Interaction of CArG Elements and a GC-rich Repressor Element in Transcriptional Regulation of the Smooth Muscle Myosin Heavy Chain Gene in Vascular Smooth Muscle Cells*

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We have previously shown that maximal expression of the rat smooth muscle myosin heavy chain (SM-MHC) gene in cultured rat aortic smooth muscle cells (SMCs) required the presence of a highly conserved domain (nucleotides -1321 and -1095) that contained two positive-acting serum response factor (SRF) binding elements (CArG boxes 1 and 2) and a negative-acting GC-rich element that was recognized by Sp1 (Madsen, C. S., Hershey, J. C., Hautmann, M. B., White, S. L., and Owens, G. K. (1997). J. Biol. Chem. 272, 10932-10940). In this study, to better understand the functional role of these three cis elements, we created a series of SM-MHC reporter-gene constructs in which each element was mutated either alone or in combination with each other and tested them for activity in transient transfection assays using primary cultured rat aortic SMCs. Results demonstrated that the most proximal SRF binding element (CArG-box1) was active in the absence of CArG-box2, but only upon removal of the GC-rich repressor. In contrast, regardless of sequence context, CArG-box2 was active only when CArG-box1 was present. We further demonstrated using electrophoretic mobility shift assays that Sp1 binding to the GC-rich repressor element did not prevent SRF binding to the adjacent CArG-box2. Thus, unlike other proteins reported to inhibit SRF activity, the repressor activity associated with the GC-rich element does not appear to function through direct inhibition of SRF binding. As a first step toward understanding the importance of these elements in vivo, we performed in vivo footprinting on the intact rat aorta. We demonstrated that both CArG boxes and the GC-rich element were bound by protein within the animal. Additionally, using the rat carotid injury model we showed that Sp1 protein was significantly increased in SMCs located within the myointimal lesion, suggesting that increased expression of this putative repressor factor may contribute to the decreased SM MHC expression within SMCs found in myointimal lesions.

Vascular lesions resulting from either atherosclerotic disease or restenosis are characterized by the significant presence of SMCs† that are phenotypically distinct from fully differentiated medial SMCs (2-4). Major modifications include decreased expression of smooth muscle isoforms of contractile proteins, increased matrix production, altered growth regulatory properties, altered lipid metabolism, and decreased contractility (5). The process by which SMCs undergo such changes is referred to as "phenotypic modulation" (6). Importantly, these alterations in SMC protein expression patterns cannot simply be viewed as a consequence of vascular disease, but rather are likely to contribute to its progression. The identification of the molecular mechanisms that regulate protein expression in the normal SMC should provide for a better understanding of how these processes are altered in phenotypically modified SMCs.

SM-MHC represents one of the best studied markers of the SMC (5). Four SM-MHC isoforms (SM-1A, SM-1B, SM-2A, and SM-2B) have been identified (7-9), all of which are derived from alternative splicing of a single gene product (10), that is exclusively expressed in SMCs (11). Expression of the SM-MHC gene has been shown to be regulated in a manner that exquisitely "marks" the differentiated state of the SMC both during normal vascular development and in vascular disease. In humans and rabbit, SM-1 is expressed during early fetal development with SM-2 appearing only after birth in what is thought to be the fully differentiated or mature SMC (12-14). Moreover, several studies have shown that SM-MHC expression is significantly decreased in vascular lesions (12, 15), and the loss of this marker protein has been used to evaluate the differentiated state of the SMC as related to vascular disease progression (16, 17). Thus, SM-MHC represents an excellent gene for delineating the transcriptional regulatory mechanisms that define the differentiated state of the SMC.

The 5'-flanking regions of SM-MHC genes cloned from rat (18), rabbit (19), and mouse (20), all contain a highly conserved 227-bp domain located between nucleotides -1321 and -1095 in rat (1). In a recent study, we demonstrated that this domain was required for maximal SM-MHC promoter activity in vascular SMCs, and that present within this domain were two positive-acting CArG boxes that were specifically recognized by SRF, and a negative-acting GC-rich element that was recognized by Sp1 (1). In a separate investigation of the rabbit SM-MHC promoter, transient transfection studies and analysis of protein-DNA interactions revealed that an approximately 100-bp segment of the conserved domain, located just upstream from the TATA box, was critical for maximal promoter activity in vascular SMCs.

