

Myosin Light-chain Expression during Avian Muscle Development

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ABSTRACT Monoclonal antibodies to adult chicken myosin light chains were generated and used to quantitate the types of myosin light-chain (MLC) isoforms expressed during development of the pectoralis major (PM), anterior latissimus dorsi (ALD), and medial adductor (MA) muscles of the chicken. These are muscles which, in the adult, are composed predominantly of fast, slow, and a mixture of fiber types, respectively. Three distinct phases of MLC expression characterized the development of the PM and MA muscles. The first identifiable phase occurred during the period of 5–7 d of incubation *in ovo*. Extracts of muscles from the pectoral region (which included the presumptive PM muscle) and early limb bud or thigh (which included the presumptive MA muscle) contained only fast MLC isoforms. This period of exclusive fast light-chain synthesis was followed by a phase (8–12 d of incubation *in ovo*) in which coexpression of both fast and slow MLC isoforms was apparent in both PM and MA muscles. During this period, the composition of fast and slow MLC isoforms in the PM and MA muscles was identical. Beginning at day 12 *in ovo*, the ALD was also subjected to immunochemical analyses. The proportion of fast and slow MLCs in this muscle at day 12 was similar to that present in the other muscles studied. The third development phase of MLC expression began at ~12 d of incubation *in ovo* and encompassed the transition in MLC composition to the isoform patterns typical of adult muscle. During this period, the relative proportion of slow MLC rose in both the MA and ALD and fell in the PM. By day 16, the third fast light chain, LC_{3f}, was apparent in extracts of both the PM and MA. These results show that there is a developmental progression in the expression of MLC in the two avian muscles studied from day 5 *in ovo*; first, only fast MLCs are accumulated, then both fast and slow MLC isoforms are expressed. Only during the latter third of development *in ovo* is the final MLC isoform pattern characteristic of a particular muscle type expressed.

The diversity of adult skeletal muscle fiber types arises, in part, from differences in the contractile protein isoforms present in individual fibers (2, 10, 11). With respect to myosin, two isoform types are recognized and designated as fast and slow (2, 3, 18). Although exceptions do exist (10, 11, 20; this report), individual adult muscle fibers generally express only one of these isoform types.

The nature of the myosin isoforms expressed by developing muscle and the relationship of this pattern of expression to the diversity of fiber types seen in the adult is a matter of controversy. Some investigators (28) contend that all muscle fibers, regardless of their anatomic origin or adult pattern of expression, initially synthesize only fast isoform types. This contention is based predominantly, although not exclusively, on the staining patterns of early fibers with antisera directed against

adult myosin. Yet, others have reported that early embryonic muscles contain both fast and slow isoform types. Their conclusions are based on the identification of myosin light chains in muscle extracts by either gel electrophoresis (22, 31) or immunoelectrophoresis with antisera to the MLCs (22) and also on the staining pattern of embryonic fibers with antisera to adult myosin (12).

The discrepancy between various laboratories may be related to the animal species employed, to the developmental ages studied, and to quantitative differences in the affinity of various antisera for the myosin isoforms. In addition, it is becoming increasingly clear from independent studies on myosin heavy-chain isoforms that distinct embryonic forms exist in developing muscle (37, 38). Conclusions about the myosin isoform composition of fibers may be misleading when they are based

solely on immunocytochemical reactions, since the antibodies may cross-react with the embryonic myosin heavy-chain isoforms.

To circumvent some of these difficulties, we have examined the isoform composition in developing muscles of the chicken using monoclonal antibodies directed solely against the myosin light chains (MLCs). We have characterized the specificity of these antibodies using a technique which does not require the antigen in a purified form (Western blotting; 7). The use of this technique and the fact that monoclonal hybridomas secrete a single antibody species means that no assumptions need to be made concerning the purity or isoform composition of the myosin used to generate or purify the antibodies. With antisera, such assumptions must be made when adsorbing determinants common to all myosin types. We have studied chicken muscle development since, unlike developing mammalian muscle in which distinct embryonic isoforms of both heavy and light chain exist (36, 37), the light chains of adult and embryonic avian muscle are indistinguishable on the basis of both electrophoretic behavior and immunological cross-reactivity (15, 30, 31). Finally, we have employed these antibodies in quantitative immunoassays thereby permitting calculation of both absolute and relative amounts of the various light chains during muscle development.

Our results show that those muscle groups of the pectoral and hindlimb region that contained skeletal muscle specific MLC at 5–7 d *in ovo* expressed only the fast MLC isoforms. Subsequently, the differentiation of both the PM and MA was characterized by a period (8–12 d) during which both fast and slow MLC isoforms were synthesized. The transition in isozyme expression to the specific pattern characteristic of the adult began as early as day 12 and was virtually complete by day 16.

MATERIALS AND METHODS

Muscle Proteins and Cellular Extracts: Myosin from adult White Leghorn chickens was obtained by repeated cycles of high salt solubilization-low salt precipitation as described by Trayer and Perry (32). Myosin light chains were obtained from the partially purified myosin by the procedure of Perrie and Perry (26). Myosin was extracted from adult pectoralis major (PM), anterior latissimus dorsi (ALD), and the medial adductor (MA) muscles. These muscles are composed predominantly of fast, slow, and a mixture of fiber types, respectively.

These same muscles were identified and dissected from chick embryos of the appropriate ages. The total muscle proteins were either solubilized in SDS lysis buffer (18) or subjected to myosin extraction (32) before analysis. The PM and MA muscles were studied from day 8 of incubation through hatching and in the adult, while the ALD, because of its small size, was studied only from day 12 through hatching and in the adult. To obtain tissue for analysis from younger embryos (5–7 d), we studied the entire musculature of the pectoral region and upper hindlimb (or limb bud). All dissections excluded the "red" portion of the PM adjacent to the clavicle (10, 14).

Monoclonal Antibody Production: Monoclonal antibodies to chicken myosin light chains were produced by hybridoma formation between the spleen cells of BALB/c mice immunized with purified light chain fractions from either the PM or MA muscles and a nonsecreting myeloma cell line (P3-NS1/1Ag4-1). Immunization, hybridoma fusion, and selection were performed according to a modification of procedures of Oi and Herzenberg (23). The supernatants from the hybridoma cultures were initially screened in a plate-binding assay using purified light-chain fractions passively adsorbed to polyvinylchloride microtiter plates (33). Positive hybridoma supernatants were further screened on electrophoretic transfers of SDS PAGE fractionated proteins (see below). Hybridomas with positive supernatants were subcloned twice by limiting dilution on a thymocyte feeder layer (23). Supernatants from the subcloned cultures were used as the source of antibody throughout this study.

