Hydrophobicity Variations along the Surface of the Coiled-Coil Rod May Mediate Striated Muscle Myosin Assembly in *Caenorhabditis elegans*

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**Abstract.** *Caenorhabditis elegans* body wall muscle contains two isoforms of myosin heavy chain, MHC A and MHC B, that differ in their ability to initiate thick filament assembly. Whereas mutant animals that lack the major isoform, MHC B, have fewer thick filaments, mutant animals that lack the minor isoform, MHC A, contain no normal thick filaments. MHC A, but not MHC B, is present at the center of the bipolar thick filament where initiation of assembly is thought to occur (Miller, D.M., I. Ortiz, G.C. Berliner, and H.F. Epstein. 1983. *Cell.* 34:477–490). We mapped the sequences that confer A-specific function by constructing chimeric myosins and testing them in vivo. We have identified two distinct regions of the MHC A rod that are sufficient in chimeric myosins for filament initiation function. Within these regions, MHC A displays a more hydrophobic rod surface, making it more similar to paramyosin, which forms the thick filament core. We propose that these regions play an important role in filament initiation, perhaps mediating close contacts between MHC A and paramyosin in an antiparallel arrangement at the filament center. Furthermore, our analysis revealed that all striated muscle myosins show a characteristic variation in surface hydrophobicity along the length of the rod that may play an important role in driving assembly and determining the stagger at which dimers associate.

*Myosin* is the motor that drives all actin-based motility. In recent years, an increasingly diverse array of myosins have been identified that participate in a variety of cellular processes (for review see Mooseker and Cheney, 1995). Myosin II is the conventional double-headed myosin. Cytoplasmic myosin IIs participate in dynamic processes, such as cytokinesis and morphogenesis, that require the assembly of transient myosin minifilaments in different subcellular locations at specific times during the cell cycle or during development. In contrast, striated muscle myosin contributes to a highly ordered filament lattice that is important for efficient contraction. We are interested in the mechanisms by which myosin is assembled into the thick filament structure. The identification and characterization of functional domains within the myosin heavy chain (MHC) molecule is important for the eventual understanding of the development, action, and functional regulation of muscle. In addition, these studies may contribute to the understanding of the molecular events that are required for assembly of other molecules, including other types of myosin, into ordered structures.

The myosin heavy chain molecule is a large, multifunctional protein (see Fig. 1a). The NH$_2$ terminal globular portion contains actin-binding and ATPase activities that move thin filaments past thick filaments to yield muscle contraction, and is the site of light chain association. The rod, where the two heavy chains dimerize by forming an $\alpha$-helical coiled coil, is the structural component that is responsible for the assembly of myosin into thick filaments. In addition, some myosins, including *Caenorhabditis elegans* body wall muscle myosins, have a COOH-terminal nonhelical region containing phosphorylation sites (Castellani et al., 1988). The other major structural protein in the thick filaments of *C. elegans* and other invertebrates is paramyosin, a coiled-coil protein homologous to the COOH-terminal three fourths of the myosin rod (Fig. 1a) (Kagawa et al., 1989). Paramyosin and associated minor proteins form the core of the thick filament (Deitiker and Epstein, 1993) upon which myosin assembles.

The two MHC isoforms that coassemble in *C. elegans* body wall muscle have specialized to play functionally distinct roles in filament assembly. Using isoform-specific antibodies, Miller et al. (1983) showed that the minor isoform, MHC A, forms the central 2 $\mu$m of the 10-$\mu$m-long bipolar thick filament. This central 2 $\mu$m includes at its center the bipolar region of the thick filament where myosin molecules associate in an antiparallel (tail-to-tail) fashion, as well as extensive polar regions where myosin molecules associate in parallel fashion, and is the site at which

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1. **Abbreviation used in this paper:** MHC, myosin heavy chain.

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myosin with the globular head, which contains the motor region of paramyosin (Schriefer and Waterston, 1989). Apart from the short, nonhelical, NH₂-terminal segment, paramyosin is largely a coiled coil, and by sequence identity, aligns with the COOH-terminal, nonhelical tailpiece of C. elegans MHC contains three potential phosphorylation sites, based on the known in vivo phosphorylation of the similar NH₂-terminal region of paramyosin (Schriefer and Waterston, 1989). Apart from the "prehinge region," the COOH-terminal three fourths of the myosin rod, as shown here; the corresponding portion of the MHC rod is in effect the light meromyosin sequences that form the backbone of the thick filament (for review of myosin structure see Squire, 1981). Based on the "hinge" definition of Lu and Wong (1985), the NH₂-terminal portion of the MHC rod that is not contained in the shorter paramyosin molecule, the "prehinge region," is part of heavy meromyosin subfragment 2 (S2). (b) Schematic diagram of a longitudinal section of the central region of the bipolar thick filament. The simplified molecules represent the dimeric paramyosin protein, and the MHC complex with associated light chains. The precise overlaps of the molecules assembled into the thick filament are unknown. Myosin and paramyosin must associate in an antiparallel or tail-to-tail fashion in the center of the filament (black), whereas elongation of the two filament arms occurs by parallel addition (gray). NH₂ termini are indicated by the myosin head for MHC and as a pointed end for paramyosin. In C. elegans, only MHC A is present in the central region. The "bare zone" refers to that central portion of the filament devoid of myosin heads.

filament initiation is thought to occur. The major isoform, MHC B, forms the remainder of the thick filament by adding in a parallel manner to either side of this central region (Fig. 1 b). The paramyosin-containing core extends almost the entire length of the filament.

