Evolutionarily Conserved Promoter Region Containing CArG*-Like Elements Is Crucial for Smooth Muscle Myosin Heavy Chain Gene Expression

A. Zilberman, V. Dave, J. Miano, E.N. Olson, M. Periasamy

Abstract—In recent years, significant progress has been made toward understanding skeletal muscle development. However, the mechanisms that regulate smooth muscle development and differentiation are presently unknown. To better understand smooth muscle–specific gene expression, we have focused our studies on the smooth muscle myosin heavy chain (SMHC) gene, a highly specific marker of differentiated smooth muscle cells. The goal of the present study was to isolate and characterize the mouse SMHC gene promoter, since the mouse promoter would be particularly suited for in vivo promoter analyses in transgenic mice and would serve as a tool for targeting genes of interest into smooth muscle cells. We report here the isolation and characterization of the mouse SMHC promoter and its 5′ flanking region. DNA sequence analysis of a 2.6-kb portion of the promoter identified several potential binding sites for known transcription factors. Transient transfection analysis of promoter deletion constructs in primary cultures of smooth muscle cells showed that the region between −1208 and −1050 bp is critical for maximal SMHC promoter activity. A comparison of SMHC promoter sequences from mouse, rat, and rabbit revealed the presence of a highly conserved region located between −967 and −1208 bp. This region includes three CArG/CArG*-like elements, two SP-1 binding sites, a NF-1–like element, an Nkx2–5 binding site, and an Elk-1 binding site. Gel mobility shift assay and DNase I footprinting analyses show that all three CArG/CArG*-like elements can form DNA-protein complexes with nuclear extract from vascular smooth muscle cells. Protein binding to the CArG* elements can be competed out by either serum response element or by an authentic CArG element from the cardiac α-actin gene. Using a serum response factor (SRF) antibody, we demonstrate that SRF is part of the protein complex. In addition, we show that cotransfection with the SRF dominant-negative mutant expression vector abolishes SMHC promoter activity, suggesting that SRF protein plays a critical role in SMHC gene regulation. (Circ Res. 1998;82:566-575.)

Key Words: smooth muscle cell • myosin heavy chain • gene expression

Smooth muscle cells have been the subject of intense study because abnormal growth and proliferation of SMCs are involved in the pathogenesis of atherosclerosis and other forms of vascular disease. Various proximal signals that influence growth and differentiation of SMCs, including growth factors, their receptors, and the intracellular signaling pathways, have been extensively studied. However, the cellular and molecular basis of smooth muscle differentiation remains poorly understood. More important, the transcription factors responsible for smooth muscle commitment and differentiation are not yet defined.

The differentiation of smooth muscle (cells) involves expression of specific protein markers and acquisition of functional properties characteristic of a fully mature SMC phenotype. To date, several smooth muscle–specific protein markers have been identified, including smooth muscle α-actin, γ-actin, smooth muscle calponin, SM22α, h-caldesmon, myosin light chains, and SMHCs (SM1 and SM2). Several of these SMC markers, including smooth muscle α-actin, SM22α, and calponin, are expressed in developing skeletal and cardiac muscle tissues and then become restricted to SMCs in the adult stages. Thus, these markers and their genes provide unique reagents to study how they become restricted to SMCs during development. On the other hand, the expression of SMHC isoforms SM1 and SM2 is found only in smooth muscle tissues. SM1 and SM2 are the products of a single myosin heavy chain gene generated by alternative RNA splicing, and their expression is developmentally regulated. The SM1 isoform is expressed both in embryonic and adult stages, whereas the expression of the SM2 isoform is restricted to fully differentiated/mature SMCs. Recent analyses of SMHC gene expression during mouse embryogenesis using in situ hybridization demonstrated that SMHC gene expression is restricted to SMCs and is not found in other cell types, including cardiac and skeletal muscle cells, at any stage of development. The expression of SMHC mRNA was shown to appear at 10.5 dpc...
in the dorsal aorta and at 11.5 dpc in the outflow tract and to remain confined to the SMC lineage as development progressed, with peripheral blood vessels of the head, musculature, and intersomitic region initially displaying a positive signal at 13.5 to 14.5 dpc and the esophagus, bladder, and ureter showing intense labeling at 17.5 dpc. These results established SMHC as a highly specific marker for the SMC lineage.

