Identification of Functional Promoter Elements in the Rabbit Smooth Muscle Myosin Heavy Chain Gene*

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Youichi Katoh, Evgenii Loukianov, Elizabeth Kopras, Alla Zilberman, and Muthu Periasamy†

From the Molecular Cardiology Laboratory, Division of Cardiology and Cardiovascular Research Center, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0842

Despite the importance of smooth muscle cell proliferation in vascular pathophysiological states, the mechanisms regulating smooth muscle cell growth and differentiation are poorly understood. Previous studies have shown that adult rabbit smooth muscles express two types of myosin heavy chain (MHC) isoforms, SM1 and SM2, which are generated through alternative RNA splicing from a single smooth muscle MHC (SMHC) gene. In the present study, we isolated and characterized the rabbit SMHC gene promoter. DNA sequence analysis of the upstream region of the SMHC gene revealed several putative cis-DNA regulatory elements proximal to the transcription start site. Most notably, cis-acting regulatory elements that closely resemble CC(A/T)GG (CAG box) and myocyte enhancer binding factor 2 (MEF-2)-type sequence motifs were found in the SMHC 5′-flanking region. In addition, six E-box motifs were found in the 5′-flanking region of the SMHC gene between −374 and −2109 base pairs from the transcription start site. A series of transient transfection assays using SMHC promoter deletion constructs indicated that a promoter fragment extending to −2266 base pairs upstream of the transcription start site has the highest reporter activity in cultured rat aortic smooth muscle cells. Gel mobility shift analyses using the MEF-2-like sequence located at −1540 revealed a specific DNA protein complex, whereas the CARG-like element located at −1275 did not show protein binding. The SMHC promoter construct, p509-CAT, which included neither the CARG- nor MEF-2-type motifs, conferred 32% of chloramphenicol acetyltransferase activity in the same cells, whereas the construct p188-CAT, which contained the minimal promoter elements (TATA box), was significantly less active (7%; 2-fold over background). This is the first report describing the promoter elements of a gene whose expression is restricted to smooth muscle cells.

Myosin, the major protein constituent of the contractile apparatus in all muscle tissues, is composed of two heavy chains and four light chains. The myosin heavy chain (MHC) molecule contains an ATPase that acts as the chemo-mechanical energy transducer during muscle contraction. The MHC isoforms are encoded by a large multigene family, the expression of which is regulated in a tissue-specific and developmental stage-specific manner (Mahdavi et al., 1986; Gullick et al., 1987; Kropp et al., 1987). In smooth muscle cells, there are at least four types of MHC, two of which are smooth muscle specific (SM1, 204 kDa, and SM2, 200 kDa) and two of which are expressed in both smooth muscle and nonmuscle tissues (NMHC-A, 196 kDa, and NMHC-B/SMemb, 200 kDa). We have shown that the SM1 and SM2 myosin isoforms are products of the same MHC gene generated by alternative RNA splicing (Babij and Periasamy, 1989, Nagai et al., 1989). During early stages of smooth muscle development, SM1 myosin is predominantly expressed, and SM2 myosin appears only during the postnatal period (Nagai et al., 1989). Recent studies have shown that the nonmuscle myosin NMHC-B/SMemb is also expressed in smooth muscle cells during early stages of development (Kuro-o et al., 1991). In the adult stage, however, SM1 and SM2 are the only two myosin isoforms detectable in smooth muscle tissues of rat and rabbit (Kuro-o et al., 1989; Borrione et al., 1989). Interestingly, SM1 but not SM2 myosin is expressed in proliferating smooth muscle cells of arteriosclerotic neointimal lesions (Kuro-o et al., 1991). In addition, we have shown that primary cultured smooth muscle cells express only the SM1-type MHC, and down-regulate the SM2 isoform (Babij et al., 1992). Although the physiological significance of these isoforms is not understood at this time, it is likely that differential expression of the SM1 and SM2 isoforms may have important roles in smooth muscle development and function.

