Expression of the Smooth Muscle Myosin Heavy Chain Gene Is Regulated by a Negative-acting GC-rich Element Located between Two Positive-acting Serum Response Factor-binding Elements*

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To identify cis- and trans-acting factors that regulate smooth muscle-specific gene expression, we studied the smooth muscle myosin heavy chain gene, a rigorous marker of differentiated smooth muscle. A comparison of smooth muscle myosin heavy chain promoter sequences from multiple species revealed the presence of a highly conserved 227-base pair domain (nucleotides −1321 to −1095 in rat). Results of a deletion analysis of a 4.3-kilobase pair segment of the rat promoter (nucleotides −4220 to +68) demonstrated that this domain was necessary for maximal transcriptional activity in smooth muscle cells. Gel-shift analysis and site-directed mutagenesis demonstrated that one true CArG and another CArG-like element contained within this domain were both recognized by the serum response factor and were both required for the positive activity attributable to this domain. Additional studies demonstrated that mutation of a GC-rich sequence within the 227-base pair conserved domain resulted in a nearly 100% increase in transcriptional activity. Gel-shift analysis showed that this GC-rich repressor element was recognized by both Sp1 and Sp3. These data demonstrate that transcriptional control of the smooth muscle myosin heavy chain gene is highly complex, involving both negative and positive regulatory elements, including CArG sequences found in the promoters of multiple smooth muscle differentiation marker genes.

Intimal migration and proliferation of vascular smooth muscle cells (SMCs)1 are known to play an integral role in development of atherosclerotic disease (1, 2), and numerous factors have been identified that have growth promoting and/or chemotactic activity for SMCs (2). An additional feature of SMCs within atherosclerotic lesions is that cells exhibit marked differences in morphology and protein expression patterns as compared with normal medial SMCs (3–6), a process referred to as “phenotypic modulation” (7). This is characterized by decreased expression of proteins that are characteristic of differentiated SMCs, including the SM isoforms of contractile proteins, as well as altered growth regulatory properties, lipid metabolism, matrix production, and decreased contractility (reviewed in Ref. 8). Of particular significance, many of these phenotypic alterations in intimal SMCs cannot simply be viewed as a consequence of atherosclerotic disease, but rather are likely to play a major role in its development and/or progression.

Although the SMC exhibits a high degree of plasticity and, unlike skeletal and cardiac myocytes, does not undergo terminal differentiation, it is a very specialized cell, whose differentiated function is dependent upon the coordinate regulation of a large number of cell type-specific/selective products, including contractile proteins, receptors, signal transduction molecules, and ion channels (8). Contractile proteins that are highly abundant and specific to the SMC represent obvious candidates for studying molecular control of SMC differentiation. Consequently, it is not unexpected that the best studied SM-specific marker is smooth muscle α-actin, which first appears in the putative SMCs that envelope the dorsal aorta (9). Although it is transiently expressed during development in other mesodermally derived tissues (10) and in fibroblasts during wound repair (11), smooth muscle α-actin is normally expressed only in SMCs and SMC-like cells in the adult (12). A second well characterized SM-specific contractile protein is smooth muscle myosin heavy chain (SM-MHC). The SMC contains four isoforms of SM-MHC (SM-1A, SM-1B, SM-2A, SM-2B) (13–15), which are all derived from a single gene via alternative splicing (16). In situ hybridization and RNase protection analysis of SM-MHC gene expression during mouse embryogenesis revealed that SM-MHC mRNAs were present only in smooth muscle-containing tissues; no transcripts were detected in the developing brain, heart, or skeletal muscle (17). Immunohistochemical studies that discriminated between the SM-1 and SM-2 isoforms revealed that, in both rabbit and human aortae, SM-MHC protein was specific to the SMC and that SM-1 predominated early during embryonic development, with SM-2 appearing postnatally (18, 19). These studies indicate that, similar to smooth muscle α-actin, SM-MHC represents a highly rigorous SMC marker and a good candidate gene for discerning transcriptional mechanisms important for maintenance of the differentiated SMC phenotype.
The promoters of the rat, mouse, and rabbit SM-MHC genes have been recently cloned and partially characterized. A reporter construct containing 2266 bp of the 5'-flanking region of the rabbit SM-MHC gene was shown to be highly active in cultured rat aortic SMCs and only minimally active in other cell types (20). In a separate study on the rabbit SM-MHC promoter, Kallmeier et al. (21) identified a 107 bp fragment (nucleotides −1332 to −1225) that enhanced transcription in what appeared to be a highly SMC-specific manner. Analysis of the rat SM-MHC promoter in multiple cell types indicated that a 68 bp domain (nucleotides −1249 to −1317) was important for restriction of expression to the SMC (22). Constructions longer than nucleotide −1317 were found to be significantly active only in the SMC. Another recent report on mouse SM-MHC showed that, similar to rat and rabbit, 1500 bp of the mouse SM-MHC promoter was strongly active only in cultured SMCs (23). In all of the above studies, several potential cis-elements, including multiple E boxes, GC boxes, and CArG or CArG-like boxes, were identified based on sequence similarities to known cis-elements. Some of these potential elements were also present in regions with functional activity. However, only in the mouse promoter study by Watanabe et al. (23) were any potential cis-elements specifically mutated and tested for function. They demonstrated by site-directed mutagenesis that two CCTCCC boxes located proximal to the TATA box were bound by Sp1 and functioned as positive-acting cis-elements.

