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Biochemistry

Myosin light chains and the developmental origin of fast muscle

(skeletal muscle/pectoral muscle/slow and fast fiber type/differentiation)

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ABSTRACT **Physiological characteristics of embryonic and fetal fast muscle function are similar to those of adult slow muscles, whereas most biochemical data suggest that embryonic and fetal fast muscles contain only fast muscle myosin. In the studies reported here, myofibrillar preparations from developing avian pectoral muscle (fast muscle) were isolated and analyzed for myosin light-chain type and synthesis. These analyses show that early in development avian fast muscle synthesizes and assembles myofibrils with light chains of both slow and fast myosins. Later in development, fast muscle no longer assembles myofibrils containing slow myosin light chains due to the cessation of synthesis of slow myosin light chains in mid-development. These *in vivo* studies indicate that the more developmentally primitive type of skeletal muscle is one that synthesizes both slow and fast myosin light chains independent of its anatomic location, and an event(s) late in fast muscle development results in the repression of synthesis of slow myosin light chains.**

Developing fast muscle has physiological characteristics similar to those of slow muscle of the adult (1, 2); however, most biochemical analyses of fetal fast muscle indicate the presence of only fast myosin (3–6). The basis for the discrepancy between physiological and biochemical measurements is complex, because of the varied developmental ages that have been studied and the age-related differences in stoichiometry of the proteins comprising the myofibrillar apparatus during embryonic development (7, 8). Furthermore, recent observations indicate that there are embryonic forms of both myosin heavy chain and light chain not found in the adult (9–11).

Gauthier *et al.* (12) report that development of a mammalian fast muscle (the diaphragm) is characterized by the presence of immunologically reactive slow and fast myosin within every muscle fiber, whereas, after birth, nearly every fiber contains only fast myosin. However, in analyzing a different fast muscle (avian pectoral muscle), Hoh (7) and Rubinstein and coworkers (4, 5) report that only fast myosin and no slow myosin is found in embryonic or fetal stages of pectoral muscle development. The latter investigators propose that the more developmentally primitive muscle synthesizes fast myosin and that the initiation of slow myosin synthesis is a consequence of developmental events imposed upon the muscle, such as innervation. However, the recent work of Keller and Emerson (13) and of Stockdale *et al.* (14) demonstrates that innervation is not required for initiation of slow myosin synthesis; myoblasts from embryonic muscle differentiating in cell culture in the complete absence of nerves synthesize slow myosin light chains. This paper extends these observations to show that early in development avian fast muscle synthesizes and assembles myofibrils with both slow and fast myosin light chains. Later in development, fast muscle no longer assembles myofibrils containing slow myosin light chains due to the cessation of synthesis of slow myosin light chains in mid-development.

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MATERIALS AND METHODS

Muscle Tissue. Muscle was prepared from White Leghorn chickens or White Leghorn chicken embryos. Fast muscle (pectoral muscle) or slow muscle (lateral adductor of the thigh or anterior latissimus dorsi) was dissected from embryonic or adult birds and cleaned, and myosin was extracted. Fast muscle development was studied from day 9 of incubation through day 1 after hatching and in the adult; slow muscle was studied in 13-day embryos through hatching and in the adult.

Tissue Culture. For explant cultures, muscles were dissected from chicken embryos of the required age, cleaned, and cut into pieces less than 1 mm^3 in size. Ten to 15 explants were placed on stainless steel grids in Cooper dishes containing culture medium (80% minimal essential medium, 15% horse serum, and 5% embryo extract and antibiotics) and 40–60 μCi of [^{35}S]methionine per ml (specific activity, 1200 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels). Explants were incubated for 6 hr at 37°C and then washed three times with Hanks' solution. Explants were either directly lysed in lysis buffer as whole cell extracts or pooled, homogenized, and treated for extraction of myosin as described below.

Preparation of Myosin Extracts. For analysis of embryonic myosin light chains in myofibrils, explants or tissue was collected in a solution of glycerol and a low ionic strength buffer (20 mM NaCl/2 mM sodium phosphate, pH 7.0), 1:1, (wt/vol) and stored for up to 3 weeks at −20°C. This preparation was washed free of glycerol with low ionic strength buffer, minced, and homogenized by shearing through a hypodermic needle. The pellet was collected by centrifugation and washed three times with low ionic strength buffer. The pellet was then briefly dissolved in 20 mM sodium pyrophosphate/1 mM $MgCl_2$, pH 9.5, and centrifuged at 200,000 × *g* for 1 hr. The supernatant was substantially enriched in myosin. All procedures were carried out at 4°C. Total protein content was estimated by the method of Lowry *et al.* (15).

