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Expression of myosin light chains during fetal development of human skeletal muscle

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The expression of myosin light chains (MLCs) during the development of human skeletal muscle was investigated by using two different two-dimensional electrophoretic techniques. In both electrophoretic systems the predominant light chain 1 (LC1) expressed during the whole fetal period was found to co-migrate with the adult fast LC1 (LC1F). The main LC2 expressed during the whole fetal period was found to be different from the main fast LC2 (LC2F) and slow LC2 (LC2S) usually present in adult muscle, but co-migrated with a minor component often present in adult muscle. This fetal LC2 was phosphorylatable, and the phosphorylated form co-migrated with the main component of LC2F expressed in the adult. The adult fast LC3 appeared as early as week 20 of gestation, whereas the adult slow light chains (LC1S and LC2S) appeared only during the late fetal period. A minor component of LC1, previously described in humans as an 'embryonic LC' ($LC_{emb.}$) [Strohman, Micou-Eastwood, Glass & Matsuda (1983) Science **221**, 955–957], was only expressed in the early fetal period and was found to co-migrate with atrial LC1 (ALC1). We discuss the expression of these specific developmental forms of MLCs co-existing with immature myosin heavy chains during fetal life.

INTRODUCTION

The sequential expression of different types of isomyosins during skeletal-muscle development has been extensively studied in several avian and mammalian species, including man. Myosin-heavy-chain (MHC) transitions from embryonic, and fetal–neonatal, to adult slow or fast forms have been well established by different approaches (Fitzsimons & Hoh, 1981; Whalen *et al.*, 1981; Bandman *et al.*, 1982; Lowey *et al.*, 1983; Maréchal *et al.*, 1984; Schiaffino *et al.*, 1986; Pons *et al.*, 1986). The nature and sequential expression of the myosin light chains (MLCs) associated with these different immature MHCs are less documented. Only a myosin LC1 component that is different from any adult skeletal MLCs has been found in embryonic skeletal muscle (Whalen *et al.*, 1978; Strohman *et al.*, 1983). This embryonic myosin light chain, $LC_{emb.}$, co-migrates with ALC1. In the early period of muscle development, neither the adult slow myosin light chains nor myosin LC3 have been detected. Some discrepancies between the few results may arise from the fact that the sequence of events is identical, but not their timing relative to birth (Syrovy, 1978; Volpe *et al.*, 1981; Biral *et al.*, 1982, 1984; Srihari *et al.*, 1982; Cummins, 1983; Lowey *et al.*, 1983; Crow *et al.*, 1983; Strohman *et al.*, 1983).

In a previous study we demonstrated that urea/PAGE differs from SDS/PAGE with respect to the separation of myosin light chains (Pons *et al.*, 1983). In the present work we again applied both electrophoretic approaches to monitor myosin-light-chain expression in human skeletal muscles during fetal life and to compare it with the myosin-light-chain composition of adult muscles. We observed that the main LC2 component expressed in the fetal period is electrophoretically different from the main LC2F expressed in adult fast muscle and is phosphorylatable. We confirm the co-migration of the main component of LC1, expressed in the fetal period, with adult LC1F. The minor component of LC1 ($LC_{emb.}$) co-migrated with ALC1 and disappeared in the late fetal period.

MATERIALS AND METHODS

Tissue sources

Biopsies of human adult skeletal muscle (vastus lateralis) were obtained from five 40–60-year-old patients undergoing surgery. Fetal muscles (vastus lateralis) were obtained from twelve fetuses legally aborted for therapeutic reasons, unrelated to muscle disease, or from spontaneous abortion and ranged in age from 10 gestational weeks to term, as shown in Table 1.

Preparative procedure

Myosin was prepared from human fetus muscle by the method of Kielly & Bradley (1956) in the presence of various proteinase inhibitors (pepstatin, 1 mg/ml; leupeptin, 20 μg/ml; soya-bean trypsin inhibitor, 0.5 μg/ml). Crude muscle extracts were prepared by tissue homogenization at room temperature in a 'lysis' buffer as described by O'Farrell (1975) containing 9.5 M-urea, 6% (v/v) Ampholines (LKB) (2% over the pH range 3.5–10, 2% over the pH range 4–6 and 2% over the pH range 5–7), 5% (v/v) 2-mercaptoethanol, 10% (v/v) Nonidet P40 (Sigma). The ratio of tissue to buffer

Abbreviations used: MLC, myosin light chain; F, fast; S, slow; $LC_{emb.}$, embryonic light chain; ALC, atrial light chain; MHC, myosin heavy chain; PAGE, polyacrylamide-gel electrophoresis; i.e.f., isoelectric focusing; PMSF, phenylmethanesulphonyl fluoride; TM, tropomyosin; MLCK, myosin-light-chain kinase.

