Cloning and Characterization of Mammalian Myosin Regulatory Light Chain (RLC) cDNA: The RLC Gene Is Expressed in Smooth, Sarcomeric, and Nonmuscle Tissues

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Abstract. The 20-kD regulatory light chain (RLC) plays a central role in the regulation of smooth muscle contraction. Little is known about the structure or expression of smooth muscle myosin light chain (MLC) genes. A cDNA library was constructed in the expression vector, λgt-11, with mRNA derived from cultured rat aortic smooth muscle cells. Using antibody generated against tracheal smooth muscle myosin, three cDNA clones encoding a RLC were isolated, one of which, SmRLC-2, represents a full-length transcript of the RLC mRNA. The derived amino acid sequence shows 94.2% homology with the chicken gizzard RLC, and 70 and 52% homology with the rat skeletal and cardiac muscle MLC-2 proteins, respectively. Thus, the gene encoding the putative smooth muscle RLC appears to have originated by duplication of the same ancestor that gave rise to the sarcomeric MLC-2 genes. Contrary to the stringent tissue-specific expression of sarcomeric MLC-2 genes, RNA blot hybridization and S1 nuclease mapping demonstrates that the putative smooth muscle RLC gene is expressed in smooth, sarcomeric, and nonmuscle tissues at significant levels. Primer extension analysis suggests that the same promoter region is used in these different tissues. Thus the putative smooth muscle RLC gene appears to be a gene that is constitutively expressed in a large variety of cells and has a differentiated function in smooth muscle.

THE contractile elements of smooth muscle are arranged in a unique pattern of filaments dispersed throughout the cytoplasm (for a review, see 10). Myosin is a major component of these filaments and is comprised of two identical 200-kD heavy chains and two sets of light chains of 20 and 17 kD (for a review, see 22). The 20 kD, or regulatory light chain (RLC),[1] plays a central role in the regulation of smooth muscle contraction (for a review, see 1, 23). The RLC is phosphorylated on a specific serine (serine 19) (31) by the enzyme myosin light chain (MLC) kinase in the presence of calcium and calmodulin. This phosphorylation is responsible for the increase in actin-activated Mg ATPase activity of smooth muscle myosin (9, 49, 51). A strong correlation has been found between RLC phosphorylation and the initiation of muscle contraction in a number of smooth muscle tissues (3, 16, 24, 50). RLC phosphorylation causes the unfolding of smooth and nonmuscle myosin and regulates the assembly of these myosins into filaments in vitro (14, 48, 53). RLC phosphorylation also appears to affect the interaction of actin and myosin by regulating the movement of smooth muscle myosin on actin filaments (47). Recently, it has been shown that MLC kinase also phosphorylates the RLC at threonine 18 (25). This second phosphorylation also increases the actin-activated ATPase activity. In addition, the RLC is phosphorylated at other sites by a number of enzymes including epidermal growth factor (21) and protein kinase C (38). The consequences of these phosphorylations are not well understood.

Both skeletal and cardiac muscle also contain phosphorylatable RLCs. These RLCs have amino acid compositions flanking the phosphorylated serine residue similar to that of the smooth muscle RLC (42). However, phosphorylation of the serine does not result in the dramatic changes in ATPase activity and does not appear to be necessary for skeletal or cardiac muscle contraction (see 1). Thus, the RLC appears to play a unique role in smooth muscle. It would be expected that this role is at least partially determined by the primary structure of the smooth muscle RLC. In addition, the regulation of smooth muscle RLC gene(s) expression might play an important role in regulating smooth muscle contraction.

There is limited information concerning the primary structure of the smooth muscle RLC. Maita et al. reported the sequence of the chicken gizzard RLC derived from sequencing tryptic peptides (31). Pearson et al. (42) assigned a different arrangement of tryptic peptides comprising the first 20 amino acids of the amino terminus of the chicken gizzard RLC. Although RLC genes have been isolated for both

1. *Abbreviations used in this paper*: MLC, myosin light chain; RASM, rat aortic smooth muscle and; RLC, regulatory light chain.

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skeletal (39) and cardiac (28, 59) muscle, the smooth muscle RLC gene(s) has yet to be characterized.

To better understand the structure of the smooth muscle RLC, to allow for comparisons between skeletal, cardiac, and smooth muscle genes, and to elucidate the molecular mechanisms responsible for smooth muscle RLC gene expression, cDNA clones encoding a RLC were isolated from a λgt-11 rat aortic smooth muscle (RASM) library. We report the characterization of these clones and compare the derived amino acid sequence of the coding region with those reported for rat cardiac and skeletal muscle. In addition, RNA blot hybridization, S1 nuclease mapping, and primer extension analyses are presented and demonstrate that the putative smooth muscle RLC gene is expressed in smooth, cardiac, skeletal, and nonmuscle tissues.

Materials and Methods

Reagents

Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA), New England Nuclear (Boston, MA), and Bethesda Research Laboratories (Gaithersburg, MD). Double-stranded M13 sequencing vector M13mp18 was obtained from New England Nuclear and the M13 sequencing kit from New England Biolabs. Radioisotopes were purchased from Amersham Corp. (Arlington Heights, IL).

Rat Aortic Smooth Muscle (RASM) Cell Culture

Cultures of vascular smooth muscle cells isolated from rat thoracic aorta by enzymatic dissociation (8) were provided by Drs. R. Wayne Alexander and Michael A. Gimbrone, Jr. Cells were grown in DME supplemented with 10% heat-inactivated calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and serially passaged upon reaching confluence. Cells in subculture passage 11 were used to generate RNA for the cDNA library and from passages 7–22 for other experiments.