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1 The abbreviations used are: SMC, smooth muscle cell; SM-MHC, smooth muscle myosin heavy chain; SRF, serum response factor; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; DMS, dimethyl sulfate; TE, Tris-EDTA buffer; LMPCR, ligation-mediated polymerase chain reaction; PCR, polymerase chain reaction; bp, base pair(s).
of CARG-box2, contained multiple enhancer elements each interacting with unidentified factors, two of which were thought to be SM-specific (21). An additional study of rat SM-MHC promoter activity in a variety of cell types, revealed that the region spanning nucleotides –1317 to –1249 was important for the restriction of gene expression to SMCs (18). Thus, this very complex and highly active domain appears to contain a multitude of cis elements that interact with multiple factors, of which only a few have been putatively identified. In addition to this conserved 227-bp domain, two tandemly oriented CTCCTCC elements, located proximal to the transcriptional start site, have been identified and also shown to be important for SM-MHC expression (20).

The CARG box motif has been demonstrated to be important for expression of multiple SM-specific genes including SM-MHC (1), SM α-actin (22), SM-22α (23), and telokin (24). Thus, a better understanding of CARG-dependent regulation for any one of these genes could lead to the identification of an important common transcriptional mechanism. In the present study, we generated a series of combinatorial mutants to better understand how CARG boxes 1 and 2 functioned within the SM-MHC promoter in vascular SMCs and how a GC-rich element, which is located between the two CARGs and which is specifically recognized by Sp1, influenced their activity. These data revealed that, upon removal of the GC-rich repressor, CARG-box1 could sustain activity in the absence of CARG-box2. In view of the fact that both CARGs in the SM α-actin promoter were absolutely required for activity (22), this finding suggests that activation through the CARG motif may involve different gene-specific mechanisms. Furthermore, unlike other factors shown to inhibit SRF activity, gel-shift analysis of Sp1 and SRF binding to the juxtaposed GC-rich repressor and CARG-box2 elements, revealed that the mechanism of repression was not likely to be mediated through direct inhibition of SRF binding. To assess their likely importance for SM-MHC expression in the fully differentiated vascular SMC, we performed in vivo footprinting on the intact rat aorta to demonstrate that protein-DNA interaction occurred at both CARG boxes and the GC-rich repressor element. Finally, we found that Sp1 presence was increased in myointimal SMCs in a rat carotid injury model, suggesting that the GC-rich repressor element may contribute to reduced SM-MHC protein expression in dedifferentiated myointimal SMCs.

EXPERIMENTAL PROCEDURES

Rat SM-MHC Promoter-Chloramphenicol Vector Chimeric Constructs—The cloning, determination of the +1 start site, and sequencing of the rat SM-MHC promoter have been previously described (1, 18). The construction of pCAT-1346, pCAT-1182, and pCAT-1102 promoter-chloramphenicol reporter plasmids containing progressive deletions of rat SM-MHC 5′-flanking DNA was described previously (1). Site-directed mutagenesis of the pCAT-1346 construct was performed using the Ex-site mutagenesis kit according to the manufacturer’s instructions (Stratagene). The integrity of each mutated construct was determined by dideoxy sequencing (25).

Cell Culture and DNA Transfection—SMCs from rat thoracic aortas were isolated and cultured under conditions that result in retention of expression of multiple SMC differentiation marker proteins including SM1 (26). SMCs (passage 10–22) were seeded for transient transfection assays into six-well plates at a density of 2 × 10^4. These densities were chosen so that the cells would be at 70–80% confluence at the time of transfection (24 h after plating). Transfections of the CAT-reporter gene constructs, subsequent growth conditions of the SMCs, and determination of CAT activities, were all done as described previously (1). The promoter construct was 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 one. Additionally, a 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. 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 Recombinant SRF, Sp1, and Electrophoretic Mobility Shift Assays (EMSA)——Recombinant SRF was generated using an in vitro transcription and translation kit (Stratagene) as described previously (27). Oligonucleotide affinity-column purified Sp1 was obtained from a commercial source (Santa Cruz).