Abnormal neointimal smooth muscle cell proliferation appears to be a common cause of vascular disease such as atherosclerosis or restenosis after coronary angioplasty (Ross, 1986). During this process, smooth muscle cells become more secretory and less contractile and reach a dedifferentiated state. The expression level of myosins is altered, involving a complete loss of SM2 myosin. Several of protein marker proteins have been identified in smooth muscle cells, including α-smooth muscle actin, SMHCs, heavy caldesmon, calponin, and others (Skalli et al., 1986; Nagai et al., 1989; Giukhova et al., 1988; Frid et al., 1992). Several of these markers have been found to be expressed in other tissues, however. For example, α-smooth muscle actin is found in myofibroblasts, skeletal, and cardiac muscle; in cells of the eye lens and hair follicles; and in bone marrow stroma (Sappino et al., 1990; Schmitt-Graff et al., 1990; Jahoda et al., 1991; Peled et al., 1991). On the other hand, the SMHC gene is expressed highly specific in smooth muscle tissues, and its expression pattern correlates well with smooth muscle growth and differentiation. Therefore, the SMHC gene is a good candidate for studying and characterizing factors that influence growth and differentiation specific to smooth muscle cells.

The purpose of this study was to isolate and characterize the rabbit SMHC gene promoter, including its 5′-upstream regions.
required for transcriptionsal activation in smooth muscle cells. In this study, a genomic clone containing the SMHC gene promoter and 8 kb of 5'-flanking region was isolated. DNA sequence analyses revealed several putative cis-regulatory elements including MEF-2-like and CAG-like box elements found in other muscle-specific genes. Gel mobility shift analysis using the MEF-2-like sequence located at -1540 revealed a specific DNA protein complex, whereas the CARG-like element located at -1275 did not show protein binding in our study. Studies using a series of SMHC promoter deletion constructs transiently transfected into primary smooth muscle cells demonstrate that a 2266-bp promoter fragment is capable of high level expression in smooth muscle cells. On the other hand, the same SMHC promoter constructs were unable to drive high levels of chloramphenicol acetyltransferase (CAT) expression in other cell types such as NIH3T3 and C127 cells. These studies demonstrate that the SMHC gene contains promoter elements that are highly tissue specific.

**EXPERIMENTAL PROCEDURES**

**Screening of the Rabbit Genomic Library**—A rabbit genomic library constructed in the cloning vector EMBl-3 SP6/CTT (Clontech Laboratories Inc.) containing 1.4 x 10^9 independent clones and with an insert size range of 8 to 22 kb was used. Screening of the genomic library was carried out as described previously under conditions of high stringency (Zarain-Herzberg et al., 1990) using a -1.3-kb fragment from the SMHC genomic clone A4G4 as a probe (Babjak et al., 1991). The clones were digested with restriction endonucleases, and selected genomic DNA fragments were subcloned into pBluescript M13+ and pJRCATX vectors (Robbins et al., 1989). For Southern blot analysis, restriction endonuclease digests of the genomic clones were separated by electrophoresis in 0.8% agarose gel and blotted onto nitrocellulose (Maniatis et al., 1989). The filters were hybridized with DNA fragments labeled with 32P by random primer extension. The SMHC genomic DNA probes that were used to determine the gene map (see Fig. 1) were as follows: -2.6 kb EcoRI-HindIII fragment containing the TATA box and the first intron exon and a -1.3-kb Sall-EcoRI fragment from the 5'-upstream region of the SMHC genomic DNA (Babjak et al., 1991). DNA sequencing was performed by the procedure of Sanger et al. (1977) using the Sequenase kit (U.S. Biochemical Corp.).

**Construction of the SMHC Gene Promoter-CAT Chimeric Constructs**—A -3.3-kb DNA fragment containing 2266 bp of the 5'-flanking region of the SMHC gene was excised from the clone A4G4 using Sall and HindIII endonucleases. The -3.3-kb fragment was blunt-ended with Klenow enzyme and cloned into the HindIII linker using standard methods (Maniatis et al., 1989). The resulting fragment was ligated in a 5' to 3' orientation into the unique HindIII site of the CAT expression vector pJRCATX (Robbins, 1989). The resulting vector, p2266-CAT (including -2266 bp of 5'-flanking region), was used as a template for 5' to 3' exonuclease III digestion (Erase-a-Base kit, Promega). A series of unidirectionally deleted promoter constructs (see Fig. 3), which were used in transfection studies. The 5'-ends of the deleted constructs were confirmed by dideoxy nucleotide sequencing (Sanger et al., 1977). In addition, the longest construct, p4.2-CAT, was created using a KpnI-HindIII fragment from the genomic clone A4G4.