Isolation of RNA

Total RNA was extracted from rat tissues by the hot phenol procedure (52). Total cytoplasmic RNA was isolated from confluent cultures of RASM, L_6E_9 myotubes and myoblasts, or rat 3T3 fibroblasts as described (4). To eliminate DNA, the RNA was precipitated once with guanidine hydrochloride (13) and ethanol. Poly(A^+) RNA was purified from RASM RNA by affinity chromatography on oligo(dT)-cellulose (2). This fraction represented ~1% of total cytoplasmic RNA.

Antiserum Preparation

The IgG fraction derived from rabbit antiserum made against bovine tracheal smooth muscle myosin was a generous gift of Dr. Robert Adelstein. Before use, the IgG was adsorbed with λgt-11-infected *Escherichia coli* Y1090 lysates on nitrocellulose filters in order to remove antibodies to both *E. coli* and λgt-11. A dilution corresponding to 1:1000 of the original IgG fraction was used for subsequent screenings.

Construction of a cDNA Library in γgt-11

Double-stranded cDNA was synthesized using 5 μg of RASM poly(A^+) RNA as template and 2.4 μg of oligo-dT(12–18) as primer following the procedures of Okayama and Berg (40). The cDNA was methylated using the procedure of Maniatis et al. (33). An equal mass of Eco RI linkers were then ligated to the cDNA using T4 DNA ligase (0.25 U/ng cDNA) and the double-stranded DNA was digested with Eco RI to create cohesive ends. The cDNA was separated from excess Eco RI linker monomers by chromatography on Sepharose CL4B. λgt-11 DNA was digested with Eco RI and treated with 0.025 U calf intestine alkaline phosphatase (Boehringer Mannheim Diagnostics, Inc., Houston, TX)/μg DNA for 1 h. cDNA was ligated to λgt-11 arms using the procedure of Dugaiczyk et al. (17). The recombinant DNAs were packaged into phage using packaging extracts from Amersham Corp. The library contained 2.2 × 10^6 members with 33% containing inserts. The library was subsequently amplified at 42°C in *E. coli* strain Y1088 (32).

Screening of the cDNA Library

The library was screened with anti-myosin antibody and ^{125}I-labeled protein A using procedures described by Young and Davis (61, 62). A single positive clone (SmRLC-1) was purified to homogeneity by repeated plating. An isolated plaque was picked, amplified, and used for large scale preparation of phage (33, 60). The cDNA insert was isolated from the λgt-11 clone by digestion with Eco RI endonuclease followed by electrophoresis on an 0.8% agarose gel. The insert was then subcloned into M13mp18 and single stranded DNA was prepared (35). The complementary strand was then labeled using the M13 universal primer, [α-^{32}P] dATP and dCTP, and DNA polymerase 1 (Klenow fragment). The uniformly labeled insert was then excised with Eco RI endonuclease, separated on a 5% acrylamide gel, and electroeluted. Using this labeled fragment, the cDNA library was rescreened by the colony filter hybridization procedure of Benton and Davis (5). Filters were prehybridized in 5 × SSC (0.15 M NaCl, 0.015 M sodium citrate), 0.025 M sodium phosphate, pH 6.5, 0.1% SDS, 50 μg/ml calf thymus DNA, 10× Denhardt's solution (15), and 25% formamide at 42°C for 4 h. Hybridization was overnight at 42°C in the same buffer, but containing 25% dextran sulfate and 2× Denhardt's solution. The hybridized filters were washed repeatedly in 1× SSC, 0.1% SDS at 65°C. The filters were air dried and exposed for autoradiography on Kodak X-Omat R film using Picker Max intensifying screens.

Nucleic Acid Sequence Determination

DNA sequence determination was performed by the dideoxy-sequencing method of Sanger (46) using the commercially available universal M13 pentadecamer primer (No. 1211; New England Biolabs, Beverly, MA) to prime second-stranded synthesis. Portions of the sequence were confirmed by the method of Maxam and Gilbert (34).

RNA Blot Hybridization

10 μg of total RNA was size fractionated by electrophoresis on 1% agarose gels in 200 mM 4-morpholinepropanesulfonic acid, pH 7.4, 1 mM EDTA, and 3% formaldehyde. Transfer to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) and hybridization to nick-translated (43) ^{32}P-labeled DNA was as described (55). At the end of hybridization, the filters were washed in 0.1× SSC, 0.1% SDS at 65°C.

S1 Nuclease Mapping

Restriction endonuclease digestions were performed under the conditions recommended by the suppliers.

The Rsa I restriction fragment was prepared from SmRLC-6 as follows. The SmRLC-6 insert was subcloned into M13mp18 and the recombinant phage restricted with Nco I and Pst I. A 372-bp fragment was separated on a 6% acrylamide gel and electroeluted. After ethanol precipitation, the sample was restricted with Rsa I, ethanol precipitated, and then labeled at the 3′ end with [α-^{32}P]cordycepin and terminal transferase (56). The labeled fragment was then purified on an 8% acrylamide gel, strand separated on a 5% acrylamide gel, and the strand complementary to the RNA was purified (34).

The Pst I/Nco I fragment was prepared from SmRLC-2 as follows. The RLC-2 insert was subcloned into M13mp18 and the recombinant phage restricted with Nco I. The Nco I site was labeled on the recessed 3′ end with [α-^{32}P] dATP using the Klenow fragment of *E. coli* DNA polymerase I (32), and then digested with Pst I. The Pst I cleaved in the M13 polylinker to yield a 485-bp fragment. This double-stranded fragment, labeled only on the strand complementary to the RNA, was purified by electrophoresis on a 5% polyacrylamide gel and boiled for 5 min immediately before hybridization with RNA.

S1 nuclease mapping was carried out with 25 μg of RNA using a modification of the Berk and Sharp technique (6) as previously described (30). Samples were analyzed on 5 (for experiments using the Pst I/Nco I fragment) or 8% (Rsa I fragment) polyacrylamide gels.