The oligonucleotides used for EMSAs were purchased commercially (Operon Technologies, Inc.) and included the following: C2 oligo, 5′-ctgctggctttttttgtgtggtggt-3′; GC oligo, 5′-ggtttcgctcgcagggcgc-3′; and C2 + GC oligo, 5′-ctgctggctttttttgtgtggtggtggtggtggt-3′. EMSAs were performed with 20 μl of binding reaction that included ~30 pg of 32P-end-labeled annealed oligonucleotides, SRF and Sp1 as indicated, and 0.25 μg of poly(dI-dC) in 1 × binding buffer (12 mM HEPEs (pH 7.9), 100 mM KCl, 5 mM MgCl₂, 4 mM 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 electrophoreses 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 × 0.45 mM Tris borate and 1 mM EDTA. Gels were dried and exposed to film for 24–48 h.

The anti-Sp1 and anti-SRF antibodies used for EMSA supershift 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 were added to the mixture; and the reaction was incubated for another 15 min at room temperature and then loaded on the gel for electrophoresis.

In Vivo Footprinting of the Intact Rat Aorta—The thoracic aortas from eight adult Sprague-Dawley rats were dissected individually from each animal and immediately placed in 5 ml of 37 °C phosphate-buffered saline (PBS) containing 0.5% dimethyl sulfoxide (DMS). The aortas were incubated for 2 min with constant gentle shaking and rinsed three times in 50 ml of ice-cold PBS containing 50 mM Tris-HCl (pH 8.0). Aortas were stored in this same solution until all dissections were completed. The adventitial and endothelial layers of the aortae were mechanically removed to minimize contribution of non-SMC DNA in the subsequent polymerase chain reactions. The aortae were then cut into small pieces, pooled, and placed in a lysis solution containing 50 mM NaCl, 50 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% sodium dodecyl sulfate, 0.3 mg of proteinase K per ml. Following an overnight room temperature incubation, chromosomal DNA was isolated by extraction with phenol (pH 8.0) (twice), phenol-chloroform-isomyl alcohol (twice), chloroform (twice) with gently mixing by inversion. After adjusting the NaCl concentration to 0.2 M, the DNA was precipitated with the addition of 2.5 volumes of ethanol, and the precipitate was transferred to 0.5 ml of Tris-EDTA buffer (TE) in a new tube, resolved, and precipitated again with ethanol. The precipitate was dissolved in 0.4 ml of TE, and the concentration was measured by optical density at 260 nm. For the in vitro methylated controls, 100 μg of Sprague-Dawley rat kidney DNA was extensively deproteinized as described above with phenol and chloroform, modified with 0.5% DMS, and precipitated with ice-cold DMS stop solution. A plasmid containing the SM-MHC promoter (pCAT-1346) was also subjected to DMS treatment and used as an additional in vitro control and also for sequence verification. An approximate equivalent amount of in vivo methylated DNA was also precipitated with DMS stop solution. The samples were dissolved in 180 μl of H₂O, adjusted to a concentration of 10% piperidine, and incubated at 90 °C for 30 min. The reaction products were cooled on ice and precipitated with the addition of 0.2 ml sodium acetate (pH 7.0) and ethanol. After two more rounds of ethanol precipitation, the samples were dried overnight, precipitated again, and resuspended in TE to 0.4 mg/ml.

The ligated-mediated polymerase chain reaction (LMPCR) performed on the DMS-treated DNA samples was as described previously (28), with minor exceptions. First strand synthesis and subsequent PCRs were performed with Taq polymerase using the reaction buffer supplied by the manufacturer (Perkin-Elmer Corp.). Following the first strand synthesis step and prior to the ligation reaction, samples were extracted with phenol-chloroform and precipitated with ethanol. This additional extraction step was performed to increase ligation efficiency. Three sets of oligonucleotide primers (nine total) were utilized to analyze the CARG-box2, GC-rich-, and CARG-box1-containing regions of the 227-bp conserved element. These were as follows: MHCP1, 5′-ggatccctttctttaaaggttggacgagg-3′; MHCP2, 5′-tcttcatagatgaggtggtggtggtggtggtggtggtggtggtggtggtc-3′; MHCP3, 5′-aggatctggggtggtggtggtggtggtggtggtggtggtggtggtc-3′; MHCP4, 5′-aggagatgaggtggtggtggtggtggtggtggtggtggtggtggtc-3′; MHCP5, 5′-gaggttgcgcagggaggagggcgc-3′; MHCP6, 5′-gaggttgcgcagggaggagggcgc-3′; MHCP7, 5′-gaggttgcgcagggaggagggcgc-3′; and MHCP8, 5′-gaggttgcgcagggaggagggcgc-3′. The restriction of gene expression to SMCs (18). Thus, this very complex and highly active domain appears to contain a multitude of cis elements that interact with multiple factors, of which only a few have been putatively identified. In addition to this conserved 227-bp domain, two tandemly oriented CTCCTCC elements, located proximal to the transcriptional start site, have been identified and also shown to be important for SM-MHC expression (20).
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c-3'; MHCPT, 5'-aggaggacaccctgagga-3'; MHCp8, 5'-ctcagaactgggctgcggc-3']; MHCp9, 5'-ctcagaactgggtgcggc-3'.

