the end assemblies of

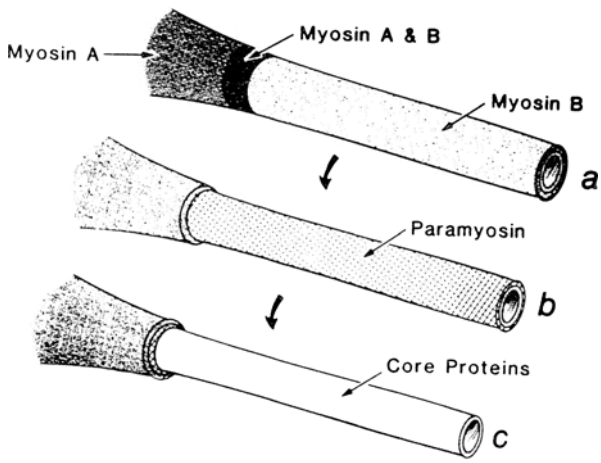

Fig. 3. The substructural domains of invertebrate thick filaments based primarily on work in the nematode *C. elegans*. Hemifilaments from central to polar zones are drawn. Reprinted from Epstein et al. (1988).

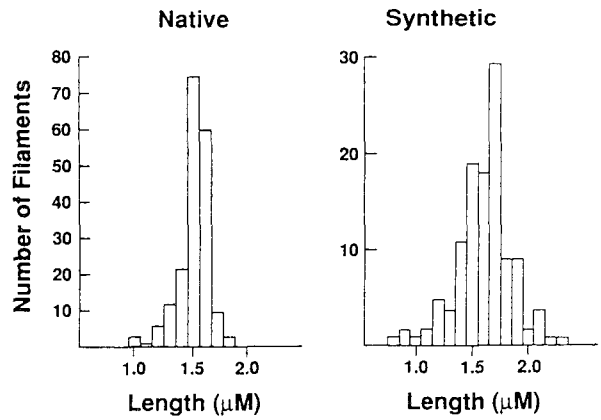

Fig. 4. The lengths of native and synthetic thick filaments. Native replotted from Morimoto and Harrington (1973), and synthetic replotted from Chowrashi and Pepe (1986). The mean lengths and standard deviations for native filaments are 1.53 ± 0.17 μm and for synthetic filaments are 1.7 ± 0.3 μm.

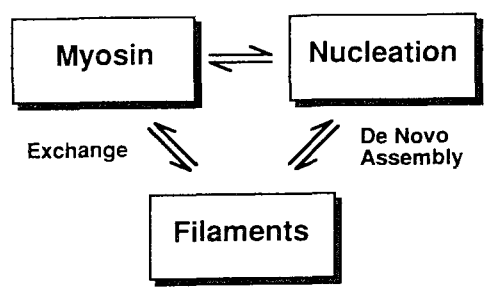

Fig. 5. Scheme of alternative modes of assembly of myosin in vertebrate striated muscles. Note that *de novo* assembly would require a nucleation step in contrast to exchange assembly.

adenylate deaminase, these filaments are 1.69 μm long (Cooper and Trinick, 1984). In contrast, various invertebrate thick filaments average between 2.8–40 μm in length. Two mechanistic questions arise: What determines the precision of length of thick filaments in a given muscle, and what underlies the differences in mean length of thick filaments from different muscles? Although careful biochemical experiments with purified skeletal muscle myosin can produce thick filaments of restricted length and approximate native dimensions (Morimoto and Harrington, 1973; Chowrashi and Pepe, 1986) (Fig. 4), there is no general agreement as to the assembly mechanism active within cells (Davis, 1988). The polymerization of myosin in vitro requires first the formation of dimers of myosin molecules in parallel (Harrington et al., 1972). These myosin dimers then pack in an antiparallel fashion to form the central bare zone (Reisler et al., 1980). This bare zone assemblage has been proposed as the nucleation complex about which further polymerization of myosin to form filaments occurs (Niederman and Peters, 1982). Self-assembly, coassembly of myosin and nonmyosin proteins as mutually regulating verniers (Huxley, 1963), and a core molecular template (Epstein et al., 1985) have all been postulated as determinants of thick filament length. It should be noted that at least two distinct pathways and mechanisms for myosin and other thick filaments may exist. In this review, I am focusing on *de novo* assembly of thick filaments. However, a potentially distinct "exchange" assembly may also be physiologically important (Saad et al., 1987; Wenderoth and Eisenberg, 1987) (Fig. 5). A mechanistic distinction between *de novo* and exchange assembly is that the initiation of new filaments would require the formation of nucleation complex, whereas exchange between

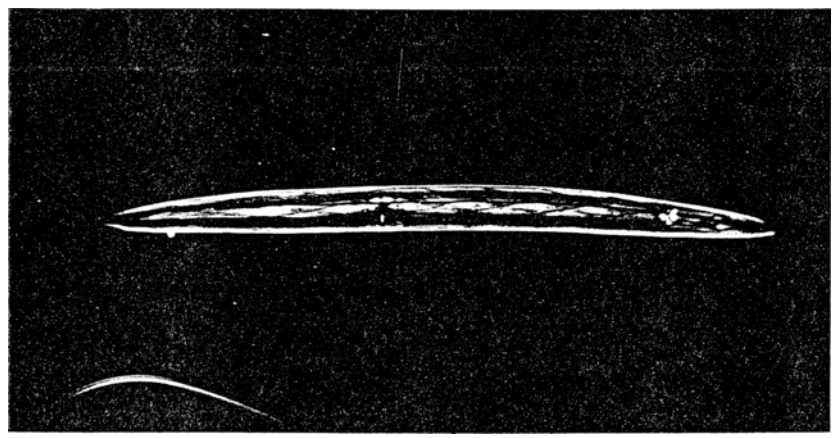

Fig. 6. Polarized light micrograph of an adult hermaphrodite nematode showing the predominant body-wall muscles.

existing filaments would not require a nucleation step.

The assembly and disassembly of thick filaments in the skeletal muscles of higher vertebrates are likely to be regulated in several ways. During development, thyroxine and specific innervation control the expression of stage-specific myosin isoforms, and this differential expression correlates with marked increases in filament assembly and muscle growth (Whalen et al., 1986). In homeostasis, myosin and other muscle filament proteins serve as rapidly mobilizable sources of amino acids, which can then serve as substrates for intermediary metabolism in the liver (Goldberg and Chang, 1978). The ketosis of starvation and diabetes is related to this physiological function (Goldberg et al., 1977). The ability of myosin to exchange between filamentous and monomeric states may be critical to these regulated changes in assembly (Saad et al., 1987; Bouché et al., 1988). The synthesis of new myosins during growth and development and the degradation of myosin in starvation and diabetes strongly suggest that the coupling of synthesis with assembly and degradation with disassembly are underlying mechanisms of the regulatory phenomena.

The rest of this discussion will concern myosin assembly in the nematode *C. elegans* and, more specifically, the behavior of myosin in the body-wall muscle cells of this organism. Recent reviews concerning the assembly of myosin in vertebrate muscle (Davis, 1988) and the molecular genetics of myosin in several systems (Emerson and Bernstein, 1987; Anderson, 1989) provide complementary discussion of relevant fields.

Thick Filament Proteins of *Caenorhabditis elegans*

The nematode *C. elegans* (Fig. 6) produces four isoforms of myosin heavy chain, each of which is the product of a unique gene (Miller et al., 1986) (*see* Table 2). Two of these isoforms, the *myo-3* product A chain and the *unc-54* product B chain, are expressed in the striated body-wall muscles (Miller et al., 1983). The heavy chains appear to self-associate to form homodimeric myosin molecules by biochemical experiments (Schachat et al., 1977) (Fig. 7); no heterodimers are detected immunochemically (Schachat et al., 1978). The complete amino acid sequences have been de-

Table 2
Expression of Thick Filament Proteins in *Caenorhabditis elegans*

Polypeptide	Gene	Muscle cell localization
Myosin heavy chain A	*Myo-3* V	Body wall, sex, anal
Myosin heavy chain B	*Unc-54* I	Body wall, sex, anal
Myosin heavy chain C	*Myo-2* X	Pharynx
Myosin heavy chain D	*Myo-1* I	Pharynx
Twitchin	*Unc-22* IV	Body wall
paramyosin	*Unc-15* I	All muscle cells
Putative core proteins	Not known	Body wall

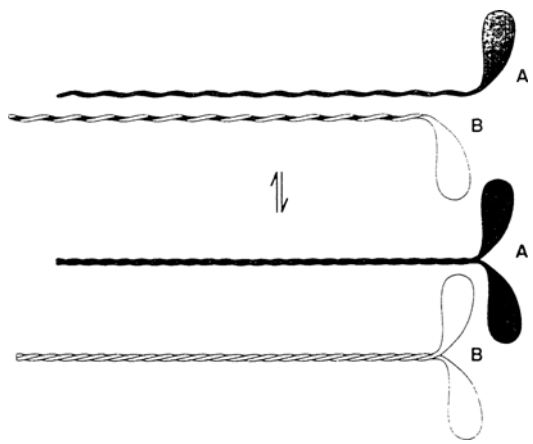

Fig. 7. Scheme of self-association of myosin heavy chains A and B in *C. elegans* to produce homodomeric myosin molecules.

rived from the DNA sequences of cloned genes (Dibb et al., 1989).

In *C. elegans*, body-wall thick filaments, paramyosin is a major component; indeed, it is the most prominent protein on a molar basis (Waterston et al., 1974; Mackenzie and Epstein, 1981; Epstein et al., 1988). The *unc-15* gene encodes all paramyosins of *C. elegans* (Waterston et al., 1977; Waterston and Brenner, 1978). Two posttranslational modifications have been demonstrated. The amino terminus is blocked, and there is specific phosphorylation of two serines (Schriefer and Waterston, 1989). The complete amino acid sequence has been determined from DNA sequencing of the cloned gene (Kagawa et al., 1989).

Two types of light chain are associated with both myosin A and B (Harris and Epstein, 1977). Genes for two light chains have been cloned and sequenced (Cummins and Anderson, 1988), but it is not known whether these code for the light chains of the body-wall myosins.