Although studies in skeletal and cardiac muscle cells have identified several transcription factors that play a critical role in the formation of these cell types, the mechanisms regulating smooth muscle development and differentiation are poorly understood. In recent years, skeletal muscle development has become the paradigm for understanding tissue-specific gene activation and cell differentiation because of the discovery of master regulatory genes, namely, the MyoD family. However, the MyoD family of genes or related helix-loop-helix proteins that regulate skeletal muscle differentiation are not expressed in SMCs.20 However, the role of MEF-2 proteins in SMC differentiation is yet to be established.

We believe that the cloning and identification of smooth muscle–specific genes and their promoters will contribute to the dissection of the molecular mechanisms controlling smooth muscle myogenesis. The recent cloning and identification of smooth muscle–specific genes, including SMHC, α- and γ-actins, SM22α, SM22β, calponin, and α-smooth muscle actin, provide unique reagents toward understanding smooth muscle–specific gene expression. Our laboratory has previously reported the isolation and characterization of the rabbit SMHC gene promoter.21 The goal of the present study was to isolate and characterize the mouse SMHC, mSMHC, gene promoter. The mSMHC gene promoter would be particularly suited for in vivo promoter analyses using transgenic mice and would serve as a powerful tool for targeted expression of specific proteins into SMCs. To this effect, we have isolated an mSMHC genomic clone that contains 9.5 kb of upstream promoter region and characterized an ≈2.6 kb of the promoter region by DNA sequence analysis. While this work was in progress, Watanabe et al22 reported the characterization of 1.5 kb of the mSMHC gene promoter and showed that −188 bp is sufficient for high-level expression in SMCs. In the present study, we provide additional data demonstrating that a highly conserved region located between −1208 and −1050 bp is critical for the SMHC promoter activity. This region shows a high degree of sequence similarity between mouse, rat, and rabbit SMHC promoters and includes two CArG*-like elements, one authentic CArG element, two SP-1 binding sites, and two NF-1–like binding sites. Using GMSA and DNAase I footprinting, we demonstrate that CArG*-like elements bind specific SRF-containing protein complexes in SMCs. In the present study, we provide evidence that SRFs play a critical role in regulating SMHC promoter activity.

**Materials and Methods**

**Screening of the Mouse Genomic Library**

To screen the mouse genomic library, a PCR probe corresponding to the mSMHC gene promoter was generated using two primers. Oligo 5′-GTTGGATTACGAGAGAAGCACCGGATG-3′ corresponds to the mSMHC 5′ untranslated region sequence; oligoB 5′-GACTCTTTTATGGCTGG-3′ corresponds to a highly conserved promoter region found in the rabbit and rat SMHC gene promoters (−1072 to −1053 bp in the rabbit promoter). PCR amplification of the mouse genomic DNA with the above primers yielded a 1.1-kb DNA fragment; DNA sequence analysis of this fragment showed that it contained 63 bp of the 5′ untranslated region plus ≈1.0 kb of the 5′ flanking region of the mSMHC promoter, with a canonical TATA box at −28 bp. The 1.1-kb genomic fragment was used to screen a mouse (Svj 129) genomic library constructed in the A Dash II vector containing 1.1×10⁹ independent clones with insert sizes ranging from 17 to 21 kb. Phage screening yielded three positive clones, mSMHC-614, mSMHC-723, and mSMHC-813. Genomic clones were mapped by Southern blotting analysis.20 DNA sequencing was performed according to the procedure of Sanger et al21 and by automated DNA sequencing.22 Nucleotide sequence data were analyzed using MacDNAsis software (Hitachi).

**Construction of the mSMHC Gene Promoter–CAT Chimeric Constructs**

A 2.8-kb promoter fragment containing 2565 bp of the 5′ flanking region, 90 bp of exon I, and 140 bp of intron I was excised from the clone AmSMHC-813 using XhoI endonuclease. The fragment was ligated in a 5′ to 3′ orientation into a unique XhoI site of pBlCAT6 expression vector,23 and orientation was confirmed by DNA sequencing.24 A 140-bp fragment was removed from the 2.8-kb CAT construct using BglII endonuclease. The resulting 2.6-kb mSMHC-CAT construct was used for producing additional SMHC gene promoter–CAT deletion constructs. The SMHC SMHC promoters (p781CAT, p1208CAT, p1128CAT, p1050CAT, p366CAT, and p121CAT) were made using native restriction sites in the 5′ flanking region (StuI, EcoRI, XhoI, BglII, and PstI, respectively).