Gel Electrophoresis and Electrophoretic Transfer of Proteins to Nitrocellulose: One-dimensional SDS PAGE was performed as described by Laemmli (17) on 12.5% gels. The electrophoretic transfer of proteins from SDS gels to unmodified nitrocellulose was performed overnight at 8–9 V/cm, as described by Burnette (7), in a buffer consisting of 10 mM

cyclohexylaminopropane sulfonic acid (pH 11.0) and 20% methanol. For immunodetection of the transferred proteins, the procedure of Burnette (7) was followed, except that the second antibody was linked to horseradish peroxidase [HPO-rabbit anti-mouse immunoglobulin (Dako, Accurate Scientific Corp., Santa Barbara, CA)]. Antigen-antibody complexes were visualized by reacting the bound HPO with diaminobenzidine and H_2O_2 (13).

Immunocytochemistry: Tissues were frozen in melting isopentane, and serial transverse sections were cut at 10 μ m and transferred to gelatin-coated glass slides. The sections were air-dried for 1 h at room temperature and fixed with N-ethyl-N'-(3-dimethyl-aminopropyl)-carbodiimide as described by Billeter et al. (6). Endogenous peroxidase was blocked with methanol/ H_2O_2 . The slides were first incubated in 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min and then with the hybridoma supernatants for 2–4 h. After washing, the sections were incubated with the HPO-conjugated second antibody for an additional 4 h. The sections were developed with the diaminobenzidine solution as described above and then permanently mounted.

Competitive Enzyme-linked Immunoassays: PROCEDURE: This assay relied on the ability of light chains present in either standardized solutions or muscle extracts to compete with light chains bound to a solid phase for antibody binding. The monoclonal antibodies used in these assays, their specificity for light chains, and the appropriate assay conditions are given in Table I. The following general protocol was employed. Purified light chains were covalently bound to the walls of polyvinylchloride flat-bottom microtiter plates (50 μ l/well) (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, VA), using a modification of the glutaraldehyde conjugation procedure of Parsons (25). Light chains were diluted in 0.5 M sodium phosphate (pH 6.0) containing 0.02% glutaraldehyde and incubated for 1 h at 37°C. An equal volume of 0.5 M sodium phosphate (pH 8.5) was then added and the solution plated into the wells of the test plate. Adsorption of the light chains to the walls of the microtiter plate occurred during an additional hour of incubation at 37°C. The amount of MLCs used in the plating procedure was chosen as the minimum amount that gave a signal of sufficient intensity (0.5 OD₄₀₅) in an assay in which no competitor was present. Following adsorption, the wells were then incubated with a 2% solution of BSA in PBS to block additional protein binding sites.

In a separate vessel, the antibody was mixed at room temperature for 2 h with serial twofold dilutions of either a standardized solution of light chains (see CALIBRATION) or a muscle extract diluted into the appropriate buffer (Table I). To maintain association of the light and heavy chains, we solubilized extracts in high salt extracting solution (32) containing 2% BSA. To dissociate the light chains from the heavy chains, we first solubilized extracts in SDS sample buffer (18) and then diluted them with 1% Triton X100–1% deoxycholate–2% BSA in PBS. After incubation, the mixtures were transferred to the precoated microtiter wells and incubated with shaking for an additional 2 h. The plates were washed and reacted for 2 h with β -galactosidase-conjugated F(ab)₂ fragments of sheep anti-mouse antibody (BRL; Gaithersburg, MD). Following another series of washes, the plates were developed with p-nitrophenyl- β -D-galactoside according to the manufacturer's instructions (BRL) and read at 405 nm on an automated plate scanner (Dynatech Laboratories, Inc.).

ANALYSIS: All measurements were performed in triplicate and, in the case of muscle extracts, on at least three separate samples. When plotted on a semi-logarithmic scale, each set of serial dilutions yielded an inverse sigmoid curve. These data were transformed to a linear form using the logit function of Robdard and Lewald (27) and the linear regression parameters determined for each curve. The dilution at which the logit function was zero represented the 50% inhibition point on the curve and was used as the reference point in the comparisons of all

TABLE I
Specificity and Immunoassay Conditions for Myosin Light-chain Monoclonal Antibodies

Hybridoma clone	Light-chain specificity	Buffer*† Composition	Sensitivity§ $\times 10^{-2}$ (μ g/ml)
F310	LC1f/3f	Triton-deoxycholate	0.5–5
S21	LC1s	NaCl-Na ₄ P ₂ O ₇	1.0–10
T76	LC1s/1f/3f	Triton-deoxycholate	0.5–5
T14	LC1f/2f/2s	—	—

* Triton-deoxycholate buffer: samples were first dissociated in SDS sample buffer (17) and then diluted with 1% Triton \times 100 – 1% deoxycholate – 2% BSA in PBS.

† NaCl-Na₄P₂O₇: samples were diluted in 0.5 M NaCl, 0.02 M Na-phosphate (pH 7.4), 0.02 M Na₄P₂O₇ (32), and 2% BSA.

§ Useful range of sensitivity of assay in μ g $\times 10^{-2}$ ml. Sensitivity is defined as the light chain concentration necessary to inhibit 50% of antibody binding to the solid phase.

analyses. To estimate the amount of light chains present in a muscle extract, we obtained the dilution of the extract at which the logit was equal to zero from the regression data. This value was then multiplied by the amount of purified light chain that, in parallel standard curves, also resulted in a logit of zero. The results from 4 to 10 such determinations were used in the statistical analysis of the data. All assays were referenced to the standard curve performed with each plate. All data are expressed as the mean of these determinations \pm the standard deviation of the mean.

CALIBRATION: A purified light-chain fraction from the pectoralis major served as a source of light chain for generating standard curves for the fast (F_{310}) and total (T_{76}) MLC immunoassays. These antibodies recognized the fast MLCs, LC_{1f} and LC_{3f} , which comprised $\sim 50\%$ of the total light-chain fraction. The exact proportions of these light chains in the purified fraction was obtained by densitometry of SDS PAGE fractionated light chains stained with Coomassie Blue R250.

