

# Neonatal and Adult Myosin Heavy Chains Form Homodimers during Avian Skeletal Muscle Development

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**Abstract.** Myosin isoforms contribute to the heterogeneity and adaptability of skeletal muscle fibers. Besides the well-characterized slow and fast muscle myosins, there are those isoforms that appear transiently during the course of muscle development. At a stage of development when two different myosins are coexpressed, the possibility arises for the existence of heterodimers, molecules containing two different heavy chains, or homodimers, molecules with two identical heavy chains. The question of whether neonatal and adult myosin isoforms can associate to form a stable heterodimer was addressed by using stage-specific

monoclonal antibodies in conjunction with immunological and electron microscopic techniques. We find that independent of the ratio of adult to neonatal myosin, depending on the age of the animal, the myosin heavy chains form predominantly homodimeric molecules. The small amount of hybrid species present suggests that either the rod portion of the two heavy chain isoforms differs too much in sequence to form a stable  $\alpha$ -helical coiled coil, or that the biosynthesis of the heavy chains precludes the formation of heterodimeric molecules.

THE MHC of vertebrate skeletal muscles exhibits a large number of isoforms that can be grouped into a "fast" or "slow" class, depending on the rate of ATP hydrolysis, immunological reactivity, and electrophoretic mobility in polyacrylamide gels (Bandman, 1985a; Lowey et al., 1986; Stockdale and Miller, 1987). Each class contains more than one developmentally regulated myosin: for instance, there are a minimum of three fast MHC isoforms in certain developing muscles of the rat (Whalen et al., 1981) and the chicken (Bader et al., 1982; Bandman et al., 1982; Lowey et al., 1983) designated as embryonic, neonatal (or posthatch), and adult myosin. These fast myosins have a high degree of sequence homology (Robbins et al., 1986; Nguyen et al., 1982) and are similar in ATPase activity (Lowey et al., 1986), but their physiological significance is largely unknown.

Polymorphism is not limited to the myosin heavy chain, but was in fact first discovered for the light chains (see review by Lowey, 1986). Two variants of a fast myosin light chain (LC), alkali 1 and alkali 2, are bound to the head region of chicken pectoralis myosin, forming a light chain homodimer (each head has the same LC) or heterodimer (each head has a different LC) (Lowey et al., 1979). Evidence favors a random association of the light chains with the heavy chains, but a more complex mechanism cannot be excluded. No striking difference in activity or assembly properties has been found between the alkali 1 or alkali 2 homodimers (Pastra-Landis et al., 1983).

The myosin molecule is generally considered to consist of two similar heavy chains, whose sequence in the rod region is constrained by the special geometry of the  $\alpha$ -helical coiled coil (Cohen and Parry, 1986). There are, however, examples of myosins formed from two different heavy chains, the best documented case being cardiac myosin (Hoh et al., 1978). The origin of the heterogeneity in cardiac myosin lies in two heavy chain isoforms,  $\alpha$ - and  $\beta$ -MHC, which are encoded by two distinct genes in tandem (Mahdavi et al., 1984). Hoh et al. (1979) first observed that rat ventricular myosin separated into three bands, V1, V2, and V3, on nondenaturing gels, and suggested that these bands correspond to  $\alpha_2$ -MHC homodimer,  $\alpha, \beta$ -MHC heterodimer, and  $\beta$ -MHC homodimer, respectively. This assignment has been confirmed by direct visualization of the three species by immunoelectron microscopy (Dechesne et al., 1987). Unlike the fast skeletal muscle myosin isoforms, the cardiac myosins have unique enzymatic properties, which correlate well with the contractile performance of heart muscle (Swynghedauw, 1986).

The evidence for a heterodimeric species in smooth muscle myosin is less firm: Rovner et al. (1986) showed that smooth muscle myosin migrated as two equal bands by SDS-PAGE, but only one band was visible in nondenaturing gels. They hypothesized that smooth muscle myosin isoforms may exist as a heterodimeric population of molecules, although alternative models could not be excluded. More recently, two isoforms of the smooth muscle myosin heavy chain have been identified by cDNA cloning, and the difference between

them has been shown to reside exclusively in the tailpieces at the 3' carboxy termini of the heavy chains (Nagai et al., 1989).

Isoform switching and transient gene expression are phenomena associated with muscle development. The overlap in temporal expression of the embryonic, neonatal, and adult myosins raises the question of whether the heavy chains can associate into hybrid or heterodimeric molecules. The three developmental isoforms were first detected in rat muscles by virtue of their distinct peptide maps and mobilities in nondenaturing gels (Whalen et al., 1981). At a stage in development where both neonatal and adult forms are coexpressed, no additional band indicative of a heterodimeric molecule was observed in the pyrophosphate gels; however, the absence of discrete bands in nondenaturing gels can never be taken as definitive evidence for the absence of a particular species of myosin (Deschesne et al., 1987).

Developing chickens muscles show an even greater degree of polymorphism than rat muscles, and as many as four fast myosin isoforms have been detected at the protein level (Lowe et al., 1986; Hofmann et al., 1988). These avian developmental myosins are indistinguishable on nondenaturing gels (Lowe et al., 1983), and therefore the question of the distribution of the heavy chains during periods of coexpression had to be addressed by immunological methods. Using monoclonal antibodies specific for neonatal and adult myosin, we find that the myosin isoforms expressed after hatching associate predominantly into homodimeric molecules consisting of identical heavy chains.

