Embryonic Chicken Skeletal, Cardiac, and Smooth Muscles Express a Common Embryo-specific Myosin Light Chain

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ABSTRACT It has been demonstrated that embryonic chicken gizzard smooth muscle contains a unique embryonic myosin light chain of 23,000 mol wt, called L_{23} (Katoh, N., and S. Kubo, 1978, *Biochem. Biophys. Acta*, 535:401–411; Takano-Ohmuro, H., T. Obinata, T. Mikawa, and T. Masaki, 1983, *J. Biochem.* (*Tokyo*), 93:903–908). When we examined myosins in developing chicken ventricular and pectoralis muscles by two-dimensional gel electrophoresis, the myosin light chain (L_e) that completely comigrates with L_{23} was detected in both striated muscles at early developmental stages. Two monoclonal antibodies, MT-53f and MT-185d, were applied to characterize the embryonic light chain L_e of striated muscles. Both monoclonal antibodies were raised to fast skeletal muscle myosin light chains; the former antibody is specific to fast muscle myosin light chains 1 and 3, whereas the latter recognizes not only fast muscle myosin light chains but also the embryonic smooth muscle light chain L_{23}. The immunoblots combined with both one- and two-dimensional gel electrophoresis showed that L_e reacts with MT-185d but not with MT-53f. These results strongly indicate that L_e is identical to L_{23} and that embyronic chicken skeletal, cardiac, and smooth muscles express a common embryo-specific myosin light chain.

Multiple myosin isoforms exist among various vertebrate muscle tissues, and each myosin isoform contains a specific combination of light chains. It has been demonstrated that myosin light chain expression changes during skeletal muscle development: the chicken skeletal muscle at embryonic stages contains both fast- and slow-type light chains (7, 12, 19, 25), and, in addition, mammalian fetal skeletal muscle contains an embryo-specific light chain (28, 30), although the vast majority of adult myofibers contain predominantly either fast light chains or slow light chains. Alteration in myosin light chains has also been observed during cardiac muscle development and some similarity in light chain expression has been shown between embryonic skeletal and embryonic cardiac muscles. An embryonic light chain identical to the embryonic skeletal light chain was detected in mammalian fetal cardiac muscle (29), and a small amount of fast skeletal light chain 1 was detected in embryonic chicken cardiac muscle (20). Furthermore, the transition of myosin light chain expression during the development of chicken gizzard smooth muscle has been reported (11, 27). Adult chicken smooth muscle contains two different myosin light chains, L_{20} and L_{17}, the molecular weights of which are 20,000 and 17,000, respectively, but in the embryonic gizzard muscle, L_{17} is present in only a small amount, and another embryo-specific smooth muscle light chain, designated L_{23} (23,000 mol wt), is expressed. None of the reports has demonstrated any similarity in myosin expression between developing smooth muscle and striated muscles. In this report, we present evidence that an embryonic smooth muscle light chain (L_{23}) is also expressed in embryonic skeletal and cardiac muscles.

MATERIALS AND METHODS

Preparation of Myosin: We prepared myosin from chicken ventricular and pectoralis muscles of various developmental ages as previously described (18, 19) by slightly modifying the method of Perry (23). Embryonic and adult chicken gizzard muscle myosins were prepared by a slight modification of the method described by Ebashi (4).

Antibodies: Polyclonal antibody against cardiac myosin light chain 1 (L_{c1}) was prepared by injecting the immunogen to rabbits, and the immunoglobulin was purified by Sepharose 4B coupled to L_{c1} as previously described (18, 20). Monoclonal antibodies (mAb's)[1] to chicken myosin light chains were produced by hybridoma formation between the spleen cells of BALB/c mice immunized with the light chain fraction from chicken pectoralis muscle and a nonsecreting myeloma cell line P_3-X63-Ag8-U1 using the techniques of Galfre et al. (6) as modified by Gefter et al. (8). mAb's to smooth muscle myosin heavy chain were prepared by the same procedure using chicken gizzard myosin

[1] *Abbreviation used in this paper:* mAb, monoclonal antibody.

as an immunogen (Tanaka, T., and T. Masaki, unpublished observation). The supernatants from the hybridoma cultures were initially screened by a plate-binding assay using purified myosin passively absorbed to polyvinylchloride microplates. Positive hybridoma supernatants were further screened by immunoblots. Hybridoma cells giving positive supernatant were subcloned twice using methylcellulose. Supernatants from the subcloned cultures were used as the source of antibody throughout this study.