For the slow alkali MLC antibody (S_{21}), this approach could not be used since, in solution, these antibodies recognized slow light chains only when they were attached to the heavy chain. The standard employed in these assays was a purified myosin preparation from the mixed fiber type MA muscle. The slow light-chain content of this preparation was determined in the following way. The amount of fast light chain was first measured by immunoassay using antibody F_{310} . The slow light-chain content was then determined from the relative proportion of fast and slow light chains as determined by densitometry following fractionation and staining of myosin standards by SDS PAGE. Slow light-chain content was also measured by subtracting the results of immunoassay with antibody F_{310} , which measured fast light-chain content, from the results with antibody T_{76} , which measured total (fast and slow) light-chain content.

Miscellaneous Procedures: Protein content was measured either by the method of Lowry et al. (19) or by the method of Zaman and Verwilghen (39). Regression analyses were performed using a Gauss-Newton method of analysis (9). Statistical calculations were performed according to Armitage (1).

RESULTS

Antibody Specificity

The antibodies used in this study reacted with the myosin light chains (MLCs) of skeletal muscle. Their specificity was established by a number of criteria and is summarized in Table I. Fig. 1 shows their reaction with fractionated proteins electrophoretically transferred to nitrocellulose (7). Fig. 1A is the Coomassie Blue-staining pattern obtained following electrophoresis of SDS-solubilized extracts of the PM (lane 1), ALD (lane 2), and MA (lane 3) muscles of the adult chicken. The

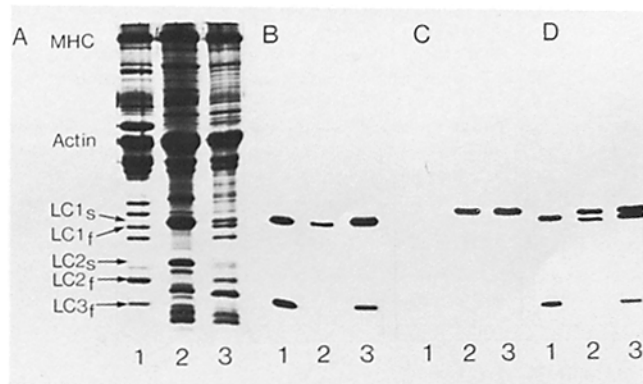


FIGURE 1 Specificity of MLC monoclonal antibodies. SDS PAGE and electrophoretic transfers of adult pectoralis major (PM) (lane 1), anterior latissimus dorsi (ALD) (lane 2), and medial adductor (MA) (lane 3) muscles were performed as described under Materials and Methods. 40 μ g total protein was applied to all lanes. The positions of the marked contractile proteins were determined by co-electrophoresis of marked purified standards. MHC: Myosin heavy chain. (A) Coomassie Blue R250-stained gel. (B) Nitrocellulose replicate of the gel reacted with antibody F_{310} . Only LC_{1f} and LC_{3f} reacted. (C) Nitrocellulose replicate of the gel reacted with antibody S_{21} . Only LC_{1s} reacted. (D) Nitrocellulose replicate of the gel reacted with antibody T_{76} . This antibody recognizes the total alkali light chains (LC_{1s} , LC_{1f} , and LC_{3f}).

light-chain types present in these electrophoretograms verified that these muscles contained predominantly fast, slow, and a mixture of fast and slow MLC isoform types, respectively. Fig. 1B shows the results of reaction between antibody F_{310} and a nitrocellulose transfer of a replicate gel. Of the many proteins present in these extracts, only LC_{1f} and LC_{3f} were recognized by this antibody. Cross-reaction between LC_{1f} and LC_{3f} is not unexpected since $\sim 70\%$ of the primary structure of LC_{1f} is homologous with the lower molecular weight LC_{3f} (21). Both the PM (lane 1) and MA (lane 3) extracts showed reaction with this antibody. Unexpectedly, extracts of the ALD (lane 2) also showed a reaction that was limited to LC_{1f} ; no LC_{3f} was evident.

The reaction between antibody S_{21} and a nitrocellulose transfer of a similar gel is shown in Fig. 1C. This antibody was specific for LC_{1s} . Extracts of both the ALD (lane 2) and the MA (lane 3) reacted with this antibody, while extracts of the PM did not (lane 1). Fig. 1D shows the reaction of antibody T_{76} with another nitrocellulose transfer. This antibody reacted with LC_{1f} , LC_{1s} , and LC_{3f} and provided additional evidence for the presence of LC_{1f} in the ALD (lane 2) and the lack of LC_{1s} in the PM (lane 1).

The reaction of fast light chain antibody F_{310} and slow light chain antibody S_{21} with serial cross-sections of adult skeletal muscle is shown in Fig. 2. The fibers of the PM stained uniformly with antibody to fast MLCs (Fig. 2a) and showed no staining with the antibody to slow light chains (Fig. 2b). On the other hand, most of the fibers of the ALD stained with both fast and slow MLC antibodies. While the staining pattern for slow light chains (Fig. 2d) was uniform throughout the section, the staining intensity for fast light chains varied from fiber to fiber (Fig. 2c). The presence of fast MLCs in the ALD is not without precedent; however, the number of fibers in the ALD that reacted with the fast light-chain monoclonal antibody was far greater than has been shown with antisera (10).

A fundamentally different staining pattern was observed in the MA. Not all the fibers in this muscle were of uniform diameter. The large-diameter fibers stained exclusively with the antibody to slow light chains (Fig. 2f); the fast light-chain antibody did not stain these fibers (Fig. 2e). The smaller-diameter fibers that border the fascicles of this muscle stained with antibodies to both fast and slow MLCs. These fibers were equivalent in diameter to those in the ALD and PM.

Developmental Sequence of Light-chain Expression

5-8 D OF INCUBATION: The light-chain isoform types present in developing avian muscle were examined for their reaction with the light-chain specific antibodies on nitrocellulose transfers of total muscle extracts. Extracts were prepared from the pectoral region and hindlimb (limb bud or thigh) of 5- and 7-d embryos and from the PM and MA of 8-d embryos. These results are shown in Fig. 3.