**Cell Culture**

SMCs from rat thoracic aorta were isolated and cultured as described in Katoh et al.25 Briefly, rat thoracic aortas were excised, washed in HBSS, cleaned from adhering fat and connective tissue, and opened with a stainless-steel screen, and fetal calf serum was added to a final concentration of 30%. After the isolated cells were collected by automated DNA sequencing,22 Nucleotide sequence data were analyzed using MacDNAsis software (Hitachi).
DNA Transfections and CAT Assays
mSMHC promoter constructs (10 μg) containing CAT reporter genes were co-transfected with MSV-β-Gal (5 μg) into cultured rat aortic SMCs, Sol 8, and NIH 3T3 cells by the calcium phosphate coprecipitation method. Cells were incubated with DNA for 5 hours, then washed twice with PBS, and glycerol-shocked for 1 to 2 minutes. Fresh growth medium containing 10% FBS was added after two washes with PBS. Cultured rat aortic SMCs and NIH 3T3 cells were harvested 48 to 72 hours after transfection. Sol 8 myogenic extract was induced to differentiate by switching to a medium containing 5% horse serum (Life Technologies, Inc) supplemented with 10% fetal calf serum. Sol 8 cells were also harvested 48 to 72 hours after transfection. Myoblasts were induced to differentiate by switching to a medium containing 5% horse serum (Life Technologies, Inc) supplemented with 10% fetal calf serum. Cells after the first or the second passage have been used for in vitro transfection studies and for preparation of nuclear extracts. Sol 8, a mouse soleus muscle cell line, was maintained in DMEM (Life Technologies, Inc) supplemented with 10% fetal calf serum. Sol 8 myoblasts were induced to differentiate by switching to a medium containing 5% horse serum (Life Technologies, Inc). NIH 3T3, a mouse fibroblast cell line, was maintained in DMEM supplemented with 10% fetal bovine serum.

DNA Gel Mobility Shift Assay
Nuclear extract from cultured SMCs were prepared according to Gossett et al. The protein concentration was determined by the Lowry method. Each DNAse I mapping assay contained 5 fmol (≈10 000 cpm) of the end-labeled fragment in 50 μL of 2× binding buffer. Rat aortic nuclear extract was added at increasing concentrations of 100 and 200 μg, and the binding reaction was carried out for 10 minutes on ice. DNAse I (50 μL, Worthington) at a concentration of 5 μg/mL in 10 mmol/L Tris-Cl, pH 8.0, 10 mmol/L MgCl2, and 1 mmol/L CaCl2 was added to the mixing buffer and incubated for 30 seconds at room temperature. The reaction was terminated by the addition of 100 μL of the stop solution (200 mmol/L NaCl, 30 mmol/L EDTA, and 1% SDS), phenolized, and ethanol-precipitated. The samples were heat-denatured in the loading dye containing 80% formamide and loaded on a 6% sequencing gel. The A+G ladders were generated by the Maxam-Gilbert chemical sequencing method.

Results
Isolation of the mSMHC Gene Promoter
A mouse genomic library (Syg 129) was screened with a 1.1-kb PCR-amplified DNA fragment (described in “Materials and Methods”) containing 1 kb of the mSMHC gene promoter and part of the first exon (63 bp). Screening of 1.1X106 independent plaques yielded three different genomic clones: AmSMHC-614, AmSMHC-723, and AmSMHC-813. Restriction mapping and Southern blotting revealed that the genomic clones AmSMHC-614 and AmSMHC-813 overlapped and contained the promoter region. The genomic clone AmSMHC-614 carried an ≈18-kb insert, which included 9.5 kb of 5′ flanking sequence, exon 1, and ≈9 kb of the intron (Fig 1). The clone AmSMHC-813 contained only 2.6 kb of the 5′ flanking sequence but included exon 1, the entire first intron (≈18 kb), and exon 2. The first exon encodes a portion of the 5′ untranslated region (91 nt), and the sequence is homologous (90%) to the rat gene. Interestingly, the size of the first intron (18 kb) in the mouse SMHC gene is very similar to that of the rabbit SMHC gene.