Gel Electrophoresis: SDS PAGE was performed as described by Laemmli (13) on 15% acrylamide gels. Two-dimensional gel electrophoresis, a combination of isoelectric focusing and SDS PAGE was carried out according to O'Farrell (22) except that the scale of the system was miniaturized as described by Mikawa et al. (15). The pattern of myosin light chains was visualized by means of a slight modification (15) of the highly sensitive silver stain according to Oakley et al. (17). Pyrophosphate acrylamide gel electrophoresis was carried out according to Hoh et al. (9). To analyze the type of myosin light chains present in each myosin isozyme, myosin isozyme bands separated on pyrophosphate gel were cut out and subjected to SDS PAGE after the treatment with an SDS solution containing 2% SDS, 2% 2-mercaptoethanol, and 20 mM Na-phosphate, pH 7.0, for about 5 min as described previously (26).

Immunoblots: The electrophoretic transfer of proteins from SDS polyacrylamide gels to nitrocellulose was performed as described by Reinach et al. (24). The nitrocellulose paper was treated with 1% bovine serum albumin (24) and then incubated for 15 min each in mAb then in ^{125}I-goat anti-mouse IgG (1 μg IgG/ml and 3 × 10^3 cpm/μl). After immunoreaction, the paper was washed with Tris-buffered saline (10 mM Tris, 0.15 M NaCl, pH 7.5) containing 0.5% Tween 80 for 20 min then rinsed with Tris-buffered saline without the detergent. Antigen was detected by autoradiography with x-ray film.

Assay of Protein Concentration: Protein concentration was determined photometrically at 310 nm using a biuret reaction according to Itzhaki and Gill (10).

RESULTS

Fig. 1 shows the changes in myosin light chain pattern during the development of chicken breast muscle. In the adult, three light chains are present (Fig. 1*a*). At late embryonic stages, light chain 3 is missing (3), and therefore only two light chains are present (Fig. 1*b*). At younger embryonic stages, for example at day 13 in ovo, two more light chains are detectable (Fig. 1*c*); these were previously identified as slow-type or cardiac-type light chains 1 and 2 (19). When the breast muscle at earlier embryonic stages was examined, one more minor spot, designated here L_e, was detected in the myosin light chain region on two-dimensional gel (Fig. 1*d*). This minor spot in the embryonic skeletal muscle (L_e) comigrated with the embryo-specific smooth muscle light chain (L_{23}) (Fig. 1, *e* and *f*) that was previously discovered in gizzard smooth muscle of chicken embryo (11, 27) upon two-dimensional gel electrophoresis, indicating that both of them have the same molecular weight and isoelectric point. The spot corresponding to L_e was also faintly observed in the myosin from a 13-d-old chick embryo (Fig. 1*c*).

The two-dimensional gel electrophoresis patterns of myosin light chains present in developing chicken ventricular muscle are shown in Fig. 2. Adult and 12-d-old embryonic chicken ventricular myosins contain two myosin light chains (L_{c1} and L_{c2}) (Fig. 2, *a* and *b*), but at young embryonic stages, for example at day 6 or day 8 of incubation, two more minor spots (L_{f1} and L_e) were detected in the myosin light chain region (Fig. 2, *c* and *d*). One of them (L_{f1}) was previously identified as fast skeletal myosin light chain 1 (20). When the mixture of embryonic ventricular myosin and embryonic gizzard myosin was examined (Fig. 2*e*), the other spot, L_e, exhibited the same mobility on two-dimensional gel as the embryonic smooth muscle light chain (L_{23}).