Purification of Myosin. Adult or embryonic tissue was washed in cold 0.1 M NaCl/1 mM $MgCl_2$/0.1 mM EDTA/5 mM sodium phosphate, pH 7.0, and homogenized with a Dounce or a Polytron homogenizer (16). The myofibrils were collected by centrifugation at 10,000 × *g* for 30 min and washed four times with the same buffer. The myofibrillar pellet was weighed and suspended in the same buffer at 100 mg/ml, and 3 M NaCl was added to give a final concentration of 0.6 M NaCl. After brief solubilization at 4°C, the solution was brought to 5 mM $MgCl_2$, 2 mM ATP, and 2 mM sodium pyrophosphate. This solution was centrifuged at 200,000 × *g* for 3 hr to remove aggregates and actin. The supernatant was collected, and myosin was precipitated by reduction of ionic strength by dialysis against 20 mM NaCl/5 mM sodium phosphate, pH 6.3, and collected by centrifugation. The myosin pellet was washed with 10 vol of dialysis buffer, dissolved in 0.6 M NaCl/50 mM Tris·HCl, pH 7.5, and centrifuged at 40,000 × *g* to clarify the solution. The absorbance of this solution was measured at 280

nm, and the concentration of myosin was estimated by using an absorption coefficient of $A_{280}^{1\%} = 5.6$ (17). When required, light chains were isolated by precipitation of myosin heavy chains in the presence of 5 M guanidine·HCl, followed by dialysis against 20 mM NaCl/5 mM sodium phosphate, pH 6.3 (16).

Two-Dimensional Gel Electrophoresis. Myosin extracts of muscle tissue, [^{35}S]methionine-labeled explants, or whole-cell lysates were analyzed by electrophoresis on two-dimensional gels by the procedure of O'Farrell (18). Samples in lysis buffer [9.5 M urea/2% (wt/vol) Nonidet P-40/2% (vol/vol) Ampholines, pH 4–6/5% (vol/vol) 2-mercaptoethanol] were loaded on 2.5 × 100 mm isoelectric focusing gels containing a pH gradient of 4–6. This gradient proved optimal for resolving the more acidic light-chain proteins. Equilibrated isoelectric focusing gels were placed in agarose on top of a 12.5% acrylamide/$NaDodSO_4$ slab gel with a uniform thickness of 1 mm topped with a 4.75% acrylamide stacking gel. The buffers used were those described by O'Farrell (18). The gels were subjected to 4–5 hr of electrophoresis at a constant current of 20 mA/gel and a programmed maximal voltage of 130 V (Hoeffer Scientific Instruments PS-DC power supply, San Francisco). They were stained with Coomassie blue, destained in methanol and acetic acid (19), and then dried under reduced pressure. Myosin light chains were identified in these extracts by their mobility in one-dimensional $NaDodSO_4$ gels, with ribonuclease A, chymotrypsinogen *a*, and ovalbumin as molecular weight markers. Usually, 50–180 μg of protein were placed on two-dimensional gels. For autoradiography, extracts containing 0.1–2 × 10^6 cpm were applied to the gels, depending upon whether the extracts were crude or purified. Autoradiograms were prepared by exposure of the dried gels to Kodak No-Screen NS2T x-ray film.

For determining rates of synthesis of various myosin light chains in explant cultures, the stained spots corresponding to each light chain were excised from the two-dimensional gels. The pieces of gel were hydrated, material was extracted in NCS solubilizer, and incorporation of [^{35}S]methionine into each light chain was determined by scintillation spectrometry. A portion of the gel with no stainable protein served as a control.

