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Characterization of Human Myosin Light Chains 1sa and 3nm: Implications for Isoform Evolution and Function

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We have isolated a cDNA clone for the human slow-twitch muscle isoform myosin light-chain 1slow-a (MLC1sa) from a skeletal muscle library and for the human nonmuscle isoform myosin light-chain 3nonmuscle (MLC3nm) from a fibroblast library. The nucleotide sequence of both isoforms was determined, and isoform-specific probes were constructed. In addition, MLC1sa was subsequently isolated from the fibroblast library. MLC1sa and MLC3nm were found to be very closely related to each other and distant from all other myosin light-chain isoforms so far described. We concluded that MLC1sa arose by duplication of MLC3nm rather than from any other isoform. A comparison was made between all human myosin light chains described to date and a model proposed for the evolution of this multigene family. A comparison between human and chicken myosin light-chain isoforms showed that human isoforms are more similar to their chicken counterparts than to human MLC1sa. The expression of MLC1sa and MLC3nm was studied in humans, rabbits, mice, and rats. MLC1sa was detected at the onset of both human and murine myogenesis in vitro. With development, MLC1sa may be replaced by the other slow-twitch muscle isoform, 1sb, in slow-twitch skeletal muscle, but the proportion of MLC1sa to 1sb expression varies between different species. MLC1sa was detected in nonmuscle cells in humans, mice, and rats. MLC3nm was the major nonmuscle alkaline myosin light chain in all species tested, but its pattern of expression in nonmuscle tissues was not identical to that of β- or γ-actin. We have shown that in the human, as in the chicken, one exon is spliced out of the MLC3nm transcript in smooth muscle to give an alternative product. We concluded that all alkali myosin light-chain isoforms may be functionally different.

Myosin, the thick filament protein in the sarcomeres of muscle, is composed of two types of subunits, myosin heavy chains (MHCs) (of approximately 220 kilodaltons [kDa]) and myosin light chains (MLCs) (17 to 23 kDa). In muscle, contraction occurs when the globular heads of MHCs undergo a conformational change and cause relative movement of the thin actin filaments to decrease sarcomeric length. MLCs are associated with MHC heads in muscle and nonmuscle, though the nature of the association and the function of the MLCs is unclear.

The MLC proteins (reviewed in reference 1) are divided into two classes which indicate the conditions required to dissociate them from the MHCs. One class is composed of those MLCs which are dissociated from MHCs at high pH and are called the alkaline MLCs; these are the MLC1s (21 to 23 kDa) and the MLC3s (17 kDa). The other class is dissociated from the MHCs in the presence of 5,5′-dithiobis-(2-dinitrobenzoicacid); these are the MLC2s (20 kDa). These are also known as the regulatory MLCs because their phosphorylation state affects the interaction between the MHC heads and actin (29).

As for all contractile proteins, there are a number of alkaline MLC isoforms. The fast-muscle isoforms of MLCs are MLC1F and MLC3F, which are derived from the same gene (34). Embryonic and atrial muscle express the same isoform, MLC1A (19). In slow-twitch muscle there are two isoforms, MLC1sb, which is also the form expressed in ventricular muscle (19), and MLC1sa. One nonmuscle MLC isoform has been described in the chicken (23), and one has been described in the human (36). Why multiple isoforms exist is not yet understood. The isoforms may be functionally equivalent, or each may function slightly differently.

In this work we describe an MLC1sa cDNA clone and characterize the expression of this isoform in the adult in various species and in myogenesis in the human and the mouse. Previously, no nucleotide or amino acid sequence data existed for this isoform, except for two short thiol peptide sequences in the rabbit (40). Little was known of the expression of its mRNA or protein in vivo or in vitro. In characterizing MLC1sa, we have also isolated and characterized a novel nonmuscle alkaline MLC which we call MLC3nm. There is a high degree of sequence similarity between MLC1sa and MLC3nm, and we suggest that MLC1sa arose from MLC3nm by gene duplication. We propose a model for the evolution of the MLC multigene family, with MLC3nm as the most probable ancestral gene. In addition, data presented here strongly suggest that different MLC isoforms fulfill different functions in muscle cells.

MATERIALS AND METHODS

Materials. All restriction endonucleases, radionucleotides, and Hybond-N were obtained from Amersham Australia Pty. Ltd., North Ryde, New South Wales, Australia. Random prime DNA labeling kits and M13 sequencing kits were also obtained from Amersham. Reverse transcriptase was obtained from GIBCO-BRL, Glen Waverley, Victoria, Australia. Sequenase DNA sequencing kits were obtained from Trace Scientific Pty. Ltd., Baulkham Hills, New South Wales, Australia. All chemicals for oligonucleotide synthesis were obtained from Applied Biosystems (Australia) Pty. Ltd., Burwood, Victoria, Australia.

DNA and RNA blot hybridizations. All hybridizations and

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prehybridizations were performed in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate)–5× Denhardt solution (9)–50 mM NaH_2PO_4 (pH 7.0). For hybridizations, 10% (wt/vol) dextran sulfate was included. Prehybridization and hybridization temperatures were identical (65°C unless otherwise stated), and these temperatures were used for a minimum of 16 h. Random primed DNA probes, approximately 10^9 dpm/μg, were hybridized at 10^6 dpm/ml. After hybridization, filters were washed at the desired stringency: for blots of human nucleic acid, this was 0.5× SSC–0.1% sodium dodecyl sulfate (SDS) at 65°C for MLC1sa and 0.2× SSC–0.1% SDS at 65°C for MLC3nm; for hybridizations to RNA from other species, this was 0.5× SSC–0.1% SDS at 55°C. Colony screening was performed, using the method of Grunstein and Hogness (14). For the human adult skeletal muscle library (16) and the low-stringency screening of the transformed human fibroblast cDNA library (26), hybridization was at 60°C and washing was in 0.5× SSC–0.1% SDS at 40°C. A total of 4,000 colonies from the skeletal muscle library and 1,600 colonies from the fibroblast library were screened with their respective probes. When the fibroblast library (26) was screened at higher stringency (using the MLC1sa-specific probe), hybridization was performed at 65°C and washing was in 0.5× SSC–0.1% SDS at 55°C. Approximately 40,000 colonies were screened in this way.

Probe subcloning. All probe fragments were subcloned into pGEM-3 and were released by digestion with *Eco*RI and *Hin*dIII.

The probe designated as the MLC1sa-specific probe was a 229-base-pair (bp) *Bal*I-*Taq*I fragment of pHMMLC1sa (see Fig. 1, map coordinates 14 to 243).

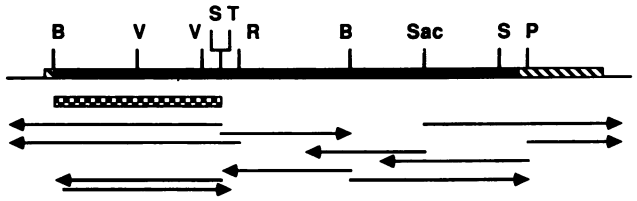
The less-specific MLC1sa probe was a 413-bp *Bal*I fragment of pHMMLC1sa (see Fig. 1, map coordinates 14 to 427). This region of the plasmid included the MLC1sa-specific 5′ terminus of the cDNA but also much of the central coding region and could recognize other MLC isoforms at appropriate stringencies.