These results indicate that the myosin light chain–like spot (L_e) detected in embryonic chicken breast and ventricular

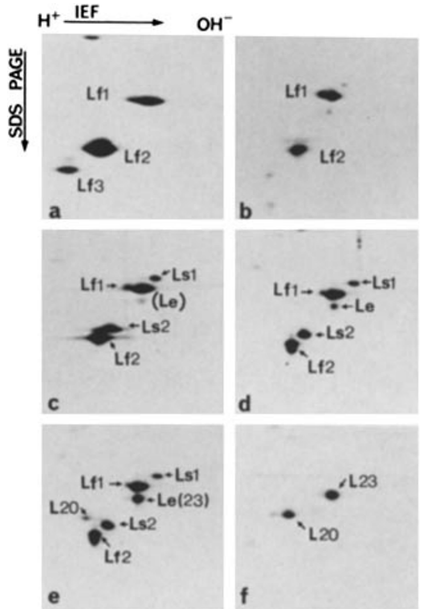

FIGURE 1 Two-dimensional electrophoresis patterns of myosin light chains present in developing chicken breast muscles. *IEF*, isoelectric focusing. (*a–d*) Adult, and 15-d-old, 13-d-old, and 10-d-old embryo chicken breast muscle myosin, respectively. (*e*) 10-d-old embryo breast muscle myosin + 12-d-old embryo gizzard muscle myosin. (*f*) 12-d-old embryo gizzard muscle myosin. 1 μg protein was applied for each myosin. Proteins were stained with silver. L_{f1}, L_{f2}, and L_{f3}, fast skeletal myosin light chains 1, 2, and 3, respectively; L_{s1} and L_{s2}, slow skeletal myosin light chains 1 and 2; L_{20}, gizzard myosin light chain; L_{23}, embryonic gizzard myosin light chain. In young embryonic chicken breast muscle, an embryonic myosin light chain that comigrates with L_{23} was detected in addition to fast and slow myosin light chains. L_e, embryonic light chain.

muscles could be the same as the embryonic smooth muscle light chain (L_{23}); in other words, L_{23} light chain may also be expressed in embryonic skeletal and cardiac muscles.

We further confirmed the expression of the embryo-specific smooth muscle light chain (L_{23}) in developing striated muscles by immunoblots using two mAb's.

The specificities of the two monoclonal antibodies (MT-53f and MT-185d) that were raised against chicken fast light chains were examined by immunoblots (Fig. 3). The myosin light chains of adult chicken breast, ventricle, gizzard muscles, and embryonic gizzard muscle were displayed on SDS polyacrylamide gel and transferred to nitrocellulose paper, reacted with two monoclonal antibodies, then treated with iodinated anti–mouse IgG. Two monoclonal antibodies reacted with both fast light chains 1 and 3 (L_{f1} and L_{f3}, respectively, in Fig. 3 *A*), but no reaction with adult cardiac and gizzard light chains was observed (Fig. 3, *B* and *C*). MT-185d, but not MT-53f, strongly reacted with embryonic smooth muscle light chain L_{23} (Fig. 3*D*). Neither MT-53f nor MT-185d exhibited positive reaction with myosin light chains in the monolayer cultures of fibroblasts from embryonic chicken skin.

With these monoclonal antibodies and polyclonal antibody to cardiac light chain 1, myosin light chains present in the ventricular muscle of 8-d-old embryo and the breast muscle of 10-d-old embryo were examined by immunoblots. The antibody to cardiac light chain 1 reacted positively with both embryonic skeletal and cardiac muscle myosins to give one band at the electrophoretic position corresponding to cardiac light chain 1 or slow light chain 1 (Fig. 4, lanes *b* and *h*), as

