

Distribution of Fast Myosin Heavy Chain Isoforms in Thick Filaments of Developing Chicken Pectoral Muscle

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Abstract. Colloidal gold-conjugated monoclonal antibodies were prepared to stage-specific fast myosin heavy chain (MHC) isoforms of developing chicken pectoralis major (PM). Native thick filaments from different stages of development were reacted with these antibodies and examined in the electron microscope to determine their myosin isoform composition. Filaments prepared from 12-d embryo, 10-d chick, and 1-yr chicken muscle specifically reacted with the embryonic (EB165), neonatal (2E9), and adult (AB8) anti-myosin gold-conjugated monoclonal antibodies, respectively. The myosin isoform composition was more complex in thick filaments from stages of pectoral muscle where more than one isoform was simultaneously expressed. In 19-d embryo muscle where both embryonic and neonatal isoforms were present, three classes of filaments were found. One class of filaments reacted only with the embryonic antibody, a

second class reacted only with the neonatal-specific antibody, and a third class of filaments were decorated by both antibodies. Similar results were obtained with filaments prepared from 44-d chicken PM where the neonatal and adult fast MHCs were expressed. These observations demonstrate that two myosin isoforms can exist in an individual thick filament in vivo. Immunoelectron microscopy was also used to determine the specific distribution of different fast MHC isoforms within individual filaments from different stages of development. The anti-embryonic and anti-adult antibodies uniformly decorated both homogeneous and heterogeneous thick filaments. The neonatal specific antibody uniformly decorated homogeneous filaments; however, it preferentially decorated the center of heterogeneous filaments. These observations suggest that neonatal MHC may play a specific role in fibrillogenesis.

THE basic structural unit of the skeletal muscle myofibril is the sarcomere, an interdigitating array of actin-containing thin filaments and myosin-containing thick filaments (22). The initial formation of myofibrils has been examined using biochemical, ultrastructural and immunochemical methods. Coinciding with terminal differentiation of the myoblast and fusion to form myotubes, the contractile proteins of the myofibril accumulate (11). Immunohistochemistry and electron microscopy of developing myotubes revealed the simultaneous appearance of thin and thick filaments after their organization into sarcomeres (17). Stress fibers in developing muscle cells have been proposed to act as templates or nucleation sites along which nascent myofibrils are formed (12, 34). However, recent studies suggest that nascent myofibrils may also form by a mechanism independent of stress fibers (31a, 43, 43a).

Sarcomeric thick filaments are composed primarily of myosin, a ubiquitous contractile protein, which is represented by a large multigene family (33, 36, 58). With the exception of *Drosophila* (4, 39), the diversity of sarcomeric myosin expression observed in eukaryotes is the result of differential gene expression (7, 27, 32, 37).

Myosin will polymerize in vitro to form filaments similar

in structure to those isolated from muscle (20, 21). However, synthetic thick filaments lack many proteins associated with native filaments. Recent studies suggest that C, H, and X proteins are localized at specific sites along the surface of the thick filament (3). Therefore, the assembly of native thick filaments involves not only myosin polymerization but interactions with other proteins that may themselves be organized in specific structural arrays.

Fluorescence energy transfer studies indicate that myosin monomers can rapidly exchange with synthetic myosin filaments (40). Smooth muscle myosin has also been found to be in equilibrium with smooth muscle thick filaments in vitro (30, 53). If a similar process occurs in native thick filaments in vivo, changes in myosin heavy chain (MHC)¹ expression would result in the insertion of new isoforms into preexisting filaments. Support for this proposal comes from studies of fluorescein-conjugated myosin microinjected into muscle cells containing organized myofibrils. In these studies, the myosin rapidly associated with A bands irrespective of the type of myosin injected (23a).

1. *Abbreviations used in this paper:* MHC, myosin heavy chain; PM, pectoralis major.

To provide more information about the assembly of thick filaments *in vivo*, we used isoform-specific gold-conjugated monoclonal antibodies and immunoelectron microscopy to analyze the isoform composition of native thick filaments isolated from developing chicken pectoralis major (PM). Our data demonstrate that during periods of development where two fast MHC isoforms were expressed, homogeneous filaments composed of either of the two isoforms and heterogeneous filaments composed of both isoforms could be isolated. Furthermore, we observed that in heterogeneous thick filaments containing embryonic and neonatal myosins, and in heterogeneous thick filaments containing neonatal and adult myosins, the neonatal-specific antibody decorated the center of many thick filaments. Thus, our results provide evidence that a specific fast myosin isoform may play a unique role in thick filament assembly.

Materials and Methods

Preparation of Native Myosin Thick Filaments

Native thick filaments were prepared from the PM of White Leghorn chickens at different ages as previously described (49). The PM was incubated for 15–30 min in relaxation buffer (100 mM KCl, 10 mM MgCl₂, 5 mM ATP, 6 mM KH₂PO₄, 1 mM EGTA, 0.1 mM PMSF, pH 7.0). The muscle was teased into thin strips, tied on wooden applicators, and incubated overnight in 50% glycerol in relaxation buffer at 4°C. After a change of buffer, the muscle was stored overnight at –20°C in 50% glycerol in relaxation buffer. The glycerinated muscle was rinsed twice with relaxation buffer and homogenized three times with a glass Dounce homogenizer in 3 vol of relaxation buffer. The homogenate was filtered through four layers of cheese cloth and the filtrate was centrifuged at 12,000 *g* for 20 min at 4°C. The supernatant containing thick filaments was used in subsequent experiments.

Myosin Preparations

Myosin was extracted from the PM of 16-d embryo, 8-d chicken, and 1-yr-old adult chickens as described earlier (48). The concentration of proteins was determined by the Bradford protein assay (5).

Preparation of Synthetic Myosin Thick Filaments

In vitro-reassembled thick filaments were prepared from myosin extracts (35, 40). Filaments were assembled by dialyzing purified myosin (0.5–1.0 mg/ml) overnight at 4°C in a low salt buffer consisting of 0.1 M KCl, 5 mM ATP, 0.2 mM DTT, 0.1 mM PMSF, 10 mM KH₂PO₄ pH 6.9. The filament solution was centrifuged at 2,000 *g* for 3 min to separate the assembled filaments from soluble myosin. Pelleted filaments were resuspended in an equal volume of low salt buffer and stored at 4°C.

