

# Immunochemical Analysis of Myosin Heavy Chain during Avian Myogenesis In Vivo and In Vitro

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**ABSTRACT** Monoclonal antibodies (McAbs) against the myosin heavy chain (MHC) of adult chicken pectoralis muscle have been tested for reactivity with pectoralis myosin at selected stages of chick development in vivo and in vitro. Three such McAbs, MF 20 and MF 14, which bind to light meromyosin, and MF 30, which binds to myosin subfragment two (S<sub>2</sub>), were used to assay the appearance and accumulation of specific MHC epitopes with: (a) indirect, solid phase radioimmune assay (RIA), (b) immunoautoradiography, (c) immunofluorescence microscopy. McAb MF 20 bound strongly and equivalently to MHC at all stages of embryonic development in vivo. In contrast, the MF 30 epitope was barely detectable at 12 d of incubation but its concentration rose rapidly just before hatching. No detectable binding of MF 14 to pectoralis myosin could be measured during myogenesis in vivo until 1 wk after hatching. Immunofluorescence studies revealed that all three epitopes accumulate in the same myocytes of the developing pectoralis muscle. Since all three McAbs bound with high activity to native and denatured forms of myosin, it is unlikely that differential antibody reactivity can be explained by conformational changes in myosin during development in vivo. When myogenesis in vitro was monitored using the same McAbs, MF 20 bound to the MHC at all stages tested while reactivity of MF 30 and MF 14 with myosin from cultured muscle was never observed. Thus, this study demonstrates three different immunochemical states of the MHC during development in vivo of chick pectoralis muscle and the absence of later occurring immunochemical transitions in the MHC of cultured embryonic muscle.

Isoforms of myosin have been demonstrated in adult (1, 3, 10, 19, 28, 37) and embryonic (11, 12, 20, 27, 30, 35, 36) striated muscles of both birds and mammals. Three experimental formats have been used to substantiate these myosin variants within embryonic muscles. First, heterogeneity of myosin can be detected by electrophoresis on native pyrophosphate gels (14). Secondly, differences exist in the peptide maps obtained by limited proteolysis of adult and embryonic myosins (27, 35, 36). Finally, immunological dissimilarities exist between myosins isolated from homologous adult and embryonic muscles (11, 12, 20, 26, 35, 36). Masaki and Yoshizaki (20) have demonstrated that myofibrils from embryonic chick pectoralis muscle bind antibodies specific for myosin of adult cardiac muscle, whereas similar myofibrils from adult pectoralis muscle do not. It was later proven that these cross-reactive determinants reside in the myosin heavy chain (MHC) subunit. More

recently, Gauthier et al. (11, 12) and Rubinstein and Kelley (26) have shown that developing rat skeletal myofibers contain MHCs which share antigenic homology with myosin isolated from both adult fast- and slow-twitch muscles. However, the adult myofibers bound specifically to either the "anti-slow" myosin or "anti-fast" myosin antiserum preparations. Immunochemical differences between myosin preparations purified from homologous embryonic, neonatal and adult rat muscles have also been demonstrated by Whalen et al. (36).

Until recently, immunochemical studies of myosin have been limited by the heterogeneous nature of the antisera even after extensive cross-absorption. Such heterogeneity severely hampers the detection and quantitation of subtle changes within specific domains of the MHC during development. With the advent of monoclonal antibody (McAb) technology, it is now feasible to generate homogeneous populations of antibodies

the epitopes<sup>1</sup> of which can be mapped to specific domains of a macromolecule. To this end, McAbs against adult chicken pectoralis myosin have been used to analyze immunological changes in the MHCs of the pectoralis muscle during development *in vivo* and *in vitro*. Our results demonstrate two transitions among three antigenic states of the MHC during myogenesis *in vivo*. Evidence is presented that these transitions represent changes in MHC primary structure rather than conformational changes of the myosin molecule. In contrast to myogenesis *in vivo*, these immunochemical transitions in the MHC were not observed during myogenesis *in vitro* even after long periods of cell culture.