## Materials and Methods

### Protein Preparation

Adult myosin was prepared from the pectoralis muscles of White Rock chickens, age 3–4 mo, by the method of Margossian and Lowey (1982). "Neonatal myosin" was prepared from the pectoralis muscles of 12-, 19-, 31-, and 54-d-old White Leghorn chickens by a minor modification of the method of Lowey et al. (1983). After precipitation overnight at low ionic strength, the myosin was resuspended in buffered 1 M NaCl, and ATP/MgCl<sub>2</sub> was added to a final concentration of 10 mM ATP, 12–15 mM MgCl<sub>2</sub>. After clarification by ultracentrifugation, the supernatant was dialyzed against 40 mM sodium pyrophosphate, pH 7.5, and purified by ion-exchange chromatography as described by Margossian and Lowey (1982). Myosins prepared by the above methods showed no contaminating proteins when analyzed by SDS-PAGE.

Protein concentration was determined by the method of Bradford (1976) using adult myosin as the standard curve, or by absorbance using an extinction coefficient (1%, 280 nm) of 5.0 and 14.0 for myosin and IgG, respectively.

### Antibody Preparation

Monoclonal antibodies specific for the myosin head (12C5) and rod (5C3) were those described by Winkelmann et al. (1983). The monoclonal antibody (2E9) specific for neonatal myosin is described by Bandman (1985b). The monoclonal antibody, 5B4, was prepared from mice immunized with myosin purified from the pectoralis of 19-d chickens as described above. The procedures used for fusion, subcloning of cell lines, and ascites production were identical to those described by Winkelmann et al. (1983). The specificity of the antibody was determined by solution competition ELISA using purified 19-d posthatch myosin as the solid-phase antigen, and myosin purified from day-10 and day-18 embryonic pectoralis muscles, day-12 and day-19 posthatch muscles, and adult muscles as the competing antigen in solution. The specificity of 5B4 was clearly for neonatal myosin, with very little affinity for embryonic (data not shown) or adult myosin (see Fig. 2). An estimate of the relative affinity of a monoclonal antibody for its immunogen can be obtained from the concentration of myosin in the soluble phase

needed to inhibit binding of antibody to the solid-phase antigen by 50%. By that criteria, 5B4 has about a 10-fold higher affinity for myosin than 12C5 or 5C3. Antibody 2E9 could not bind to soluble neonatal myosin in the presence of plate bound myosin, and therefore could not be used in a competition ELISA.

### Binding Assays by ELISA

Antibody binding to myosin was determined by direct solid-phase ELISA. The adsorption of antigen to the microtiter plate and the washing procedure between steps was performed according to Winkelmann et al. (1983). The antibody was threefold serially diluted from 6 µg/ml. Antibody binding to antigen was detected with biotinylated anti-mouse IgG, followed by streptavidin-β-galactosidase, and the substrate *p*-nitrophenyl-β-D-galactopyranoside (Bethesda Research Laboratories, Gaithersburg, MD). Absorbance at 405 nm was read on a plate reader (model EL308; Bio-Tek Instruments, Inc., Burlington, VT).

For the solution-competition assay, a constant amount of antibody was mixed with twofold serial dilutions of myosin, starting from ~0.2 mg/ml. The antibody concentration in the competing solution was that which gave half-maximal color by direct solid-phase ELISA (~0.1 µg/ml). The amount of free antibody in the antibody-antigen mixtures was determined by binding to the solid-phase antigen as in a direct ELISA.

### Immunoaffinity Chromatography

Purified antibodies were coupled to Sepharose 4B as described by Silberstein and Lowey (1981). CNBr-activated Sepharose 4B was supplied by Pharmacia (Piscataway, NJ). Column dimensions were 4–6 ml Sepharose containing 6–10 mg of coupled antibody. Typically, 0.5–1 mg of myosin was applied to the column in 0.5 M NaCl, 25 mM imidazole, pH 7, 4°C, and the bound myosin was eluted with 4 M guanidine hydrochloride. Recovery of protein from the void and eluted volumes was 70–80% of the applied protein.

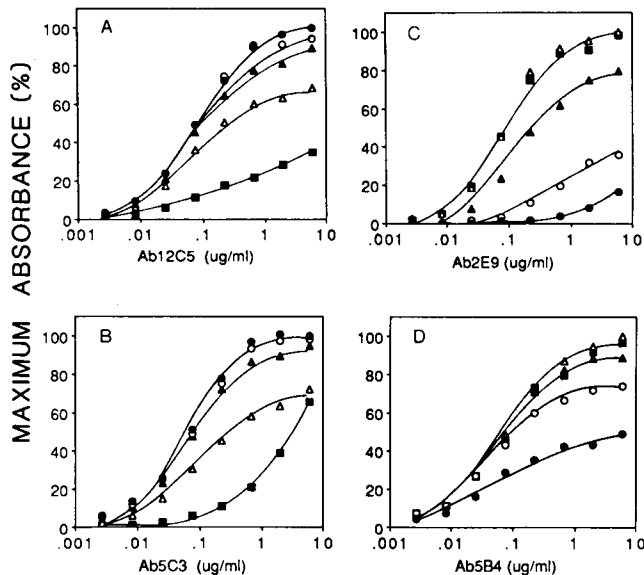
### Immunoelectron Microscopy

Myosin in 0.4 M NaCl, 10 mM NaPi, pH 7.5, at a concentration of ~1 mg/ml, was incubated with an equimolar ratio of antibody for 15–30 min at room temperature, before being diluted 100-fold into 0.5 M ammonium acetate, pH 7.2, 66% glycerol. The complexes were rotary shadowed with platinum as described by Winkelmann et al. (1983). Electron microscopy was performed on a Philips EM301 electron microscope operated at 60 kV.