In this study, we utilized ~4.2 kilo-base pairs of the rat SM-MHC gene 5'-flanking region and created a series of mutated CAT fusion constructs to identify functionally important cis-elements. We specifically targeted those sequences that exhibited homology to known cis-elements and that were also conserved between species. A sequence alignment of the rat, mouse, and rabbit SM-MHC promoters revealed a highly conserved 227 bp domain that spanned nucleotides −1321 to −1095 in rat. Deletion analysis showed that this domain was required for maximal promoter activity in SMCs. Site-directed mutagenesis was utilized to further demonstrate that two of the three CArG or CArG-like boxes contained within the 227-bp domain functioned as positive-acting cis-elements recognized by the serum response factor (SRF) or an SRF-like protein. A GC-rich sequence located within this domain was determined by inclusion on the gel a dideoxy sequence marker. These densities were chosen so that the cells would be 70–80% confluent at the time of transfection (24 h after plating). Transfections of the CAT reporter gene constructs, subsequent growth conditions of the SMCs and L6 myoblasts, and preparation of the cell extracts for measurement of CAT activity were all performed as described previously (24), with only slight modification, namely, the amount of plasmid DNA added per well was 4 μg, and N-[1-2,3-dioleoyloxypropyl]-N,N,N-trimethylammonium methylsulfate (7.5 μg/μl of DNA; Boehringer Mannheim) was utilized as the transfection reagent. All CAT activity values were normalized to the protein concentration of each cell lysate as measured by the Bradford assay (25). Early transfection experiments included a β-galactosidase-plasmid as a cotransfection partner for measurement of transfection efficiency. However, the β-galactosidase measurements did not result in qualitative changes in the data, nor did they affect experimental variability. Since the cotransfections conferred no advantage in the reduction of experimental error and had the potential of competing for limited trans-acting factors that regulate SM-MHC transcription, the β-galactosidase cotransfections were discontinued. In each experiment, the promoterless pCAT construct was also transfected into triplicate wells to serve as the base-line indicator of CAT activity, and the activity of each promoter construct is expressed relative to the promoterless construct set to 1. Additionally, an SV40 promoter-CAT construct with enhancer (Promega) served as a positive control of transfection and CAT activity. All SMC CAT activity values represent at least three independent experiments, with each construct tested in triplicate per experiment. L6 myoblasts and myotube CAT activity values represent two independent experiments. Relative CAT activity data are expressed as the means ± S.D. computed from the results obtained from each set of transfection experiments. One-way analysis of variance followed by the Newman-Keuls’s multiple range test were used for data analysis. Values of p < 0.05 were considered statistically significant.