## MATERIALS AND METHODS

### Protein Preparation

Myosin was prepared from the pectoralis muscle of adult chickens and of developing chickens at selected days before and after hatching, and from primary muscle cell cultures. Muscles from developing birds were homogenized in 100 mM NaCl, 10 mM sodium phosphate buffer, 0.1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, MO), pH 7.4, in a Waring blender at 4°C. After low-speed centrifugation (16,000 g for 10 min), the pellet was resuspended in 0.6 M NaCl, 10 mM sodium phosphate buffer, 0.1 mM PMSF, pH 7.4 (1:3, initial muscle wt/vol), extracted for 12 min, and centrifuged (16,000 g for 10 min). The resulting supernatant was brought to  $i < 0.05$  with cold distilled water and centrifuged (16,000 g for 10 min) to collect the crude myosin pellet. The precipitated myosin was resuspended in 0.4 M NaCl, 10 mM sodium phosphate buffer, 0.1 mM PMSF, pH 7.4 and, after the addition of MgCl<sub>2</sub> and sodium ATP to 5 mM, spun at 160,000 g for 1.5–2 h. The supernatant was collected and subjected to one additional low salt-high salt cycle and stored in 0.3 M NaCl/50% glycerol at –20°C. Protein concentrations were determined by the Lowry technique (18), and purity of the preparations was estimated by SDS PAGE. Myosin was prepared from the adult pectoralis muscle (major and minor without the red strip) by mincing the tissue in a meat grinder followed by immediate extraction in Guba-Straub solution (0.3 M KCl, 0.1 M KH<sub>2</sub>PO<sub>4</sub>, 50 mM K<sub>2</sub>HPO<sub>4</sub>, 1:3 wt/vol) for 15 min at 4°C. Extraction was terminated by threefold dilution in water. After filtration through gauze and precipitation by dilution ( $i < 0.05$ ), myosin was collected by centrifugation (14,000 g for 10 min). The pellet was resuspended in 0.5 M KCl, 5 mM MgCl<sub>2</sub>, 5 mM ATP from a 100 mM stock (pH 7.0) and cleared by centrifugation at 140,000 g for 2 h. Myosin was recovered from the resulting supernatant by centrifugation (14,000 g for 10 min) after low ionic strength precipitation, redissolved in 0.6 M KCl, and stored in 0.3 M KCl/50% glycerol at –20°C. For preparation of myosin from primary cell cultures, the monolayers were extracted on ice for 1–3 min with 1% Triton X-100 in 150 mM NaCl, 10 mM sodium phosphate, 0.1 mM PMSF, pH 7.5. For each preparation, ten 100-mm plates were scraped, cytoskeletons were disrupted in a Dounce homogenizer (7 ml, 20 strokes), and the resulting supernatant was centrifuged (16,000 g for 10 min). The pellet was extracted in 0.6 M NaCl, 10 mM sodium phosphate, 5 mM MgCl<sub>2</sub>, 5 mM ATP, 0.1 mM PMSF, pH 7.2, for 15 min (5 ml/10 100-mm plates). Extraction was terminated by centrifugation (16,000 g for 10 min) and the supernatant containing myosin was subjected to one low salt-high salt cycle. These preparations were used for radioimmunoassay and immunautoradiography. Chymotryptic subfragments of adult pectoralis myosin were prepared (as described in reference 34) and were used in RIA experiments.

### Monoclonal Antibodies

Noncolumn-purified, adult chicken pectoralis myosin was used as the immunogen with or without prior SDS treatment (17). C3H or BALB/C mice were immunized subcutaneously and intraperitoneally with 100 µg of adult chicken pectoralis myosin emulsified in complete Freund's adjuvant followed by two injections of the same protein sample (100 µg) suspended in incomplete Freund's adjuvant. Isolated splenocytes were fused with a P3U-1 myeloma cell line (the gift of Dr. J. Unkeless, The Rockefeller University, New York, NY) according to Galfré et al. (9) as modified by Geffer et al. (13). Hybridoma cells were screened for anti-myosin immunoglobulin secretion with an indirect, solid phase radioimmuno assay. Positive wells were cloned twice in soft agarose (Sea Plaque).

<sup>1</sup> Epitope: Structural unit within an antigen recognized by a clonal immunoglobulin.

## Primary Cell Culture Techniques

Cell cultures were prepared from pectoralis muscles of 11-d chicken embryos using trypsin (29) or mechanical (4) dissociation at a density of  $3 \times 10^6$  cells/100-mm Falcon tissue culture dish (Falcon Labware, Oxnard, CA) coated with 1% gelatin. No differences in the immunochemical properties of the two preparations were observed. The culture medium consisted of Dulbecco's modified Eagle's medium (MEM; Gibco, Grand Island Biological Co., Grand Island, NY), glucose (4.5 g/liter), horse serum (10%; Gibco lot #34P5105) and chick embryo extract (5%). Cultures were maintained in 5% CO<sub>2</sub> and medium replaced every other day.

## Solid Phase Radioimmunoassay

A radioimmunoassay (RIA) was used for screening cell line supernatants for immunoglobulin production and for subsequent antibody titrations. A known concentration of myosin in 0.4 M NaCl was immobilized by drying in polyvinyl chloride microtiter wells. The microtiter wells were rinsed in 150 mM NaCl, 10 mM sodium phosphate, pH 7.4 (PBS), followed by another wash in PBS containing 0.1% bovine serum albumin (BSA, Sigma Chemical Co., Cohn Fraction V), and blotted dry. 30 µl of conditioned media were added to the microtiter wells for 30 min at room temperature, the wells were washed with PBS and subsequent PBS/0.1% BSA solutions, and then reacted with 50 µl of an affinity-purified <sup>125</sup>I-labeled goat anti-mouse IgG (GAM, Cappel Laboratories, Cochranville, PA, 50,000 cpm/well) as described by Reinach et al. (24). Bound <sup>125</sup>I-GAM was assayed by  $\gamma$ -spectrometry after extensive washing in PBS. The conditions of this assay system provide a qualitative indication of the amount of antigen present (22). A McAb against dog kidney-cell surface antigens (the gift of Drs. D. Herzlinger and G. Ojakian) was used as a control. Backgrounds were routinely less than 200 cpm/well.