The MLC3nm-specific probe was a 260-bp *Ava*II fragment of pHFMLC3nm (see Fig. 3, map coordinates 283 to 543).

Preparation of plasmid DNA and DNA fragments. Plasmid DNA was prepared from overnight cultures, using lysis by boiling and then banding in CsCl gradients (21). Enzyme digestions were performed according to the recommendations of the manufacturer, and DNA fragments were separated by agarose gel electrophoresis and electroeluted into dialysis bags (21).

Preparation and electrophoresis of RNA. Total RNA was prepared from muscle tissues by the phenol-chloroform method described by Palmiter (27). RNA from human muscle and primary human cultures was isolated previously (15). Total cellular RNA was isolated from fibroblasts and nonmuscle tissues by the guanidine hydrochloride procedure (35). Cells were cultured as previously described (5). Samples of RNA were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde and transferred to Hybond-N as described by Maniatis et al. (21). Integrity of RNA and efficiency of transfer were ensured for each panel by hybridization to a total actin probe (7) or an 18S ribosomal RNA probe, after stripping of bound MLC probe.

DNA sequencing. DNA sequence was obtained by the dideoxy method of Sanger et al. (33), using [α-^{35}S]dATP. Restriction fragments were subcloned into M13 phage vectors mp10 and mp11 in a site-directed manner. Additional primers used were synthesized on a 380B Applied Biosystems DNA synthesizer (Applied Biosystems Australia Pty. Ltd., Burwood, Victoria, Australia), using cyanoethyl phosphoamidite chemistry. The oligonucleotides were cleaved from the column, desalted according to the recommendations of the manufacturer, and used directly in sequencing reactions.

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                                                    ACACTGTCCTTTGGCCACCGGACATC

 27 ATGCCTCCCAAGAAGGATGTTCCCGTGAAGAAACCAGCAGGGCCCTCCATCTCCAAACCT
    MetProProLysLysAspValProValLysLysProAlaGlyProSerIleSerLysPro

 87 GCTGCTAAGCCAGCAGCAGCAGGGGCTCCTCCAGCCAAGACCAAAGCTGAGCCAGCTGTC
    AlaAlaLysProAlaAlaAlaGlyAlaProProAlaLysThrLysAlaGluProAlaVal

147 CCCCAGGCCCCTCAGAAAACCCAGGAGCCTCCAGTCGATCTCTCCAAAGTGGTGATCGAG
    ProGlnAlaProGlnLysThrGlnGluProProValAspLeuSerLysValValIleGlu

207 TTTAACAAGGACCAGCTGGAGGAGTTCAAGGAGGCCTTCGAGCTGTTTGACCGAGTGGGG
    PheAsnLysAspGlnLeuGluGluPheLysGluAlaPheGluLeuPheAspArgValGly

267 GATGGCAAGATCCTGTACAGCCAGTGTGGGGACGTGATGAGGGCCCTGGGCCAGAACCCC
    AspGlyLysIleLeuTyrSerGlnCysGlyAspValMetArgAlaLeuGlyGlnAsnPro

327 ACCAACGCCGAGGTGCTCAAGGTCCTGGGGAACCCCAAGAGTGATGAGCTGAAGTCGCGG
    ThrAsnAlaGluValLeuLysValLeuGlyAsnProLysSerAspGluLeuLysSerArg

387 CGTGTGGACTTTGAGACTTTCCTGCCCATGCTCCAGGCAGTGGCCAAGAACCGAGGCCAA
    ArgValAspPheGluThrPheLeuProMetLeuGlnAlaValAlaLysAsnArgGlyGln

447 GGCACATATGAGGACTACTTGGAGGGGTTTCGTGTGTTTGACAAGGAGGGGAACGGCAAA
    GlyThrTyrGluAspTyrLeuGluGlyPheArgValPheAspLysGluGlyAsnGlyLys

507 GTCATGGGAGCAGAGCTCAGACATGTTCTCACCACCCTTGGAGAGAAGATGACTGAGGAG
    ValMetGlyAlaGluLeuArgHisValLeuThrThrLeuGlyGluLysMetThrGluGlu

567 GAGGTGGAGACCGTTCTGGCAGGACACGAGGACAGCAACGGCTGCATCAACTACGAGGCC
    GluValGluThrValLeuAlaGlyHisGluAspSerAsnGlyCysIleAsnTyrGluAla

627 TTCTTGAAACACATCCTAAGCGTCTGAGTGCTGCAGATCCAGTGGGGTCCGGACACTGGG
    PheLeuLysHisIleLeuSerVal***

687 CCCCGCAGGCGAAAGCACGTTCCAGCCACCAGGAGGCCACCTATTGTTTCAAAATAAAGA
747 CTGGGTTCCTC
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FIG. 1. Human MLC1sa from skeletal muscle. cDNA map, sequencing strategy, nucleotide sequence, and deduced amino acid sequence of clone pHMMLC1sa are shown. Vector sequence (thin black line), UTRs (hatched boxes), and coding sequence (filled box) are indicated. Checked box below cDNA map indicates region of the cDNA used as the MLC1sa-specific probe. Selected restriction enzyme sites are shown. Abbreviations: B, *Bal*I; V, *Pvu*II; R, *Rsa*I; T, *Taq*I; S, *Stu*I; Sac, *Sac*I; P, *Pst*I. Arrows indicate fragment lengths and direction of sequencing. Amino acid residues are denoted with the three-letter codes. The termination codon (***) is indicated. The polyadenylation signal is underlined.

Densitometry. Autoradiographs were scanned, using a Hoefer GS 300 scanning densitometer and a Spectra-Physics SP4270 integrator.

Computer analysis. Protein sequences were analyzed by the methods of Chou and Fasman (6) and Garnier et al. (12) for secondary structure predictions and the method of Kyte and Doolittle (20) for hydrophilicity. The GenBank database (release number 59) was searched, using the program of Pustell (30). All programs were contained within the IBI Pustell Sequence Analysis package. Alignment of protein sequences was performed with the Multalin program (8) and structured to maximize homology between all isoforms.

Amplification of DNA by PCR. For the sample of human smooth muscle (colon), RNA was reverse transcribed, using 200 U of enzyme and 10 μg of total RNA per reaction. This cDNA was then used in the polymerase chain reaction (PCR) with 1 μg of each oligonucleotide primer. For amplification of the region from the plasmid pHFMLC3nm, 500 ng of

plasmid was used with 1 μg of each oligonucleotide primer. All PCRs were performed on a Hybaid heating block (Integrated Sciences, Crows Nest, New South Wales, Australia), using buffer recommended by Perkin-Elmer. After incubation at 95°C for 5 min, the reaction mix was run through 40 cycles of denaturation (1 min at 92°C), annealing (1 min at 60°C), and extension (1 min at 72°C) and was finished with a 10-min incubation at 72°C. The 5′ oligonucleotide primer covered the sequence 5′-ATGACAGAGGAAGAAGTAGA-3′ (see Fig. 3, nucleotides 398 to 418), and the 3′ primer covered the sequence 5′-GTTTGCCGTCAGCATTCACC-3′ (see Fig. 3, nucleotides 641 to 661). These primers were expected to generate a fragment of 284 nucleotides when the MLC3nm plasmid was used as the template. One-half of the total PCR mix for human colon and one-fifth of the total PCR mix from the plasmid were size fractionated on a 1.5% agarose gel.

