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Characterization of a mammalian smooth muscle myosin heavy chain cDNA clone and its expression in various smooth muscle types

(cDNA cloning/DNA sequence/evolution)

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ABSTRACT **A cDNA clone, SMHC-29, encoding the light meromyosin of smooth muscle myosin heavy chain (MHC), was isolated from a rabbit uterus cDNA library constructed in phage λgt11. This smooth muscle MHC cDNA demonstrates significant nucleotide and amino acid sequence homologies with known sarcomeric MHC genes from rabbit, rat skeletal, and nematode body wall myosin, and even with nonmuscle MHC gene from a slime mold (*Dictyostelium discoideum*), suggesting that smooth muscle, striated muscle, and nonmuscle MHC genes diverged from a common ancestor. The deduced amino acid sequences of the smooth muscle light meromyosin show very similar periodic distributions of hydrophobic and charged residues as found for the light meromyosin of striated muscle MHCs together with a high potential for α-helical formation, indicating an α-helical coiled-coil structure for the smooth muscle light meromyosin sequences. Furthermore, S1 nuclease mapping has revealed that this smooth muscle MHC gene for SMHC-29 is specifically expressed in smooth muscles of vascular and nonvascular types but not in the striated muscles or nonmuscle cells.**

Myosin, one of the major contractile proteins in muscles as well as in nonmuscle cells, is involved in conversion of chemical energy into mechanical work. A myosin molecule consists of two heavy chains (about 200 kDa) and two pairs of light chains. Myosin heavy chain (MHC) is an asymmetric protein with a globular head and an α-helical rod. The rods of two MHCs intertwine to form a coiled-coil structure and the light meromyosin (LMM) becomes the structural backbone of thick filament assembly in striated muscles. In vertebrate striated muscles, MHCs are encoded by a highly conserved multigene family of at least 10 members and exist in several isoforms, the expression of which is regulated developmentally, hormonally, and in a tissue-specific manner (1–6).

Little is known about smooth muscle regarding myosin structure, thick filament assembly, and the diversity of MHC molecules. It has been reported that vertebrate smooth muscle myosin monomers form longer thick filaments (2.2 μm) (7) than striated muscle myosins (1.5–1.6 μm). Smooth muscle myosin has a unique morphological characteristic that has not been observed in striated muscle myosin. At high salt concentrations, smooth muscle myosin monomer is an asymmetric molecule with an extended rod region. At low salt concentration, on the other hand, myosin takes a less asymmetric form in which the LMM region of the rod is bent back on the subfragment 2 (8, 9). Antibodies against smooth muscle MHC also do not cross-react with striated muscle MHC (10). Moreover, the contraction of smooth muscle MHC is activated mainly through phosphorylation of the myosin light chains, whereas the actin-linked troponin system regulates contraction in striated muscles (11). Thus, it is obvious that there are intrinsic structural differences between smooth muscle and striated muscle MHCs in spite of their morphological similarities.

To understand the molecular structure of smooth muscle myosin, we have characterized cDNA clones encoding a smooth muscle MHC§ and analyzed its expression in different smooth muscle tissues.

METHODS

Extraction of RNA. Total RNA was isolated by the hot phenol method (12) from aorta, urinary bladder, trachea, esophagus, stomach, small intestine, large intestine, skeletal muscle, and brain of 7- to 10-week-old male New Zealand White rabbits. Total RNA of uterus was isolated from a gravid rabbit. Oligo(dT)-cellulose chromatography was used to obtain poly(A)$^+$ RNA as described (13).

Construction and Screening of λgt11 cDNA Library. A λgt11 expression cDNA library was constructed by using rabbit uterine poly(A)$^+$ RNA as described (14, 15). The library was screened with the IgG fraction of rabbit polyclonal antibody against human uterus MHC (16, 17).