Mutations that produce alterations or deficiencies in myosin heavy chains A and B and paramyosin have been defined (Epstein et al., 1974, 1987; MacLeod et al., 1977a,b,1981; Harris and Epstein, 1977; Waterston et al., 1977; Anderson and Brenner, 1984; Bejsovec et al., 1984; Dibb et al., 1985; Eide and Anderson, 1985; Bejsovec and Anderson, 1990); in fact, *C. elegans* was the first organism in which mutations affecting these proteins were dis-covered (Fig. 8). Several genes related to thick filament structure and assembly have been identified by mutation. Specific mutations affecting *unc-54* myosin heavy chain B, *unc-15* paramyosin, *myo-3* myosin heavy chain A, *unc-82* putative paramyosin kinase, *unc-22* twitchin, *unc-45* putative myosin B assembly factor, and *unc-87* (unknown product and function) have been identified (Waterston et al., 1980; Zengel and Epstein, 1980a; Dibb et al., 1985). Lethal mutations in *unc-54, myo-3* , and *unc-45* have been reported (Waterston, 1988). Genetically altered proteins produced in specific mutants of *unc-54, unc-15,* and *unc-22* have been studied. This variety of mutations affecting thick filament-related genes is the basis of experimental approaches to understanding myosin assembly in *C. elegans.*

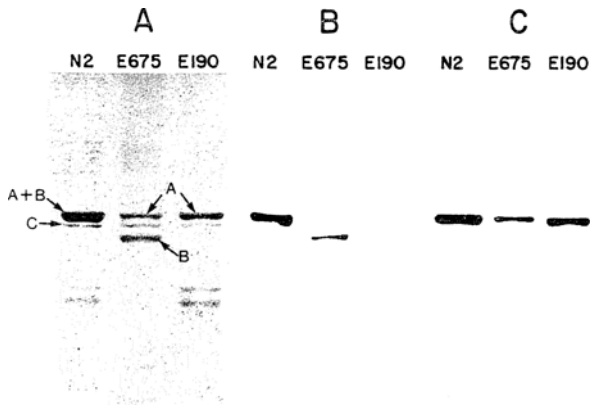

Fig. 8. Mutants affecting myosin heavy chain B. N2 is wild-type in which myosin heavy chains A and B comigrate in 4.5% polyacrylamide-sodium dodecyl sulfate gels (A) as verified by reaction with monoclonal antibodies specific to the BN chain (B) and A chain (C). The *unc-54* mutants E675 and E190 produce a truncated B chain and no B chain, respectively, as verified by the specific antibody reaction (B). Reprinted from Miller et al. (1983).

Thick Filament Structure in *C. elegans*

The thick filaments in nematode body-wall muscle (Fig. 9) are about 10.0 μm long and taper in diameter from 33.0 nm in the central zone to 14.0 nm in the terminal regions (Waterston et al., 1974; Epstein et al., 1985). Myosins A and B are present in all of the thick filaments in body-wall muscle cells of wild-type *C. elegans* (Mackenzie et al., 1978a; Miller et al., 1983). Within each thick filament the two myosins assemble into distinct regions (*see* Fig. 10). The myosins were localized by specific monoclonal antibodies under stringent conditions of specificity (Epstein et al., 1982b; Miller et al., 1983). The localizations were performed by immunofluorescence of muscle A bands in whole nematode mounts and by electron microscopy of isolated thick filaments. By both methods, myosin A is restricted to a 1.7–1.8 μm central zone, whereas myosin E is present in the flanking polar zones. Two small zones (~0.4 μm each) at the junctions of the central and polar regions contain both myosins A and B. These localizations were the first demonstration that specific myosin isoforms assemble differentially. Recent evidence suggests that distinct myosins in cardiac and skeletal muscle can also assemble differentially (Wenderoth and Eisenberg, 1987; Taylor and Bandman, 1989).

In the nematode, the thick filaments exhibit other structural properties consistent with molecular differentiation requiring a sequence of reactions in their assembly. Myosin B may be solubilized stepwise from filament ends towards the center by incubation with progressively higher concentrations of KCl (Epstein et al., 1985). Under intermediate conditions, the paramyosin substratum is exposed. However, the paramyosin, too, becomes solubilized between 0.35–0.45*M* KCl, pH 6.4, but a clear 15 nm wide tubular structure remains (Fig. 11). We have named this newly identified entity the polar core structure. Since myosin and paramyosin had been almost entirely solubilized from these structures, it is likely that nonmyosin proteins constitute the core structure.

In the central 1.0 μm zones of the salt-treated filaments, myosin A remains. The native diameter of the central zone is also preserved, about 32.0–33.0 nm. Here, the structure is electron dense across the entire diameter by positive staining and does not permit penetration of uranyl acetate under negative staining conditions. In contrast, the polar core structures appear hollow by positive staining and permit penetration of uranyl acetate. Therefore, we propose that the interior of the central zones is composed of protein structures distinct from the polar cores. Since most of the paramyosin had been solubilized and because paramyosin-deficient mutant filaments still exhibit wide diameter, electron dense central zones, additional protein components are likely to be required in the center.

Fig. 9. Thick filaments of *C. elegans* body-wall muscle. Electron microscopy of negatively stained filaments. Bar=1.0 μm.

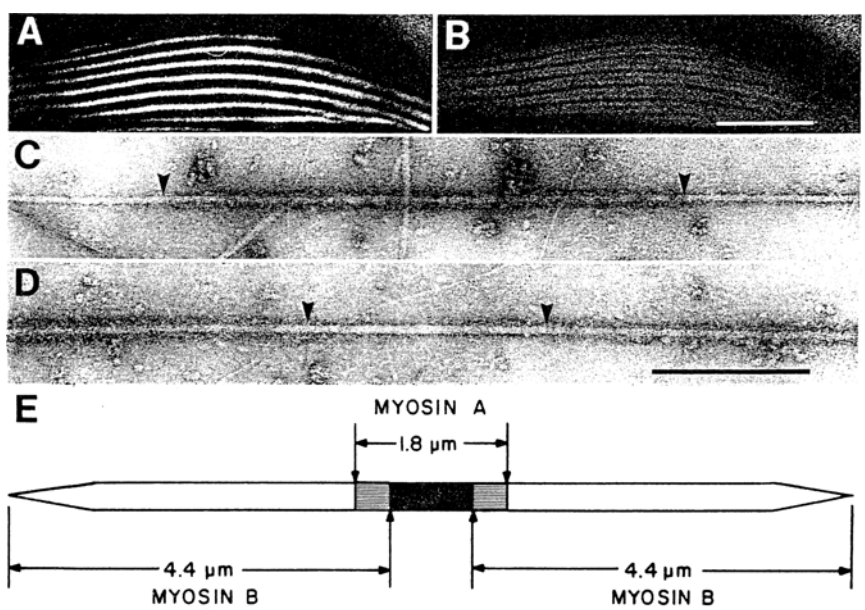

Fig. 10. Localization of myosin isoforms in nematode body-wall muscle (A,B) represent immunofluorescence of body-wall muscle labeled with antimyosin A and antimyosin B, respectively. Bar is 5 μm. (C,D) represent electron microscopy of negatively stained complexes of antimyosin A and antimyosin B with isolated thick filaments, respectively. Bar is 0.5 μm. (E) is a schematic diagram of the distribution of myosins A and B in an individual thick filament based on observations in (A–D). Adapted from Miller et al. (1983).

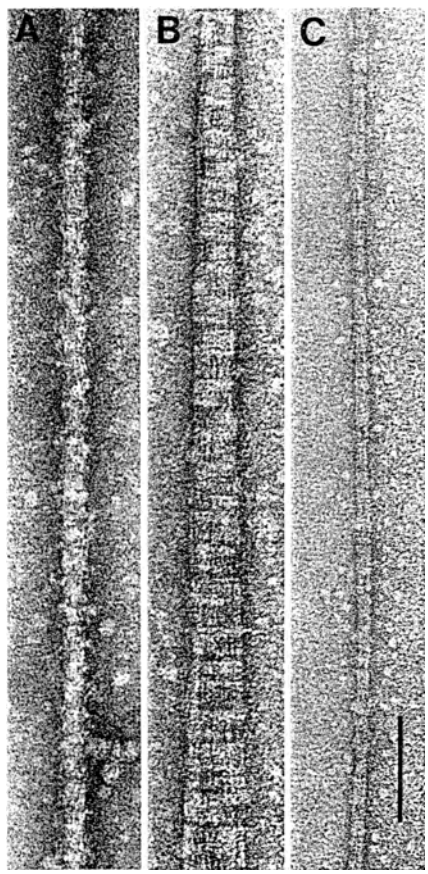

Fig. 11. Dissociation of nematode thick filaments to reveal substructural domains. A. Native filament exhibiting myosin heads. B. Partially dissociated filaments revealing paramyosin substructure. C. Further dissociated filament exhibiting polar core structures. Bar is 0.1 μm.

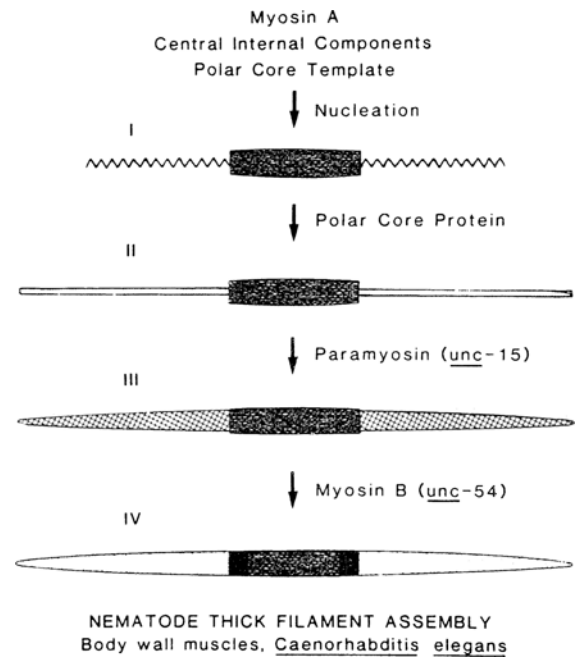

Fig. 12. Linear sequence of assembly reactions. Reprinted from Epstein et al. (1985). This sequence is based primarily upon dissociation experiments. The central and polar core components and the length-determining template are hypothetical.