**Materials and Methods**

**Construction of Rat SM-MHC Promoter-CAT Expression Plasmids—**

The cloning, determination of the +1 start site, and nucleotide sequence (nucleotides −1699 to +121) of the rat SM-MHC promoter have been previously reported (22). In this study, we ligated a 4308-bp BglII fragment, spanning nucleotides −4220 to +88, into a partially filled-in SalI site of the pCAT-Basic reporter vector (Promega). This clone (pCAT−4220) was sequenced by the dye-exchange method of Sanger et al. (25) using either an automated sequencer (Applied Biosystems Inc.) or a Sequenase kit (U. S. Biochemical Corp.). The sequence from nucleotides −4220 to −2400 was generated from analysis of only a single strand, whereas both strands were sequenced from nucleotides −2400 to +88. Serial deletion constructs were generated from the pCAT−4220 clone using exonuclease III and the protocol provided by the manufacturer (Stratagene). Deletion clones that targeted specific sites were generated using T7 polymerase and a thermal cycler (Perkin-Elmer). Oligonucleotide primers were purchased from a commercial source (Optron Technologies, Inc.) and contained HindIII and XbaI linkers such that PCR products could be directionally cloned into the pCAT-Basic vector (Promega). Site-directed mutagenesis of the pCAT−1348 construct was performed using the Ex-site mutagenesis kit according to the manufacturer’s instructions (Stratagene). The orientation and integrity of the mutated constructs were determined by dideoxy sequencing (25).

**Comparative Sequence Analysis—**The nucleotide sequences of the rat (nucleotides −2366 to +1) (20), mouse (nucleotides −1500 to +1) (23), and rabbit (nucleotides −4220 to +1) SM-MHC promoters were analyzed with computer assistance using sequence similarity programs (Genetics Computer Group, Madison, WI).

**Cell Culture, Transient Transfections, and Reporter Gene Assays—**

SMCs from rat thoracic aorta were isolated and cultured as described previously (26). Rat L6 skeletal myoblasts were cultured as described previously (24) and fused into myotubes using the protocol of Yaffe (27). SM/M6 myoblasts (10–22) and L6 myoblasts were seeded for transient transfection assays into 6-well plates at a density of 2 × 10⁴. These densities were chosen so that the cells would be 70–80% confluent at the time of transfection (24 h after plating). Transfections of the CAT reporter gene constructs, subsequent growth conditions of the SMCs and L6 myoblasts, and preparation of the cell extracts for measurement of CAT activity were all performed as described previously (24), with only slight modification, namely, the amount of plasmid DNA added per well was 4 μg, and N-[1-2,3-dioleoyloxypropyl]-N,N,N-trimethylammonium methylsulfate (7.5 μg/μl of DNA; Boehringer Mannheim) was utilized as the transfection reagent. All CAT activity values were normalized to the protein concentration of each cell lysate as measured by the Bradford assay (25). Early transfection experiments included a β-galactosidase-plasmid as a cotransfection partner for measurement of transfection efficiency. However, the β-galactosidase measurements did not result in qualitative changes in the data, nor did they affect experimental variability. Since the cotransfections conferred no advantage in the reduction of experimental error and had the potential of competing for limited trans-acting factors that regulate SM-MHC transcription, the β-galactosidase cotransfections were discontinued. In each experiment, the promoterless pCAT construct was also transfected into triplicate wells to serve as the base-line indicator of CAT activity, and the activity of each promoter construct is expressed relative to the promoterless construct set to 1. Additionally, an SV40 promoter-CAT construct with enhancer (Promega) served as a positive control of transfection and CAT activity. All SMC CAT activity values represent at least three independent experiments, with each construct tested in triplicate per experiment. L6 myoblasts and myotube CAT activity values represent two independent experiments. Relative CAT activity data are expressed as the means ± S.D. computed from the results obtained from each set of transfection experiments. One-way analysis of variance followed by the Newman-Keuls’s multiple range test were used for data analysis. Values of p < 0.05 were considered statistically significant.

**Preparation of SMC Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSA)—**

Nuclear extracts were prepared from rat aortic SMCs grown to confluency under the same media conditions utilized for the transfection assays. The early preparative method used was essentially that of Dignam et al. (29); protease inhibitors were added as described previously (24).

The oligonucleotides used for EMSAs were purchased commercially (Optron Technologies, Inc.) and included the following: CArG3, 5'-ggggacatatattagtcagg-3'; CArG2, 5'-ctgctgtttttggtggtt-3'; CArG1, 5'-ctgcttttattagctga-3'; GC-rich, 5'-ggtttgttcccagcaggcc-3'; CArG2Mut, 5'-ctgcttttttggtggtt-3'; GC-richMut, 5'-ggtttctgctttggtggtt-3'; and SRE, 5'-gggcttcattagctga-3'. EMSAs were performed with 20 μl of binding reaction that included ~30 μg of SMC nuclear extract (unless otherwise indicated), and 0.25 μg of poly(dI-dC) in 1× binding buffer (12 mM HEPES (pH 7.9), 100 mM KCl (50 mM for GC-rich oligonucleotide EMSA), 5 mM MgCl₂, 4 μM Tris-HCl (pH 7.5), 0.6 mM EDTA, 0.6 mM dithiothreitol, and 10% glycerol). Following a 20-min incubation at room temperature, the samples were subjected to electrophoresis on a 5% polyacrylamide gel, which had been pre-run at 170 V for 1 h. Electrophoresis was performed at 170 V in 0.5 × 45 mM Tris-borate and 1 mM EDTA. Gels were dried and exposed to film for 24–72 h.