RESULTS

Myosin Light Chains in Developing Fast Muscle. The types of myosin light chains that accumulate in developing pectoral muscle were determined in chicken embryos from 9 through 19 days of incubation, after hatching, and in the mature adult. Fig. 1 shows a two-dimensional gel of extracts of adult pectoral muscle, a fast muscle. Only fast myosin light chains ($LC1_f$, $LC2_f$, and LC3) were present. Fig. 2 shows two-dimensional gels of pectoral muscle extracts at 9, 11, 13, and 16 days of embryonic and fetal development. Both the 9- and 11-day pectoral muscle contained $LC1_s$, $LC1_f$, $LC2_s$, and $LC2_f$ myosin light chains. The 13-day pectoral muscle nearly lost the first and second slow myosin light chains, whereas the 16-day pectoral muscle contained only the fast myosin light chains $LC1_f$ and $LC2_f$. The third myosin light chain of the adult fast muscle was not seen at these stages. For purposes of comparison, the myosin found in the latissimus dorsi muscle of a 16-day chicken embryo, a slow muscle in the adult, contained both fast and slow myosin light chains, as shown in Fig. 3. This comparison demonstrates that, in 9- through 13-day embryos, fast muscle contains the same myosin light chains as slow muscle.

Slow Myosin Light Chains in 11-Day Embryonic Myofibrillar Protein. To demonstrate that the slow myosin light chains seen in early stages of fast muscle development are myofibrillar proteins, we isolated and analyzed breast muscle from

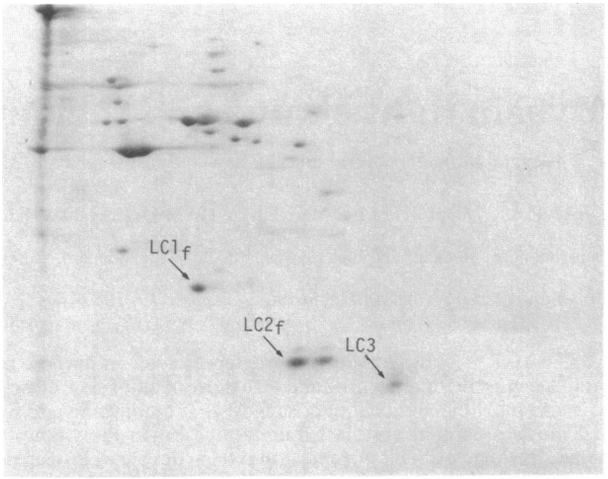

FIG. 1. Adult chicken pectoral muscle. Myosin was extracted and chromatographed on two-dimensional gels, which were stained with Coomassie blue. Myosin light chains $LC1_f$, $LC2_f$, and LC3 are labeled. One hundred micrograms of protein was applied to the gel.

240 11-day chicken embryos. Eleven grams of tissue was obtained. Myofibrils were washed exhaustively in low ionic strength buffer and then dissolved in high ionic strength buffer. The actomyosin was precipitated by reduction of ionic strength, dissolved, combined with ATP, and then centrifuged to reduce actin content. The high-speed supernatant from this preparation was subjected to electrophoresis on two-dimensional gels (Fig. 4). Purified myofibrillar preparations from 11-day fast muscle contained both $LC1_s$ and $LC2_s$ in addition to the fast myosin light chains.

Synthesis of Slow Myosin Light Chains by Early Embryonic Fast Muscle. To demonstrate that the early stages of fast muscle synthesize the slow myosin light chains, we determined the types of myosin synthesized by explant cultures of 10-day embryonic pectoral muscle. Small pieces of 10-day embryonic pectoral muscle were incubated as explants for the first 6 hr after excision from the embryo in the presence of 60 μCi of [^{35}S]methionine per ml. Myosin synthesis in these explants was analyzed both in whole-cell lysates and in extracts of purified myofibrils. These preparations were coelectrophoresed in two dimensions with purified adult lateral adductor myosin so that the myosin light chains could be identified in the autoradiograms. Two-dimensional gels of both whole-cell lysates and purified myosin extracts of 10-day embryonic pectoral muscle contained $LC1_s$, $LC1_f$, $LC2_s$, and $LC2_f$. Fig. 5*A* is an autoradiogram of the myosin light-chain region of a two-dimensional gel of purified myosin from pectoral muscle explants of 10-day embryos.