RNA and DNA Blot Analysis. RNA gel electrophoresis and hybridization were performed as described (1). Chromosomal DNA from rabbit spleen was prepared as described by Wu *et al.* (18) and 10 μg of DNA was digested with restriction endonucleases *Bam*HI, *Eco*RI, and *Hin*dIII, electrophoresed on a 0.8% agarose gel, and transferred to a nitrocellulose filter. The filter was hybridized to 10^7 cpm of ^{32}P-labeled SMHC-29 insert and washed in several changes of 75 mM NaCl/7.5 mM sodium citrate/0.1% $NaDodSO_4$ at 47°C.

Nucleic Acid Sequence Analysis. Two of 30 positive clones, SMHC-29 and SMHC-30, harboring the longest cDNA inserts were subcloned into the *Eco*RI site of pUC18 and M13-mp18 cloning vectors and sequenced by the chemical cleavage method of Maxam–Gilbert (19) and the dideoxy method of Sanger *et al.* (20).

S1 Nuclease Mapping. S1 nuclease mapping was carried out by modifying the protocol of Berk and Sharp (21, 22). A 260-base-pair (bp) *Pst* I/*Pst* I restriction fragment from the 3′ end of SMHC-29 clone in pUC18 was 3′ end-labeled with [α-^{32}P]cordycepin (Amersham) and strand separated, and the strand complementary to the mRNA was purified. This

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Abbreviations: MHC, myosin heavy chain; LMM, light meromyosin; nt, nucleotide.

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§The sequence reported here is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03614).

probe contains the 103 nucleotides (nts) of coding region, 117 nts of the 3′ untranslated region, and 40 nts of the polylinker of the pUC18 cloning vector.

RESULTS AND DISCUSSION

Isolation of Smooth Muscle MHC cDNA Clones. Thirty positive clones were isolated from 6×10^5 recombinant λgt11 cDNA clones screened with anti-human uterus MHC antibody. Two λgt11 clones, SMHC-29 and SMHC-30, were subcloned into pUC18 vector. Inserts of these cDNA clones hybridized to an ≈32S mRNA from smooth muscle that appeared to be large enough to code for a protein of ≈200 kDa. SMHC-29 and SMHC-30 were subjected to nucleotide sequencing analysis and identified to encode the LMM region of smooth muscle MHC based on the following findings: (*i*) nucleotide and amino acid homology with skeletal muscle MHC and (*ii*) predicted α-helical coiled-coil structure of the deduced protein sequence. These findings are discussed below.

Nucleotide and Amino Acid Sequence Analysis of SMHC-29. The partial restriction map, nucleotide sequences, and deduced amino acids of SMHC-29 are shown in Fig. 1. SMHC-30 was slightly shorter than SMHC-29 and its whole nucleotide sequence was included in SMHC-29. SMHC-29 is 1571 nts long, including a 3′ untranslated region (117 bp), and encodes 484 amino acids contained in the LMM region of the protein. Comparison of nucleotide sequences in this LMM region with rat skeletal muscle MHC (23) and nematode *unc*-54 MHC (24) showed 47.8% and 43.5% homology, respectively.

Direct comparison of the amino acid sequences deduced from SMHC-29 with rabbit skeletal muscle MHC (3, 25), rat skeletal muscle MHC (amino acids 1451–1934) (23), nematode *unc*-54 MHC (amino acids 1462–1945) (24), and nonmuscle MHC of a slime mold (*Dictyostelium discoideum*; amino acids 1627–2103) (26) is presented in Fig. 2. The homology between this LMM region of rabbit smooth muscle MHC and the partially known rabbit skeletal MHC amino acid sequence (25) is 31%, as is the case with rat skeletal MHC or with nematode *unc*-54 MHC. Surprisingly, the rabbit smooth muscle MHC shows 25% homology even with the nonmuscle myosin LMM of *Dictyostelium* (26).

Based on the nucleotide and amino acid sequence homologies, we conclude that the smooth muscle, striated muscle, and nonmuscle MHC genes must have diverged from a common ancestor. Thus, the noted homologies raise intriguing questions about the origin and evolution of the MHC genes in general and in particular the date of gene duplication and divergence. Further characterization of the MHC genes is required for understanding the evolutionary relationships of smooth muscle MHC with striated muscle and nonmuscle MHCs.