The Sp1, Sp3, SRF, and CTF/NF1 antibodies used for EMSA super-shift experiments were all purchased commercially (Santa Cruz). EMSA binding reactions were set up as described above and incubated for 20 min; 2 μl of the antibody was added to the mixture; and the reaction was incubated for a further 15 min at room temperature and then loaded on to the gel for electrophoresis.

**DNase I Footprinting—**Binding conditions were as described for EMSAs, except that 40 μg of SMC nuclear extract was used per reaction. DNase I digestions and electrophoresis of the samples were performed as described previously (30). Location of the footprinted regions was determined by including on the gel a dideoxy sequence marker.
Identification of a 227-bp Sequence Domain Conserved between the SM-MHC Promoters of Rat, Mouse, and Rabbit—The rat SM-MHC promoter has been recently cloned and sequenced from bp −1699 to +121 (22). To assist in the identification of potential cis-elements, we sequenced an additional 2500 bp of the rat SM-MHC promoter (up to nucleotide −4220) and compared the rat promoter sequence with the published sequences of rabbit (−2266 bp) (20) and mouse (−1500 bp) (23). We identified only two domains that shared obvious sequence similarities among all three species (Fig. 1A). The distal (relative to the +1 start site) conserved domain was located in the rat promoter between nucleotides −1321 and −1095 (Fig. 1B). Within this 227-bp domain, we identified five known cis-elements, all of which were totally conserved between the three species. These elements included two CArG-like boxes and one true CArG box (hereafter referred to as CArG boxes 1, 2, and 3, in order of proximal to distal), a GC-rich element, and a CTF/NF1 site. These three elements represent potential binding sites for SRF (31), Sp1 (32), and CTF/NF1 (33), respectively. It should be noted that the centrally located nucleotides of the 13-bp CTF/NF1 site (TGCC(N)5GCCA) contain sequence differences between the three species; however, these residues are not considered to be important for factor binding (34). The second conserved domain encompassed the TATA box and extended to nucleotide −128 in rat. Contained within this domain were two conserved CCTCCCC elements that were previously shown in transient transfection studies to function as positive-acting cis-elements in the mouse SM-MHC promoter (23).

Deletion Analysis of the Rat SM-MHC Promoter Revealed That the Conserved 227-bp Domain Is Required for Maximal Activity—To identify functional elements contained within the rat SM-MHC promoter, a 4308-bp BglII fragment (bp −4220 to +88) was cloned into a pCAT reporter vector. A series of deletion constructs were created by treating the pCAT−4220 construct with exonuclease III and, when targeting specific regions of interest, by PCR (see “Materials and Methods”). Transient transfection of these constructs into cultured rat aortic SMCs revealed that pCAT−1346, which contained both of the conserved domains, was the most active construct, with an activity level 4-fold over promoterless pCAT (Fig. 2). The pCAT−1346 construct contained a more promoter 5′-flanking DNA reduced transcriptional activity to 29-fold (pCAT−3443) and 24-fold (pCAT−4220) over promoterless pCAT. This observed decrease in activity suggests that other regulatory elements exist upstream of nucleotide −1346. The pCAT−1182 construct, which is missing CArG boxes 3 and 2 and the GC-rich element, yielded an activity 34-fold over promoterless pCAT. When the entire 227-bp conserved domain was deleted (pCAT−1102), transcriptional activity further decreased to 17-fold over promoterless pCAT. The activity of the pCAT−562 construct was found to be approximately equal (14-fold over promoterless pCAT) to that of the pCAT−1102 construct. The notable difference in activity between pCAT−1346 and pCAT−1102 suggests that a positive-acting element(s) resides within the 227-bp conserved domain. The incremental gain of activity noted for the pCAT−1182 and pCAT−1346 constructs indicates that more than one positive-acting cis-element is present within this region.