Preparation of Colloidal Gold-conjugated Monoclonal Antibodies

Three monoclonal antibodies EB165, 2E9, and AB8 that recognize the embryonic, neonatal, and adult fast myosin heavy chain isoforms (9) were conjugated to colloidal gold. Ammonium sulfate (40%) precipitated antibodies were further purified by DEAE Affi-gel blue chromatography (6). The purified monoclonal antibodies were conjugated to 5-nm (EB165 and AB8) or 15-nm (2E9) colloidal gold particles (Janssen Life Sciences Products, Piscataway, NJ) as previously described (38).

Immunogold Electron Microscopy

Myosin thick filaments were decorated with gold-conjugated antibodies as described below. 200-mesh copper grids were coated with 0.25% Formvar in dichloroethane. The Formvar-coated grids were coated with carbon by vacuum evaporation. Filaments were allowed to adsorb to the grid surface for 1–2 min. The grids were incubated with 2% BSA in relaxation buffer for 1 h at 4°C. Filaments were reacted overnight at 4°C with a 1:10–1:100

dilution of gold-conjugated monoclonal antibody in 2% BSA in relaxation buffer. For double labeling, the two gold-conjugated antibodies were combined in solution before incubating with the filaments bound to the grid. Unreacted antibody was removed by floating the grids sequentially on six drops of relaxation buffer. Filaments were fixed with 1% glutaraldehyde, washed on six drops of relaxation buffer, washed on six drops of distilled water, and finally negative stained with 1% uranyl acetate. Grids were observed with an electron microscope (H-600; Hitachi Ltd., Tokyo) at an accelerating voltage of 75 kV.

Quantitation of Immunogold Labeling

To quantitate specific gold decoration of isolated thick filaments, electron micrographs were taken of randomly selected regions of the grid. Samples for each experiment consisted of at least 600 thick filaments photographed from 5–10 grids. Only those particles distributed along the surface of the filament were counted, and a filament was considered labeled when at least five gold beads were observed on a filament. Filaments were considered double labeled when at least five 5-nm gold beads and five 15-nm gold beads decorated the same filament.

Results

Specificity of Gold-conjugated Monoclonal Antibodies

Monoclonal antibodies that recognize the three stage-specific fast MHC isoforms identified in chicken PM were conjugated to either 5- or 15-nm colloidal gold as described in Materials and Methods. Western blots demonstrated that conjugation to colloidal gold had no effect on antibody specificity (data not shown). Table I summarizes the specificities of the gold-conjugated monoclonal antibodies.

Immunogold EM of Myosin Thick Filaments

Native thick filaments were prepared from PM of 12-d chicken embryos, 19-d chicken embryos, 10-d chickens, 44-d chickens, and 1-yr chickens for use in immunogold labeling experiments. At all stages of development, thick filaments had similar structure consisting of finely tapered ends, smooth central bare zone, rough surface projections, and an average length of 16 μ m (data not shown).

To optimize the decoration of thick filaments with gold-conjugated monoclonal antibodies, a series of dilution experiments were performed for each antibody. An example of these results is shown in Fig. 1 where 15-nm gold-conjugated 2E9 antibody was incubated with thick filaments isolated from 10-d chicken PM. Filaments were decorated along their length with gold particles (Fig. 1). Dense staining protein aggregates and background staining were also observed. Since unpolymerized myosin is present in our samples and would be adsorbed to the grid, some antibody binding to the grid surface would be expected. At high antibody concentrations dense decoration of the filaments was observed (Fig. 1, *C* and *D*). However, this often results in large, darkly stained aggregates, which makes visualization of the filament and identification of different size gold beads in the same fila-

Table I. Specificity of Gold-conjugated Monoclonal Antibodies to Chicken Pectoral Muscle Myosins

Stage of development	EB165	2E9	AB8
12-d embryo	+	–	–
10-d chick	+ / –	+	–
1-yr chicken	+	–	+

