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Ventricular myosin light chain 1 is developmentally regulated and does not change in hypertension

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ABSTRACT

Cardiac myosin heavy chain (MHC) isoform distribution has been shown to undergo changes during development, in response to hormonal stimuli, and during pathologic states like hypertension. We initiated a study of myosin light chain 1 (MLC1) expression in cardiac tissue to determine whether MLC1 undergoes changes similar to those seen for MHC. We isolated a full length cDNA for the predominant MLC1 sequence in rat hearts. This gene is expressed in ventricular tissue at much higher levels than in atrial tissue. Based on its expression pattern and sequence homology, this cDNA encodes the rat ventricular MLC1 and has been named RVMLC1. RVMLC1 is expressed at very low levels in cardiac tissue during early development and is expressed abundantly after birth and in adult hearts. The expression of RVMLC1 was found not to change in the hearts of rats with renovascular hypertension.

INTRODUCTION

Myosin, a predominant component of sarcomeres, hydrolyzes ATP and interacts with actin to generate the force of muscle contraction. Myosin consists of two heavy chains, approximately 200 kDa, and two pair of light chains, 16-25 kDa. There are two classes of myosin light chains, LC1-3 (also called alkali or essential) and LC2 (also called DTNB or regulatory). The light chains are thought to play a role in regulating the ATPase of the heavy chain. LC2 is phosphorylated in smooth and nonmuscle myosins by a calcium/calmodulin dependent myosin light chain kinase which regulates the calcium sensitivity of the actin-activated ATPase of myosin (for review, 1,2). The role of the alkali light chain is less clear. Structural studies have shown the MLC1 in smooth muscle myosin is located very near the ATP binding site (3). In skeletal muscle, the shortening velocity of the muscle was correlated with specific isoforms of MLC1 (4).

In vertebrates, both MHC and MLC are the products of multigene families whose members show tissue-specific and developmental-stage specific expression (5-7). In the heart, two genes, alpha and beta, encode the MHC. The genes encoding alpha and beta MHC are closely linked in the genome of both rat and man (8,9) and have tightly regulated expression in response to a variety of stimuli including thyroid hormone (10). Alpha and beta myosin have different ATPase activity *in vitro* (11) and *in vivo*, beta-MHC-predominant hearts, with lower ATPase activity, display a reduced contractility (12).

MHC isoform switches have been well documented, however changes in MLC isoforms may also contribute to the myosin isozyme switches which are seen on non-denaturing gels (11). Previously, it was observed that the MLC2 isoform does not change qualitatively in the hearts of spontaneously hypertensive rats (SHR), a genetically inbred strain which develops hypertension (13). Recently, ventricular MLC2 mRNA was observed to increase in the atria of SHR rats (14). The mRNA changes preceded the onset of hypertension.

The two cardiac MLC1 isoforms, the atrial and ventricular MLC1, are distinct from the fast skeletal muscle isoforms, MLC1 and MLC3. The molecular genetic characterization of the fast skeletal muscle MLC1-3 gene has been described for chicken (15), rat (16), mouse (17) and human (18) genes. In these organisms, one gene undergoes promoter selection and alternate splicing to produce MLC1, 27 kDa, and MLC3, 17 kDa. Fast skeletal muscle MLC1 and MLC3 are identical over their carboxyl two-thirds, but additional exons found at the 5′ end of the mRNA encode the additional amino acids found at the amino terminus of MLC1 which are missing from MLC3.

The two heart MLC1 isoforms are products of two separate genes. Partial cDNAs encoding the mouse atrial and ventricular isoforms were isolated and shown to be encoded by two genes (19,20). Sequence data from one of these cDNAs, the atrial MLC1, was recently presented (21). Using the mouse cDNAs, it was been shown that the adult ventricular isoform is also expressed in slow skeletal muscle. It has also been shown that the atrial isoform is identical to the predominant MLC1 of fetal hearts (22).