Genetic analysis has confirmed the functional differences suggested by the differential subcellular localization of the two isoforms, and has demonstrated that MHC A is uniquely capable of thick filament initiation. Many mutations in the unc-54 locus, the structural gene for MHC B (Epstein et al., 1974; Waterston et al., 1982), have been isolated in screens for uncoordinated animals. Animals homozygous for null mutations in unc-54 are paralyzed, though viable and fertile, and exhibit disorganized body wall muscle containing a reduced number of thick filaments. These filaments can be of normal length, and they contain MHC A along their entire length (Epstein et al., 1986). In contrast, myo-3 alleles (mutations that eliminate MHC A) cause embryonic paralysis and death due to the absence of a functional myofilament lattice, and mutant animals contain no normal thick filaments (Waterston, 1989). Therefore, despite that fact that it comprises only 20–30% of body wall myosin, MHC A is required for viability, and apparently plays a unique and essential role in the initiation of thick filament assembly. The unique function of MHC A has been confirmed by transformation experiments (Fire and Waterston, 1989) in which introduced copies of myo-3 (MHC A) were able to improve the motility of unc-54 mutants, consistent with results obtained earlier with chromosomal duplications (Riddle and Brenner, 1978; Waterston et al., 1982; Otsuka, 1986; Miller and Maruyama, 1986). In the reciprocal test, however, additional copies of the unc-54 gene were unable to rescue myo-3 lethality.

Paramyosin is encoded by the unc-15 gene (Waterston et al., 1977). Mutant animals that lack paramyosin are paralyzed, and contain abnormally short and wide thick filaments (Mackenzie and Epstein, 1980) in which the distinct localization of MHC A and B is lost (Epstein et al., 1986).

The absence of the major isoform, MHC B, at the filament center, as well as the lethality associated with mutations that eliminate MHC A, suggest that the B isoform has specialized and apparently lost the ability to participate in some aspect of filament formation thought to be common to all myosins. The localization of the minor isoform, MHC A, to the center of the bipolar thick filament, where initiation of assembly may occur, as well as the genetic evidence that normal thick filaments are absent in mutants lacking MHC A, suggest that "A-specific function" is required in the early steps of filament formation. MHC A and MHC B protein sequences are 65% identical, and comparisons (Dibb et al., 1989) do not readily identify features or regions particularly different between the two that might be responsible for the unique function of MHC A.

The functional specialization of the two MHC isoforms that coassemble in C. elegans body wall muscle provides a unique opportunity to probe in vivo the molecular events involved in the initiation of thick filament assembly in striated muscle. To elucidate MHC A-specific function and thereby learn about the molecular events of thick filament initiation, we used the molecular genetics of C. elegans to map the sequences that are important for MHC A function in vivo. Our results identify regions of the rod that are important for MHC A function. Detailed comparisons of the differences between isoforms A and B in these two regions provide new insights into the characteristics of coiled coils that are important for assembly of myosin dimers into the highly ordered structure of the thick filament. In addition to electrostatic interactions analyzed in earlier work (Parry, 1981; McLachlan and Karn, 1982), our results suggest that characteristic variations in hydrophobicity on the rod surface also play a role in determining which dimer–dimer interactions occur in vivo.
Materials and Methods

Worm Cultures

General methods for culturing and handling C. elegans have been described (Brenner, 1974; Wood, 1988). N2 wild type was the parent of all strains used in this work. Strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota St. Paul, MN).

Injection

Transformed lines carrying extrachromosomal arrays were generated as described (Mello et al., 1991) using a 50:1 ratio of DOL-mycosin DNA at 200 pg/µl in 10 mM Tris, 1 mM EDTA, pH 8. Each construct was tested for the rescue of myo-3(e376) by injecting DNA into heterozygous animals [sma-1(e30) myo-3(e376)(myo-288) GA] that carry a chromosome bearing a myo-3 mutation linked to a marker gene sma-1 (small), balanced by the eDfI chromosome (which suppresses recombination in the myo-3 internal). The F1 progeny were scored both for rescued small animals and for rolling heterozygotes. If small animals were obtained, rescue was confirmed by observing their offspring: unstable transformation events yield hermaphrodites that give rise to only mutant progeny, thus confirming that rescue, rather than recombination, occurred. The F1 rolling heterozygotes were picked to establish heritably transformed lines. If myo-3 rescue was not obtained, the negative result was confirmed by establishing lines in which the union of myo-3 fragments pPH23aPI Sall-SnBaI, pJK26a SnaB-I-XbaI and pJK26a XbaI-ClaI with an unc-54 Clal-APaI fragment from chimera 1.

Chimeras 4 and 5. The 2.2-kb Sphi-APaI fragment from pPHM7 was replaced by SOE PCR. PCR 1: B0153 CGCAGAAGAAGAGGA/ 928 TAGGTAGCCATTGGGACCGTGCTTC TT on pJK26a. PCR 2: 929 CTCGACAGATGCAGCACAAATATCGCTGTTG/GGAGGAGCACGCG (the original stop was changed to TAG).

Chimera 5. The 1.6-kb Mdi-ApaI fragment of pJK28b was replaced by SOE PCR. PCR 1: A037 GCTCGAACACCGGCTGCA/ A039 CTTTTACAGCTTGGGAGAGAGAGT on pJK26b. PCR 2: A034 TCGAGGTCCTAAAGACAGTGCTGCA/679 GGGCGTGAGC (unc-54 codon K1935 to TAA was inserted into pPH24aPI to make chimera 6 (the original stop was changed to TAG)).

