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cDNA recombinant plasmid complementary to mRNAs for light chains 1 and 3 of mouse skeletal muscle myosin

(cDNA cloning/alkali myosin light chain RNA)

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ABSTRACT **A recombinant plasmid with a cDNA sequence transcribed from mouse skeletal muscle RNA is shown to hybridize with mRNAs for myosin light chains $LC1_F$ and $LC3_F$. The inserted fragment corresponds exclusively to the 3′-noncoding region of the mRNA. It hybridizes almost exclusively with the two light chain messengers from fast skeletal muscle RNA of adult mouse. Slight hybridization is seen with RNA from heart muscle and embryonic skeletal muscle. The implications of the conservation of the 3′-noncoding regions between the two mRNAs are discussed.**

Myogenesis is characterized by the expression of a number of well-defined proteins (e.g., α-actin, myosin, α- and β-tropomyosin, M-creatine phosphokinase, and the acetylcholine receptor) (1). In the last few years, it has become clear that different types of muscle and nonmuscle cells contain different isoforms of the contractile proteins, encoded by similar but nonidentical genes, which form multigene families. Analysis of the organization of these multigene families, different components of which may be coordinately expressed to give rise to a specific phenotype (2–4), should provide some insight into the way in which expression of eukaryotic genes is regulated. We have, therefore, undertaken the cloning of cDNA probes of the sequences coding for mouse muscle contractile proteins. We have already reported the characterization of a muscle actin plasmid (5). We describe here the cloning and characterization of a recombinant plasmid that hybridizes specifically with the messengers coding for the muscle myosin light chains LC1 and LC3.

Myosin from fast skeletal muscle is constituted of two heavy chains and four light chains; these light chains belong to two different functional classes: the alkali-isolated light chains $LC1_F$ and $LC3_F$ (M_r, ≈21,000 and 17,000, respectively) and the dithionitrobenzoic acid light chain LC2 (M_r, ≈19,000) (6). Different isoforms of myosin alkali-isolated light chain LC1 are expressed in different muscle and nonmuscle tissues (7–9) and at different stages in the development of skeletal (10–12) and heart muscle (13). In the cases that have been investigated, these isoforms of the alkali-isolated light chains show common structural features, reflecting a probably common evolutionary origin. Complete amino acid sequence data are available for skeletal fast muscle and cardiac muscle from the chicken (14, 15): in this case, the degree of conservation is ≈70%. It is striking that the two types of alkali-isolated light chains in fast skeletal muscle, LC1 and LC3, share the same COOH-terminal sequence of 141 [rabbit (16)] or 142 [chicken (14)] amino acids. Nevertheless, LC3 is not a fragment of LC1 since it has 8 residues at the NH_2 terminal that differ from the corresponding residues in the 49 amino terminal sequence of LC1.

MATERIALS AND METHODS

Preparation of Poly(A)$^+$RNA and Synthesis of cDNA. Muscles from the hind legs of 8- to 10-day-old mice were dissected, RNA was extracted by the LiCl/urea precipitation technique, and poly(A)$^+$RNA was fractionated on 5–20% sucrose gradients as described (5). Yields were ≈10 μg of poly(A)$^+$RNA/g of muscle. A light chain-enriched size cut of poly(A)$^+$RNA (5 μg) was transcribed using avian myeloblastosis virus RNA-dependent DNA nucleotidyltransferase (reverse transcriptase) (a gift from J. Beard, Life Sciences, St. Petersburg, FL) as described (5). The resulting cDNA was sedimented in a 5–20% sucrose gradient in 0.1 M NaOH/0.9 M NaCl. The cDNA (1.25 μg) longer than 300 base pairs (bp) was used for replication with DNA polymerase I (Boehringer Mannheim). Double-stranded cDNA was treated with nuclease S1 (a gift from M. Jacquet, University of Paris, Orsay, France) and size selected on a 5–20% sucrose gradient in 10 mM Tris·HCl, pH 7.5/100 mM NaCl/1 mM EDTA.