To evaluate the potential for reaction variability, multiple LMPCRs were performed on the in vivo aortic, in vitro control kidney, and SM-MHC plasmid DNAs. All samples were subjected to electrophoresis on 8% sequencing gels along with an additional SP-end-labeled molecular weight ladder (Life Technologies, Inc.) and exposed to film. We observed that band intensities were highly consistent between individual LMPCRs, and that the in vitro control kidney DNA and the SM-MHC plasmid DNA both yielded essentially identical patterns of methylation reactivity (not shown). Quantitation and identification of protected or hypermethylated guanine residues was as described previously (29).

Balloon Catheter Injury of Rat Carotid Arteries and Sp1 Immunohistochemistry—Rat carotid arteries injured by balloon catheterization were prepared by Zivic-Miller Laboratories. Fourteen days after injury the left and the right uninjured carotid vessels were perfusion fixed with 4% paraformaldehyde and processed for routine histology. Six-mm sections were cut and placed on ProBond slides (Fisher).

Paraffin-embedded sections were deparaffinized with xylene (three changes, 10 min each) and rehydrated through a graded ethanol series of 100, 95, and 70% (two changes each at 3 min per change). Endogenous peroxidase activity was blocked by treating with 0.3% H2O2 in methanol for 30 min at room temperature. Blocking of nonspecific binding was accomplished with a 1-h incubation in 2.0% goat serum and 3% bovine serum albumin in PBS. The slides were incubated overnight in the primary anti-Sp1 antibody (Santa Cruz) diluted 1:1000 in PBS. Binding of the primary antibody was detected with a biotinylated goat anti-rabbit antibody that was subsequently detected with an avidin-biotin complex with horseradish peroxidase (Vectostain Elite ABC kit, Vector Laboratories) using diaminobenzidine tetrahydrochloride as the peroxidase substrate.

RESULTS

Combinatorial Mutation Analysis of CArG-box1, CArG-box2, and the GC-rich Repressor Element—The SM-MHC, SM-22α, and SM α-actin promoters all contain a set of paired CArG boxes each of which has been demonstrated to specifically bind SRF (1, 22, 23). For all three promoters, both CArG elements were shown to be required for maximal transcriptional activity in cultured vascular SMCs. However, in contrast to the SM-MHC and SM α-actin promoters where mutation of either CArG element resulted in complete abrogation of all CArG-dependent activity, the most proximal CArG box in the SM-22α promoter was capable of sustaining activity on its own, albeit at a reduced level. Thus, although the transcriptional mechanisms that utilize this dual-CArG motif are likely to have several features in common, other gene-specific differences may also exist.

To better understand CArG-dependent regulation of the SM-MHC gene and to gain insight regarding the role of the GC-rich repressor element, we analyzed the activity of these elements in a variety of sequence contexts. From our previous deletion analysis (1), we determined that a SM-MHC reporter-gene construct containing nucleotides −1346 to +88 (pCAT-1346), that included the entire 227-bp conserved domain, was sufficient for maximal transcriptional activity in vascular SMCs. Using this pCAT-1346 construct as a template, both CArG boxes and the GC-rich repressor element were mutated either alone or in combination with each other, and tested for alterations of transcriptional activity in transient transfection assays using cultured vascular SMCs (Fig. 1). For comparative purposes wild-type deletion constructs pCAT-1102, pCAT-1182, and pCAT-1346 were also included in the analysis. For ease of reference we refer to activity levels at or below 20-fold over promoterless pCAT (the level of pCAT-1102 construct which does not contain the 227-bp domain) as minimal.