There is one additional set of observations relevant to thick filament structure. Similar to the tapering from the central zones to the ends of the filaments, the electron density of the cores changes along the lengths. These phenomena are observed in cross-sections of positively stained nematode muscles. The central zones are highly electron dense and most polar regions are hollow, but the regions in between show intermediate levels of density in the cores of the filaments. One explanation is that a macromolecular structure in the centermost space of the filament is changing properties or that the numbers of its constituent molecules diminish along the length of each half of the filament.

The existence of three coaxial domains within nematode thick filaments, myosin, paramyosin, and core structures, and the differentiation of the myosin domains into myosin A-containing central and myosin B-containing polar zones strongly suggest that a sequence of reactions takes place during assembly. Different proteins would necessarily interact with specific intermediates in assembly. Figure 12 shows a scheme for such a sequence of reactions (Epstein et al., 1985; Epstein, 1986). In this model, the components of the bipolar central zone, myosin A and central core components, and a polar core template that determines overall filament length may be related to the changing core density and contribute to a nucleation center (I). The polar core structure (II) would be

formed by polymerization of its protein components about the polar core template. Paramyosin, the *unc-15* gene product, assembles onto the polar core structure (III). Myosin B then interacts with paramyosin to form the completed thick filament (IV). This model is based primarily upon the known facts about the final structure of nematode thick filaments, experiments on the dissociation of the filament, and previous considerations of myosin assembly (Huxley, 1963; Harris and Epstein, 1977; Davis, 1988). The model emphasizes the role of stoichiometric components and the sequence of their addition. The control of the initiation and overall rate of assembly, potential coupling to the biosynthesis of components, and the regulation of the polymerization of individual components at specific steps are not explicitly addressed. More recent studies of mutants have indicated that important modifications of this linear sequential model may be necessary.

Structural Analysis of Myosin and Paramyosin

The small size of its genome, about 8×10^7 basepairs of DNA (Sulston and Brenner, 1974; Coulson et al., 1986), and the availability of deletions and transposable elements to mark functional genes (Moerman et al., 1986) have permitted rapid cloning and detailed molecular analysis in *C. elegans*. For these reasons, the first complete amino acid sequence of a myosin heavy chain was derived from molecular cloning and DNA sequencing of the *unc-54* gene (Karn et al., 1982; McLachlan and Karn, 1983). More recently, the complete primary sequences of all four sarcomeric heavy chains (Dibb et al., 1989) and of paramyosin (Kagawa et al., 1989) have been similarly obtained. These results have been compared with available sequences of vertebrate skeletal and smooth muscle heavy chains, unicellular eukaryotic myosin heavy chains, and schistosome paramyosin. I will focus upon the detailed analysis of the *unc-54* myosin heavy chain B, *myo-3* myosin heavy chain A, and *unc-15* paramyosin derived amino acid sequences (McLachlan and Karn, 1983; Dibb et al., 1989; Kagawa et al., 1989).

It must be emphasized that the theoretical analyses of rod structure in these proteins in terms of α-helical repeats within single polypeptides, coiled-coils of α-helices within the dimeric protein rods, and interactions between coiled-coils within the thick filament are based primarily on one-dimensional comparisons between polypeptide chains. The physical relations tested are principally short-range residue-to-residue pairings for either attractive or repulsive electrostatic interactions between charged amino acid side chains and nonpolar interactions between hydrophobic amino acid side chains. Most experimental structural data relevant to two- or three-dimensional analysis are relatively low resolution. Since no high-resolution three-dimensional data presently exist for the coiled-coil structures of myosin or paramyosin rods, the heuristic insights of Karn, McLachlan, and their colleagues provide us with our best available picture.

The derived amino acid sequences of myosin heavy chains A and B show identities of 603/850 amino acids in the head (71.28%) and of 684/1117 amino acids in the rod (61.23%). Paramyosin is homologous to residues 267–1089 of myosin heavy chain B. The heavy chains possess a nonhelical tailpieces at their *C* terminals that show the multiple serines typical of nonmuscle myosins (Karn et al., 1985; Dibb et al., 1989). Paramyosin has nonhelical regions at both the *N* and *C* terminals. Residues 1–24 of paramyosin possess seven serines, two of which are phosphorylated in *C. elegans* (Schreifer and Waterston, 1989). The *N* terminals of all these proteins are blocked.

The derived amino acid sequences of all three body-wall thick filament proteins are consistent with long rod-like domains of coiled-coils of α-helices. The α-helical regions demonstrate a repeat of nonpolar amino acids leading to long

stretches of hydrophobic seams along the coiled-coils. There is a longer, 28 amino acid repeat of charged amino acids. The interaction between myosin rods in parallel occurs optimally at a stagger of 96 residues, whereas paramyosin molecules interact optimally in parallel at a 493 amino acid stagger. These predicted staggers correspond to distances of 14.55 nm for myosin and 72.0 nm for paramyosin, assuming a rise per residue of 0.148 nm. These distances agree with experimental data. The well-known 14.3–14.5 nm repeat of myosin cross-bridges is determined by X-ray diffraction of muscle fibers and electron microscopy of isolated thick filaments, and the 72.0 nm repeat in paracrystals is observed by electron microscopy (Huxley, 1969; Cohen et al., 1971). Both myosin and paramyosin also interact with each other in parallel, at optimal staggers of –98 and –298 amino acids. The latter stagger would have the amino end of paramyosin near the myosin head.

The rods must also interact in antiparallel in the same zone of the thick filament. The optimum situation (Fig. 13) is myosin to paramyosin, parallel 296 amino acid stagger, paramyosin to paramyosin antiparallel 1–593 amino acid stagger, and paramyosin to myosin, parallel 296 amino acid stagger. In this scheme, the antiparallel myosin stagger would be 0. This prediction agrees with the morphological features of bipolar myosin aggregates (Harris and Epstein, 1977).

The limitation of these theoretical considerations is that no prediction can be made as to whether myosin A or B would preferentially pack in antiparallel within the thick filament center. Kagawa et al. (1989) have analyzed both the myosin–myosin and myosin–paramyosin interactions for each myosin isoform. Their considerations concern interactions of single molecules of myosin and myosin or myosin and paramyosin. Experimental studies of myosin polymerization in vitro have shown that dimers of myosin molecules in parallel are the species that polymerizes (Harrington et al., 1972).

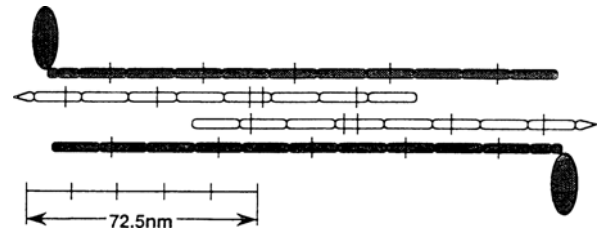

Fig. 13. Interactions of myosin and paramyosin molecules in the structural bare zone of nematode body-wall thick filaments. Adapted from Kagawa et al. (1989). The structures are the results of calculations based upon amino acid sequences that, in turn, are predicted from DNA sequences of cloned genetic material. The dark structures are myosin rods and heads (amino terminus). The white structures are paramyosin rods (arrow end is amino terminus). The segments are 14.5 nm long repeating units in the rod. Shorter helical repeats exist between rod and head in myosin. The vertical black lines are "skip" residues that do not fit the seven amino acid (heptad) repeat of α-helices *28 amino acid repeat within α-helices.*

Therefore, it is the interaction of dimers to dimers that may be the proper focus of modeling isoform specificity. As discussed above, only myosin A is present in the central zone of nematode body-wall thick filaments (Miller et al., 1983). The diameters and cross-sectional densities of thick filaments in the central bipolar zone are similar in wild-type and paramyosin-deficient mutant strains (Epstein et al., 1986). These observations suggest the possibility that paramyosin is not present in the central zones.

Genetic Alterations of Myosin Assembly

Mutations that disrupt thick filament assembly and organization in *C. elegans* have been found in genes encoding the myosin heavy chains, paramyosin, and functions that interact with the myosins or paramyosin. These genetic results have been particularly useful in outlining the properties of myosin and related proteins in assembly. An early finding of signifi-

cance is that overall sarcomere organization and function are not required for the assembly of individual filament structures. In general, nematode mutants that produce fewer thick filaments and abnormal thick filament-related structures have mutations in one of the three classes of genes mentioned above.

Mutants Affecting Myosin

Mutants of the *unc-54* I locus were critical in demonstrating that this gene encodes myosin heavy chain B (Epstein et al., 1974; MacLeod et al., 1981; Miller et al., 1986). Waterston (1988) outlines four classes of *unc-54* mutant alleles—null recessives, lethal semidominants, weakly dominants, and missense recessives. The first three classes produce various defects in assembly, whereas the recessive missense mutants vary from nearly normal function to stiff, slowly moving animals of normal size with near normal to fully wild-type morphology. The primary defects are amino acid substitutions in the myosin head regions that may affect the sites for ATP hydrolysis, actin binding, or mechanochemical transduction between the two sites.

The null recessives are a large class of *unc-54* mutations whose defects are clearly observed in homozygous animals. The primary defect is absence of myosin heavy chain B owing to either nonsense mutations leading to premature termination, e.g., *e1214* (Waterston and Brenner, 1978), internal deletions producing either out-of-frame reading, e.g., *e190* (Dibb et al., 1985), or deletions of the entire gene, e.g., *eDf10* (Anderson and Brenner, 1984). The A bands of the body-wall muscles are severely disorganized and decreased numbers of thick filaments are detected in these mutants (Epstein et al., 1974; MacLeod et al., 1977b). However, thick filaments of normal size and morphology may be isolated from *unc-54* null mutants (Mackenzie and Epstein, 1980; Epstein et al., 1986; Epstein, 1986). In these myosin B deficient mutants, myosin A is localized along the entire length of the thick filament by labeling with antibodies specific to myosin A whereas no specific anti-myosin B antibody reacts.

The class of semidominant alleles is characterized by slow-moving, muscle-defective heterozygotes, and severely paralyzed or embryonic lethal homozygotes (MacLeod et al., 1977b; Dibb et al., 1985). The *e1152* allele showed no reduction of myosin heavy chain B, but reduced numbers of thick filament and myofilament disorder. However, greater numbers of these mutants have been more recently studied; the most severe alleles, such as *r342*, accumulate less than 2% of normal heavy chain B (Bejsovec and Anderson, 1988). The morphology of the severe mutants has been studied as heterozygotes with wild-type and null alleles of *unc-54*. When *unc-54* is represented only by dominant mutant heavy chain B, very few thick filaments are formed (Fig. 14).