**Cell Culture**—Smooth muscle cells from rat thoracic aorta were isolated and cultured by a modification of the procedures described by Owens et al. (1986). Male Sprague-Dawley rats (6-8 weeks old) were anesthetized with an intraperitoneal injection of sodium pentobarbital, and the descending thoracic aorta was excised aseptically. Two vessels were placed in Hanks' balanced salt solution (Life Technologies, Inc.) with 1% Antibiotic-Antimycotic (Life Technologies, Inc.), cleaned from adhering fat and connective tissue by blunt dissection, and opened longitudinally. After perfusion of the vessels for 15-25 min at 37 °C in a 5% CO2/95% air atmosphere in Hanks' balanced salt solution in the presence of 0.05 mg/ml collagenase (210 units/mg, Worthington) and 0.16-0.2 mg/ml elastase (4.2 units/mg, Worthington) with 1% Antibiotic-Antimycotic, the adventitia was carefully stripped off under a dissecting microscope, and the luminal surface was scraped with the convex side of curved forceps to remove endothelial cells. The resulting aorta pieces were placed into fresh collagenase-elastase solution, minced into 1-2-mm pieces, and incubated at 37 °C for 15-25 min. After 5-10 min, the confluent cultures were usually obtained after 4-8 days. The confluent cultures were then cultured in a humidified atmosphere of 5% CO2/95% air with medium changes every 2-3 days. Confluent cultures were transfected with 1 x phosphate-buffered saline, harvested with 0.05% trypsin, and reseeded at a 1:5 or 1:6 split ratio into 90-mm tissue culture plates. Since it has been reported that SM1 and SM2 myosin disappeared in cultured rat aortic smooth muscle cells after several passages and was replaced by a 190-kDa nonmuscle MHC (NMCH-A) (Kawamoto et al., 1993), we chose to use rat aortic smooth muscle cells after the first passage for our in vitro transfection studies and nuclear extraction.

**DNA Transfections**—Transient transfections of the SMHC promoter constructs were performed using the calcium phosphate co-precipitation method (Brent et al., 1987). Briefly, duplicate dishes of cells (5 x 10^5 cells/dish) were transfected with 15 μg of each of the SMHC-CAT constructs plus 5 μg of MSVpGAL DNA. DNA for transfections was purified by two successive C127 density gradient centrifugations. After 8-14 h, cells were washed with PBS buffer (137 mM NaCl, 27 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4) and glycerol shocked for 1-2 min. Cells were then washed twice with PBS, and fresh growth medium (10% fetal calf serum) was added. For cultured rat aortic smooth muscle cells and NIH3T3 fibroblasts, cells were harvested 48 h posttransfection. For C127 myotubes, myoblasts were allowed to propagate in growth medium for 10-12 h and then were replaced with differentiation medium to induce myotube formation. C127 myotubes were harvested 8-10 days following medium change.

**CAT Assays**—The transfected cells were rinsed twice with cold Hank's solution and harvested in release buffer (40 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl). The cells were lysed in 250 mM Tris-HCl, pH 7.5, by three cycles of freeze-thawing, and the cell lye was purified by precipitation with the excess sodium acetate eluted from the oxalocolic acid sepharose CL-6B column (Young et al., 1984) were performed on 30% of the cell extract to provide values of relative transfection efficiency. The resulting values were used to normalize the amount of extract added to the subsequent CAT assays. The reaction mixture contained 20 μl of the extract in a 150-μl assay containing 4 mM acetyl coenzyme A (Sigma) and 0.05 μCi of [*3H]chloramphenicol (54 mCi/mmol, Amersham Corp.) in 250 mM Tris-HCl, pH 7.8. The reaction was stopped and extracted with 1 ml of ethyl acetate. The organic phase was dried in 40-80 μl of chloroform-methanol (95:5 system) and then was extracted onto a silica gel thin layer chromatogram. The chromatogram was developed in a chloroform/methanol (95:5) system, dried, and autoradiographed. For quantitating the conversion of chloramphenicol to its acetylated forms, the spots were excised, and the radioactivity was measured in a liquid scintillation counter. All constructs were tested in at least three separate transfection experiments with at least two different plasmid preparations. The construction of the SMHC gene was used as a positive control (Gorman et al., 1982). Experiments in which the transfection efficiencies varied more than 10% between the cultures were discarded.