Transcriptional activity of the SM-MHC promoter has been measured in several other non-SMC types, including L8 skeletal myoblasts (22), NIH3T3 mouse fibroblasts (29), and C6G12 mouse myoblasts (20). For comparative purposes, the deletion constructs were tested for activity in L6 myoblasts and myotubes (Fig. 3). Transient transfection analysis of L6 myotubes revealed that all constructs were only minimally active relative to SMCs, ranging from 8 to 14-fold activity over promoterless pCAT. Similar results were seen in L6 myoblasts, except that the range in activity (4–7-fold over promoterless pCAT) was about half that of myotubes. In contrast to the SMC data, the addition of the 227-bp domain failed to enhance transcription in either L6 myoblasts or myotubes, suggesting that the activity of this domain is regulated in a cell type-specific manner.

Multiple Sites of Protein-DNA Interaction Are Present within the 227-bp Conserved Domain—To identify potential cis-elements within the 227-bp domain, DNase I footprint experi-
ments were performed using nuclear extracts from cultured rat aortic SMCs. Using a probe that extended from nucleotides −21346 to −1071, we identified three domains of protein binding (Fig. 4). These same three domains were also identified in a DNase I analysis of the antisense strand (data not shown). A summation of the footprint data shows that footprint 3 extended from nucleotides −1234 to −1206 and encompassed CArG box 2 and an adjacent GC-rich element. Footprint 2 extended from nucleotides −1146 to −1121 and encompassed the CTF/NF1 site. Footprint 1 extended from nucleotides −1115 to −1098 and encompassed CArG box 1. The identification of multiple sites of protein-DNA interaction is consistent with the promoter deletion data that indicated the presence of multiple regulatory elements within this region.

CArG Boxes 1 and 2 Are Positive-acting cis-Elements, and the GC-rich Sequence Functions as a Negative-acting cis-Element—To determine if the sites of protein-DNA interaction described in Fig. 4 represented functional elements, we mutated each of the four known cis-elements contained within the 227-bp domain and tested for functional activity. Although CArG box 3 did not display any protein binding activity, we mutated this element anyhow due to its similarity to other SRF recognition sites and sequence conservation among all three species. Transient transfection of the mutated constructs into rat aortic SMCs revealed that the SM-MHC promoter was regulated by both positive- and negative-acting cis-elements. As shown in Fig. 5A, CArG box 3 was totally deleted, and CArG boxes 1 and 2 were mutated at the conserved 5′-CC doublet. The presence of these two cytosine residues has been previously shown to be necessary for SRF binding and functional activity (24). Transfection analysis of the three CArG boxes showed that deletion of CArG box 3 had virtually no effect on functional activity relative to wild-type pCAT−21346 (Fig. 5B). However, mutations of CArG boxes 1 and 2 resulted in a 50% decrease in transcriptional activity (20- and 23-fold over promoterless pCAT, respectively) when compared with wild-type pCAT−21346 (48-fold over promoterless pCAT). The reduced activity of pCAT-CArG1Mut and pCAT-CArG2Mut suggests that a major portion of the positive activity of the 227-bp conserved domain (the difference in activity between pCAT−1102 and pCAT−21346) is dependent on the presence of CArG boxes 1 and 2.