## Immunautoradiography After SDS PAGE (Immunoblots)

Myosin samples were separated by electrophoresis in 7.5% SDS PAGE using the buffer system of Laemmli (16), and the protein subunits were electrophoretically transferred to nitrocellulose paper according to Towbin et al. (32) using 40 mM Tris, 240 mM glycine, 20% methanol as the transfer buffer with a constant current of 100 mA for 3 h. SDS (0.1%) was added to the cathodal backing sponge and filter paper to facilitate transfer of the MHC. After incubation for 10 min in PBS/1% BSA, the nitrocellulose papers were reacted sequentially with McAb (5 min) and <sup>125</sup>I-GAM (5 min) and air dried after extensive PBS washing. Autoradiograms were produced with Kodak X-ray film X-OMAT-AR5 (Eastman Kodak) after 6- to 48-h exposures with an image intensifying screen (Dupont Lightning Plus). To insure that equivalent amounts of MHC were used in immunautoradiographic experiments, samples were displayed in 7.5% gels, stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA), 0.4% in 7% acetic acid/50% methanol for 15 min, and destained with 7% acetic acid overnight. The 200-kdalton (kd) MHC band was cut from gels and extracted overnight in 25% pyridine (8). Extracted dye was measured electrophoretically at 605 nm, and sample concentrations were adjusted accordingly.

## Immunofluorescence Study of Developing Muscle Cells *In Vivo* and *In Vitro*

Transverse sections (10 µm) of the pectoralis muscle from chick embryos and from posthatch and adult chickens were cut at –20°C, affixed to glass slides, and treated with acetone (–20°C) for 1 min. Muscle cell cultures grown on 1% gelatinized polystyrene coverslips (30 mm, Flow Laboratories, Rockville, MD) were extracted with 0.1% Triton X-100 in PBS for 1 min, followed by fixation in 100% methanol for 2 min. From this point, sections and coverslips were treated identically for immunofluorescence. Samples were washed with PBS/1% BSA and reacted sequentially with McAb and fluorescein-labeled GAM (FITC-GAM, Cappel Laboratories; commercial FITC-GAM was affinity purified with a Sepharose 4B-mount IgG column in our laboratory). PBS washes followed both antibody treatments. Samples were fixed in 4% formalin before mounting in 90% glycerol, 10 mM Tris HCl (pH 8) and examined by epifluorescence microscopy. Controls were similar to the RIA studies.

## RESULTS

### McAb Reactivity with Adult Myosin

The three McAbs used in this study were generated in two separate cell fusions, one with splenocytes from a C3H mouse,

the other with a BALB/c mouse. The former immunization, resulting in clones MF 14 and MF 20, used adult pectoralis myosin solubilized in high salt buffer and then emulsified in Freund's adjuvant. MF 30 resulted from an immunization with identical myosin but first denatured in 1% SDS/5%  $\beta$ -mercaptoethanol (17). All three McAbs react with the MHC (Fig. 1), while occasional and slight reactivity with smaller molecular weight bands is thought to be due to McAb binding to cleavage products of the MHC in stored myosin samples. Chymotryptic subfragments of myosin were reacted with the three McAbs by RIA and, as shown in Fig. 2, MF 14 and MF 20 reacted strongly with rod and LMM whereas MF 30 bound to rod and subfragment 2 (S-2). The slight cross-reactivity of MF 20 with the S-2 preparation was due to minor contamination of S-2 with LMM peptides as revealed by immunoblots with MF 14 and MF 20 (Bader, unpublished results). Immunofluorescence studies of adult pectoralis muscle have proven that all myofibers and all A-bands of all myofibrils react with these McAbs (data not shown).

Immunofluorescence microscopy, RIA and immunofluorography were used to establish exclusive binding of these McAbs to striated muscle, to the protein myosin, and to the MHC subunit. When myosin from the pectoralis muscle of adult chicken was displayed by SDS PAGE, and MHC subunits were transferred to nitrocellulose, all three McAbs bound to the 200-kd MHC peptide (Fig. 1). If a fixed amount of adult myosin was reacted by solid phase RIA with serial dilutions of these three McAbs, each antibody exhibited a similar plateau binding value of  $^{125}$ I-GAM at high titer levels of McAb (Fig. 3). Furthermore, each had half-maximal binding values at nearly equivalent dilutions of the McAb solutions. If one interprets the half-maximal binding value for each McAb as an index of its relative affinity for its epitope, then it is likely that the epitopes bound by that antibody during development are identical to those in adult MHC.

To determine whether antigen recognition by the McAbs was dependent on higher order structure of the myosin molecule, myosin from the adult pectoralis muscle was denatured by boiling the protein in 1% SDS/5%  $\beta$ -mercaptoethanol or dissolving it in 8 M guanidine HCl. These samples were then dried in microtiter wells at known concentrations, and the excess denaturant was removed by extensive washing with PBS. Reactivity of the McAbs to denatured myosin was then compared with antibody binding to untreated myosin by solid phase RIA (see Materials and Methods). As shown in Fig. 4, MF 20 bound strongly to denatured and nontreated myosin preparations. McAbs MF 14 and MF 30 also exhibited high binding for the denatured myosin samples although more variability in the saturation binding values was observed than

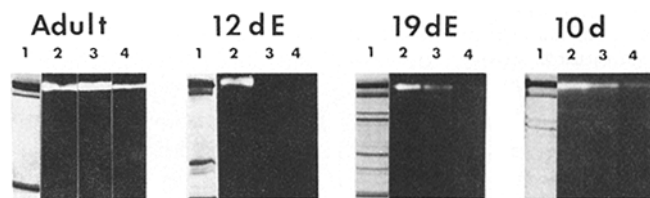


FIGURE 1 Immunofluorography of myosin from adult, 12 and 19-d embryonic and 10-d posthatching chicken pectoralis muscles. Immunoblots with MF 20 (2), MF 30 (3) and MF 14 (4) were performed as described in Materials and Methods. The amount of protein added to each gel was adjusted so that equivalent amounts of MHC ( $\pm 12\%$ ) was present as determined according to (8).