Characterization of the SMHC Gene Promoter Region by DNA Sequence Analysis and Comparison of the Promoter Elements Between Mouse, Rat, and Rabbit SMHC Genes
The mSMHC promoter was recently cloned and sequenced from −1526 to +47 bp. In the present study, we performed additional sequence analysis up to −2565 bp of the mouse promoter. The nucleotide sequence of the mSMHC promoter region (−2565 bp from the transcription initiation site) is shown in Fig 2. The start site of transcription was determined by an RNase protection assay and found to map precisely to the site reported by Watanabe et al. (data not shown). Sequence analysis revealed a canonical TATA box at −95 bp.
TATAAA box at position 28 bp, a GATA box motif at position 243 bp, and two CArG boxes: one at 2977 bp (CArG 1, 5′-CCTTTTATGG-3′) and one at 29132 bp (CArG 2, 5′-CCAAAATAGG-3′). Also, two CArG*-like elements were found: one at 21102 bp (CArG*I, 5′-CCTTTTATGG-3′) and one at 211320 bp (CArG*II, 5′-CCAAAATAGG-3′). An MEF-2–like sequence was identified at 21374 bp, and two A/T-rich elements were also found at -2563 bp and -2085 bp. Sequence comparison with rabbit SMHC and rat SMHC gene promoters revealed that within the first 1208 bp of the proximal promoter, there was a high degree of sequence homology: mouse compared with rat was 81%, and mouse compared with rabbit was 65%. When compared with the rat promoter, the mouse promoter showed 65% homology within -1208 bp, whereas comparison with the rabbit promoter showed 55% homology within -1800 bp.

In addition, we identified a highly conserved region located between -947 bp and -1208 bp in all three SMHC promoters. This region contains CArG*II at -1179 bp (5′-CCATTTTAG-3′), CArG*I at -1102 bp (5′-CCCTTTGGG-3′), and one authentic CArG 1 box at 2977 bp (5′-CCAAAATAGG-3′). The two CArG*-like boxes were found in mouse, rat, and rabbit SMHC promoters and were identical in their nucleotide sequence. These CArG*-like elements occurred at the same position and showed similar spatial organization in all three promoters. In addition, three conserved SP-1 binding sites (5′-GGGAGG-3′ at -1195 and -1069 bp and 5′-CCCGCCC-3′ at -1086 bp) and two NF-1–like binding sites (at -1147 and -1002 bp) were found in this region. Although the overall sequence conservation between different SMHC gene promoters is poor, the 1208- to 947-bp sequence shows 90% of sequence homology between mouse and rat and 70% between mouse and rabbit promoters.

Functional Characterization of the SMHC Gene Promoter in Primary Vascular Smooth Muscle Cultures and Its Tissue Specificity

To characterize important cis elements regulating SMHC gene expression, the SMHC-promoter deletions were linked to the CAT reporter vector, pBLCAT6 (Fig 4). Transient transfection analyses into primary cultures of rat aortic SMCs revealed that the promoter–CAT construct mSMHC-1208 was the most active (30-fold over pBLCAT6) and therefore was treated as 100% activity for comparison with other constructs. Inclusion of more upstream sequences (p1831CAT and p2565CAT) decreased promoter activity (Fig 5). The shortest promoter construct, p121CAT, which contained the TATA box and two SP-1 binding sites, produced only 4% of the maximal CAT activity, whereas the p366CAT produced 35% of CAT activity (Fig 5).
To investigate the functional importance of the highly conserved promoter region (−1208 to −947 bp), two additional deletion constructs were produced. The construct p1128CAT was created by deleting the CArG*II region. In addition, we created the p1050CAT construct, in which both the CArG*II and CArG*I regions were deleted. Interestingly, when transfected into primary vascular SMCs, the p1128CAT construct produced 57% of maximal CAT activity (Fig 5), whereas the deletion of both CArG*II and CArG*I resulted in a drastic reduction of the reporter activity (only 13% of maximal activity). These results suggest that both CArG*I and CArG*II elements are important for maximal promoter activity.