Tailing and Transformation. Twenty-five nanograms of double-stranded cDNA (longer than 400 bp) was elongated with an average of 100 deoxycytidine residues per cDNA extremity by using terminal deoxynucleotidyltransferase from calf thymus (Bethesda Research Laboratories) as described (5). Supercoiled plasmid pBR322 was restricted with *Pst* I (Bethesda Research Laboratories) and ≈25 deoxyguanosine residues were similarly added to the 3′-OH termini. Ten nanograms of tailed cDNA was mixed with 80 ng of elongated plasmid (mol/mol, 1:1, considering 500 bp as the mean size for the cDNA), hybridized, and used to transform *Escherichia coli* C_{600} ($r_k^-m_k^-$) by the $CaCl_2$ method (17). The transformed bacteria were selected on agar/L broth containing tetracycline (10 μg/ml; Sigma).

All manipulations with recombinant bacteria were carried out in a category 2 containment laboratory as stipulated by the French Commission de Classement des Recombinaisons Génétiques *in vitro*.

***In Situ* Hybridization.** Transformed colonies of bacteria were grown overnight on a nitrocellulose filter (Schleicher and Schuell); the filters were then treated according to Grünstein and Hogness (18) and hybridized to ^{32}P-labeled cDNA as described (5).

Preparation of Plasmid DNA. Plasmid DNA was prepared from small quantities of bacterial cultures (1.5 ml) by the rapid method of Birnboim and Doly (19). Large amounts were prepared from cleared lysates by phenol/chloroform extraction and purification on cesium chloride gradients as described by Minty *et al.* (5). For restriction, enzymes were obtained from Bethesda Research Laboratories and used according to their instructions.

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Abbreviations: bp, base pair(s); DBM, diazobenzyloxymethyl.

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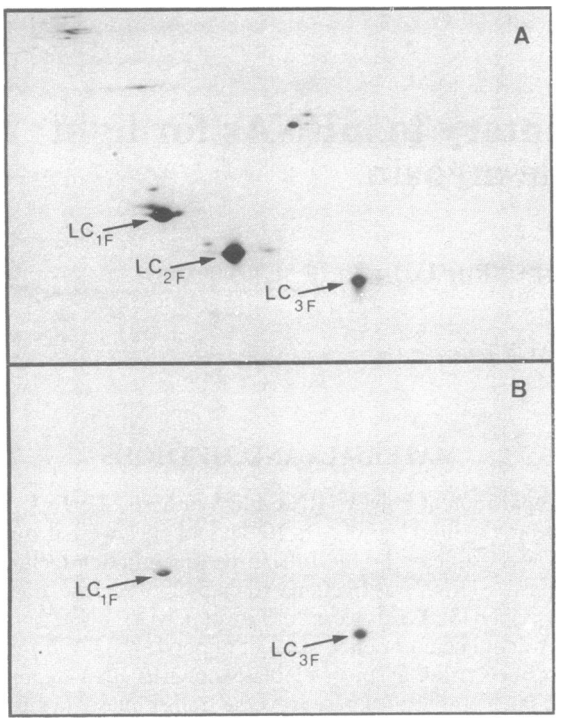

FIG. 1. Two-dimensional gel analysis of translation products of mouse muscle RNA hybridized to DBM-bound plasmid 161 DNA. (*A*) Photograph of Coomassie blue-stained gel, showing the light chains of purified myosin from mouse fast skeletal muscle ($LC1_F$, $LC2_F$, and $LC3_F$) comigrating with the translation products. (*B*) Twenty-four-hour autoradiograph of the same gel. First dimension, isoelectric focusing at pH 4–6; second dimension, 17.5% acrylamide/$NaDodSO_4$ gel.

Preparation and Hybridization of Diazobenzyloxymethyl (DBM) Filters Containing Plasmid DNA. Sonicated plasmid DNA (20 μg) was fixed to 1-cm^2 circles of DBM paper as described by Stark and Williams (20). Hybridization of poly(A)$^+$RNA (2.5 μg), washing of the filters, and elution of the bound RNA was carried out as described by Smith *et al.* (21). Bound and unbound RNAs were concentrated by alcohol precipitation and translated in a nuclease-treated reticulocyte lysate (22). Electrophoresis of the products was carried out as described by O'Farrell (23).