Oligonucleotide Synthesis and Primer Extension

21-mer and 39-mer single-stranded DNA oligonucleotides (Fig. 1 *B*, underlined regions) were synthesized using the DNA synthesizer according to the protocols recommended by the supplier (model 8600; Biosearch, San

Rafael, CA). The oligomers were purified on a 20% polyacrylamide gel. The primer extension was done as described (7) except that annealing was performed using 5 ng of end-labeled primer and 30 μg of RNA in 50 mM NaCl, 34 mM Tris-HCl, pH 8.3, 6 mM $MgCl_2$, and 5 mM dithiothreitol at 65°C for 15 min and 42°C for 15 min. Extension reactions were performed using 2.5 mM dGTP, dTTP, dCTP, and dATP, and 28 U of avian myeloblastosis virus reverse transcriptase for 1 h at 42°C. Extended products were analyzed on 6% polyacrylamide-urea gels.

Results

Isolation and Characterization of RASM RLC cDNA Clones

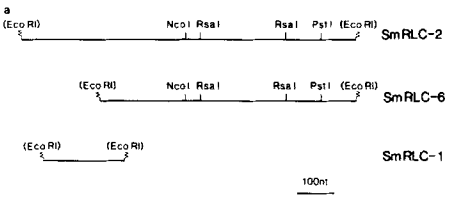
To isolate cDNA clones encoding the smooth muscle myosin RLC, a library was constructed in λgt-11 from RASM poly-(A^+) RNA. 5×10^5 recombinants were screened using IgG directed against bovine tracheal myosin. A single positive clone was purified after three rounds of repeat plating. This clone, SmRLC-1, proved to have an insert of 234 bp. After being excised from λgt-11 and subcloned into the Eco RI site of M13mp18, the insert was sequenced and identified as a RLC (Fig. 1).[2]

The SmRLC-1 insert was labeled with ^{32}P and then used to rescreen the RASM library. 10 additional cDNA clones were detected by positive hybridization to the SmRLC-1 insert. Seven clones contained inserts of 927 bp (SmRLC-2, 3, 5, 7, 9, 10), two of 680 bp (SmRLC-6,8), and one of 234 bp (SmRLC-4). These inserts were also excised from λgt-11 and subcloned into M13mp18. Restriction endonuclease mapping demonstrated that all clones were derived from the same mRNA and were overlapping (Fig. 1 *a*). By sequencing the inserts from the overlapping clones SmRLC-1, SmRLC-2, and SmRLC-6, the complete nucleotide sequence for the 927-bp insert was obtained. Fig. 1 *b* shows the nucleotide and derived amino acid sequence for the putative smooth muscle RLC cDNA. The cDNA contains a 69-nucleotide 5′ untranslated region, a 516-nucleotide (172 amino acids) coding region, and a 342-nucleotide 3′-untranslated region ending in a short 17-nucleotide poly-A tail.

Fig. 1 *c* shows a comparison of the derived amino acid sequence for the RASM RLC with published sequences derived from rat skeletal (39) and cardiac (28) muscle MLC-2 cDNA's and from direct sequencing of tryptic peptides isolated from chicken gizzard RLC (31, 42). It can be seen that the RLCs expressed in smooth muscle are highly conserved between species. From amino acid 18 to 171, there are only nine differences between the chicken gizzard and rat aortic RLC. Of these, six are conservative substitutions. Two previous reports (31, 42) have presented differing sequences for the first 17 amino acids of the chicken gizzard RLC. The sequence derived from the rat RLC cDNA is generally in agreement with the sequence published by Pearson et al. (42), showing one amino acid substitution in the rat. However, by rearranging the tryptic peptides designated by Maita et al. (31) as T3-T7, all three sequences can be shown to be in good agreement. It is this rearranged chicken gizzard sequence that is shown in Fig. 1 *c*.

As demonstrated in Fig. 1 *c*, there are a number of areas that are highly conserved among the three rat RLCs. These same regions are also conserved among the chicken RLC

2. This sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00622.



b

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GCG GCG GCA AAG CTT CGC AGA GAC GCT CAC TCT TGG TTC TCG CGG CTG    48

AGC AGG GAT TTA ACC GCC ACC ATG TCG AGC AAA AGA GCG AAG ACC AAG    96
                               met ser ser lys arg ala lys thr lys

ACC ACC AAG AAG CGC CCT CAG CGC GCA ACG TCC AAC GTG TTC GCC ATG   144
thr thr lys lys arg pro gln arg ala thr ser asn val phe ala met

TTT GAC CAG TCC CAG ATC CAG GAG TTC AAA GAG GCC TTC AAC ATG ATC   192
phe asp gln ser gln ile gln glu phe lys glu ala phe asn met ile

GAC CAG AAC CGG GAC GGC TTC ATC GAC AAG GAG GAC CTG CAC GAT ATG   240
asp gln asn arg asp gly phe ile asp lys glu asp leu his asp met

CTG GCT TCA ATG GGA AAA AAT CCA ACT GAT GAA TAC CTG GAC GCC ATG   288
leu ala ser met gly lys asn pro thr asp glu tyr leu asp ala met

ATG AAT GAG GCC CCG GGC CCC ATC AAT TTC ACC ATG TTC CTC ACC ATG   336
met asn glu ala pro gly pro ile asn phe thr met phe leu thr met

TTT GGA GAA AAG CTG AAC GGC ACC GAC CCT GAG GAC GTC ATC AGA AAT   384
phe gly glu lys leu asn gly thr asp pro glu asp val ile arg asn

GCC TTC GCT TGC TTC GAT GAG GAA GCA ATC GGC ACC ATC CAG GAG GAT   432
ala phe ala cys phe asp glu glu ala ile gly thr ile gln glu asp

TAC CTG AGG GAG CTG CTC ACC ACC ATG GGC GAC CGC TTC ACA GAT GAG   480
tyr leu arg glu leu leu thr thr met gly asp arg phe thr asp glu

GAA GTG GAT GAG CTG TAC AGG GAG GCC CCC ATC GAC AAA AAG GGG AAT   528
glu val asp glu leu tyr arg glu ala pro ile asp lys lys gly asn

TTC AAC TAC ATC GAG TTC ACG CGC ATC CTC AAG CAC GGA GCG AAA GAC   576
phe asn tyr ile glu phe thr arg ile leu lys his gly ala lys asp

AAA GAT GAC TGA AGA GCT GTG GCT TCC AGC CAA ATG TCC CTG TTG CCA   624
lys asp asp  .