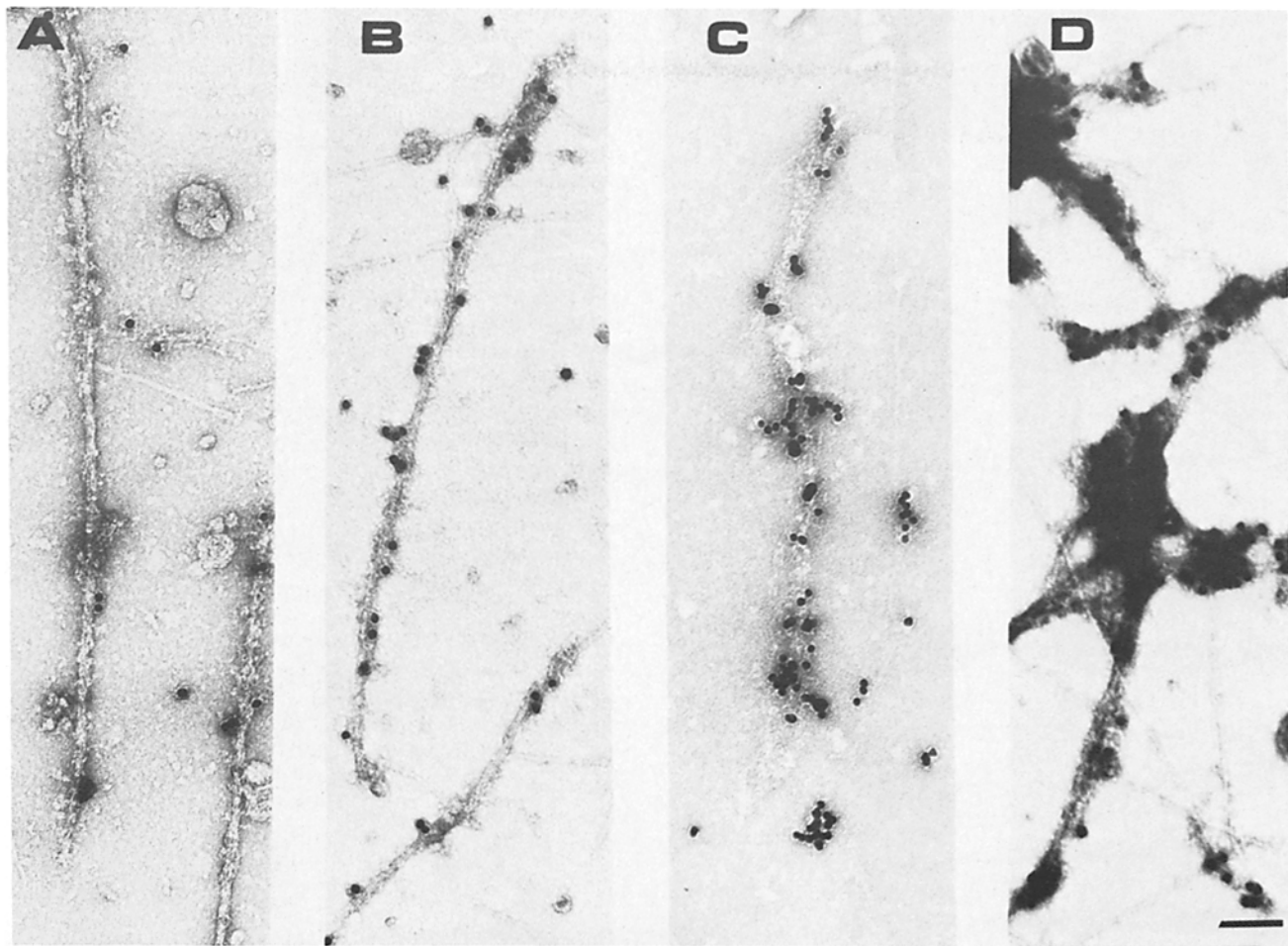


Figure 1. Thick filaments isolated from 10-d chicken PM decorated with anti-neonatal MHC antibody. Thick filament preparations from 10-d chick PM were incubated with 15-nm gold-conjugated 2E9 antibody at (A) 1:100, (B) 1:50, (C) 1:10, and (D) 1:5 dilutions. At a dilution of 1:100, the majority of gold beads decorated the filaments while background labeling was minimal. Increased antibody concentration results in more filament decoration (B and C) with an increase in background as well. At a 1:5 dilution of antibody, filament decoration was very dense resulting in the formation of large aggregates which obscured the filament. Bar, 100 nm.

ment difficult. Thus, we used antibody concentrations (1:50–1:100) that decorated >90% of the filaments when they contained the appropriate myosin (see Fig. 2 and Table II). This allowed us to easily identify filaments that reacted with two different antibodies.

Thick filaments isolated from the PM of 12-d embryonic chicken, 10-d chicken, and 1-yr chicken were reacted with EB165, 2E9, and AB8 gold-conjugated monoclonal antibodies. Filaments prepared from the PM of 12-d chicken embryos were decorated with 5-nm gold-conjugated EB165 but not with gold-conjugated 2E9 or AB8 antibodies (Fig. 2 A). This is the expected result if only embryonic myosin is expressed at this stage of development. As shown in Fig. 2 B, 15-nm gold-conjugated 2E9 antibody reacts with the majority of filaments from the PM of 10-d chickens, while 5-nm gold-conjugated AB8 antibody does not. A few filaments react with 5-nm gold-conjugated EB165 at this stage of development. This is consistent with previous studies showing the persistence of a small amount of embryonic fast MHC in the PM of neonatal chickens (9). Native filaments prepared from the PM of 1-yr chickens were decorated with both gold-conjugated EB165 and gold-conjugated AB8 antibodies but not

with gold-conjugated 2E9 antibody (Fig. 2 C). This is consistent with previous studies that showed only the adult isoform of MHC is present at this stage of maturation (9). Table II summarizes the percentage of filaments decorated with each gold-conjugated monoclonal antibody at these stages of

Table II. Single Immunogold-labeled Thick Filaments

Stage	Antibody	Total filaments counted	Percent labeled
12-d embryo	EB165	495	98
	2E9	500	0
	AB8	450	0
10-d chick	EB165	600	3
	2E9	605	90
	AB8	590	0
1-yr chicken	EB165	600	93
	2E9	550	0
	AB8	595	93

The percentage of thick filaments decorated with each of the gold-conjugated antibodies at different stages of PM muscle development is summarized from observations on filaments like those illustrated in Fig. 4. A filament was considered labeled when at least five gold beads were present along the length of the filament.