Fig. 3A shows the reaction of antibody T_{76} with a nitrocellulose replicate of an SDS polyacrylamide gel of these extracts. As shown above, antibody T_{76} reacted with both fast (LC_{1f} , LC_{3f}) and slow (LC_{1s}) MLCs of adult chicken myosin. In extracts of pectoral and hindlimb muscle from day-5 and -7 embryos, only LC_{1f} was detected on the nitrocellulose replicates. By day 8, however, both slow (LC_{1s}) and fast (LC_{1f}) MLCs were apparent. Reaction of the slow alkali light-chain antibody S_{21} with a similar nitrocellulose replicate (Fig. 3B)

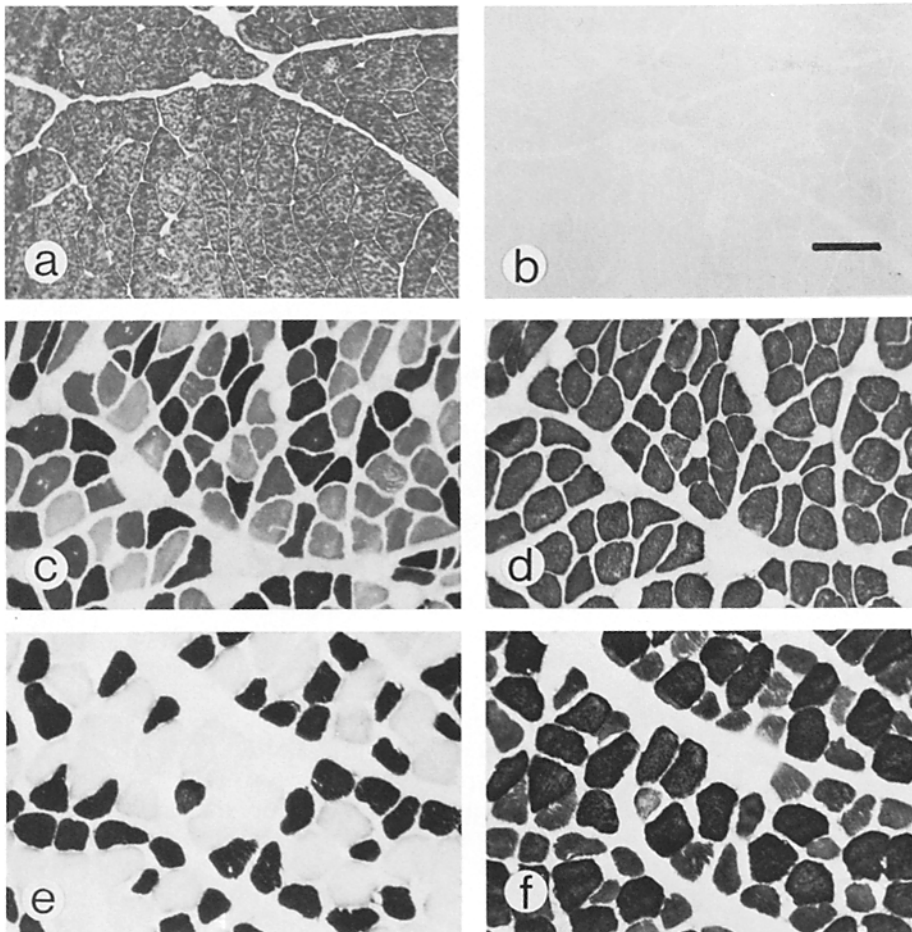


FIGURE 2 Immunocytochemical staining of serial cross sections of adult chicken skeletal muscle with monoclonal antibodies. Frozen serial sections of the adult PM (a and b), ALD (c and d), and MA (e and f) muscles were cut at 10 μm and treated as outlined in Materials and Methods. In panels a, c, and e, sections were reacted with fast light-chain antibody F_{310} , while panels b, d, and f show the staining pattern obtained with the slow light-chain antibody S_{21} . Bright-field illumination. All panels shown at same magnification. Bar, 100 μm . $\times 90$.

confirmed that LC_{1s} was present only in extracts of 8-day muscles.

The MLCs recognized by antibodies T_{76} , S_{21} , and F_{310} are the alkali MLCs of chicken myosin (18, 35). As a group, these light chains share a number of chemical similarities with homologous MLCs of other species (21, 34). The other MLCs of chicken muscle myosin, LC_{2s} and LC_{2f} , are members of the DTNB (18) or regulatory (16) class of MLCs. In adult muscle, there are 2 moles of both alkali and DTNB/regulatory light chains per mole of myosin (19). These are distributed so that one member of each light-chain class is associated with each myosin S_1 head (35). To determine whether light chains from both the alkali and DTNB/regulatory groups were present in early embryonic muscle groups, we compared the reaction of monoclonal antibody T_{14} on another series of nitrocellulose replicates (Fig. 3C) with the reactions described above. This antibody reacted with the fast alkali light chain, LC_{1f} , the regulatory MLCs, LC_{2f} and LC_{2s} , and, to a lesser extent, the slow alkali light-chain, LC_{1s} , of adult chicken myosin. The results of reaction with embryonic muscle extracts confirmed that only LC_{1f} was present in the 5-d muscle groups examined; neither regulatory MLC was present. By day 7, extracts of the thigh and pectoral region contained both alkali (LC_{1f}) and regulatory (LC_{2f}) fast light-chains. By day 8, both fast (LC_{1f}) and slow (LC_{1s}) (Fig. 3B) alkali light-chains as well as fast (LC_{2f}) and slow (LC_{2s}) (Fig. 3C) regulatory light-chains were present in extracts of the PM and MA.

9 D OF INCUBATION THROUGH THE ADULT: The reaction of antibodies F_{310} and S_{21} with extracts from the PM, ALD, and MA muscles over the period of 9 d of incubation to

the adult is shown in Fig. 4. Both fast and slow myosin light-chains were present in extracts of all these muscles from day 9 through day 14 *in ovo*. In the PM (Fig. 4A and D), slow light chains decreased below the level of detection by day 16. Coincident with this disappearance was the appearance of the fast alkali light-chain, LC_{3f} (Fig. 4A). Thereafter, no further qualitative changes in the light-chain isoform types were observed in this muscle. While the transfer shown in Fig. 4D does show a transient increase in slow light-chain content around the time of hatching (day 20), this increase was not observed in the competitive immunoassays (see below).