The amino acid composition of the smooth muscle LMM in the region encoded by cDNA SMHC-29 reveals a high content of positively (arginine and lysine) and negatively (aspartic and glutamic acid) charged residues. The α-helix-breaking amino acid proline, on the other hand, does not exist except at the very carboxyl-terminal end. Proline residues at the tail end are an interesting feature of smooth muscle MHC because this has not been observed in any known vertebrate sarcomeric MHCs (2, 3, 23, 25), whereas a predicted nonhelical tail piece containing proline does occur at the carboxyl termini of striated muscle MHCs in invertebrates, such as nematode (24) and *Drosophila* (27, 28).

Structure of Smooth Muscle LMM and Thick Filament Assembly. Secondary structure predicted by the conformational parameters of the 20 amino acids as described by Chou and Fasman (29) shows the LMM of smooth muscle MHC has a high potential for an α-helix conformation except for the last several amino acids at the carboxyl terminus (Fig. 3*a*). In addition to an α-helical structure, the LMM of smooth muscle MHC reveals a periodic seven-residue repeat in which hydrophobic residues occur at alternate intervals of three and four residues, thus occupying positions a and d when the repeat is expressed as $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$ (Fig. 3*b*). This characteristic amino acid distribution in an α-helical structure facilitates two such α-helical molecules to be held together through hydrophobic interactions in which hydrophobic residues become the packed interface. The two strands intertwine around each other because the average position of each hydrophobic residue is shifted approximately 10° radially from the one preceding it, establishing a coiled-coil structure with a pitch equal to 36 turns of the minor helix (30). These data demonstrate that the smooth muscle LMM has an α-helical coiled-coil structure as described for the LMM of striated muscle MHC (2, 3, 23, 25, 32), nonmuscle MHC (26), or other fibrous proteins (33, 34).

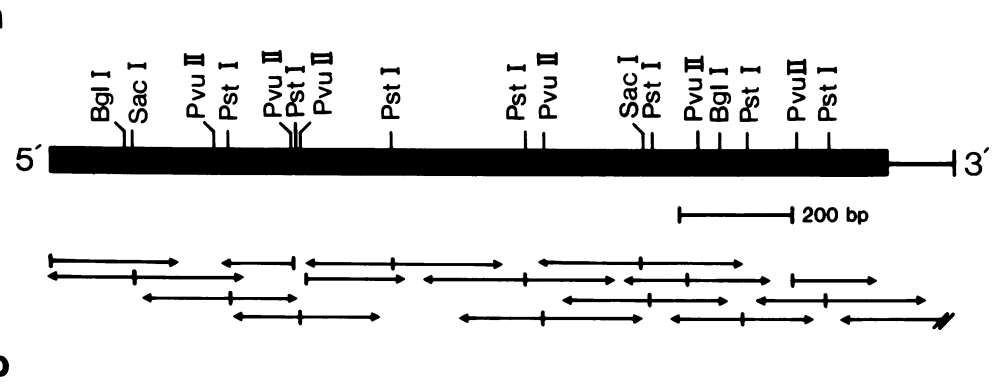
Furthermore, in the striated muscles, the LMM is known to have a 28-residue repeat pattern as originally pointed out by Parry (32) and later detailed for nematode *unc*-54 MHC by McLachlan and Karn (31). In a 28-residue repeat unit, positively and negatively charged residues tend to cluster in the former and the latter half of the unit, respectively. Thus, the LMM possesses the positive and the negative charge cluster alternating every 14 residues along its entire region. This alternate charge distribution favors adherence of adjacent myosin LMMs through electrostatic interactions.