DNA Sequence Analysis. For 3′ labeling, *Pst* I-restricted DNA fragments (30 pmol of 3′ termini) were incubated for 10 min at 37°C with 80 pmol of [α-^{32}P]cordycepin 5′-triphosphate (Amersham; 3,000 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) in the buffer described by Roychoudhury and Wu (24) in a final volume of 50 μl. Twenty units of terminal deoxynucleotidyltransferase (Boehringer Mannheim) was added, and the mixture was incubated for 20 min at 37°C; then, 20 units more was added, and incubation was continued for a further 20 min. The reaction was stopped with 5 mM EDTA and the nucleic acids were alcohol precipitated. They were then digested with a second restriction enzyme (*Sau*3A), and the fragments were separated in 5% polyacrylamide gels and eluted according to Maxam and Gilbert (25). The isolated fragments were subjected to chemical degradation as described by Maxam and Gilbert (25) and analyzed on thin (0.35-mm) polyacrylamide gels as described by Sanger and Coulson (26).

RNA Fractionation and Transfer to DBM Paper. Polyadenylated RNAs (1 μg) from various sources were denatured and subjected to electrophoresis on 1.5% agarose gels according to MacMaster and Carmichael (27). They were transferred to DBM paper, and the blots were hybridized with 5 × 10^6 cpm of plasmid DNA [^{32}P labeled by nick-translation (5)] and treated as described by Alwine *et al.* (28).

Purification of Myosin. Myosin was extracted from 10- to 15-day mouse embryo or adult leg muscles as described by Whalen *et al.* (10) except that chromatography on Sepharose 2B was omitted.

RESULTS

Cloning of a cDNA Complementary to the Light Fractions of mRNA from Mouse Skeletal Muscle. RNA extracted from skeletal muscle of 8- to 10-day-old mice contains as relatively major species the mRNAs coding for muscle specific proteins: namely, myosin heavy chain, α-actin, tropomyosins, and the myosin light chains LC1, LC2, and LC3, as judged by *in vitro* translation and two-dimensional gel analysis (figure 1 of ref. 5). The only isoforms of myosin light chains detected in this analysis are those of the adult phenotype of fast skeletal muscle. To prepare specific probes against the mRNA coding for the smaller contractile proteins (LC1, LC2, LC3, and the troponins) we used a size-selected fraction of poly(A)$^+$RNA as a matrix for synthesis of a double-stranded cDNA. This was inserted in the *Pst* I site of plasmid pBR322, and recombinants were selected by bacterial cloning. From 80 ng of hybrid plasmid, we obtained 750 independent clones. Parallel transformations gave 1.5 × 10^6 transformants per μg of supercoiled pBR322 and none with 10 ng of deoxyguanosine-elongated plasmid.

Identification of a Plasmid. The transformant clones were first screened by the Grünstein and Hogness *in situ* hybridization procedure (18), with a ^{32}P-labeled cDNA synthesized from the mRNA used in the cloning. Clones giving a strong signal (≈200) were selected as good candidates for carrying a sequence abundant in the starting RNA. From a rapid analysis of small aliquots of 30 colonies, 16 were selected that had functional *Pst* I sites and the largest insertions. Plasmid 161 bound to DBM paper, when hybridized with RNA from muscle of 8- to 10-day-old mice, retained a mRNA that, after *in vitro* translation, gave two peptides comigrating on a two-dimensional gel with myosin $LC1_F$ and $LC3_F$ (Fig. 1). Binding of the RNAs coding for $LC1_F$ and $LC3_F$ is very selective; migration of the translation products on a nonequilibrium two-dimensional gel (29) confirmed that these are the only peptides synthesized (30). The fact that this plasmid hybridizes to two different mRNAs is consistent with the very high homology between the two proteins [which share an identical sequence over their COOH-terminal 141 residues (16)].

Restriction analysis of plasmid 161 shows that it contains a 380-bp insert. The sequence of this insert has been partially determined from the 3′ termini toward the *Sau*3A site (Fig.

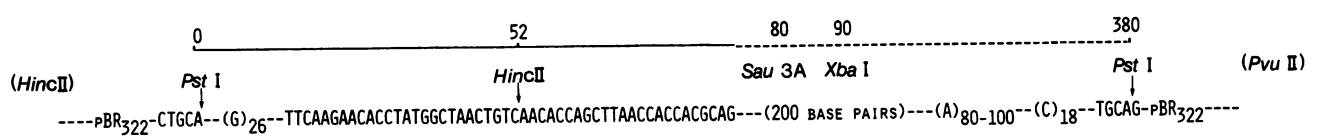

FIG. 2. Partial restriction map and nucleotide sequence of plasmid 161. The coordinates are those of the insert and are oriented from the 5′ end to the 3′ end of the mRNA. The orientation of the insert in the plasmid is given relative to the *Pvu* II (2,067) and *Hin*cII (3,908) restriction sites of pBR322 (31).