In the ALD, the most noticeable feature revealed by this method of immunodetection was the presence of fast alkali light chains (LC_{1f}) at day 12 and in all subsequent stages (Fig. 4B). LC_{3f} , however, was not detected. Slow alkali light-chains (LC_{1s}) were present at all stages in development of this muscle (Fig. 4E). The persistence of LC_{1f} into the adult ALD is consistent with the immunocytochemical staining of adult tissue sections (Fig. 2c and d). In the developing MA, the only conspicuous change in light-chain types was the appearance at 16 d of LC_{3f} (Fig. 4C). Fast and slow light chains were present in this muscle over the entire developmental period from day 9 to the adult (Fig. 4C and F).

Competitive Immunoassay

EXTRACTS OF ADULT MUSCLE: To quantitate the amount of fast and slow MLCs present in extracts of developing muscle, we developed competitive immunoassays. These assays were first characterized using extracts of adult skeletal muscle. The results are shown in Fig. 5.

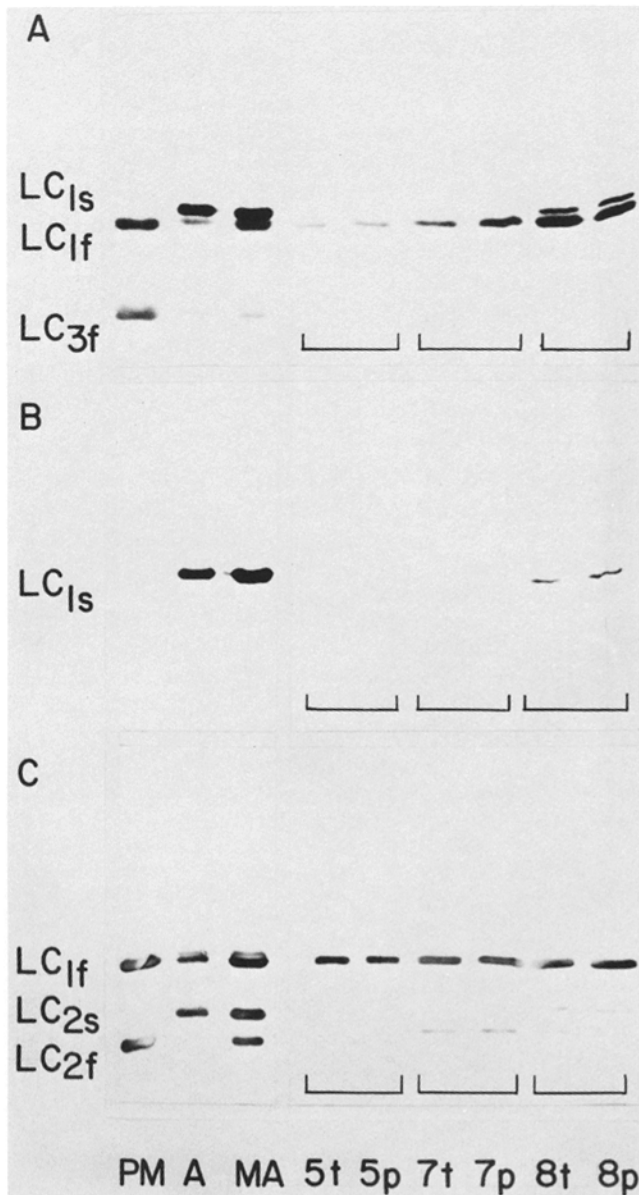


FIGURE 3 Reaction of MLC monoclonal antibodies with extracts of embryonic muscles: 5–8 d of incubation *in ovo*. Extracts of muscle from the pectoral region and hindlimb (limb bud or thigh) of 5- and 7-d embryos and the PM and MA of 8-d embryos were subjected to SDS PAGE and then electrophoretically transferred to nitrocellulose. The nitrocellulose replicates were then reacted with antibodies T₇₆ (A), S₂₁ (B), or T₁₄ (C). The reaction of these antibodies with purified myosin from PM, ALD, and MA muscles is shown to the left of each panel. Numbers indicate the days of incubation *in ovo*. Abbreviations: p, muscle extracts from the pectoral region (5 and 7 d) and the PM (8 d); t, muscle extracts from the limb bud (5 d), hindlimb (7 d), or MA (8 d); PM, pectoralis major; A, anterior latissimus dorsi; MA, medial adductor. The bars indicate the lanes containing pectoral and hindlimb extracts from each day.

Fig. 5A shows the results of the competitive immunoassay for fast alkali light chains using antibody F₃₁₀. While this antibody recognized fast alkali light chains both associated with and dissociated from the heavy chain, prior treatment with SDS to dissociate the light chains from the heavy chains was routinely employed since the sensitivity of the assay was higher under such conditions. The fast alkali light-chain con-

tents of the adult PM, ALD, and MA muscles are given in Table II.

Fig. 5B shows the results of the competitive immunoassay for slow alkali light chains of these same adult muscles using antibody S₂₁. In contrast to antibody F₃₁₀, S₂₁ recognized these light chains only in extracts solubilized in high salt buffer (dashed lines, Fig. 5B). Light chains that were dissociated from the heavy chain by SDS treatment as well as purified light chains in high salt buffer were not recognized by the antibody. This immunoassay showed that little or no slow light chain could be detected in adult PM extracts—the slow alkali light-chain content of the adult PM was below the limit of detection by this assay (see Table II). The ratio of fast to slow light chains in this muscle was, therefore, greater than 2,000 to 1. The slow alkali MLC content of the ALD and MA are all given in Table II. While significant amounts of fast light chain were detectable in adult ALD extracts, slow light chains were the dominant light-chain type and outnumbered fast light chains by approximately 23 to 1.

Fig. 5C shows the results of the competitive immunoassay for total alkali MLC content in these same muscles using antibody T₇₆. The requirements for light-chain presentation by this antibody were similar to those of F₃₁₀. The results of these assays confirmed that in adult muscle extracts the total alkali light-chain content measured by T₇₆ was equal to the sum of the fast (F₃₁₀) and slow (S₂₁) alkali MLCs (Table II).

EXTRACTS OF DEVELOPING MUSCLE: The immunoassays described above were applied to extracts of developing avian muscle. The total light-chain content normalized to total protein increased ~10–20-fold over the developmental stages examined (8 d of incubation to adult). The alkali light chains comprised ~0.15% of the total protein in extracts of 8-d PM and MA muscles; in the adult, this value increased to 3.5% and 2.5%, respectively.