Table 1. Ages, numbers and origins of fetuses used as sources of fetal muscle

Two adult atrial muscle samples were obtained from autopsies, one within 12 h of death and the other immediately after death (transplant donor). All muscle samples were stored as previously described (Pons *et al.*, 1983). Abbreviations used: S, spontaneous abortion; T, therapeutic abortion.

Age in gestational weeks...	10	11	17	18	20	22	23	24	26	27	36
No.	1	1	1	1	2	1	1	1	1	1	1
Origin	T	T	T	T	T/S	T	T	T	S	T	S

was 1:30 (w/v) in the case of adult extract, and 1:10 (w/v) in the case of fetal extract. The homogenate was centrifuged at 900 *g* for 2 min and the supernatant was directly applied to i.e.f. gels. When the phosphorylation was performed before electrophoresis, the extraction procedure was carried out at 4 °C in the following buffer: 60 mM-KCl/40 mM-imidazole/1 mM-dithiothreitol/ 1 mM-PMSF), pH 7.4.

Light-chain phosphorylation procedure

Myosin light chains were phosphorylated in crude muscle extractor in myosin samples in the above-mentioned buffer. The preparation of pure myosin was complemented with MLCK contained in a small amount of crude extract sufficient to allow phosphorylation of MLC under the conditions described by Cardinaud (1986). The amount of the additional myosin from this extract was less than 10% of the myosin analysed. The reaction was started by adding 2.5 mM-ATP, 15 mM-$MgCl_2$ and 0.2 mM-$CaCl_2$ and continued for 10 min at 23 °C. The reaction was stopped by adding 8 M-urea, and the samples were ready to be applied to i.e.f. gels after adding all constituents of the 'lysis' buffer.

Two-dimensional gel electrophoresis

The first electrophoretic separation of the proteins was carried out by using O'Farrell's (1975) method on an i.e.f. gel, separating the proteins according to their charge. For the second dimension we used two procedures: SDS/PAGE, to separate proteins according to their M_r (Laemmli, 1970), and urea/PAGE, to separate the proteins according to both their M_r and their charge (Perrie & Perry, 1970). Portions (10–40 μl) of muscle extracts containing 40 μg of myosin were applied to cylindrical i.e.f. gels (2 mm × 100 mm). Focusing was performed for 16 h at 400 V and finally for 1 h at 500 V. In the SDS/PAGE system, the i.e.f. gels were then equilibrated in SDS running buffer [0.1% (w/v) SDS/50 mM-Tris/150 mM-glycine, pH 8.6], loaded and run for 5 h at 220 V in slab gels (1.5 mm × 100 mm) with 15% (w/v) polyacrylamide for the running gel and 5% (w/v) polyacrylamide for the stacking gel. In the urea/PAGE system the i.e.f. gels were equilibrated in the running buffer (20 mM-Tris/125 mM-glycine, pH 8.6), loaded, and run in slab gels (1.5 mm × 100 mm) containing 10% (w/v) acrylamide, 0.3 M (w/v) bisacrylamide, 8 M-urea, 20 mM-Tris and 125 mM-glycine, pH 8.6. Gels were run at 300 V for 7 h. The gels were usually stained with Coomassie Brilliant Blue.

RESULTS

MLC analysis of fetal and adult skeletal muscles

The MLC composition of fetal and adult muscles was compared in muscle extracts by using two different two-dimensional electrophoretic systems, SDS/PAGE and urea/PAGE. As Figs. 1(*a*) and 1(*a'*) show, since normal human adult muscles contain fast and slow types of fibres, the corresponding total muscle extracts contained both types of MHC and myosin light chains. The identification of the various myosin-light-chain spots, with regard to the fast and slow myosins, has previously been established by comparison with fast and slow rabbit myosins (Pons *et al.*, 1983). Slow and fast MLCs had inverse mobilities in the two systems. The slow and fast components of LC1 were present in nearly equal amounts (Figs. 1*a* and 1*a'*). Six spots of variable intensity were usually observed in the LC2 region. Three of them were LC2S and the three others were LC2F. For convenience we refer to the LC2F components as LC2Fa, LC2Fb and LC2Fc in order of their electronegativity. In most of the normal adult muscle extracts the main LC2F component present was LC2Fb and, to a lesser extent, LC2Fc; the LC2Fa spot was faint or often absent. The more acidic LC3 spot migrated faster in both systems and was associated with the fast type of myosin.