**Preparation of Nuclear Extracts and Gel Mobility Shift Assays**—Nuclear extracts from rat aortic cultured smooth muscle cells were prepared essentially as described by Dignam et al. (1983). The protein concentration was determined by the Bradford assay (Bradford, 1976). Nuclear extracts were prepared by a centrifugation assay, as previously described by Sambrook et al. (1989). Oligonucleotide was end-labeled with [*32P]ATP, using T4 polynucleotide kinase. Synthetic oligonucleotide top strands were annealed with a 3-fold molar excess of the corresponding bottom strand unlabeled oligonucleotide. Briefly, 2-10 μg of nuclear extracts was incubated in a total volume of 30 μl for 30 min at room temperature in the presence of 1 ng of radioactively double-stranded oligonucleotides added over 30 μl of reaction volume subsequent to addition of [*32P]-labeled oligonucleotide probe, and it incubated at 4 °C for 1 h. Following electrophoresis, the gels were stained in 10% acetic acid, 30% methanol, dried, and autoradiographed.
RESULTS

Isolation of the Rabbit SMHC Gene Promoter and Its 5'-Upstream Region—To initiate studies on the control elements regulating SMHC gene expression, the rabbit SMHC gene promoter and its 5'-flanking region were isolated. A rabbit genomic library constructed in EMBL-3 SP6/T7 was screened with a -1.3-kb SalI-HindIII fragment from the 5'-end of clone ARG4 (Babij et al., 1991) under conditions of high stringency. This fragment was previously identified to contain the TATA box and transcription initiation site. Screening of 2 x 10⁶ independent plaques yielded five positive recombinant phages. These genomic clones were subjected to further analysis by restriction enzyme mapping and Southern blotting. A single clone of interest (ARG8), estimated to contain an 8-kb upstream region of the SMHC gene promoter, was chosen for detailed analysis (Fig. 1).

Sequence Analysis of the SMHC Gene Promoter and Its 5'-Flanking Region—To map precisely the promoter and regulatory sequences in the genomic clone, ARG8 restriction fragments were subcloned into the M13mp19 vector, and DNA sequence analysis was performed. The nucleotide sequence of the SMHC promoter region extending to -2266 bp upstream of the transcription initiation site is shown in Fig. 2. Detailed analysis of the nucleotide sequence upstream of the transcription initiation site revealed a canonical TATA box (5'-TATAAA-3') sequence at position -26 bp (Breathnach and Chambon, 1981). The CAAT box motif usually found around -80 bp was not found. The consensus sequence for Sp1 binding site 5'-GGGGCGG-3' (present in constitutively expressed genes (Dynan and Tjian, 1985)) is represented 3 times at positions -2225 to -2220, -1956 to -1951, and -1502 to -1497; and the complementary sequence 5'-CCGCCCGG-3' is found 3 times at positions -1177 to -1172, -579 to -574, and -523 to -518. Several DNA sequences previously reported to bind specific trans-acting factors are also present in this gene. Two AP2 binding sites are found at positions -48 to -41 (5'-CCGCGGGC-3') and -1248 to -1241 (5'-CCCCGGGGC-3') (Imagawa et al., 1987); and two CF1 recognition sites are found at positions -847 to -842 and -1852 to -1847 (5'-ANATGG-3') (Khoury-Christianson et al., 1992) (Fig. 2). Two CArG box-like motifs, previously identified in skeletal and cardiac muscle actin genes (Minty and Kedes, 1986), are present at -1275 to -1266 (5'-CCATATTGAG-3') and -1194 to -1185 (5'-CTTCTTGGGG-3') upstream of the transcription initiation site. Interestingly, a myocyte enhancer binding factor consensus (MEF-2) (Gossett et al., 1989) like sequence was found at -1540 to -1530 (5'-TATTTAATATAA-3'), and a DNA sequence resembling the MEF-2 site is located at -789 to -779 (5'-TATTAATATAA-3').