## Results

### Antibody Characterization

The specificities of monoclonal antibodies 12C5 and 5C3 for adult chicken fast skeletal muscle myosin heavy chain have been previously described (Winkelmann et al., 1983). Antibody 12C5 binds to an epitope present in the head (S1) portion of myosin, while antibody 5C3 binds to an epitope present in the rod near the COOH-terminus of the heavy chain. The specificity of antibody 2E9 for neonatal myosin has also been described (Bandman, 1985b); and its epitope resides within the LMM region of myosin. While antibody 2E9 could discriminate among different myosin isoforms by direct solid-phase ELISA (Fig. 1), it could not be used in a competition assay. The antibody bound more strongly to the myosin adsorbed to a microtiter plate than to the competing myosin in solution. This low affinity of 2E9 for myosin in solution also precluded its use as an immunoaffinity adsorbent, and its use for labeling myosin molecules in immunoelectron microscopy. These problems were circumvented by isolation of a second monoclonal antibody to neonatal myosin, 5B4, which bound with much higher affinity to the native form of neonatal myosin. As shown in Fig. 1, the specificity of 5B4 is qualitatively similar to 2E9 in that both antibodies react



**Figure 1.** Reactivity of monoclonal antibodies with myosin isolated from different stages of chicken pectoralis muscle development by direct ELISA. Antibodies 12C5 (A) and 5C3 (B) react primarily with adult myosin, whereas antibodies 2E9 (C) and 5B4 (D) are specific for neonatal myosin. When smooth muscle myosin was used as the antigen, none of the antibodies showed any reaction, and therefore gizzard myosin served as a control in the assay. Myosins isolated from posthatch muscles at day 12 (■), day 19 (△), day 31 (▲), day 54 (○), and from adult muscle (●) were adsorbed to microtiter plates, and analyzed for adult and neonatal epitopes.

most strongly with myosins from the pectoral muscles of chickens 12–31 d after hatching. However, the relatively stronger binding of 5B4 to adult myosin than 2E9 (compare Fig. 1 D with C) reflects the higher affinity of 5B4 antibody for the few percent of neonatal myosin remaining in adult myosin.

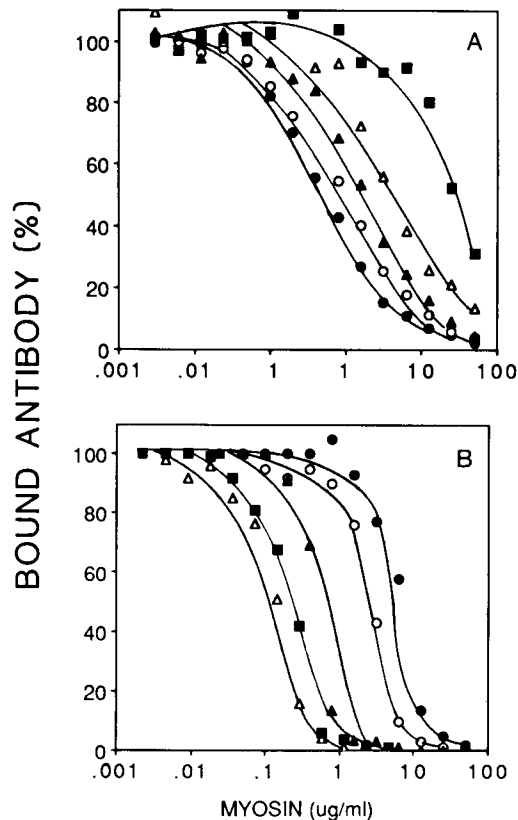
#### Quantitation of Neonatal and Adult Myosin Epitopes in Developing Muscle

Solution-competition ELISA was used to estimate the amount of neonatal and adult myosin present in pectoral muscles between day-12 posthatch and the adult (Fig. 2 A). Myosin samples of different ages were incubated with antibody 12C5, and the ability of the myosin to competitively inhibit the binding of antibody to the solid-phase adsorbed adult myosin was measured. The competition curve generated by adult myosin as the competing antigen served as a standard curve for comparison with the test samples. The percent adult epitope at each age was determined from the concentration of myosin required to inhibit binding of antibody to the solid-phase antigen by 50% relative to the standard curve. The amount of adult myosin present after hatching was ~2% at day 12, ~15% at day 19, 30–40% at day 31, and >60% at day 54. Similar values were obtained in a competition ELISA using the other adult specific antibody 5C3 (data not shown). These observations are also consistent with earlier experiments using radioimmunoassays to quantitate the amount of adult myosin expressed during development (Winkelmann et al., 1983). The complementary experiment was done with antibody 5B4 in a competition

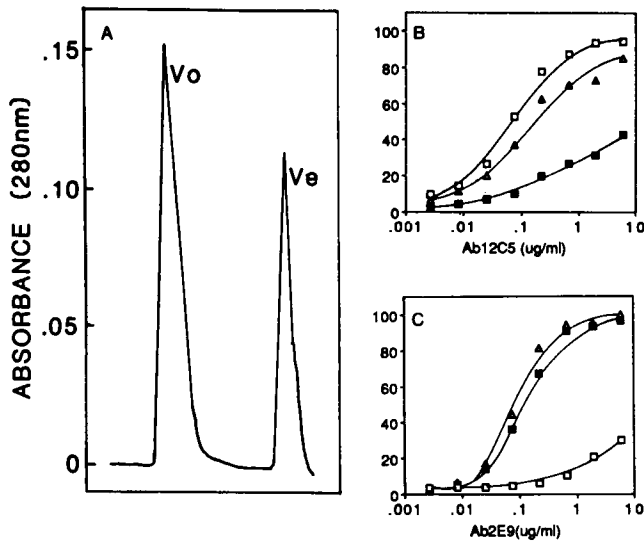
ELISA to follow the disappearance of neonatal myosin epitopes in the same myosin samples (Fig. 2 B). Although the results were qualitatively similar to those obtained with the adult specific antibodies, the absolute values for the concentration of adult myosin epitopes at a particular age were somewhat higher. Given the theoretical limitations of any binding isotherm involving a solid phase, the two sets of data are in reasonable agreement.