The most dramatic changes in MLC composition were associated with changes in slow alkali light-chain content (Fig. 6A). In the MA, slow alkali MLCs increased ~400-fold over the period of 8 d of development *in ovo* to the adult. In both the PM and MA, slow MLC content rose 20-fold over the period of 8–12 d *in ovo*. In contrast, fast alkali MLC content in these same muscles increased only two- or three-fold during the same period and paralleled the general rise in total MLC content associated with development (Fig. 6B). Beginning at day 12, slow light chain content fell in the PM and rose in MA. A similar rise in slow light chain content was also observed in the ALD beginning at day 12 (the first day this muscle was studied).

In Fig. 6C, the results of the immunoassays were summarized by calculating the slow alkali light-chain content relative to total alkali light-chain content as a function of developmental age. As alluded to previously, the MLC compositions of the PM and MA during the period of 8–12 d *in ovo* were identical. Slow alkali MLCs (LC_{1s}) constituted only ~2.5% of the total alkali MLC content in these muscles at day 8 but rose gradually over the period of 8–12 d *in ovo* to 12% of the total alkali light-chain content. The transition to the isoform pattern characteristic of adult muscle began at ~12 d *in ovo*. By day 16, all three muscles had assumed a light-chain composition similar to that of the adult. The fast to slow light-chain ratio in the PM had increased from 9:1 at day 12 to 138:1 by day 16 (in the adult, this ratio was 2,340:1), while in the MA this ratio decreased from 8.2:1 at day 12 to 1.4:1 at day 16 (in the adult, this ratio was 0.52:1). In the ALD, the value of this same ratio fell from

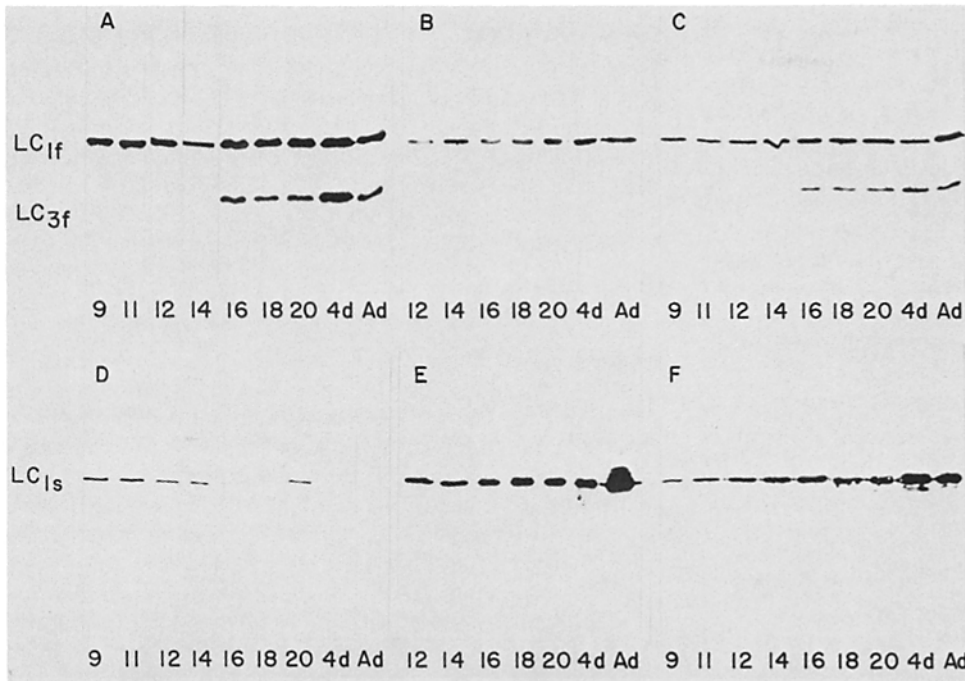


FIGURE 4 Reaction of MLC monoclonal antibodies with extracts of embryonic muscle: 9 d of incubation to the adult. Extracts of the PM (A and D), ALD (B and E), and MA (C and F) muscles from embryonic, posthatch, and adult chickens were subjected to SDS PAGE and then electrophoretically transferred to nitrocellulose. The nitrocellulose replicates were reacted with antibodies F₃₁₀ (A, B and C) and S₂₁ (D, E and F). 25 μ g total protein was applied to all lanes. Numbers indicate the days of incubation *in ovo* (9–20); 4d, 4 d posthatch; ad, adult muscle.

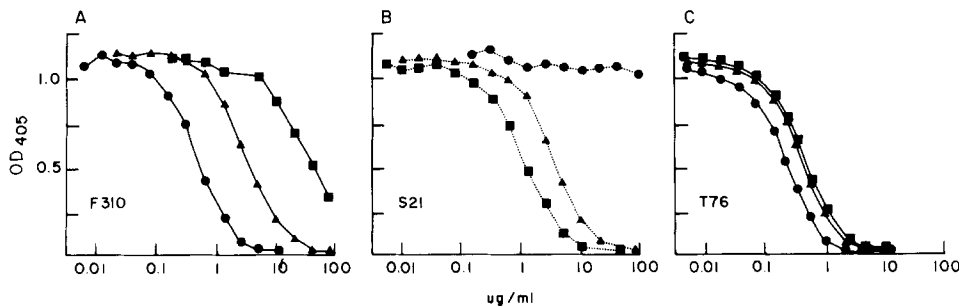


FIGURE 5 Competitive immunoassays of MLCs in adult muscle extracts. The abscissa shows the protein concentration of serial twofold dilutions of muscle extracts and the ordinate is the optical density at 405 nm. High values for the ordinate indicate the failure of extracts to bind antibody and compete with light chains absorbed to the solid phase, while low values indicate effective binding and competition. Extracts of adult PM (●), ALD (■),

and MA (▲) muscles solubilized and diluted in either high salt extracting solution (33) (---) or a combination of SDS and detergents (—) (see Table I). (A) Immunoassay with fast alkali MLC antibody F₃₁₀. (B) Immunoassay with slow alkali MLC antibody S₂₁. (C) Immunoassay of total alkali light chains with antibody T₇₆. The standard deviation of all measurements was contained within the area of the symbols.

3.5:1 at day 12 to 0.48:1 by day 16 (in the adult, this ratio was 0.04:1).