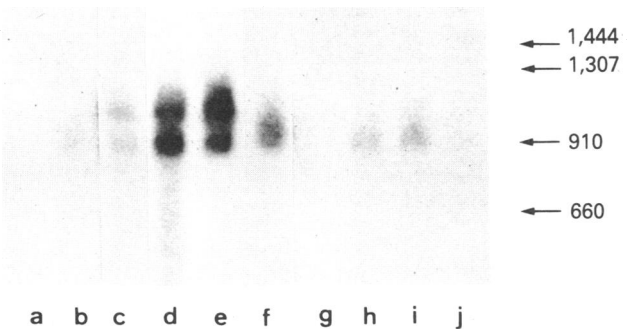

FIG. 3. Blot analysis of myosin light chain mRNAs of various origins. RNAs (1 μg) from dividing L6 myoblasts (lane a), fused L6 myotubes (lane b), newborn rat skeletal muscle (lane c), newborn mouse skeletal muscle (lane d), embryonic mouse skeletal muscle (lane e), fused T984 myotubes (lane f), dividing T984 myoblasts (lane g), adult mouse heart muscle (lane h), embryonic mouse heart muscle (lane i), and adult mouse stomach (lane j) were analyzed on the same 1.5% agarose gel. Lanes c–f were exposed for 14 hr and lanes a, b, and g–j were exposed for 44 hr. Markers (nucleotides) were *Alu* I and *Taq* I digests of pBR322 DNA (31).

2). The longest fragment contains a long (80–100) stretch of poly(dA)poly(dT) which corresponds to the poly(A) of the mRNA, from which the reverse transcription is initiated. Thus any coding region of the mRNA should be located at the opposite end of the insertion. The sequence at this end, however, translated according to any of the three possible reading frames gives rise to an amino acid sequence that bears no relationship to the published data for the COOH termini of LC1 and LC3 (14, 16). These published sequences are those of the rabbit and chicken light chains and the corresponding mouse sequence is not known but, in view of the very high conservation of the sequences between the chicken and the rabbit (only 1 amino acid is changed over the last 20 COOH-terminal residues), it is highly probable that the mouse sequence is not significantly different. Our conclusion is that the inserted cDNA represents exclusively a sequence from the 3′-noncoding region of the mRNA coding for LC1 or LC3 and that this 3′-noncoding sequence is highly conserved, if not identical, between the messengers coding for the two light chains.

mRNAs Coding for the Alkali-Isolated Light Chains of Skeletal Muscle Myosin. We have used the LC1/LC3 recombinant plasmid to look at the mRNAs coding for the alkali-isolated light chains in different tissues by the RNA blotting technique (28). In the RNA from 8- to 10-day-old mouse skeletal muscle, two bands were detected, as expected with this plasmid, corresponding to 1,050 and 900 nucleotides (Fig. 3, lane d). Both RNAs are large enough to encode either of the proteins since, according to the amino acid sequence data for the rabbit light chains (16), only 570 and 447 nucleotides are necessary for the coding sequences of LC1 and LC3, respectively. It should be noted, however, that the difference in size between the two mRNAs (≈150 nucleotides) is consistent with the difference in the coding sequences required for these proteins [123 nucleotides in the case of the rabbit (16)]. This suggests that there are ≈450 nucleotides of noncoding sequence, including the poly(A), in these mRNAs.

To evaluate the extent of homology between the 3′-noncoding sequences of these two mRNAs, we have compared the thermal stability of the hybrids that these form with plasmid 161. A blot of mouse skeletal muscle RNA was hybridized with labeled plasmid 161 and then washed under increasingly stringent conditions (Fig. 4). The two bands revealed in this RNA by plasmid 161 begin to decrease in parallel at temperatures >50°C. At 60°C, the amount of hybrid remaining is small but both bands are still present. At 65°C, no hybridization was detectable even after exposing the blot for 100 hr. The autoradiograms of three such blots were scanned with a Vernon densitometer (Fig. 4*B*). No significant difference in the ratio between the two bands was detected with increasing temperature, suggesting a similar melting temperature for the two hybrids. We thus conclude that the homology between the 3′-noncoding sequences of the two messengers is very high or complete.