TTG GGT ATT TCT GAG ATT TTC CTC CTG GAG CGG TCG GCT GCC CTT GCT   672

TTT CTG CCT TTT GCT TCC CTT GTT TTG TAT TTA TTC TCA GCC ACT TTG   720

GGC CAC GTG TAC CTT CAT CAT CAG ACT GGA AAC GGG ACT TTC TGT CAT   768

TGT TCG ATG AGA ACG TAA GGT AAT TTA ACT TAC AGA CAG TCT TGT CCC   816

TTG TAA TAA CTG CAG CCA CAG AGT CAG TAT ATT TTT TCA GAG AAA GTT   864

ATC CAC TCA ATT TTT TCT GAA TGA TAA TTA AAC TTT CTG ATA AAA TAA   912

AAA AAA AAA AAA AAA                                              927
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c

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RASM    SSKRAKTKTTKKRPQ*RATSNVFAMFDQSQIQEFKEAFNMIDQNRDGFIDKEDLHDMLASM   60
CGSM    ------A--------*------------------------------------------------
RCM     *****SP-KA---LE*GGS----S--E-T---------TIM----A-----N--R-TF-AL
RSM     *****AP-KA-R-AAAEGS----S----T---------TV-------I------R-TF-A-

RASM    GK*NPTDEYLDAMMNEAPGPINFTMFLTMFGEKLNGTDPEDVIRNAFACFDEEAIGTIQED  120
CGSM    --*-------EG--S-------------------------------------------S-F-H--
RCM     -RV-VKN-EI-E-IK---------V-----R---K-AG--ETLL---KV-APRRRI-GA*-
RSM     -RL-VKN-E-----K--S------V---------K-A------TG--KVL-P-GK---KKQ

RASM    YLRELLTTMGDRFTDEEVDELYREAPIDKKGNFNYIEFTRILKHGAKDKDD             171
CGSM    H------------------M-------------V---------------
RCM     CVQ-M---QAE--SK--T-QMFAAF-PNVT--LD-KNLVH-IT--EEKD**
RSM     F-E-----QC---SQ--IKNMWAAF-P-VG--VD-KNICYVIT--DAKDQE
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Figure 1. Partial restriction maps (*a*) of cDNA clones SmRLC-1, SmRLC-2, and SmRLC-6. The restriction sites in parenthesis represent the linkers used for cloning of the cDNA into the λgt-11 vector. Nucleotide and derived amino acid sequences (*b*) of SmRLC-2. The sequences are written 5′ to 3′ (NH_2- to COOH-terminal). Underlined areas are those that correspond to the 39-mer and 21-mer oligonucleotides synthesized for primer extension analysis (see Fig. 4). Amino acid sequence comparisons (*c*) among putative rat aortic smooth muscle RLC (RASM, this report), chicken gizzard smooth muscle RLC (CGSM, reference 31), rat cardiac muscle MLC-2 (RCM, reference 28), and rat skeletal muscle MLC-2 (RSM, reference 39). The tryptic peptides designated as T_3-T_7 by Maita et al. (31), representing amino acids 5–16, are rearranged to agree with the amino-terminal sequence of Pearson et al. (42). Numbering is based upon the RASM sequence, with the initiating methionine absent. (−) Represents amino acids identical to that of the RASM RLC; (*) is used to denote positions where amino acids are not present.

proteins. Although there are large differences at both the amino- and carboxy-terminal ends, between serine 19 and glutamine 137 there is 70 and 52% homology between the amino acid sequence of the smooth muscle-derived RLC sequence and those of the rat skeletal and cardiac muscle MLC-2, respectively. This is also seen on the nucleotide level, where the homologies are 71 and 65%. As expected, the serine phosphorylated by MLC kinase (serine 19) is found in all three rat RLC's. However, threonine 18, which is also phosphorylated by myosin light chain kinase in chicken gizzard (25), is only present in the RLC isolated from RASM.

The Gene Encoding the Putative Smooth Muscle RLC Is Expressed in Smooth, Cardiac, and Skeletal Muscle, and in Nonmuscle Tissues

The contractile protein genes of skeletal and cardiac muscle are expressed in a developmental and tissue-specific manner (see reference 18). Little is known about the expression of smooth muscle contractile protein genes. To examine the expression of the gene encoding the RLC mRNA represented by the cDNA clones described here, RNA blot analyses were undertaken using total RNA isolated from a variety of rat skeletal, cardiac, smooth, and nonmuscle tissues, as well as total cytoplasmic RNA from RASM, L_6E_9 (36), and 3T3 fibroblast cells. Fig. 2 depicts two typical experiments (*top* and *bottom* panels) in which the above RNAs were hybridized with the ^{32}P-labeled 927-bp cDNA insert from SmRLC-2.