The activity levels of pCAT-CArG1Mut, pCAT-CArG2MutA, and pCAT-GC-richMutA were previously described (1), however, because CArG-box2 (CC(TTTTT)GG) does not conform to the classic CArG consensus site (CC(A/T)6GG) (30), we generated a different mutation within this element (pCAT-CArG2MutB) that was previously shown to be important for SRF binding (22, 31). Also for verification purposes, we created an additional mutant construct that targeted the GC-rich repressor element (pCAT-GCrichMutB) at nucleotide positions known to be required for Sp1 binding (32). As shown in Fig. 1, the activity levels of the pCAT-CArG2MutA and pCAT-CArG2MutB constructs were approximately equal (20- and 17-fold over promoterless pCAT, respectively). This similar decrease in activity levels suggests that both mutations equally inhibited binding of the same positive acting factor, most likely SRF. Likewise, the similar increases in activity levels observed for pCAT-GCrichMutA (87-fold over promoterless pCAT) and pCAT-GCrichMutB (94-fold over promoterless pCAT), indicated the abrogation of binding of a common negative-acting factor, potentially Sp1 or a related family member.

Based on the juxtapositioning of the GC-rich repressor element and CArG-box2, we originally hypothesized that the primary mechanism of repression was likely to be mediated through factor binding to the GC-rich sequence leading to inhibition of SRF binding to CArG-box2. However, the combinatorial mutation data strongly suggests that the presence of the GC-rich element can also negatively regulate CArG-box1 activity. As shown in Fig. 1, the activity of pCAT-1182 which contains CArG-box1 was greater than that of pCAT-1102 (34-versus 20-fold over promoterless pCAT). This finding suggested that CArG-box1 contained within the pCAT-1102 construct was capable of enhancing transcription above the 20-fold minimal level in the absence of CArG-box2. However, when CArG-box2 was mutated (pCAT-CArG2MutA and -MutB) within the context of the pCAT-1346 construct, transcriptional activity decreased to the 20-fold level of pCAT-1102 and not the expected 34-fold level observed for the pCAT-1182 construct, even though CArG-box1 remained intact. These data suggested that a repressor element located between nucleotides −1182 and −1346 negatively influenced CArG-box1 activity. To test if the previously identified GC-rich repressor element was responsible for the putative decrease in CArG-box1 activity, we generated a construct in which both CArG-box2 and the GC-rich repressor element were mutated (pCAT-CArG2/GC-richMut). Mutation of CArG-box2 along with the GC-rich element allowed us to rule out the possibility that any noted increases in activity could be due to alleviation of repressor effects on CArG-box2 activity. The activity level of this construct was determined to be 35-fold over that of promoterless-pCAT, essentially identical to the 34-fold activity level of the pCAT-1182 construct. Activity returned to a minimal level when CArG-box1 was mutated along with the CArG-box2 and GC-rich elements (pCAT-CArG1/CArG2/GC-richMut). These data strongly suggest that the GC-rich element, located greater than 100 bp upstream, negatively regulated CArG-box1 activity.

To determine if we could similarly measure alterations of CArG-box2 activity by mutating the GC-rich repressor, we created a construct in which CArG-box1 and the GC-rich repressor element were both mutated (pCAT-CArG1/GC-richMut) and compared its activity to the CArG-box1 only mutant (pCAT-CArG1Mut). Both of these constructs were determined to have only minimal levels of activity (below that of pCAT-1102). The inactivity of CArG-box2 even with removal of the GC-rich repressor suggested that in contrast to CArG-box1, CArG-box2 required the presence of an additional CArG element to potentiate transcription. Unfortunately, due to the required presence of CArG-box 1, any potential negative effects of the GC-rich element on CArG-box2 activity could not be directly measured. However, a comparison of activity levels of pCAT-CArG2/GCrichMut (34-fold), pCAT-1346 (47-fold), and
pCAT-GCrichMutA and MutB (−90-fold), suggests that the GC-rich element was capable of repressing the synergistic activation resulting from the presence of both CArG elements. Whether or not both CArG-boxes were directly affected by the presence of the GC-rich element remains to be determined.

To test for the presence of additional potential cis-elements whose positive activity may also have been repressed by the presence of the GC-rich element, we created a double CArG box mutant (pCAT-CArG1/CArG2), and compared its activity to that of pCAT-CArG1/CArG2/GC-richMut. Both of these constructs were determined to have only minimal levels of activity.