To examine the cluster of charged residues in the rabbit smooth muscle LMM, we made a histogram of the net charge in the 28-residue repeat (Fig. 3*c*). The net charge was calculated by subtracting the total number of negative residues from the number of positive residues at each position of 28 longitudinal columns of rabbit smooth muscle MHC as segmented in Fig. 2. As shown in Fig. 3*c*, the LMM of smooth muscle MHC has a structure of alternating positive and negative charges across the 28-residue unit as is found with striated muscle MHC.

When aligned into 28-residue units, the repeat pattern of smooth muscle LMM sequences is interrupted at two positions by the insertion of one extra "skip" residue (31). These extra residues occur in smooth muscle MHC at identical positions as noted for sarcomeric MHCs of rabbit, rat, chicken, and nematode (3, 23, 31).

To further analyze the effects of this segmented charge distribution on assembly of smooth muscle thick filaments, we calculated the charge interaction between two parallel rods by the method of McLachlan and Karn (31). Our calculation predicts that the strongest attraction between two rods will occur with a stagger of 97 residues, which would correspond to a distance of 144 Å, assuming 1.485 Å per residue for a helical pitch (33) (Fig. 3*d*). This distance is very well matched to the 144-Å periodicities of crossbridge projections along the native smooth muscle thick filaments (35). These analyses predict overall that smooth muscle rods should have a packing scheme similar to that of striated muscle.

Expression of Smooth Muscle MHC in Various Tissues. Transfer blot analysis using total RNA from vascular (aorta) and nonvascular (uterus, urinary bladder, trachea, and gastrointestinal tract) smooth muscle, skeletal muscle, and nonmuscle (brain) tissue was performed to examine the tissue-specific expression of the gene represented by the SMHC-29 cDNA clone (Fig. 4*a*). The SMHC-29 cDNA hybridized to ≈32S mRNA from aorta, uterus, urinary bladder, trachea, esophagus, small intestine, and large intestine and weakly to mRNA from skeletal muscle. The weak hybridization to skeletal muscle mRNA is probably due to nucleotide homology between smooth and skeletal muscle MHC. It is interesting to note that two RNA species were



b

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AT CAG TTG TTA GCC GAG GAA AAG AAC ATC TCT TCC AAG TAT GCG GAT GAA AGG GAC CGA GCC GAG GCT GAA GCA AGG GAA    80
   Q  L  L  A  E  E  K  N  I  S  S  K  Y  A  D  E  R  D  R  A  E  A  E  A  R  E

AAG GAA ACC AAG GCC TTG TCC CTG GCT CGG GCC CTC GAG GAG GCC TTG GAG GCC AAA GAG GAG CTC GAG AGA ACC AAC AAA   161
 K  E  T  K  A  L  S  L  A  R  A  L  E  E  A  L  E  A  K  E  E  L  E  R  T  N  K

ATG CTC AAG GCC GAG ATG GAA GAC CTC GTC AGC TCC AAG GAC GAC GTG GGC AAG AAC GTC CAT GAG CTG GAG AAG TCC AAG   242
 M  L  K  A  E  M  E  D  L  V  S  S  K  D  D  V  G  K  N  V  H  E  L  E  K  S  K

CGG GCC CTG GAG ACA CAG ATG GAG GAG ATG AAG ACG CAG CTG GAA GAG CTA GAG GAC GAG CTG CAG GCC ACC GAG GAC GCC   323
 R  A  L  E  T  Q  M  E  E  M  K  T  Q  L  E  E  L  E  D  E  L  Q  A  T  E  D  A

AAG CTG CGG TTG GAG GTC AAC ATG CAG GCC CTC AAA GTC CAG TTC GAG CGG GAT CTC CAG GCC CGG GAT GAG CAG AAC GAG   404
 K  L  R  L  E  V  N  M  Q  A  L  K  V  Q  F  E  R  D  L  Q  A  R  D  E  Q  N  E

GAG AAG AGG AGG CAG CTG CAG AGG CAG CTG CAT GAG TAC GAG ACG GAA CTG GAA GAC GAG CGC AAG CAG CGG GCC CTG GCC   485
 E  K  R  R  Q  L  Q  R  Q  L  H  E  Y  E  T  E  L  E  D  E  R  K  Q  R  A  L  A

