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Isolation of the cDNA Encoding Rat Skeletal Muscle Myosin Light Chain Kinase

SEQUENCE AND TISSUE DISTRIBUTION*

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A cDNA clone encoding skeletal muscle myosin light chain kinase (MLCK) was isolated from a rat skeletal muscle library using oligonucleotide probes. The total length of the rat skeletal muscle MLCK cDNA was 2823 base pairs with an open reading frame of 1830 base pairs. The deduced sequence of the 610-amino acid protein exhibited 96% amino acid identity to rabbit skeletal muscle MLCK in the carboxyl-terminal portion of the molecule, which contains the catalytic and the calmodulin-binding domains, and 58% identity in the amino-terminal region. Analysis of total rat mRNA revealed a single mRNA species of 3.4 kilobases that was unique to skeletal muscle. Further analysis of skeletal muscle tissue using fast-twitch glycolytic, fast-twitch oxidative glycolytic, and slow-twitch oxidative fibers isolated from rat leg revealed that the mRNA level for MLCK varied among the three fiber types. The results of kinase assays performed on the fibers showed that MLCK activity levels paralleled the MLCK mRNA levels found in each of the three types of skeletal muscle fibers studied. Fast-twitch oxidative glycolytic (gastrocnemius red) and slow-twitch oxidative (soleus) exhibited 60 and 13%, respectively, of the enzymatic activity present in fast-twitch glycolytic (gastrocnemius white) fibers.

Myosin light chain kinase (MLCK)[1] was first described and purified to homogeneity from rabbit skeletal muscle by Pires and Perry (1). The enzyme catalyzes the phosphorylation of a specific class of myosin light chain known as the phosphorylatable or P-light chain. MLCK exists as several distinct molecular species. Separate forms are present in skeletal muscle (1), heart (2), and smooth muscle or non-muscle tissue (3, 4). In all tissues, however, the kinase exhibits a complete dependence of activity on Ca^{2+}-calmodulin (CaM) and a high degree of substrate specificity, phosphorylating only the P-light chain at significant rates. The functional significance of myosin light chain phosphorylation is different in striated (skeletal and cardiac) and nonstriated (smooth and non-muscle) muscle tissue. In smooth muscle, P-light chain phosphorylation is required for the initiation of contraction (5), whereas in skeletal muscle this function is performed by the troponin-tropomyosin system (6). Myosin phosphorylation in striated muscle is thought to play a regulatory role, such as the potentiation of isometric twitch tension (7). Besides differing in their physiological functions, the smooth and skeletal muscle forms also differ in their physicochemical properties. MLCKs purified from skeletal muscle are smaller (M_r = 65,000–90,000) than those purified from smooth muscle (M_r = 130,000–150,000) (8). Antibodies to either species cross-react with the other weakly, if at all (9, 10).

Skeletal muscle MLCK has been characterized extensively. The complete amino acid sequence of the purified M_r = 65,000 rabbit kinase has been determined (11, 12), and the functional domains of the enzyme have been elucidated (13). The CaM-binding region has been identified (14), and structural studies have been carried out with monoclonal antibodies to further define various domains of the kinase (15). Furthermore, using a combination of partial proteolytic digestion and hydrodynamic studies, Mayr and Heilmeyer (16) were able to construct a three-dimensional model for the enzyme.

Somewhat less is known about the structure of smooth muscle MLCK than the skeletal muscle enzyme; however, considerable progress has also been made in characterizing this kinase. Lukas *et al.* (17) have identified the cAMP-dependent protein kinase phosphorylation site in the enzyme that is protected by calmodulin binding. When a synthetic peptide analogue of this fragment was produced, it was shown to be a high-affinity CaM-binding peptide. A cDNA, from chicken gizzard, encoding the carboxyl-terminal segment of smooth muscle MLCK has been characterized and sequenced by Guerriero *et al.* (18). The amino acid sequence derived from this partial cDNA clone showed significant identity (54%) to the amino acid sequence of rabbit skeletal muscle MLCK. Further characterization of the cDNA using a series of deletion mutants in an expression vector system identified a 192-base pair fragment coding for the CaM-binding domain of this enzyme; this domain overlapped that identified by Lukas *et al.* (17).

In order to further extend our knowledge of the structure, mechanism of action, and physiological significance of skele-

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[1] The abbreviations used are: MLCK, myosin light chain kinase; CaM, calmodulin; kb, kilobase; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TEMED, N,N,N′,N′-tetramethylethylenediamine; MOPS, 4-morpholinepropanesulfonic acid; l, liter.

tal muscle MLCK, we have cloned and sequenced a cDNA encoding the rat skeletal muscle enzyme. With this cDNA we have also determined the size and distribution of the mRNA for skeletal muscle MLCK and identified the gene copy number and approximate gene size.

EXPERIMENTAL PROCEDURES

Materials

Herring sperm DNA and polyadenylic acid (both dissolved in distilled water at 10 mg/ml) were purchased from Boehringer Mannheim. Lithium chloride was obtained from J. T. Baker (Phillipsburg, NJ). The enzyme "Sequenase" was purchased from United States Biochemical Corp. (Cleveland, OH) and used according to their instructions with the included buffers and reagents. The agarose used was "SeaKem Le" obtained from FMC BioProducts (Rockland, ME). The paper used in blotting and for filter replication was Whatman 3MMChr (Whatman Ltd., Maidestone, United Kingdom). The Nytran membrane used for the northern filter analysis and the nitrocellulose used for Southern filter analysis and colony screening were purchased from Schleicher and Schuell. Guanidine isothiocyanate, acrylamide, bis-acrylamide, TEMED, ammonium persulfate, 5-bromo-4-chloro-3-indoyl β-D-galactoside, and isopropyl-1-thio-β-D-galactopyranoside were purchased from Bethesda Research Labs. Restriction enzymes, DNA Polymerase I large fragment (Klenow), DNA polymerase I, polynucleotide kinase, and T4 DNA ligase were purchased either from New England Biolabs or Bethesda Research Labs and used according to the manufacturer's directions unless otherwise noted. Tryptone and yeast extract were produced by Difco. Other chemicals were obtained from Sigma and were reagent grade or better.