#### Analysis of Myosin Isoforms by Affinity Chromatography

To determine the heavy chain composition of myosin molecules during periods of coexpression of two isoforms, the myosin population was fractionated on an immunoadsorbent prepared from the adult-specific monoclonal antibody, 12C5. A typical elution pattern is shown in Fig. 3 A for myosin extracted from day-19 posthatch chicken pectoralis. The immunoadsorbent bound ~10% of the applied myosin, after cor-



**Figure 2.** Determination of the concentration of adult and neonatal myosin epitopes at different ages by solution competition ELISA. Antibody binding to the solid-phase adsorbed adult myosin (A) or neonatal myosin (B) was competitively inhibited by incubation with myosin samples of varying ages. Bound antibody was measured as in the direct ELISA, and is expressed as a percent normalized to the binding in the absence of competing antigen. Displacement of the curve to the right indicates a lower concentration of a particular epitope. Antibodies specific for adult myosin, 12C5 (A), and for neonatal myosin, 5B4 (B) were incubated at a fixed concentration with increasing amounts of adult myosin (●), day-12 (■), day-19 (△), day-31 (▲), and day-54 (○) posthatch myosin.



**Figure 3.** Fractionation of day-19 posthatch myosin on an immunoabsorbent prepared from antibody 12C5 specific for the head region of adult myosin. A typical chromatogram in *A* shows the unretained protein (*Vo*), and the bound myosin (*Ve*) eluted with 4 M guanidine hydrochloride. The starting material ( $\Delta$ ), the bound fraction ( $\square$ ), and the unretained fraction ( $\blacksquare$ ) were reacted with adult-specific antibody 12C5 in *B*, and with neonatal-specific antibody 2E9 in *C*. The lack of reactivity of the bound protein with 2E9 antibody shows the absence of neonatal epitopes in the bound fraction. The areas under the two peaks are not strictly proportional to concentration, due to a refractive index change upon the application of guanidine hydrochloride.

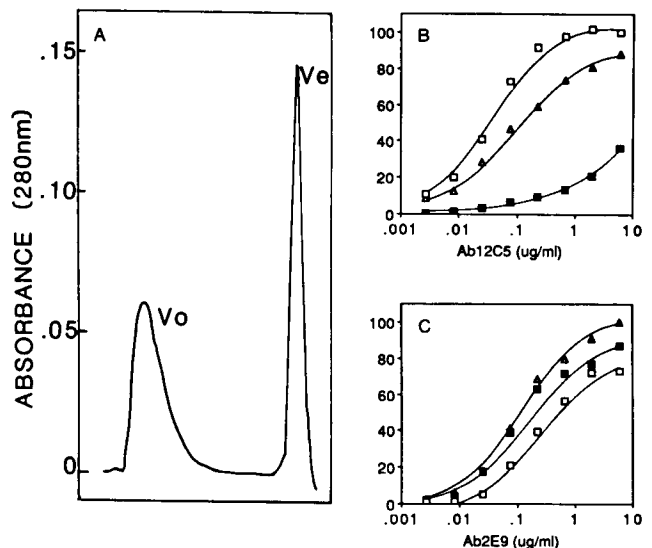
recting for 80% recovery of the applied protein in the eluted and void volume fractions. A similar fractionation of myosin prepared from day-31 pectoral muscles gave  $\sim 30\%$  of the applied protein bound to the immunoabsorbent (data not shown). These values are in good agreement with the percent myosin determined from competition assays.

The bound and unretained fractions were analyzed by solid-phase ELISA using 12C5 and 2E9 antibodies to detect adult and neonatal epitopes, respectively (Fig. 3, *B* and *C*). At 19 days' posthatch, the 12C5 antibody reacted strongly with the bound myosin (*Ve*), as would be expected, and showed little reactivity with the unretained void volume (*Vo*) fraction. The neonatal-specific antibody, 2E9, showed no reaction with the bound myosin (*Ve*), but gave a strong reaction with the unretained myosin (*Vo*). Since day-19 myosin has only  $\sim 15\%$  adult myosin, the absence of adult epitopes in *Vo* is not surprising. What is remarkable is the total absence of neonatal epitopes in *Ve*, since one might have expected at least some nonspecific binding to the column from such a high concentration of neonatal myosin. Similar results were obtained with myosin isolated from day-31 posthatch myosin. Again, the bound fraction reacted only with the adult-specific antibody, 12C5 (data not shown). The complete absence of neonatal epitopes in the retained fraction suggested that adult and neonatal myosin heavy chains are assembled predominantly as homodimeric molecules. Before reaching this conclusion, however, it seemed prudent to test the fractionation with another adult-specific antibody, 5C3. It is possible that 12C5 antibody immobilized on an insoluble matrix might not bind a single myosin head, such as

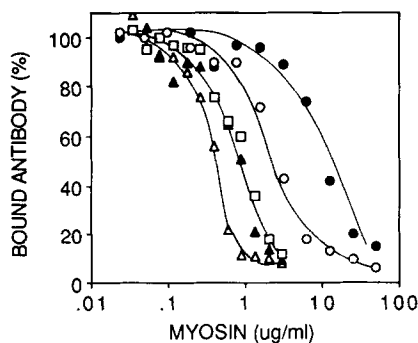
would be present in adult/neonatal heterodimer, with the same avidity as a two-headed myosin homodimer. In that case, a small population of heterodimeric molecules among the major homodimeric species could escape detection.

