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Complete Nucleotide Sequence, Structural Organization, and an Alternatively Spliced Exon of Mouse *h1*-Calponin Gene

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From a CC1.2 embryonic stem cell genomic library, we isolated and sequenced a 10.4-kb DNA segment (GenBank/EMBL Data Bank accession number L49022) containing the entire gene encoding mouse *h1*-calponin, an actin-associated smooth muscle-specific protein and a potential modulator of contraction. Sequence data revealed that there are seven exons and six introns in the *h1*-calponin gene. Determined by primer extension mapping of the RNA transcripts, the transcription of *h1*-calponin gene initiates at the same site in stomach, urinary bladder and pregnant uterus smooth muscles. The genomic organization suggests that the previously identified α- and β-calponin isoforms are produced by splicing of exon 7 at two alternative acceptor sites. Isolation and structural characterization of the *h1*-calponin gene provides information to further investigate the expression regulation of this smooth muscle-specific gene.

Calponin is a recently identified smooth muscle-specific protein associated with the thin filament (1) and has been demonstrated to bind in vitro to actin (1–3), Ca^{2+}-calmodulin (1,4), tropomyosin (4,5), and myosin (6,7). Calponin inhibits the actin-activated Mg^{2+}-ATPase activity of phosphorylated smooth muscle myosin and this inhibition is regulated by phosphorylation-dephosphorylation of calponin (2,3,8,9). Consistently, in vitro motility assays have demonstrated that calponin inhibits the relative movement of actin and myosin filaments (10,11). The association of calponin with the actin filament and its regulation of actomyosin ATPase activity suggests that this protein may represent an actin thin filament-based regulatory system (similar to striated muscle troponin) in smooth muscle (12,13) to modulate contractility in addition to the myosin light chain phosphorylation mechanism (14–16). cDNAs encoding two smooth muscle calponins (*h1* and *h2*, 17–19) and an acidic calponin (20,21) have been isolated. The cDNA cloning and sequencing data suggest that the *h1*-, *h2*- and acidic calponins are encoded by different genes. In addition, cDNAs encoding α- and β-isoforms of *h1*-calponin have been found as potential products of alternative mRNA splicing (19). *h1*-calponin is the major calponin found in smooth muscle and has been analyzed for its function in the previous studies (1–16). Regulated expression of calponin in smooth muscle during differentiation/development has been observed (22–25), suggesting its importance in the functional maturation of smooth muscle myofilaments. However, the functional significance and expression regulation of the calponin isoforms remain to be established. This present study isolated and sequenced the entire mouse *h1*-calponin gene, revealing its structural organization and alternative splicing pattern for further studies of the expression regulation of this smooth muscle-specific gene.

MATERIALS AND METHODS

The basic recombinant DNA techniques applied in this study were described previously (26–29) or can be found in Ausubel et al. (30) and Sambrook et al. (31).

Preparation of mouse h1-calponin cDNA probes. To obtain specific probes to identify and isolate mouse genomic DNA clones spanning the *h1*-calponin gene, full length *h1*-calponin cDNA was cloned by reverse transcriptase-polymerase chain

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The abbreviations used are: bp, base-pair; kb, kilo-base; nt, nucleotide; RT-PCR, reverse transcriptase polymerase chain reaction; TE buffer, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA; TM buffer, 10 mM Tris-HCl, pH 8.0, 10 mM $MgSO_4$.

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reaction (RT-PCR). Total RNA was extracted from pregnant Balb/c mouse uterus with the TRIZOL reagent (Gibco/BRL) according to the manufacturer's protocol. The specific 5′- and 3′-flanking primers were synthesized according to the previously published sequences (17) and Moloney murine leukemia virus reverse transcriptase (Pharmacia Biotech Inc.) was used for the first strand cDNA synthesis (30). The double-stranded cDNA amplified by RT-PCR was cloned into the pCRII vector (Invitrogen, San Diego, CA) and fully sequenced after subcloned as different restriction endonuclease fragments. The mouse *h1*-calponin cDNA inserts were isolated by preparative agarose gel electrophoresis and purified by the Prep-A-Gene matrix binding method (BioRad Lab.) according to the manufacturer's protocol. The cDNA fragments were then labeled with [^{32}P]-dCTP by the random priming synthesis method for use as probes in the genomic DNA library screening.