The primary defect of *e1152* is the substitution of a gly-lys pair of amino acids by arg-met near the head-rod junction of myosin heavy chain B (Dibb et al., 1985). Recent experiments by Bejsovec and Anderson (1990) show that most semidominant mutations are owing to missense substitutions in the head of the myosin molecule. Surprisingly, the major sites for these mutations are the putative ATP and actin binding sites. These important observations raise the possibility that actin-myosin-ATP cycling may be necessary for filament assembly. The dominance of myosin B mutations may imply not only an inability of the affected isoform to assemble properly, but an interference of the mutant myosin B with the assembly of the normal myosin A in the same muscle cell (Bejsovec and Anderson, 1988).

The class of weakly dominant alleles has two members, *e675* and *s29l*. These homozygous mutants show near paralysis and a fine twitch. Reduced numbers of thick filaments, severe A band disorganization, and significant numbers of "large diameter filaments" are observed

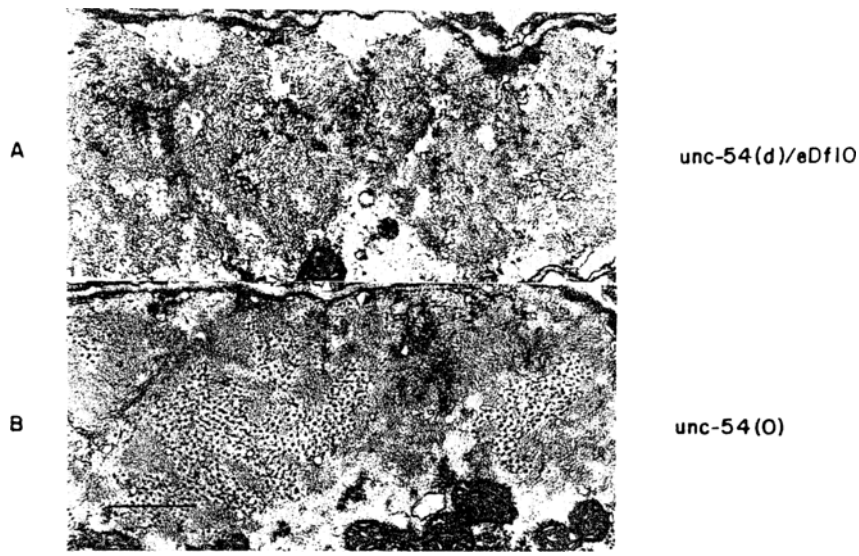

Fig. 14. Mutants of myosin heavy chain B. Electron micrographs of cross-sectional nematode body-wall muscle cells. *A.* The heterozygote of a dominant *unc-54* allele and a deletion overlapping wht *unc-54* gene (null). *B.* Homozygote of a null *unc-54* allele that produces no myosin heavy chain B. The effect of the dominant mutation in disrupting thick filaments in (A) is greater than the effect of a complete absence of myosin B. The thick filaments in (B) are composed of myosin A only. The dominant mutation is in the globular head of myosin, yet interferes with the assembly of rods not only of its own myosin molecule, but of the normal myosin A molecules. Reprinted from Bejsovec and Anderson (1988). Bar is 0.5 μm.

(Waterston, 1988). The heterozygotes show more moderate disorganization and slow movement.

The primary defect in both alleles is a deletion within the gene that preserves the reading frame so that a truncated polypeptide chain is produced. The shortened B myosin heavy chain produced by *e675* provided the first clue that *unc-54* encodes that polypeptide (Epstein et al., 1974). The absent polypeptide sequence is in the rod portion of the myosin molecule (MacLeod et al., 1977a; Dibb et al., 1985).

The gene encoding myosin heavy chain A is denoted *myo-3*. It was identified by molecular cloning, sequencing, and expression of partial polypeptide sequences in bacteria (Miller et al., 1986). The specific monoclonal antibodies to myosin heavy chain A reacted only with *myo-3* expression clones. *Myo-3* was localized to chromosome V by *in situ* hybridization (Albertson, 1985).

Recently, lethal alleles of *myo-3* have been isolated (Waterston, 1989). Little or no movement of body-wall muscles takes place. No myosin heavy chain A is detected. Very few, if any, normal thick filaments are observed by electron microscopy. Instead, structures of variable diameter that exhibit axial striations along their length are observed as well as thin filaments. These "large diameter filaments" appear similar to the multifilament assemblages of *unc-15* and *unc-82* mutants (*see below*). Duplications of *myo-3* have been studied as suppressors of *unc-54* null alleles (myosin heavy chain B) and of *unc-15* missense alleles (paramyosin) (Riddle and Brenner, 1978; Miller and Maruyama, 1986). The primary effect of these duplications is a twofold increase in myosin A levels (Waterston et al., 1982; Otsuka 1986). Increased numbers of thick filaments are produced in *unc-54* (0) *sup-3* homozygotes compared to *unc-54* (0) mutants. The *e1407* allele in a wild-type background produces thick filaments in which myosin A is localized to a central zone of 3.6 μm in length by

electron microscopy of specific antibody complexes (Epstein et al., 1986). In contrast, wild-type has a central myosin A zone 1.75 μm long (Miller et al., 1983).

Similar spreading of myosin A is observed in thick filaments from *unc-52* mutants (Epstein et al., 1986; Epstein, 1986). In the *e444* and *e664* alleles, myosin heavy chain B synthesis is decreased up to twofold (Zengel and Epstein, 1980b). The mechanism of *unc-52* effects is unknown. Concomitant with this decreased myosin B synthesis and myosin A spreading is an overall decrease in myofilament number (Mackenzie et al., 1978b). These results suggest that the levels of myosin heavy chains A and B produced by protein synthesis may be coupled to their differential assembly properties and to the rate of thick filament assembly.

Mutants Affecting Paramyosin

Mutants of the *unc-15* locus have been important in identifying paramyosin as the most likely gene product (Waterston et al., 1977; Waterston and Brenner, 1978). Two types of mutant alleles are noted—presumptive null alleles and missense alleles. The null allele, *e1214*, fails to accumulate detectable paramyosin caused by premature polypeptide chain termination. Missense alleles, for example *e73*, produce relatively normal levels of paramyosin. The assembly of thick filaments is severely impaired in both kinds of mutants, providing formal evidence that paramyosin is required for normal myosin assembly in *C. elegans*.

The *e1214* allele produces thick filament-like structures with central myosin-containing zones about 1.5–2.0 μm long and polar hollow structures about 15 nm wide (Epstein et al., 1986). The polar structures appear to be very fragile, leading to many free bipolar myosin zones, hollow structures in isolated preparations (Mackenzie and Epstein, 1980), and possibly to collections of hollow structures in the muscles in vivo (Waterston et al., 1977). Within these myosin zones, both myosins A and B appear coextensive by antibody labeling (Epstein et al., 1986) (Fig. 14). Careful examination of the labeling of *e1214* filaments indicates that both myosins A and B can pack antiparallel in the structural bare zone as well as in parallel in more polar regions (Fig. 14). Further, the presence of paramyosin appears to affect not only overall myosin assembly, but the specific assembly of the two isoforms to different locations.

The presumptive missense alleles that synthesize paramyosin, such as *e73*, produce a variety of thick filament-related structures. These include both filaments similar to wild-type and structures like those in null alleles. Most striking, however, are the "multifilament assemblages" (Epstein et al., 1987). These structures were originally detected in electron micrographs of *e73* muscle sections by their clear striations, which are similar to the periodicities within paracrystals of paramyosin (Waterston et al., 1977). However, when isolated, it is clear that thick, filament-like structures emanate from the central paracrystalline region (Fig. 15). Myosin B is localized to these short protruding filaments, whereas myosin A may be absent or at the junctions between the paracrystalline and filament structures. The exact number of polar filaments and the size of the assemblages vary considerably. Assemblages may be 3–20 μm long; they may have 1–10 filaments extend from either one or both poles.

Mutant effects of the *e73* and similar *unc-15* alleles are markedly suppressed by *sup-3* alleles, such as *e1407*. The number of thick filaments increases and the number of abnormal structures, including multifilament assemblages, decreases (I. Ortiz and H. F. Epstein, unpublished results). These results indicate that increased levels of myosin A lead to increased assembly of the mutant paramyosin and the myosins. Interestingly, *sup-3* alleles have little or no effect on paramyosin null alleles, such as *e1214*.

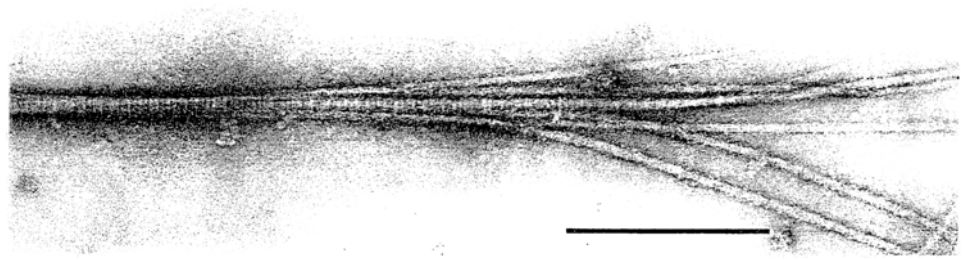

Fig. 15. Multifilament assemblage. Electron microscopy of negatively stained multifilament assemblage isolated from a semidominant myosin mutant. Note multiple myosin-containing filaments emanating from a single paramyosin-containing paracrystalline structure. Bar is 0.5 μm.

Mutants Affecting Other Assembly Functions

Mutants in several additional genes have effects upon thick filament assembly. These effects appear to be specific to either the *unc-54* product myosin heavy chain B or to the *unc-15* product paramyosin. The assembly functions encoded by these additional genes may be either additional protein components of the thick filaments, enzymes that catalyze modifications of myosin or paramyosin, or as yet unspecified activities required for the proper assembly of the major proteins.