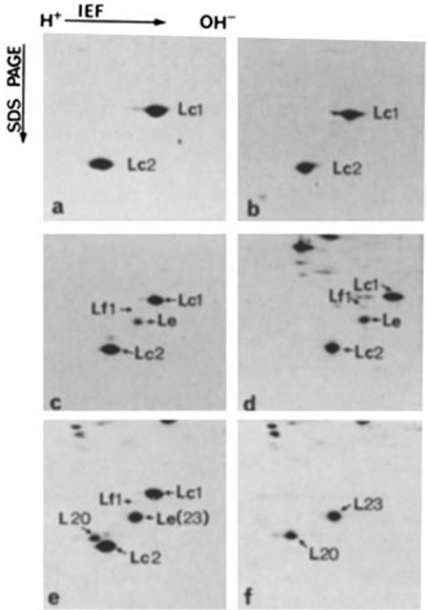

FIGURE 2 Two-dimensional electrophoresis patterns of myosin light chains present in developing chicken cardiac muscles. *IEF*, isoelectric focusing. (*a–d*) Adult, and 12-d-old, 8-d-old, and 6-d-old embryo cardiac muscle myosin, respectively. (*e*) 8-d-old embryo cardiac muscle myosin + 12-d-old embryo gizzard muscle myosin. (*f*) 12-d-old embryo gizzard muscle myosin. 1 μg protein amount was applied for each case. Proteins were stained with silver. Embryonic ventricular myosin contained embryonic light chain which comigrates with L_{23}. L_{c1} and L_{c2}, myosin light chains. Other labels are explained in legend to Fig. 1.

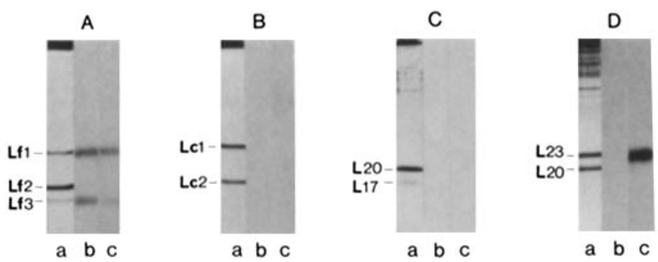

FIGURE 3 The specificities of two mAb's to fast skeletal myosin light chains. The light chains of adult chicken breast muscle myosin (*A*), adult chicken cardiac muscle myosin (*B*), adult chicken gizzard muscle myosin (*C*), and 12-d-old embryo gizzard muscle myosin (*D*) were displayed on an SDS polyacrylamide gel, then transferred to nitrocellulose paper. Separate lanes of each specimen were reacted with either MT-53f (lane *b*) or MT-185d (lane *c*). One lane of each specimen (a) was stained with Coomassie Brilliant Blue to locate the position of light chains. MT-53f was specific to L_{f1} and L_{f3}, but MT-185d positively reacted not only with L_{f1} and L_{f3} but also with L_{23}. L_{17}, 17,000-mol-wt myosin light chain. Other labels are explained in the legends to Figs. 1 and 2.

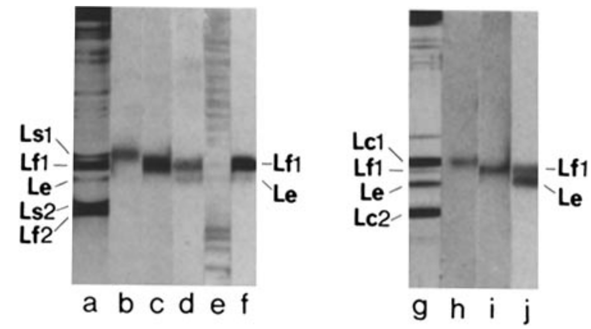

FIGURE 4 Immunoblot analyses of myosin light chains present in 10-d-old embryo breast muscle (lanes *a–d*), breast muscle culture (lanes e and *f*), and 8-d-old embryo cardiac muscles (lanes *g–j*). To examine myosin light chains in the muscle culture (lanes *e* and *f*), the whole protein extract with 2% SDS and 2% 2-mercaptoethanol was used instead of myosin in the case of embryonic muscles (lanes *a–d* and *g–j*). Separate lanes of each specimen were treated with anti-L_{c1} polyclonal antibody (lanes *b* and *h*), MT-53f (lanes *c* and *i*), and MT-185d (lanes *d*, *f*, and *j*). One lane of each specimen (lanes *a*, *e*, and *g*) was stained with Coomassie Brilliant Blue to locate the positions of myosin light chains. Labels are explained in the legends to Figs. 1 and 2.