Chimera 7. The 1.1-kb Staf-Eal fragment from chimera 6 was replaced with a pJK28b PCR product: B0601 CTGAAGGACGCGCCGGCCGCC (adds an SphI site and destroys a HindIII site, no coding changes)/A731 GGTGGGAGCACAGGGAG. DNA sequencing revealed a change in L1929 (to CT).

Chimera 8. The 854-bp XbaI-ClaI pPHU3 fragment was ligated into XbaI-ClaI-chimera 7. The XbaI-Apaf fragment from the chimera was cloned into XbaI-Apaf-cut pPHUM7. The XbaI-Apaf fragment from the resulting clone was then inserted into pPH24aPI.

Chimeric Myosin Constructs

All portions generated by PCR and all chimeric borders were verified by DNA sequencing unless indicated otherwise. Bases italicized in oligonucleotides do not match the wild-type sequence. All portions generated by PCR and all chimeric borders were verified by DNA sequencing unless indicated otherwise. Bases italicized in oligonucleotides do not match the wild-type sequence.

Chimeras 1 and 3. The myo-3 XbaI site formed by the ST795 and R796 codons served as junction site in both chimeras. The XbaI site was introduced into the unc-54 clone pJK28b (A794 to T, ST795 to R) using two sets of oligonucleotide primers: 92 GAGAGCATGGAACCGGCTCTCTCAGGAGCTGCAAGCT (adds XbaI site) and 92 CGCAGTCGGATCCCTCTATTAGGAGAGAGAGAT (adds XbaI site). The construct failed to rescue unc-54 and backcrossed into SmaI/Apaf-cut pPHUM7 (myo-3). The 1.6-kb Mdi-ApaI fragment of pJK28b was replaced by SOE PCR. PCR 1: A037 GCTCGAACACCGGCTGCA/ A039 CTTTTACAGCTTGGGAGAGAGAGT on pJK26b. PCR 2: A034 TCGAGGTCCTAAAGACAGTGCTGCA/679 GGGCGTGAGC (unc-54 codon K1935 to TAA was inserted into pPH24aPI to make chimera 6 (the original stop was changed to TAG)).

Chimera 9. A polylinker of annealed A411 AGCTGGGCCCCTGCGCACCGAATGAACTCGTAAAGTCGGAGACA (adds an SphI site and destroys a HindIII site, no coding changes)/A731 GGTGGGAGCACAGGGAG. DNA sequencing revealed a change in L1929 (to CT).

Chimera 10. The 3.2-kb BstXI-ApaI chimera 8 fragment was replaced with unc-54 sequences from clone pPHU5bXBS (see chimera 9). This construct failed to rescue unc-54 animals. The complete unc-54 construct pPHUM7 (made by inserting pPHU5bXBS and reducing the pPH24aPI stop codon) also failed to rescue, indicating that a mutation had occurred. We mapped the mutation using a combinatorial overlapping restriction fragments, with vector sequences intact, from pPHXBS and wild-type unc-54, relying on in vivo recombination to form a gene complete during extrachromosomal array formation. The fragments were injected into e190 homozygotes at a total DNA concentration of 150 ng/µl, with the 5' fragment as 0.5%, the 3' fragment as 10%, and total DNA as 90% of total DNA. The first set of injections combined a 5'-AflI fragment with a 3' BamHI-Sall fragment. Rescue was obtained with pPHXBS Apal-AflI fragment/wild-type BanHI-Sall fragment, but not wild-type Apal-AflI fragment/pPHXBS BanHI-Sall, indicating the mutation lay 3' to the AflI site at 5983. The next set combined a 5'-Apal-Sphi fragment with a 3'-AspI-Sall fragment. Both combinations of fragments produced rescuing arrays, mapping the mutation to the region of overlap between the two fragments, unc-54 6410-6949. DNA sequencing revealed a single basepair deletion (G6751) in the R1544 codon. To generate a functional construct, we cojected re-
mal PCR (Williams et al., 1992) across the recombination border using F0219 ACTCTCTTACCGCTTG (myo-3)/F0548 CTTTCCCTTTAAGCTTCG (unc-54).

**Chimera 11.** The 2.4-kb XbaI-SphI pPHUM7 fragment was replaced by the pJK26a PCR product: 91 B0417 GAAAGCATAGTTTCCAAGGCGGCCTG (adds an Sppl site, no coding change). The SnaBl-Apal fragment from this was ligated into pPH24aP1.

**Chimera 12.** The 1.1-kb SphI-EagI chimera 11 fragment was replaced by SOE PCR. PCR 1: A298 CCAGGTGAAATTTCCF/F0562 GGCCTCCTCCAGCTTGCTAGTGGCCCG (unc-11) on pJK26a. PCR 2: B0651 GACGTCAAAGGCTGAGGACGGCCCTCAAGA/B0702 on pJK26a. PCR 3: B0417 GAACTTGAAGAGATACC/A732 GAGCTCAAGAACGCTGAGGAGCGCTCCAAG (adds an SphI site, no coding change). The subsequent cDNA was cut with SphI and HindIII to remove 0.8 kb, which was replaced by SOE PCR. PCR 1:A298 CCAGGTGAAATTTCCF/F0562 GGCCTCCTCCAGCTTGCTAGTGGCCCG (unc-11) on pJK26a. PCR 2: B0600 CTCAAGAGTACAAGGCCCGCCCTGAAGAGAAGGACAA/B0601 on pJK26a. From this, the Sppl and Apal fragment was introduced back into chimera 11.