In the early period of human fetal muscle development, the MLC pattern consisted of two LC1 and two LC2 of unequal intensity in each case (Figs. 1*b* and 1*b'*). The main LC1 present in the fetal extract co-migrated in both systems with adult LC1F (Figs. 1*c* and 1*c'*). The minor spot LC1 ($LC_{emb.}$) was heavier than the main LC1 spot at about the same pI (see the arrowhead in Fig. 1*b*) and migrated faster in the urea/PAGE system (arrowhead, Fig. 1*b'*). The main LC2 component present in the early-fetal muscle extract co-migrated with LC2Fa in both systems. This spot, which was faint or often absent in adult muscles, migrated at the same level as tropomyosin (TM) in the urea/PAGE sytem. The minor fetal LC2 spot, which was more acidic and migrated faster on urea/PAGE, co-migrated with LC2Fb in both techniques. The same myosin-light-chain patterns were observed in the preparations of fetal myosin (see Fig. 3 below). To summarize, the myosin-light-chain pattern in the early-fetal muscle extracts was characterized by the presence of a main LC1 co-migrating with fast adult LC1F, a main LC2F which was different from the main adult form, a small amount of $LC_{emb.}$ and no slow LC1S, LC2S, or LC3.

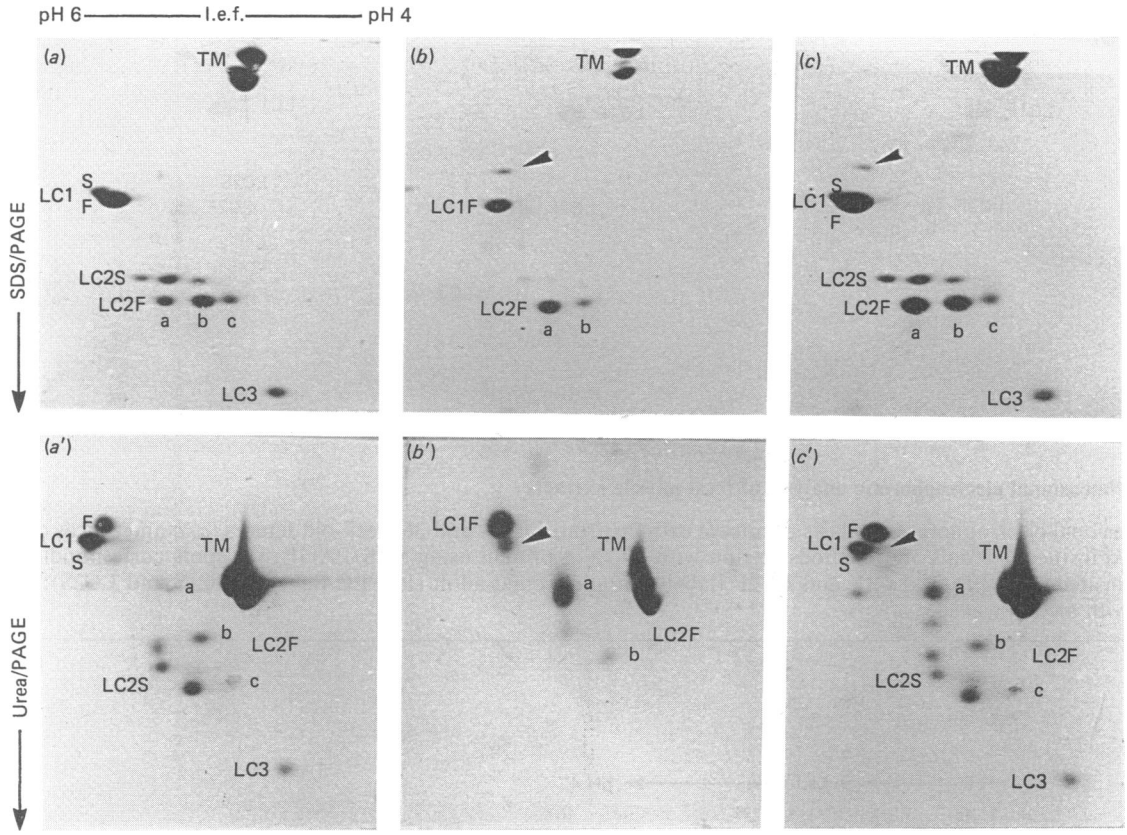

Fig. 1. Two-dimensional electrophoretic analysis of fetal and adult muscle extracts