There was disagreement when the total alkali light-chain content in muscle extracts from 8–16 embryos was measured directly by immunoassay with antibody T₇₆ or calculated from the sum of the results of assay with antibodies S₂₁ and F₃₁₀. Table II shows these values for muscles from 12-d embryos. Significantly more alkali light chain was detected by direct immunoassay. There was no evidence from immunodetection on nitrocellulose gel replicates for an unidentified pool of MLCs present in 8–16-d extracts that could account for this discrepancy. The discrepancy may be due to failure of antibodies F₃₁₀ and S₂₁ to recognize some of the alkali light chains in extracts of early embryos. The conformational specificity of antibody S₂₁ suggests that slow MLCs present in extracts from early embryos may not be recognized either because most of these light chains were not associated with heavy chains or because their association with “embryonic” myosin heavy chains resulted in altered recognition by this antibody.

DISCUSSION

Rationale

We have focused our study of myosin isoform expression in developing muscle on the changes in myosin light-chain types that occur during development. Unlike the myosin light chains of developing mammalian muscle which are present in distinct embryonic isoforms (36), the light chains of developing avian muscle are apparently identical with those of the adult. This conclusion is based on their immunological cross-reactivity with adult light-chain antibodies (22, 30) and their co-migration in two-dimensional SDS PAGE with purified adult light chains (15, 31). In contrast, the myosin heavy chains of embryonic muscle are present as distinct isoforms (29, 37, 38). Since the total number and types of distinctive embryonic heavy chains that may cross-react with antisera to adult myosin remain unknown, interpretations based solely on immunochemical reactions alone may be misleading.

We have characterized the antibodies used in this study by

a number of methods, the most definitive of which was the reaction of antibody with nitrocellulose replicas of SDS PAGE fractionated muscle extracts ("Western" blots). The technique combines two powerful analytical methods (immunochemical detection and characterization by electrophoretic mobility) and has many advantages over more traditional methods of analysis, such as Ouchterlony diffusion, in that analysis does not require either purified antigen or the ability of the antibody to form immunoprecipitating complexes. Reaction of the monoclonal antibodies on "Western" blots showed that they were specific to skeletal muscle MLCs and that type-specific antibodies could be obtained. This method was also used in the analysis of light-chain expression in developing muscle since it provided additional evidence that the light chains detected by the antibodies that were present in embryonic muscle extracts were identical in their electrophoretic behavior to adult MLCs.

TABLE II
Myosin Light-chain Composition of Adult and 12-Day Embryonic Muscles

	(1) LC1f/3f*	(2) LC1s† (µg/mg protein)	(3) LC1f/3f + LC1s§
Adult			
PM	24.1 ± 0.26 (4)**	0.02 ± 0.01 (4)	23.9 ± 0.11†† (4)
ALD	1.02 ± 0.04 (4)	23.3 ± 0.08 (4)	23.9 ± 0.22†† (4)
MA	11.3 ± 0.45 (4)	12.8 ± 0.41 (4)	24.3 ± 0.17†† (4)
12-Day			
PM	5.67 ± 0.19 (4)	0.62 ± 0.02 (8)	6.82 ± 0.72§§ (4)
ALD	1.98 ± 0.11 (4)	0.55 ± 0.03 (4)	3.38 ± 0.2§§ (4)
MA	4.95 ± 0.08 (4)	0.71 ± 0.07 (9)	7.31 ± 0.71§§ (4)

* Results of competitive immunoassay with McAb F310.

† Results of competitive immunoassay with McAb S21.

§ Results of competitive immunoassay with McAb T14.

** Number of separate determinations of muscle extracts.

†† Values for immunoassay with McAb T14 not statistically different than sum of values for immunoassay with McAbs F310 and S21 at the level ($p > 0.10$).

§§ Values for immunoassay with McAb T14 statistically different than sum of values for immunoassay with McAbs F310 and S21 at the level ($p < 0.005$).

Fiber Composition of Adult Chicken Muscle

Extracts of the pectoralis major (PM), anterior latissimus dorsi (ALD), and medial adductor (MA) muscles of the chicken were chosen for analysis of light-chain expression during development since numerous biochemical and immunocytochemical observations had suggested that in the adult these muscles are composed predominantly of fast, slow, and a mixture of fiber types, respectively. Analysis of light-chain composition of these muscles with monoclonal antibodies confirmed these observations and, in addition, revealed a number of unexpected findings.

Most muscle fibers in the chicken are of a uniform diameter (30–50 µm) and react with either fast light-chain antibody alone or both fast and slow light-chain antibodies. We have shown that the adult PM is composed of a homogeneous group of such fibers containing exclusively fast type MLCs, by both immunocytochemistry (Fig. 2a and b) and immunoblotting (Fig. 1), in agreement with the findings of others (2, 10). On the other hand, these same methods revealed the presence of significant amounts of fast light chain in the adult ALD (Figs. 1 and 2c and d). This was not due to fiber contamination from other muscles since fibers containing fast light chains could be shown by immunocytochemistry to be distributed throughout the entire cross-section of the muscle. Histochemical studies have also demonstrated that the slow-type fibers of the adult ALD comprise a heterogeneous population. Ovalle (24) found that while the fibers of the adult ALD exhibited a uniform histochemical reaction for acid-stable (slow) myosin ATPase, the intensity of reaction for alkaline-stable (fast) myosin ATPase varied from fiber to fiber and more recently, Billeter et al. (5), using two-dimensional gel electrophoresis, demonstrated the presence of both fast and slow MLCs in single adult human fibers that were histochemically typed as slow. Of the adult muscles studied, only the MA contained fibers which reacted exclusively with slow light-chain antibodies. On average, these fibers were twice as large (50–80 µm) as those of either the ALD, PM, or other fibers in the MA which contained both fast and slow MLCs.