To ascertain whether the mSMHC promoter is smooth muscle specific, the promoter deletion CAT constructs were transiently transfected into NIH 3T3 fibroblasts and Sol 8 myotubes. As shown in Fig 5, none of the promoter constructs produced significant CAT activity. However, the control plasmid, PSV.CAT, gave a high level of CAT activity (30-fold over background) in these cell types (data not shown). These results support our previous observations that SMHC gene expression is restricted to SMCs.21

DNase I Footprinting Reveals Protection of CArG*I and CArG*II-Like Elements

Our transient transfection analysis revealed that deletion of the 160 bp of the promoter sequence between −1208 and −1050 bp decreased CAT activity from 100% to 13%. An inspection of the deleted sequence revealed the presence of two CArG-like boxes (CArG*I sequence [5′-CCTTTTTGGG-3′] and CArG*II sequence [5′-CCATATTTAG-3′]), one consensus-inverted Elk-1 site49 (5′-CAGGAAT-3′), three SP-1 sites (two identical 5′-GGGAGG-3′ sites and one 5′-CCCGCC-3′ site), and one NF-1–like site (5′-TGGTATGCCC-3′). To determine the nature of protein binding sites within the −1208- to −1050-bp promoter region, DNase I footprinting was performed using SMC nuclear extract (Fig 6). DNase I footprinting of the sense strand showed strong protection of the CArG*II sequence (footprint IV). In addition, footprinting was also observed with the CArG*I binding site (footprint I). A significantly large area of protection was also observed at footprint III (5′-CACGCTGGAATTCCTG-3′). A close examination of this region reveals a putative protein binding palindromic sequence, 5′-TGGAATTCCT-3′. Interestingly, this protected region contains a consensus-inverted Elk-1 site, 5′-CAGGAAT-3′. Footprint II indicated protection of 5′-TTTCGAGAATTGCGCC-3′. A transcription factor bind-
ing-site search using the program TFSEARCH (version 1.3) showed maximum homology between the Nkx2-5 binding site \( \text{CGATATTG} \) and footprint II with a G instead of a T (shown in bold).

**CArG*-Like Elements Form Specific Protein Complexes With Vascular Smooth Muscle Nuclear Extract**

To determine precisely the nature of protein binding in the highly conserved region (from \(-1208\) to \(-947\) bp), we performed GMSAs using \(^{32}\)P-labeled oligonucleotides corresponding to CArG*I, CArG*I, and CArG 1 elements (Fig 7). An oligonucleotide containing SRE derived from the human c-fos promoter was used as a positive control for SRF protein binding and for competition analysis. As shown in Fig 7, CArG*I, CArG*I, and c-fos SRE form a similar-sized DNA-protein complex. However, CArG*I (lanes 1 to 4) and CArG*I (lanes 5 to 8) sequences consistently produced more abundant complexes compared with the CArG 1 element (lanes 9 to 12) and the c-fos SRE (lanes 13 to 15).

Interestingly, 200-fold molar excess of c-fos SRE oligo was able to compete protein binding to CArG*I (lane 4) and to CArG 1 (lane 12) sequences. In contrast, SRE oligo was unable to compete out protein binding to the CArG*II sequence (lane 8). This result was unexpected.

In addition, the cardiac \( \alpha \)-actin CArG element (shown to bind SRF) was used as a competitor (Fig 8). Interestingly, the cardiac actin CArG box was able to compete protein binding to the CArG*II element when added in the range of 100- to 200-fold molar excess (Fig 8, lanes 3 to 5). Similarly, unlabeled CArG*II-like oligonucleotide added in 100- to 200-fold molar excess could compete protein binding to the cardiac actin CArG element.
Cardiac α-actin CArG box (Fig 8, lanes 8 to 10). However, the cardiac actin CArG box produced a protein complex that is distinct from that of the CArG*I-like element in vascular SMCs (Fig 8, lanes 1 and 6). These results suggest that the CArG elements from different promoters may bind different SRF-containing protein complexes.