Homologies of Plasmid 161 with Other Myosin RNAs. Plasmid 161 does not hybridize with mRNA from stomach muscle, which contains a form of LC1 similar or identical to the nonmuscle type (32) (Fig. 3, lane j), nor with mRNA from mouse brain (not shown) nor mRNA from undifferentiated cells of myogenic cell lines (Fig. 3, lanes a and g). With mRNA from adult heart muscle, there is a faint hybridization with an RNA migrating slightly slower than the 900-nucleotide band detected in skeletal muscle RNA (Fig. 3, lane h). A similar, slightly stronger band is seen with embryonic heart RNA (Fig. 3, lane i). This may represent some cross-hybridization with the mRNA coding for the cardiac alkali light chain $LC1_{card}$, which has some amino acid sequence homology with $LC1_F$ from fast skeletal muscle (15). Alternatively, it may reflect the presence of the

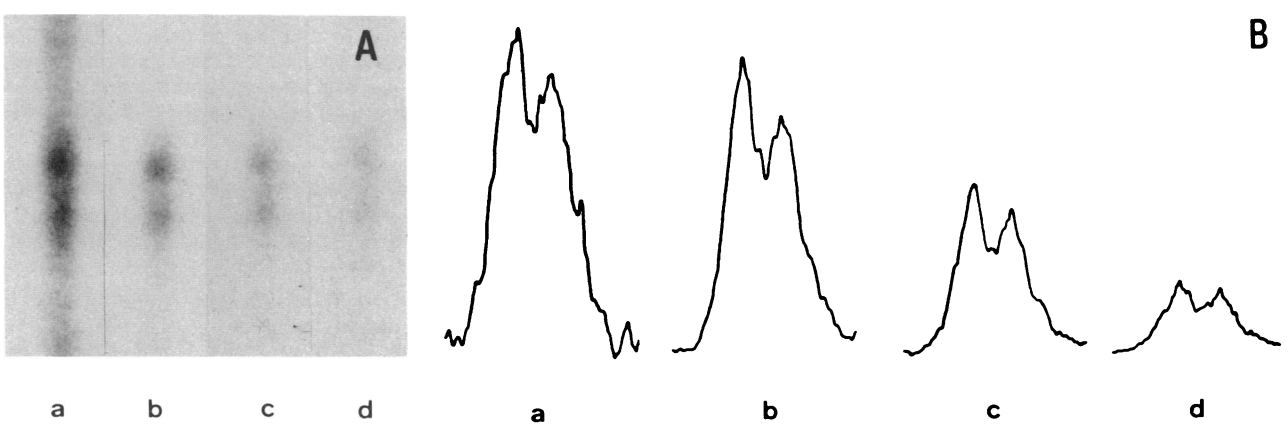

FIG. 4. Thermal stability of the hybrids formed between LC1 and LC3 mRNAs and plasmid 161. RNA from skeletal muscle of newborn mice was separated on a 1.5% agarose gel and transferred to DBM paper as in Fig. 3. After hybridization, the blot was washed in 2% NPE buffer (0.9 M NaCl/50 mM sodium phosphate, pH 7.0/5 mM EDTA) for 30 min at room temperature (lane a), 50°C (lane b), 55°C (lane c), and 60°C (lane d). (*A*) Autoradiograms of the blot. Exposures were for 24 hr, except for lane d, which was exposed for 66 hr. (*B*) Densitometric tracings of the region corresponding to the light chain mRNAs on the autoradiograms. The higher molecular weight RNA is on the left.

embryonic light chain $LC1_{emb}$ present with $LC1_{card}$ in the ventricles of embryonic heart muscle (13) and retained in the atria but not the ventricles of the adult heart (33). This hypothesis is supported by the fact that the RNA hybridizing with plasmid 161 in embryonic and adult heart preparations migrates in the same position as the mRNA for $LC1_{emb}$ (Fig. 3, lane b, and see below), but we cannot exclude the possibility that the messengers for adult cardiac LC1 and embryonic LC1 have a similar size and some homology with plasmid 161.