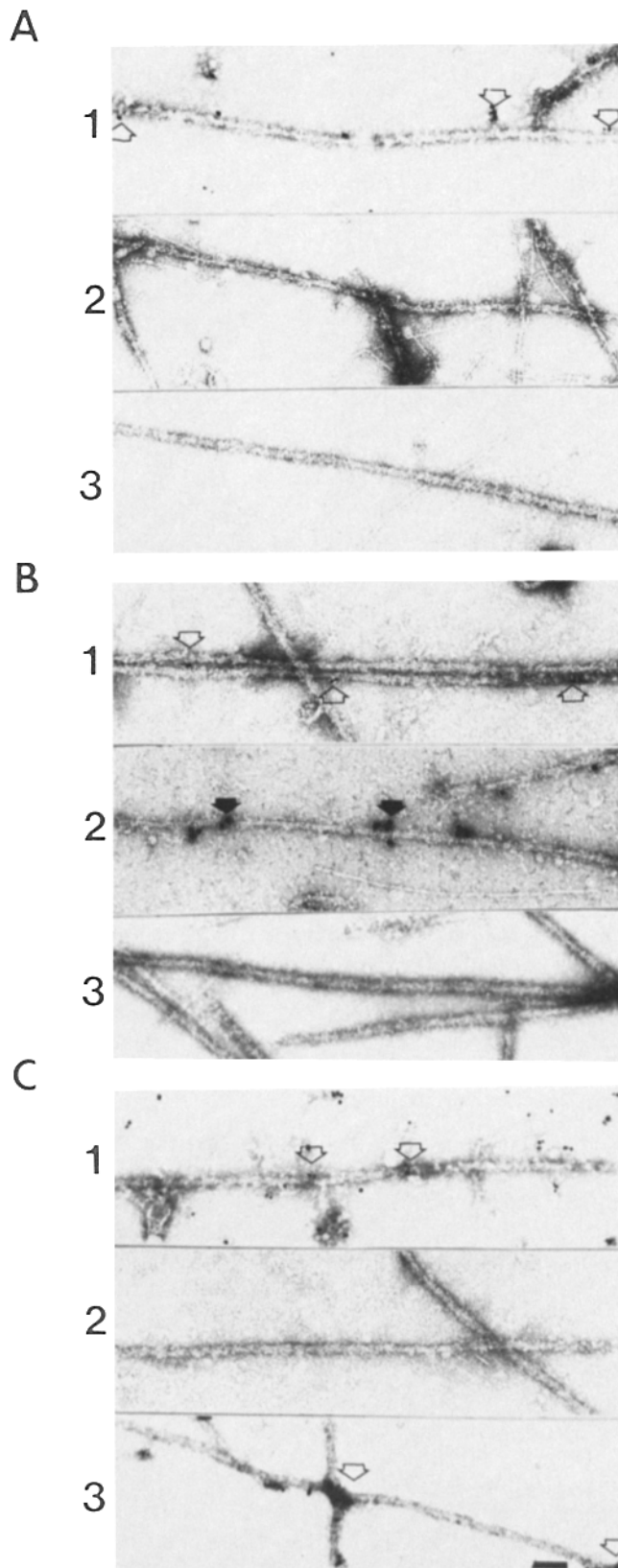


Figure 2. Immunogold labeling of thick filaments from the PM of 12-d chicken embryos, 10-d chicks, and 1-yr chickens. Thick filaments were prepared from the PM of 12-d chicken embryos (*A*), 10-d chicks (*B*), and 1-yr chickens (*C*). Filaments from each stage were reacted with 5-nm gold-conjugated EB165 (*A* 1, *B* 1, and *C* 1), 15-nm gold-conjugated 2E9 (*A* 2, *B* 2, and *C* 2), and 5-nm gold-conjugated AB8 (*A* 3, *B* 3, and *C* 3). Filaments from the embryo

PM development. From these results and the known specificity of our antibodies, filaments from the PM of 12-d embryo, 10-d chicken, and 1-yr chicken are homogeneous and composed of embryonic myosin, neonatal myosin, and adult myosin, respectively. Although 3% of the filaments from the PM of 10-d chickens were decorated with EB165 antibody, in these experiments, we cannot determine whether embryonic and neonatal myosins are in the same or different filaments.

To determine the myosin composition of filaments from stages of PM development where multiple fast MHCs are simultaneously expressed within the same muscle fiber, we reacted isolated filaments with a mixture of gold-conjugated antibodies and examined them by EM. Thick filaments were prepared from the PM of 19-d chicken embryos, a stage of development where neonatal fast MHC is rapidly accumulating in muscle fibers comprised of embryonic myosin (9). When filaments were incubated with both 5-nm gold-conjugated EB165 antibody and 15-nm gold-conjugated 2E9 antibody, three classes of filaments were observed. One class of filaments was decorated with the embryonic antibody (Fig. 3 *A*); a second class reacted only with the neonatal antibody (Fig. 3 *B*); and a third class was labeled by both antibodies (Fig. 3 *C*).

Similar results were obtained with thick filaments isolated from the PM of 44-d-old chickens in which both the neonatal and adult MHCs are expressed. These filaments were incubated with 15-nm gold-conjugated 2E9 antibody and 5-nm gold-conjugated AB8 antibody and examined by EM. Again, three classes of filaments were identified based on their reactivity with these antibodies. One class reacted with 2E9 antibody (Fig. 4 *A*), the second class was decorated with AB8 antibody (Fig. 4 *B*), and the third class reacted with both antibodies (Fig. 4 *C*).

Table III summarizes our observations on filaments from the PM of 19-d embryos and 44-d chickens. At both stages of PM development, approximately half of the filaments were labeled by two antibodies clearly demonstrating that two fast MHC isoforms can exist in the same native thick filament. However, in addition to heterogeneous filaments, there remain filaments that react only with a single antibody.

To determine whether both homogeneous and heterogeneous filaments would form from myosins polymerized *in vitro*, we prepared synthetic thick filaments from a mixture of purified embryonic and neonatal myosins as described in Materials and Methods. In electron micrographs of negatively stained filaments, the synthetic filaments had tapered ends and rough surface projections similar to native filaments but exhibited a greater variability of filament length (Fig. 5 *A*). These filaments were then incubated with 5-nm gold-conjugated EB165 and 15-nm gold-conjugated 2E9 antibodies and examined by EM. In contrast to native filaments, only a single class of filaments (>98%) decorated with both antibodies was observed (Fig. 5 *B*).

reacted only with EB165 antibody. The majority of filaments from 10-d chicks were decorated by 2E9, but a few (~3%) reacted with EB165. No filaments from the 10-d chicks reacted with AB8. Filaments from the 1-yr adult reacted with both EB165 and AB8, but not with 2E9. Closed arrows indicate 5-nm gold beads and open arrows indicate 15-nm gold beads. Bar, 100 nm.