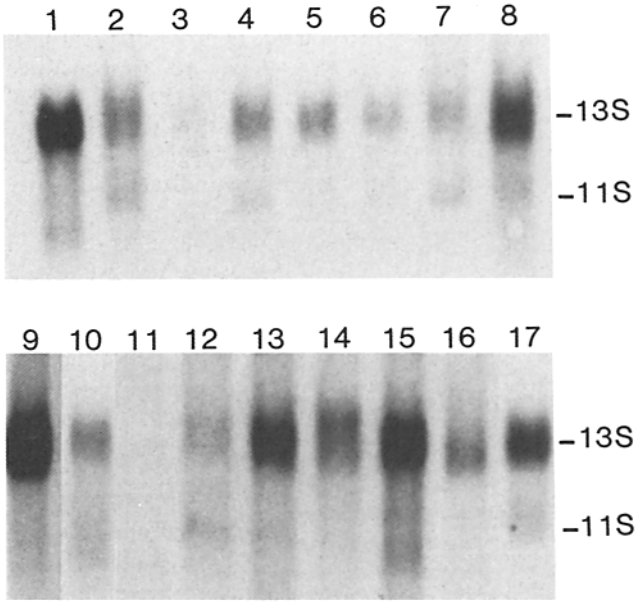

Figure 2. Pattern of expression of the gene encoding the RLC from RASM. RNA blot hybridizations using ^{32}P-labeled SmRLC-2 insert were done as described in Materials and Methods using 15 μg of RNA from the following rat tissues: (lane *1*) postconfluent RASM cultures; (*2*) fetal cardiac muscle; (*3*) adult atrium; (*4*) adult ventricle; (*5*) fetal skeletal muscle; (*6*) newborn skeletal muscle; (*7*) adult skeletal muscle (leg); (*8*) adult skeletal muscle (soleus); (*9*) 3T3 fibroblasts; (*10*) brain; (*11*) liver; (*12*) esophagus; (*13*) placenta; (*14*) uterus; (*15*) postconfluent RASM cultures; (*16*) lung; and (*17*) L_6E_9 myoblasts. The relative mobilities of the major hybridizing species were calculated based on the mobilities of 28 and 18 S ribosomal RNA and are indicated.

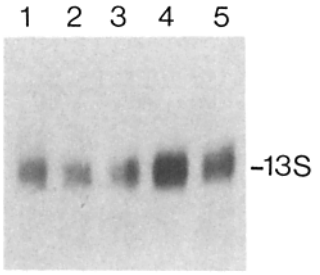

Figure 3. The effect of RASM cell density on the expression of the gene encoding the RLC from RASM. RNA blot hybridizations using ^{32}P-labeled SmRLC-2 insert were done as described in Materials and Methods using 15 μg of cytoplasmic RNA from cultures of RASM (passage 10) at (lane *1*) 50; (*2*) 75; (*3*) and 100% confluence; and (*4*) 2-d postconfluence. Lane *5* contained 15 μg of total RNA from rat aortic tissue.

Filters were washed at high stringency (0.1× SSC, 0.1% SDS at 65°C). A hybridizing species migrating at ~13 S can be observed in all tissues tested with the exception of rat liver. This species has the expected size for the putative smooth muscle RLC. A second and weaker hybridizing species migrating at ~11 S can be identified in many RNAs. Although its identity is not known, its size is appropriate for either skeletal or cardiac muscle MLC-2 or the 17-kd smooth muscle MLC. It should also be noted that the 11 S species present in smooth muscle is slightly smaller than that seen in skeletal or cardiac muscle, suggesting that they do not represent the same RNA. The level of expression of the 13 S species varies markedly in different tissues and is as high or higher in some skeletal and nonmuscle tissues as in smooth muscle. Of particular interest is the high level of expression in the 3T3 fibroblast and L_6E_9 cell lines, neither of which are smooth muscle cells. Thus, the presence of high levels of the 13 S species is not dependent upon the existence of the smooth muscle contractile phenotype.

It has been recently demonstrated that nonmuscle isoform(s) of the myosin heavy chain (26, 44) and α-actin (41) predominate in subconfluent or newly confluent cultures of vascular smooth muscle cells, while smooth muscle isoforms are found primarily in postconfluent cultures. Fig. 3 represents a blot hybridization using 10 μg of RNA isolated from RASM at varying cell densities and from intact aorta. Similar levels of hybridization were seen in subconfluent and newly confluent cells and in intact aorta, while postconfluent cells contained much larger amounts of hybridizing RNA. Thus, induction at postconfluency of the RLC mRNA represented by the cDNA clones described here is similar to that described for other smooth muscle contractile proteins. Nevertheless, as shown here for RLC, there is a significant amount of hybridizing RNA present even in subconfluent cultures. There appears to be no significant differences in the amount of hybridizing RNA found in postconfluent cells taken from the 7th through 22nd passages.

The RNA blot and sequence analyses suggest that the RNA represented by the RLC cDNA clones isolated from smooth muscle is indeed coding for the smooth muscle RLC. However, the fact that this mRNA appears to be expressed in a wide variety of muscle and nonmuscle tissues is an unexpected finding. To further examine this phenomenon and to get a better understanding of the nature of the hybridizing species seen on the RNA blots, S1 nuclease mapping was performed using RNA from the same tissues and cells as de-