It was previously shown that the 5′-CC(A/T)6AG-3′ sequence present in *Xenopus* MyoDβ promoter does bind MEF-2 protein.5 However, in our experiments the consensus oligo for MCK MEF-2 (100-fold and 200-fold molar excess) did not compete CArG*I protein binding, suggesting that this sequence does not bind MEF-2 protein in vascular SMCs. The SP-1 consensus DNA fragment also failed to compete protein binding.

**SRF Protein Is Part of the DNA-Protein Complex Formed With CArG*I or CArG*II Elements**

To determine whether SRF is part of the protein complex that binds to the CArG*I or CArG*II element, we performed GMSA with an SRF antibody.43 For comparative analysis, we included c-fos SRE, which is demonstrated to bind SRF protein. Our results show (Fig 9) that addition of SRF antibody produced a supershift with CArG*I, CArG*II, and c-fos SRE. However, the antibody did not supershift the entire complex. This is probably due to the low concentration of SRF antibody used in this assay, or, possibly, other DNA binding proteins that are part of this complex interfere with the antibody’s access to SRF protein. Furthermore, we found that in vitro–translated SRF protein binds to both CArG*I and CArG*II elements (data not shown).

**Cotransfection of Dominant-Negative SRF to Vascular SMCs Decreased SMHC Promoter Activity**

To determine whether the smooth muscle myosin promoter was a direct target for SRF activity, the SRFpm1 mutant expression vector was cotransfected with the p1208CAT SMHC promoter construct into primary vascular smooth muscle cultures as described in “Materials and Methods.” The SRFpm1 mutant was previously demonstrated to block the endogenous SRF DNA-binding activity by forming heterodimers with any available wild-type SRF.52 In response to SRFpm1 overexpression, the SMHC promoter activity was decreased significantly.

At concentrations 1 μg and higher, the SRF mutant vector abolished 85% of SMHC promoter activity (Fig 10). This result demonstrates that SRF proteins are critical for the SMHC promoter activity.
Discussion
The goal of the present study was to isolate and characterize the mouse SMHC (mSMHC) gene promoter. We report here the isolation of the 9.5-kb mSMHC gene promoter and its functional characterization in primary cultures of SMCs. The mSMHC promoter is active only in SMCs but not in Sol 8 or NIH 3T3 cells, which is consistent with our earlier findings for the rabbit SMHC promoter.21 Our transient transfection analysis using the mSMHC promoter deletion constructs identified a region between −1208 and −1050 bp in the SMHC promoter as highly important for its expression in SMCs. This region includes two CArG-like elements (CArG*I [5′-CCATATTTAG-3′] and CArG*II [5′-CCTTTTTGGG-3′]), two SP-1 binding sites (both 5′-GGAAGG-3′), and an NF-1-like element (5′-TGGTATGCCAC-3′). The two CArG*-like elements do not conform to the authentic CArG box, CC(T/A)6GG. Interestingly, this region is highly conserved between mouse, rat, and rabbit promoters, suggesting its importance for SMHC gene expression. In the present study, we demonstrate that CArG*I-like and CArG*II-like elements form specific DNA-protein complexes that include SRF protein as part of the complex. In addition, we demonstrate that expression of mutant SRF protein abolishes SMHC promoter activity in SMCs. These results provide an important first step toward further elucidating SMHC gene regulation both in vitro and in vivo using transgenic mice.

In a recent study, Watanabe et al22 reported that −188 bp of the mouse SMHC gene promoter (which includes two sets of CCTCCC elements located at −89 bp and −61 bp) is sufficient for high-level expression in SMCs. These elements were shown to bind SP-1. However, this region does not provide tissue-specific expression when linked to a heterologous promoter. In contrast, our results show that the proximal −121 bp of the SMHC promoter that includes the CCTCCC elements was the least active (4% of maximal activity) in primary cultures of SMCs. Our transient transfection analysis demonstrates that the −1208-bp promoter construct gave the maximal activity in SMCs. Our data also indicate that deletion of approximately 160 bp between −1208 and −1050 bp drastically decreased the SMHC promoter activity (to 13% of maximal activity) in primary SMCs. The incremental loss of activity observed for the constructs p1128CAT and p1050CAT indicates that more than one positively acting cis element is present within this region. Deletions in the corresponding region of the rat23,53 and rabbit24 SMHC promoter also produced a significant decrease in promoter activity, suggesting that elements located in this region play an important role in SMHC gene expression, whereas studies of Watanabe et al22 showed that deletions between −1226 bp and −188 bp of the 5′ flanking sequence of the mSMHC gene did not affect the promoter activity. The discrepancy between these two findings may be attributed to differences in the cell culture systems used in these studies. It is well known that when SMCs are grown in culture, they readily dedifferentiate and often down-regulate SMHC gene expression. Therefore, depending on the differentiated state of SMCs, the SMHC promoter activity may vary.