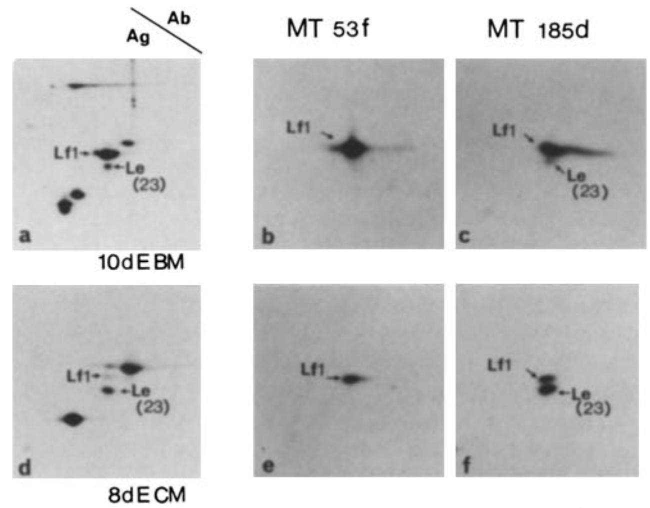

FIGURE 5 Analyses of myosin light chains present in embryonic breast and cardiac muscles by immunoblots combined with two-dimensional gel electrophoresis. Myosin light chains from 10-d-old breast (*10dEBM*) and 8-d-old cardiac (*8dECM*) muscles were displayed on two-dimensional gel as shown in *a* and *d*, respectively, transferred to nitrocellulose paper, and then reacted with either MT-53f (*b* and *e*) or MT-185d (*c* and *f*). (*a* and *d*) Electrophoretic patterns stained with silver; (*b* and *c*) autoradiograms with embryonic breast muscle myosin; (*e* and *f*) autoradiograms with embryonic cardiac muscle myosin. *Ag*, antigen. *Ab*, antibody. Other labels are explained in the legend to Fig. 1.

previously demonstrated (19, 20). MT-53f also reacted with both embryonic myosins to give one band and this band was located at the electrophoretic position of fast light chain 1 (Fig. 4, lanes *c* and *i*). In contrast, MT-185d gave two bands when reacted with embryonic breast and ventricular myosins. One corresponds to fast light chain 1 and the other corresponds to embryonic L_{23} light chain, as judged by their electrophoretic mobilities (Fig. 4, lanes *d* and *j*). The extracts from the myotubes cultured for 5 d in vitro, which were originated from 11-d-old embryonic chicken breast muscle, similarly gave two bands when treated with MT-185d (Fig. 4*f*).

Immunoblotting combined with two-dimensional gel electrophoresis gave us more conclusive data. Myosin light chains in 10-d-old embryonic breast muscle and 8-d-old embryonic cardiac muscle were displayed on two-dimensional gels, transferred to nitrocellulose paper, and reacted with the two mAb's. Both myosins positively reacted with MT-53f to give one spot that corresponds to fast light chain 1 (Fig. 5, *b* and *e*). When the myosins from both embryonic striated muscles were reacted with MT-185d, two spots were obtained: two major spots in the case of embryonic cardiac myosin (Fig. 5*f*), and one major and one minor spot in the case of embryonic breast muscle myosin (Fig. 5*c*). By comparing the two-dimensional gel electrophoresis patterns of myosin light chains, we ascertained that these spots were raised by the reaction of MT-185d with fast light chain 1 and embryonic light chain (L_{23}), respectively.

To examine whether the embryonic myosin light chain is incorporated into myosin molecules in developing cardiac and skeletal muscles, native myosin isozymes of embryonic skeletal and ventricular muscles were isolated on pyrophosphate acrylamide gel; myosin isozyme bands were sliced out, and then light chains present in myosin isozymes were examined by SDS PAGE. The light chain distributions in embryonic skeletal and cardiac myosins are shown in Fig. 6. The distribution of light chains in the embryonic skeletal myosin differed from that in adult skeletal myosin: a tiny amount of embryonic L_e (L_{23}) light chain as well as two fast light chains and two slow light chains were detected in the myosin from 10-d-old chicken embryo breast muscle (Fig. 6, lanes *a* and *b*), whereas adult fast skeletal myosin contained three fast light chains (Fig. 6, lanes *c* and *d*). Two cardiac light chains (L_{c1} and L_{c2}) were present in adult cardiac myosin isozyme (Fig. 6, lanes *g* and *h*), but small amounts of two additional light chains (L_{f1} and L_e or L_{23}) were also detected in the embryonic cardiac myosin isozyme (Fig. 6, lanes *e* and *f*). Based on these observations, embryonic light chain (L_e or L_{23}) is probably associated with heavy chains to form myosin molecules in embryonic skeletal and cardiac muscles. Embryonic myosin isozymes may be a mixture of isozymes that contain different combinations of light chains.