To ensure that excision of the tissue and its placement in explant culture did not induce the synthesis of light chains $LC1_s$ and $LC2_s$ or that these two light chains were synthesized at later stages but not assembled into myofibrils, we also carried out explant experiments on 14-day embryonic pectoral muscle. Fourteen-day pectoral muscle tissue was selected because *in vivo* it accumulated only fast myosin light chains. Unlike myofibrils isolated from 10-day explants, myofibrils from 14-day explants were found to contain only newly synthesized fast myosin light chains $LC1_f$ and $LC2_f$. Whole-cell lysates of these explants contained only newly synthesized fast myosin light chains $LC1_f$ and $LC2_f$; if excess acid-precipitable radioactivity was added to the gels, very trace amounts of $LC2_s$ but not $LC1_s$ could be seen (Fig. 5*B*). Explants of 14-day slow muscle (lateral adductor), on the other hand, synthesized all four of the light

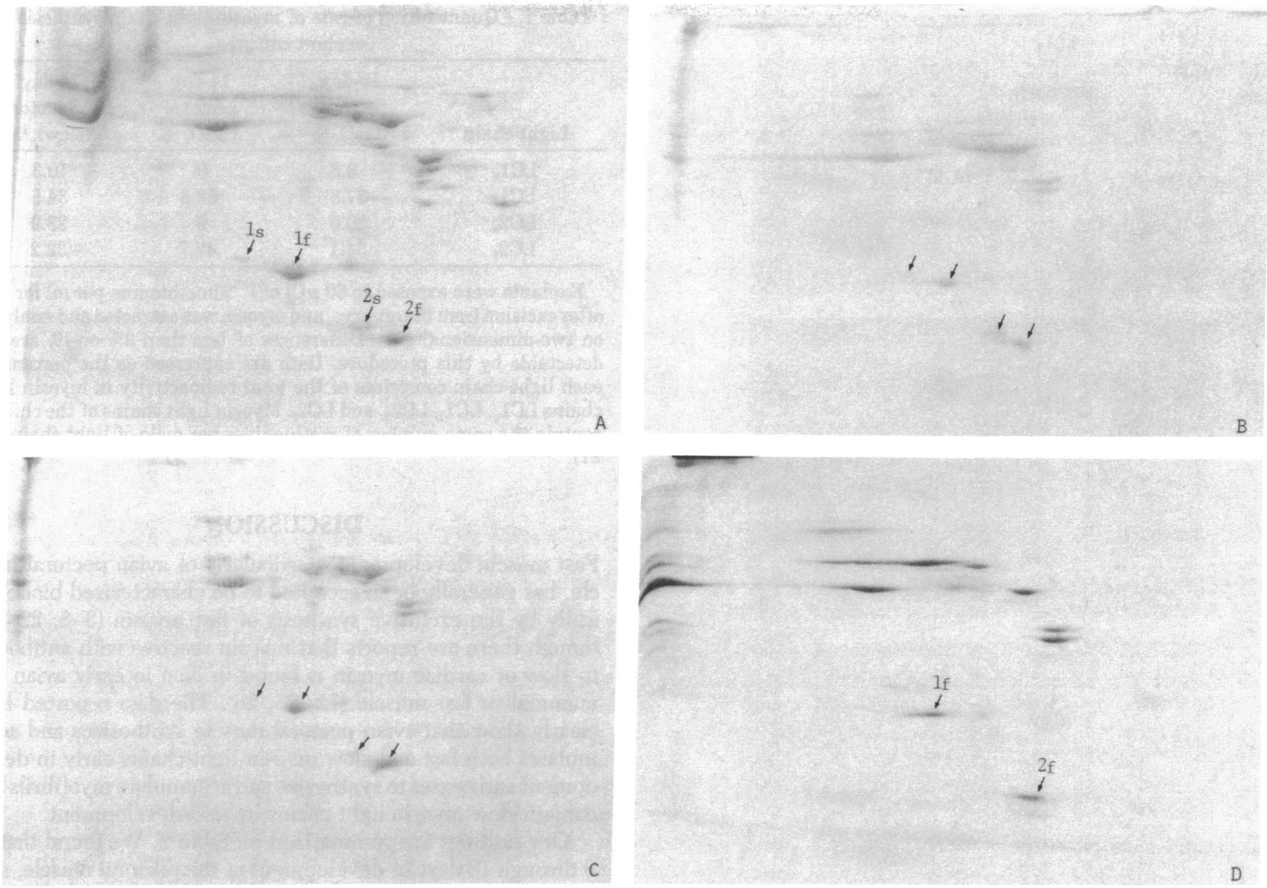

FIG. 2. Embryonic chicken pectoral muscle. Myosin was extracted and chromatographed on two-dimensional gels, which were stained with Coomassie blue. (*A*) Nine-day pectoral muscle; (*B*) 11-day pectoral muscle; (*C*) 13-day pectoral muscle; (*D*) 16-day pectoral muscle. Myosin light chains $LC1_s$, $LC1_f$, $LC2_s$, and $LC2_f$ are seen in 9-, 11-, and 13-day pectoral muscle, whereas only $LC1_f$ and $LC2_f$ are seen in 16-day pectoral muscle. The following amounts of protein were applied to the gels: (*A*) 180 μg; (*B*) 100 μg; (*C*) 100 μg; (*D*) 100 μg.

chains when analyzed as either whole-cell lysates or as extracts of purified myofibrils (Fig. 5*C*).