The most striking feature of the SMHC gene promoter is the high degree of sequence similarity in the −1208– to −947-bp region between mouse, rat, and rabbit. In particular, a set of three CArG/CArG*-like elements is preserved in this region (Fig 3) in mouse, rat, and rabbit promoters.22–24

In a recent study describing the rat SMHC promoter, Madsen et al53 reported that CArG 1 and CArG*I elements (in their nomenclature CArG 1 and CArG 2, respectively) functioned as positive-acting cis elements. Their study also demonstrated that CArG 1 and CArG*I (CArG 2) elements formed DNA–protein complexes that contained a factor antigenically related to SRF. However, their study did not find the CArG*II (CArG 3) element as important for maximal SMHC promoter activity, and they failed to detect any protein binding to the CArG*II-like (CArG 3) element. In the present study, we found that the CArG*II-like element was equally important for SMHC promoter activity.

In the present study, we show that deletion of this element reduces SMHC promoter activity significantly (Fig 5, construct p1128CAT), whereas deletion of both CArG*I and CArG*II decreases promoter activity to a low level. We also show that all three CArG elements can form similar DNA–protein complexes with SMC nuclear extract. Interestingly, protein binding to the CArG*II-like element can be competed out by the cardiac α-actin CArG box, but not by c-fos SRE. Using SRF antibody, we have demonstrated that SRF is part of the protein complex produced by both CArG*I and CArG*II elements. These data suggest that the CArG*II-like element is equally important for SMHC promoter activity.

In the present study, we also found that SRF pure protein can bind directly to each of these CArG*-like elements. However, our GMSAs with SMC nuclear extract indicate that SRF is not the only protein binding to the CArG*II-like or CArG*I-like element. On the basis of the abundance of the DNA–protein complex formed with individual CArG elements, it appears that additional proteins are binding to these sites. We interpret these data to suggest that protein binding to...
a CArG* element may be influenced by the core and flanking nucleotides of a CArG element. Future studies will focus on how the flanking nucleotides determine protein binding to the CArG element.

Our cotransfection analyses with dominant-negative SRF expression vector provide additional evidence for the role of SRF proteins in the regulation of the SMHC promoter. The studies by Madsen et al35 on the rat promoter and our studies on the murine SMHC promoter strongly suggest that CArG/CArG*-like elements and CArG-SRF interaction are critical for the tissue-specific expression of the SMHC gene.

To date, transcription factors that are unique to SMCs have not been identified, and smooth muscle–specific gene expression remains underexplored. However, the recent isolation and characterization of the smooth muscle–specific gene promoters for α-smooth muscle actin,26-27,54 γ-smooth muscle actin,28 SM22α,15,29,55 calponin,17,18 and SMHC provide unique opportunities for dissecting the various cis- and trans-regulatory factors involved in SMC-specific gene expression. Studies using the above-mentioned promoters have identified a number of known cis elements, including the CArG box, E box, GATA-binding site, AP2, SP-1, CACCC boxes, and A/T-rich element. In particular, CArG elements that bind SRF have been shown to be important for smooth muscle α- and γ-actin gene expression.25,26,54 Similarly, CArG box elements are found in the SM22α proximal promoter and have proved to be indispensable for its regulation in vascular SMCs.29,55,56 These studies suggest that CArG elements play important roles in directing the expression of a subset of smooth muscle–specific genes. However, the precise role of SRF transcription factors in smooth muscle–specific gene expression remains to be explored. Future experiments will be directed toward identifying the role of SRF and its associated factors in the transcriptional regulation of SMHC gene expression.

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