In this report, we describe a full length cDNA encoding the rat ventricular MLC1, RVMLC1. This was obtained by screening a cDNA library constructed from rat heart with a portion of the rat skeletal MLC1-3 cDNA. The tissue-specific and developmental-stage specific expression of RVMLC1 was studied using both the complete cDNA and an oligonucleotide specific for the 3' untranslated region. RVMLC1 was abundantly expressed in ventricular tissue and was expressed at much lower levels in atrial tissue. RVMLC1 was expressed in slow but not fast skeletal muscle. RVMLC1 is expressed at very low levels in fetal hearts and is abundantly expressed in neonatal and adult hearts. The expression of RVMLC1 did not change in hearts of rats with renovascular hypertension. Hybridization to total genomic DNA shows one prominent sequence which is homologous to RVMLC1.

MATERIALS AND METHODS

cDNA library construction and isolation of RVMLC1.

Total RNA was extracted from rat hearts (23). Poly $(A)^+$ RNA was isolated using oligo dT column chromatography and cDNA was synthesized using AMV reverse transcriptase (Life Sciences, St. Petersburg, FL) according to the method of Leonard (24) with the exception that EcoRI-digested and phosphatased λgt10 and lambda packaging extracts were purchased from Stratagene (LaJolla, CA). The gt10 library had approximately 10^6 pfu prior to amplification. Insert sizes ranged from several hundred to approximately 5000 bp. The library was screened using standard methods (25). A 400 bp internal EcoRI fragment of the rat skeletal muscle MLC1-3 clone (the complete LC1-3 cDNA was generously provided by B. Nadal-Ginard) was used as a probe (26). This 400bp EcoRI fragment was electrophoresed into 0.7% SeaPlaque (FMC) low melt agarose and labelled by random primer labelling (Pharmacia) according to suggested protocols. Several positive clones were plaque purified and characterized.

Sequence of RVMLC1

A λgt10 clone with a 1.0 Kb insert was subcloned into ptz19r (US Biochemicals) and overlapping clones for sequencing were generated with the Erase-a-Base (Promega) exonuclease III/ nuclease S1 system (27). The exonuclease treated DNA was ligated

and transformed into E. coli strain DG101 and minilysate DNA was subjected to dideoxy sequencing using Sequenase (US Biochemicals) (28). The sequences were read with an IBI gel reader and analyzed with the Staden programs. Because of the nested deletion approach used to sequence this clone, each base was determined at least two times.

RNA analysis

RNA was prepared from pooled rat hearts at various developmental stages and from the hearts of individual normotensive or hypertensive adult female Wistar rats. Rats were made hypertensive by unilateral renal artery clipping using a silver clip with an internal diameter 0.21-0.23 mm according to previously described methods (29). Blood pressure was determined daily after surgery by the tail cuff method under light ether anesthesia. Animals were allowed to remain hypertensive for 2 days or 2-3 weeks prior to sacrifice. Hearts were dissected from ether anesthetized animals and immediately frozen in liquid nitrogen. The soleus muscle and medial and lateral heads of the gastrocnemius muscle were dissected from the hind limb of a normotensive rat and processed in a similar fashion. RNA was prepared by the method of Chirgwin (23) with the following modifications. The tissue was homogenized with a polytron in 4 M guanidium thiocyanate, 50 mM EDTA, 100 mM Tris, pH 7.4, and 1 M beta-mercaptoethanol, and layered on a 5.7 M CsCl, 50 mM EDTA cushion and centrifuged in a SW41 at 175,000 x g for 24 hours. The RNA pellets were resuspended in 10 mM Tris, pH 8.0, 0.1 mM EDTA, extracted 2 times with a 1:1 mixture of phenol and chloroform and once with chloroform and then precipitated twice with 100% ethanol. 10 ug was electrophoresed in 1.0% Seakem GTG (FMC) agarose gel in MOPS-formaldehyde and transferred by capillary action onto Genescreen (Dupont) or nitrocellulose (Schleicher & Schuell) in 10X SSC (1X SSC is 150 mM NaCl, 15mM sodium citrate, pH 7.0) (25). Probes used were either the random primer labelled RVMLC1 insert or an oligonucleotide complementary to the 3'untranslated region of RVMLC1. The 3' untranslated region oligonucleotide (5'-AGATGGCATGTCCCCAGCTTG-3') was radiolabelled with polynucleotide kinase (Pharmacia). Hybridization conditions were as follows: 5X SSC, 50 mM $NaPO_4$, 1X Denhardts, 10% dextran

sulfate and 50 ug/ml salmon sperm DNA for 14 hours at 65°C with 2 x 10^5 cpm/ml probe. Hybridization washes were at 65°C for one hour in 2X SSC, 0.2% SDS. Hybridizations with the oligonucleotide probe were washed in 5X SSC, 0.2% SDS at 25°C followed by a 5-10 minute wash at 65°C.