RESULTS

Isolation and characterization of MLC1sa. A human adult skeletal muscle cDNA library (16) was screened, using a human MLC1sb cDNA probe. This probe contained most of the coding and 3′ untranslated regions of the MLC1sb cDNA described by Wade et al. (38). Screening was conducted at low stringency to facilitate cross-isoform hybridization. Seven positive clones were grouped on the basis of restriction analysis and size, and these groups were compared with clones known to be MLC1sb and MLC1F/MLC3F (38). On the basis of this analysis, the probe detected one clone identical to itself and two clones identical to MLC1F/MLC3F; the remaining group of four clones was presumed to be MLC1sa.

The longest cDNA, pHMMLC1sa, was selected from the putative MLC1sa group and was restriction mapped and sequenced (Fig. 1). The cDNA contains 624 bp of coding sequence, 26 bp of 5′ untranslated region (UTR), and 105 bp of 3′ UTR. The amino acid sequence derived from pHMMLC1sa encodes a protein of 208 residues with a molecular mass of 22.7 kDa and a pI of 5.42, making it the largest and most basic alkaline MLC described. It shows the enrichment of lysine, proline, and alanine residues at the amino terminus that is characteristic of the alkaline MLCs, and computer predictions based on sequence data indicate a protein that is largely hydrophilic and helical (6, 12, 20). Other reported studies of the MLC1sa protein support the conclusion that pHMMLC1sa encodes this isoform; amino acid residues 87 to 93 and 187 to 198 of pHMMLC1sa correspond to two thiol peptides of MLC1sa sequenced in the rabbit (40), and MLC1sa has been demonstrated to be larger and more basic than other alkaline MLCs (4, 13, 32, 41). Unlike rabbit MLC1sa (17), the human protein cannot contain trimethylalanine as the first amino acid after the initiation codon because the residue in this position is a proline.

The amino acid sequence derived from pHMMLC1sa was compared with those of other alkaline MLCs. It was evident that the first 60 amino acids of MLC1sa have negligible homology to the other alkaline MLCs and that the amino terminus is therefore specific for this isoform (see Fig. 4). This region of the cDNA was selected as an MLC1sa-specific probe which was used in all subsequent experiments and is indicated in Fig. 1. The probe spans 12 bp of 5′ UTR and the first 217 nucleotides of the MLC1sa coding sequence.

To confirm the identity of pHMMLC1sa as MLC1sa, we studied its expression in the rabbit, using the MLC1sa-specific probe. In the rabbit, expression of MLC1sa is known to be high in the predominantly slow-twitch skeletal muscle, the soleus (1). Total RNA was prepared from rabbit slow-twitch (soleus), fast-twitch (psoas, gastrocnemius, and extensor digitorum longus), and cardiac muscles (atrium and ventricle) and kidney. This was size fractionated on a denaturing gel and then hybridized to the MLC1sa-specific probe. The probe showed preferential hybridization to an mRNA in slow-twitch skeletal muscle (this mRNA is approximately 900 bp) (Fig. 2). In particular, the failure of the probe to hybridize to transcripts in the ventricle distinguishes the expression pattern of this cDNA from that of MLC1sb (19) and confirms that pHMMLC1sa encodes MLC1sa. In the rabbit, this isoform was detected primarily in slow-twitch muscle, with 10% of this level of expression in gastrocnemius and a trace detectable in both the extensor digitorum longus and psoas muscles.

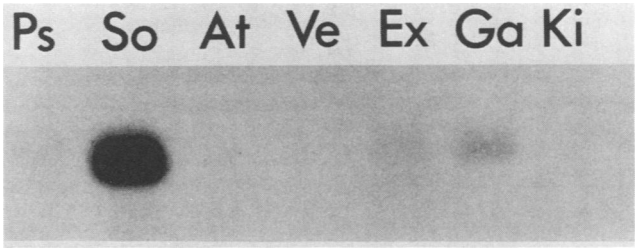

FIG. 2. Hybridization of MLC1sa-specific probe to RNA from rabbit muscle and kidney (10 μg of total RNA per lane). Abbreviations: Ps, psoas; So, soleus; At, atrium; Ve, ventricle; Ex, extensor digitorum longus; Ga, gastrocnemius; Ki, kidney.

Both the MLC1sa-specific probe and the less-specific probe were used to study MLC1sa expression in the human (results not shown). Two significant observations were made. First, hybridization of both probes to an mRNA in human fibroblasts, primary myoblasts, and fetal muscle in addition to the predicted occurrence of MLC1sa in adult muscle was observed. This indicated that MLC1sa mRNA is not exclusive to mature muscle. Second, the intensity of the signal from each sample was greater when the less-specific probe was used. This suggested that, in addition to MLC1sa, these cells express a second product with strong homology to the central region of MLC1sa. Thus, it became necessary to define precisely what alkaline MLC mRNAs exist in nonmuscle cells.

Isolation of MLC1sa and MLC3nm from human fibroblasts. The less-specific MLC1sa probe was used to screen a size-fractionated, transformed human fibroblast cDNA library (26) at the low stringency previously used. The probe detected a single clone, pHFMLC3nm, which was restriction mapped and sequenced. The cDNA map and nucleotide and deduced amino acid sequence of pHFMLC3nm are shown in Fig. 3. The molecular mass of this protein is 16.9 kDa; pHFMLC3nm therefore encodes an MLC3 isoform. The pI of the protein is 4.31, which makes it the most acidic human alkaline MLC described and reflects the absence of the highly basic amino terminus found in all other MLCs. In the coding region, pHFMLC3nm showed 91% identity at the amino acid level and 80% identity at the nucleotide level to the nonmuscle MLC from chicken fibroblast described by Nabeshima et al. (23). A probe specific for MLC3nm was selected and is indicated in Fig. 3. This probe spans 210 bp of the coding sequence and 50 bp of 3′ UTR and, at the stringency used in subsequent experiments, cannot hybrid-

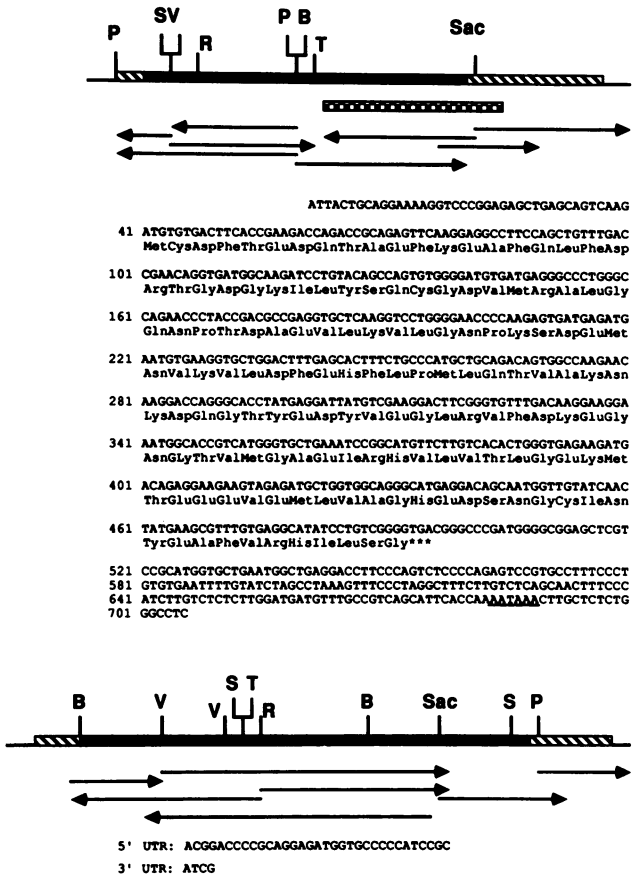

FIG. 3. Human MLC3nm and MLC1sa from fibroblasts. Symbols and abbreviations are as described in the legend to Fig. 1. Checked box below the cDNA map of pHFMLC3nm indicates the region of the cDNA used as the MLC3nm-specific probe. Complete nucleotide sequence and deduced amino acid sequence of pHFMLC3nm and additional residues to the UTRs of pHFMLC1sa (compared with those of pHMMLC1sa) are shown.