Functional Characterization of the SMHC Gene Promoter in Rat Aortic Smooth Muscle Cultures—To test whether the cloned gene carried a functional promoter and the appropriate regulatory sequences, we subcloned two restriction endonuclease fragments containing upstream regions -2266 bp and -1223 bp into the CAT reporter vector, pJRCATX (Fig. 3). Transient transfection analyses of these two promoter constructs into smooth muscle cells showed that the construct p2266 produced high levels of CAT activity, whereas the p1223 construct produced low levels of CAT activity (Fig. 4). To delineate further the boundaries of the functional promoter and regulatory elements within the 5'-flanking region of the SMHC gene, we prepared a series of deletion constructs using the p2266-CAT construct and exonuclease III (Fig. 3). The resulting constructs were verified by DNA sequencing analysis before transfection. Each construct contained different lengths of the 5'-flanking region, the first exon (78 bp, 5'-untranslated), and 787 bp of the first intron linked to the CAT reporter gene. The following deletion constructs were chosen for further analysis: p1947-CAT, p1548-CAT, p1392-CAT, p1042-CAT, p753-CAT, p509-CAT, and p188-CAT (Fig. 3). In addition, we created a larger promoter construct p4.2-CAT, which contained 4.2 kb of the upstream region (Fig. 3).

To define promoter elements, the above described constructs were transfected into primary cultures of rat aortic smooth muscle cells. We have previously shown that these cultures are myosin positive and express high levels of SMHC transcripts (Babij et al., 1992). As shown in Fig. 4, the highest CAT activity was observed with the p2266 SMHCEAT construct and, therefore, this was treated as 100% for comparison with other constructs. The larger promoter construct, p4.2-CAT, produced only 14% of the maximal CAT activity.

The construct p188-CAT, which contained the minimal promoter elements including the TATA box, produced low levels of CAT activity (7% of maximal activity), whereas the promoter construct p509-CAT produced 32% of maximal CAT activity showing a 5-fold increase over p188-CAT. Therefore, the region between -188 and -509 may contain important positive regulatory elements promoting transcription of the SMHC gene. Inclusion of additional upstream sequences from -509 up to -1223 produced a significant decrease in reporter activity. The CAT constructs p753-CAT, p1042-CAT, and p1223-CAT, gave only 12, 11, and 10% of maximal activity, respectively, suggesting that the region between -509 and -1223 acts as a negative regulator on this promoter. Interestingly, the addition of further upstream sequences located between -1223 and -2266 produced significant increases in CAT activity, as seen with promoter constructs p1392-CAT (30%), p1548-CAT (62%), and p1947-CAT (65%). The CAT activity reached the maximum with p2266-CAT (100%). Therefore, a second positively acting upstream region is located between -1392 and -2266. In contrast, the largest construct (p4.2-CAT) produced only 15% of...
Fig. 2. Nucleotide sequence analysis of the rabbit SMHC gene promoter. The putative cis-acting elements are boxed and labeled. Asterisk denotes a mismatch with the consensus sequence. The TATAA element is boxed. The 5'-untranslated region exon is boxed and shaded. E1-E6, E boxes.
Fig. 3. Schematic representation of the SMHC promoter-CAT fusion constructs. The relative positions of the putative cis-activating regulatory elements, TATA box, and 5'-untranslated region (5'-UT) are indicated. Each SMHC-CAT construct is named based on the number of nucleotides upstream of the transcriptional initiation site. Constructs were generated by exonuclease III and S1 digestion (see “Experimental Procedures”) and fused to the CAT expression vector pJRCATX. E, E-box.

maximal CAT activity, suggesting that inclusion of further upstream regions act negatively in our in vitro assays.

Transfection of SMHC-Promoter/CAT Constructs into NIH3T3 Fibroblasts and C2C12 Skeletal Muscle Cells—To determine whether the SMHC gene promoter is specific for smooth muscle cells or if it is active in other cell types, the SMHC promoter deletion-CAT constructs were transiently transfected into NIH3T3 fibroblasts and C2C12 myotubes. As
shown in Fig. 5, these constructs did not yield significant CAT activity in NIH3T3 or C2C12 cells (maximal CAT activity was 3-fold over background; whereas the CAT activity produced by pSV$_C$CAT was more than 60-fold over background, data not shown) as compared with smooth muscle cells. These results are in agreement with our in vivo observations, where SMHC gene expression is limited to smooth muscle cells (Nagai et al., 1989).