The sequence identified as the GC-rich element contains a core Sp1 site (CCCGCCC); thus, the central nucleotides of this sequence were specifically mutated such that any putative Sp1 binding would be abrogated (32). Alteration of this element resulted in a marked increase in transcriptional activity (Fig. 5B). The actual level of activity (94-fold over promoterless pCAT) was approximately double that of wild-type pCAT−21346 and nearly equal to that of the powerful SV40 viral promoter (pSV40-CAT). This result suggests that the GC-rich element located at nucleotide −1215 functions as a negative-acting cis-element. The CTF/NF1 site in pCAT-NF1Mut was completely deleted due to previous reports indicating that this factor is capable of binding to either half of its palindromic recognition sequence (TGGC(N)5GCCA) (35). The activity of
CArG Boxes 1 and 2 Function as Recognition Sites for SRF or an SRF-like Protein—EMSA and antibody supershift analysis were utilized to determine if the three CArG boxes identified in Fig. 1 functioned as SRF recognition sites. Individual oligonucleotides containing CArG boxes 1–3, as well as 5 bp of 5’- and 3’-flanking sequence, were labeled, incubated with 10 μg of SMC nuclear extracts, and analyzed by EMSA (Fig. 6A). Duplicate reactions that included an SRF antibody were also electrophoresed on the same gel (Fig. 6A, right panel). For comparison, we included a similarly sized oligonucleotide that contained the well-characterized SRF-binding site present in the c-fos promoter, the SRE (31). Similar to a previous study on SRF binding to the SM α-actin CArG boxes (24), we identified two closely migrating protein-DNA complexes with the CArG1 probe. The mobilities of these two bands were supershifted upon SRF antibody addition. Two bands were also identified with the SRE probe that comigrated along with the CArG1-SRF complexes and supershifted upon SRF antibody addition. These data demonstrate that the protein-CArG1 complexes contain SRF or an SRF-like factor. No protein-DNA complexes whose mobilities supershifted upon addition of SRF antibody were detected with either the CArG box 3 or box 2 probes.

Transfection data of pCAT-CArG3Mut indicated that CArG box 3 was nonfunctional; as such, we did not further pursue experimentation on protein binding to this site. However, we did perform further EMSAs on CArG box 2. By increasing the amount of SMC nuclear extract from 10 to 30 μg in the gel-shift reaction, we were able to detect a band that comigrated with the SRE-SRF complex and also supershifted upon SRF antibody addition (Fig. 6B). We have previously demonstrated that mutation of the 5′-CC doublet of the CArG box completely abrogated SRF binding (24). However, given the amount of extract needed for detection of the CArG2-SRF (or SRF-like) complex, we wanted to verify that, under these conditions, SRF did not bind to the mutated CArG2 oligonucleotide. A CArG2Mut oligonucleotide that duplicated mutations created in the pCAT-CArG2Mut construct was synthesized and tested for SRF binding. As shown in Fig. 6C (right lane), the CArG2Mut probe failed to generate a band that comigrated with the SRE-SRF complex, even when 30 μg of SMC extract was used. In conjunction with the transfection data, these protein binding studies suggest that SRF interaction with CArG boxes 1 and 2 functions to activate transcription of the SM-MHC gene. Although the binding affinities for the three CArG boxes were not determined, the data presented in Fig. 6 suggest that the binding affinity of SRF, or an SRF-like protein, is much greater for CArG1 than for CArG2.

The Negative-acting GC-rich Element Functions as a Sp1/Sp3 Recognition Site—Due to the considerable sequence similarities between the negative-acting GC-rich element located within the 227-bp conserved domain and the published Sp1-binding site, we wanted to determine if this element functioned as a Sp1 recognition site. Using a labeled oligonucleotide that encompassed the SM-MHC GC-rich element, EMSAs were performed in the presence of different unlabeled competitors (Fig. 7). The GC-rich probe formed three predominant protein-DNA complexes (bands 1–3) in the presence of SMC nuclear extract. Bands 1 and 2 exhibited very similar mobilities and migrated through the gel slower than did band 3. This particular migration pattern of protein-DNA complexes was similar to the pattern previously described for Sp1 and Sp3 binding; using antibody supershift analysis and a Sp1 probe, Dennig et al. (36)
demonstrated that band 1 contained Sp1 protein and that bands 2 and 3 represented Sp3-probe complexes. It should be noted that with longer electrophoresis times, we were able to better resolve bands 1 and 2 (data not shown). Further analysis of Fig. 7 shows that the consensus Sp1 oligonucleotide competed very efficiently for factor binding, even more so than did the wild-type GC-rich competitor. The fact that all bands disappeared equally with addition of the Sp1 competitor oligonucleotide is consistent with Sp1 and Sp3 binding since both factors bind to the same element with equal affinities (37). Nucleotide alterations in the GC-rich Mut oligonucleotide were created to duplicate mutations made in the pCAT-GC-rich Mut construct. No competition was evident for the GC-rich Mut oligonucleotide even at the highest concentration utilized (250 μ excess). Antibody supershift analysis were employed to determine if the bands that were competed away with the Sp1 consensus element truly represented Sp1- and Sp3-containing complexes (Fig. 8). Similar to the results found in the aforementioned study, the addition of Sp1 antibody resulted in the disappearance of band 1 and the formation of supershifted complexes. Likewise, the addition of Sp3 antibody resulted in the disappearance of bands 2 and 3 and the formation of supershifted complexes. The data from Figs. 7 and 8 demonstrate that the GC-rich element located within the 227-bp conserved domain functions as a recognition element for both Sp1 and Sp3. Furthermore, the lack of competition with the GC-rich Mut oligonucleotide suggests that any potential Sp1/Sp3 binding at this site would have been abolished in the highly active pCAT-GC-rich Mut construct.