GCG GCA GCC AAG AAG AAG CTG GAG GGG GAC CTG AAA GAC CTG GAG CTT CAG GCG GAC TCC GCC ATC AAA GGG CGG GAG GAA   566
 A  A  A  K  K  K  L  E  G  D  L  K  D  L  E  L  Q  A  D  S  A  I  K  G  R  E  E

GCC ATC AAG CAG CTT CTG AAA CTG CAG GCT CAG ATG AAG GAC TTC CAG AGA GAA CTG GAA GAT GCC CGT GCC TCC AGA GAC   647
 A  I  K  Q  L  L  K  L  Q  A  Q  M  K  D  F  Q  R  E  L  E  D  A  R  A  S  R  D

GAG ATC TTT GCC ACA GCC AAG GAG AAC GAG AAG AAA GCC AAG AGT CTG GAG GCA GAC CTC ATG CAG CTA CAA GAG GAT CTG   728
 E  I  F  A  T  A  K  E  N  E  K  K  A  K  S  L  E  A  D  L  M  Q  L  Q  E  D  L

GCC GCG GCA GAG AGG GCT CGC AAA CAG GCA GAC TTG GAG AAG GAG GAG CTG GCC GAG GAG CTG GCC AGC AGC TTG TCC GGA   809
 A  A  A  E  R  A  R  K  Q  A  D  L  E  K  E  E  L  A  E  E  L  A  S  S  L  S  G

AGG AAC GCG CTG CAG GAT GAG AAG CGC CGC CTG GAG GCC CGG ATC GCA CAG CTG GAG GAG GAG CTG GAG GAG GAA CAG GGC   890
 R  N  A  L  Q  D  E  K  R  R  L  E  A  R  I  A  Q  L  E  E  E  L  E  E  E  Q  G

AAC ATG GAG GCA ATG AGC GAC CGC GTC CGC AAG GCT ACG CAG CAG GCC GAG CAG CTC AGC AAC GAG CTG GCC ACA GAG CGC   971
 N  M  E  A  M  S  D  R  V  R  K  A  T  Q  Q  A  E  Q  L  S  N  E  L  A  T  E  R

AGC ACA GCC CAG AAG AAT GAG AGC GCA CGG CAG CAG CTC GAG CGG CAG AAC AAG GAG CTC AAG AGC AAG CTG CAG GAG ATG  1052
 S  T  A  Q  K  N  E  S  A  R  Q  Q  L  E  R  Q  N  K  E  L  K  S  K  L  Q  E  M

GAG GGG GCA GTC AAG TCC AAG TTC AAG TCC ACT ATC GCG GCG CTG GAG GCC AAG ATT GCG CAG CTG GAG GAG CAG GTT GAG  1133
 E  G  A  V  K  S  K  F  K  S  T  I  A  A  L  E  A  K  I  A  Q  L  E  E  Q  V  E

CAG GAG GCC AGA GAG AAG CAG GCG GCC GCC AAG GCG CTG AAG CAG AGG GAC AAG AAG CTG AAG GAG ATG CTG CTG CAG GTG  1214
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CTG GAG GAG GCC GAG GAG GAG TCG CAG CGC ATC AAC GCC AAC CGC AGG AAG CTG CAG CGG GAG CTG GAC GAG GCC ACG GAG  1376
 L  E  E  A  E  E  E  S  Q  R  I  N  A  N  R  R  K  L  Q  R  E  L  D  E  A  T  E

AGC AAC GAG GCC ATG GGC CGC GAG GTG AAC GCG CTC AAG AGC AAG CTC AGA GGG CCC CCC CCA CAG GAA ACT TCG CAG TGA  1457
 S  N  E  A  M  G  R  E  V  N  A  L  K  S  K  L  R  G  P  P  P  Q  E  T  S  Q  *