Two-dimensional electrophoretic analysis of crude skeletal muscle extract was carried out as described in the Materials and methods section, with about 40 μg of proteins using either SDS/PAGE (*a*, *b* and *c*), or urea/PAGE (*a'*, *b'* and *c'*). The separated light chains were stained with Coomassie Brilliant Blue. Only the light chain and the TM region are shown. Normal adult skeletal-muscle extract is shown in *a* and *a'*; skeletal-muscle extract from a 17-week-old fetus is shown in *b* and *b'*; the co-electrophoresis of adult and fetal muscle extracts is shown in *c* and *c'*. The spots corresponding to the adult fast type of light chain are shown: LC1F, LC2F (labelled a, b, and c) and LC3. The spots corresponding to slow light chains are labelled LC1S. The $LC_{emb.}$ is indicated with an arrowhead.

Developmental expression of MLCs

The timing of myosin-light-chain expression during fetal life was analysed by two-dimensional SDS/PAGE of crude muscle extracts from twelve fetuses of different ages. The most characteristic steps are shown in Fig. 2. In the four younger fetuses, 10–20 gestational weeks of age, the pattern was the same as in Fig. 1: two main MLCs, LC1F and LC2Fa, and two minor spots, $LC1_{emb.}$ (see arrowhead) and LC2Fb (Fig. 2*a*).

In the middle period of fetal development from 20 to 25 weeks of gestation (five fetuses) the major feature was the appearance of LC3. The main LC1 and LC2 spots were the same as in the preceding period (Fig. 2*b*). The relative proportion of $LC_{emb.}$ was lower at this stage than in the preceding period.

In the later fetal period, from 25 weeks to term (three fetuses), the MLC pattern was characterized by the appearance of spots co-migrating with adult LC1S and LC2S. However, this pattern differed from that of adult light chains (for comparison, see Fig. 1*b*): the LC2S were present in smaller amounts, and the main fast LC2F component was still LC2Fa, with a small amount of LC2Fb and no LC2Fc. At this stage, as in the adult pattern, $LC_{emb.}$ was nearly undetectable and LC3 was present in substantial amounts.

Phosphorylation of LC2

To investigate the possibility that different phosphorylation states of LC2 are responsible for the presence of the different LC2 components in adult and fetal muscle extracts, we carried out phosphorylation experiments *in vitro* in which pure fetal myosin was phosphorylated by MLCK contained in a small amount of crude fetal muscle extract. The results were analysed in both electrophoretic systems (Fig. 3). After phosphorylation, the main fetal spot, LC2Fa, decreased. Another spot appeared, which was more acidic and migrated faster in urea/PAGE; this spot co-migrated with adult LC2Fb. However, no LC2Fc spot appeared in any case. The phosphorylation of adult LC2 was performed similarly by adding ATP, Mg^{2+} and Ca^{2+} to crude muscle extracts. Two-dimensional analysis showed a similar transition of the main LC2Fb to LC2Fc (results not shown). In the adult muscles the LC2Fb spot could be either the phosphorylated form of LC2Fa or the unphosphorylated form of LC2Fc.

Myosin-light-chain analysis in fetal skeletal muscle and in adult atrium

The presence of an $LC_{emb.}$ in embryonic striated (cardiac or skeletal) muscles, has been demonstrated in