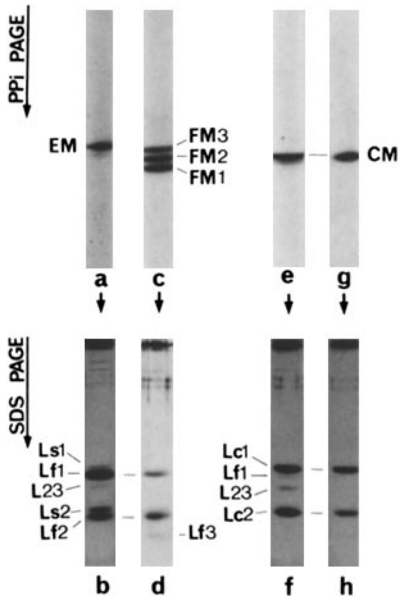

FIGURE 6 The compositions of myosin light chains present in native myosin isozymes of embryonic chicken skeletal and cardiac muscles. Myosin isozymes were separated by pyrophosphate acrylamide gel electrophoresis (*PPi PAGE* [*top*]) and the light chain compositions in myosin isozyme bands were examined by SDS PAGE (*bottom*). Lanes *a* and *c*, 10-d-old embryo (*EM*) and adult breast muscle myosin (FM_1, FM_2, and FM_3) isozymes, respectively; lanes e and g; 8-d-old embryo and adult cardiac muscle isozyme (*CM*), respectively; lanes *b* and *d*, light chain compositions of 10-d-old embryo (*EM*) and adult breast (FM_2) myosin isozymes, respectively; lanes *f* and *h*, the compositions of light chains in 8-d-old embryo and adult cardiac muscle myosin, respectively. Embryonic light chain (L_{23}) was detected in the myosin isozymes from embryonic skeletal and cardiac muscles, suggesting that this light chain is incorporated into myosin molecules in the embryonic tissues. Labels are explained in the legends to Figs. 1 and 2.

We examined whether the myosin from embryonic breast and cardiac muscles is contaminated by smooth muscle myosin derived from blood vessels using the monoclonal antibody (G 3–6) specific to smooth muscle myosin heavy chain (Fig. 7). This antibody reacts with the myosin heavy chains of both gizzard and aorta smooth muscles (Tanaka, T., and T. Masaki, unpublished observation). As shown in Fig. 7, neither embryonic cardiac nor embryonic breast muscle myosin exhibited any positive reaction with G 3–6. This antibody failed to stain skeletal muscle cultures, as determined by an indirect immunofluorescence method. That we did not detect smooth muscle myosin isozyme or smooth muscle myosin heavy chain in the breast and the ventricular muscles of embryonic chicken by pyrophosphate acrylamide gel electrophoresis and by peptide mapping (21) is inconsistent with these observations.

The time course of the expression of the embryonic myosin light chain (L_{23}) during the development of three different chicken muscle tissues is schematically shown in Table I. During development, the embryonic light chain is transiently expressed in all of these muscles, but the time points when the embryonic light chain disappears are not the same in all of these muscles: L_{23} is expressed in the striated muscles only at early embryonic stages, but it continues to be expressed in gizzard smooth muscle until neonatal ages.

DISCUSSION

Using two-dimensional electrophoresis and immunoblot analysis, we have demonstrated that an embryonic myosin light chain (L_{23}), which was originally discovered in embryonic chicken gizzard smooth muscle (11, 27), is expressed in young embryonic chicken skeletal and cardiac muscles. This light chain was also detected in embryonic chicken atrium and aorta muscles (data not shown), but not in adult muscle tissues; therefore we can conclude that L_{23} is a light chain that is generally expressed in embryonic smooth and striated muscles at certain developmental stages. We can eliminate the possibility that L_{23} light chain present in embryonic striated muscles might originate from blood vessel smooth muscle that may coexist in the embryonic tissues for two reasons: First, we detected neither smooth muscle myosin isozyme, which is easily distinguishable from striated muscle myosin