**Chimera 13.** The Sppl-Apal subclone of the chimera 13 COOH terminus was cut with Sppl and HindIII to remove 0.7 kb, which was replaced by SOE PCR. PCR 1: A109 GAACTTGAAGAGATACC/A732 GAGCTCAAGAACGCTGAGGAGCGCTCCAAG (adds an SphI site, no coding change). The result of the sequence was designed in Cotton et al. (1988) and Montandon et al. (1989).

**Chimera 15.** The 2.3-kb SphI-Apal subclone of chimera 13 was cut with BstXI and EagI and replaced 0.7 kb, which was replaced by SOE PCR. PCR 1: B0651 GACGTCAAAGGCTGAGGACGGCCCTCAAGA/B0702 TTCCAGGGATACGCGAGAACAAGCTGGG/329 on pJK26a. The resulting subclone was cut with Sppl and Apal, and introduced back into chimera 13.

**Heteroduplex Mismatch Analysis**

Oligo pairs 324 CCCAAATGCCATCTGATITG/325 GCGAACAA- 
CTTGGGAGCTTCCAGCTCTGC/326 on chimera 13. PCR 2: B0703 CATCAAGAGCACCACAAAGACGCTG/ 
A731 on pJK26a. This was replaced in chimera 13.

**Sequencing**

Double-stranded templates were sequenced using reagents and methods in the Sequenase Kit (U.S. Biochemical Corp., Cleveland, OH) or Taq Dye Deoxyterminators Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA).

**Nucleotide and Protein Sequences**

GenBank/EMBL/DDBJ accession numbers for C. elegans protein files are as follows: MHC A, P12844; MHC B, P02566; paramyosin, P10567 (however, the NH2 terminus is incorrect, see Kagawa et al. [1989] for correct sequence). GenBank/EMBL/DDBJ accession numbers for DNA files are as follows: unc-54, J01500; myo-3, X00807. The 1,088-residue rod sequence from the following striated muscle myosins were aligned without gaps beginning at the rod-initiating proline: Drosophila, M61229; scallop, P24733; chicken fast skeletal, PIR JX0178; human perinatal, M36769; rat skeletal, A24922.

**Results**

**MHC A-specific Function Maps to the Rod**

We have used chimeric constructs in an in vivo assay to identify regions of the MHC that are important for A-specific function. The constructs have used genomic DNA fragments, thus testing native regulatory sequences as well as protein-coding portions of the gene. The in vivo assays involved introducing the recombinant genes into C. elegans as extrachromosomal arrays along with a marker construct to allow the identification of transformed animals independently of myosin construct function. Activity was determined in an unc-54 loss-of-function background to assure that a functional MHC was produced, and in a myo-3 null background to assess the MHC A-specific activity (see Materials and Methods for details).

The first set of chimeric constructs, illustrated in Fig. 2, involved substituting large portions of A sequence in the B gene or vice versa, and allowed us to map A-specific function to the α-helical coiled-coil rod. Chimera 1, which contains sequences coding for most of the A head with the remainder of the protein specified by B sequences, and chimera 2, which contains the entire A head and most of the prehinge region of the rod (see Fig. 1a), failed to rescue myo-3 mutations, but did rescue unc-54. The gene expression in these constructs must be driven by myo-3 regulatory sequences since all known unc-54 regulatory sequences have been replaced in these constructs. A third construct, chimera 3, containing the B head and the A rod and nonhelical tailpiece, rescued unc-54 and myo-3 mutations. Taken together, these results show that the sequences encoding the NH2-terminal half of MHC A, including regulatory sequences as well as coding sequences for the globular head and the light chain-binding sites, are neither necessary nor sufficient for A-specific filament initiation function.

We then tested the role of the nonhelical tailpiece region, which we define here as M1934 (beginning of Mclachlan and Karn [1982] zone 40) to the end of the coding region so as to include all three potential phosphorylation sites (Schriefer and Waterston, 1989), and therefore including a small part of what is formally the COOH terminus of the rod. Chimera 4, specifying B head, A rod, and B tailpiece rescues myo-3; by contrast, chimera 5 specifies B head, B rod, and only the tailpiece from A, and fails to rescue myo-3 (it does rescue unc-54). Chimera 6, a construct that specifies the B head and A rod but has a stop codon introduced before the sequences specifying the tailpiece, rescues myo-3 lethality. Transformants carrying this construct, however, show disorganized muscle structure, a property we are currently characterizing further. Taken together, these results show that the rod alone is both necessary and sufficient for A-specific activity, and that the tailpiece is not essential for thick filament initiation, but is required for normal filament structure.