Genomic DNA analysis.

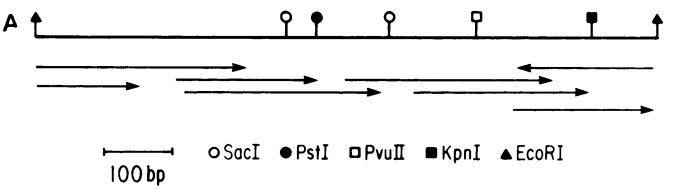
One rat liver was homogenized in 20 mls of 0.5 M EDTA, pH 8.0, 0.5% Sarkosyl and total DNA was prepared according to Maniatis (25). 30 ug of DNA was digested and electrophoresed on a 0.7% agarose gel in Tris Acetate according to the method of Southern (30).

RESULTS AND DISCUSSION

A full length cDNA for rat ventricular MLC1.

A rat cardiac λgt10 cDNA library was constructed from poly $(A)^+$ RNA prepared from an entire rat heart. This library was screened with an internal portion of the rat skeletal MLC1-3 cDNA, pLC84 (26). The rat skeletal MLC1-3 probe that was used to screen the library starts at amino acid 50 and terminates 6 bp beyond the termination codon. This probe was selected because it is homologous to the most conserved portion of the MLC sequences and does not contain the repetitive element present in the 3' untranslated region (3'UTR) of the rat skeletal MLC1-3 cDNA. The rat skeletal MLC1-3 probe hybridized well to the cardiac library and several cDNAs were obtained which appeared to be full length (~1 kB). One was selected, subcloned into pTZ19R and was subjected to dideoxy sequencing. The restriction map, strategy for sequencing, nucleotide sequence, and predicted amino acid sequence are shown in Figure 1.

The RVMLC1 cDNA is 890 bp in length excluding the poly $(A)^+$ tail. The open reading frame spans from nucleotide 46 to nucleotide 646 and the 3'UTR is 240 bp long. The open reading frame encodes a protein that is 200 amino acids in length and of predicted molecular mass 22,150 kDa. MLC1 purified from rat ventricles migrates at ~27 kDa on SDS-PAGE. To investigate the difference between the predicted and observed molecular masses, this cDNA was subcloned into an *E. coli* expression vector that directs the synthesis of a native sequence although without post-



B

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                                                          M  A  P  K  K
ACTCTCTTCTCTGCTTCTTCCCGCCTCTCTGTGATTACAGCTCCAATGGCCCCCAAAAAG
        10        20        30        40        50        60

 P  E  P  K  K  D  D  A  K  T  A  A  P  K  A  A  P  A  P  A
CCAGAGCCCAAGAAGGACGATGCCAAAACCGCTGCCCCCAAAGCAGCTCCGGCCCCTGCG
        70        80        90       100       110       120

 A  A  P  A  A  A  P  E  P  E  R  P  K  E  A  E  F  D  A  S
GCTGCACCTGCGGCTGCACCTGAGCCCGAACGCCCTAAGGAAGCAGAGTTTGATGCCTCC
       130       140       150       160       170       180

 K  I  K  I  E  F  T  P  E  Q  I  E  E  F  K  E  A  F  Q  L
AAGATTAAGATTGAGTTCACGCCTGAACAGATTGAAGAGTTCAAGGAGGCCTTCCAACTG
       190       200       210       220       230       240

 F  D  R  T  P  K  G  E  M  K  I  T  Y  G  Q  C  G  D  V  L
TTTGACCGCACACCTAAGGGCGAGATGAAGATCACGTACGGGCAGTGTGGGGATGTCCTG
       250       260       270       280       290       300

 R  A  L  G  Q  N  P  T  Q  A  E  V  L  R  V  L  G  K  P  K
CGGGCTCTGGGACAGAATCCTACCCAGGCAGAGGTGCTCCGCGTCTTGGGGAAGCCCAAA
       310       320       330       340       350       360