When day-19 posthatch myosin was applied to an immunoabsorbent comprised of 5C3 antibody,  $\sim 20\%$  of the recovered protein bound to the column (Fig. 4 *A*). This represents about twice as much binding as was observed for the 12C5 immunoabsorbent. Furthermore, the bound fraction (*Ve*) showed for the first time some reactivity with the anti-neonatal 2E9 antibody (Fig. 4 *C*). This raised the question of whether the 5C3 antibody column was more efficient at binding heterodimers than the 12C5 column, or whether it simply attracted more nonspecific binding. To resolve this problem, the void volume from the first passage (*Vo*), which presumably was devoid of adult epitopes, was passed through the column a second time. The amount of bound protein was negligible, suggesting that the binding of neonatal heavy chain to the 5C3 column could not be attributed to nonspecific binding. It appears more likely that myosin expressed at day-19 posthatch contains some adult/neonatal heterodimers in addition to the homodimers of adult myosin and neonatal myosin. Similar results were obtained in the analysis of day-31 posthatch myosin: The bound fraction, representing  $\sim 50\%$  of the applied protein, reacted with the neonatal-specific antibody (data not shown).

Although direct ELISA indicates a small population of heterodimers exists together with the homodimers, it is difficult to quantitate the amount of each species by this technique. The myosin fraction bound to the adult-specific immunoabsorbent (5C3) was, therefore, further analyzed in a



**Figure 4.** Fractionation of day-19 posthatch myosin on an immunoabsorbent prepared from antibody 5C3 specific for the rod region of adult myosin. The chromatogram in *A* shows the unretained protein (*Vo*), and the bound myosin (*Ve*) eluted with 4 M guanidine hydrochloride. The starting material ( $\Delta$ ), the bound fraction ( $\square$ ), and the unretained fraction ( $\blacksquare$ ) were reacted with 12C5 in *B*, and with 2E9 in *C*. Note that the bound fraction now reacts with neonatal-specific antibody, whereas in Fig. 3 there was no reaction. The concentration of neonatal epitopes in *Ve* was determined by competition ELISA; see Fig. 5.



**Figure 5.** Determination of the concentration of neonatal epitopes in myosin fractionated by immunoadsorption. Myosin isolated from day-19 and day-31 posthatch muscles was applied to an adult-specific immunoadsorbent (5C3), and the bound fractions ( $\square$ , day 19;  $\circ$ , day 31) were analyzed by competition ELISA. Antibody specific for neonatal myosin, 5B4, was used in the assay. Curves generated by unfractionated day-19 ( $\Delta$ ), day-31 ( $\blacktriangle$ ), and adult ( $\bullet$ ) myosin are included as references. All the myosin antigens were briefly exposed to 4 M guanidine hydrochloride before analysis in a benign solvent to facilitate comparison.

competition ELISA using the neonatal-specific antibody, 5B4 (Fig. 5). Since the myosin had been eluted from the immunoadsorbent with guanidine hydrochloride, all the antigens used in the competition assay were exposed to 4 M guanidine hydrochloride before dialysis into a benign buffer. Myosin exposed to guanidine hydrochloride gave a similar distribution of developmental epitopes to that obtained previously (compare Fig. 5 with Fig. 2 B); the principal difference was a weaker affinity of myosin for antibody, as evidenced by the shift of the competing myosin to higher concentrations. The fractions eluted from the immunoadsorbents (Ve) gave values of  $\sim 30\%$  and  $10\%$  neonatal epitopes for day-19 and day-31 myosin, respectively, by competition ELISA (Fig. 5). Given that  $\sim 20\%$  of day-19 and  $\sim 50\%$  of day-31 myosin bound to the 5C3 column,  $<10\%$  of the total myosin expressed at any age during development was in the form of heterodimers.

#### Localization of Myosin Epitopes by Immunoelectron Microscopy

The most direct approach to determine the amount of adult/neonatal heterodimers in a population of myosin molecules is to simply add a mixture of adult-specific and neonatal-specific antibodies to day-31 myosin (an age when both epitopes are expressed in approximately equal amounts), and look for myosin molecules with two bound antibodies by electron microscopy. When antibody 5B4 was incubated with neonatal myosin, a high percentage of the myosin molecules had a globular mass bound at the COOH-terminal end of the tail (Fig. 6 A). It is noteworthy that the adult-specific antibody, 5C3, was mapped previously to the same COOH-terminal region of the myosin rod (Winkelmann et al., 1983). This portion of myosin seems to be particularly variable in sequence, which may account for its immunogenic character (Periasamy et al., 1984). The similarity in epitope location between 5C3 and 5B4 precluded their simultaneous use in double-labeling experiments, and instead we chose 12C5 antibody whose epitope lies in the NH<sub>2</sub>-terminal 25-kD re-