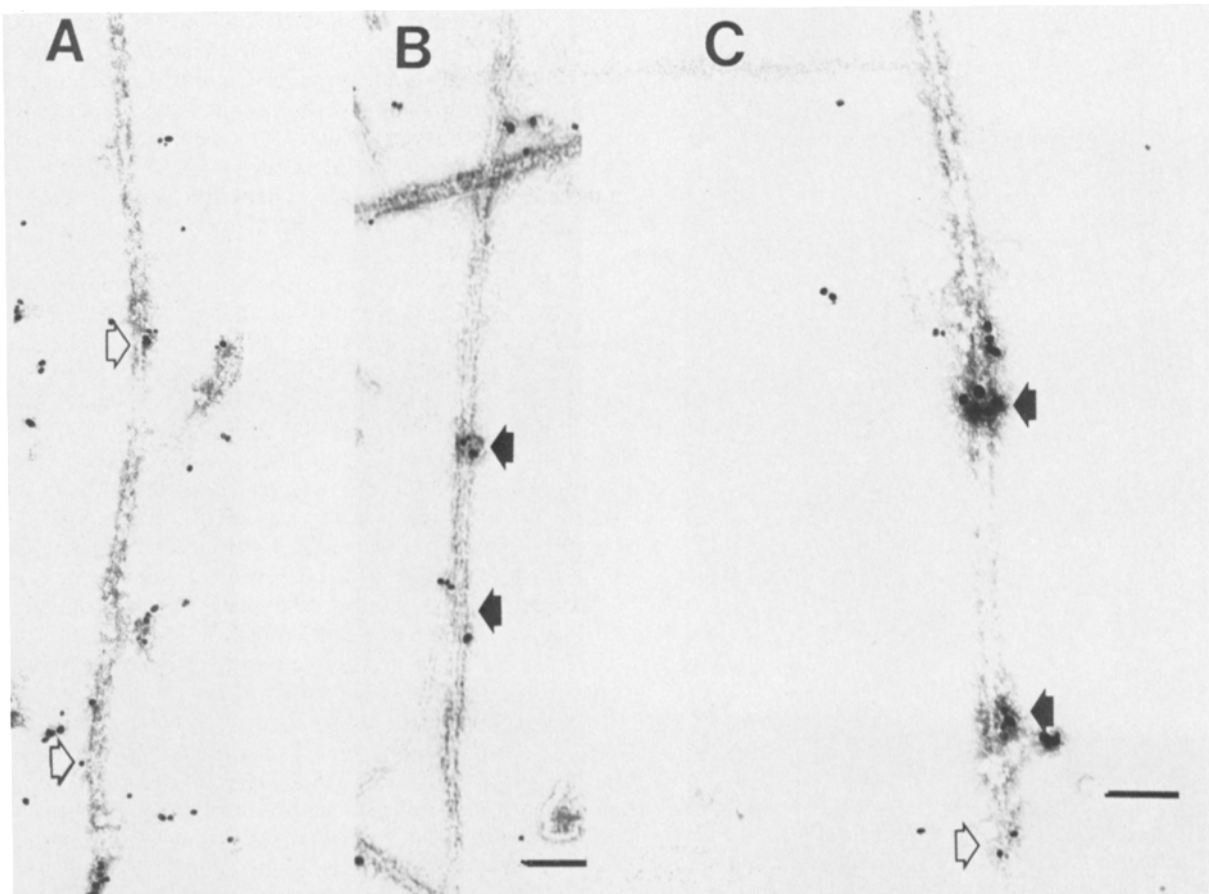


Figure 3. Native thick filaments isolated from the PM of 19-d embryos reacted with gold-conjugated anti-embryonic and gold-conjugated anti-neonatal antibodies. Filaments from PM of 19-d embryos were labeled with 5-nm gold-conjugated EB165 antibody and 15-nm gold-conjugated 2E9 antibody as described in Materials and Methods. Filaments were labeled with EB165 (A), 2E9 (B), or both antibodies (C). White arrows indicate the position of 5-nm gold beads and black arrows indicate the position of 15-nm gold beads. Bar, 100 nm.

Distribution of Different MHC Isoforms in Native Thick Filaments

To determine the distribution of MHCs within individual thick filaments, a grid was constructed that divided a filament into five equal segments (Fig. 6). This grid was placed over an electron micrograph of a filament and the number of gold particles decorating each of the five regions was determined. If filaments were randomly decorated, each region would then contain ~20% of the gold labeling.

The distribution of immunogold labeling was first analyzed on homogeneous filaments from the PM of 12-d embryos, 10-d chickens, and 1-yr chickens. As seen in Fig. 7, ~20% of the gold particles counted were localized in each region for filaments prepared from 12-d embryo incubated with the anti-embryonic MHC antibody. Similar results were obtained with filaments from the PM of 10-d chickens reacted with the anti-neonatal MHC gold-conjugated antibody and with filaments from the PM of 1-yr chickens reacted with anti-adult MHC gold-conjugated antibody. Based on these results, it appears that the embryonic, neonatal, and adult antibody gold probes uniformly decorate filaments composed of embryonic, neonatal, and adult MHCs, respectively. This

uniform labeling indicates that the epitopes for these antibodies were accessible along the entire length of the filament.

This same analysis was then performed on thick filaments from the PM of 19-d chicken embryo that reacted with both EB165 and 2E9 antibodies. As shown in Fig. 8, filaments were uniformly decorated with gold-conjugated anti-embryonic MHC antibody. However, the anti-neonatal MHC antibody preferentially decorated the center of filaments. More than 42% of the gold beads counted decorated this region while the remainder of the beads were uniformly distributed among the other four regions. The data suggest that while the embryonic fast myosin isoform was randomly distributed in filaments from 19-d embryonic PM, the neonatal isoform was preferentially localized in the center of filaments. In filaments from the PM of 44-d posthatch chicken that contained both neonatal and adult MHCs, gold-conjugated anti-adult MHC antibody uniformly decorated the thick filaments while antineonatal MHC antibody again was observed to preferentially decorate the center of the filaments (Fig. 9). Thus, the neonatal fast MHC isoform exhibited a preferential localization at the central region of heterogeneous filaments irre-