To localize further the sequences important for A-specific function, we made chimeras in which only a portion of

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Figure 2. MHC A-specific function maps to the rod. The MHC A sequences required for filament initiation function were mapped by constructing chimeric myosins of MHC A and MHC B, and testing these in vivo via germline transformation for their ability to substitute for the native MHC A and MHC B. For each chimera (1–6, left column) the precise MHC A amino acids included in each construct are indicated (second column) along with a schematic myosin molecule showing the portions of MHC A (black) and MHC B (gray). The last two columns summarize the results of in vivo rescue experiments testing the construct for filament initiation function (defined as ability to rescue *myo-3* mutations) and general myosin function (defined as ability to rescue *unc-54* mutations). The top two lines illustrate the results obtained by Fire and Waterston (1989) demonstrating that MHC A alone is able to rescue the lethality of *myo-3* mutations, while transgenic copies of either isoform can compensate for loss of *unc-54* gene activity. The six chimeric constructs demonstrate that the MHC A rod alone is necessary and sufficient for A-specific function. *R1934 is the last MHC A residue, but an additional N residue from MHC B is present at the COOH-terminus; ND, not done.

the MHC B rod was replaced by MHC A sequences. Our results, illustrated in Fig. 3, revealed that two distinct regions of the rod are independently sufficient to rescue *myo-3* mutations. The five constructs in the top portion of Fig. 3 define a 264-residue region in the central portion of the rod (region 1) that is sufficient to confer A-specific function. The five constructs drawn in the bottom portion of the figure define a 170-residue region at the COOH terminus of the rod (region 2) that is sufficient. Attempts to further refine the region failed: the two constructs chimera 14 and chimera 15, each of which contain one half of the 170-residue COOH-terminal region, did not contain full rescuing activity. Few of the lines obtained upon injection into *unc-54* and then crossed into a *myo-3* background to test for rescue. The partial activity of each construct, which was not seen in other constructs, suggests we have divided the active region. It is unclear without further experiments how widely the activity is distributed along the rod. Because these results indicate that the activity required for A-specific function must be a characteristic of a rod section rather than a single difference between isoforms A and B, we undertook a comparative sequence analysis of the two rod regions we had defined in the above chimeras to look for consistent differences in rod amino acid composition that correlate with the observed difference in function.

**Comparison of Residues in the A and B Rods**

In our analysis of the two sequences, we took advantage of the known structure of the myosin rod, a coiled-coil domain formed by the \( \alpha \) helices of the two heavy chain monomers. Protein sequences that adopt a coiled-coil structure display a characteristic seven-residue repeat pattern, designated *a–g* (Fig. 4), where residues *a* and *d* form the core of the coiled coil, an internal hydrophobic seam. Other residues are next-to-core (*e* and *g*), or coat (*b*, *c*, and *f*).
Comparison of the two isoforms is most effectively done by considering residues at similar positions within the repeat.

Comparison of Charged Residues in MHC A and MHC B

Charge interactions are postulated to play a fundamental role in myosin assembly, both providing the driving force and determining the stagger at which two rods associate. Analysis of the distribution of basic and acidic residues reveals a 28-residue repeat unit or “zone” in which the strongest peak of positive charge is spaced 14 residues away from the strongest peak of negative charge. During the assembly of dimers into the thick filament, neighboring rods will associate at stagger distances that pair the peaks of positive and negative charge (Parry, 1981; McLachlan and Karn, 1982).

Because of the known importance of electrostatic interactions in the assembly of myosin dimers into the thick filament structure, we compared the content of charged residues in MHC A and B. The two isoforms are quite similar in total number of charges found in the rod (acidic: A = 249, B = 251; basic: A = 196, B = 200). To determine whether differential charge distribution could account for the differences in isoform function, we compared the location of charged residues in the rods of isoforms A and B. At each position within the rod (defined here as in Kagawa et al. [1989] as P851 to R1938, corresponding to zones 1 through part of zone 40 of McLachlan and Karn [1982]), we compared the charged residues in the two isoforms, and determined whether the charge at any one position was of the same or opposite sign. Of 414 positions in the 1,088-residue rod where both MHCs A and B contained a charged residue, charges of the same sign were found in 412 positions, while charges of opposite sign were found in only 2. “Unmatched” charges, those found at a particular position in only one isoform, totaled 28 in MHC A and 34 in MHC B. Many of these positions (Fig. 5), as well as the two positions that contained opposite charges, are found in the prehinge region, the portion of the rod that is most variable (Dibb et al., 1989). The remaining charge differences are distributed fairly evenly along the rest of the rod, without an apparent concentration in the regions containing MHC A–specific activity (Fig. 5).

To determine how the differences in charge distribution in MHC A and B compare to other myosins, we examined the charge content in other myosins at the rod positions in which either A or B contained an unmatched charge. Most myosins are thought to be capable of both parallel and antiparallel assembly, and that is clearly the case for Drosophila melanogaster, where there is only one gene (alternative splicing does not affect the two regions of the rod defined in our study [George et al., 1989]). Our analysis re-
vealed that within the whole rod, and in both regions sufficient for A-specific function, the MHC A charge distribution is more divergent (Fig. 5). One model for A-specific function might have been that MHC B has specialized to perform only parallel assembly, and has thus lost a part of the charge pattern that is required only for antiparallel assembly. Because charge placement in MHC B is more similar to the consensus pattern, this model is an unlikely explanation for A-specific function.

The one aspect of charge distribution in MHC A that could be correlated with A-specific function was a reduction in surface charge. In both regions sufficient for A-specific function, the majority of unmatched charges in MHC B (5/6 and 5/7) are conserved residues found in coat positions, whereas few of the unmatched changes in the same regions of MHC A (1/4 and 1/4) fall into coat positions, resulting in a net loss of charge from the MHC A coat. Reduction of surface charge may also occur by a second mechanism in region 2, where unmatched charges in next-to-core positions result in a net gain in the number of favorable 1g-2e charge interactions within the MHC A coiled coil. Within region 2, MHC A loses one unfavorable interaction because of loss of a consensus charge, and gains two favorable salt bridges because of charges in atypical positions (Fig. 5). Because the additional intradimer salt bridges in MHC A are formed by atypical charges pairing with and therefore attenuating a conserved residue of opposite charge, we believe these changes may result in a reduction of the effective charge seen at the rod surface.