The *unc-22* IV gene has many alleles, all of which exhibit a characteristic twitch within the muscle cells. Different mutant alleles vary considerably in their effects on muscle movement and structure, ranging from *e105*, which has normal movement and structure, to *s32*, which is paralyzed and has disorganized A bands (Waterston, 1988). Genetic experiments in which *unc-54* alleles affecting myosin B heads suppressed the twitch of *unc-22* alleles provided the first clue for the potential interaction of the gene products (Moerman et al., 1982). Portions of the *unc-22* gene were cloned by tagging with the transposable element Tcl (Moerman et al., 1986). A 60,000 dalton *unc-22*-encoded polypeptide was expressed as part of a fusion protein with β-galactosidase. Antiserums to the protein chimera were developed, and a specific anti-*unc-22* antibody was prepared by immunoabsorption against β-galactosidase (Moerman et al., 1988). The antibody reacts with a protein of about 500–600 kDa in immunoblots of nematode homogenates and labels the A bands of wild-type body-wall muscles in a pattern very similar to that of anti-myosin B (Miller et al., 1983). Therefore, functional genetic and immunological localization experiments suggest that the *unc-22*-encoded protein interacts and possibly coassembles with *unc-54*-encoded myosin heavy chain B. Specific *unc-22* mutants can disrupt thick and thin filament organization. Large regions of the *unc-22*-encoded protein twitchin have now been sequenced. The protein has a mass of greater than 665 kDa and shows sequence homology in one motif to myosin light chain kinase and in another motif to the immunoglobulin superfamily (Benian et al., 1989).

The *unc-45* III gene is unusual for *C. elegans* in having several temperature-sensitive alleles (Epstein and Thomson, 1974; Zengel and Epstein, 1980a). Lethal alleles have been recently found (Waterston, 1988). At the restrictive temperature, 25°C, *e286* shows reduced numbers of thick filaments and disorganized A bands. Recent unpublished work reviewed in Waterston (1988) suggests that the *unc-45*-encoded product interacts with myosin B during assembly. Double homozygotes of *unc-54* (0)/*unc-45* (ts) are less paralyzed and show more organized

myofibrillar structure than the parent *unc-54* (0) or *unc-45* (ts) strains. The triple homozygote strain *sup-3 (e1407)/unc-54* (0)/*unc-45* (ts) shows nearly wild-type myofibrillar organization and assembly of myosin A by immunofluorescence microscopy using specific antimyosin A monoclonal antibody. In *unc-45* (ts) mutants or in *unc-45* (ts/*sup-3* double homozygotes, myosin B is not assembled into the same structures as myosin A, nor is myosin B assembly improved by *sup-3*. The conclusion from all of these results is that wild-type *unc-45* activity appears to be required for normal myosin B assembly; mutant *unc-45* does not affect myosin A assembly.

The *unc-82* IV locus includes a set of mutant alleles whose phenotypes resemble less severe versions of *unc-15* missense alleles (Waterston et al., 1980). The most prominent aspect of the mutant phenotype is the occurrence of multifilament assemblages. The properties of these structures overlap those of *unc-15 (e73)*. The structures on the whole are smaller than the corresponding *unc-15* assemblages (Epstein et al., 1987). Double homozygotes of *unc-15 (e73)/unc-82 (e1323)* show suppression of the severe paralysis of *unc-15;* the null *unc-15 (e1214)/ unc-82 (e1220)* shows the *unc-15* phenotype; *e1214* and *e1323* double homozygotes are lethal (Waterston et al., 1980). Recent biochemical work has shown that the phosphorylation of the *unc-15*-encoded paramyosin is deficient in the *unc-82* mutant (Schreifer and Waterston, 1989). This result suggests that *unc-82* is related to a protein kinase, possibly a paramyosin-specific enzyme. The missense mutant paramyosins of *unc-15* alleles may interact differently with the underphosphorylated *N*-terminal regions of neighboring paramyosin molecules.

Isolation of revertants of *unc-15 (e73)* includes intragenic revertants, suggesting interallelic complementation between paramyosin molecules *sup-3* V alleles and the *m210* allele of *sup-19* (Brown and Riddle, 1985). The suppressed strain, *unc-15 (e73)sup-3(e1407)* was mutagenized to yield mutations interfering with suppression. Two recessive mutations of *sus-1* III block the suppression. The increase of myosin A owing to *sup-3* is maintained in the presence of mutant *sus-1*. The *sus-1* mutant alleles do not interfere with the effects of *sup-3* on *unc-54* myosin B mutants. The *sup-19* and *unc-82* alleles do not suppress *unc-54* mutant alleles. Therefore, the effects of *sus-1, sup-19,* and *unc-82* appear to be specific to the mutant paramyosins of *unc-15* missense alleles. The suggestion is that all three genes encode products that either modify or interact with paramyosin prior to its assembly.

Expression of Myosin and Paramyosin

Myosin heavy chains A and B and paramyosin are synthesized in the 95 body-wall muscle cells, for alimentary tract cells, and 16 reproductive muscle cells of the adult hermaphrodite of *C. elegans* (Ardizzi and Epstein, 1987). The ratios of A and B chains synthesized and accumulated during the larval stages (LI–L4) through early adulthood do not change significantly, although the amount of myosin in each nematode increases about 40-fold (Garcea et al., 1978; Epstein et al., 1982a).

Reexamination of the expression of the myosin forms and paramyosin has been performed using monoclonal antibodies specific to each polypeptide and genomic sequences specific to the corresponding mRNAs (Honda and Epstein, 1990). Homogenates of L2 and L4 nematodes, developed from a synchronous population of eggs (Garcea et al., 1978; Zengel and Epstein,1980b; Gossett et al., 1982), were electrophoresed on 3.0–7.5% polyacrylamide-sodium dodecyl sulfate gels, blotted in nitrocellulose, and reacted with antimyosin A, antimyosin B, and antiparamyosin monoclonal antibodies (Epstein et al., 1982b; Miller et al., 1983; Ardizzi and Epstein, 1987) (Fig. 16). The paramyosin reactivity served as an internal

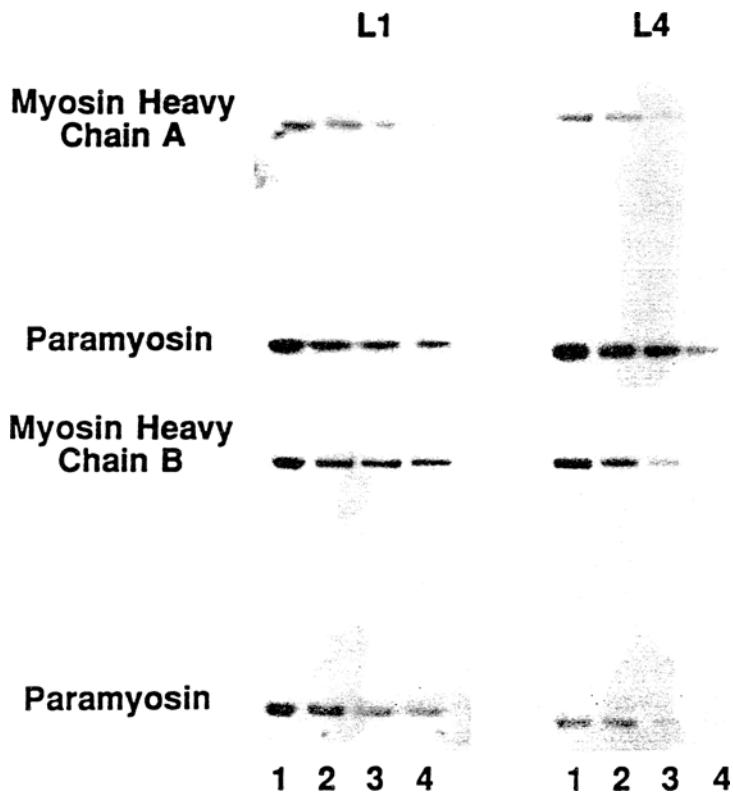

Fig. 16. Expression of thick filament proteins in *C. elegans*. Specific monoclonal antibodies to myosin heavy chains A and B and paramyosin were reacted with nitrocellulose blots of polyacrylamide-SDS electrophoretograms, homogenates of L1 and L4 larvae, with paramyosin as an internal standard. The ratios of myosin heavy chains B and A were determined to be between 1.7–2.4:1. Reprinted from Honda and Epstein (1990).

standard for comparison of the myosin isoform reactivities that were assumed equal on a molar basis. The ratios of heavy chains A and B were 1:1.7 and 1:2.4. for L2 and L4, respectively. The weight ratio of myosin to paramyosin has been determined in homogenates (Waterston et al., 1974) and at several steps in the purification of thick filaments (Epstein et al., 1988) to be 1:1.5. Therefore, the molar ratios of paramyosin (*unc-15*), myosin heavy chain B (*unc-54*), and myosin heavy chain A (*myo-3*) are 4:1.7:1 and 5.1:2.4:1 for L2 and L4, respectively. If one averages the larval data, the overall molar stoichiometry of polypeptides would be 4.5:2.0:1. The overall weight ratio of 1.5:1 paramyosin:myosin and the measured lengths of wild-type nematode body-wall thick filaments, 10 μm (Mackenzie and Epstein, 1980,1981), is consistent with analogous comparisons of muscles from a variety of invertebrate phyla (Levine et al, 1976). The molar stoichiometries of the two myosin isoforms are also consistent with their assembly along different lengths of the surface domain of a tapering filament and of paramyosin in the interior structures of the same filament (Miller et al., 1983; Epstein et al., 1985).