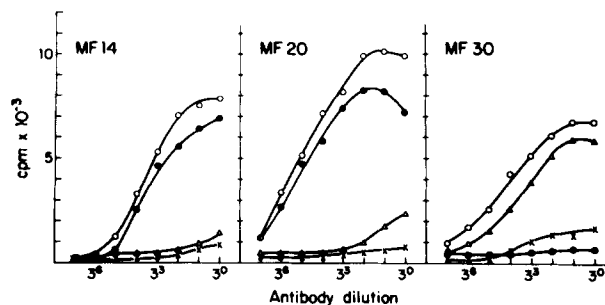


FIGURE 2 RIA analysis of chymotryptic subfragments of adult myosin. McAbs in 1:3 dilutions from supernatant were reacted with 1  $\mu$ g of each antigen in solid phase, indirect RIA. Rod (O), S1 (X), LMM (●), and S2 ( $\Delta$ ) were prepared according to (34).

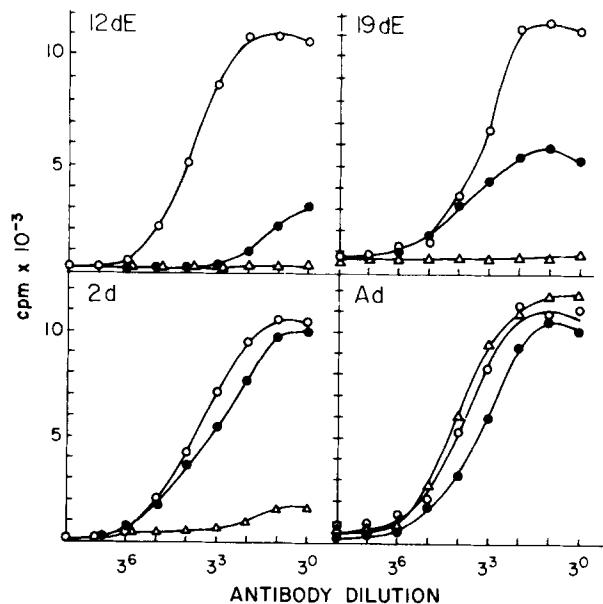


FIGURE 3 RIA analysis of myosin with MF 20, MF 30, and MF 14. 1  $\mu$ g of myosin from 12 and 19-d embryonic, 2-d posthatching and adult pectoralis muscles was reacted with McAbs in 1:3 dilutions of the hybridoma supernatants. All points represent the average of two analyses. MF 20 (O); MF 30 (●), and MF 14 ( $\Delta$ ).

with MF 20. We interpret these results to indicate that higher order conformation of the myosin molecule is not a major factor in determining the specificity of these three McAbs for their respective epitopes.

### Immunochemical Analysis of Myosin during In Vivo Myogenesis

To compare the reactivities of MF 20, MF 30, and MF 14 with myosin during development, this protein was prepared from pectoralis muscles at 12, 15, 17, and 19 d of egg incubation and 2, 10, 14, and 28 d posthatching. The samples were then tested for McAb binding by solid phase RIA and immunofluorographic procedures. In the RIA experiments, fixed concentrations of the various myosin samples were immobilized to the microtiter wells and reacted with serial dilutions of each McAb followed by  $^{125}$ I-GAM. The half-maximal and saturation-binding values were then compared for each of the assay curves (Fig. 3). McAb MF 20 reacted equivalently with myosin at all stages of development; and no significant differences were seen in plateau values of  $^{125}$ I-GAM bound to the microtiter wells at saturating levels of MF 20. Thus, the epitope