ize to other MLC isoforms. Analysis of nucleotide sequence data demonstrated first that MLC3nm and MLC1sa, though related and exceptionally similar at the amino acid level, are not alternative products of the same gene. Second, because it has no region of similarity to the amino terminus of MLC1sa, MLC3nm could not hybridize to the MLC1sa-specific probe. This meant that we had isolated an MLC which accounted for the cross-hybridization observable to the less-specific probe but had not explained the signal observable in fibroblasts and myoblasts when using the MLC1sa-specific probe. Thus, another MLC with homology to the amino terminus of MLC1sa must also be expressed in fibroblasts.

We therefore screened the fibroblast library, using the MLC1sa-specific probe to isolate this other MLC. High-density screening of bacterial colonies gave six positive clones, which were processed to purity and screened again. The longest clone, pHFMLC1sa, was then restriction mapped and sequenced according to the strategy shown in Fig. 3. The MLC isolated in this way was identical to pHMMLC1sa, except that it contained an additional 33 nucleotides at the 5′ end of the 5′ UTR and 4 nucleotides at the 3′ end of the 3′ UTR; these extra bases are noted in Fig. 3. Thus, MLC1sa is expressed in these transformed human fibroblasts in a form identical to that in which it is expressed

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HMLC1sa   MPPKKDVPVK  KPAGPSISKP  AAKPAAAGAP  PAKTKAEPAV  PQAPQKTQEP
HMLC3nm
HMLC1F    .A.....--.  ..VAAAAAA.  .PA..P.P..  ----------  --..A.PK.E
HMLC1sb   .A...PE.-.  .DDAKAAP.A  .PA..PPPE.  ----------  ----ERPK.V
HMLC1A    .A...PE.-.  .E.AKA-PA.  .PA..P.P..  ----------  ----EAPK..

HMLC1sa   PVDLSKVVIE  FNQDQLEEFK  EAFELFDRVG  --DGKILYSQ  CGDVMRALGQ
HMLC3nm         MCD   .TE..TA...  ...Q....T.  --........  ..........
HMLC1F    KI...AIKM.  .SKE.QD...  ....L....T.  --.S..IL..  V...L....T
HMLC1sb   EF.A..IK..  .TPE.I....  ...M....TP  KCEM..T.G.  ....L.....
HMLC1A    AF.PKS.K.D  .TAE.I....  ...S....TP  TGEM..T.G.  ....L.....

HMLC1sa   NPTNAEVLKV  LGNPKSDELK  SRRVDFETFL  PMLQAVAKNR  GQGTYEDYLE
HMLC3nm   ...D......  ........MN  VKVL...H..  ....T....K  D.......V.
HMLC1F    .......R..  ....SNE..N  AKKIE..Q..  ..M..ISN.K  D.A....FV.
HMLC1sb   ...Q....R.  ..K.RQE..N  TKMM......  ....HIS..K  DT.....FV.
HMLC1A    ........R.  ..K..PE.MN  VKML......  .I..HISR.K  E...ED.FV.

HMLC1sa   GFRVFDKEGN  GKVMGAELRH  VLTTLGEKMT  EEEVETVLAG  HEDSNGCINY
HMLC3nm   .L........  .T.....I..  ..V.......  .....MLV..  ..........
HMLC1F    .L........  .T........  ..A......K  .....ALM..  Q.........
HMLC1sb   .L........  .T........  ..A....RL.  .D...KLM..  Q.........
HMLC1A    .L...E..S.  .TD.......  ..A.......  .V...QL...  Q..A......

HMLC1sa   EAFLKHILSV
HMLC3nm   ...VR....G
HMLC1F    ...V...M.I
HMLC1sb   ...V...M.S
HMLC1A    ...V...M.G
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FIG. 4. A comparison of human alkaline MLC amino acid sequences with MLC1sa. Amino acid residues are denoted by using the single-letter amino acid code. Sequences were aligned by using the Multalin program (8) to give maximum homology between all isoforms. A dot indicates a residue identical to that in MLC1sa; a dash indicates a space introduced to maximize homology. The sequence for MLC1F is from reference 34, and the sequences for MLC1A and MLC1sb are from reference 19.

in muscle. We conclude that the hybridization we had observed to the MLC1sa-specific probe in human fibroblasts and myoblasts was due to the presence of MLC1sa mRNA in these cells.

Comparisons of MLC amino acid sequences. Figure 4 compares the amino acid sequences of all human alkaline MLCs described to date. Sequences were aligned by computer to maximize identity between all isoforms. Similarity between all isoforms is evident in the first seven amino acids, but the isoform specificity of the MLC1sa amino terminus and the degree of sequence similarity between MLC1sa and MLC3nm are immediately evident. An analysis of the amino acid sequence difference between the human isoforms, over the length of MLC3nm, is presented in Table 1. Only MLCs 1sb and 1A are more similar to each other than MLC1sa and MLC3nm are to each other, which suggests a close evolutionary relationship between each of these pairs. Note that whereas MLC1sa and MLC3nm show only a 20% difference from each other at the amino acid level, MLC1sa shows more than a 30% difference and MLC3nm shows more than a 25% difference from all other members of the family (Table 1). This leads to the surprising conclusion that MLC1sa was generated by duplication of MLC3nm rather than by duplication of any other alkaline MLC. MLC1sa is not notably similar to MLC1sb; indeed, it shows greater identity to the

TABLE 1. Differences between human MLC isoforms at the amino acid level[a]

Isoform	% Difference between isoform:				
	1sa	3nm	1F[b]	1sb[c]	1A[c]
MLC1sa		20.0	30.0	30.3	32.9
MLC3nm	20.0		27.3	27.0	27.6
MC1F[b]	30.0	27.3		25.0	29.6
MLC1sb[c]	30.3	27.0	25.0		17.7
MLC1A[c]	32.9	27.6	29.6	17.7	

[a] Comparison conducted over length of MLC3nm.
[b] Sequence from reference 34.
[c] Sequences from reference 19.