**Differences in Hydrophobicity in Coat Positions**

The overall content of hydrophobic residues (defined as those having a positive value on a hydrophobicity scale) is quite similar in the rods of MHC A and MHC B (Kyte and Doolittle, 1982: MHC A = 349, MHC B = 340; Engelman et al., 1986: MHC A = 470, MHC B = 466). Throughout the rod, however, the overall distribution of hydrophobic residues is not as well conserved between the two isoforms as the distribution of charged residues. Comparisons using the Kyte and Doolittle (1992) scale as a reference find 267 positions at which both isoforms have a hydrophobic residue, 82 positions that contain a hydrophobic residue in MHC A only, and 73 that contain a hydrophobic residue in MHC B only. Comparisons using the Engelman et al. (1986) scale find 394 positions in which both isoforms contain a hydrophobic residue, 76 with a hydrophobic residue only in MHC A, and 72 with a hydrophobic residue only in MHC B.

To investigate whether these differences were important for MHC A–specific function, we compared the hydrophobic content of the two isoforms in the three types of positions within the heptad repeat (core, next-to-core, and coat, see Fig. 4). The most striking difference in sequence composition was a statistically significant increase in the hydrophobicity of the MHC A coat residues only in those regions containing A-specific function (Fig. 6 b). Within both of these regions, the MHC A values are similar to those of paramyosin, for which a more hydrophobic coat has been correlated with its more internal position within the thick filament (Cohen et al., 1987).

To determine how the coat hydrophobicity values of MHC A and B compare to those of other myosins, we performed the same calculations on selected striated muscle myosins from vertebrates and other invertebrates (Fig. 6 c). In both regions sufficient for function, but not in other rod regions, the MHC A value is the highest and the MHC B value is the lowest. This strengthens our conclusion that isoforms A and B differ significantly in coat hydrophobicity in these regions, and strongly suggests that this observed difference is important for A-specific function.

Our results suggest that differences in coat hydrophobicity in certain rod segments, defined by the borders chosen for our chimeric constructs, are important in at least one aspect of myosin assembly. To examine the variation in coat hydrophobicity in a less restricted fashion, we calculated hydrophobicity in a smaller window of sequence moved along the length of the rod (Fig. 7). This analysis revealed a characteristic hydrophobicity profile shared by MHCs A and B (Fig. 7 a). The regions where the two isoforms show the greatest difference in coat hydrophobicity are contained within the sequences we identified as important for MHC A–specific function. We then performed this same analysis on other myosins in the public databases, representatives of which are shown in Fig. 7 c, and found that the characteristic coat hydrophobicity profile is common to striated muscle myosins in vertebrates and invertebrates alike, suggesting that variations in hydrophobicity of the rod surface may play an important role in assembly of all myosins. Interestingly, when the hydrophobicity profiles for MHC A and paramyosin are compared, they are very similar overall, and particularly within the regions sufficient for MHC A–specific function (Fig. 7 b).

Because of the similarity of MHC A and paramyosin coat hydrophobicity scores, we examined other aspects of sequence composition that are known to differ between most myosins and paramyosin (Cohen et al., 1987; Kagawa et al., 1989). Paramyosin is thought to form a more rigid coiled-coil rod partly because of a reduced number of glycine residues. In addition, paramyosin sequences have a higher R/K ratio. When we compared isoforms A, B, and paramyosin in the region that all are homologous, we found that MHC A, like paramyosin, had fewer glycines (PM = 9, A = 15, B = 29) and a higher R/K ratio (PM = 1.1, A = 0.92, B = 0.83) than MHC B, indicating that the A rod is likely to be more rigid. Thus, in addition to its higher coat hydrophobicity, MHC A sequences have diverged along the length of the rod in ways that make it more similar to paramyosin.

**Discussion**

In our experiments, we have exploited a difference in the genetic properties of two MHC isoforms to elucidate aspects of the molecular basis for thick filament initiation. Using chimeric myosin constructs, we have mapped the sequences within MHC A that confer A-specific function in vivo. Neither the head nor the nonhelical tailpiece provide this activity; in fact, constructs that contain the MHC A rod and lack a tailpiece altogether rescue loss-of-function mutations in MHC A. Instead, the rescuing activity maps to two distinct regions of the coiled-coil rod, either of which is sufficient in chimeric myosins for the initiation function. Comparison of the MHC A and MHC B sequences
within these two regions revealed a marked difference in the hydrophobicity of the coat residues, those exposed on the rod surface where they can mediate protein–protein interactions during assembly. We propose that sequences within these regions play an important role in the intermolecular interactions that are critical for the initiation of thick filament assembly. Furthermore, analysis of the coat positions along the length of the entire rod revealed a characteristic hydrophobicity profile that is conserved in all striated muscle myosins. We propose that this variation in coat hydrophobicity plays an important role in thick filament assembly.

Differences in Coat Hydrophobicity Correlate with Differences in Assembly Behavior

Within the two regions sufficient for A-specific function, the coat hydrophobicity of MHC A is higher than that of MHC B and closely approximates that of paramyosin, a molecule that can be viewed as a fragment of myosin that has specialized to perform a strictly structural role in the formation of the large thick filaments that are present in invertebrate muscle. In addition, other aspects of the MHC A rod sequence, such as glycine content, are more similar to paramyosin. These sequence similarities, particularly within the critical regions, suggest that MHC A has specialized structural roles that may be shared with paramyosin. MHC B may be specialized for other structural roles, such as elongation in parallel assembly.