The buffers and solutions used were as follows: TBE buffer (135 mM Tris, 45 mM boric acid, and 2 mM EDTA adjusted to pH 9.0); TAE buffer (8 mM Tris and 1 mM EDTA titrated to pH 8.0 with sodium acetate); Northern-formamide gel buffer (40 mM MOPS, pH 7.2, 10 mM sodium acetate, and 1 mM EDTA); 1 × SSC (0.15 M sodium chloride and 15 mM sodium citrate); 50 × Denhardt's solution (1% (w/v) each ficoll, polyvinylpyrrolidone, and bovine serum albumin); denaturing solution (0.5 M sodium hydroxide and 1.5 M sodium chloride); neutralizing solution (1 M Tris, pH 7.0, and 1.5 M sodium chloride); 2 × Stark's buffer (10 × SSC, 50 mM sodium phosphate, pH 6.5, 20 μg/ml polyadenosine, 10 × Denhardt's solution, 500 μg/ml herring sperm DNA); and 1 × SSPE (0.18 M sodium chloride, 10 mM sodium phosphate, pH 7.7, and 1 mM EDTA). Luria broth (LB) was made with 10 g/l tryptone, 5 g/l sodium chloride, and 10 g/l yeast extract with the addition of 15 g/l agarose for plates and 100 mg/l ampillicin for drug selection.

Methods

Identification of cDNA Clones Encoding Rat Skeletal Muscle MLCK—The construction of the rat skeletal muscle cDNA library, using pUC8 and pUC9 has been previously described (19). Ampillicin-resistant colonies were plated on nitrocellulose filters, the colonies lysed, and the filters baked for 2 h at 80 °C. The filters were placed in prehybridization buffer (6 × SSC, 50 mM sodium phosphate, pH 6.7, 1 × Denhardt's solution, 0.5% SDS, and 100 μg/ml sheared and denatured herring sperm DNA) for 4–16 h at 37 °C. Hybridization was performed at 37 °C overnight using hybridization buffer (prehybridization buffer lacking SDS) in the presence of a mixture of oligonucleotides end-labeled with (γ-^{32}P)ATP by polynucleotide kinase (20). Nitrocellulose filters were washed at 37 °C for 1 h in 6 × SSC, 0.5% sodium phosphate before washing at 42 (low stringency) or 49 °C (high stringency) for 10 min.

Subcloning and Sequencing of the cDNAs—DNA from positive clones was isolated by the alkaline lysis method (21) and the two *Eco*RI/*Sal*I inserts excised by restriction enzymes. The inserts, MLCK-1 and MLCK-2, were subcloned into m13mp18 and m13mp19 (22). The cDNA was sequenced by the dideoxy chain termination method of Sanger (23). The sequence was obtained using oligonucleotide primers generated from previous data. The gels were visualized by autoradiography with Kodak XAR-5 film.

Southern Transfer Analysis—High molecular weight rat DNA was isolated from rat liver as described previously by Maniatis *et al.* (24). After restriction enzyme digestion, the DNA was electrophoresed on a 1.4% agarose gel in TAE buffer and blotted to nitrocellulose (25). The filter was baked for 2 h at 80 °C, prehybridized in 2 × Stark's buffer and 50% (v/v) deionized formamide, and then hybridized overnight at 42 °C with the addition of nick-translated probe. The nick-translated hybridization probes (26) consisted of the *Eco*RI-*Sal*I inserts from both MLCK-1 and MLCK-2. The filter was washed at 55 °C using 0.1 × SSC and 0.1% SDS and exposed to x-ray film (Kodak XAR-5) with an intensifying screen (Dupont Cronex) at −70 °C.

Northern Transfer Analysis—Total cellular RNA was prepared from rat aorta, brain, heart, intestine, kidney, liver, lung, skeletal muscle, gastrocnemius white, gastrocnemius red, and soleus (27). The tissues were excised from freshly killed rats, immediately frozen at −70 °C, and then homogenized using a Polytron (Kinetimatica GmbH) for 1 min at setting of 5. The RNA was electrophoresed on a 1.0% agarose-formaldehyde gel (Ref. 28, as modified in Ref. 24) in RNA buffer and transferred to Nytran (29). The filters were prehybridized in 0.25 ml/cm^2 50% (v/v) deionized formamide, 5 × Denhardt's solution, 0.1% (w/v) SDS, 150 μg/ml sheared and denatured herring sperm DNA, and 5 × SSPE for 4–24 h at 42 °C. Hybridization was performed at 42 °C overnight with 50% (v/v) formamide, 1.5 × Denhardt's solution, 150 μg/ml sheared and denatured herring sperm DNA, 0.1% SDS, and 5 × SSC in the presence of nick-translated (26) *Eco*RI-*Sal*I inserts isolated from MLCK-1 and MLCK-2. The filter was washed twice for at least 15 min with 5 ml/cm^2 6 × SSC, 0.1% SDS at room temperature, then at 37 °C for 2 × 15 min in 1 × SSC, 0.5% SDS. The final wash was for 1 h at 42 °C in 0.1 × SSC, 1% SDS after which the filter was exposed to Kodak XAR-5 film with an intensifying screen (Dupont Cronex) at −70 °C.

In order to control for the intactness of the RNA samples, for possible gel loading errors, and for completeness of transfer to the Nytran filters several controls were done. First, all gels were run in duplicate such that one set of RNA samples were stained and the other set transferred to Nytran; following transfer, the gel was stained to identify any untransferred RNAs. Second, the filters were probed with a mouse actin probe and stained with methylene blue (24) following autoradiography to ascertain whether the amount of total RNA on the filter was equal in each lane.