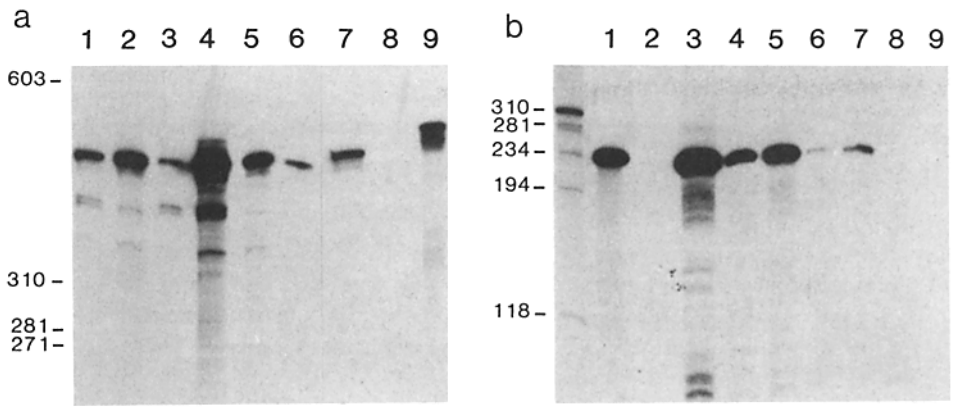

Figure 4. S1 nuclease mapping analysis of RLC cDNA isolated from RASM. 25 μg of RNA and 75 U of S1 nuclease were used per assay. Fragments derived from cDNA clones SmRLC-2 and SmRLC-6 were prepared as described in Materials and Methods. (*a*) 5% polyacrylamide gel of digests using the 5′-end Pst I/Nco I fragment and the following rat RNAs: (lane *1*) adult cardiac muscle; (*2*) neonatal skeletal muscle; (*3*) adult skeletal muscle (leg); (*4*) L_6E_9 myotubes; (*5*) L_6E_9 myoblasts; (*6*) 3T3 fibroblasts; (*7*) 90% confluent RASM cultures; and (*8*) tRNA. The intact Pst I/Nco I probe is shown in lane 9. (*b*) 8% polyacrylamide gel of digests using the 3′-end Rsa I fragment. Molecular weight markers are shown on the extreme left. (Lane *1*) Intact Rsa I probe; (*2*) tRNA; (*3*) L_6E_9 myotubes; (*4*) adult cardiac muscle; (*5*) adult skeletal muscle (leg); (*6*) uterus; (*7*) 90% confluent RASM cultures; (*8*) brain; and (*9*) liver. It should be noted that a signal can be seen with brain RNA when the autoradiogram is exposed for longer periods of time, but none is detected with liver.

scribed above. ^{32}P-labeled probes were constructed from restriction fragments of SmRLC-2 and SmRLC-6 as described in the Materials and Methods section. The Pst I/Nco I fragment (Fig. 4 *a*) was comprised of 30 nucleotides of the M13 polylinker, the entire 5′-untranslated region and the first 386 nucleotides of the coding region. The Rsa I fragment (Fig. 4 *b*) was comprised of the final 89 nucleotides of the coding region and 145 nucleotides of 3′-untranslated cDNA. With the exception of rat liver, all cardiac, skeletal, smooth, and nonmuscle tissues tested contained RNA that fully protected both the 3′- (234 bp) and 5′- (485 bp) end probes. It should be noted that the 5′-end Pst I/Nco I probe is larger than the "fully-protected" species seen in Fig. 4 *a* because it contains a 30-nucleotide stretch of the M13 polylinker which is cleaved by S1 nuclease after hybridization with homologous RNA. The S1 nuclease mapping demonstrates the presence of RNA homologous to the RASM RLC cDNA in cardiac, skeletal, and nonmuscle tissues. This result, together with the RNA blot data, supports the conclusion that the expression of the putative smooth muscle RLC gene(s) is ubiquitous. Aside from the fully protected fragments, partially protected fragments are produced by hybridization of the Pst I/Nco I probe to most of the RNAs tested (Fig. 4 *a*). The identity of these fragments is not known. It is possible that they result from partial S1 nuclease digestion, although this interpretation is unlikely since the same banding pattern is seen at high enzyme concentrations. Whether these additional RNAs represent the products of other MLC genes or are the products of differential splicing has not yet been elucidated.

It may be noted that the cardiac and skeletal muscle tissues produced bands of greater intensity than that seen for RASM in the S1 mapping experiments while the reverse appeared true in the RNA blot hybridizations. This is due in large part to differences in the RNA used for each type of experiment. In particular, the RASM RNA used in the blot hybridizations was isolated from cultures that were postconfluent and thus as noted in Fig. 3 contained large amounts of the RLC RNA while that used for the S1 mapping was isolated from 90% confluent cultures and thus contained smaller amounts. Similarly, different preparations of skeletal and cardiac muscle were used for the blot hybridizations and S1 nuclease analyses.

RLC mRNA's Expressed in Muscle and Nonmuscle Cells Are Transcribed from the Same Promoter Region

The finding that RNA homologous to the RLC cDNA was present in sarcomeric, smooth muscle, and nonmuscle tissues was unexpected. To determine whether this RNA was derived from the same promoter(s) or from different promoters specific to each cell type, primer extension analysis was performed. A 21-mer oligonucleotide was synthesized that corresponded to nucleotides 181–201 of the noncoding strand of SmRLC-2 (Fig. 1 *b*, underlined region). This probe was used for primer extension after hybridization to 25 μg of RASM, L_6E_9, and 3T3 RNA. Elongated products were examined on 6% acrylamide sequencing gels. As shown in Fig. 5, two species of ~194 and 218 nucleotides were seen in all three cultures tested. Similar results were found with all three cultures using a 39-mer corresponding to nucleotides 35–73 (Fig. 1 *b*, underlined region; results not shown). As described in the previous section, the S1 nuclease mapping experiments showed that all three cell types contained RNAs that fully protected the 5′-end cDNA probe. This probe contained the entire 5′-untranslated region and therefore included the entire sequence comprising the smaller (194 nucleotide) primer extension product and all but the last 17 nucleotides of the larger (218 nucleotide) product. While it is possible that there is sequence divergence in those 17 nucleotides and that the identical sizes of the larger extension products among the three cell types tested are coincidental, taken in concert with the results of the S1 nuclease mapping experiments, the most likely explanation for the results of the primer extension analyses are that the same RLC message is produced in all three cell types and that this message derives from the same promoter in RASM, 3T3 fibroblasts, and L_6E_9 myoblasts. The shorter primer extension product likely represents a point of premature termination of the reaction, but the possibility that it represents an additional start site(s) has not been formally excluded. In fact, heterogeneity