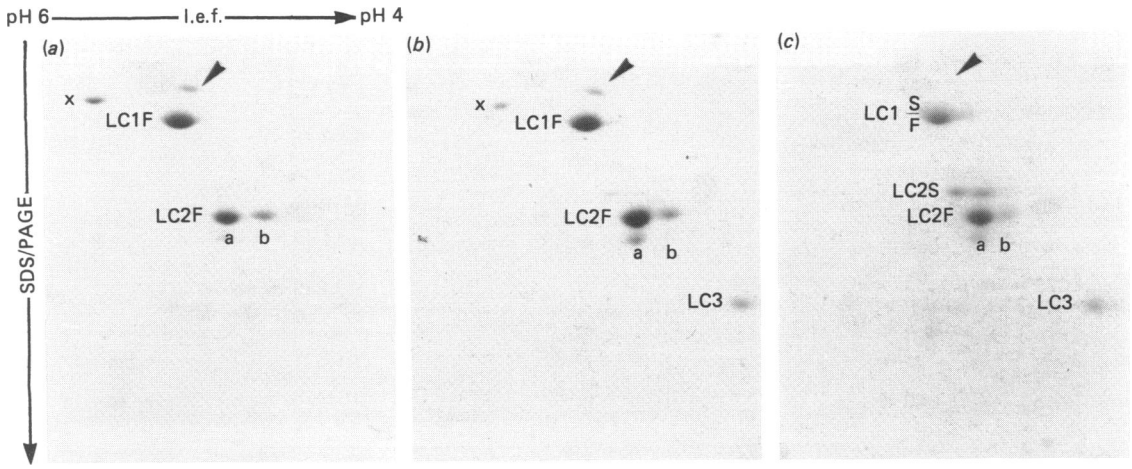

Fig. 2. Two-dimensional electrophoretic analysis of fetal muscle extracts

Two-dimensional electrophoretic analysis of muscle extracts from 17–23- and 36-week-old fetuses (*a*, *b* and *c*) was carried out as described in the Materials and methods section with 40 μg of protein using SDS/PAGE; the spots corresponding to fetal LC are indicated as in Fig. 1: LC1F and LC2F (labelled a and b) and adult slow LC (labelled LC1S and LC2S); $LC_{emb.}$ is indicated with an arrowhead.

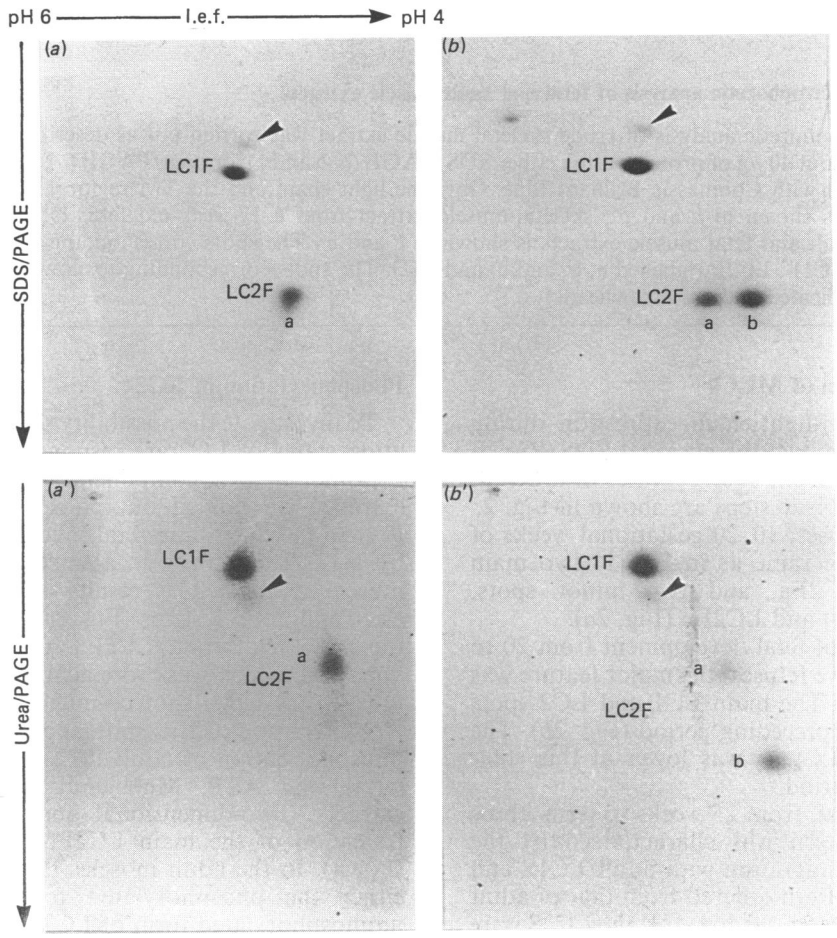

Fig. 3. Two-dimensional electrophoretic analysis of myosin from a 17-week-old fetus before (*a* and *a'*) and after (*b* and *b'*) phosphorylation

The analysis was performed as described in the Materials and methods section by using either SDS/PAGE (*a* and *b*) or urea/PAGE (*a'* and *b'*).