MLCK Enzyme Assays—Kinetic assay performed on the isolated rat skeletal muscle fiber types were performed with a peptide substrate (30) following the assay procedure described earlier (31). The frozen tissue was prepared by homogenization using a Polytron (Kinematica GmbH) for 2 min at a setting of 5 in cold homogenization buffer (50 mM MOPS, pH 7.0, 1 mM dithiothreitol, 2 mM EGTA, 50 mM NaCl, 5 mg/l leupeptin, and 1 mM phenylmethylsulfonyl fluoride).

RESULTS

Isolation and Characterization of Rat Skeletal Muscle Myosin Light Chain Kinase Clones—The oligonucleotide probes used to isolate cDNA clones for rat skeletal muscle MLCK were based on the rabbit skeletal muscle MLCK amino acid sequence. The probes were generated from functionally important domains, regions that were assumed to have the greatest probability of being conserved between the two species. The location of the probes was further defined by utilizing regions of low-codon degeneracy; for the degenerate amino acids codon bias tables were used to predict the third base position. Oligonucleotide probe 1 consisted of two 44-mers derived from amino acids 332–348 of the rabbit sequence. Codon bias tables were consulted, and the only degenerancy allowed was at position 1 of Lysine 339. Probe 2 contained 48 20-mers encoding amino acids 570–576. Of the 200,000 colonies screened, 38 clones hybridized to both oligonucleotides. Additional screening of these colonies with two other oligonucleotide probes, derived from amino acids 407–411 and amino acids 509–516 of rabbit skeletal muscle MLCK, identified four colonies that hybridized to all four probes. The clone containing the largest cDNA insert, 1.8 kb, was selected and purified. Sequence analysis showed that this clone, MLCK-1, contained an open reading frame encoding a sequence of amino acids closely corresponding to those of positions 299–603 of rabbit skeletal muscle MLCK. Partial sequence analysis of the other three positive colonies revealed that they all terminated at the same *Eco*RI site, leading to

the conclusion that the message contained an internal *Eco*RI site that had not been methylated during library construction. Therefore, we rationalized that the 5′ region of the mRNA could be present in the library as a separate clone, since any full-length cDNA would have been cleaved when the excess *Eco*RI linkers were removed. The library was rescreened with oligonucleotide probe 5 (amino acids 261–272, unique sequence) and oligonucleotide probe 6 (amino acids 48–55, 16-fold degenerate). This screening identified a single positive colony, MLCK-2, 947 base pairs in length.

The two cDNAs, MLCK-1 and MLCK-2, were sequenced on both strands. Together, the two clones were found to encode the entire rat skeletal muscle MLCK. The internal *Eco*RI site was found to be located at amino acids 296 and 297 (of the rat sequence). These two clones were ligated together at the *Eco*RI site, so that the coding region would be intact. Their composite restriction map is shown in Fig. 1. The DNA sequence and deduced amino acid sequence are shown in Fig. 2. MLCK-2 contained 59 bases of 5′-untranslated region, the initiation codon, and nucleotides corresponding to the first 295 amino acids of rat skeletal muscle MLCK. MLCK-1 comprised 1876 bases and encoded the last 314 amino acids of rat skeletal muscle MLCK and part of the 3′-untranslated region, which contains a polyadenylation signal at position 2695–2700 (see Fig. 2). The possibility remains that there could be a fragment of the message missing if there were two *Eco*RI sites located close together in this region. However, when the rat and rabbit sequences are compared (see Fig. 3), this region of the protein is seen to be highly conserved, supporting the theory that the entire message has been cloned.

Comparison of Rat and Rabbit Sequences—In Fig. 3, the amino acid sequence predicted from the rat skeletal muscle MLCK cDNA is compared to that of rabbit skeletal muscle MLCK (11, 12). In the carboxyl-terminal segment (residues 266–610 of the rat sequence), both proteins are highly homologous, and many of the differences represent conservative changes; there are only two cases where the amino acid changes could not be regarded as conservative, one at residue 391 where histidine is changed to glutamine and at residue 361 where an alanine is changed to a serine. The CaM-binding domains (amino acids 580–606) were identical; however, rat skeletal muscle MLCK contained an additional four carboxyl-terminal amino acids not found in the rabbit enzyme.

Takio *et al.* (11) defined residues 302–508 (of the rabbit sequence) as the catalytic domain based on sequence identity to other protein kinases. The corresponding region of the predicted rat sequence was also compared to other protein kinases and as expected showed a high degree of homology. In contrast, the amino-terminal sequence showed greatly reduced homology between the rat and the rabbit. It is known (12) that the amino-terminal domain of rabbit skeletal muscle MLCK has an unusual amino acid composition, being especially rich in glutamic acid, glycine, alanine, and proline. Although the predicted amino-terminal rat protein does not share high sequence identity with the rabbit sequence, it does contain a similar composition of amino acids as seen in Table I.

Determination of mRNA Size and Tissue Distribution—MLCK-1 and MLCK-2 cDNAs were first hybridized to total rat RNA isolated from aorta, intestine (smooth muscle sources), and skeletal muscle. This experiment identified a single transcript of 3.4 kb (as determined by comparison to size markers) which was present only in skeletal muscle (Fig. 4*A*). Since the cDNA contains 2823 base pairs including a polyadenylation signal in the 3′-untranslated region, it is reasonable to assume that a significant portion (up to 600 base pairs) of the 5′-untranslated region of the mRNA is missing from the cDNA. This problem is currently being investigated using genomic clones which contain DNA located upstream of the 5′ end of the cDNA.