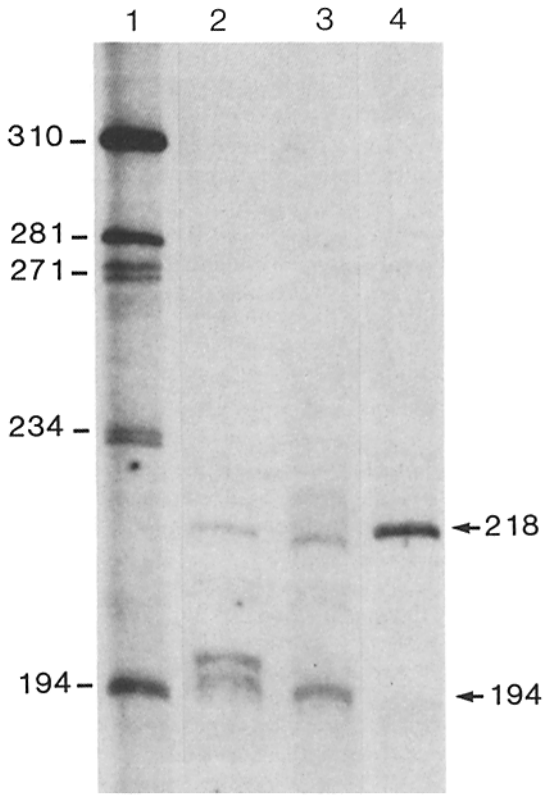

Figure 5. Primer extension analysis of the RLC 5′ cDNA sequences using RNA from sarcomeric, smooth, and nonmuscle cells. A 21-mer oligonucleotide (see Fig. 1 *b* for its location on the RLC cDNA) was synthesized as described in Materials and Methods, hybridized to cytoplasmic RNA from L_6E_9 myotubes (lane *2*), RASM cells (lane *3*), and 3T3 fibroblasts (lane *4*), and extended with reverse transcriptase. Extended products were analyzed on a 6% polyacrylamide-urea gel along with molecular weight markers (lane *1*). Molecular weights of the two initiation sites are noted by arrows and were calculated from their relative positions with respect to the markers.

in the transcription start site is a common feature of housekeeping promoters. The length of the longer primer extension product suggests that most of the 5′-untranslated region is contained within clone SmRLC-2. Since that clone also contains a small poly-A tail, SmRLC-2 represents a virtual full length transcript of the RLC mRNA. Sequencing of the primer extension product was not possible due to the small amount of extended DNA produced. However, analysis of rat genomic clones recently isolated by hybridization with SmRLC-2 cDNA should allow for further elucidation of the 5′ end.

Discussion

We report the isolation of cDNA clones encoding the 20-kD smooth muscle RLC. Clone SmRLC-2 is a near full-length transcript of the RLC mRNA, containing 5′- and 3′-untranslated regions of 69 and 342 nucleotides, respectively. The derived amino acid sequence of the coding region shows striking homology with that of the chicken gizzard RLC, with only 10 substitutions out of 171 amino acids, suggesting that the cDNA encodes a smooth muscle RLC isoform.

Work from several laboratories has demonstrated that there are a number of myosin heavy chain isoforms expressed in cultured smooth muscle cells (26, 29, 44). Nonmuscle myosin heavy chain appears to be the predominant species present in subconfluent and newly confluent cultures, whereas significant amounts of smooth muscle isoform(s) are present only in postconfluent cultures. Similar induction of smooth muscle actin isoforms has been seen in postconfluent RASM cultures (41). Unlike these instances, where multiple isoforms are known to exist in culture, only a single RLC isoform has been detected in RASM (19). One possible explanation is that only a smooth muscle RLC isoform is present in adult RASM and a separate nonmuscle isoform is present in nonmuscle cells. A second explanation is that both smooth and nonmuscle isoforms are present in RASM but are so similar in sequence that they cannot be resolved by gel electrophoresis or immunoprecipitation. A third possibility, which we favor, is that the smooth muscle and nonmuscle RLC's are identical and are encoded either by the same gene or by different genes containing identical coding regions. The RLC encoded by the cDNA clones described in this study has striking sequence homology to the chicken gizzard RLC and thus likely represents a smooth muscle isoform. However, the lack of available nonmuscle RLC sequence prevents us from formally proving this point. Accordingly, we refer to the clone as a "putative" smooth muscle RLC.

The conservation of primary structure between the chicken gizzard RLC and the putative rat smooth muscle RLC (94.2%) is somewhat higher than that reported for species differences among the light chains of cardiac and skeletal muscle. This may reflect the importance of the primary structure in providing the smooth muscle RLC with its unique properties in regulating both actin-activated myosin ATPase activity and smooth muscle contraction. In contrast, there are significant differences between the derived amino acid sequences of the putative rat smooth muscle RLC and that of rat skeletal and cardiac muscle MLC-2. The most striking differences are found at both the amino and carboxy termini. Kemp et al. (27) have reported that the sequence at the amino terminus of the chicken smooth muscle RLC is critical to the activity of the smooth muscle MLC kinase on the RLC. It would thus not be surprising that this area is highly conserved between the rat and chicken smooth muscle RLCs. In contrast, the differences in this region between smooth and sarcomeric muscle RLCs might provide a means by which the light chains can be distinguished by their respective kinases. The role of the carboxy terminus in smooth muscle RLC function is unclear. Here again, the level of conservation between chicken and rat is in distinct contrast to the almost total lack of amino acid sequence homology between the putative rat smooth muscle RLC and the RLCs of rat skeletal and cardiac muscle. This raises the question as to whether the structure of the carboxy terminus is important in providing the smooth muscle RLC with its differentiated functions. Despite the differences between the sarcomeric and smooth muscle RLCs at the carboxy and amino termini, there are a number of areas (amino acids 19–53 and 75–99) where the sequences are highly conserved (>75% homology). This argues strongly that the gene(s) encoding the putative smooth muscle RLC originated by duplication of the same ancestor that gave rise to the MLC-2 genes.