 Q  E  E  L  N  S  K  M  M  D  F  E  T  F  L  P  M  L  Q  H
CAGGAAGAGCTCAACTCCAAGATGATGGATTTTGAAACGTTCCTGCCCATGCTGCAGCAC
       370       380       390       400       410       420

 I  S  K  N  K  D  T  G  T  Y  E  D  F  V  E  G  L  R  V  F
ATCTCCAAGAACAAAGACACGGGCACGTATGAGGACTTCGTGGAGGGGCTGCGGGTCTTC
       430       440       450       460       470       480

 D  K  E  G  N  G  T  V  M  G  A  E  L  R  H  V  L  A  T  L
GACAAGGAGGGCAACGGAACTGTTATGGGTGCAGAGCTCCGTCATGTGCTGGCCACGCTG
       490       500       510       520       530       540

 G  E  R  L  T  E  D  E  V  E  K  L  M  A  G  Q  E  D  S  N
GGTGAGAGGCTGACAGAAGACGAGGTAGAGAAACTGATGGCTGGTCAAGAAGACTCCAAT
       550       560       570       580       590       600

 G  C  I  N  Y  E  A  F  V  K  H  I  M  A  S  *
GGCTGCATCAACTATGAAGCATTTGTGAAGCACATCATGGCCAGCTGAGCCTCTCAGGAA
       610       620       630       640       650       660

GCCCAGGGCAGGCCAAGCTGGGGACATGCCATCTCCCAACCATAATGCTGACACCATGTC
       670       680       690       700       710       720

TGGAGCTTCGGGAAGGAAAGGAGTGTCCAGACTCCAGCACATGGTGCAGATCACTCCGTG
       730       740       750       760       770       780

TGCGGTGGTCCTCGCTGGCCCCTGCTAGGTATGTGCTTGCCTACCCGTGGTACCCTTTCT
       790       800       810       820       830       840

GTCTCCACCCTGCGGCCTTATGaataaaTGATTTCCTTCCTTCTGAAAAAAAAAAAAAAA
       850       860       870       880       890       900
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Figure 1. A) The restriction map and strategy for sequencing of RVMLC1 are shown. B) The nucleotide sequence and the predicted amino acid sequence of RVMLC1. Underlined are the sequences complementary to the 3'UTR oligonucleotide that was used as a probe for RNA analysis. Shown in lower case letters is the poly-adenylation site. Number below refer to nucleotide positions.

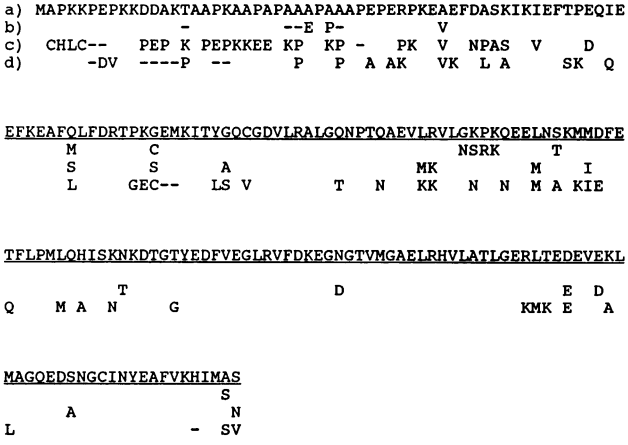

Figure 2. Amino acid sequence comparison of RVMLC1 to other MLC1 sequences. a) RVMLC1; b) human ventricular MLC1 (31); c) chicken cardiac MLC1 (32); d) rat fast skeletal muscle MLC1 (16). Non-identical amino acid residues are shown. A blank space indicates identity. A dash indicates missing residues. The homologous region of RVMLC1 to the portion of the fast skeletal MLC1-3 cDNA that was used as a probe to screen the library is shown underlined.

translational modifications. Like the MLC1 expressed in rat ventricles, the E. coli expressed RVMLC1 protein migrates at 27 kDa on SDS-PAGE (data not shown). Thus, there is a discrepancy between the predicted molecular mass of this protein and its migration on SDS-PAGE.