The cDNA clones were next used to survey total RNA prepared from rat brain, heart, kidney, liver, and lung. Skeletal muscle MLCK mRNA was not detectable in any of these tissues (Fig. 4*B*), and even after extended exposure, no additional mRNAs were found to hybridize to the probes. The mRNA levels were then studied in three types of muscle fibers isolated from rat leg: 1) gastrocnemius white (fast-twitch glycolytic), 2) gastrocnemius red (fast-twitch oxidative glycolytic), and 3) soleus (slow-twitch oxidative) (32). Fig. 4*C* shows that the levels, but not the size, of skeletal muscle MLCK mRNA differ between the three fiber types. Expres-

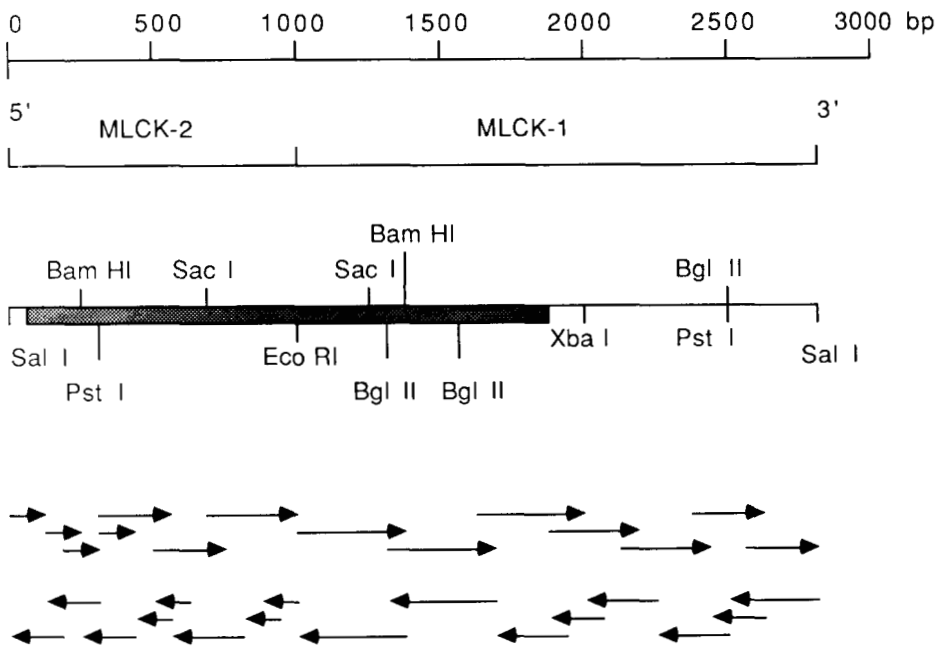

FIG. 1. **Partial restriction map of MLCK-2 and MLCK-1.** The restriction maps of MLCK-2 and MLCK-1 cDNA clones were deduced from the nucleotide sequence. Restriction sites are indicated at approximate positions as is the *thick bar* representing the protein coding region. The *thin lines* represent the 3′- and 5′-untranslated regions. The sequencing reactions used to determine the base pair sequence are denoted by the *directional arrows*.

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-59 ggtcgaccagctagaacgccttgagttcagacacgcagaagcacacgcctacctacctc ATG GCT ACA GAA AAC GGA GCA GTT GAG CTG GGA ACC CAG    39
                                                                Met Ala Thr Glu Asn Gly Ala Val Glu Leu Gly Thr Gln

AGT CTG TCA ACA GAC CAC CCA CCT ACA GAC GCA GCA GGT GAT GGA TCT CCA GCT TCA GAG AAA GAA CCC AGC CTC CCA GAC ACA GAG  126
Ser Leu Ser Thr Asp His Pro Pro Thr Asp Ala Ala Gly Asp Gly Ser Pro Ala Ser Glu Lys Glu Pro Ser Leu Pro Asp Thr Glu

AAA GAC CTG GGC CCC ACT AAC ACG AAG AAA GAC CCT GGC GCG CCG GAT CCA AAG AAA AAT CCA GAT CCA CCC TCC CTG AAG AAA ACT  213
Lys Asp Leu Gly Pro Thr Asn Thr Lys Lys Asp Pro Gly Ala Pro Asp Pro Lys Lys Asn Pro Asp Pro Pro Ser Leu Lys Lys Thr

CCC GAA GCC CCT GGT CCA GAG AAA AAG GGT GAC TCG GCC CCA GCT TCA GCC AGC AAC CAG GGC CCT TCA GGA GAG GGT GAT GGA GGT  300
Pro Glu Ala Pro Gly Pro Glu Lys Lys Gly Asp Ser Ala Pro Ala Ser Ala Ser Asn Gln Gly Pro Ser Gly Glu Gly Asp Gly Gly

GGG GGC CCT GCT GAA GGC GGT ACG GGG CCT CCT GCA GTC TTG CCC CAG CCC ACT GCC ACA GCT GAT GCC AGC ATC CAG AAG CTG GAC  387
Gly Gly Pro Ala Glu Gly Gly Thr Gly Pro Pro Ala Val Leu Pro Gln Pro Thr Ala Thr Ala Asp Ala Ser Ile Gln Lys Leu Asp

GCC ACG CAA GAG CCC TCG GGC AAC CAG GAA TCT GGA GAG GCC AAG GCA GGC AAG AAG GCA GCA GAA TGC CGA GAA GCA GGA CGA AGG  474
Ala Thr Gln Ala Pro Ser Gly Asn Gln Glu Ser Gly Glu Ala Lys Ala Gly Lys Lys Ala Ala Glu Cys Arg Glu Ala Gly Arg Arg

GGC TCA CCC GCC TTT CTT CAC AGC CCT AGC TGC CCC GCG ATC ATC TCC TGT TCT GAG AAG ACA CTG GCC ATG AAG CCC TTA AGC GAG  561
Gly Ser Pro Ala Phe Leu His Ser Pro Ser Cys Pro Ala Ile Ile Ser Cys Ser Glu Lys Thr Leu Ala Met Lys Pro Leu Ser Glu

ACA ACA GAG CTC ATC TTT GCA GGG GTG TCT GAA ACC CCT GAT CCC CAA GAT CCT GGT CCA GCT AAG GAC GAA GGA GGG ACG AAC ACG  648
Thr Thr Glu Leu Ile Phe Ala Gly Val Ser Glu Thr Pro Asp Pro Gln Asp Pro Gly Pro Ala Lys Asp Glu Gly Gly Thr Asn Thr