To look more closely at the situation with the messenger for this embryonic light chain, we used blots of RNA from embryonic muscle and muscle cell lines synthesizing this isoform. The major bands seen on hybridization of RNA from skeletal muscle of 15- to 20-day mouse fetuses with plasmid 161 correspond to those seen with newborn skeletal muscle RNA (Fig. 3, lane e), although these muscles contain some $LC1_{emb}$ protein together with the adult alkali-isolated light chains $LC1_F$ and $LC3_F$ (Fig. 5*B*). With RNA from myotubes of the rat muscle cell line L6 (Fig. 3, lane b), which synthesize almost exclusively this embryonic form of LC1 (10), no cross-hybridization with RNAs of these sizes is seen but a very faint band is detectable migrating more slowly than the lighter skeletal muscle mRNA, whereas RNA from rat skeletal muscle cross-hybridizes with the probe, showing the two adult light chain mRNAs (Fig. 3, lane c). We conclude that the faint band with L6 RNA is probably the mRNA for $LC1_{emb}$, which shows some slight hybridization with plasmid 161 under RNA blot conditions. This provides further evidence that $LC1_{emb}$ originates from a separate gene and is not a modified form of $LC1_F$. RNA from differentiated cultures of the mouse muscle cell line T984 (34) shows a diffuse band migrating as far as the lighter of the two skeletal muscle RNAs (Fig. 3, lane f). Very little hybridization is seen in the region of the heavier band. RNA from fused cultures of this mouse line directs the synthesis in a reticulocyte lysate of the alkali-isolated myosin light chains LC3, $LC1_{emb}$, and small amounts of $LC1_F$ (Fig. 5*A*). When this RNA is hybridized to DBM-immobilized plasmid 161, and the translation products are analyzed on two-dimensional gels with myosin markers, LC3 is the major species synthesized. Trace amounts of $LC1_F$ are detectable, but no peptide that comigrates with $LC1_{emb}$ is found (Fig. 5*C*). We therefore conclude that the smaller messenger seen on the RNA blots probably codes for LC3. It seems likely that the smear behind the main band seen with RNA from T984 (Fig. 3, lane f) is also due to the messenger for mouse $LC1_{emb}$. The apparent discrepancy in the detection of $LC1_{emb}$ RNA between the blot and the DBM filter hybridization experiments probably reflects the more sensitive conditions used in the former.

In addition to its tissue specificity, plasmid 161 is also species specific. Apart from some cross-hybridization with RNA from newborn rat skeletal muscle (Fig. 3, lane c), which gives bands similar to, but fainter than, those seen with mouse muscle (Fig. 3, lane d) (suggesting that the 3′-noncoding sequence of LC1/LC3 has diverged, even between these two closely related species), no cross-hybridization is seen with chicken or human skeletal muscle RNA under blot conditions (data not shown).

DISCUSSION

We have characterized a recombinant plasmid (plasmid 161) that contains a cDNA sequence hybridizing with the messengers coding for the adult myosin light chains $LC1_F$ and $LC3_F$ from mouse fast skeletal muscle with apparently equal affinity. Some cross-hybridization is seen with the messengers for the corresponding rat fast light chains. A very faint reaction is seen with the messenger for embryonic light chain $LC1_{emb}$ expressed in the rat cell line L6 and with embryonic and adult heart RNA.