**DISCUSSION**

In the present study, we have isolated and characterized the rabbit SMHC gene promoter. Analysis of the immediate 5'-upstream sequences reveals a canonical TATA box sequence at -26 bp (5'TATAAA-3') (Breathnach et al., 1981). The CAAT box usually located around -80 bp is not found. The proximal 5'-flanking region of the SMHC gene is highly GC-rich. The SP-1 binding site (5'GGCCGG-3') described in promoters of several genes (Dynan and Tijan, 1985) is present 4 times. The rabbit SMHC gene also contains two CArG box-like motifs. The CArG motif at -1194 position differs from the CC(A/T)$_4$GG configuration since only 5 of the 6 core A/T residues are present. The second CArG motif that is found at position -1275 also differs from the consensus in that it ends in AG nucleotides instead of GG. The CArG motif, which was first described in the 5'-regulatory region of striated muscle a-actin genes, is also found in several other muscle-specific genes (Zakut et al., 1982; Bergsma et al., 1986; Miwa and Kedes, 1987; Sternberg et al., 1988). The importance of CArG elements in the regulation of the smooth muscle a-actin promoter has been established (Carroll et al., 1986). Another important regulatory element, namely the MEF-2-like site, is situated at positions -1540 and -789 bp in the SMHC upstream region. MEF-2 is a trans-acting factor and is expressed abundantly in cardiac, skeletal, and smooth muscle tissues (Yu et al., 1992; Gossett et al., 1989). MEF-2 recently has been shown to be important for the transcriptional regulation of a number of skeletal and cardiac muscle-specific genes and to act in concert with myogenic regulatory proteins such as myogenin in myogenesis (Edmondson et al., 1992). In addition, six E-box motifs are found in the 5'-flanking region between -374 and -2109. E-box motifs have been shown to bind muscle-specific and basic helix-loop-helix trans-activating factors (Olson, 1990) and are known to be important for muscle-specific gene expression (Blackwell and Weintraub, 1990). The exact role of these sequences in the SMHC gene regulation is yet to be defined.

Our transient transfection analyses have identified multiple positive and negative regulatory elements in the 5'-flanking region of the SMHC gene. Most notably, the promoter element located between -1392 and -2266 produced the highest reporter CAT activity upon transfection into smooth muscle cells. Another region of importance is located between -188 and -509, since inclusion of this region produced a large increase in CAT activity over that of the minimal promoter. These two
regions act as positive regulatory elements. Interestingly, progressive deletions in the upstream region from -2266 bp to -1392 resulted in a gradual loss of CAT activity from 32 to 10%. This finding suggests that there are multiple positive regulatory elements in this region, and that maximal expression depends upon cooperative interactions between these elements. Actually, four out of six E-boxes are found within the upstream region from -2266 to -1392, which exhibits the highest promoter activity.

Our deletion analysis also revealed negative elements in the SMHC promoter. In particular, when the DNA sequence between -509 and -753 bp is included in the promoter construct, there is a significant decrease (~2.7 fold) in activity in comparison with the promoter construct that includes only 509 bp of upstream DNA.

Interestingly, the deletion of a MEF-2-like site present at -1540 bp results in a significant loss of CAT activity (from 62 to 32%). However, deletion of the second MEF-2-like site present at -789 does not modify CAT activity. The deletion of the CArG-like motif, present at -1275 results in a loss of CAT activity (from 32 to 10%), whereas the deletion of the CArG motif at -1194 did not affect CAT activity.

Gel mobility shift analyses using the MEF-2-like (A/T-rich) sequence (5'-TATTAATATAA-3') located at -1540 to -1530 showed that this element binds to a specific protein complex in nuclear extracts from vascular smooth muscle cells. However, this binding was not competed out by the muscle creatine kinase MEF-2 consensus element. Also, the protein binding to this element was not modified by a MEF-2 antibody, suggesting that the nuclear factor binding to this A/T-rich sequence is not a MEF-2 protein. Further analyses will be needed to define the dissimilarity between this factor/A-T-rich element binding protein and another already known proteins that bind to some A/T-rich elements such as related to serum response factor (SRSP) (Pollock and Treisman, 1991) or Antp-type homeoproteins (Beachy et al., 1988; Muller et al., 1988). On the other hand, the CArG-like element (5'-CCATATTAG-3') found at -1275 to -1266 did not demonstrate any specific protein binding. To establish firmly the functional role of these and other elements in the SMHC gene, it will be necessary to perform additional experiments.