CTG GCA GAC GGG AAA GAA GAG GCA GAG GCA GGC CAG GCT GAG CAG GCT AAG GTG CAA GGG GAC ACC TCC CAG AGG ATC GGG TTC CAG  735
Leu Ala Asp Gly Lys Glu Glu Ala Glu Ala Gly Gln Ala Glu Gln Ala Lys Val Gln Gly Asp Thr Ser Gln Arg Ile Gly Phe Gln

GCT GTT CCC TCA GAG AGA GCA GAG GTG GGG CAG GCC CTC TGT CTC ACG GCC AAG GAG GAA GAC TGC TTC CAA ATT CTG GAT GAT TGT  822
Ala Val Pro Ser Glu Arg Ala Glu Val Gly Gln Ala Leu Cys Leu Thr Ala Lys Glu Glu Asp Cys Phe Gln Ile Leu Asp Asp Cys

CCA CCA CCC CCA GCC CCC TTC CCA CAC AGG ATC GTG GAG CTG AGG ACC GGA AAT GTC AGC AGT GAA TTC AGC ATG AAC TCA AAG GAG  909
Pro Pro Pro Pro Ala Pro Phe Pro His Arg Ile Val Glu Leu Arg Thr Gly Asn Val Ser Ser Glu Phe Ser Met Asn Ser Lys Glu

GCA TTG GGA GGT GGC AAG TTT GGA GCT GTG TGC ACC TGC ACG GAG AGA TCC ACA GGC CTC AAG CTG GCA GCC AAG GTC ATC AAG AAA  996
Ala Leu Gly Gly Gly Lys Phe Gly Ala Val Cys Thr Cys Thr Glu Arg Ser Thr Gly Leu Lys Leu Ala Ala Lys Val Ile Lys Lys

CAG ACC CCC AAG GAC AAG GAA ATG GTG TTG CTG GAG ATC GAG GTC ATG AAC CAG CTG AAC CAC CGC AAT TTG ATC CAG CTG TAC TCA 1083
Gln Thr Pro Lys Asp Lys Glu Met Val Leu Leu Glu Ile Glu Val Met Asn Gln Leu Asn His Arg Asn Leu Ile Gln Leu Tyr Ser

GCC ATT GAG ACC TCT CAC GAG ATC ATC CTG TTC ATG GAG TAC ATT GAG GGC GGC GAG CTC TTT GAG AGG ATC GTG GAT GAG GAC TAC 1170
Ala Ile Glu Thr Ser His Glu Ile Ile Leu Phe Met Glu Tyr Ile Glu Gly Gly Glu Leu Phe Glu Arg Ile Val Asp Glu Asp Tyr

CAA CTG ACG GAA GTG GAT ACC ATG GTG TTC GTC AGG CAG ATC TGC GAC GGG ATC CTC TTC ATG CAC AAG ATG AGG GTC CTG CAC TTG 1257
Gln Leu Thr Glu Val Asp Thr Met Val Phe Val Arg Gln Ile Cys Asp Gly Ile Leu Phe Met His Lys Met Arg Val Leu His Leu

GAC CTC AAG CCA GAG AAC ATC CTG TGT GTG AAT ACC ACC GGG CAC TTG GTG AAG ATA ATT GAC TTC GGC CTG GCA AGA AGG TAT AAC 1344
Asp Leu Lys Pro Glu Asn Ile Leu Cys Val Asn Thr Thr Gly His Leu Val Lys Ile Ile Asp Phe Gly Leu Ala Arg Arg Tyr Asn

CCC AAT GAG AAG CTG AAG GTG AAT TTT GGG ACC CCG GAG TTC CTG TCA CCA GAG GTG GTG AAC TAT GAC CAG ATC TCT GAC AAG ACG 1431
Pro Asn Glu Lys Leu Lys Val Asn Phe Gly Thr Pro Glu Phe Leu Ser Pro Glu Val Val Asn Tyr Asp Gln Ile Ser Asp Lys Thr

GAC ATG TGG AGT CTT GGG GTG ATC ACC TAC ATG CTA CTG AGT GGC CTC TCT CCC TTC CTG GGC GAC GAT GAC ACG GAG ACC CTA AAC 1518
Asp Met Trp Ser Leu Gly Val Ile Thr Tyr Met Leu Leu Ser Gly Leu Ser Pro Phe Leu Gly Asp Asp Asp Thr Glu Thr Leu Asn

AAC GTC CTG TCT GCC AAC TGG TAC TTT GAT GAG GAG ACT TTT GAA GCT GTG TCA GAC GAG GCC AAA GAC TTC GTT TCC AAT CTT ATC 1605
Asn Val Leu Ser Ala Asn Trp Tyr Phe Asp Glu Glu Thr Phe Glu Ala Val Ser Asp Glu Ala Lys Asp Phe Val Ser Asn Leu Ile

ACG AAG GAC CAG AGT GCC AGA ATG AGT GCT GAG CAG TGC CTC GCC CAC CCC TGG CTC AAC AAC CTG GCA GAA AAA GCC AAG CGC TGC 1692
Thr Lys Asp Gln Ser Ala Arg Met Ser Ala Glu Gln Cys Leu Ala His Pro Trp Leu Asn Asn Leu Ala Glu Lys Ala Lys Arg Cys

AAC CGT CGT CTC AAG TCC CAG ATC CTG CTT AAG AAA TAC CTC ATG AAG AGG CGC TGG AAG AAA AAC TTC ATT GCT GTC AGC GCA GCC 1779
Asn Arg Arg Leu Lys Ser Gln Ile Leu Leu Lys Lys Tyr Leu Met Lys Arg Arg Trp Lys Lys Asn Phe Ile Ala Val Ser Ala Ala