Quantitation of Myosin Light-Chain Synthesis. The amount of myosin light chains synthesized in pectoral muscle was determined in explant cultures of embryonic muscle (Table 1). Explants from 10- and 14-day embryonic pectoral muscle and 14-day lateral adductor muscle were exposed for 6 hr to 60 μCi of [^{35}S]methionine per ml, and myosin was extracted from myofibrils. The extracts were run on two-dimensional gels; those spots corresponding to the myosin light chains were excised,

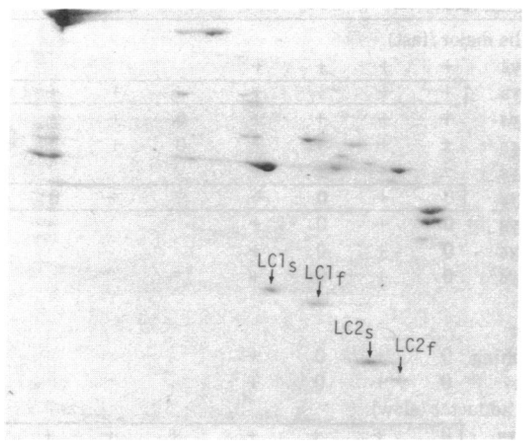

FIG. 3. Myosin was extracted from 16-day anterior latissimus dorsi muscle of chicken embryo and chromatographed on a two-dimensional gel, which was stained with Coomassie blue. Myosin light chains $LC1_s$, $LC1_f$, $LC2_s$, and $LC2_f$ are seen. Forty-eight micrograms of protein was applied to this gel.

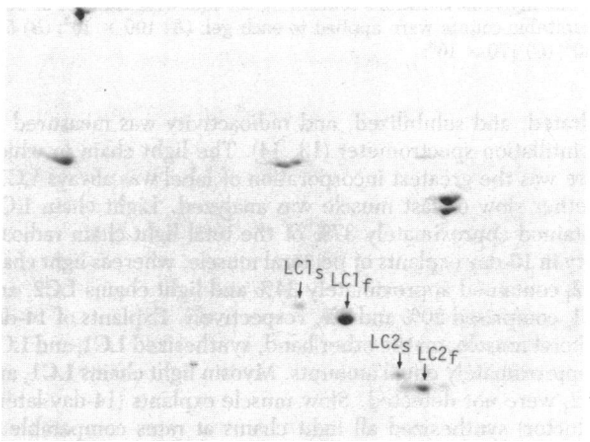

FIG. 4. Two-dimensional gel of myosin extracted from purified myofibrils isolated from 11-day pectoral muscle. The gel was stained with Coomassie blue. Myosin light chains $LC1_s$, $LC1_f$, $LC2_s$, and $LC2_f$ are present. Eighty micrograms of protein was applied to this gel.