CTCAGCAGGCGAGGAAACGAGACCTCGTTCGTTCCTACCAGAAGGTCTGGAGGGCGTAGAGTTATTGAAAACGCAGATGGCTCTGAGGAGGAAGTGGACGCTCGCGA  1564

CGCAGAG  1571
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FIG. 1. Restriction map (*a*) and nucleotide and deduced amino acid sequence (*b*) of the cDNA clone SMHC-29. The protein coding region is indicated by a closed box. The arrows below the map refer to the sequencing strategy for SMHC-29.

detected in the esophagus and the lower band comigrated with MHC mRNA in skeletal muscle. Since it has been known that esophagus has a mixed population of striated muscle and smooth muscle, this lower band appears to be due to cross-hybridization with striated muscle MHC. However, this lower band corresponding to skeletal muscle MHC mRNA disappears with high-stringency washes. We did not observe any hybridization with brain mRNA, confirming that our cDNA clone does not encode nonmuscle MHC.

Since we observed hybridization of the SMHC-29 probe to all smooth muscle mRNA in transfer blot analysis, we adopted a more sensitive and discriminative S1 nuclease mapping technique to determine the tissue-specific expression of this MHC gene. Our S1 nuclease mapping analysis, using a probe containing the 3′ coding and untranslated region, demonstrates a fully protected fragment of 220 bases and a partially protected fragment of about 90 bases long in all smooth muscle tissues examined but not in skeletal muscle and brain (Fig. 4*b*). The presence of a fully protected probe (220 nts) in all smooth muscle types indicates that the