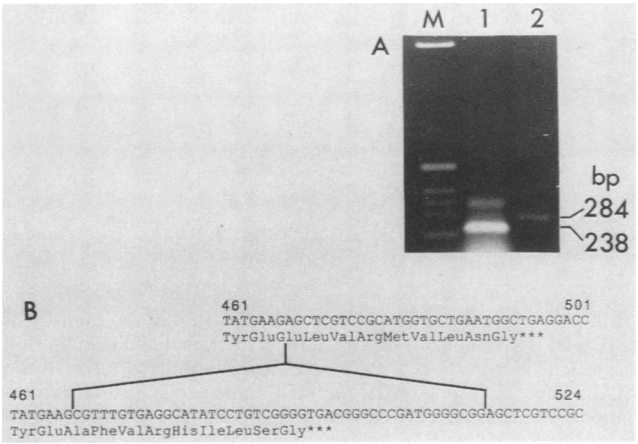

FIG. 5. Alternative splicing of MLC3nm in human smooth muscle; amplification of all MLC3nm-derived products, using the PCR. (A) PCR products for human smooth-muscle RNA (lane 1) and plasmid pHFMLC3nm (nonmuscle; lane 2) were separated on an agarose gel. Sizes of major products are indicated at right. Markers (lane M) are pBR322 DNA cut with *Hin*fI. (B) Nucleotide sequence of the smooth-muscle MLC3nm-derived fragment over the splice junction (upper sequence) and its relationship to MLC3nm (lower sequence, numbered as described in the legend to Fig. 3).

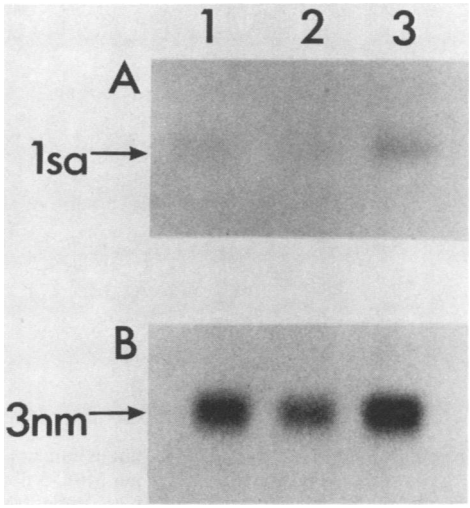

FIG. 6. Expression of MLC1sa and MLC3nm in normal and transformed human fibroblasts. Total RNA from MRC-5 (10 μg, lane 1), HuT-12 (10 μg, lane 2), and HuT-14 (20 μg, lane 3) was size fractionated on denaturing agarose gels, transferred to nylon, and hybridized to (A) the MLC1sa-specific probe or (B) the MLC3nm-specific probe.

fibroblast MLC isoform of the chicken (75% over the last five exons) than to human MLC1sb (70% over this region). The sequence disparity between the two slow mammalian MLC isoforms makes it unlikely that, as had previously been supposed, they are functionally equivalent.

Alternative splicing of MLC3nm. The MLC3nm isolated from chicken fibroblast by Nabeshima et al. (23) is alternatively spliced in smooth muscle to give a second, smaller product. To determine whether this also occurs in the human, we used the PCR to amplify all the products in smooth muscle that are derived from the MLC3nm gene. Primers specific for an area of the coding region which, in the chicken, is just 5′ of the splice site and for the extreme 3′ end of the 3′ UTR were designed. Total RNA was prepared from a sample of human colon and was reverse transcribed to give cDNA. This cDNA and, for comparison, the plasmid cDNA pHFMLC3nm were then each subjected to PCR, and the products were separated on a 1.5% agarose gel (Fig. 5A). Human smooth muscle shows a smaller MLC3nm-derived product than that found in nonmuscle (238 bp compared with 284 bp). An additional, minor product is also amplified but has not been examined. To determine the nature of the smooth-muscle MLC3, the 238-bp fragment amplified by PCR was subcloned and sequenced (Fig. 5B). Nucleotide numbers for MLC3nm are the same as those shown in Fig. 3 and, for MLC3smooth, correspond to their positions in the smooth-muscle mRNA. One 46-bp exon of MLC3nm, including the termination signal of the nonmuscle protein, is spliced out in smooth muscle so that an alternative 3′ coding sequence and translation termination site are used. The region of MLC3smooth that forms the final coding exon represents part of the UTR of the nonmuscle form; thus, the transcripts for the two MLC3s are different sizes. This splicing pattern exactly matches that in the chicken (23), except that in the chicken the omitted exon is 40 bp. Both proteins encoded by pHFMLC3nm consist of 151 amino acid residues, but the smooth-muscle form has a slightly higher molecular mass (17 kDa) and is slightly more acidic (pI of 4.22) than the nonmuscle protein.

Expression of MLC1sa and MLC3nm in human tissues. Since MLC1sa and MLC3nm had been isolated from a transformed human fibroblast library, it was of interest to examine their expression in normal human fibroblasts. Total RNA from cultured normal human fibroblasts (MRC-5), transformed human fibroblasts (HuT12), and transformed, tumorigenic fibroblasts (HuT14) was hybridized separately to the two isoform-specific probes; results are shown in Fig. 6 (note that lane 3 is double loaded compared with lanes 1 and 2). MLC1sa is detected in normal fibroblasts, and thus aberrant gene expression resulting from transformation was not responsible for its appearance in the fibroblast library. MLC1sa therefore represents a genuine, though minor, nonmuscle MLC isoform. It appears to be expressed at approximately equal levels in all fibroblast types. MLC3nm was detected at high levels in normal human fibroblasts and is clearly the major human nonmuscle alkaline MLC isoform. MLC3nm expression is similar in all fibroblast types.

Total RNA from cultures of human fibroblasts, primary myoblasts, and primary myotubes was then used to examine expression of the two isoforms in nonmuscle, muscle progenitor cells, and developing muscle to determine the regulation pattern of these MLC isoforms in human myogenesis. Total RNA from human fetal and adult skeletal muscle (15) was included as reference points for in vivo development and maturity. The results of the hybridization of these samples to the isoform-specific probes are shown in Fig. 7. Note that MLC1sa expression is not detected in the fibroblast lane because this panel carries only 5% of the amount of RNA per lane as that in lanes 1 and 2 of Fig. 6. The two isoforms appear to show reciprocal expression patterns. MLC1sa expression is induced as myoblasts fuse to form myotubes during differentiation, the level of expression in myotubes being about 70% of the maximum adult level. MLC1sa expression varies with muscle development in vivo, as demonstrated by its expression in muscle from a 24-week-old fetus at 20% of the adult level. In contrast, MLC1sb has been shown to be present at detectable levels only in adult skeletal muscle (38). Predictably, MLC3nm is

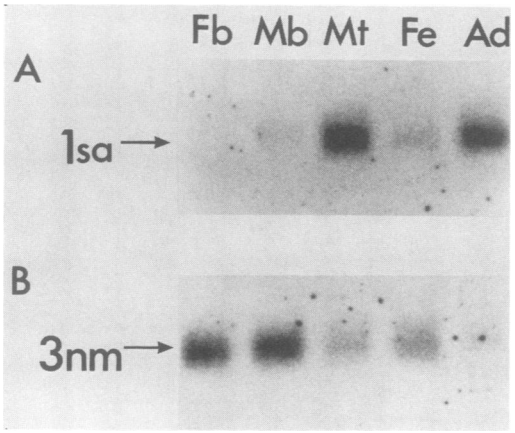

FIG. 7. Expression of MLC1sa and MLC3nm in human myogenesis. A 0.5-μg portion of total RNA from human MRC-5 fibroblasts (Fb), 40% confluent myoblasts (Mb), day 4 myotubes (Mt), 24-week-old fetal muscle (Fe), and adult skeletal muscle (Ad) was size fractionated on denaturing agarose gels, transferred to nylon, and hybridized to (A) the MLC1sa-specific probe or (B) the MLC3nm-specific probe.

most readily detected in fibroblasts and myoblasts, with fetal muscle expressing this isoform at about 50% of that level. Expression in myotubes is slightly less than that in fetal muscle and is just detectable in adult skeletal muscle. The expression pattern of MLC3nm in myogenesis is similar to that of β-actin, which also shows down-regulation of expression in myotubes compared with fibroblasts and myoblasts, slightly higher levels in fetal muscle than in mature myotubes, and a lower level in adult muscle (P. Gunning, M. Gordon, R. Wade, R. Gahlmann, C.-S. Lin, and E. Hardeman, Dev. Biol., in press). However, MLC3nm is expressed at higher levels than is β-actin in all samples, compared with their maxima in fibroblasts.