The relative levels of the mRNAs for the thick filament genes are markedly different than the protein stoichiometry. RNA antisense copies (riboprobes) of short genomic DNA sequences specific

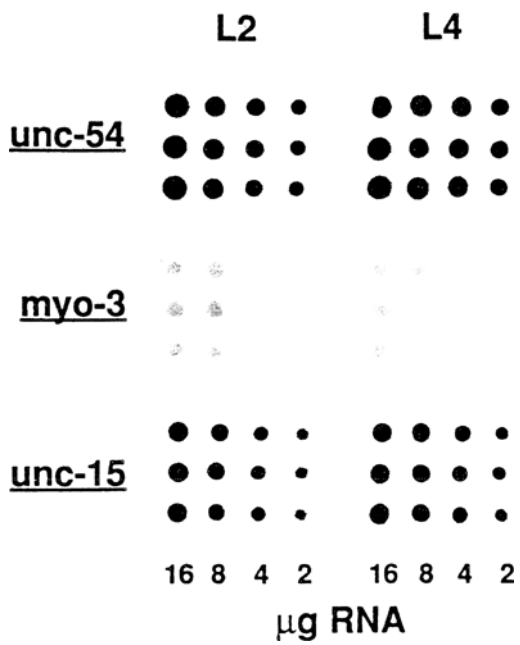

Fig. 17. Expression of thick filament mRNAs. Riboprobes specific to *unc-54*, *myo-3*, and *unc-15* were hybridized to dot blots of total RNA from L2 and L4 larvae. The ratios of *unc-54* and *myo-3* mRNAs determined by this method were about 27–50:1. Reprinted from Honda and Epstein (1990).

to each of the three genes were synthesized with [α-^{32}P]-CTP. The specificities were verified by Northern blots of electrophoretically separated total RNAs. Dot blots showed that *unc-54* (myosin B) and *unc-15* (paramyosin) mRNAs were within twofold of each other, but *myo-3* (myosin A) mRNA was as much as 50-fold lower (Fig. 17). The expression at the protein and mRNA levels appears, therefore, to be markedly discoordinate. Although myosin heavy chain A is two- to fourfold lower than the other major thick filament proteins, the mRNA required for its synthesis is severely limiting. Presumably, the translation or translatability of these mRNAs must be differentially regulated to permit the observed stoichiometry of polypeptide synthesis and assembly. Biochemical and genetic data suggest that myosin accumulation and assembly in *C. elegans* are coordinate and coupled (Epstein et al., 1982a, 1986).

In other assembly systems, including bacteriophage proteins (Geisselsoder et al., 1978; Showe and Onarato, 1978), acetylcholine receptor subunits (Merlie and Lindstrom, 1983; Kordeli et al., 1989), sodium pump subunits (Taormino et al., 1989), and sarcoplasmic reticulum proteins (Jorgenson et al., 1979), mRNA or nascent protein and the final stoichiometry of assembly also appear discoordinate. Some common phenomenon in the control of expression in terms of specific coupling between synthesis and assembly may be present in these examples. Alternatively, the ratio of actual concentrations required for the proteins to assemble in terms of required rates or extents may be different from their assembled stoichiometries. These two mechanisms are not mutually exclusive.

Assembly Pathway for Myosin

Multiple features of thick filament structure, genetic perturbation of muscle organization in mutants, and the patterns of specific mRNA and isoform synthesis indicate that a complex pathway of reactions coupled with the regulated synthesis of component proteins must take place in the assembly of thick filaments in the nematode *C. elegans*. Two models have been proposed: A sequential pathway based on a specific nucleation center for the assembly of each filament (Epstein et al., 1985; Epstein, 1986); and a cyclic pathway in which a regenerating assemblage produces multiple filaments (Epstein, 1988a,b) (Fig. 18).

In the linear sequential model (Fig. 12), myosin A, a polar core template, and proteins specific to the core structure of the central zone of the thick filament are required for formation of the nucleation center (I). Myosin A is implicated in the nucleation step because of its position in the central bipolar region, which appears to be the nucleation center in myosin polymerization (Davis, 1988), and because of its apparent limiting activity upon thick filament number de-

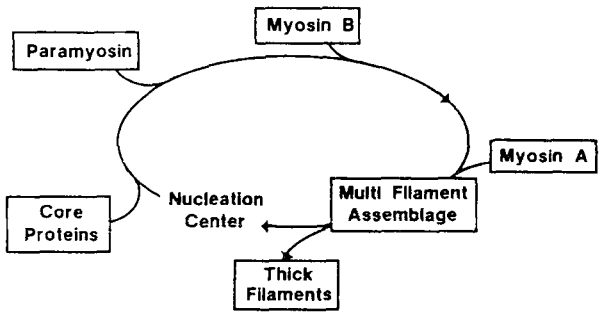

Fig. 18. Scheme of thick filament assembly cycle. The multifilament assemblage produces thick filaments and regenerates centers for nucleation of nascent thick filament structures. Myosin A availability is rate-limiting to this cycle.

duced from the genetic data. Further, the central zone and myosin A are the least soluble components by salt treatment. The polar core template is the postulated length-determining molecule of the polar filament structures. The template mechanism is hypothesized because it is the only length determination mechanism in a biological system for which there is direct evidence (Katsura, 1987). The central core proteins are proposed because the diameter of the central zone is maintained even in the genetic absence of paramyosin, and intact myosin molecules could not pack to fill its 32–33 nm diameter (Wray, 1979).

Polar core proteins polymerize about the template (II) to form the observed core structure. Candidate proteins for the core structures have been identified (Epstein et al., 1988), but direct evidence for their localization to the core structure by specific antibodies and characterization of their native structure has not yet been accomplished.

Paramyosin, the *unc-15* gene product, modified by the action of *unc-82*-mediated phosphorylation and possibly the action of *sus-1* and *sup-19* gene products, polymerizes about the core structure (III). It should be noted that paramyosin is the most abundant protein on a mole basis in *C. elegans* body-wall thick filaments as in many other invertebrate systems. This fact emphasizes the specialization inherent in myosin assemblies.

The final step is the polymerization of myosin B. The myosin B heavy chains, products of *unc-54*, require the activity of *unc-22* and *unc-45* gene products for their proper assembly. Clearly, myosin A, the *myo-3* product, can substitute for myosin B in this last step; however, it does not require *unc-22* or *unc-45* activity to do this. Perhaps, as yet unknown analogous functions are required for *myo-3* assembly.

In the linear model, myosin A synthesis would be rate-limiting in thick filament assembly because of its role in the initial nucleation step. The cyclic model is hypothesized chiefly upon two sets of observations—the properties of multifilament assemblages and the ability of myosin A to participate in what appear to be both initial and terminal steps in thick filament assembly of *C. elegans*. The multifilament assemblages of wild-type and mutants exhibit structural properties unprecedented from studies of purified myosin and paramyosin. The generation of two or more thick filaments from a single filament-like structure is not observed in any of the structural polymorphisms of purified myosin, paramyosin, or their combination (Harris and Epstein, 1977).

Different levels of myosin A influence the extent of thick filament assembly. Decreased availability of myosin A in *unc-54* (0) alleles leads to decreased numbers of thick filaments, and increased total myosin A in *sup-3* strains leads to increased thick filament numbers in both *unc-54* null and missense mutants. All of these results are consistent with a role of myosin A in filament initiation. The expanded central zones containing myosin A in *sup-3* and *unc-52* strains suggest that myosin A can assemble in locations usually specific to myosin B dependent upon the relative levels of myosin A to B. Myosin A also appears to participate in the terminal steps of assembly.

Preliminary in vitro experiments suggest that solutions containing multifilament assemblages exhibit increased numbers of thick filaments upon incubation; solutions containing only thick filaments do not show such increases (K. C.

Lerner, G. C. Berliner, and H. F. Epstein, unpublished experiments). The appearance of "large or variable diameter filaments" in specific *unc-54* (K. C. Lerner, I. Ortiz, and H. F. Epstein, unpublished results) and lethal *myo-3* mutants (Waterston, 1989) suggests that the blocks of thick filament assembly owing to absence of myosin A or alteration of myosin B may also lead to accumulation of multifilament assemblages. "Large diameter filaments" have also been observed in specific myosin heavy chain mutants of *Drosophila* (O'Donnell and Bernstein, 1988).

The cyclic model contains the reactions of the linear models, but differs in two critical aspects. Myosin A interacts with the multifilament assemblage to generate thick filaments and to reactivate the assemblage to enter another cycle of assembly reactions. In both models, myosin A synthesis and accumulation would be critical kinetically. The potential significance of actin filaments and their ATP-mediated interaction with myosin during thick filament assembly has been indicated by the recent molecular genetic experiments of Bejsovec and Anderson (1990).

Summary

The nematode muscle field has matured to where experiments can be designed to establish detailed mechanisms operating in physiological assembly. Such experiments will be based on the genetic, biochemical, and structural observations outlined. This body of work has clearly demonstrated that myosin assembly into thick filaments results from a complex sequence of reactions involving multiple gene products including component proteins and noncomponent assembly functions.

Acknowledgments

I am indebted to my colleagues who have collaborated in many experiments and who have shared with me their results and ideas. In particular, I wish to thank Philip Anderson, Amy Bejsovec, Guy Benian, Sanford Bernstein, Patrick O'Donnell, and Robert Waterston for their generous sharing of information before publication. Irving Ortiz and Lois Smith greatly assisted me in the preparation of this manuscript. The research in my laboratory is supported by grants from the National Institute of General Medical Sciences and the Muscular Dystrophy Association.

References

Albertson D. G. (1985) Mapping muscle protein genes by *in situ* hybridization using biotin-labeled probes. *EMBO J.* **4,** 2493–2498.

Anderson, P. (1989) Molecular genetics of nematode muscle. *Annu. Rev. Genet.* **23,** 507–525.

Anderson P. and Brenner S. (1984) A selection for myosin heavy-chain mutants in the nematode. *Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA* **81,** 4470–4474.

Ardizzi J. P. and Epstein H. F. (1987) Immunochemical localization of myosin heavy chain isoforms and paramyosin of developmentally and structurally diverse muscle cell types of the nematode *Caenorhabditis elegans. J. Cell Biol.* **105,** 2763–2770.

Bejsovec A. and Anderson P. (1988) Myosin heavy chain mutations that disrupt *Caenorhabditis elegans* thick filament assembly. *Genes Devel.* **2,** 1307–1317.

Bejsovec A. and Anderson P. (1990) Function of the myosin ATPase and actin binding sites is required for *C. elegans* thick filament assembly. *Cell* **60,** 133–140.

Bejsovec A., Eide D., and Anderson P. (1989) Genetic techniques for analysis of nematode muscle, in *Molecular Biology of the Cytoskeleton,* Borisy G. et al., eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 267–273.

Benian G. M., Kiff J. E., Neckelmann N., Moerman D. G., and Waterston, R. H. (1989) Sequence of an unusually large protein implicated in regulation of myosin activity in *C. elegans. Nature* **342,** 45–50.

Bennett P., Craig R., Starr R., and Offer G. (1986) The ultrastructural localization of C-protein, X-protein and H-protein in rabbit muscle. *J. Muscle Res. Cell Motil.* **7,** 556–567.