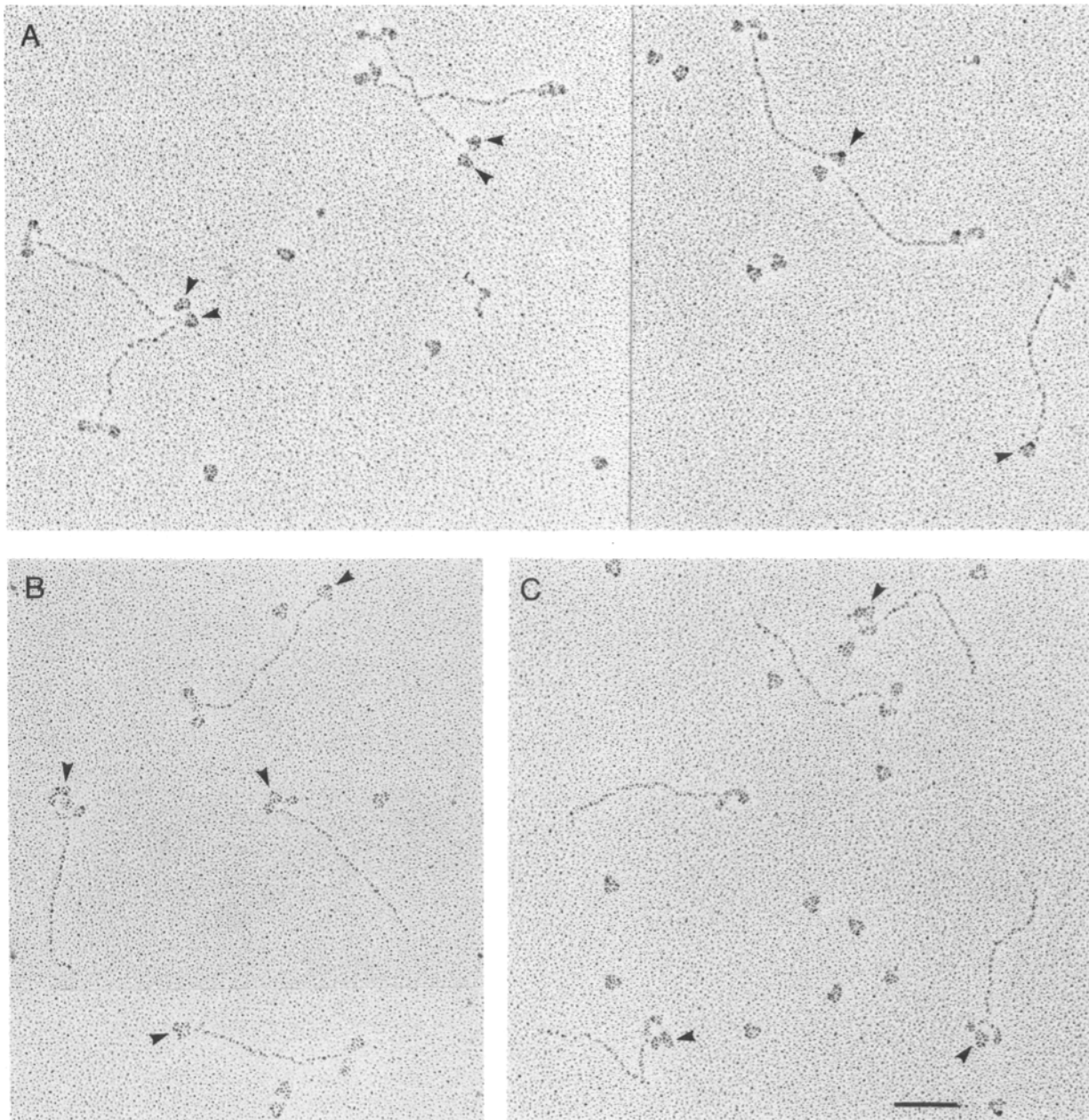
gion of the myosin head. When 12C5 and 5B4 antibodies were added together to day-31 posthatch myosin, bound antibody was seen on the myosin head or at the end of the tail, but rarely at both locations on a single myosin (Fig. 6 B). When day-12 myosin was used, antibodies were seen binding to the end of the tail (Fig. 6 A), and in the case of adult myosin, antibodies bound predominantly to the head (Fig. 6 C).

Based on a sampling of several hundred myosin molecules, we found that at day 12  $> 98\%$  of the bound antibody was located at the end of the tail; at day 19,  $\sim 80\%$  of the bound antibody mapped to the tail, and the rest mapped to the head; in the adult over  $90\%$  of the bound antibody was located on the head and about  $6\%$  on the tail. At day 31 about equal amounts of antibody were seen binding to either the head or the rod (see histograms in Fig. 7). The number of molecules with antibody bound at both the head and the rod was  $<4\%$  at any age, and therefore not statistically significant. The distribution of isoforms by immunoelectron microscopy agreed qualitatively with that determined by the immunological assays, and confirmed that the amount of heterodimers is small compared with the homodimer population.