We further characterized the nature of the isoforms in the human by determining the gene copy number of each. Figure 8 shows the hybridization of the isoform-specific probes to human genomic HeLa DNA restricted with various endonucleases. MLC1sa appears not to be a single-copy gene (Fig. 8A). Probes to 5′ and 3′ regions of MLC1sa gave the same banding pattern, except where the DNA contained an exonic site for the enzyme with which the genomic DNA was

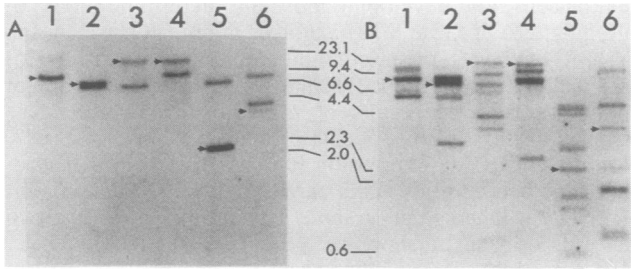

FIG. 8. Genomic DNA from HeLa cells was restricted with endonucleases and hybridized to isoform-specific probes. Lane 1, *Bgl*II; lane 2, *Bam*HI; lane 3, *Eco*RI; lane 4, *Hin*dIII; lane 5, *Pst*I; lane 6, *Pvu*II. DNA (5 μg per lane) was size fractionated on agarose gels, transferred to nylon, and hybridized to (A) the MLC1sa-specific probe or (B) the MLC3nm-specific probe. Arrows indicate a band which hybridized to both probes (see text for details). Marker bands (λ DNA cut with *Hin*dIII) are indicated.

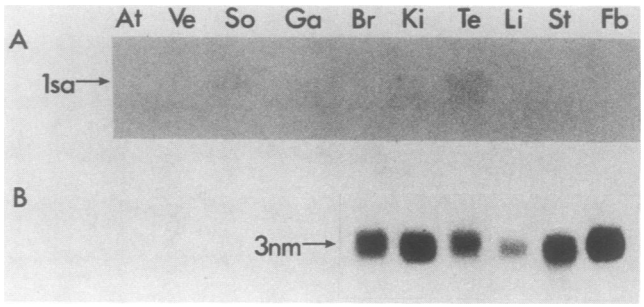

FIG. 9. Expression of MLC1sa and MLC3nm in the mouse (10 μg of total RNA per lane). RNA was size fractionated on denaturing agarose gels, transferred to nylon, and hybridized to (A) the MLC1sa-specific probe or (B) the MLC3nm-specific probe. Abbreviations: At, atrium; Ve, ventricle; So, soleus; Ga, gastrocnemius; Br, brain; Ki, kidney; Te, testes; Li, liver; St, stomach; Fb, NIH 3T3 fibroblasts.

digested (*Pst*I and *Pvu*II). In these cases, two hybridizing bands for the two probes were identical, and one was of a different size. The MLC3nm probe hybridized to a large number of genomic fragments, indicating that MLC3nm is not a single-copy gene. Therefore, it is likely that for both MLC3nm and MLC1sa, sequences which are not identical to the sequence encoding the protein exist within the genome. Such elements probably correspond to pseudogenes, in the form of cDNA copies of mature mRNA reinserted into the genome. These sequences cannot give rise to functional proteins and so can diverge freely. It should be noted that although there is little similarity between the probe sequences, both isoform-specific probes hybridized to a band of the same size (Fig. 8, arrows). When panel A was hybridized to the MLC3nm-specific probe, the band indicated in Fig. 8A hybridized to MLC3nm and corresponded to that in Fig. 8B (arrows). This indicates that a human genomic locus which contains sequences related to both MLC1sa and MLC3nm exists. This result was confirmed, using probes for the 3′ UTRs of MLC1sa and MLC3nm. Such probes are completely specific in their hybridization, and yet both still hybridized to the band indicated by arrows (results not shown).

Expression of MLC1sa and MLC3nm in rodents. Previous reports (2) had suggested that MLC1sa is not present in the mouse in vivo, and no data exists for nonmuscle MLC expression. We used our isoform-specific probes to analyze the expression of these MLCs in this species. Of the muscles tested in the mouse, MLC1sa was detected only in the soleus (Fig. 9A). The level of expression is so low as to make its detection exceptionally difficult, and this explains the failure of other groups to see MLC1sa expression on two-dimensional gels. In nonmuscle tissues, MLC1sa was detected in the testes at levels approximately equal to that in the soleus and in the kidney at lower levels. Expression was also just detectable in the brain, though this signal was too weak to withstand photographic reproduction. Thus, in the mouse, MLC1sa is a nonmuscle MLC but is not significantly elevated in adult striated muscle.

The result obtained in adult mouse muscle raised the possibility that the mouse never expresses MLC1sa at a high level at any point in muscle development. Accordingly, expression was examined in the mouse myogenic cell line C_2C_{12} (5, 42) in which myoblasts can be differentiated to myotubes in culture. MLC1sa is expressed in myotubes in

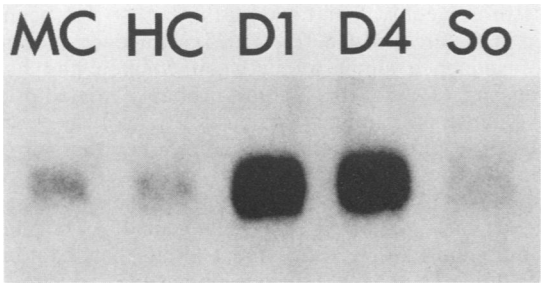

FIG. 10. Expression of MLC1sa in murine myogenesis and adult mouse slow-twitch muscle (10 μg of total RNA per lane). RNA was size fractionated on denaturing agarose gels, transferred to nylon, and hybridized to the MLC1sa-specific probe. Abbreviations: MC, moderate-confluence myoblasts; HC, high-confluence myoblasts; D1, day 1 myotubes; D4, day 4 myotubes; So, soleus.

vitro at 15 times the level in the soleus in vivo (Fig. 10). MLC1sa is primarily associated with early and mature myotubes; thus, in the mouse as in the human, MLC1sa is expressed at a high level at the onset of muscle differentiation. In addition, this isoform is expressed prior to differentiation, since expression in myoblasts is also higher than in the adult soleus. The mouse does not significantly utilize MLC1sa in mature muscle; this represents the major difference between these organisms in the expression of this isoform. The mouse does not express MLC1sb during myogenesis in culture (38), but MLC1sb is the preferred isoform in slow-twitch muscle on maturation.