**DISCUSSION**

The goal of this study was to identify cis-elements and trans-acting factors important for transcriptional regulation of the SM-MHC gene. By comparing the 5'-flanking sequences of the
FIG. 6. Binding of SM nuclear proteins to the three SM-MHC CArG motifs. A, radiolabeled 20-bp oligonucleotides encompassing CArG box 1, 2, or 3 or the SRE were incubated with 10 μg of SMC nuclear extract only (lanes 1–4) or with extract plus SRF antibody (Ab; lanes 5–8) and subjected to EMSA. The two highest mobility bands that correspond to CArG-SRF complexes are indicated by arrows. Supershifted bands are indicated by SS next to the arrow. B, EMSA was performed on radiolabeled SRE oligonucleotides incubated with 5 μg of SMC nuclear extract only (lane 1) or extract plus SRF antibody (lane 2) and on radiolabeled CArG2 oligonucleotides incubated with 30 μg of SMC nuclear extract only (lane 3) or extract plus SRF antibody (lane 4). C, radiolabeled CArG2 (left lane) and CArG2Mut (right lane) oligonucleotides were incubated with 30 μg of SMC nuclear extract.

rat, mouse, and rabbit SM-MHC genes, we identified two domains that were conserved among all three species. The most proximal domain encompassed the TATA box and contained two conserved CCTCCC elements. This region was previously studied in mouse, and the CCTCCC elements were shown to be necessary for the basal promoter activity ascribed to this domain (23). Therefore, we focused our studies on the distally conserved domain located between nucleotides −1095 and −1321 in rat. From deletion analysis, we determined that inclusion of this 227-bp domain was necessary for maximal expression of the SM-MHC gene in cultured rat aortic SMCs. We further demonstrated that several of the known cis-elements present within this domain were important for SM-MHC expression, both as positive and negative regulators.

The presence of this 227-bp domain failed to enhance transcription in either rat L6 myoblasts or myotubes. This result suggests that the positive effects attributable to this region are potentiated in a cell type-specific manner. This observation is in agreement with an earlier SM-MHC promoter mapping study by Kallmeier et al. (21). They demonstrated that a rabbit SM-MHC construct containing 1332 bp of 5′-flanking DNA (including the 227-bp conserved region) was highly active in primary cultures of rabbit aortic SMCs (6-fold over the basal activity level of the −112 promoter construct). This −1332 construct was only minimally active (basal level activity) in five other non-SMC types. However, a complete review of the literature indicates that there exist several discrepancies with respect to certain SM-MHC deletion constructs and their activity levels in both SMC and non-SMC types. Katoh et al. (20) analyzed the rabbit promoter in primary cultures of rat aortic SMCs and found that −509 and −1392 deletion constructs were both at −25% relative CAT activity when compared with the −2266 construct (100% CAT activity), their most active construct. All of the constructs tested were only minimally active in other non-SMCs. In contrast, White and Low (22) analyzed the rat SM-MHC promoter in primary cultures of rat aortic SMCs and reported that a −1249 deletion construct was the most active construct, exhibiting twice the level of activity of the next most active construct, −602 (26- versus 12-fold activity over promoterless plasmid). More important, they found that with the addition of 68 bp of more 5′-flanking sequence (the −1327 construct), transcriptional activity decreased to one-half that of the −602 construct. A significant decrease in activity was also noted when the −1249 and −1327 constructs were compared in rat L6 myoblasts. The noted high level of activity (150-fold over promoterless plasmid) of the −1249 construct in L6 myoblasts and the subsequent drop in activity (barely detectable levels) with the addition of 68 bp of 5′-flanking sequence led the authors to conclude that the domain was likely to contain a repressor element(s) necessary for cell type-specific expression. In another study, Watanabe et al. (23) reported that a mouse −188 deletion SM-MHC promoter construct, when tested in primary cultures of rabbit aortic SMCs, was the most active construct. The addition of up to 3 kilobase pairs of more 5′-flanking sequence only led to a reduction in transcriptional activity. No increase in activity was observed with inclusion of CArG boxes 1 and 2.