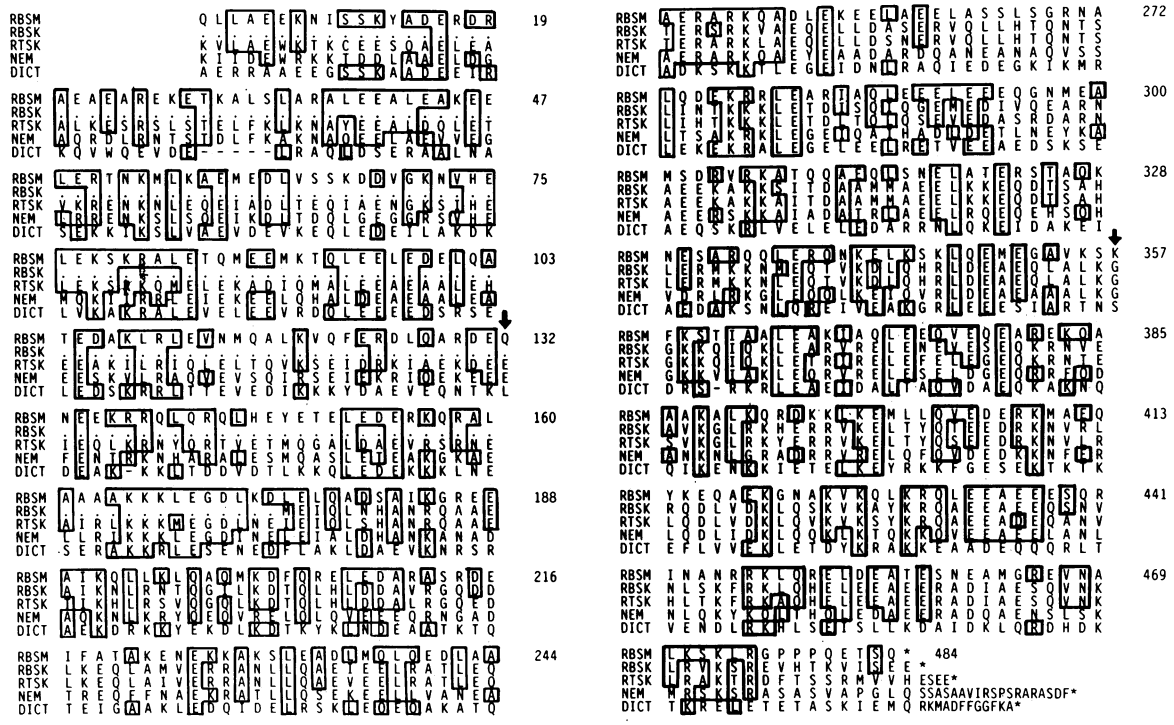

FIG. 2. Comparison of the amino acid sequences of rabbit smooth muscle (RBSM) MHC with rabbit skeletal muscle (RBSK) (25), rat embryonic skeletal muscle amino acids (1451–1934) (RTSK) (23), nematode *unc*-54 (amino acids 1462–1945) (NEM) (24) MHC, and nonmuscle MHC of a slime mold (*Dictyostelium discoideum*; amino acids 1627–2103) (DICT) (26). Amino acid sequences are formulated into repeating 28-residue units. Arrows mark skip residues at positions 132 and 357. In display of *Dictyostelium* MHC, gaps were inserted for the best fit.

gene for SMHC-29 is expressed in all of them. The smaller fragment (≈90 nts) may suggest the existence of another MHC mRNA species partially homologous to SMHC-29 with divergence in the 3′ untranslated region. Thus, the S1 nuclease protection results suggest the presence of two MHC mRNA species that may correspond to two different protein isoforms as shown in protein studies (36, 37). It also is intriguing to note that the SMHC-29 gene is ubiquitously expressed in such functionally different smooth muscle types as vascular and nonvascular systems.

Genomic Complexity of the Smooth Muscle MHC Gene. We attempted to determine the genomic complexity of smooth

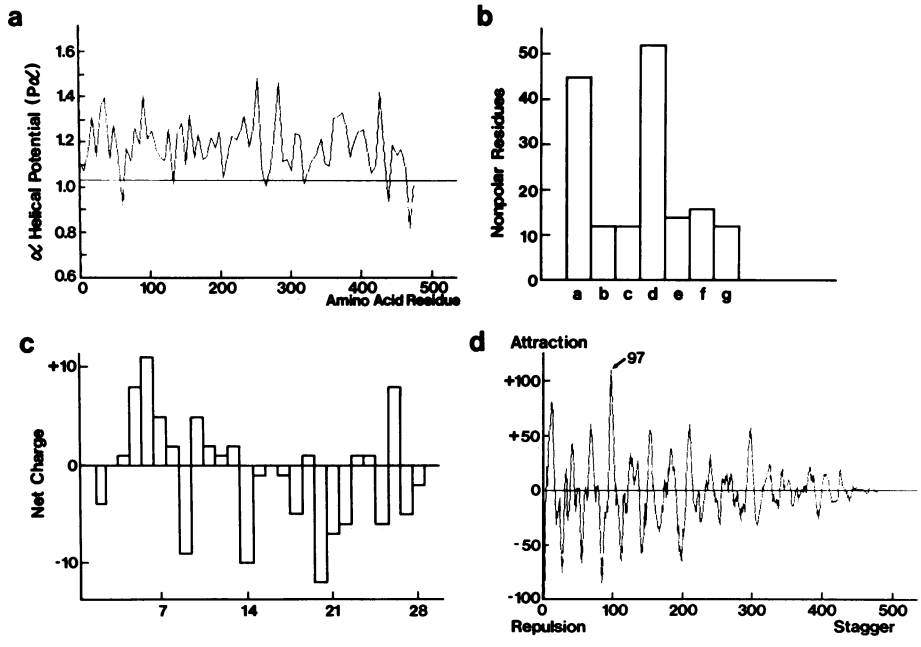

FIG. 3. Structural characteristics of smooth muscle LMM. (*a*) Propensity for α-helix formation as a sliding average by the method of Chou and Fasman (30). The horizontal line represents Pα of 1.03, any number above which indicates a high probability for α-helix formation. (*b*) Histogram showing more frequent distribution of hydrophobic residues at positions a and d in the seven-residue periodicity. (*c*) Distribution of charged residues across the 28-residue periodical structure in rabbit smooth muscle LMM. The net charge at each of the 28 columns in Fig. 2 was calculated by subtracting the total number of negatively charged residues from the number of positively charged residues. (*d*) Charge interactions between two parallel rods of 484 residues length. Attractions minus repulsions were computed within an axial range of ±2 residues and plotted vertically on the graph against the stagger of the residues (horizontal) as described by McLachlan and Karn (31).