In the rat, the two slow MLC isoforms are characterized as MLC1s (equivalent to MLC1sb) and MLC1s′ (13, 41). It was likely that the isoform called MLC1s′ was equivalent to MLC1sa; this was examined with the MLC1sa-specific probe and total RNA from adult rat tissues (Fig. 11A). MLC1sa is easily detectable in the rat soleus, with about 5% of this level of expression detected in gastrocnemius and a trace detected in extensor digitorum longus. It is presumably equivalent to MLC1s′, since two-dimensional gel analysis of rat MLCs has shown that the isoform described as MLC1s′ is larger and more basic than MLC1sb (13, 41); this correlates with the size and pI data obtained for MLC1sa. In addition, the level of MLC1sa expression observed in the rat is high enough for it to be detected simply and unlikely to be missed. In the nonmuscle tissues of the rat, MLC1sa was detected in the testes at about twice the level observable in

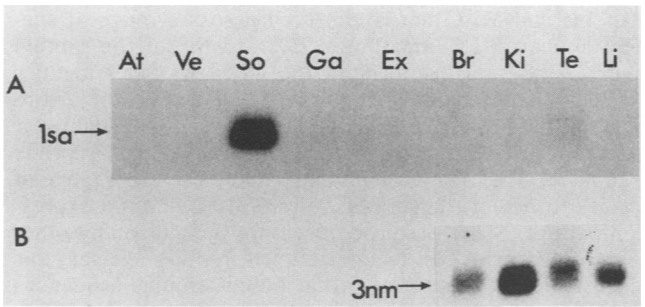

FIG. 11. Expression of MLC1sa and MLC3nm in the rat (10 μg of total RNA per lane). RNA was size fractionated on denaturing agarose gels, transferred to nylon, and hybridized to (A) the MLC1sa-specific probe or (B) the MLC3nm-specific probe. Abbreviations: At, atrium; Ve, ventricle; So, soleus; Ga, gastrocnemius; Ex, extensor digitorum longus; Br, brain; Ki, kidney; Te, testes; Li, liver.

TABLE 2. MLC3nm and β- and γ-actin levels in mouse and rodent tissues

Animal	Tissue	Transcript level[a] of:		
		MLC3nm	β-Actin	γ-Actin
Mouse	Brain	0.44	0.86	0.51
	Kidney	0.67	0.76	0.58
	Testes	0.26	0.69	0.83
	Liver	0.16	0.35	0.004
	Stomach	0.57	0.38	0.17
	Fibroblast	1.00	1.00	1.00
Rat	Brain	0.32	0.66	0.71
	Kidney	1.00	1.00	1.00
	Testes	0.45	0.81	1.46
	Liver	0.46	0.35	0.11

[a] Relative to mouse fibroblast and rat kidney.

the gastrocncemius and in trace amounts in the kidney. This demonstrates that, as in other species, MLC1sa is a genuine nonmuscle MLC in the rat.

Figures 9B and 11B show results obtained by using the MLC3nm-specific probe in the nonmuscle tissues of the mouse and rat, respectively. It was not possible to use this probe as an isoform-specific probe in the skeletal and cardiac tissues of these animals because of cross-hybridization experienced between the probe and other isoforms. However, MLC3nm is clearly a major nonmuscle isoform in these rodent species, and its pattern of expression is quite different from those of β- and γ-actin in these tissues (Table 2). Transcript levels for these nonmuscle isoforms relative to the same tissue for all isoforms (mouse fibroblasts and rat kidney) are presented. This demonstrates that there is no single program of expression of nonmuscle gene products at a quantitative level in the tissues of these animals.

DISCUSSION

The human alkaline MLC gene family. MLC1sa has been demonstrated to be a genuine nonmuscle isoform in all species tested, although MLC3nm is the major nonmuscle isoform. MLC1sa is expressed in nonmuscle tissue in a form identical to that in which it is expressed in slow-twitch skeletal muscle. The expression of MLC1sa is increased over this basal level at the onset of myogenesis, and in the adult it is expressed specifically in slow-twitch skeletal muscle. A regulatory MLC, MLC2s′, was similarly first detected in slow-twitch skeletal muscle and has since been shown also to be expressed in smooth muscle and nonmuscle tissues (36). Another contractile protein isoform, troponin C slow, has also been detected in fibroblasts (11). Therefore, three isoforms which were thought to be slow-twitch muscle specific but which are also detectable in nonmuscle have now been detailed. This suggests that slow-twitch muscle isoforms are less restricted in their expression than are fast isoforms, which have not so far been detected in nonmuscle. The function of some slow-twitch isoforms may thus not relate exclusively to the muscle state.

MLC1sa and MLC3nm are closely related to each other and less related to other MLCs so far described (Table 1). This has resulted in our proposal that MLC1sa arose by duplication of MLC3nm. Splicing patterns of MLCs appear universal, on the basis of data from the mouse (3, 31), the chicken (22, 24), the rat (28), and the human (10), in which the N terminus of MLC isoforms is encoded by a single

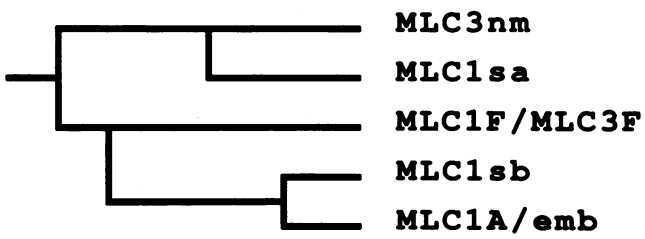

FIG. 12. Proposed model for the evolution of the MLC gene family. Vertical lines to the left represent early duplications which give rise to isoforms which now show less identity to each other than do those isoforms arising from duplications to the right.

exon. Therefore, we presume that the first exon of MLC1sa would span the first 58 amino acids of the protein and that the second exon of MLC3nm would begin at residue number 2 (cysteine). The sequence evidence that the body of the MLC1sa gene is derived from duplication of the MLC3nm gene is strong. However, according to this proposal, sequences which now form the MLC1sa-specific first coding exon had also to be incorporated into the MLC1sa locus. No significant similarity between the sequences of exon 1 of MLC1sa and any other sequences in the GenBank database, including other MLC isoforms, is found. We therefore view as unlikely the idea that the first exon of MLC1sa is derived from another MLC isoform and has diverged at an accelerated rate, although we cannot eliminate this possibility. We feel it more likely that these sequences were acquired independently. In addition to exon 1, it seems that sequences which govern expression of this gene in slow-twitch skeletal muscle would have been acquired. Generation of MLC1sa in this way would be consistent with its observed expression in nonmuscle, since it may reflect the continued presence of MLC3nm promoter elements directing expression in nonmuscle cells.

By analogy to the evolution of other contractile protein multigene families (37), MLC3nm is the most likely ancestral gene of the alkaline MLC gene family. Our model, based on comparisons of sequence identity (Table 1), is outlined in Fig. 12. We propose that MLC3nm was duplicated and, with some additional sequences, gave rise to MLC1F/MLC3F in muscle. Duplication of MLC1F sequences could then give rise to either MLC1A or MLC1sb, and duplication of this locus could produce the other gene (for MLC1sb or MLC1A, respectively). The duplication of MLC3nm to give MLC1sa was most probably a totally separate event from those which gave rise to the other striated muscle MLC isoforms.