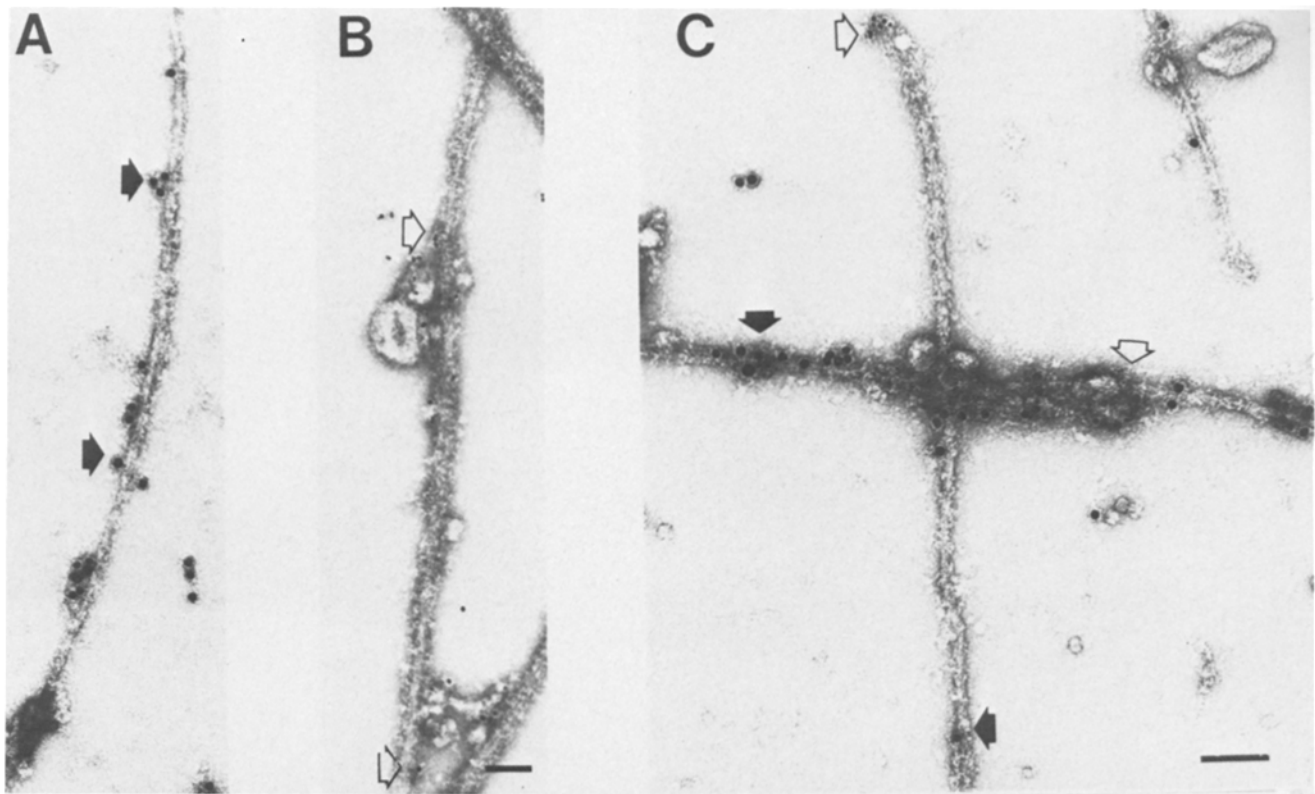


Figure 4. Native thick filaments isolated from the PM of 44-d chickens reacted with gold-conjugated anti-neonatal and gold-conjugated anti-adult MHC antibodies. Filaments from the PM of 44-d chickens were labeled with 15-nm gold-conjugated 2E9 and 5-nm gold-conjugated AB8 antibodies as described in Materials and Methods. Filaments were labeled with 2E9 (A), AB8 (B), or both antibodies (C). White arrows indicate the position of 5-nm gold beads and black arrows indicate the position of 15-nm gold beads. Bar, 100 nm.

spective of whether the other isoform was the embryonic or adult MHC.

Discussion

We have demonstrated that when two MHC isoforms are expressed in chicken PM, thick filaments may contain either one or both MHCs. Isolated thick filaments from 19-d embryonic PM may be grouped into three classes based on their MHC content. As shown in Fig. 3, thick filaments may be

composed of embryonic MHCs, neonatal MHCs, or both MHCs. Since our filament preparations are derived from whole muscle homogenates, we have not demonstrated that all three classes of filaments are found in the same fiber. However, at this early stage of pectoral muscle development, all of the fibers react with the anti-embryonic MHC antibody and >90% of the fibers also react with the anti-neonatal MHC antibody (reference 9; data not shown). Since no fibers are present that react with only the anti-neonatal MHC antibody, the filaments composed of pure neonatal myosin must arise from fibers that also contain embryonic myosin. Similar observations with filaments from 44-d muscle, a stage at which virtually all cells contained both neonatal and adult MHCs, also support the conclusion that all myosin filaments within a single fiber are not identical.

Although 33% of the filaments isolated from 19-d embryonic pectoral muscle reacted only with anti-neonatal MHC antibody, double antibody sandwich ELISA (8) suggests that only 10–15% of the myosin present in the pectoral muscle at this stage of development is the neonatal isoform (data not shown). This discrepancy may be the result of a differential release of thick filaments from myofibrils. The majority of preexisting thick filaments in embryonic muscle would contain embryonic MHC. Thus, neonatal MHC-containing thick filaments would represent a larger percentage of newly assembled thick filaments than of total filaments in 19-d embryonic muscle. It has been reported that homogeni-

Table III. Double Immunogold-labeled Thick Filaments

Stage	Antibody	Percent labeled
19-d embryo (<i>n</i> = 934)	EB165	14.3
	2E9	33.7
	EB165 + 2E9	44.9
44-d chicken (<i>n</i> = 999)	2E9	23
	AB8	21.4
	2E9 + AB8	54.8

Filaments from the PM of 19-d embryos were incubated with a mixture of 5-nm gold-conjugated EB165 and 15-nm gold-conjugated 2E9 antibodies. Filaments from the PM of 44-d chickens were incubated with a mixture of 15-nm gold-conjugated 2E9 and 5-nm gold-conjugated AB8 antibodies. The percentage of filaments decorated with either a single or both antibodies is summarized from observations on filaments similar to those illustrated in Figs. 5 and 6. *n*, number of filaments counted.