The predicted amino acid sequence for RVMLC1 shows greatest homology to the human ventricular MLC1 sequence, 91% (31). RVMLC1 shows 80% homology to the chicken cardiac MLC1 sequence (32) and 70% homology to the rat skeletal MLC1-3 sequence (16). The fact that the homology is greater to the chicken cardiac sequence than to the rat skeletal MLC1 sequence suggests that the cardiac specific sequences have been selected for and may have functional significance. Figure 2 highlights the amino acids which are different among the sequences discussed. The greatest homology is seen over the carboxy half of the MLC1 sequence which includes the domains which have been implicated in divalent cation binding (33). The region of the rat skeletal MLC1-3 sequence that was used as a probe to obtain the rat cardiac MLC1

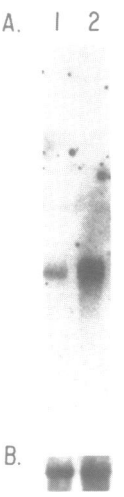

Figure 3. Hybridization of RVMLC1 to atrial and ventricular RNA. A, lane 1, atrial RNA and lane 2, ventricular RNA. A gene-specific 3'UTR oligonucleotide (see text) was hybridized to total RNA prepared from adult rat hearts. B, shows the same lanes hybridized to a probe for 18S ribosomal RNA to indicate loading (36).

sequence is also included in the more highly conserved regions and is underlined in Figure 2.

The nucleotide sequence of 3'UTRs of the rat skeletal MLC1-3 and RVMLC1 sequences are similar in size, but do not show any sequence homology. The rat skeletal MLC1-3 sequence has a repetitive element in its 3'UTR. RVMLC1 does not show any homology to this repetitive sequence. This suggests that the cardiac and skeletal MLC1 genes diverged before the likely transposition of the repeated DNA sequence to the rat skeletal MLC1-3 locus. Because these two genes do not share any sequence homology in the 3'UTRs, the 3'UTR of MLCs are gene specific. Most of the 3'UTRs of MHC genes have been shown to be gene specific and conserved through evolution (5). Because the ventricular MLC1 3'UTR sequence is not available from any other species, we cannot comment on the conservation of the MLC 3'UTRs between organisms. A 3'UTR oligonucleotide was synthesized to use as a gene specific probe. It is the inverse complement of nucleotides 675 to 695 and is underlined in Figure 1B.

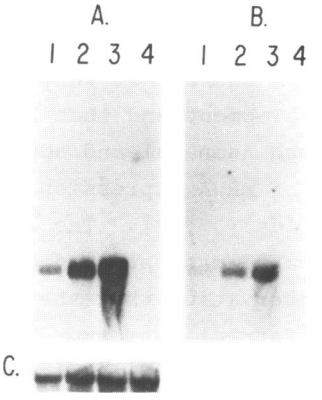

Figure 4. Developmental expression of RVMLC1. Total RNA was prepared as described from day 16 gestation fetal hearts, 5 day neonatal hearts, adult ventricles and adult liver, and was hybridized to either the complete cDNA insert of RVMLC1 or the 3'UTR oligonucleotide. A) RVMLC1 cDNA hybridized to lane 1, fetal heart, lane 2, neonatal heart, lane 3, adult ventricle, and lane 4, adult liver RNA. B) The 3'UTR oligonucleotide hybridized to the same lanes. C) Hybridization of the same lanes to the ribosomal 18S probe to compare loading (36).

RVMLC1 is expressed primarily in ventricles.

The abundance of the RVMLC1 sequence in the cardiac library and the sequence comparisons shown in Figure 2 suggest that the clone we obtained is the ventricular MLC1 isoform. To confirm this, we studied its expression in different tissues. Total RNA was prepared from the atria and ventricles of adult rats and was hybridized to the 3'UTR oligonucleotide. Figure 3A shows the results of hybridization to the 3'UTR oligonucleotide. RVMLC1 is expressed to a much greater extent in ventricles that in atria, although low levels of expression are also seen in the atrial tissue. Some of this hybridization may be due to contamination of the atrial tissue with ventricular tissue or may reflect the microheterogeneity of myosin distribution described in the peri-valvular region of the heart (34,35). Hybridization to a ribosomal 18S RNA probe (36) is shown in the lower panel to demonstrate any variability in loading.