It is difficult to reconcile the discrepancies in the data presented in the above studies, not only with respect to our data, but also with respect to each other. Particularly perplexing is the result in mouse, where maximal activity in SMCs was achieved with a −188 construct. One possible explanation for many of the observed differences between these studies may be differences in the phenotypic state of the SMC in which the activity of the SM-MHC promoter was assessed. It is well documented that SMCs undergo rapid phenotypic modulation when placed in culture (38). In particular, there is an immediate and significant decrease in expression of the SM-MHC gene (39). However, we have demonstrated that rat aortic SMCs used in our studies re-express the SM-MHC gene upon reaching confluency (39). Western blot analysis of the SMCs utilized in this study showed that, although there was a marked de-
crease in SM-2 protein, the SM-1 isoform was present in confluent cells up to passage 22, the last passage analyzed. Moreover, we have shown by protein electrophoretic analysis, Northern analysis, and nuclear run-on analysis (39, 40) that the endogenous SM-MHC gene is actively transcribed in our SMC cultures under the exact transfection conditions used in the present study. In all but one of the studies cited above (22), it was not made clear if the endogenous SM-MHC gene was determined to be active at the time when the SM-MHC promoter-reporter gene fusion constructs were analyzed. Based on our observations, it is possible that differences in confluency at the time of transfection and harvest and differences related to culture methodology could account for the noted differences in expression. Additionally, inherent phenotypic differences between cultured rat and rabbit SMCs may represent another possible explanation. Despite several attempts, we have been unable to culture rabbit aortic SMCs such that they continue to express their endogenous SM-MHC gene, even at early passages. Potential effects due to phenotypic differences become even more plausible in view of the observation that SM-MHC deletion constructs were differentially expressed in SMCs isolated from either rat aortic or tracheal tissues. Finally, sequence differences (resulting from either construct design or species divergence) in the deletion constructs being compared could certainly explain some of the differences in expression. However, it is doubtful that this explanation could account for the mouse data, where the addition of CArG boxes 1 and 2 or any other 5′-flanking sequence failed to enhance transcription above the level of the –188 construct. Clearly, discerning the many factors likely to be involved in the expression of the SM-MHC gene in cultured SMCs will require further careful study in which each of the potential mitigating factors is analyzed. This study demonstrated through site-directed mutagenesis that SM-MHC CArG boxes 1 and 2 functioned as positive-acting cis-elements. We further demonstrated that both CArGs, in the presence of aortic SMC nuclear extract, formed protein-DNA complexes that contained a factor antigenetically related to SRF. These data provide evidence that CArG-SRF interaction within the vascular SMC contributes significantly to the expression of the SM-MHC gene. Our laboratory previously demonstrated by site-directed mutagenesis that two CArG boxes located at nucleotides –112 and –62 in the rat SM α-actin promoter were absolutely required for transcriptional activity in cultured SMCs. Thus, similar to several cardiac- and skeletal muscle-specific genes (42–44), CArG-SRF interaction appears to serve as a common transcriptional activation mechanism for multiple SM-specific genes.

It was somewhat surprising that mutation of the highly conserved CTF/NF1 site present within the 227-bp domain did not result in any significant alteration in activity. However, several studies indicated that, at least in some circumstances, CTF/NF1 functions to regulate transcription via a mechanism...
that is likely to involve higher order chromatin structures (45, 46). Such an activity would likely be missed in a transient transfection reporter gene analysis where “naked” plasmid DNA is utilized (35).

Another important regulatory element identified in this study is the GC-rich repressor that was recognized by both Sp1 and Sp3. Multiple mechanisms can be envisioned whereby Sp1 and/or Sp3 binding could inhibit transcription. The fact that binding of Sp3 appears to negatively regulate transcription (48–50). It will be of interest in the future to investigate how the elements identified in this study, as well as their contrast to Sp1, a number of recent promoter studies indicate that binding of Sp3 appears to negatively regulate transcription (48–50). It will be of interest in the future to investigate how the elements identified in this study, as well as their potential binding factors, are involved in the altered expression of the SM-MHC gene in phenotypically modified SMCs found in atherosclerotic lesions in man (51).

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