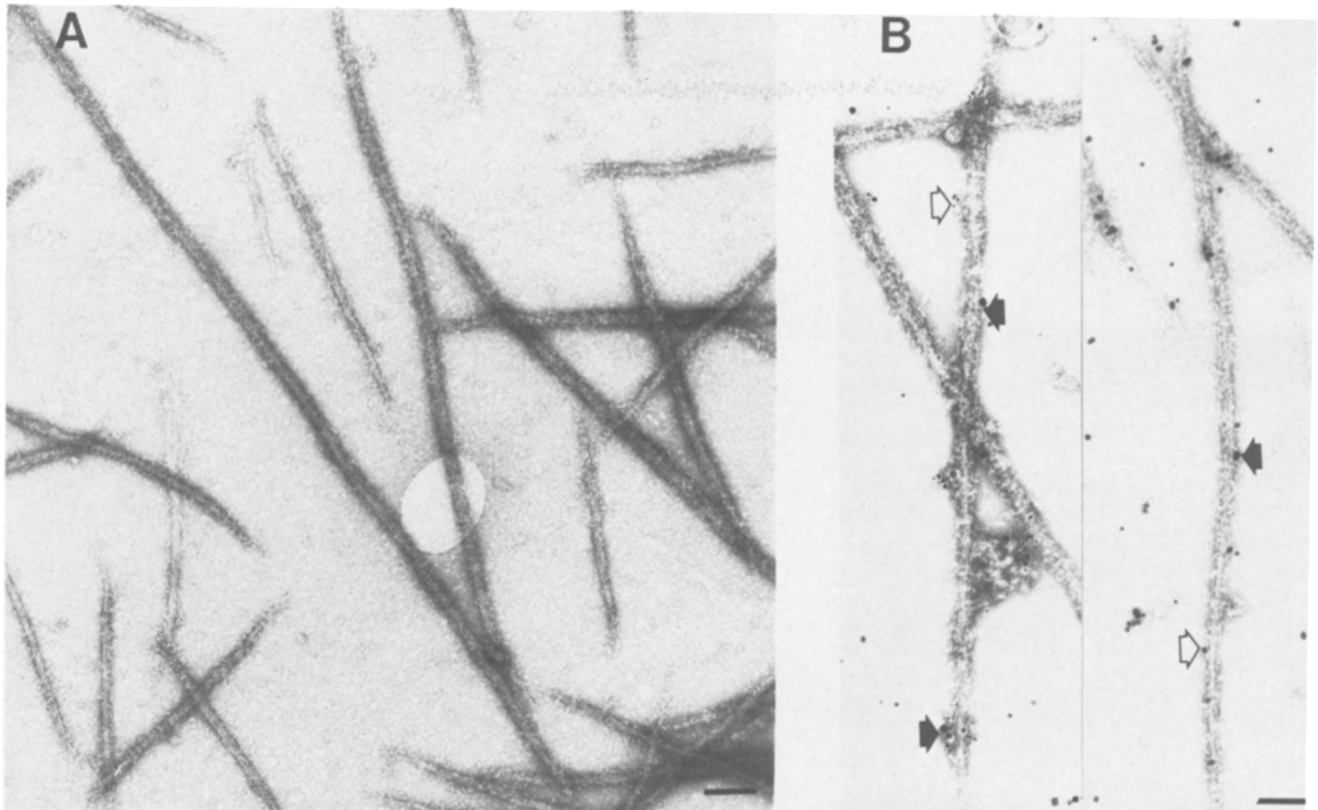


Figure 5. Synthetic filaments prepared from embryonic and neonatal MHCs reacted with gold-conjugated anti-embryonic and gold-conjugated anti-neonatal MHC antibodies. Synthetic filaments were prepared from embryonic and neonatal myosin as described in Materials and Methods. (A) A representative sample of assembled filaments is shown. (B) Filaments were incubated with a mixture of 5-nm gold-conjugated EB165 antibody and 15-nm gold-conjugated 2E9 antibody. All synthetic filaments were decorated with both antibodies. White arrows indicate the position of 5-nm gold beads and black arrows indicate the position of 15-nm gold beads. Bar, 100 nm.

zation of muscle in ATP-containing buffers may preferentially release newly added thick filaments (54). Thus, our observations may be explained if recently added myosin filaments enriched in neonatal MHC are at the periphery of the myofibril and are more easily extracted by our procedures. If this hypothesis is correct, it may be possible, by using im-

munolectron microscopy, to demonstrate directly the peripheral localization of neonatal MHC at this stage of maturation.

Since three classes of filaments were isolated from developing pectoral muscle, some form of compartmentalization of myosins or their mRNAs may exist in muscle cells.

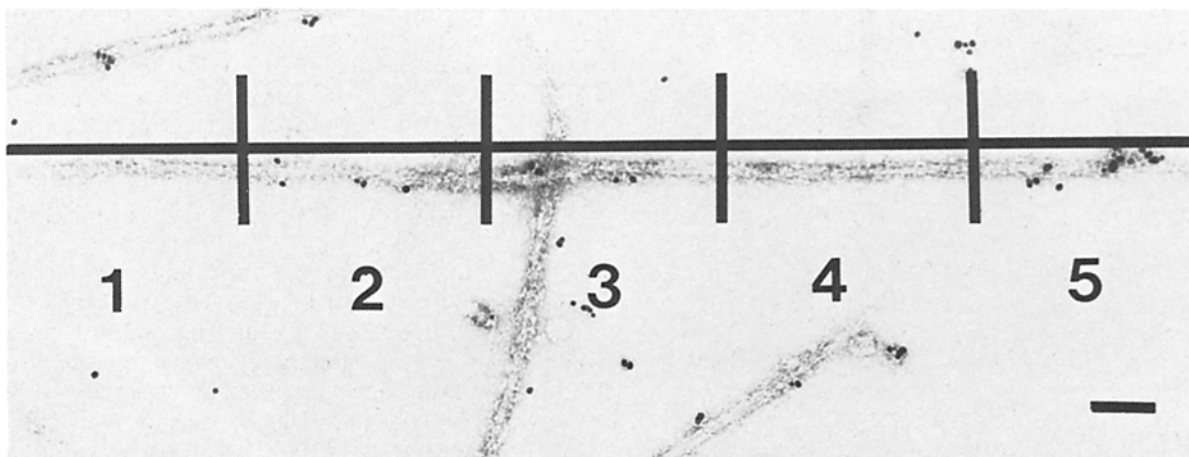


Figure 6. Immunogold labeled thick filaments divided into five regions. A grid was constructed that when overlaid on micrographs of native thick filaments divided them into five equal regions, ends (1 and 5), mid-portions (2 and 4), and central zone (3) are indicated. The number of gold beads was counted in each region of the labeled filaments. Bar, 100 nm.

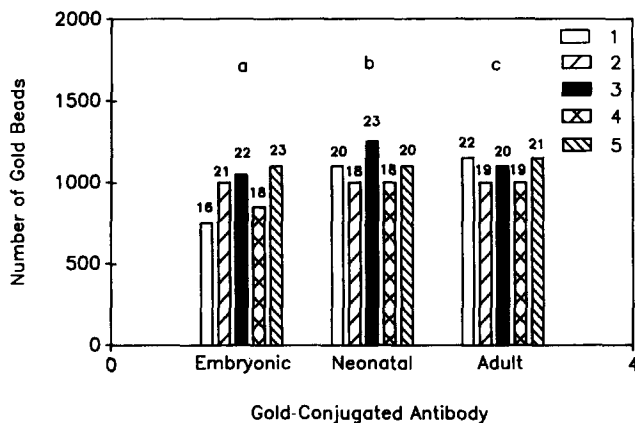


Figure 7. Distribution of gold beads in homogeneous filaments. (a) Filaments from the PM of 12-d embryos were reacted with gold-conjugated EB165 and the number of gold beads in each of the five regions described in Fig. 6 was determined. The number above each of the bars in the histogram represents the percentage in that region of the total beads counted; (b) filaments from the PM of 10-d chicks reacted with gold-conjugated 2E9 antibody; and (c) filaments from the PM of 1-yr chickens reacted with AB8 antibody were analyzed as described above. A total of 795 filaments were counted in *a*, 787 in *b*, and 750 in *c*. The data indicate that the epitope recognized by each antibody is equally accessible along the length of the filament.