Developmental expression of RVMLC1.

To study the developmental regulation of expression of the

RVMLC1, total RNA was prepared from midgestation fetal, 5 day neonatal and adult hearts and was hybridized to the insert of the RVMLC1 cDNA. Figure 4A shows that very little RVMLC1 is expressed early in development and that RVMLC1 is expressed at very high levels in both neonatal and adult hearts. This developmental pattern of mRNA expression is consistent with the presence the MLC1 peptide (37,38). The low level expression of RVMLC1 mRNA in fetal cardiac tissue and adult atrial tissue is consistent with the observation that the fetal MLC1 isoform is identical to the adult atrial isoform. Figure 4 shows that some RVMLC1 is expressed in fetal cardiac tissue. The 3'UTR oligonucleotide showed a similar pattern of hybridization as the complete cDNA (Figure 4B) insert indicating that the complete insert is gene specific under the conditions used. Figure 4C shows hybridization to the 18S ribosomal probe (36).

Expression of RVMLC1 in slow skeletal muscle.

The ventricular MLC1 is also present in slow but not fast skeletal muscle. To confirm that RVMLC1 mRNA shows a similar pattern of expression, the RVMLC1 cDNA was hybridized to total RNA from the soleus muscle and the medial and lateral heads of the gastrocnemius muscle. The results are shown in Figure 5A. Lanes 1 and 3 show the expression in the soleus and the lateral head of the gastrocnemius. Both of these lanes represent slow skeletal muscle. Lane 2 shows the hybridization to the medial head of the gastrocnemius, a fast skeletal muscle. Weak cross-hybridization to the fast skeletal muscle MLC1 and MLC3 is seen in lane 2. A portion of the MLC1-3 cDNA was used as the probe to isolate RVMLC1. Figure 5B shows the hybridization to the 18S ribosomal RNA to confirm that the lanes were equally loaded.

Expression of RVMLC1 in hypertension.

To study the expression of RVMLC1 in hypertension, total cardiac RNA was prepared from rats with renovascular hypertension. Fig 6A, lanes 1-3, shows that RVMLC1 expression appears to be unchanged in both early (lanes 1-2) and late (lane 3) stage hypertensive animals. To control for variable loading in the lanes, the filter was hybridized to 8B, a housekeeping gene which does not change expression under a variety of conditions (39) (Figure 6B). Densitometric analysis showed that RVMLC1 varied in

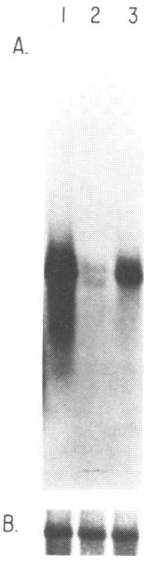

Figure 5. Expression of RVMLC1 in slow and fast skeletal muscle. A) Hybridization of RVMLC1 cDNA to skeletal muscle RNA. Lane 1 contains soleus muscle RNA, slow skeletal muscle. Lane 2 contains the medial head of the gastrocnemius, fast skeletal muscle. Lane 3 contains the lateral head of the gastrocnemius, slow skeletal muscle. RVMLC1 is abundant in the two slow skeletal samples and only weakly cross-hybridizes to the fast skeletal muscle MLC1-3 mRNA. B) Hybridization to 18S RNA to control for loading variability.

parallel with 8B indicating that any variation in the intensity of the signal reflects variability in loading. Duplicate slot blots containing these same RNA samples were analyzed by hybridization to RVMLC1 cDNA, the 8B sequence and oligonucleotides specific for alpha and beta MHC. Figure 6C shows the results of these hybridizations. THe decrease in alpha MHC expression and the increase in beta MHC expression are apparent. RVMLC1 does not change its level of expression. Thus, while it has been shown previously that alpha and beta MHC isoforms change their ratios in hypertrophic states (40), it appears that RVMLC1 does not change its level of expression during hypertension.

Hybridization of RVMLC1 to genomic DNA.