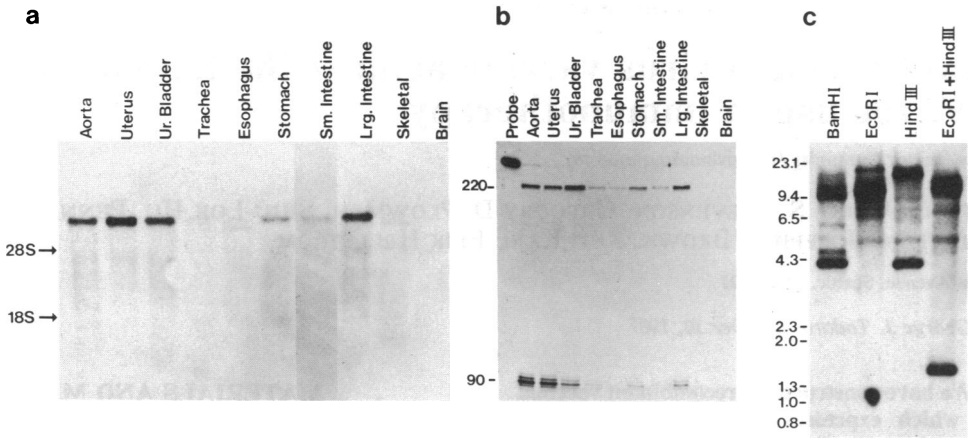

FIG. 4. Analysis of SMHC-29 gene expression in different muscle tissues and hybridization to rabbit genomic DNA. (*a*) Detection of smooth muscle MHC mRNA by transfer blot analysis. Fifteen micrograms of total RNA from various smooth muscle tissues [aorta, uterus, urinary (Ur.) bladder, esophagus, trachea, stomach, small (Sm.) intestine, and large (Lrg.) intestine], skeletal muscle, and brain were size-fractionated on a 0.8% denaturing agarose gel, transferred to a nitrocellulose filter, and hybridized to ^{32}P-labeled SMHC-29 cDNA. (*b*) S1 nuclease protection analysis performed with the same RNA samples as used for RNA transfer blot analysis. (*c*) Southern blot analysis of rabbit genomic DNA.

muscle MHC sequences by chromosomal DNA blot hybridization. Rabbit genomic DNA digested with restriction enyzmes *Bam*HI, *Eco*RI, and *Hin*dIII gave multiple hybridizing bands with cDNA SMHC-29 with washing at high stringency (Fig. 4*c*). Considering that the cDNA clone does not have these restriction sites, those multiple bands may correspond to different genes. Alternatively, the hybridization pattern could be produced by a single gene, interrupted by intervening sequences with appropriate restriction sites, or else be due to cross-hybridization with nonmuscle MHCs. Further studies are required to answer this question, including isolation of smooth muscle and nonmuscle MHC genes.

In summary, the results presented here clearly demonstrate that the smooth muscle myosin molecule has similar characteristics in the rod structure as does sarcomeric myosin, suggesting that smooth muscle myosin molecules might follow a similar packing scheme to form thick filaments. It is apparent from this study that MHC genes of smooth muscle, striated muscle, and nonmuscle cells constitute a superfamily of MHC genes. Our study also demonstrates that the SMHC-29 gene is expressed in all vascular and nonvascular smooth muscle tissues. Development of smooth muscle MHC cDNA probes as we reported here should make it feasible to further investigate the molecular diversity and the organization of MHC gene families.

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