In the smooth muscle α-actin gene, a proximal E-box has been reported to be important for expression of the gene (Carroll et al., 1988). Although E-box motifs are present both in the proximal and distal 5'-upstream regions, the exact function of these E-box elements in the SMHC promoter is unclear. However, the presence of constitutively expressed basic helix-loop-helix transcription factors in smooth muscle cells (Blackwell and Weintraub, 1990) suggests that E-boxes may have important roles in SMHC gene regulation.

In this study, we show that the SMHC promoter constructs, when transfected into NIH3T3 fibroblasts and C2C12 muscle cells, did not produce significant CAT activity. This further demonstrates that the SMHC gene contains promoter elements that restrict its expression to smooth muscle cells. This is in contrast to the smooth muscle actin promoter, which has been shown to be expressed not only in smooth muscle cells but also in fibroblasts and myoblasts (Carroll et al., 1988).

In summary, the results of this study demonstrate that the 5'-flanking region of the rabbit SMHC gene contains multiple positive and negative regulatory elements extending to -2266 bp upstream of the transcription start site. Furthermore, we demonstrate that the SMHC promoter directs high levels of CAT expression only in cultured rat smooth muscle cells but not in NIH3T3 fibroblasts or C2C12 myotubes. Therefore, our in vitro transfection studies confirm our earlier observations that the SMHC gene expression is highly restricted to smooth muscle cells. This is also the first study describing functional elements of a smooth muscle-specific promoter. Of particular interest in future studies will be the definition of discrete DNA elements controlling SMHC gene expression in smooth muscle cells. The promoter provides an important tool toward understanding mechanisms regulating smooth muscle cell growth and differentiation and may serve to target expression of specific gene products into smooth muscle cells.