These data suggested that either no other positive elements were present or that at least one of the CArG boxes was necessary for enhanced activity. These data also indicated that the negative influence of the GC-rich element appeared to be limited to the conserved domain, since its presence did not significantly affect activity associated with promoter sequences from −1102 to +88.

Analysis of SRF and Sp1 Binding to the Juxtaposed CArG-box2 and GC-rich Elements—Previous studies of the cardiac α-actin gene revealed that the positive SRF activity associated with the most proximal CArG element was subject to repression by a specific factor known as YY1 (33). Electrophoretic mobility shift assays were utilized to demonstrate that repression occurred through SRF and YY1 competition for overlapping binding sites. The fact that CArG-box2 and the GC-rich repressor element are only separated by six nucleotides suggests that analogous to YY1, Sp1 binding may also directly inhibit SRF binding. As previously noted, because CArG-box2 was inactive in the absence of CArG-box1, we could not directly determine if the GC-rich repressor element negatively affected CArG-box2 activity within the cell. Therefore, we employed EMSA to directly test if Sp1 binding to the GC-rich repressor element could inhibit SRF binding to CArG-box2 in vitro. For these studies we utilized three double-stranded synthetic oligonucleotides that contained either CArG-box2 (C2 oligo), the GC-rich element (GC oligo), or both elements (C2 + GC oligo) (Fig. 2A). It should be noted that specificity of SRF and Sp1 binding to the CArG-box2 and GC-rich elements contained within the C2 and GC oligonucleotides (respectively) was shown in our previous study (1).

As shown in Fig. 2B, the three oligonucleotide probes were included in separate reaction mixtures containing either SRF (lanes 1–3), purified Sp1 (lanes 4–6), or both SRF and Sp1 (lanes 7–9). As expected, SRF-DNA interaction was observed only with the C2 and C2 + GC oligonucleotide probes, and...
Sp1-DNA interaction was observed only for the GC and C2 + GC probes (lanes 1–6). The protein-DNA complexes labeled as either SRF or Sp1 containing were identified as such by their ability to form higher order complexes with either anti-SRF- or anti-Sp1-specific antibodies (1). The weak intensity of the SRF-DNA complex shown in lanes 1 and 7 is due to the fact that CArG-box2, in contrast to CArG-box1, functions as a weak binding site for SRF (1). Interestingly, the increase in SRF binding activity observed for the longer C2 + GC probe (lane 3) relative to the C2 probe (lane 1), suggested that SRF binding could be enhanced with the addition of more flanking DNA. The slightly faster mobilities of the C2 + GC-SRF (lane 3) and C2 + GC-Sp1 (lane 6) complexes relative to the C2-SRF (lane 1) and GC-Sp1 (lane 4) complexes, are most likely the result of DNA bending resulting from factor binding. Importantly, a comparison of all protein-DNA reaction combinations (lanes 1–9) revealed that a unique higher ordered complex formed only when SRF and Sp1 were added together in the presence of the C2 + GC probe (lane 9).

We performed antibody supershift EMSA to determine if the unique protein-DNA complex that formed with the C2 + GC probe contained both SRF and Sp1 (Fig. 3A). To serve as mobility reference points we included reactions that contained the GC probe and either Sp1 alone (lanes 1 and 3) or Sp1 plus anti-SRF antibody (lane 5). A comparison of lanes 2 and 4 demonstrated that addition of anti-SRF antibody resulted in decreased abundance of the unique complex (arrow) and formation of higher order supershifted (SS) complexes. This result demonstrated that SRF represented at least one component of the unique C2 + GC complex. Due to the similar mobilities of the Sp1 supershifted complexes (lanes 5 and 6) and the unique C2 + GC complex (lane 2), we could not readily determine if the band representing the unique complex diminished with addition of anti-Sp1 antibody to the reaction mixture. The use of different reaction conditions, polyacrylamide gel mixtures and/or electrophoresis times failed to provide better resolution of these complexes. Therefore, we employed an alternative method to determine whether Sp1 was part of the unique C2 + GC complex. We reasoned that, if Sp1 was a component of the unique complex, then based on simple binding kinetics and as long as probe concentration was not limiting, more of this complex should be generated with the addition of more Sp1 protein. As shown in Fig. 3B (lanes 1–3), in the presence of constant amounts of excess C2 + GC probe and SRF, increased amounts of Sp1 protein added to the binding reaction resulted in a corresponding increase in formation of the unique complex.
Thus, these data together with the data presented in Fig. 3A, demonstrated that SRF and Sp1 were able to simultaneously bind to the C2 + GC oligonucleotide. This finding indicates that, in contrast to YY1, the putative Sp1 repressor activity associated with the GC-rich element is probably not mediated through direct inhibition of SRF binding. Inhibition of SRF binding to CarG-box1 is also very unlikely given the fact that it is located approximately 100 bp away from the GC-rich element.