Our observation that the MHC A rod sequence shares characteristics in common with the paramyosin sequence correlates well with the similar in vitro behavior of MHC A and paramyosin. Biochemical experiments in which native thick filaments were dissociated by treatment with increas-
ing concentration of KCl, which disrupts electrostatic interactions, revealed that the salt concentrations that completely removed MHC B resulted in core structures composed of paramyosin and minor associated proteins (Deitiker and Epstein, 1993), with MHC A located in the central region (Epstein et al., 1985). Because hydrophobic interactions are resistant to high salt, this observation is consistent with an important role of increased MHC A coat hydrophobicity in the assembly of this structure. When similar experiments were performed with thick filaments isolated from a mutant that lacks MHC B and thus has MHC A along the entire length of the filament, the central MHC A zone remaining on the core structure was expanded, suggesting that this increased interaction is not restricted to the smaller portion of the filament core where MHC A associates in wild type (Epstein et al., 1986). These experimental observations support our hypothesis that the higher coat hydrophobicity in regions 1 and 2 has functional consequences for MHC A assembly, and suggest that these differences result in more stable association of MHC A with itself and/or with paramyosin.

In addition, genetic experiments indicate that MHC A and paramyosin share a close interaction in vivo, perhaps mediated by their matching hydrophobic coat. Genomic duplications that increase MHC A expression suppress the defects in structure and motility associated with e73, a missense allele of paramyosin (Riddle and Brenner, 1978; Waterston et al., 1982; Brown and Riddle, 1985). Homozygous e73 animals accumulate wild-type levels of paramyosin protein (Waterston et al., 1977), but move poorly and show disrupted muscle structure in which aberrant accumulations of paramyosin appear at the ends of muscle cells (Waterston et al., 1977; Epstein et al., 1987; Gengyo-Ando and Kagawa, 1991). Transgenic copies of MHC A, but not MHC B, can also suppress e73, and this A-specific suppression maps to the rod (Hoppe, P.E., and R.H. Waterston, unpublished data). Thus, sequences within the rod allow MHC A to interact strongly enough with paramyosin, such that increased concentrations can drive assembly of the mutant protein into a more wild-type structure.

Given the hypothesis that MHC A and paramyosin share assembly behaviors because of their very similar coat hydrophobicity profiles (Fig. 7 b) and the sequence alignment of paramyosin with the light meromyosin portion of myosin, it is tempting to speculate that myosin and

Figure 7. Analysis of coat hydrophobicity along the length of the coiled-coil rod. The hydrophobicity score was calculated by summing the Kyte and Doolittle (1982) scores for all residues in coat positions within each zone, and then determining the average sum within a three-zone interval. ("Zone" refers to the 28-residue repeat defined by McLachlan and Karn [1982]). The three-zone window of sequence was shifted along the length of the rod from zone 2 through 39 in one-zone increments. Similar graphs were obtained using the Engelman et al. (1986) scale. The two horizontal black bars indicate regions of the graph that contain at least one zone of sequence from one of the regions found sufficient for MHC A-specific function. (a) The graphs of the hydrophobicity scores of MHC A and MHC B reveal that coat hydrophobicity varies along the length of the rod, and that the overall pattern of this fluctuation is quite similar in both isoforms. The two areas of the graph where the scores of MHC A and B are most different (arrows) are contained within those regions we have identified as important for thick filament initiation in vivo. (b) Comparison of the hydrophobicity scores of MHC A and paramyosin reveals that the graph of the paramyosin sequence, when aligned by homology, is quite similar to the graph of MHC A, particularly within the regions we have defined as important for A-specific function. (c) Striated muscle myosins from vertebrates and invertebrates show a common overall hydrophobicity profile, particularly in the region from repeat 11 to the end of the rod. The two prominent peaks (arrows) are separated by ~10-11 zones (~280-308 residues or 416-460 A), which is close to the 430-Å axial spacing that is observed by x-ray diffraction of muscle and associated with the packing of the myosin molecule into the thick filament (Huxley and Brown, 1967).
paramyosin assemble with equivalent staggers. In this light, it is interesting to note that all missense mutations that disrupt paramyosin assembly (Gengyo-Ando and Kagawa, 1991) fall within the regions of paramyosin that are homologous to those we have defined as important for myosin assembly.

What is the Structural Basis for MHC A-specific Function? The in vitro and in vivo observations discussed above suggest that MHC A forms more stable associations with itself and/or with paramyosin than does MHC B. Previous models of myosin–myosin and myosin–paramyosin interaction in assembly have focused on the matching of charged residues. Recent work examining the solubility of mutant fragments in which the charge pattern had been disrupted indicates, however, that a simple summing of charge–charge interactions along the length of the rod is not sufficient to explain the association of myosin in vitro (Atkinson and Stewart, 1992). Below, we present two models in which the relatively greater coat hydrophobicity within MHC A regions 1 and 2 confers A-specific activity by contributing to more stable association of dimers within the thick filament.

In the first model, the increased coat hydrophobicity is critical to filament initiation because it contributes to a stronger interaction between MHC A dimers in antiparallel association; because of reduced coat hydrophobicity, antiparallel MHC B dimers are not sufficiently stable to promote filament assembly. In this light, it is interesting to note that MHC B is by far the least hydrophobic of any myosin in region 2, and is the only myosin known not to be capable of filament initiation. Because the rescue of A-specific function is dominant (that is, a chimeric myosin requires MHC A sequences in either region 1 or region 2, but not both), this model requires that the high hydrophobic content of a single region is sufficient to provide the necessary (although perhaps not optimal) stability. The partial function observed when we divided region 2 sequences (Fig. 3) would then reflect the formation of an antiparallel dimer that was of intermediate stability, close to the threshold level required for filament initiation.