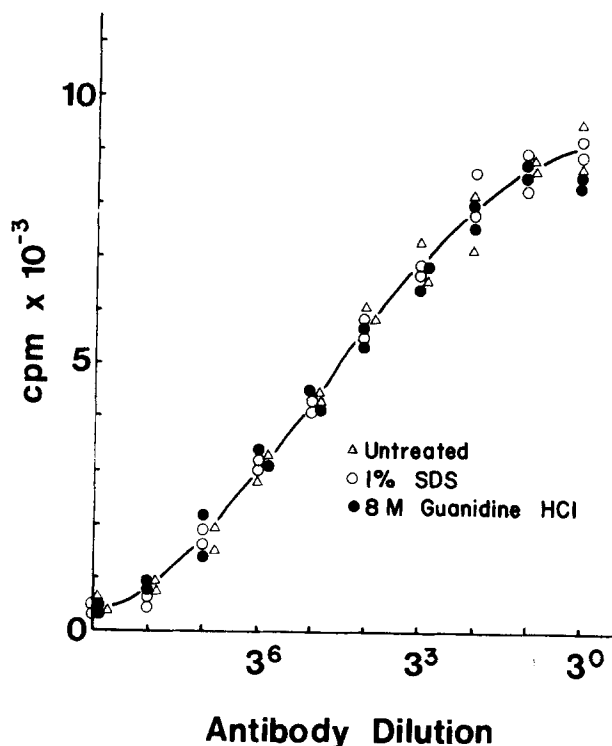


FIGURE 4 RIA analysis of 1% SDS denatured, 8 M guanidine-HCl denatured, and untreated adult chicken pectoralis myosin with MF 20. 1  $\mu$ g of myosin with or without prior denaturation was reacted with MF 20 (1:3 dilutions from supernatant) and McAb binding measured by RIA. All myosin samples were dissolved in 0.6 M NaCl and either plated in microtiter wells directly ( $\Delta$ ) or denatured in 1% SDS/5%  $\beta$ -mercaptoethanol ( $\circ$ ) or 8 M guanidine HCl ( $\bullet$ ).

specified by MF 20 in the LMM region of myosin is present in embryonic, posthatch and adult MHC, presumably at equivalent molar values. In contrast, no significant binding of MF 14 was observed with pectoralis myosin from 12-d embryos, although low but definite reactivity of MF 30 was detected with high concentrations of this McAb (Fig. 3). With progressive embryonic development there was increased binding of MF 30 to breast myosin; by 19 d of egg incubation, ~40% of adult saturation binding with this McAb was observed, and 90% of adult values were seen by 2 d after hatching. A different time course of epitope appearance was noted with MF 14. This McAb did not react with pectoralis myosin preparations until the first week after hatching, and saturation binding curves equivalent to adult myosin were not seen until 28 d posthatching. These results demonstrate the independent appearance of three separate epitopes on the MHC, that specified by MF 20, followed by that for MF 30, and lastly the binding site for MF 14.

Immunoprecipitation was done to establish whether epitopes recognized by these McAbs resided on the MHC or on some other myofibrillar protein in the developing pectoralis muscle, and, in addition, to confirm the results obtained by the RIA experiments. Immunoblots of 12-d embryonic myosin demonstrated the specificity of MF 20 for the MHC and the absence of MF 30 and MF 14 binding to this preparation (Fig. 1). When myosin prepared from embryonic pectoralis muscles at 19 d of incubation was used as the test antigen, significant reactivity of MF 20 and MF 30 was observed whereas MF 14 binding remained negative (Fig. 1). No binding to the MHC of 2-d posthatching pectoralis muscles was seen with MF 14, but

this antibody bound significantly to MHC from 10-d posthatch chickens (Fig. 1). Thus, these results confirmed the RIA analyses and demonstrated the specificity of the McAbs for MHCs at all stages of breast muscle development.

Immunofluorescence microscopy was undertaken to determine the cellular distribution of MHCs recognized by the three McAbs during myogenesis *in vivo*. As previously stated, all muscle fibers of the adult pectoralis muscle contain MHC recognized by MF 20, MF 30, and MF 14. When tissue sections of the 12-d embryonic muscle were reacted with the three McAbs, the developing muscle cells were strongly reactive with only MF 20 (Fig. 5), and it appeared, although not proven definitively, that all muscle cells bound this antibody. While most muscle cells of the 12-d pectoralis muscle were completely negative when treated with MF 30, weak fluorescence was detected in some myogenic cells, and by 19 d of incubation, all pectoralis myofibers were strongly positive for MF 30 (Fig. 5). MF 14 did not bind to any embryonic pectoralis muscle cells, and only by 7 d after hatching was definite fluorescence observed (Fig. 5). These results demonstrate histochemically that all or nearly all pectoralis myofibers undergo the epitope transitions described above.

#### Immunochemical Analysis of Myosin during *In Vitro* Myogenesis

When MF 20 was reacted with myosin prepared from cultures at 3, 6, 10, and 28 d after plating, strong binding was observed (Fig. 6). Immunoprecipitation demonstrated the specificity of MF 20 for the 200-kd MHC of cultured muscle (Fig. 7). In contrast, MF 30 bound with low apparent affinity to myosin isolated from 3-d cultures and, thereafter, MF 30 binding was always <25% of the adult value. Immunoprecipitation of MF 30 with myosin from cultured muscle were negative (Fig. 7). MF 14 binding was always <10% of adult values in RIA, and immunoprecipitation with MF 14 proved negative (Figs. 6 and 7).

Immunofluorescence studies demonstrated the specificity of MF 20 for cultured myotubes and the positive reactivity of all myotubes at all stages when tested with this McAb (Fig. 8). Conversely, MF 30 and MF 14 were always negative for myotubes in parallel cultures even after 28 d in culture. Thus, the transitions in immunochemical properties of the MHC observed during myogenesis *in vivo* were not observed during myogenesis *in vitro* under our standard culture conditions.