AAT CGC TTC AAG AAG ATC AGT AGC TCG GGG GCA CTG ATG GCT TTG GGG GTC TGA accccaggcgactaaaagcctggatgcactcgcatggtcaaggc 1877
Asn Arg Phe Lys Lys Ile Ser Ser Ser Gly Ala Leu Met Ala Leu Gly Val Term

tgaaaccagagagagccggccagatctctagagcaggctggtcaggacaaatctgcaccaggcccagaggcttggggctccgcaggtccttaggcttaccgcttcccctacgtgaa 1993

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cttataataaacacacaggttggaggcagcctccttcctctgcgcatgcaaaaaaaaaaaaaaaggtcgacctgca                                    2764
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FIG. 2. **The composite nucleotide sequence of the rat skeletal muscle MLCK cDNA clones.** The nucleotide sequence of MLCK-2 and MLCK-1 (2823 base pairs) is presented with the deduced amino acid sequence. The polyadenylation signal (AATAAA) is *underlined*. The internal *Eco*RI site delineating the end of MLCK-2 and the beginning of MLCK-1 is located at nucleotides 885–890 of the coding sequence.

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RAT    1 M A T E N G A V E L G T Q S L S T D H P P T D A A G D G S P
RABBIT   * - - - - - - - - - - I - - - - - - E A S K G - - S E E - L

      31 A S E K E P S L P D T E K D L G P T N T K K D P G A P D P K
         - A - - D - A P - - P - - G P - - S D - - Q - - D P S T - -

      61 K N P D P P S L K K T P E A P G P E K K G D S A P A S A S N
         - D A N T - A P E - G D V V - A Q P S A - G - * * * * * * *

      91 Q G P S G E G D G G G G P A E G G T G P P A V L P Q P T A T
         - - - A - - - G Q V E A - - - - S A - K - - A - - - Q - - -

     121 A D A S I Q K L D A T Q A P S G N Q E S G E A K A G K K A A
         - E - - E K - P E - E K G - - - H - D P - - P T V - - - V -

     151 E C R E A G R R G S P A F L H S P S C P A I I S C S E K T L
         - G Q A - A - - - - - - - - - - - - - - - A S T - - L P

     181 A M K P L S E T T E L I F A G V S E T P D P Q D P G P A K A
         - Q - - - - - A S - - - - E - - P A - - G - T E - - - - - -

     211 E G G T N T L A D G * K E E A E * A * G Q A E Q A K V Q G D
         - - - V D L - - E S Q - - A G - K - P - - - D - - - - - - -

     238 T S Q R I G F Q A V P S E * * R A E V G Q A L C L T A K E E
         - - R G - E - - - - - - - - R P - P - - - - - - - - P - R - -

     266 D C F Q I L D D C P P P P A P F P H R I V E L R T G N V S S
         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

     296 E F S M N S K E A L G G G K F G A V C T C T E R S T G L K L
         - - - - - - - - - - - - - - - - - - - - - - K - - - - - -

     326 A A K V I K K Q T P K D K E M V L L E I E V M N Q L N H R N
         - - - - - - - - - - - - - - - - M - - - - - - - - - - - - -

     356 L I Q L Y S A I E T S H E I I L F M E Y I E G G E L F E R I
         - - - - - A - - - - P - - - V - - - - - - - - - - - - - - -

     386 V D E D Y Q L T E V D T M V F V R Q I C D G I L F M H K M R
         - - - - - H - - - - - - - - - - - - - - - - - - - - - - - -

     416 V L H L D L K P E N I L C V N T T G H L V K I I D F G L A R
         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

     446 R Y N P N E K L K V N F G T P E F L S P E V V N Y D Q I S D
         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

     476 K T D M W S L G V I T Y M L L S G L S P F L G D D D T E T L
         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

     506 N N V L S A N W Y F D E E T F E A V S D E A K D F V S N L I
         - - - - - G - - - - - - - - - - - - - - - - - - - - - - - -

     536 T K D Q S A R M S A E Q C L A H P W L N N L A E K A K R C N
         V - E - G - - - - - A - - - - - - - - - - - - - - - - - - -

     566 R R L K S Q I L L K K Y L M K R R W K K N F I A V S A A N R
         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

     596 F K K I S S S G A L M A L G V Term
         - - - - - - - - - - -
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FIG. 3. **Comparison of rat and rabbit skeletal muscle MLCK.** The amino acid sequence predicted by the rat cDNA clone is compared to the rabbit sequence, Takio *et al.* (11, 12). Eight gaps (*) were introduced into the rabbit sequence, and five gaps were introduced into the rat sequence to maximize homology. Identity is indicated by a *dash* (—).

sion of rat skeletal muscle MLCK mRNA was highest in fast-twitch glycolytic fibers, whereas an intermediate level was found in fast-twitch oxidative glycolytic fibers and the lowest level was detected in soleus fibers. The results of kinase activity assays performed on the three fiber types are presented in Table II and it appeared that the activity of MLCK reflected the level of its mRNA in the three fiber types.

Determination of Gene Size and Number—In order to determine the number of genes that encode rat skeletal muscle MLCK, we examined the hybridization pattern on a genomic blot of rat DNA, probed sequentially with radiolabeled MLCK-1 and MLCK-2. MLCK-1 was found to hybridize to the 12-kb *Eco*RI fragment (data not shown). When MLCK-1 and MLCK-2 were used in concert, both the 12- and the 5.3-kb bands of the *Eco*RI digest hybridized to the radiolabeled probes (Fig. 5, *lane a*). This would suggest that the larger (12 kb) *Eco*RI fragment contains the 3′ region of the gene, whereas the 5′ region is contained on the 5.3-kb fragment. These results are consistent with the presence of a single skeletal muscle MLCK gene, since the cDNA contains a single *Eco*RI site.