Isolation, subcloning and sequencing of mouse genomic DNA containing h1-calponin gene. As described previously (27,28), cDNA probes were used to screen and isolate genomic DNA clones containing segments of the mouse *h1*-calponin gene from a mouse CC1.2 embryonic stem cell genomic library constructed in the λDASHII vector (generously provided by Dr. D.E. Rancourt, University of Calgary). The plaque-purified positive phages were used to infect XL1-B MRA(P2) *E. coli* grown in soft top agar on Luria broth plates to prepare high titer phage lysate. Phage particles were purified by applying the lysate directly to a CsCl step-gradient [1.3/1.5/1.7 g/ml in TM buffer (10 mM Tris-HCl, pH 8.0, 10 mM $MgSO_4$)]. After centrifugation at 26,000 rpm in a Beckman SW28 rotor for 2.5 hr, the phage band was collected and concentrated by spinning at 45,000 rpm in a Beckman SW51 rotor for 16 hr. After dialysis against TM buffer to remove CsCl, recombinant phage DNA was prepared by extraction with 1:1 phenol/chloroform followed by thorough dialysis against TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The purified phage DNA was then digested with selected restriction endonucleases to subclone the inserts into plasmid vectors for mapping and sequencing. The mouse genomic DNA insert fragments in the digestion mixture of each recombinant λ phage were ligated to the digested, dephosphorylated pGEM3Z (Promega) or pBluescript (Stratagene) plasmid vector. Through construction of restriction endonuclease fragment subclones and ExoIII nuclease serial deletion subclones using the Erase-A-Base kit from Promega, as well as specific synthetic oligonucleotide primers, a sequence of 10.4-kb mouse genomic DNA was determined. To carry out the large volume of manual DNA sequencing, recombinant plasmid DNA was prepared by a column minipreparation method (Qiagen, Chatsworth, CA) according to the manufacturer's protocol; double-stranded DNA was sequenced directly using the dideoxy chain-termination method (32) with the T7 polymerase DNA sequencing kit (Pharmacia Biotech Inc.) and [^{35}S]-dATP labeling; the DNA sequencing samples were resolved by electrophoresis on 6% polyacrylamide gels containing 7 M urea and the results were documented by autoradiography of the fixed, dried gels (27,29).

Primer extension mapping of the 5′-ends of mouse h1-calponin mRNA. As described previously (28), total RNA was prepared from adult 129 mouse stomach, urinary bladder and pregnant uterus by extracting 0.1–0.2 g of fresh or liquid nitrogen-frozen tissue with the TRIZOL reagent. A 25-nt (nucleotide) oligonucleotide primer was synthesized as the antisense sequence of part of the exon 1 of mouse *h1*-calponin gene and 5′-end-labeled with ^{32}P using T4 polynucleotide kinase. 30 μg of each total RNA was coprecipitated with 0.5 pmole of the radioactive primer in ethanol and annealed in 10 μl of 150 mM KCl, 50 mM Tris-HCl, pH 8.3, 1 mM EDTA by initial incubation at 90°C and slowly cooling down to 42°C. The primer extension reaction was carried out in 50 μl of 30 mM KCl, 50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 2 mM dNTP, 10 mM dithiothreitol, containing 50μg/ml actinomycin D (Sigma) and 10 u of Moloney murine leukemia virus reverse transcriptase (Pharmacia Biotech Inc.) at 37°C for 1 h. After RNase A treatment at 37°C for 15 min in the presence of 10 μg salmon sperm DNA, the reaction mixture was extracted by 1:1 phenol/chloroform and the cDNA synthesized was