Bouché M., Goldfine S. M., and Fischman D. A. (1988) Posttranslational incorporation of contractile proteins into myofibrils in a cell-free system. *J. Cell Biol.* **107,** 587–596.

Brown S. J. and Riddle D. L. (1985) Gene interactions affecting muscle organization in *Caenorhabditis elegans*. *Genetics* **110,** 421–440.

Bullard B., Luke B., and Winkelman L. (1973) The paramyosin of insect flight muscle. *J. Mol. Biol.* **75,** 359–367.

Chowrashi P. K. and Pepe F. A. (1986) The myosin filament XII. Effect of MgATP on assembly. *J. Muscle Res. Cell Motil.* **7,** 413–420.

Cohen C., Lanar D. E., and Parry D. A. D. (1987) Amino acid sequence and structural repeats in schistosome paramyosin match those of myosin. *Biosci. Rep.* **7,** 11–16.

Cohen C., Szent-Györgyi A. G., and Kendrick-Jones J. (1971) Paramyosin and the filaments of molluscan "catch" muscles. I. Paramyosin: structure and assembly. *J. Mol. Biol.* **56,** 223–237.

Coluccio L. M. and Bretscher A. (1987) Calcium-regulated cooperative binding of the microvillar 110 K-calmodulin complex to F-actin: formation of decorated filaments. *J. Cell Biol.* **105,** 325–333.

Conzelman K. A. and Mooseker M. S. (1987) The 100 kD protein-calmodulin complex of the intestinal microvillus is an actin-activated MyATPase. *J. Cell Biol.* **105,** 313–324.

Cooper J. and Trinick J. (1984) Binding and location of AMP deaminase in rabbit psoas muscle myofibrils. *J. Mol. Biol.* **177,** 137–152.

Coulson A., Sulston J., Brenner S., and Karn J. (1986) Towards a physical map of the genome of the nematode *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **83,** 7821–7825.

Craig R. and Megerman J. (1977) Assembly of smooth muscle myosin into side-polar filaments. *J. Cell Biol.* **75,** 990–996.

Craig R. and Offer G. (1976) The location of C-protein in rabbit skeletal muscle. *Proc. Roy. Soc. Lond. B.* **192,** 451–464.

Cummins, C. and Anderson, P. (1988) Regulatory myosin light-chain genes of *Caenorhabditis elegans*. *Mol. Cell. Biol.* **8,** 5339–5349.

Davis J. S. (1988) Assembly processes in vertebrate skeletal thick filament formation. *Annu. Rev. Biophys. Chem.* **17,** 217–235.

De Lozanne A. and Spudich J. A. (1987) Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. *Science* **236,** 1086–1091.

Dibb N. J., Brown D. M., Karn J., Moerman D. G., Bolten S. L., and Waterston R. H. (1985) Sequence analysis of mutations that affect the synthesis, assembly and enzymatic activity of the *unc-54* myosin heavy chain of *Caenorhabditis elegans*. *J. Mol. Biol.* **183,** 543–551.

Dibb N. J. Maruyama I. N., Krause M., and Karn J. (1989) Sequence analysis of the complete *Caenorhabditis elegans* myosin heavy chain gene family. *J. Mol. Biol.* **205,** 603–613.

Eide D. and Anderson P. (1985) The gene structures of spontaneous mutations affecting a *Caenorhabditis elegans* myosin heavy chain gene. *Genetics* **109,** 67–79.

Emerson C. P. and Bernstein S. I. (1987) Molecular genetics of myosin. *Annu. Rev. Biochem.* **56,** 695–726.

Epstein H. F. (1986) Differential roles of myosin isoforms in filament assembly. *UCLA Symp. Mol. Cell. Biol. New Ser.* **29,** 653–666.

Epstein H. F. (1988a) Modulation of myosin assembly. *UCLA Symp. Mol. Cell Biol. New Ser.* **93,** 207–219.

Epstein H. F. (1988b) Modulation of myosin assembly. *Bioessays* **9,** 197–200.

Epstein H. F. and Thomson J. N. (1974) Temperature sensitive mutation affecting myofilament assembly in *Caenorhabditis elegans*. *Nature* **250,** 579–580.

Epstein H. F., Berliner G. C., Casey D. L., and Ortiz I. (1988) Purified thick filaments from the nematode *Caenorhabditis elegans:* evidence for multiple proteins associated with core structures. *J. Cell Biol.* **106,** 1985–1995.

Epstein H. F., Berman S. A., and Miller D. M. III (1982a) Myosin synthesis and assembly in nematode body-wall muscle, in *Muscle Development: Molecular and Cellular Control,* Pearson M. and Epstein H. F., eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 419–427.

Epstein H. F., Miller D. M. III, Gossett L. A., and Hecht R. M. (1982b) Immunological studies of myosin isoforms in nematode development, in *Muscle Development: Molecular and Cellular Control,* Pearson M. and Epstein H. F., eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 7–14.

Epstein H. F., Miller D. M. III, Ortiz I., and Berliner G. C. (1985) Myosin and paramyosin are organized about a newly identified core structure. *J. Cell Biol.* **100,** 904–915.

Epstein H. F., Ortiz I., and Berliner G. C. (1987) Assemblages of multiple thick filaments in nematode mutants. *J. Muscle Res. Cell. Motil.* **8,** 527–536.

Epstein H. F., Ortiz I., and Mackinnon L. A. T. (1986) The alteration of myosin isoform compartmentation in specific mutants of *Caenorhabditis elegans*. *J. Cell. Biol.* **103,** 985–993.

Epstein H. F., Waterston R. H., and Brenner S. (1974) A mutant affecting the heavy chain of myosin in *Caenorhabditis elegans*. *J. Mol. Biol.* **90,** 291–300.

Gadasi H. and Korn E. D. (1980) Evidence for differential intracellular localization of the Acanthamoeba myosin isoenzymes. *Nature* **286,** 452–456.

Garcea R. L., Schachat F., and Epstein H. F. (1978) Coordinate synthesis of two myosins in wild-type and mutant nematode muscle during larval development. *Cell* **15,** 421–428.

Geisselsoder J., Chidambaram M., and Goldstein R. (1978) Transcriptional control of capsid size in the P2:P4 bacteriophage system. *J. Mol. Biol.* **126,** 447–456.

Goldberg A. F. and Chang T. W. (1978) Regulation and significance of amino acid metabolism in skeletal muscle. *Fed. Proc.* **37,** 2301–2307.

Goldberg A. L., Griffin G. E., and Dice J. F. (1977) Regulation of protein turnover in normal and dystrophic muscle, in *Pathogenesis of Human Muscular Dystrophies*, Rowland L. P., ed., Excerpta Medica, Amsterdam, pp. 376–385.

Gossett L. A., Hecht R. M., and Epstein H. F. (1982) Muscle differentiation in normal and cleavage-arrested mutant embryos of *Caenorhabditis elegans*. *Cell* **30,** 193–204.

Grove B. K., Cerny L., Perriard J-C., and Eppenberger H. M. (1985) Myomesin and M-protein: expression of two M-band proteins in pectoral muscle and heart during development. *J. Cell Biol.* **101,** 1413–1421.

Harrington W. F., Burke M., and Barton J. S. (1972) Association of myosin to form contractile systems. *Cold Spring Harbor Symp. Quart. Biol.* **37,** 77–85.

Harris H. E. and Epstein H. F. (1977) Myosin and paramyosin of *Caenorhabditis elegans:* Biochemical and structural properties of wild-type and mutant proteins. *Cell* **10,** 709–719.

Honda S. and Epstein H. F. (1990) Modulation of muscle gene expression in *Caenorhabditis elegans:* Differential regulation of transcripts, mRNAs, and polypeptides for thick filament proteins during nematode development. *Proc. Natl. Acad. Sci. USA* **87,** 876–880.

Huszar G. (1972) Amino acid sequences around the two ε-*N*-trimethyllysine residues in rabbit skeletal muscle myosin. *J. Biol. Chem.* **247,** 4057–4062.

Huszar G. and Elzinga M. (1973) Homologous methylated and nonmethylated histidine peptides in skeletal and cardiac myosins. *J. Biol. Chem.* **247,** 745–753.

Huxley H. E. (1963) Electron microscopy studies of native and synthetic protein filaments from striated muscle. *J. Mol. Biol.* **7,** 281–308.

Huxley H. E. (1969) The mechanism of muscular contraction. *Science* **164,** 1356–1366.

Jorgensen A. O., Kalinins V. I., Zubryzycka E., and MacLennan D. H. (1977) Assembly of the sarcoplasmic reticulum. *J. Cell Biol.* **74,** 287–298.

Kagawa H., Gengyo K., McLachlan A. D., Brenner S., and Karn J. (1989) The paramyosin gene *(unc-15)* of *Caenorhabditis elegans:* Molecular cloning, nucleotide sequence and models for thick fila- ment assembly. *J. Mol. Biol.* **207,** 311–333.

Karn J., Dibb N. J., and Miller D. M. (1985) Cloning nematode myosin genes, in *Cell and Muscle Motility,* Shay J., ed., vol. 6, Plenum Press, New York, pp. 185–237.

Karn J., McLachlan A. D., and Barnett L. (1982), *Unc-54* myosin heavy-chain gene of *Caenorhabditis elegans:* Genetics, sequence, structure, in *Muscle Development: Molecular and Cellular Control,* Pearson M. and Epstein H. F., eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 129–142.

Katsura I. (1987) Determination of bacteriophage l tail length by a protein ruler. *Nature* **327,** 73–75.

Kensler R. W. and Stewart M. (1983) Frog skeletal muscle thick filaments are three-stranded. *J. Cell Biol.* **96,** 1797–1802.

Knecht D. A. and Loomis W. F. (1987) Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum. Science* **236,** 1081– 1086.

Kordeli E., Cartaud J., Nghiem H-O., Devilliers-Thiérry A., and Changeux J-P. (1989) Asynchronous assembly of the acetylcholine receptor and of the 43-kD v, protein in the postsynaptic membrane of developing *Torpedo marmorata* electrocyte. *J. Cell Biol.* **108,** 127–139.

Kuczmarski E. R. and Spudich J. A. (1980) Regulation of myosin self-assembly: phosphorylation of *Dictyostelium* heavy chain inhibits formation of thick filaments. *Proc. Natl. Acad. Sci. USA* **71,** 7292–7296.