Further mapping of the gene was accomplished using restriction enzymes whose recognition sites were rare or absent from the cDNA. Any resulting fragments would therefore be due to the presence of enzyme recognition sites in introns of

TABLE I

Comparison of the amino-terminal amino acid composition of rat and rabbit skeletal muscle myosin light chain kinase

The deduced amino acid sequence from the rat skeletal muscle MLCK cDNA was compared to the first 292 amino acids of the rabbit skeletal muscle MLCK (30, 31). Three amino acids, proline, alanine, and glycine, make up 35% (40% for the rabbit sequence) of this segment.

Amino acid	Rat	Rabbit
	residues/molecule	
Aspartic acid	19	15
Glutamic acid	27	31
Arginine	7	8
Histidine	3	3
Lysine	20	20
Asparagine	7	3
Glutamine	14	17
Cysteine	6	4
Serine	23	23
Threonine	22	13
Tyrosine	0	0
Glycine	33	32
Alanine	38	43
Leucine	16	14
Isoleucine	7	7
Proline	38	43
Valine	8	12
Phenylalanine	5	5
Tryptophan	0	0
Methionine	2	0
	295	292

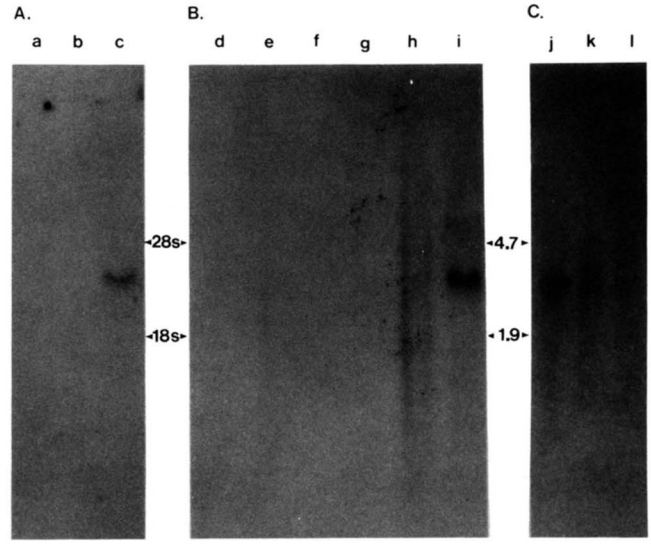

FIG. 4. **Northern transfer analysis of total RNA from rat tissue.** Total rat RNA was isolated, loaded onto a 1% agarose-formaldehyde gel, electrophoresed, and analyzed as described under "Experimental Procedures." *A*, analysis of rat smooth and skeletal muscle RNA, 50 μg of total RNA from rat aorta (*lane a*), intestine (*lane b*), and skeletal muscle (*lane c*). *B*, tissue distribution, 30 μg of total rat RNA from each of the following tissues: brain (*lane d*), heart (*lane e*), kidney (*lane f*), liver (*lane g*), lung (*lane h*), and skeletal muscle (*lane i*). *C*, analysis of rat skeletal muscle fibers, 30 μg of total rat RNA per lane, gastrocnemius white (*lane j*), gastrocnemius red (*lane k*), and soleus (*lane l*). The filters were probed with nick-translated *Sal*I-*Eco*RI inserts from MLCK-1 and MLCK-2. Probing of the filters with a mouse actin clone and staining with methylene blue showed that equal amounts of RNA were present in each lane of each respective filter (data not shown). The positions of the 28 S and the 18 S ribosomal subunits and their molecular weights (in kb) are indicated.

TABLE II

Activity of rat skeletal muscle myosin light chain kinase

The MLCK activity of the muscle fiber types was determined using an M5 peptide assay (15) following a 2-min homogenization of the tissues using a Polytron (Kinematica GmbH).

Muscle fiber type	Specific activity	Relative values
	(μmol/min mg) × 10³	
Gastrocnemius white (fast-twitch glycolytic)	1.92	1.0
Gastrocnemius red (fast-twitch oxidative glycolytic)	1.33	0.69
Soleus (slow-twitch oxidative)	0.33	0.16

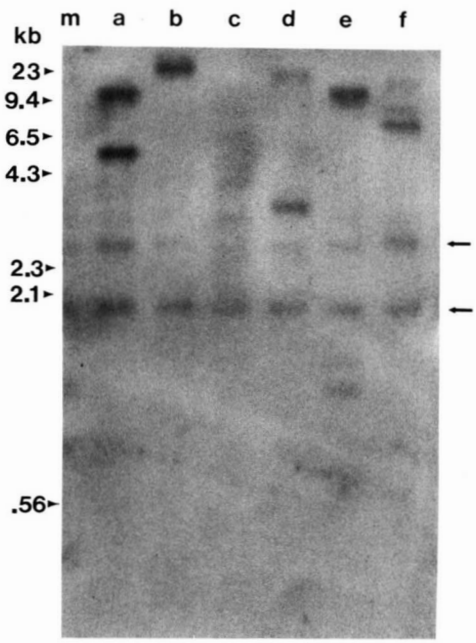

FIG. 5. **Southern transfer analysis of rat genomic DNA.** Rat liver DNA, 20 μg/lane, was digested with *Eco*RI (*lane a*), *Sal*I (*lane b*), *Kpn*I (*lane c*), *Hin*dIII (*lane d*), *Xba*I (*lane e*), and *Sca*I (*lane f*) and then run on a 1.4% agarose gel and analyzed as described under "Experimental Procedures." The filter was probed first with the nick-translated *Sal*I-*Eco*RI insert from MLCK-1 and then with MLCK-2 insert. Marker sizes (*m*, extreme *left lane*) are derived from *Hin*dIII digested λ DNA. The *arrows* denote the position of contaminant bands which are present in all lanes including the marker lane.

the skeletal muscle MLCK gene. No recognition sites for *Sal*I, *Kpn*I, *Hin*dIII, *Sca*I were contained in the cDNA, whereas a single *Xba*I site was located in the 3′-untranslated region. Therefore, the detection of multiple bands in the *Kpn*I (*lane c*), *Hin*dIII (*lane d*), *Xba*I (*lane e*), and *Sca*I (*lane f*) restriction digests, is probably due to the presence of these restriction sites within an intron or introns spanned by the MLCK cDNA.