#### DISCUSSION

By a variety of immunochemical procedures, we have demonstrated that breast muscle from 12-d-old chick embryos contains little or no myosin with the antigenic properties of the adult protein. Evidence was presented that the antigenic differences between the adult and embryonic myosins are likely to reside in primary structure of the molecule, at least in the MHC subunit. Previous investigations with polyclonal antisera have demonstrated that antibodies raised against adult fast, slow, and cardiac muscle myosin cross-react with myosin from embryonic muscles (11, 12, 20, 26, 35, 36). Peptide maps of adult and embryonic fast MHC (27) or whole myosin (35, 36) also provide evidence for both primary sequence homologies and differences. In the present study, McAb MF 20 was shown to likely recognize a primary sequence determinant common to both embryonic and adult pectoralis MHC, and other studies have proven this epitope to reside in LMM. In contrast, the

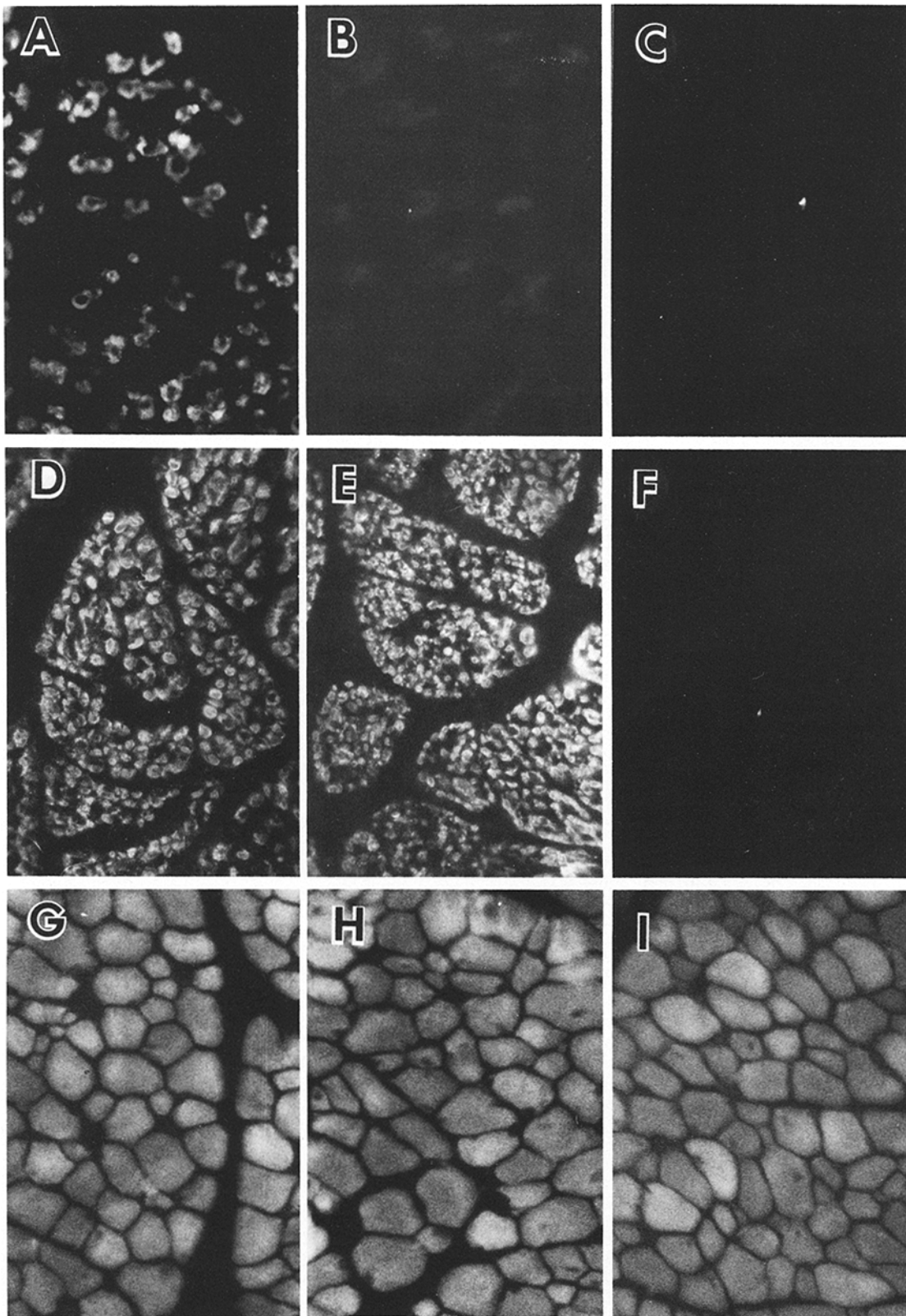


FIGURE 5 Indirect immunofluorescence microscopy of pectoralis muscle at different stages of development stained with McAbs MF 20, MF 30, and MF 14. 12-d embryonic myocytes react strongly with MF 20 (A), weakly if at all with MF 30 (B), and not at all with MF 14 (C). At 19-d of egg incubation, MF 20 (D) and MF 14 (E) react with muscle cells while MF 14 (F) is still negative. By 14 d after hatching pectoralis muscle cells are reactive with MF 20 (G), MF 30 (H), and MF 14 (I).  $\times 250$ .