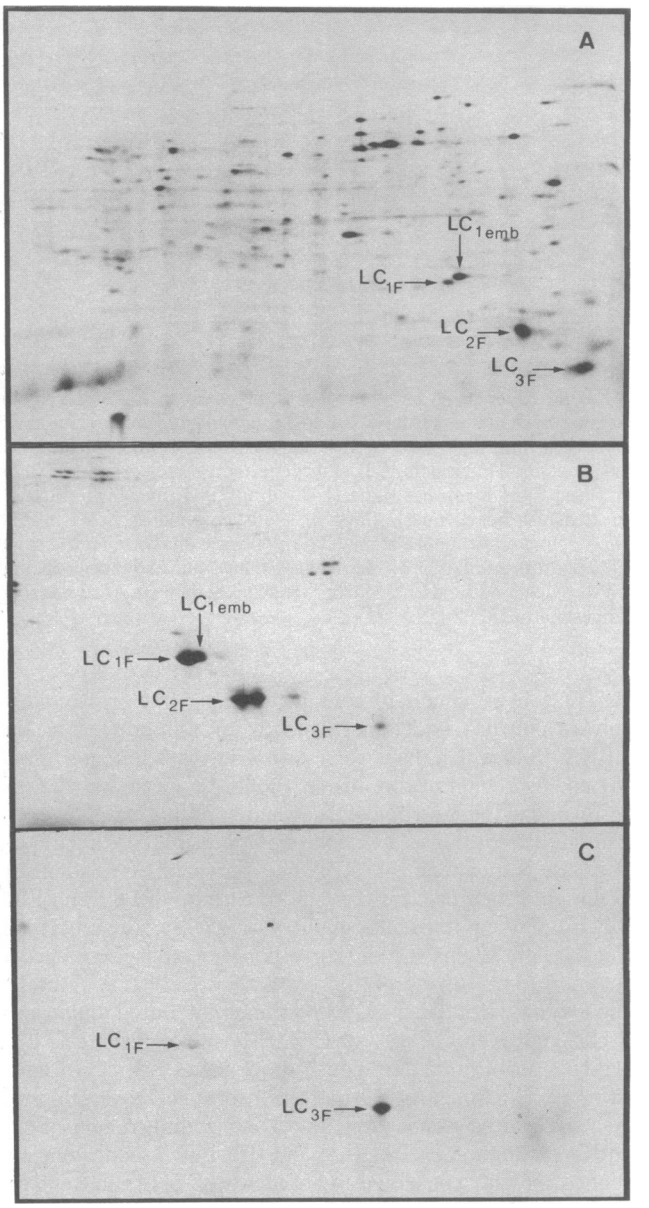

FIG. 5. Two-dimensional gel analyses. (*A*) Translation products of total poly(A)$^+$RNA from fused T984 myotubes. First dimension, isoelectrofocusing at pH 3.5–10; second dimension, 15% acrylamide/NaDodSO$_4$ gel. (*B*) Myosin from mouse embryonic muscle (Coomassie blue-stained). (*C*) ^{35}S-Labeled translation products of poly(A)$^+$RNA from fused T984 myotubes hybridized to DBM-bound DNA of plasmid 161. (*B* and *C*) First dimension, isoelectrofocusing at pH 4–6; second dimension, 17.5% acrylamide/NaDodSO$_4$ gel.

Embryonic heart muscle contains $LC1_{emb}$ (13) that is retained in the atria of adult hearts (33). This cross-hybridization may therefore be between cardiac LC1 or $LC1_{emb}$. Otherwise, the cloned sequence is apparently species and tissue specific.

Plasmid 161 was characterized by comigration of the hybridization-translation products with myosin light chains from fast skeletal muscle. DNA sequence analysis indicated that it does not correspond to a coding region of the light chains and represents part of the 3′-untranslated region. This may be the origin of its high specificity vis-à-vis the other isoforms of LC1, which are expressed in different tissues of the mouse, and of its species specificity. 3′-Noncoding sequences in mRNAs seem to be much less conserved between related genes than are coding regions. Thus, the 3′-noncoding sequences of α- (5, 35, 36),

β-, and γ(37)-actin mRNAs have been shown by methods similar to those used here to hybridize almost exclusively to their homologous RNAs while the coding sequence hybridizes with the messengers of other isoforms in the same animal (5, 35–37) and throughout evolution (36, 37).

Considering these data, the high conservation between the 3′-noncoding sequences of $LC1_F$ and $LC3_F$ was unexpected and suggests that the two genetic regions have been prevented from drifting. Matsuda *et al.* (14), on the basis of the total conservation of the COOH-terminal sequences between $LC1_F$ and $LC3_F$ in the skeletal muscle of chicken and rabbit, have proposed that the constant portion of these proteins originates from a single gene, the different NH_2 termini of LC1 and LC3 then resulting from differential splicing. Alternatively, the genes could be distinct and have been conserved by a conversion mechanism like that suggested by Slightom *et al.* (38) for the γ-globin genes in human. This would then imply a close linkage of the two genes to permit the recombination events. It should be possible to examine the number and possible linkage of the LC1 and LC3 genes by Southern blot experiments. Attempts with plasmid 161 as a probe have been unsuccessful because of the extensive poly(A) sequence that this plasmid contains. Investigation of the arrangement of the sequences coding for LC1 and LC2 by using cloned genomic fragments will distinguish between these different hypotheses.

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