#### Discussion

Overlap in the temporal expression of embryonic, neonatal, and adult skeletal myosin genes raised the question of whether the myosin heavy chains assemble into molecules with two identical chains and/or two different chains. In the case of the light chain isoforms, it was shown previously that three populations of myosins can form, depending upon whether both heads bind A1, A2, or a combination of A1 and A2 (Lowey et al., 1979). It is difficult to determine the heavy chain composition of individual myosin molecules when more than one isoform is expressed, except under the rare circumstances when the isoforms are sufficiently different in charge to permit their separation by ion-exchange chromatography, or by electrophoresis under nondenaturing conditions. Fractionation of nematode myosin from *Caenorhabditis elegans* by hydroxylapatite led to the conclusion that at least  $85\%$  of the myosin was in the form of homodimers (Schachat et al., 1977). Cardiac myosin from the adult rat was shown to have as much as  $30\%$  heterodimer based on pyrophosphate gel electrophoresis (Hoh et al., 1979). Apart from these two examples, little is known about the composition of myosin with respect to its heavy chains. Isoform-specific antibodies are usually needed to determine the distribution of heavy chains, but these are difficult to obtain because of the high degree of sequence homology among myosins. Here we used previously described monoclonal antibodies specific for neonatal myosin (Bandman, 1985b) and adult myosin (Winkelmann et al., 1983), and a newly isolated monoclonal antibody (5B4), specific for neonatal myosin.

The most direct way to demonstrate the existence of a myosin heterodimer is by visualizing two different stage-specific antibodies bound to a single myosin molecule in the electron microscope. This approach was used by Dechesne et al. (1987) to show the three myosin species formed by the  $\alpha$ - and  $\beta$ -cardiac myosin heavy chains. By simultaneously labeling cardiac myosin with two antibodies specific for  $\alpha$ - and  $\beta$ -MHC, he was able to visualize  $\alpha_2$ - and  $\beta_2$ -MHC homodimers and  $\alpha, \beta$ -MHC heterodimers. The proportions of these myosin isoforms determined by microscopy com-

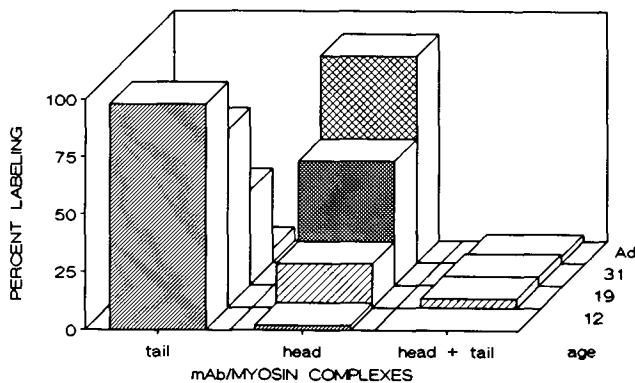


**Figure 6.** Localization of adult and neonatal epitopes by immunoelectron microscopy. (A) Metal-shadowed images of neonatal-specific antibody 5B4 bound to the COOH-terminal tail of day-12 posthatch myosin (B) When both 5B4 and 12C5 antibodies were added to 31-d myosin, antibody bound either at the head or the tail of individual myosin molecules, but rarely at both sites on the same molecule. (C) Adult-specific antibody 12C5 bound to the head of adult myosin. Arrows point to antibody. Bar, 50 nm.

pared favorably with the amounts determined by densitometric analysis of nondenaturing gels; for example, the concentration of heterodimers by gels was 29% compared to 17% by microscopy (Dechesne et al., 1987). It is unrealistic to expect a better correlation, given the limitations of the technique (high dilution of antibody-myosin complexes used in specimen preparation, shearing forces in spraying molecules onto mica). The method is only possible with very high affinity antibodies, and even then it is not always possible to achieve 100% labeling. Although the electron microscopic technique is not quantitative, it can be used to set limits to the amount of heterodimer present in a given myosin population. Within these constraints it is reasonable to conclude

that there is <20% of this species present during the coexpression of neonatal and adult myosin.

Since nondenaturing gels cannot resolve the developmental myosin isoforms, affinity chromatography was used to fractionate the different myosins (Benfield et al., 1983). The first adult-specific immunoabsorbent (12C5) bound sufficient myosin to account for the amount of adult myosin identified by solution competition ELISA. More important, the bound myosin contained no neonatal epitopes. This experiment alone, therefore, suggested that the concentration of heterodimer, if it exists at all, must be low. When a second adult-specific immunoabsorbent (5C3) was used, the bound myosin now showed some neonatal epitopes, albeit a small amount



**Figure 7.** Histogram of the types of antibody (mAb)-myosin complexes formed at different developmental ages. Antibodies specific for adult (12C5) and neonatal (5B4) myosin were added simultaneously to myosin at each age. At day 12, 98% of the images showed antibody bound exclusively to the tail, which contains the epitope for 5B4, and 2% showed antibody bound to the head, which has the epitope for 12C5 ( $n = 187$ ); at day 19, 77% of the complexes showed antibody bound to the tail, and 19% showed antibody at the head ( $n = 86$ ). At day 31, when both isoforms are present in roughly equal amounts, 54% of the images showed antibody bound to the head, and 42% showed antibody at the tail ( $n = 207$ ). Adult myosin showed antibody bound to the head in 90% of the complexes, and only 6% had antibody on the tail ( $n = 146$ ). Less than 4% of the images at all ages showed antibodies bound simultaneously to the adult and neonatal epitopes. Binding of two antibodies of the same specificity to myosin was uncommon, especially in the case of the anti-head antibody. The degree of labeling of myosin by antibody was between 70 and 90% in all experiments.

as analyzed by competition ELISA. It is difficult to explain the different behavior of the two columns. It is possible that the intrinsic association constant per site of the anti-rod antibody (5C3) is greater than the affinity per site of the anti-head (12C5) antibody for myosin. When homodimers are the interacting species, the antibody can form a bivalent complex by cross-linking the two heavy chains, and thereby acquire an apparent binding constant of high avidity. When heterodimers are present, however, binding must take place via a single site, with an accompanying loss in binding energy. This is not to imply that 12C5 antibody cannot bind a single head; when S1 was applied to the 12C5 immunoadsorbent, ~50% of the S1 bound to the column (unpublished data). Moreover, 12C5 antibody was shown in an earlier study to affect the enzymatic activity of S1, and bind to actin decorated with S1 (Winkelmann and Lowey, 1986). Therefore, we cannot readily account for the inability of the 12C5 immunoadsorbent to bind heterodimeric myosin. It is well known, however, that antibody-antigen interactions can be very dependent on the conditions of the assay, and the behavior of antibodies is not always predictable. Despite these limitations in the immunological approaches used here to analyze heavy chain distribution, we believe the combined evidence strongly supports the conclusion that the stage-specific developmental myosin heavy chains segregate primarily into homodimers, with only a small (~10%) population of heterodimers.