binding site for MF 14 is absent from 12-d-old embryonic breast myosin, and the binding site for MF 30 must be in very low concentration or in a form which confers very low antibody

affinity. Since the latter two epitopes also appear to be predominantly primary sequence in nature, we suggest that primary structural changes must occur in the MHC in both S-2 and

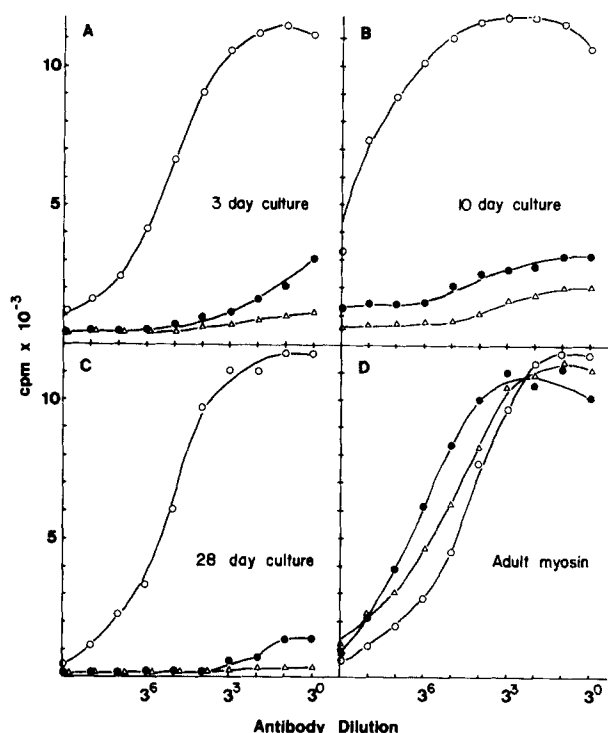


FIGURE 6 RIA analysis of myosin from cell cultures at 3, 10, and 28 d after plating with MF 20, MF 30, and MF 14. 1  $\mu$ g of myosin from cell cultures and adult pectoralis muscle was reacted with MF 20 (○), MF 30 (●), and MF 14 (△) in 1:3 dilutions of hybridoma supernatants.

LMM subfragments of the molecule during the course of muscle development.

The sequential changes in the MHC recognized by MF 20, MF 30, and MF 14 can be explained by several different hypotheses. First, it is possible that three different MHC genes are expressed in muscle development. Using immunological and biochemical means, Whalen et al. (36) have provided evidence that three different MHC isoforms are sequentially expressed during rat skeletal myogenesis and from these data proposed that embryonic, neonatal, and adult MHCs are different gene products. Evidence that embryonic pectoralis muscle contains a MHC mRNA different from that in the adult muscle has recently been reported by Bandman et al. (2). In this scheme, MF 20 would bind to embryonic, posthatch and adult MHCs, MF 30 would bind to posthatch and adult MHC, and MF 14 would recognize only adult MHC. Secondly, one might consider differential posttranslational modifications. Trayer et al. (33) and Huszar (15) have demonstrated selective posttranslational modification of the MHC in adult rabbit muscle and the absence of this modifying system in embryonic muscle. Since slight alterations in the primary structure of the antigen can dramatically affect its recognition by McAbs, the immunological changes in the MHC observed during myogenesis may reflect posttranslational modifications of the MHC. This explanation would require the sequential appearance of at least two different and selectively modifying systems in developing muscle. Finally, although it is widely assumed that MHC in adult pectoralis is homogeneous, if different heavy chains exist, the age-dependent, differential binding of the McAbs may represent the noncoordinate expression of separate "adult-type" MHC isoforms. Previous work (23, 31) has dem-

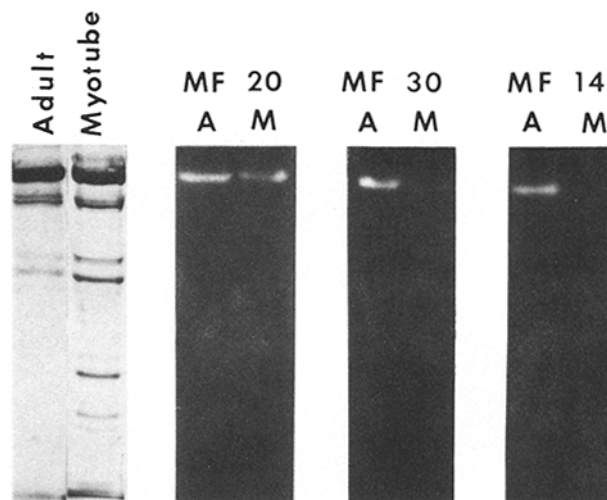


FIGURE 7 Immunoblotting of myosin from adult chicken pectoralis muscle and muscle cell cultures 10-d after plating with MF 20, MF 30, and MF 14. The gel lanes at the left are crude myosin preparations of adult muscle and myotube cultures stained with Coomassie Brilliant Blue. The same myosin samples were used for the accompanying immunoblots.

onstrated microheterogeneity in the amino terminal region of MHCs isolated from rabbit back muscle, and Zweig (39) has recently proven that MHCs from two different adult rabbit fast muscles contain unique MHC isoforms. In addition, immunological evidence for subfragment-one heterogeneity has been reported (38).