Less stringent washes revealed more bands; however, the bands migrated with the same molecular weight in each lane including that of the markers suggesting a contaminant in the loading buffer. Although the data do not rule out the presence of more than one skeletal muscle MLCK gene, it is clear that there is not a multigene MLCK family encoding proteins related to skeletal muscle MLCK.

DISCUSSION

We have isolated and sequenced two cDNAs, which taken together contain the complete coding region corresponding to the mRNA for rat skeletal muscle MLCK. There is a single open reading frame of 1830 nucleotides that translates into a protein of 610 amino acids, which by comparison to the rabbit enzyme represents rat skeletal muscle MLCK.

Comparison of the protein sequence predicted by the rat cDNA to the rabbit skeletal muscle MLCK (11, 12) showed that the catalytic and calmodulin-binding domains are highly conserved with only 11 amino acid differences, and all but two of these are conservative changes. The identity drops from 96 to 58% in the amino-terminal region, and although the sequence is not highly conserved, the unusual amino acid composition of the rabbit protein sequence (11, 12) is also present in the rat sequence. This includes a high percentage of proline (13%), alanine (13%), and glycine (11%). Mayr and Heilmeyer (16) referred to the amino-terminal region as the "tail" region because of its rod-like structure determined by sedimentation studies. If the amino-terminal region functions in the anchorage of MLCK to the muscular fibrillar substructure, then the conservation of its rod-like shape may be important.

The sequence data presented here allows a comparison to be made of CaM-binding regions of a specific enzyme isolated from two different species. Rat and rabbit skeletal muscle MLCK are 100% conserved in their CaM-binding domains. Previous comparisons of smooth muscle MLCK (17), neuromodulin,[2] the γ-subunit of phosphorylase kinase (33), and rabbit skeletal muscle MLCK (14) had shown that although their respective CaM-binding regions contain 1) clusters of basic residues, 2) hydrophobic residues adjacent to these clusters, and 3) α-helical structures, their overall sequence is not highly conserved (17, and data not shown). The current data illustrate that while the CaM-binding domain may differ from protein to protein, it is highly conserved between species for a given enzyme.

Northern transfer analysis showed that the 3.4-kb mRNA for rat skeletal muscle MLCK is distinctly smaller than the 5.5-kb mRNA reported for chicken smooth muscle MLCK (18). It has long been speculated, based upon their differences in molecular weight, that the skeletal muscle MLCK might represent a proteolytically processed fragment of the larger smooth muscle enzyme (34). The predicted high molecular weight precursor was not observed, however, when skeletal muscle tissue was probed with anti-MLCK antibodies (9). Moreover, the amino acid sequence predicted from a partial clone of chicken smooth muscle MLCK showed only 54% homology with a comparable section of sequence from rabbit skeletal muscle MLCK (18). Translation of the cDNA sequence from rat skeletal muscle MLCK predicts a protein of molecular weight 58,949, sufficient to account for the skeletal muscle enzyme, but much too small to account for a larger (M_r > 100,000) precursor. Moreover, the observation that skeletal muscle MLCK is encoded by a mRNA distinct from that of smooth muscle MLCK in both size (3.4 *versus* 5.5 kb (18)), and tissue distribution indicates that no precursor-product relationship between the smooth and skeletal enzyme exists. The lack of detectable mRNA in heart implies either that these striated enzymes are not closely related at the mRNA level or that the amount of cardiac MLCK mRNA is too low for detection by this system. The latter hypothesis would be consistent with the low levels of MLCK present in cardiac tissue (34).

Differences in the specific activity of MLCK purified from slow- *versus* fast-twitch skeletal muscle have been reported (35, 36). However, Nunnally and Stull (9) found that there was no difference in the ability of their anti-myosin light chain kinase antiserum (to rabbit skeletal muscle MLCK) to inhibit the enzyme in extracts from several skeletal muscle

[2] K. Alexander and D. Storm, personal communication.

fiber types. These researchers also demonstrated, using antiserum overlays, that there was no difference in the apparent molecular weights of MLCK in the various skeletal muscle fibers. In this report, Northern transfer analysis showed that the amount, but not the size, of mRNA for rat skeletal muscle MLCK differed between rat fast-twitch glycolytic, fast-twitch oxidative glycolytic, and slow oxidative fiber types. Using aliquots of the same tissues, this work demonstrates that the specific activity of rat skeletal muscle MLCK decreases in the same order as the amount of mRNA in the tissues. These results imply that the activity of skeletal muscle MLCK in various fiber types reflects the amount of mRNA present in each fiber type. It is interesting that more skeletal muscle MLCK mRNA was found in fast-twitch glycolytic fibers, since phosphorylation of P-light chains occurs at physiologically relevant contraction frequencies and durations only in fast-twitch and not in slow-twitch fibers (36). The phosphorylation of P-light chains is thought to be involved in contraction-induced isometric twitch potentiation in fast-twitch skeletal muscle. It appears that the message levels and the activity of skeletal muscle MLCK may reflect the level of tissue involvement in the only physiological correlate of myosin P-light chain phosphorylation known to date for skeletal muscle. In support of this theory, studies have shown that increased contractile activity of rat skeletal muscle augments the enzymatic capacity of mitochondria and suppresses the enzymatic capability of several cytoplasmic enzymes of glycolysis. Williams *et al.* (37) measured the concentrations of cytochrome *b* mRNA and aldolase A mRNA in limbs exposed to 21 days of constant electrical contraction, compared to unstimulated limbs of the same rats. They determined that the mRNA levels differed based on the contraction frequency of the muscle, suggesting that a factor(s) were regulating the expression of these two genes. Such a factor may play a role in the differential expression of skeletal muscle MLCK mRNA in fast-twitch *versus* slow-twitch skeletal muscle fibers.

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