Levine R. J. C., Elfvin M., Dewey M. M., and Walcott B. (1976) Paramyosin in invertebrate muscles II. Content in relation to structure and function. *J. Cell Biol.* **70,** 273–279.

Lowey S., Slayter H.S., Weeds A., and Baker H. (1969) Substructure of the myosin molecule. I. Subfragments of myosin by enzymic digestion. *J. Mol. Biol.* **42,** 1–29.

Mackenzie J. M., Jr. and Epstein H. F. (1980) Paramyosin is necessary for determination of nematode thick filament in vivo. *Cell* **22,** 747–755.

Mackenzie J. M., Jr. and Epstein, H. F. (1981) Electron microscopy of nematode thick filaments. *J. Ultrastruct. Res.* **76,** 277–285.

Mackenzie J. M., Jr., Schachat F., and Epstein H. F. (1978a) Immunocytochemical localization of two myosins within the same muscle cells in *Caenorhabditis elegans. Cell* **15,** 413–420.

Mackenzie J. M., Jr., Garcea R. L., Zengel J. M., and Epstein H.F. (1978b) Muscle development in *Caenorhabditis elegans* mutants exhibiting retarded sarcomere construction. *Cell* **15,** 751–762.

MacLeod A. R., Karn J., and Brenner S. (1981) Molecular analysis of the *unc-54* myosin heavy chain gene of *Caenorhabditis elegans. Nature* **291,** 386–390.

MacLeod A. R., Waterston R. H., and Brenner S. (1977a) An internal deletion mutant of a myosin heavy chain in *Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA* **74,** 5336–5340.

MacLeod A. R., Waterston R. H., Fishpool R. M., and Brenner S. (1977b) Identification of the structural genes for a myosin heavy-chain in *Caenorhabditis elegans. J. Mol. Biol.* **14,** 133–140.

Masaki T. and Takaiti O. (1974) M-protein. *J. Biochem.* (Tokyo) **75,** 367–380.

McLachlan A. D. and Karn J. (1983) Periodic features in the amino acid sequence of nematode myosin rod. *J. Mol. Biol.* **164,** 605–626.

Merlie J. P. and Lindstrom J. (1983) Assembly in vivo of mouse muscle acetylcholine receptor: identification of an a subunit species that may be an assembly intermediate. *Cell* **34,** 747–757.

Miller D. M. and Maruyama I. (1986) The *sup-3* locus is closely linked to a myosin heavy chain gene in *Caenorhabditis elegans. UCLA Symp. Mol. Cell Biol. New Ser.* **29,** 629–638.

Miller D. M. III, Ortiz I., Berliner G. C., and Epstein H. F. (1983) Differential localization of two myosins within nematode thick filaments. *Cell* **34,** 477–490.

Miller D. M., Stockdale F. E., and Karn J. (1986) Immunological identification of the genes encoding the four myosin heavy chain isoforms of *Caenorhabditis elegans. Proc. Natl. Acad. Sci.* **83,** 2305–2309.

Moerman D. G., Benian G. M., and Waterston R. H. (1986) Molecular cloning of the muscle gene *unc-22* in *Caenorhabditis elegans* by Tcl transposon tagging. *Proc. Natl. Acad. Sci. USA* **83,** 2579–2583.

Moerman D. G., Benian G. M., Barstead R. J., Schreifer L., and Waterston R. H. (1988) Identification and intracellular localization of the *unc-22* gene product of *Caenorhabditis elegans. Genes Dev.* **2,** 93–105.

Moerman D. G., Plurad S., Waterston R. H., and Baillie D. L. (1982) Mutations in the *unc-54* myosin heavy chain gene of *Caenorhabditis elegans* that alter contractility but not muscle structure. *Cell* **29,** 773–781.

Morimoto K. and Harrington W. F. (1973) Isolation and composition of thick filaments from rabbit skeletal muscle. *J. Mol. Biol.* **77,** 165–175.

Niederman R. and Peters L. K. (1982) Native bare zone assemblage nucleates myosin filament assembly. *J. Mol. Biol.* **161,** 505–517.

O'Donnell P. T. and Bernstein S. I. (1988) Molecular and ultrastructural defects in a *Drosophila* myosin heavy chain mutant in: differential effects on muscle function produced by similar thick filament abnormalities. *J.Cell Biol.* **107,** 2601–2612.

Otsuka A. J. (1986) *sup-3* Suppression affects muscle structure and myosin heavy chain accumulation in *Caenorhabditis elegans. UCLA Symp. Mol. Cell. Biol. New Ser.* **29,** 619–628.

Pollard T. D. and Korn E. D. (1973) Acanthamoeba myosin I. *J. Biol. Chem.* **248,** 4682–4690.

Reisler E., Smith C., and Seegan G. (1980) Myosin minifilaments. *J. Mol. Biol.* **143,** 129–145.

Riddle D. L. and Brenner S. (1978) Indirect suppression in *Caenorhabditis elegans. Genetics* **89,** 299–314.

Saad A. D., Pardee J. D., and Fischman D. A. (1987) Dynamic exchange of myosin molecules between thick filaments. *Proc. Natl. Acad. Sci. USA* **83,** 9483–9487.

Schachat F., Garcea R. L., and Epstein H. F. (1978) Myosins exist as homodimers of heavy chains: demonstration with specific antibody purified by nematode mutant myosin affinity chromatography. *Cell* **15,** 405–411.

Schachat F., Harris H. E., and Epstein H. F. (1977) Two homogeneous myosins in body-wall muscle of *Caenorhabditis elegans. Cell* **10,** 721–728.

Scholey J. M., Taylor K. A., and Kendrick-Jones J. (1980) Regulation of non-muscle assembly by calmodulin-dependent light chain kinase. *Nature* **287,** 233–235.

Schreifer L. and Waterston R. H. (1989) Phosphorylation of the N-terminal region of *Caenorhabditis elegans* paramyosin. *J. Mol. Biol.* **207,** 451–454.

Showe K. and Onorato L. (1978) Kinetic factors and form determination of the head of bacteriophage T4. *Proc. Natl. Acad. Sci. USA* **75,** 4165–4169.

Starr R. and Offer G. (1973) Polarity of the myosin molecule. *J. Mol. Biol.* **81,** 17–31.

Strehler E. E., Pelloni G., Heizmann C. W., and Eppenberger H. M. (1980) Biochemical and ultrastructural aspects of M 165,000 M-protein in cross-striated chicken muscle. *J. Cell Biol.* **86,** 775–783.

Sulston J. E. and Brenner S. (1974) The DNA of *Caenorhabditis elegans. Genetics* **77,** 95–104.

Szent-Györgyi A. G., Cohen C., and Kendrick-Jones J. (1971) Paramyosin and the filaments of molluscan "catch muscles." II. Native filaments: isolation and characterization. *J. Mol. Biol.* **56,** 239–258.

Taurmino J., Wolitzky B. A., Takeyasa K., Tamkun M. M., and Fambrough D. M. (1989) Up-regulation of the sodium pump in primary chick muscle cultures. *UCLA Symp. Mol. Cell Biol. New Ser.* **93,** 327–334.

Taylor L. D. and Bandman E. (1989) Distribution of fast myosin heavy chain isoforms in thick filaments of developing chicken pectoral muscle. *J. Cell Biol.* **108,** 533–542.

Trybus K. M. and Lowey S. (1987) Assembly of smooth muscle myosin minifilaments: effects of phosphorylation and nucleotide binding. *J. Cell Biol.* **105,** 3007–3014.

Turner D. C., Walliman T., and Eppenberger H. M. (1973) A protein that binds specifically to the M-line of skeletal muscle is identified as the muscle form of creatine kinase. *Proc. Natl. Acad. Sci. USA* **70,** 702–705.

Warrick H. M. and Spudich J. A. (1987) Myosin: structure and function in cell motility. *Ann. Rev. Cell Biol.* **3,** 379–422.

Waterston R. H. (1988) Muscle, in *The Nematode* Caenorhabditis elegans, W. B. Wood, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 281–335.

Waterston R. H. (1989) The minor myosin heavy chain, mhcA, of *Caenorhabditis elegans* is necessary for the initiation of thick filament assembly. *EMBO J.* **8,** 3429–3436.

Waterston R. H., Epstein H. F., and Brenner S. (1974) Paramyosin in *Caenorhabditis elegans. J. Mol. Biol.* **90,** 285–290.

Waterston R. H., Thomson J. N., and Brenner S. (1980) Mutants with altered muscle structure in *Caenorhabditis elegans. Dev. Biol.* **77,** 271–302.

Waterston R. H., Fishpool R. M., and Brenner S. (1977) Mutants affecting paramyosin in *Caenorhabditis elegans. J. Mol. Biol.* **117,** 679–697.

Waterston R. H. and Brenner S. (1978) A suppressor mutation in the nematode acting on specific alleles of many genes. *Nature* **275,** 715–719.

Waterston R. H., Moerman D. G., Baillie D. L., and Lane T. R. (1982) Mutations affecting myosin heavy chain accumulation and function in the nematode *Caenorhabditis elegans,* in *Disorders of the Motor Unit,* Schotland D. M., ed., John Wiley, New York, pp. 747–760.

Wenderoth M. P. and Eisenberg B. R. (1987) Heterogeneous distribution of nascent heavy chains into thick filaments of cardiac myocytes in thyroid treated rabbits. *J. Cell Biol.* **105,** 2971–2780.

Whalen R. G., Butler-Browne G. S., Pinset C., Toutant M., Watkins S. C., Ajioka J., Laurent C., McCormick D., and Riley G. P. (1986) Control of myosin isoform expression during skeletal muscle development in rodents. *UCLA Symp. Mol. Cell. Biol., New Ser.* **29,** 237–251.

Wray J. (1979) Structure of the backbone in myosin filaments of muscle. *Nature* **277,** 37–40.

Zengel J. M. and Epstein H. F. (1980a) Identification of genetic elements associated with muscle structure in the nematode *Caenorhabditis elegans. Cell Motil.* **1,** 73–97.

Zengel J. M. and Epstein H. F. (1980b) Mutants altering coordinate synthesis of specific myosins during nematode muscle development. *Proc. Natl. Acad. Sci. USA* **77,** 852–856.