The failure of cultured embryonic muscle cells to undergo the immunochemical transitions in the MHC recognized by MF 30 and MF 14, whether these differences reflect posttranslational modifications of the MHC or the expression of a new MHC gene product, provides strong evidence that the intrinsic, morphogenetic program of developing skeletal myocytes is not sufficient to direct their full terminal differentiation in monolayer culture. Several extrinsic influences such as innervation have been implicated in directing isoformic transitions of myosin *in vivo* (11, 36) and it is conceivable that the addition of as yet uncharacterized components to muscle cultures might serve to trigger the expression of specific MHC isoforms *in vitro*. It has been demonstrated that several structural genes encoding for myofibrillar proteins are coordinately expressed during early myoblast differentiation in cell cultures lacking nerve (5, 6). It now remains to be seen whether later biochemical and morphogenetic events of terminal muscle development (7, 21, 25) require environmental interactions with other cell types, extracellular matrices, or hormones for their normal appearances.

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## Phase

## Fluorescence

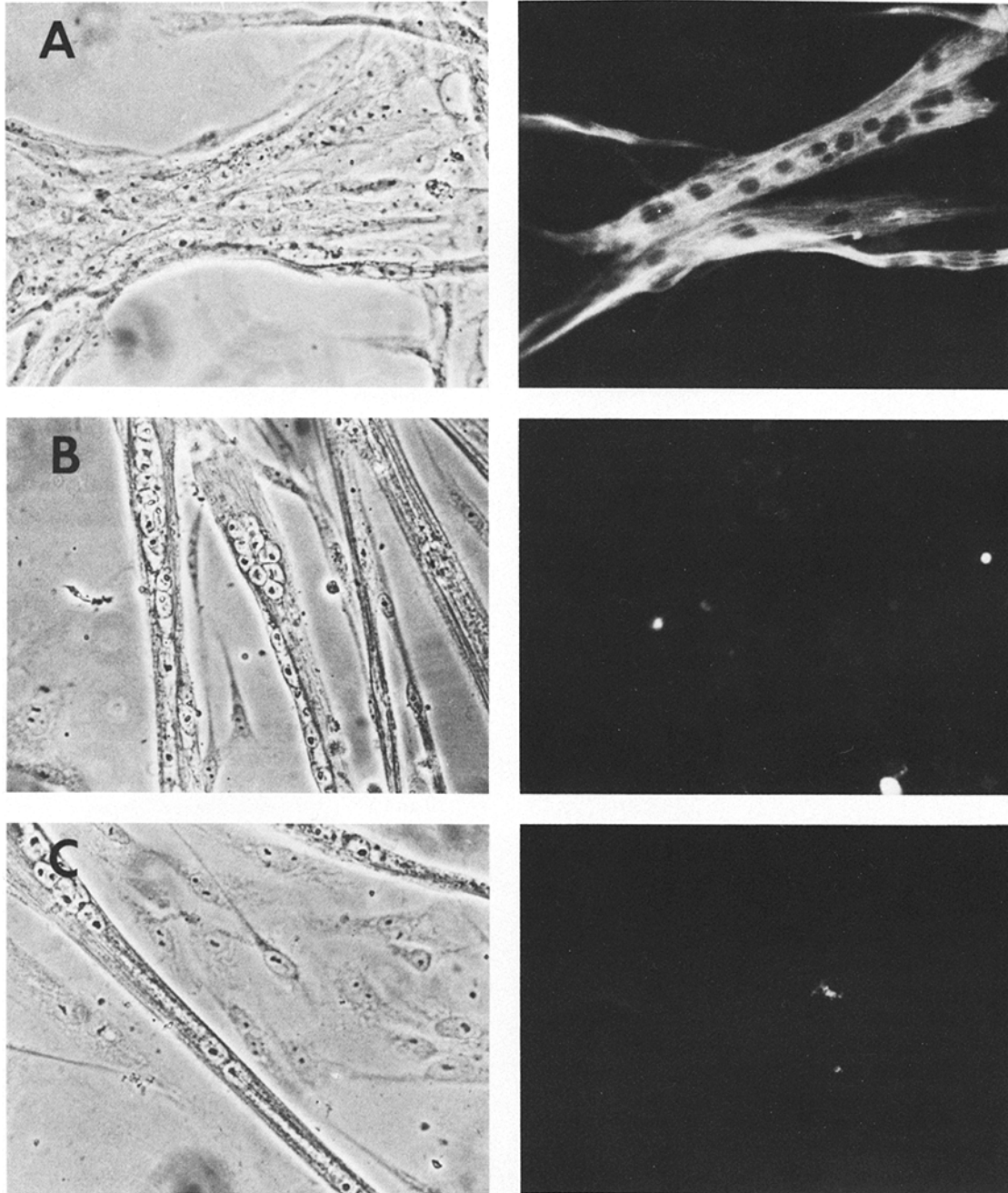


FIGURE 8 Indirect immunofluorescent analysis of cultured muscle cells 8 d after plating reacted with McAbs MF 20 (A), MF 30 (B), and MF 14 (C). Phase contrast photomicrographs are presented at the left, and the fluorescent micrographs are seen at the right.  $\times 250$ .

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