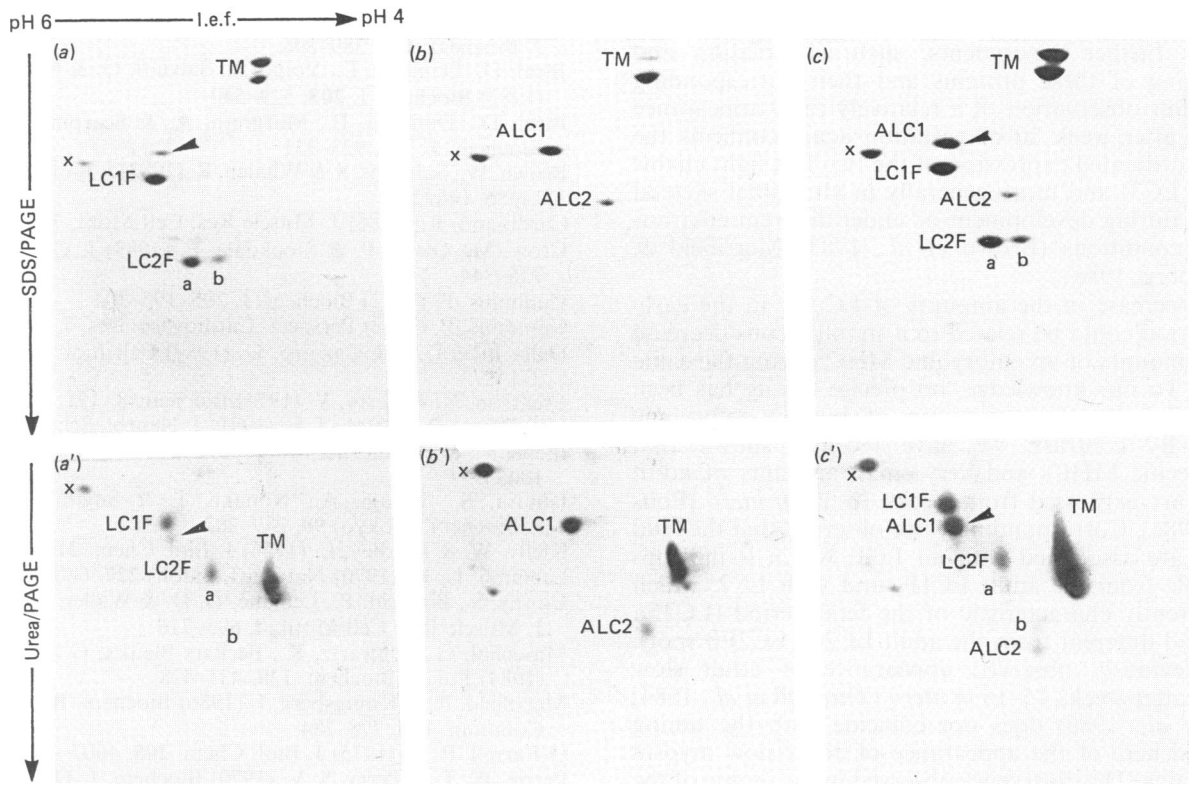

Fig. 4. Two-dimensional electrophoretic analysis of crude fetal muscle extract (*a* and *a'*), or adult atrial extract (*b* and *b'*), and co-electrophoresis of both extracts (*c* and *c'*) using SDS/PAGE (*a*, *b* and *c*) or urea/PAGE (*a'*, *b'* and *c'*)

The spots corresponding to atrial LC are labelled ALC1 and ALC2; the fetal LCs are labelled LC1 and LC2, and the minor fetal LC1 is indicated by an arrowhead. Only the light chain and TM region is shown.

several animal species, and is identical with ALC1 (Cummins, 1982; Srihari *et al.*, 1982; Whalen *et al.*, 1982; Biral *et al.*, 1984). In our comparison of human fetal skeletal-muscle extract (Figs. 4*a* and 4*a'*) with human atrial extract (Figs. 4*b* and 4*b'*), we found that the human $LC_{emb.}$ co-migrated with ALC2 (Figs. 4*c* and 4*c'*) in both electrophoretic systems. The spot marked 'X', present in the atrial extract, co-migrated with a spot marked 'X' present in the fetal extract; this spot, which was observed by Ishiura *et al.* (1981) in slow human muscle fibre, was not present in our myosin preparation of the fetal muscle (Fig. 3*a*).