To study the genomic organization of RVMLC1, total DNA was prepared from a rat liver, digested with several enzymes,

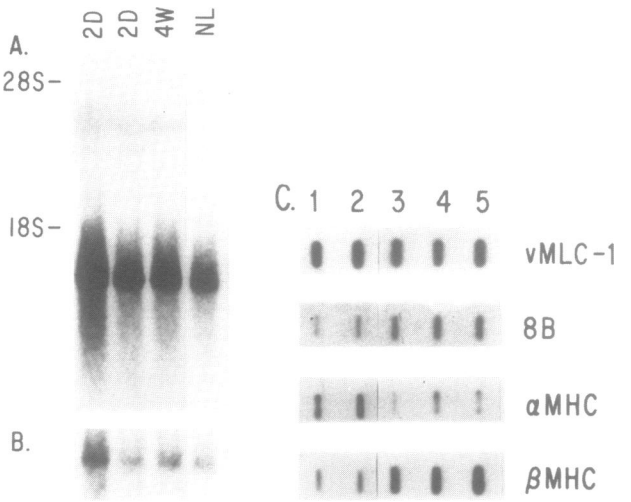

Figure 6. Expression of RVMLC1 in hypertensive animals. Total RNA was prepared from cardiac tissue. A) Hybridization to RVMLC1. Lanes 1 and 2 show hybridization of RVMLC1 to 2 day hypertensive animals, and lane 3 shows hybridization to an animal that had remained hypertensive for 2 weeks. Lane 4 shows hybridization to a clipped, normotensive control. B) Hybridization to 8B, a housekeeping gene, to show loading variability (39). C) Slot blot analysis of the same RNA samples hybridized to RVMLC1, 8B, and oligonucleotides specific for alpha MHC or beta MHC. The oligonucleotides for MHC analysis are complementary to the 3'UTRs. Slots 1 and 2 represent normotensive clipped, controls, and slots 3, 4 and 5 represent clipped hypertensive animals.

electrophoresed and transferred to nitrocellulose. Hybridization to the total insert of RVMLC1 revealed 1 or 2 prominent bands depending on the restriction enzyme used (Figure 7). On longer exposure, weak hybridization to other bands was seen. Because there is one major hybridizing sequence to this cDNA, this suggests that there is only one gene encoding RVMLC1. The much more weakly hybridizing sequences, seen on longer exposure, probably represent other MLC1 genes of lower homology, notably the gene encoding the skeletal MLC1-3 gene which was used as a probe to obtain RVMLC1.

Our initial cardiac library screening resulted in twelve clones with homology to skeletal muscle MLC1-3. Hybridization to gene specific oligonucleotides indicated that 11 of 12 were

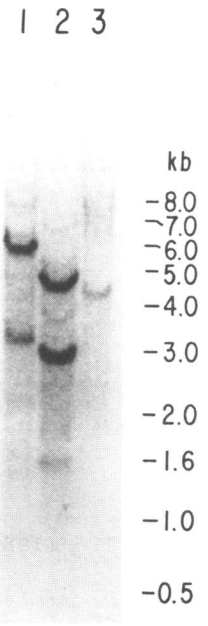

Figure 7. Hybridization of RVMLC1 to total genomic DNA. Liver DNA was digested with several different enzymes, electrophoresed, transferred and hybridized to the total insert of RVMLC1 as described. Lane 1, BamHI, lane 2, PstI, and lane 3, XbaI.

RVMLC1 sequences. One of the twelve appears to represent a second form which is not identical to RVMLC1 over the carboxy half as determined by sequence analysis and developmental expression patterns (data not shown). We are currently investigating this MLC1 sequence. This MLC1 most likely represents the atrial MLC1 because of its low abundance in the library.

In summary, we have isolated a full length cDNA for the predominant MLC1 from rat hearts. This MLC1 is abundantly expressed in rat ventricular tissue and shows 91% homology to the human ventricular MLC1 isoform. This MLC1 isoform was found to be developmentally regulated. RVMLC1 was found to be abundantly expressed in neonatal and adult hearts. Expression in fetal cardiac tissue was low but detectable. RVMLC1 is expressed in slow but not fast skeletal muscle. The expression of RVMLC1 did not change in the hearts of rats with renovascular hyper-tension.

The genomic pattern of hybridization to the RVMLC1 cDNA suggests that one gene encodes this isoform.

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