Differential distribution of myosins has been demonstrated in developing muscle cells in culture (8), in regenerating muscle fibers (24), at the neuromuscular junction (42), and in muscle fibers undergoing fiber type transformation (51). These regional differences may be the result of the expression of different mRNAs by myonuclei within the same fiber. Recently, it has been reported that mRNAs that encode

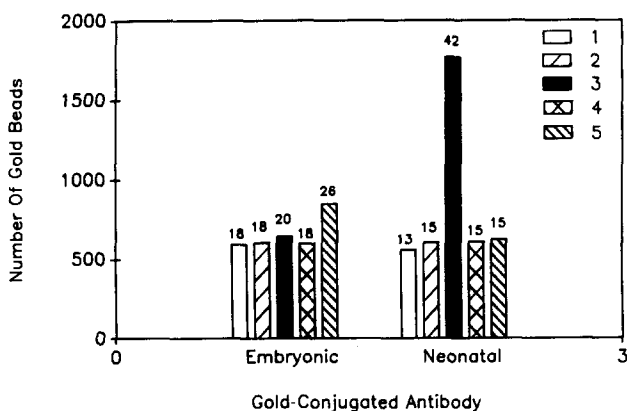


Figure 8. Distribution of gold beads in heterogeneous filaments from the PM of 19-d embryos. Myosin filaments isolated from the PM of 19-d embryos were reacted with 5-nm gold-conjugated EB165 and 15-nm gold-conjugated 2E9. The distribution of gold beads on filaments decorated with both antibodies was analyzed as in Fig. 7. The number above each of the bars in the histogram represents the percentage in that region of the total beads of each size counted. The total number of double-labeled filaments analyzed was 625. The results show that the embryonic antibody uniformly decorated filaments, while the neonatal antibody preferentially labeled the center of filaments.

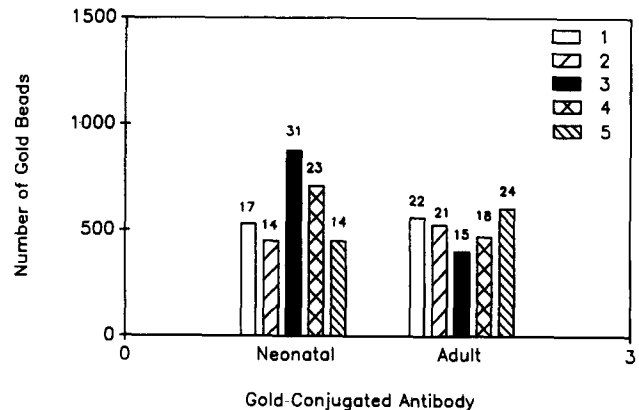


Figure 9. Distribution of gold beads in heterogeneous filaments from the PM of 44-d chickens. Myosin filaments isolated from the PM of 44-d chickens were reacted with 15-nm 2E9 antibody and with 5-nm gold-conjugated AB8 antibody. The distribution of labeling for each antibody in filaments that were decorated by both antibodies was determined as described in Fig. 7. The number above each of the bars in the histogram represents the percentage in that region of the total beads of each size counted. The total number of double-labeled filaments analyzed was 605. Although to a lesser degree than in Fig. 8, labeling with the anti-neonatal antibody in the center of the filament is greater than at the ends.

cytoskeletal proteins, such as actin and vimentin, have specific cytoskeletal localizations (25). These studies coupled with co-translational assembly of MHCs in cultured muscle cells (23) may explain the biosynthesis of discrete classes of thick filaments.

Since there is no evidence that entire myofibrils disassemble or turn over en masse (28, 59), myosin exchange has been proposed as a mechanism for replacing myosin isoforms in developing muscle (40). However, the persistence of filaments containing different MHCs argues against a rapid rate of exchange in vivo. It has been reported that the rate of exchange was significantly reduced in filaments assembled from myosin and C protein (Saad, A. D., E. Zlotchenko, and I. Tan, unpublished observations). If accessory proteins stabilize myosin filaments in vitro, one would expect a significantly slower rate of exchange within the complex structure of the myofibril.

We also observed that neonatal MHC appears to be preferentially localized in the central bare zone in thick filaments composed of more than one isoform. This is not the result of a difference in the availability of the epitope recognized by the anti-neonatal MHC antibody since the antibody reacts equally well with all regions of the myosin filament. A differential localization of different myosins has previously been reported in nematode muscle where one of the isoforms, myosin A, is also preferentially localized in the central region of the filament (14, 15, 31). Although our observation is the first direct demonstration of differential distribution of a myosin isoform in thick filaments from vertebrate muscle, it has previously been observed that cardiac myosin isoforms are differentially localized in the A band of myofibrils during thyroid hormone-induced isoform transitions (57) and that slow myosin is found in the central region of the A band of myofibrils in myotubes containing both fast and slow MHC isoforms (44).

The expression of neonatal MHC in pectoral muscle corresponds with a period of rapid fiber hypertrophy, a concomitant increase in the diameter of myofibrils, and an accumulation of myosin thick filaments (19, 45, 46). Neonatal MHC is centrally localized in both early stages of muscle maturation in which it is first being expressed (Fig. 8), and in later stages of muscle growth in which it is being replaced by the adult isoform (Fig. 9). This suggests that its specific localization in thick filaments is the result of an intrinsic property of this isoform rather than the timing of its appearance.

The localization of neonatal myosin in the central bare zone suggests that this isoform may play a specific role in thick filament assembly. If the majority of filaments that we observe are newly formed, then perhaps neonatal myosin preferentially nucleates *de novo* assembly. This would be consistent with *in vitro* studies of myosin assembly in which synthetic thick filaments are thought to form from the bare zone outward (10). However, there are other models of filament assembly. Accessory proteins appear to be involved in the nucleation of microtubules and microfilaments (18, 56). Recently, it has been proposed that there is a core structure in thick filaments from nematode muscle that acts as a template for the addition of myosin subunits (15, 16). If this proposal is correct, then the regulation of myosin assembly *in vivo* may be specified by uncharacterized proteins of the myofibril. This would explain why conclusions based on studies of synthetic filaments formed from purified myosin would differ from conclusions of studies of filaments formed *in vivo*. That factors other than the myosin precursor pool are involved in native thick filament formation is suggested from our studies since only a single homogeneous class of myosin filaments is produced when embryonic and neonatal myosin are copolymerized *in vitro* (Fig. 5).

In conclusion, we have shown that in developing pectoral muscle the myosin isoform composition of native thick filaments is more complex than would have been predicted based on studies of synthetic filaments. While our results do not rule out myosin exchange in native filaments, the heterogeneity of the filament population that we observed can only be maintained if its rate is considerably slower than predicted from *in vitro* experiments. Furthermore, we demonstrate the preferential localization of neonatal myosin to the central bare zone of many thick filaments whether it is copolymerized *in vivo* with embryonic or adult myosin. This suggests that some MHC isoforms may play specific roles in myofibrillogenesis *in vivo*.

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