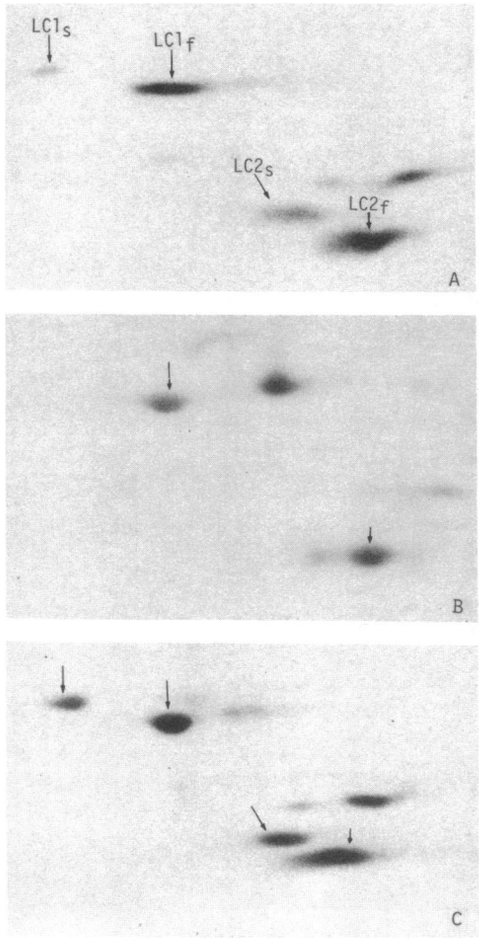

FIG. 5. Autoradiograms of two-dimensional gels of myosin extracts or whole-cell lysates from pectoral and lateral adductor muscle explants grown in tissue culture for 6 hr in the presence of [^{35}S]methionine. Shown are enlargements of that region of the gel containing the myosin light chains: (*A*) myofibrillar extract of 10-day pectoral muscle explants; (*B*) whole-cell lysates, 14-day pectoral muscle explants; (*C*) myofibrillar extract of 14-day lateral adductor muscle explants. Light chains $LC1_s$, $LC1_f$, $LC2_s$, and $LC2_f$ are seen in the myosin of 10-day pectoral muscle explants; only $LC1_f$ and $LC2_f$ and trace amounts of $LC2_s$ in the original autoradiogram are seen in whole-cell lysates of 14-day pectoral muscle explants. The 14-day explants of slow muscle (lateral adductor) contain light chains from both slow and fast myosins. Solutions containing the following number of acid-precipitable counts were applied to each gel: (*A*) 100×10^3; (*B*) 580×10^3; (*C*) 170×10^3.

hydrated, and solubilized, and radioactivity was measured in a scintillation spectrometer (13, 14). The light chain in which there was the greatest incorporation of label was always $LC1_f$, whether slow or fast muscle was analyzed. Light chain $LC1_f$ contained approximately 37% of the total light chain radioactivity in 10-day explants of pectoral muscle, whereas light chain $LC2_f$ contained approximately 34% and light chains $LC2_s$ and $LC1_s$ comprised 20% and 9%, respectively. Explants of 14-day pectoral muscle, on the other hand, synthesized $LC1_f$ and $LC2_f$ in approximately equal amounts. Myosin light chains $LC1_s$ and $LC2_s$ were not detected. Slow muscle explants (14-day lateral adductor) synthesized all light chains at rates comparable to those found in 10-day fast muscle (pectoral). The relative amounts of myosin light chains that accumulated at these ages of development *in vivo*, as estimated by stained two-dimensional gels, agree with the relative amounts synthesized in explant cultures of these same tissues.

Table 1. Quantitation of rate of myosin light-chain synthesis in explant cultures

Light chain	10-day pectoral (fast), %	14-day pectoral (fast), %	14-day adductor (slow), %
$LC1_s$	8.8	0	10.3
$LC1_f$	37.3	52.3	34.5
$LC2_s$	20.0	0	23.9
$LC2_f$	34.1	47.7	32.2

Explants were exposed to 60 μCi of [^{35}S]methionine per ml for 6 hr after excision from the embryo, and myosin was extracted and analyzed on two-dimensional gels. Differences of less than 3% or 4% are not detectable by this procedure. Data are expressed as the percentage each light chain comprises of the total radioactivity in myosin light chains $LC1_s$, $LC1_f$, $LC2_s$, and $LC2_f$. Myosin light chains of the chicken contain the same number of methionines per mole of light chain (20, 21).

DISCUSSION

Fast muscle development, particularly of avian pectoral muscle, has generally been accepted to be characterized biochemically by the exclusive synthesis of fast myosin (3–8, 22–24), though there are reports that myosin reactive with antibodies to slow or cardiac myosin is found *in vivo* in early avian and mammalian fast muscle (12, 25, 26). The data reported here clearly show that avian pectoral muscle synthesizes and accumulates both fast and slow myosin light chains early in development and ceases to synthesize and accumulate myofibrils that contain slow myosin light chains by mid-development.