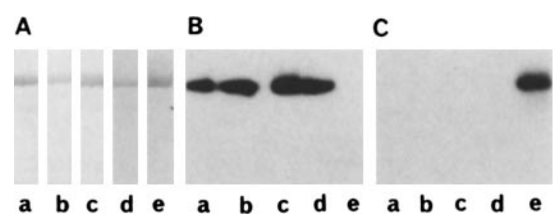

FIGURE 7 Immunoblot analyses of myosin heavy chains from embryonic breast and cardiac muscles. Myosins from breast muscle of 10-d-old embryo (lane *a*) or adult (lane *b*), ventricular muscle of 8-d-old embryo (lane *c*) or adult (lane *d*), and adult gizzard muscle (lane *e*) were treated with mAb MF-20, which binds to all striated muscles (*B*; reference 5) or mAb G 3-6 specific to smooth muscle myosin heavy chain (*C*). Electrophoresis patterns stained by Coomassie Brilliant Blue are given in *A*.

Table I

Expression of Embryonic Myosin Light Chain during Development of Chicken Skeletal, Cardiac, and Smooth Muscles

	Developmental ages (*d*)									
	Embryo							Postnatal		
	3	6	8	10	13	15	20	3	8	Adult
Cardiac muscle	+	+	+	±	−	−	−	−	−	−
Breast muscle				+	±	−	−	−	−	−
Gizzard muscle				+	+	+	+	+	−	−

+, detectable; ±, faintly detectable; −, not detectable.

isozymes by pyrophosphate acrylamide gel electrophoresis (21), nor smooth muscle-type myosin heavy chain, which is characterized by peptide mapping (21) and by the monoclonal antibody specific to smooth muscle myosin heavy chain in the embryonic striated muscle myosin preparations (Fig. 7): Second, we could detect L_{23} light chain in skeletal and cardiac muscle cells in culture, although the striated muscle cultures were not stained by mAb G 3-6 specific to smooth muscle myosin heavy chain as examined by an indirect immunofluorescence method. We did not detect L_{23} light chain in nonmuscle cells such as fibroblasts and intestinal brush border.

Burridge and Bray (1) demonstrated that myosin light chain of 23,000 mol wt exists in both embryonic and adult chicken brain. Our preliminary investigation demonstrated by two-dimensional gel electrophoresis that the brain L_{23} light chain co-migrates with embryonic smooth muscle L_{23} light chain. It may be possible that the two L_{23} light chains are identical. Further investigation is now under way to clarify whether the same L_{23} light chain is expressed in brain and embryonic muscle tissues.

In our investigation, embryonic light chain was detected together with fast and slow skeletal myosin light chains in the embryonic breast muscle. According to Crow et al. (2), at very early developmental stages, slow-type light chains do not exist in the embryonic breast muscle, although fast-type light chains are detectable. It is of interest to learn what combinations of myosin light chains are expressed at earlier developmental stages. The transition of myosin light chain expression during muscle development in vivo may be more complicated than heretofore suspected.

Chicken embryonic myosin light chain (L_{23}) is quite different from mammalian embryonic light chain, as was reported by Whalen et al. (28): They differ in both isoelectric point and molecular weight. The embryonic light chain in mammalian muscle is the major alkali light chain in fetal striated muscles (28, 29), but the L_{23} does not exist in embryonic chicken striated muscles as the major light chain component. The mammalian embryonic light chain is indistinguishable from atrium light chain 1 (30), whereas the chicken embryonic L_{23} differs from adult atrium light chains.

The embryonic light chain (L_{23}) may be categorized into catalytic or essential light chains such as gizzard L_{17} light chain and fast skeletal light chains 1 and 3, since L_{23} light chain is replaced by gizzard L_{17} light chain during gizzard muscle development (27). The embryonic L_{23} light chain exhibited immunological similarity with fast skeletal light chains 1 and 3. Therefore, structural similarity should exist among these proteins. It has been demonstrated that these two fast skeletal light chains are closely related in primary structure (14) and are the products of a single gene (16). It is of interest how the embryonic light chain is structurally related to these two fast skeletal light chains. Correlative analyses with cDNA probes and monoclonal antibodies could resolve this problem.

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