The expression of the putative smooth muscle RLC was

studied using both RNA blot analyses and S1 nuclease mapping. It has been shown that the RNA encoding the putative smooth muscle RLC is present not only in smooth muscle, but in cardiac, skeletal, and nonmuscle cells and tissues as well. This result is unexpected. Studies of adult and embryonic cardiac and skeletal muscle using two-dimensional gel electrophoresis have failed to disclose the presence of smooth muscle RLC protein in chicken sarcomeric tissues at any stage of development (54), although a 20-kD MLC electrophoretically distinct from skeletal muscle MLCs was demonstrated in "presumptive" myoblast cells (11, 12). The presence of smooth muscle RLC protein in rat sarcomeric tissues has not been demonstrated.

One possible explanation for our findings would be the presence of contaminating vascular smooth muscle in the tissues used to prepare the RNA. The amount of such contaminating material is relatively small and unlikely to give a signal of equal or greater strength than seen in tissues derived directly from smooth muscle. More importantly, the lack of a signal in several different preparations of rat liver, a highly vascular organ, strongly argues against contamination from blood vessels as being a source of the smooth muscle RNA in skeletal or cardiac tissues. The lack of hybridizing RNA in rat liver is itself intriguing, as it is the only tissue in which this has been the case. Similar results have been noted using a smooth muscle α-tropomyosin cDNA probe (Wieczorek, D. F., and B. Nadal-Ginard, manuscript in preparation), which hybridizes with all tissues except rat liver. These liver preparations do give appropriate signals when hybridized to nonmuscle probes. The presence of hybridizing RNA in cloned lines such as 3T3 fibroblast and L_6E_9 myoblasts further argues against contaminating smooth muscle elements being responsible for the signals seen in nonsmooth muscle tissues. As noted earlier, a second explanation for the ubiquitous presence of homologous RNA is that the cDNA isolated in this report either encodes the nonmuscle myosin RLC or that the nonmuscle and smooth muscle RLCs are products of the same gene or of genes with strikingly similar coding regions.

Both RNA blot analysis and S1 nuclease mapping suggest that the amount of putative smooth muscle RLC RNA is equal or greater in some sarcomeric tissues than that seen in smooth muscle tissues or cells. Were the RNAs to be translated with equal efficiency in all tissues, it would be extremely likely that the protein corresponding to the 20-kD RLC would be found in sarcomeric tissues. Nevertheless, as mentioned above, 20-kD MLCs similar to either smooth muscle or nonmuscle RLC have been found in presumptive myoblasts but not in other skeletal or cardiac tissues to any significant degree. It thus appears likely that the regulation of smooth muscle RLC synthesis is a posttranscriptional event. In sarcomeric tissues, the RNA is expressed in high levels like those of the other contractile proteins (and in the case of L_6E_9 cells is expressed in even greater amounts in myotubes than in myoblasts), but is not translated into significant amounts of stable RLC protein. In smooth muscle, the RNA is expressed at lower levels, but is translated into readily detectable RLC protein. Thus, the putative smooth muscle RLC gene(s) appears to be constitutively expressed in a large variety of cells shows neither significant tissue or developmental specificity, but has a differentiated function in smooth muscle cells.

There is limited information concerning the expression of smooth muscle contractile protein genes. α-Tropomyosin has been shown to be encoded by a single gene that is spliced alternatively to produce smooth, skeletal, and cardiac muscle forms (45) which are expressed uniquely in the corresponding tissues. As mentioned earlier, a variety of actin forms, including both smooth muscle–specific and nonmuscle actins, has been found in several smooth muscle tissues (20, 57). These are expressed in a tissue-specific manner and changes in expression are seen concomitant with changes in smooth muscle cell phenotype (19, 20, 41). The smooth muscle myosin heavy chain has yet to be well characterized and the number of smooth muscle heavy chain genes is unknown. However, it is clear that the smooth muscle myosin heavy chain(s) is antigenically (58) and genetically (37) distinct from that of skeletal or cardiac muscle. The work presented in this report demonstrates that the putative smooth muscle RLC is expressed not only in smooth muscle, but in sarcomeric muscle and in nonmuscle tissues as well. Thus, its expression appears to be distinct from the expression of other vertebrate muscle contractile proteins and together with the 23-kD embryonic light chain expressed in chicken smooth, cardiac, and skeletal muscle is the only known muscle contractile protein gene to be expressed in all types of muscle cells. In this regard, the expression of the putative smooth muscle RLC may represent an earlier stage in the evolution of contractile protein gene regulation: the smooth muscle RLC being transcribed in most cell types, but translated only in some cells, and the sarcomeric RLC being transcribed only in sarcomeric tissues.

The smooth muscle cell expresses both contractile and noncontractile phenotypes, which are both distinct and reversible (see reference 10). Changes in phenotype are seen not only in cell culture, but also in areas of atherosclerotic plaques and blood vessel injury. An important feature of this so-called "phenotypic modulation" is a change in the number and in the staining patterns of myosin-containing thick filaments (see reference 10) and in the expression of smooth muscle actins (20, 41). One hypothesis for this phenotypic modulation is that there is coordinate regulation on the transcriptional level of specific smooth muscle contractile protein genes, resulting in either a contractile or noncontractile phenotype. The finding that the putative smooth muscle RLC gene appears to be constitutively expressed in most cells suggests that the regulation of the contractile phenotype may require more than transcriptional regulation and may involve posttranscriptional or translational events as well. The recent availability of cDNA probes, not only for the RLC but for other smooth muscle contractile proteins as well, should provide a valuable tool for elucidating the molecular regulation of smooth muscle cell contraction.

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