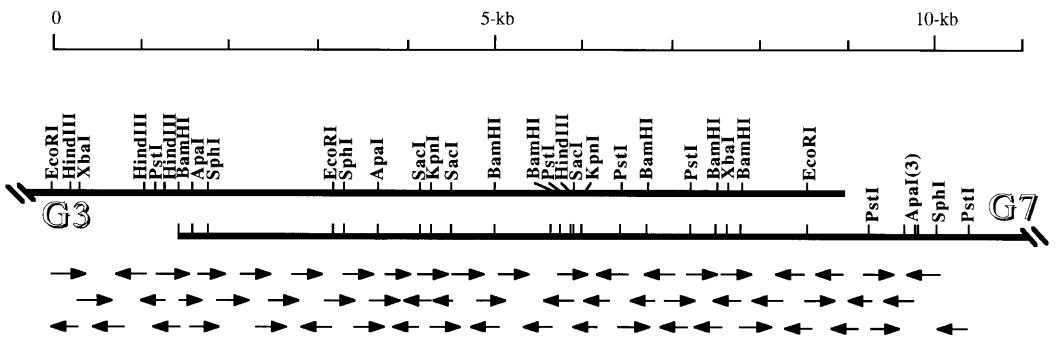

FIG. 1. Cloning and sequencing characterization of mouse *h1*-calponin gene. To isolate and map mouse *h1*-calponin gene, several RT-PCR cloned mouse *h1*-calponin cDNAs containing different portions of the exon sequences were prepared as specific probes. Among five positive phage clones screened from a λDASHII CC1.2 mouse embryonic stem cell genomic DNA library, two overlapping clones (G3 and G7, their selected restriction enzyme maps are shown as verified by sequences) were subcloned and sequenced. The arrows represent overlapping DNA sequencing reactions carried out on restriction endonuclease or ExoIII nuclease serial deletion subclones to reveal the complete sequence of mouse *h1*-calponin gene. It is noted that there is no XhoI, SaII or NotI cut in the entire mouse *h1*-calponin gene.

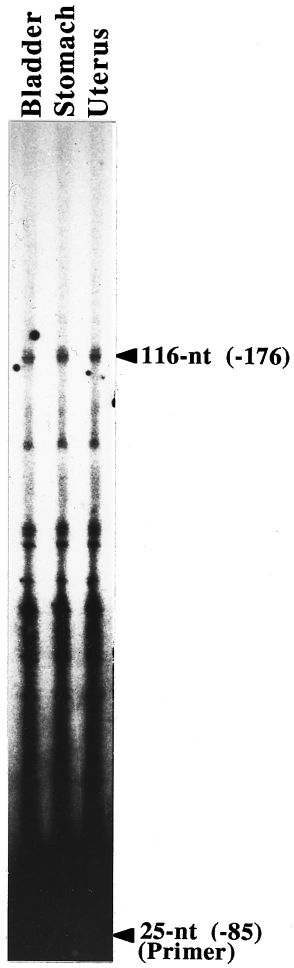

FIG. 2. Primer extension mapping of the 5′-ends of mouse *h1*-calponin mRNA. Using a [^{32}P]-labeled 25-nt antisense oligonucleotide primer corresponding to the 5′-region of the mouse *h1*-calponin cDNA, primer extension experiments were carried out to determine the 5′-ends of mouse *h1*-calponin transcript. As shown by the 6% denaturing polyacrylamide gel autoradiography, the *h1*-calponin mRNAs were extended to 116 bases from the 5′-end of the primer, corresponding to −176-bp from the translation initiation codon. *h1*-calponin mRNAs extracted from adult mouse urinary bladder, stomach and pregnant mouse uterus smooth muscles had 5′-ends of identical lengths. DNA sequencing samples from the same primer on the cloned mouse genomic DNA template containing exon 1 and 5′-upstream region of *h1*-calponin gene were used as the size marker.

precipitated by adding 1/10 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of cold 97% ethanol. The precipitates were air-dried, redissolved in 8 μl of TE buffer and 8 μl of sequencing gel loading dye (30), heated at 75°C for 3 min and chilled on ice. The samples were resolved by electrophoresis on a 6% polyacrylamide sequencing gel containing 7 M urea followed by autoradiography of the fixed, dried gel.

RESULTS AND DISCUSSION

Isolation and sequencing of mouse h1-calponin gene. By hybridization in DNA dot blotting to the [^{32}P]-labeled probes derived from different portions of the mouse *h1*-calponin cDNA, two of the positive λDASHII phage clones (G3 and G7; their selected restriction enzyme maps are shown in Fig. 1) identified with overlapping inserts containing the entire mouse *h1*-calponin gene were subcloned for sequencing analysis. As summarized in Fig. 1, the DNA sequencing reactions performed on 59 overlapping genomic DNA subclones revealed the complete nucleotide sequence

of mouse *h1*-calponin gene. The 10,373-bp nucleotide sequence has been deposited in the GenBank/EMBL Data Bank (accession number L49022).

Structural organization of mouse h1-calponin gene. The transcription initiation site of mouse *h1*-calponin gene (176-bp upstream of the translation initiation codon) was determined by primer extension mapping of the 5′-end of the RNA transcript (Fig. 2). The complete *h1*-calponin gene structure was revealed by alignment of the cDNA and genomic DNA sequences. There are a total of seven exons and six introns in this gene and the 10,373-bp genomic DNA sequenced also contains a 631-bp 5′-upstream sequence and a 212-bp 3′-downstream region. The structural organization of mouse *h1*-calponin gene is summarized in Fig. 3 together with the mRNA map to illustrate the corresponding region of each exon. There is no typical-30 TATA box found in the promoter region of mouse *h1*-calponin gene and the *cis*-regulatory elements governing transcription of *h1*-calponin gene remain to be established experimentally.