DISCUSSION

During the fetal life of human skeletal muscles we observed the persistent expression of one LCl co-migrating with adult LC1F in two different electrophoretic systems. By contrast, the embryonic $LC_{emb.}$ was only expressed in the early fetal period. Our observation of a co-migration of $LC_{emb.}$ with human atrial ALCl extends to humans the observation already reported in mammals, that both cardiac and skeletal myosins have the same LC1. Since this light chain is only expressed in adult atrium, this muscle appears to be undifferentiated from this point of view. Our analyses did not rule out the possibility of a structural difference between the $LC_{emb.}$ and ALC1 or between the main LC1 present in human fetal muscle and adult LC1F. In the mouse, Barton *et al.* (1985) have found a single gene for ALC1 and $LC_{emb.}$. In rabbits, however, Dalla Libera & Carraro (1983) detected differences between $LC1_{emb.}$ and ALC1 by peptide mapping.

Several LC2 components have also been observed in human cardiac muscle (Cummins, 1982), rabbit skeletal muscles (Frearson & Perry, 1975) and adult human skeletal muscles (Billeter *et al.*, 1981). The possible interconversion between LC2 spots has already been studied by phosphorylation *in vitro* and *in vivo*. The latter does not provide a satisfactory explanation, since a single phosphorylation can only change one spot into another spot but not into two others. The case of a double phosphorylation has only been described in smooth muscle (Ikebe & Harshorne, 1985). Faced with the same three LC2 spots in bovine hearts, Westwood & Perry (1982) carried out peptide mapping, which showed differences between two of the LC2 forms, suggesting a difference in their primary structure rather than a post-translational modification. In addition, they showed a developmental replacement of one LC2 form by another. Our results, showing that the unphosphorylated form, LC2Fa, is predominant during the whole human fetal period, instead of the adult LC2Fb or LC2Fc component, support the same hypothesis of a probable existence of two structurally different LC2F components. According to this hypothesis, the LC2Fb spot could result from the co-migration of two different LC2F components: the unphosphorylated form of 'fetal' LC2Fa and the phosphorylated form of adult-specific LC2Fc. The clear demonstration of a 'fetal' LC2

component different from the adult form obviously requires further experiments, such as isolation and sequencing of these proteins and their corresponding genes. Our observation of a relatively early appearance of LC3 after week 20 of gestation again confirms the non-co-ordinated expression of the two fast light chains (LC1F, LC3) and more generally in all animal skeletal muscles during development or under different environmental conditions (Brown *et al.*, 1983; Merrifield & Konigsberg, 1986).

The decrease in the amounts of $LC_{emb.}$ in the early fetal period could be related to a simultaneous decrease in the amounts of an embryonic MHC during the same period. To our knowledge, no precise timing has been determined for the expression of human embryonic MHC. By contrast, we have recently shown that fetal-specific MHCs and very small amounts of adult MHCs are expressed from weeks 10–12 *in utero* (Pons *et al.*, 1986). Correspondingly, we observed that the fetal MHCs are associated with an LC1, which is indistinguishable from the adult LC1F, and with LC2, which is apparently characteristic of the fetal period (LC2Fa spot) and different from the adult LC2F (LC2Fb spot). The previously observed appearance of adult slow MHCs after weeks 15–18 *in utero* (Thornell *et al.*, 1984; Pons *et al.*, 1986) does not coincide with the timing observed here of the appearance of adult slow myosin light chains. The divergence observed in the timing of the synthesis of the two slow myosin components could be due to the different sensitivities of the two detection techniques employed (immunofluorescence with high-affinity antibodies for detecting MHCs and Coomassie Blue staining on gel electrophoresis for detecting MLCs), or else the results suggest that light-chain expression is delayed with regard to the corresponding slow MHC. By contrast, the onset of fast LC3 considerably precedes that of the adult fast MHCs, which are expressed only in the last fetal period near birth in human skeletal muscles. This study again shows the asynchronous expression of both light and heavy myosin subunits during development.

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