Our findings are summarized in Table 2. We found that, at 9 through 13 days of development of the pectoral muscle, slow and fast myosin light chains were present in both whole-cell lysates and purified extracts of myofibrils. Therefore, both slow and fast myosin light chains are synthesized and assembled as part of the myofibrillar apparatus at these ages. Synthesis of slow myosin light chains in pectoral muscle begins to decline at approximately 12 days of development; by 14 days of development, only trace amounts of one slow myosin light chain,

Table 2. Accumulation and synthesis of myosin light chains in developing fast and slow muscle of the chicken

	Accumulation *in vivo*				Synthesis in explant culture			
Age	$LC1_s$	$LC1_f$	$LC2_s$	$LC2_f$	$LC1_s$	$LC1_f$	$LC2_s$	$LC2_f$
Pectoralis major (fast)								
9 days	+	+	+	+				
10 days	+	+	+	+	+	+	+	+
11 days	+	+	+	+	0	+	±	+
12 days	±	+	+	+	0	+	±	+
13 days	±	+	±	+				
14 days	0	+	0	+	0	+	0	+
15 days	0	+	0	+				
16 days	0	+	0	+				
19 days	0	+	0	+				
1 day after hatching	0	+	0	+				
Adult	0	+	0	+				
Lateral adductor (slow)								
14 days	+	+	+	+	+	+	+	+
Adult	+	+	+	+				

Data are based upon myosin extractable from myofibrils. +, Present; ±, easily seen in some preparations, trace in others; 0, absent or present in only trace amounts (less than 3–4% of total cpm in myosin light chains).

$LC2_s$, may be found in whole tissue lysates. At this time slow myosin light chains are no longer isolated from purified myofibrils. The influences that produce these changes in synthesis and accumulation into myofibrils clearly are not systemic because, within other anatomic regions (lateral adductor or anterior latissimus dorsi) of the same embryo, slow myosin light chains continue to be synthesized and to accumulate throughout development and into adulthood.

These observations raise at least two possible explanations for the developmental events during fast muscle formation. One is that early in development two separate muscle-fiber populations exist, with the slow fibers disappearing later; the other is that a single fiber population exists and, as development proceeds, it sequentially synthesizes different myosin isozymes. The former possibility cannot be ruled out in this study because analyses were done on pieces of tissue rather than on single cells. Though there are muscles composed of various fiber types and cell culture data that support the idea of several cell lineages in developing pectoral muscle (27–29), there is abundant evidence that individual fibers can synthesize both slow and fast myosin and that the synthesis of myosin type is able to be regulated within an individual fiber (12, 30–34). For example, in cross-innervation or chronic stimulation of fast muscle there is a change in myosin isozyme type in existing fibers as a response to these changing environmental stimuli (30–33). Fluorescent antibody staining of embryonic muscle fibers in culture or *in vivo* also shows that individual fibers accumulate myofibrils containing both slow and fast myosin (12, 34), and biochemical analysis of clonally derived cultures shows that a single myoblast can give rise to fibers that synthesize both slow and fast myosin (13).

Though the mechanisms that activate or inactivate the genes for the multiple myosin isozymes (9, 10, 35–40) within a muscle fiber remain unknown, the central nervous system is thought to have an important role; it has been shown to be involved in the regulation of types and amounts of muscle-specific protein synthesized (30–33). However, determination of the types of myosin synthesized by muscle cells formed in cell culture shows that the central nervous system is not required for the activation of genes for either fast or slow myosin (13, 14, 34). The data reported here extend these observations to show that, once synthesis of slow myosin light chains has been activated, some event(s) that occurs in fast, but not slow, muscle leads to a cessation of synthesis of slow muscle light chains.

In summary, the results described here and those recently reported from studies on muscle cells in culture (13, 14, 34) indicate that fast muscle development is not exclusively characterized by the synthesis of fast muscle. Indeed, it appears that the more primitive skeletal muscle is one that synthesizes both slow and fast myosin whether localized to an anatomic slow or fast muscle region. However, we show here that as fast muscle development proceeds, slow myosin light chains cease to be found in fast muscle myofibrils, whereas fast myosin persists. Because slow myosin light chains disappear from whole-cell lysates at the same time as they do from purified myofibrils, the data suggest that cessation of synthesis rather than just nonassembly of slow myosin light chains is responsible for this change. Though this selective repression of synthesis of slow myosin light chains in development may be the result of transcriptional control mechanisms, other mechanisms such as changes in mRNA processing, translation efficiency, or stability must be considered. It is not known if the mechanism(s) responsible for this selective repression is mediated via the nerves that innervate developing muscle (41), but they remain a likely candidate.

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