Transcription initiation of mouse h1-calponin gene in different smooth muscles. As shown in Fig. 2, primer extension transcript mapping showed identical results from RNA extracted from adult mouse stomach, urinary bladder and pregnant uterus smooth muscles. The results imply that *h1*-calponin, a smooth muscle-specific protein, is expressed under similar transcriptional regulation in smooth muscles of different organs or at different differentiation/development stages.

Alternative splicing of exon 7 at two different acceptor sites to produce the α- and β-isoforms of h1-calponin. It was suggested by the chicken gizzard calponin cDNA cloning/sequencing data that alternative mRNA splicing may be the mechanism to produce the α- and β-isoforms (19). The structural organization of mouse *h1*-calponin gene revealed that there is a large exon 7 in the gene containing a region encoding the peptide from Ala_{217} to the COOH-terminus (Ala_{297}) of calponin and the entire (496-bp) 3′-noncoding region (Fig. 3). Therefore, the 40 amino acids (Ala_{217}-Arg_{256}) insert found only in the α-calponin is not encoded by a separate exon. Instead, this structural difference between the α- and β-isoforms may be produced by mRNA splicing to include or exclude the exon 7a segment at alternative acceptor sites (Fig. 3 and 4). Although the α-calponin appeared as the predominant form in most smooth muscles (1,17–21), very similar consensus splicing junction sequences [$(T/C)_nN(C/T)AG$&verbarG, 33] are present at the two alternative acceptor sites together with identical sequences at the two potential exon 5′-ends (underlined by the circles in Fig. 4). The internal acceptor site in the exon 7 of mouse *h1*-calponin gene responsible for generation of the β-isoform is another example of this type of mRNA splicing regulation (34) that has been found in several other muscle specific proteins, such as cardiac troponin T (27,29).

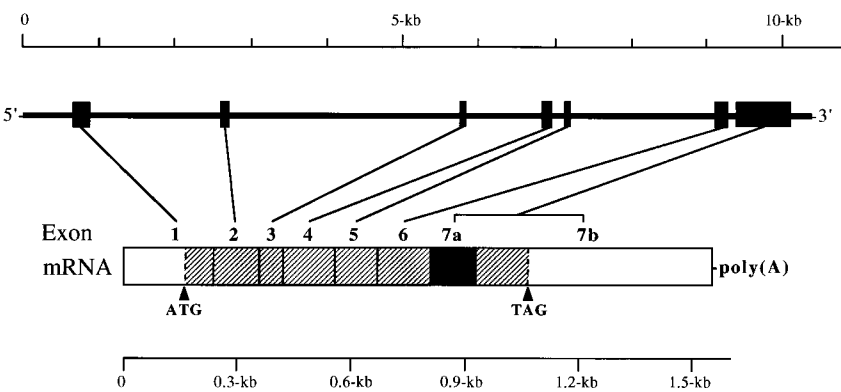

FIG. 3. Structural organization of the mouse *h1*-calponin gene. The exon-intron organization of mouse *h1*-calponin gene is derived from the 10.4-kb genomic DNA sequence. On the upper genomic map, the exons are illustrated as solid blocks relative to their sizes. On the lower mRNA map, the coding region is shadowed and the translation initiation (ATG) and termination (TAG) codons are indicated. The seven exons found in the gene are numbered accordingly and the alternatively spliced exon 7a is illustrated as a solid box.

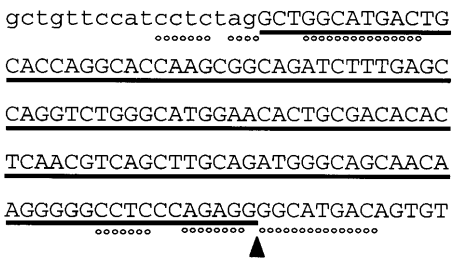

FIG. 4. Alternative splicing of the exon 7 responsible for the generation of *h1*-calponin α-and β-isoforms. Genomic DNA sequence of the 3′-portion of intron 6 (in lower case letters) and the 5′ -portion of exon 7 (in capital letters) of mouse *h1*-calponin gene is shown. The underlined sequence shows exon 7a encoding 40 amino acids corresponding to the difference between the α- and β-isoforms of chicken gizzard calponin. The boundary between exon 7a and b (Fig. 3) is indicated by an arrowhead. Very similar consensus splicing acceptor sequences (underlined by circles) are found at both alternatively spliced sites.

Regulation of the exon 7 alternative splicing and its biological significance remain to be established.

Cloning and sequence characterization of the entire mouse *h1*-calponin gene has established a foundation for further experimental analysis of the regulatory mechanisms governing smooth muscle-specific gene expression and function during differentiation, development and pathological processes.

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