In the second model, A-specific function reflects a tight association of MHC A with paramyosin via a rod surface that supports this interaction. In this case, because the paramyosin hydrophobicity profile is like that of MHC A, the dominance of A-specific rescue can be explained if one requires a good match at only one of the two hydrophobic regions during myosin–paramyosin assembly (e.g., MHC A region 1 with its paramyosin target).

What regions of MHC A or paramyosin contact the residues within regions 1 and 2 in antiparallel alignment? Models based solely upon maximization of charge interactions between antiparallel dimers favor an overlap with zero stagger (s = 0) such that the two rods associate along their entire lengths (Kagawa et al., 1989; Fig. 8 a in this paper). This stagger results in the association of region 1 of one rod with region 1 of the other, as well as the pairing of the two NH2-terminal peaks of the hydrophobicity profile (see below). However, it also results in the association of region 2 with sequences in the prehinge region of its partner molecule, the portion of the rod that is most variable in sequence (Dibb et al., 1989) and thought to be only loosely associated with the filament backbone. One intriguing alternative is that region 1 of one rod contacts region 2 of its partner molecule during filament initiation. For example, an antiparallel stagger of s = 452, which is also favored by charge interaction modeling (Kagawa et al., 1989), results in a direct association between the portions of region 1 and region 2 that are most different in hydrophobicity in MHCs A and B (Fig. 8 a). This stagger would produce a tail–tail overlap of 605 residues which, assuming a helical rise of 1.48 Å per residue (Elliot et al., 1968), gives an expected length of 898 Å, or essentially the 900-Å region of overlap measured in type II segments of vertebrate striated muscle myosin in antiparallel association in vitro (Harrison et al., 1971).

The s = 452 stagger does not involve the NH2-terminal prehinge portion of the rod, consistent with biochemical studies that demonstrate that the COOH-terminal two thirds of the rod is sufficient to form ordered aggregates, while the prehinge portion is quite soluble and unnecessary (Lowey et al., 1967). Since the prehinge region is in effect the segment lacking in paramyosin, this stagger can be used to explain both myosin and paramyosin antiparallel association (Fig. 8 a). While staggers that result in incomplete overlap of the rods might seem at odds with the observed length of the bare zone, which is ~1,600 Å and approximately equal to the length of the myosin rod, subsequent dimer–dimer association in a staggered fashion could produce a filament bare zone of smaller size in a manner similar to that proposed for minifilament assembly in Acanthamoeba nonmuscle myosin (Sinard et al., 1989).

Although A-specific function presumably involves antiparallel association, we were interested to determine where regions 1 and 2 fall in the parallel dimer. The s = 296 parallel stagger is favored by charge modeling studies, and agrees well with the 430-Å axial spacing detected by x-ray diffraction and EM (McLachlan and Karn, 1982; Kagawa et al., 1989). Pairing of myosin rods with the s = 296 stagger results in contact of regions 1 and 2, but the area of overlap does not include the portion of region 1 that differs in hydrophobicity between MHCs A and B (Fig. 8 c). Parallel association of paramyosin is postulated to involve the s = 493 (720 Å) stagger (Kagawa et al., 1989; Gengyo-Ando and Kagawa, 1991). Interestingly, like the antiparallel s = 452 stagger, assembly of myosin dimers with the parallel s = 493 stagger results in the association of the two sites that show the greatest difference in coat hydrophobicity in MHCs A and B (Fig. 8 b).

The Conserved Hydrophobicity Profile May Be Important for Determining Stagger of Assembly

Given the proposed overall importance of hydrophobicity in stabilizing dimer–dimer associations, we wanted to determine how the characteristic fluctuations in coat hydrophobicity fit with various proposed molecular staggers. As shown in Fig. 7 c, the coat hydrophobicity profile, conserved in all striated muscle myosins, contains two prominent peaks. The regions we have defined map near, but do not coincide precisely with these peaks. The more NH2-terminal peak falls in the portion of region 1 that is most different in hydrophobicity between MHCs A and B. The more COOH-terminal peak falls just outside of region 1.
Intriguingly, the $s = 296$ parallel stagger (see above) results in the direct pairing of the more NH$_2$-terminal hydrophobicity peak of one rod with the more COOH-terminal peak of its partner (Fig. 8 c). Models of thick filament structure can incorporate parallel staggerers of both $s = 296$ (430 Å) and $s = 493$ (720 Å) by having, for instance, parallel dimers assemble in one long, single-width subfilament with a 430-Å stagger, and then having side-to-side association of these subfilaments with the 720-Å stagger (Chapter 9 in Squire, 1981). The antiparallel stagger $s = 283$ results in a similar pairing of the hydrophobicity peaks of interacting rods (Fig. 8 c), and produces no overlap of the portions of Region 1 and 2 that differ in hydrophobicity in MHCs A and B. Future studies, including the characterization of hydrophobicity profiles of myosins from distantly related nematodes, as well as in vitro mutagenesis experiments in *C. elegans*, may elucidate the role of variations in coat hydrophobicity in both antiparallel and parallel assembly of myosin and paramyosin into the thick filament.

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