The finding that myosin isolated during development consists primarily of homodimers would be expected if the mRNAs of the different isoforms are spatially segregated in

the muscle fiber, and myosin subunit assembly occurred either on the polyribosome or just subsequent to the release of the nascent peptide. More difficult to understand is the presence of a small, but experimentally significant, amount of heterodimer. The question is raised as to what determines whether the population of heterodimers is very limited, as in developmental myosins, or more abundant, as in the case of rat cardiac myosin.

A clue to the mechanism of heterodimer formation can be found in the recent studies on the assembly of tropomyosin. This molecule is a coiled-coil  $\alpha$ -helix, similar to the myosin rod, which is composed of two subunits  $\alpha$  and  $\beta$ , in proportions that vary depending on the type and source of the muscle (Bronson and Schachat, 1982; Sanders et al., 1986). Tropomyosin from frog muscle has approximately equal concentrations of  $\alpha$  and  $\beta$ , which are present as an  $\alpha_2\beta$  heterodimer in the native molecule; however, equal amounts of  $\alpha_2$ - and  $\beta_2$ -homodimers can be produced in vitro after thermal unfolding and refolding at low temperatures (Lehrer et al., 1989). If these homodimers are incubated above 30°C, the native heterodimer will form by subunit exchange over long time periods. It was concluded that the formation of  $\alpha_2\beta$  is favored thermodynamically, and whatever  $\alpha_2$ -homodimer is present results primarily from an excess of  $\alpha$  over  $\beta$  expression (Lehrer et al., 1989; Bronson and Schachat, 1982). Similar results were obtained by incubating refolded homodimers of chicken gizzard tropomyosin at physiological temperatures (Graceffa, 1989; Lehrer and Qian, 1990); the heterodimer, which is the native species, formed by exchange.

The concept of heterodimer formation by dissociation and reassociation of subunits (Ozeki, S. M. E. Holtzer, and A. Holtzer, 1990. *Biophys. J.* 59:443a [Abstr.]) can be applied to the assembly of myosin from different heavy chain isoforms. Comparison of the complete nucleotide and amino acid sequence of  $\alpha$  and  $\beta$  cardiac myosin heavy chains shows them to be >93% identical; in the rod portion of the sequence, 65 out of 1,099 residues are nonidentical, of which only 20 are nonconservative substitutions (McNally et al., 1989). These differences are clustered in and near the S2 hinge region, within the center of LMM and at the carboxyl terminus of the rod. The COOH-terminal portion, in particular, has been shown to be isoform specific, and contains the epitopes for both the 5C3 and 5B4 antibodies. This high degree of homology between the  $\alpha$  and  $\beta$  chains may explain why cardiac isoforms form approximately equal amounts of heterodimers and homodimers (Dechesne et al., 1987). The  $\alpha_2\beta$  heterodimer would form by chain exchange from  $\alpha_2$ - and  $\beta_2$ -homodimers until an equilibrium distribution is reached that reflects the relative stability of the three species. In contrast, the rod sequences of MHC A and MHC B from *C. elegans* are matched to only ~61%, and form predominantly homodimers (Dibb et al., 1989), presumably because the heterodimer is too unstable. Such an exchange mechanism assumes the existence of a pool of monomeric myosin in equilibrium with filaments. Although a myosin pool has not been demonstrated in situ, there is evidence for myosin exchange from in vitro experiments (Bouché et al., 1988).

The complete rod sequence for neonatal and adult chicken myosin has not been determined, but the degree of homology between these two isoforms may be less than for cardiac myosin, and a reduction in amino acid conservation may result in the relative instability of the heterodimeric species over

homodimers of neonatal and adult myosin. This view is probably too simplistic, however, since homologous chains, as shown for  $\beta_2$ -tropomyosin, are not always the most stable form (Lehrer et al., 1989). Although it is appealing to think of equilibrium thermodynamics as determining the concentration of heterodimers arising from homodimers, we must recognize that no exchange experiments analogous to those reported for tropomyosin exist for myosin. Recent experiments using neonatal and adult myosin chymotryptic rod fragments suggest that homodimers are the thermodynamically stable form (Kerwin and Bandman, 1991). The possibility that biological factors contribute towards determining the subunit composition of myosin cannot, however, be excluded at present.

Although we can determine the distribution of myosin heavy chain isoforms during muscle development, we still don't know the biological significance of their transitory existence. The fast myosin isoforms do not show any significant differences in actin-activated ATPase activity, but a small (<20%) change in activity would probably be overlooked by these measurements (Lowey et al., 1986). New methods for determining movement, such as the in vitro motility assay (Sheetz and Spudich, 1983), or measurements of the maximum velocity of shortening of skinned single muscle fibers (Reiser et al., 1988), may reveal differences between myosin isoforms not readily detected by the more conventional solution approaches.

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