Previous studies using antisera have shown that fast myosin isoforms are present in only a few fibers of the ALD (10). From the data in Table II and Fig. 2c, it is evident that fast MLCs are present at much lower concentration than slow MLCs in the adult ALD and that these fast MLCs are distributed

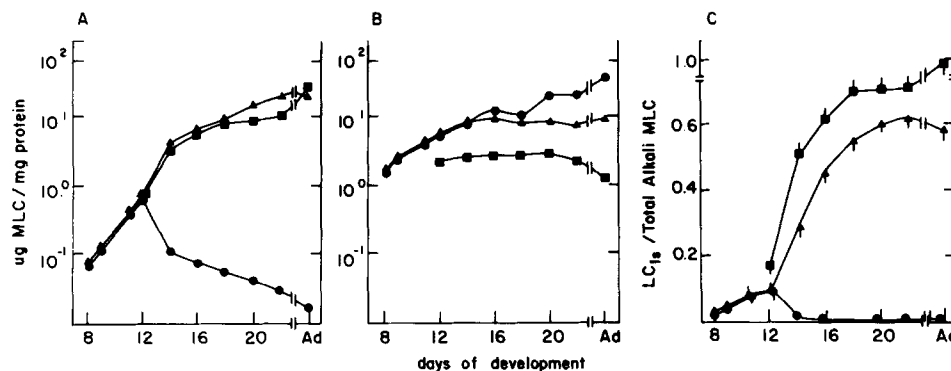


FIGURE 6 Competitive immunoassay of MLCs in embryonic muscle extracts. The MLC content in µg/mg total muscle protein is shown as function of the days of development for PM (●), ALD (■), and MA (▲) extracts from day 8 *in ovo* through the adult. (A) Slow alkali MLC content measured with antibody S₂₁. (B) Fast alkali MLC content measured with antibody F₃₁₀. (C) Slow alkali MLC content relative to total alkali MLC content measured by immunoassay with antibody T₇₆. The numbers indicate days *in ovo*; Ad, adult muscle extracts. Hatching occurred between days 20 and 21. The standard deviation of all measurements are given by vertical bars, where appropriate; in A and B, the standard deviation was contained within the area of the symbols.

unevenly among the fibers of this muscle. If the fast MLCs are associated with fast myosin heavy chains, then the quantitative discrepancy between the results shown in Fig. 2c and the results with pectoralis myosin antisera (10) may simply relate to the relative sensitivity of the two sets of antibodies. Other possibilities consistent with both findings are that fast MLCs are not incorporated into myosin heavy chains or are incorporated onto heavy chains which do not react with antisera to fast heavy chains. We have recently obtained evidence that such hybrid molecules occur *in vivo* (Crow, M. T., and F. E. Stockdale, manuscript in preparation).

The increased sensitivity and specificity of the monoclonal antibodies used in this study have demonstrated that in the adult both fast and slow MLCs are expressed in a substantial number of muscle fibers. With such reagents, it may be determined that the number of muscle fibers in other species which express both fast and slow MLCs is greater than currently believed. On the other hand, coexpression may be peculiar to chicken, since a large proportion of its adult skeletal musculature is composed of tonic-type fibers with many endplates distributed along the fiber (4).

Developmental Sequence of Light-chain Expression

Three distinct phases of MLC expression characterized the development of avian muscle. During the first phase (days 5–7), myosin extracted from the limb bud or pectoral region contained only fast MLCs. In the day-5 muscles, only light chain LC_{1f} was detected; neither LC_{1s}, LC_{2s}, nor fast light chain, LC_{2f}, was present. By day 7, however, both LC_{1f} and LC_{2f} were evident.

During the second phase of MLC expression (8–12 d *in ovo*), both fast and slow alkali (LC_{1s}, LC_{1f}) and regulatory (LC_{2s}, LC_{2f}) light chains were expressed by the PM and MA. In both muscles, slow alkali light-chain content rose steadily during this period, reaching a value of ~12% of the total alkali light-chain content by day 12. While the ALD could not be studied as a distinct muscle at earlier stages, fast and slow myosin light chains were also present in the 12-d ALD in proportions similar to those in the 12-d PM and MA.

During the third phase of MLC expression (the remaining one-third of development *in ovo*), the pattern of light-chain expression characteristic of adult muscle appeared. Beginning at day 12, the relative proportion of slow alkali light chains rose in the presumptive slow muscles (MA and ALD) and fell in the presumptive fast muscle (PM). By day 16, these muscles had assumed a light-chain composition characteristic of the adult. Day 16 also marked the appearance of LC_{3f}. Further developmental changes involved only quantitative changes in the relative proportion of the light chains. Not all muscles that expressed fast alkali light chains also expressed LC_{3f}; this light chain was conspicuously absent in the ALD even though LC_{1f} was present and easily detectable. The transfer data in Fig. 4D also show a transient increase in slow MLCs in the PM around the time of hatching (day 20). The reason for its appearance in the transfers is not clear, since no increase was observed in the analyses by competitive immunoassay (Fig. 6A). It is possible that it represents free, unassociated slow MLCs: that would be consistent with requirements of antibody S₂₁ for antigen presentation in the competitive immunoassays (see Materials and Methods).

The results reported here confirm the observations made by others on the temporal sequence of light chain expression in

avian muscle. Stockdale et al. (31) have reported that extracts of 9-d pectoral muscle contained both fast and slow light chains when analyzed by two-dimensional gel electrophoresis and that slow MLCs were no longer detectable by day 16. Similar results were reported by Obinata et al. (22) using one-dimensional gel electrophoresis and immunoelectrophoresis with antisera to the MLCs. More recently, Gauthier et al. (12) have shown that slow MLCs are present in the embryonic PM and that fast light chains are present in the embryonic ALD. Using immunocytochemistry, Rubinstein and Kelly (28, 33), have shown that in the developing distal rat hindlimb the first fibers detected with antisera to adult myosin reacted only with anti-fast myosin. Later in development these same fibers reacted with antisera to both adult fast and slow myosin, while a new group of fibers appeared which were morphologically distinct from the dual staining fibers and reacted only with anti-fast myosin. It will be of interest to determine whether all muscle fibers of the avian embryo express both light-chain types (12) or whether some or all of these fibers are restricted to the expression of a single isoform type (28). Our preliminary data suggest that muscle fibers in the avian embryo do differ in light-chain expression (8).

Our results show that while the first muscle groups in the limb and pectoral region to express skeletal muscle specific myosin light chains (5–7 d) express only fast light chains, the differentiation of muscle groups in the chick is characterized by a period (8–12 d) in which both fast and slow light chains are present. Since embryonic isoforms of the myosin heavy chains are now well documented (29, 37, 38), the developmental changes in MLC expression may be linked with the developmental sequence of heavy-chain expression. Monoclonal antibodies to specific myosin light chains are useful ligands for affinity chromatography of myosin. Such a method can be used to determine the type of heavy chain to which particular light chains are associated during development.

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