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REFERENCES

Babij, P., Kelly, C., and Periasamy, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10876-10880
Babij, P., Kawamoto, S., Adelstein, R. S., and Periasamy, M. (1992) Am. J. Physiol. 263, C607-C613
Babij, P., and Periasamy, M. (1989) J. Mol. Biol. 210, 673-679
Beachy, P. A., Krasnow, M. A., Gavis, E. R., and Hogness, D. S. (1986) Cell 55, 1069-1081
Bergsma, D. J., Grichuk, J. M., Gossett, L. M. A., and Schwartz, R. J. (1989) Mol. Cell. Biol. 9, 2859-2871
Blackwell, T. K., and Weintraub, H. (1990) Science 250, 1104-1110
Borronese, A. C., Zanellato, A. M. C., Scannapieco, G., Pauletto, P., and Sartore, S. (1988) Mol. Cell. Biol. 8, 1051
Brent, R., and Chambon, P. (1986) EMBO J. 5, 2327-2334
Breathnach, R., and Chambon, P. (1981) Annu. Rev. Biochem. 50, 349-383
Brenn, P. R., Kingston, R., Moore, D., Seidman, J., and Struhl, K. (1987) Cell 55, 143-147
Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
Breathnach, R., and Chambon, P. (1981) Annu. Rev. Biochem. 50, 349-383
Brenn, P. R., Kingston, R., Moore, D., Seidman, J., and Struhl, K. (1987) Current Protocols in Molecular Biology, Vol. 2, pp. 9.1.1-9.1.9, John Wiley and Sons, Inc., New York
Carroll, S. L., Bergsma, D. J., and Schwartz, R. J. (1986) J. Biol. Chem. 261, 8965-8976
Carroll, S. L., Bergsma, D. J., and Schwartz, R. J. (1986) Mol. Cell. Biol. 6, 2462-2475
Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475-1489
Dynan, W. S., and Tjian, R. (1985) Nature 316, 774-778
Edmondson, D. G., Cheng, T. C., Cserjesi, P., Chakraborty, T., and Olson, E. N. (1992) Mol. Cell. Biol. 12, 3685-3697
Frid, M. G., Shekhonin, O. V., and Frid, M. G. (1989) Eur. J. Biochem. 183, 180-189
Glukhova, M. A., Babij, P., Kawamoto, S., and Periasamy, M. (1991) Mol. Cell. Biol. 11, 2327-2334
Gossett, L. A., Kelvin, D. J., Sternberg, E. A., and Smirnov, V. N. (1988) Nucleic Acids Res. 15, 9543-9546
Gorman, C. M., Mofett, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044-1061
Gossett, L. A., Kelvin, D. J., Sternberg, E. A., and Olson, E. N. (1989) Mol. Cell. Biol. 9, 5022-5033
Gulick, J., Krehm, K., and Robbins, J. (1987) Eur. J. Biochem. 170, 79-84
Imagawa, M., Chiu, R., and Karin, M. (1987) Cell 56, 1-20
Imagawa, M., Chiu, R., and Karin, M. (1987) Cell 51, 251-260
Imagawa, M., Chiu, R., and Karin, M. (1987) Cell 51, 251-260
Jahoda, C. A. B., Reynolds, A. J., Chepponner, C., Forestier, J. C., and Gabbiani, G. (1991) J. Biol. Chem. 266, 9796-9802
Kawamoto, S., and Adelstein, R. S. (1987) J. Biol. Chem. 262, 7282-7288
Khoury-Christenson, A. M., King, D. L., Hatzivassiliou, E., Cesas, J. E., Hallenbeck, P. L., Nikodem, V. M., Mitsialis, S. A., and Kafatos, F. C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11055-11057
Kropp, K. E., Guldick, J., and Robbins, J. (1987) J. Biol. Chem. 262, 16536-16545
Kuro-o, M., Nagai, R., Nakahara, K., Kato, H., Tsai, R. T., Tsuchimoto, H., Yazaki, Y., Okhobo, A., and Takaku, F. (1991) J. Biol. Chem. 266, 3768-3773
Kuro-o, M., Nagai, R., Tsuchimoto, H., Kato, H., Yazaki, Y., Okhobo, A., and Takaku, F. (1989) J. Biol. Chem. 264, 19772-19775
Mahdavi, V., Strehler, E. E., Periasamy M., Wiesczrek, D., Izumo, S., Grund, S., Strehler, M. A., and Nadal-Ginard, B. (1986) UCLA (Univ. Calif. Los Angel.) Symp. Mol. Cell. Biol. New Ser. 29, 345-361
Mancini, T., Fritsch, E. F., and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, pp. 8-11, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Minty, A., and Kedes, L. (1986) Mol. Cell. Biol. 6, 2125-2136
Miwa, T., and Kedes, L. (1987) Mol. Cell. Biol. 7, 2803-2813
Muller, M., Affett, M., Leupin, W., Ottung, G., Wuthrich, K., and Gehring, W. (1988) EMBO J. 7, 4299-4304
Nagai, R., Kuro-o, M., Babij, P., and Periasamy, M. (1989) J. Biol. Chem. 264, 9734-9737
Olsen, E. N. (1990) Genes & Dev. 4, 1454-1461
Owens, G. K., Loeb, A., Gordon, D., and Thompson, M. M. (1990) J. Cell Biol. 102, 343-352
Peled, A., Zipori, D., Abramsky, O., Ovadia, H., and Sbescin, E. (1991) Blood 78, 304-309
Pollock, R., and Treisman R. (1991) Genes & Dev. 5, 2327-2341
Robins, J., Subramaniam, A., and Gulick, J. (1989) Gene (Amst.) 85, 541-544
Reus, F. (1986) N. Engl. J. Med. 314, 488-500
Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467
Sacco, R., Schiure, W., and Gabbiani, G. (1990) Lab. Invest. 63, 144-161
Schmitt-Graff, A., Pau, H., Spahr, R., Piber, H. M., Skalli, O., and Gabbiani, G. (1990) Differentiation 43, 115-122
Skalli, O., Roopra, P., Trzeciak, A., Benzonana, G., Gillessen, D., and Gabbiani, G. (1986) J. Cell Biol. 103, 2777-2780
Sternberg, E. A., Spiz, G., Perry, W. M., Vizard, D., Weil, T., and Olson, E. N. (1988) Mol. Cell. Biol. 8, 2896-2900
Sukovitch, D. A., Stabber, J., and Periasamy, M. (1993) Nucleic Acids Res. 21, 2723-2728
Yu, Y. T., Brettard, R. E., Smitt, L. B., Lee, Y., Mahdavi, V., and Nadal-Ginard, B. (1992) Genes & Dev. 6, 1783-1798
Zakut, R., Shani, M., Givol, D., Neuman, S., Yafo, D., and Nudel, U. (1982) Nature 298, 857-859
Zazzo-Heusberg, A., MacLennan, D. H., and Periasamy, M. (1990) J. Biol. Chem. 265, 4670-4677