Conservation of amino acid sequences implies that the duplication of the MLC3nm gene locus to give MLC1sa must have occurred more recently than did the duplication of MLC1F/MLC3F to give MLC1sb or MLC1A (assuming that all duplicated gene sequences diverge at the same rate). MLC1sa and MLC3nm show only 20% divergence, MLC1F/MLC3F and MLC1sb are 25% divergent, and MLC1F/MLC3F and MLC1A are nearly 30% divergent in amino acid sequence over the last five exons (Table 1). MLC1A and MLC1sb are more similar to each other than are MLC1sa and MLC3nm, which suggests that this duplication may be the most recent. Only MLC1sb and MLC1A carry an insertion of two extra amino acids in their coding sequences (Fig. 4), which emphasizes the relatedness of this pair. The closeness of the three striated muscle isoforms MLC1F, MLC1A, and MLC1sb to each other is demonstrated by the maintenance of their sequence identity over the entire length of the protein, including the amino terminus.

No isoform equivalent to MLC1sa has so far been described in the chicken (18, 22, 23, 25). However, a comparison of sequence similarity between chicken and human isoforms suggests that this isoform probably existed prior to the divergence of mammals and birds. An analysis of amino acid data of isoforms in the chicken shows that all other human alkaline MLC isoforms so far described have a corresponding isoform in the chicken. Thus, mammals and birds each have an MLC1F/MLC3F gene (22, 34) and an MLC3nm gene (23). It seems most likely that mammalian MLC1A equates to the chicken embryonic isoform L23 (described in references 18 and 24) and that mammalian MLC1sb equates to the chicken cardiac isoform MLC1 (described in reference 25). These chicken isoforms (L23 and cardiac MLC1), like their human counterparts (MLC1A and MLC1sb), contain an insertion of two extra amino acids in their coding sequences. Over exons 2 to 6, human isoforms show greater identity to the corresponding chicken isoform than to human MLC1sa, emphasizing the difference between MLC1sa and the other human MLCs (see Table 3). We have suggested that MLC1sa arose before the MLC1sb/MLC1A duplication and, since the chicken has MLC1sb and MLC1A, we presume chickens would also have MLC1sa. Chickens may have ceased to utilize this isoform, or it may be that it has not yet been detected.

Isoform function. The reason for the existence of multiple isoforms of contractile proteins is an important question in muscle development; a number of alternatives are possible. Since isoforms have their own expression patterns and tissue specificities, it seems likely that each isoform functions in a manner slightly different to all others; so, each cytoskeletal structure has a choice of a number of isoforms and can select that which is most appropriate. For the MLCs, the difference between the forms would probably relate to the isoform-specific amino termini of the proteins. Immunoelectron microscopic observations have suggested that the amino termini are located near the head-tail junction of the MHC (39); functional differences between MLC isoforms may therefore arise from their influence on the MHC head and the binding of the head to actin. Evidence presented in this work strongly supports the argument that MLC isoforms fulfill their functions slightly differently and can be seen in terms of (i) sequence conservation of isoforms across a range of species, (ii) the independent acquisition of the characteristic alkaline MLC alanine-proline-lysine-rich amino terminus by MLC1sa, and (iii) the different expression patterns of MLC1sa and MLC1sb in various species.

First, considerable sequence conservation of both MLC1sa and MLC3nm is observed across a range of species. Human MLC3nm shows 91% identity at the protein level to the same isoform in the chicken. Such identity suggests that functional constraints exist over the entire length of the protein. For MLC1sa, no data are available for other species, but we have demonstrated that a probe specific for MLC1sa in the human can be used in a range of species to detect transcripts with an MLC1sa-type expression pattern. Since the probe does not recognize any other human MLCs but can recognize itself in other species, this suggests that at least some of the amino-terminal sequences of MLC1sa are maintained in other species. In contrast, examination of the amino termini (exon 1) of human isoforms MLC1sb and MLC1A and comparison with the corresponding isoform in the chicken (Table 3) show no such conservation in this region. This is significant because these two isoforms have different expression patterns in these species: chicken embryonic isoform L23 is expressed only in the

TABLE 3. Differences at amino acid level between human MLC isoforms and their chicken counterparts and human MLC1sa

Isoform	% Difference between isoforms over region:			
	Exons 2 to 6		Exon 1	
	Human MLC1sa	Chicken counterpart[a]	Human MLC1sa	Chicken counterpart
MLC1F[b]	30.0	20.0	61.4	14.0
MLC1sb[c]	30.3	14.5	73.7	56.1
MLC1A[c]	32.9	18.4	68.4	40.9

[a] Sequence of chicken MLC1F from reference 22, sequence of chicken cardiac MLC1 from reference 25, and sequence of chicken embryonic L23 from references 18 and 24.
[b] Sequence from reference 34.
[c] Sequences from reference 19.

brains of adult chickens (whereas the mammalian embryonic isoform MLC1A is expressed in the atrium of the adult), and cardiac MLC1 is expressed in both the ventricle and the atrium in the chicken (whereas mammalian MLC1sb is expressed in slow-twitch muscle and in the ventricle). The adaptation of the embryonic alkaline MLC to a specialist atrial isoform is therefore apparently a mammalian trait. Alterations in expression patterns may thus expose isoforms to different selective pressures and may eventually result in alterations of sequence. In contrast to the situation with MLC1sb and MLC1A, MLC1F/MLC3F amino acid sequences are not vastly different in their amino termini between human and chicken, and their expression patterns are very similar. These observations are consistent with the proposal that MLC isoforms fulfill their functions slightly differently and that this relates to the amino-terminal sequence of the protein.

Second, all MLC1 proteins show enrichment of alanine, proline, and lysine at their amino termini. The MLC1sa amino terminus is compositionally but not linearly related to those of the other muscle MLCs. According to our proposed evolutionary model of the family, this reflects that MLC1sa arose via a duplication event which was independent of those which gave rise to the other alkaline MLCs. We propose that MLC1sa has acquired the enriched alanine, proline, and lysine composition because it is necessary for function and that, since its amino-terminal sequence is so different, it fulfills its function in a manner slightly different from that of the other isoforms.

Third, the expression pattern of MLC1sa is independent of MLC1sb. In all animals, MLC1sa is not detectable in the ventricle, in which MLC1sb represents a major isoform, and accumulation patterns of MLC1sb in muscles do not mirror those of MLC1sa. Therefore, each slow isoform can be specifically controlled. This is demonstrated by the variation in different animal species in the relative proportion of MLC1sa to MLC1sb mRNA in slow-twitch skeletal muscle such that there is no universal program for the level of expression of these isoforms. The selectivity of slow-twitch muscle isoform expression appears to relate to maturation rather than myogenesis, which is consistent with the proposal that MLC1sa and MLC1sb fulfill their functions differently. We have demonstrated that the onset of muscle differentiation is associated with the induction of MLC1sa expression to high levels. It appears that after birth, within a slow-twitch fiber, the mix of slow MLC isoforms established depends on the species. Our data show that a mouse will repress MLC1sa mRNA, and Barton et al. (2) have shown that, in the adult slow-twitch muscle of the mouse, high expression of MLC1sb is induced. It seems that larger species continue to express MLC1sa at high levels relative to MLC1sb. This selection of isoform level probably relates to muscle (and therefore isoform) function, since it is at this time in development that muscles are first put under load and required to do work.

With the cDNAs for the MLC isoforms now in hand, we are in a position to begin to address the questions of isoform selection and function directly. Introducing exogenous copies of these isoforms into cells in culture or the creation of transgenic animals to cause inappropriate expression of isoforms (in terms of level or developmental stage) will allow the effects of such expression to be evaluated. This may allow us to define the biological function of the MLC family and the individual isoforms.

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