In Vivo Footprint Analysis of Protein-DNA Interaction within the Intact Aorta—As a first step toward understanding the likely importance of CarG-box1, CarG-box2, and the GC-rich repressor element for SM-MHC expression within fully differentiated vascular SMCs, we performed in vivo methylation footprint analysis of the endogenous SM-MHC promoter region using intact rat aorta. DMS is a small molecule that methylates guanine residues in DNA at the N-7 position and, to a lesser extent, adenines at the N-3 position, making them sensitive to subsequent cleavage at alkaline pH and elevated temperatures (34). When bound to specific DNA residues, transcription factors can decrease or enhance purine reactivity to DMS methylation relative to naked DNA (35, 36). Although limited DMS penetration has been reported to be a hindrance in the analysis of whole tissues (37), we reasoned that the limited number of cell layers and tubular structure would make the rat aorta susceptible to DMS treatment and further in vivo footprint analysis using the LMPCR technique as originally described by Mueller and Wold (28). For in vitro control reactions we utilized naked DNA obtained from rat kidney tissue.

To obtain sufficient material for multiple LMPCRs and to reduce any potential variability resulting from the DMS treatment step, eight DMS-treated rat aortas were combined prior to the cell lysis and DNA isolation step. A schematic depicting the location of the oligonucleotide primer sets utilized for each of the three in vivo footprinting reactions is given in Fig. 4A. The presence of a repeat domain located immediately downstream of CarG-box1 did not allow for generation of template-specific primers that could be utilized in the LMPCRs. Subsequently, analysis of the sense strand encompassing this element was prohibited. The three primer sets utilized allowed for individual nucleotide analysis of the antisense strand from −1338 to −1185 (primers 1–3, Fig. 4B), sense strand from −1290 to −1190 (primers 4–6, Fig. 4C), and antisense strand from −1137 to −1044 (primers 7 and 8, Fig. 4D). The insets next to Fig. 4, B and D provide a more detailed view of the antisense strands encompassing the CarG-box2 and GC-rich elements, and CarG-box1, respectively.

As shown in Fig. 4B–D and summarized in 4E, when compared with naked DNA controls, several regions of altered methylation reactivity were identified within the conserved 227-bp region. In particular, guanine residues that exhibited either methylation protection or hypermethylation were present within or immediately adjacent to CarG-box1, CarG-box2, and the GC-rich repressor element. Within CarG-box2 both of the guanine doublets found on the sense strand (nucleotides −1223 and −1222) and antisense strand (−1230 and −1231) were protected. Guanines −1232 and −1233 located immediately adjacent to CarG-box2 were hypermethylated and protected, respectively. Protection of the two pairs of guanine doublets is identical to the methylation protection pattern previously described for SRF binding to the c-fos SRE in vivo (38). Additionally, in vitro studies have shown that these sites are likely to be in close contact with SRF and are critical for binding (33, 39). Hypermethylation can result from local conformational changes in the DNA (35), thus, hypermethylation of guanine −1232 is consistent with SRF binding, since SRF is a known DNA-bending protein (40), and has been shown to induce hypermethylation of guanine residues the lie adjacent to the c-fos SRE (39). An analysis of the guanine doublets within CarG-box1 was not as straightforward as for CarG-box2. Mainly, the presence of the repeat domain prohibited sense strand analysis and, only one of the CarG-box1 guanines (nucleotide −1111), which was protected, could be observed for the antisense strand. The absence of particular nucleotide bands is unfortunately an inherent property of this technique (41, 42), yet, it should be noted that these absences or differences in band intensities reflect differential access of DMS to guanine residues as a consequence of the DNA sequence context, and do not effect comparisons between in vitro and in vivo samples (42). It should also be noted that modification of reaction conditions and/or the use of alternative thermophilic DNA polymerases failed to enhance the intensities of faint bands observed in both in vitro and in vivo reactions. Analysis of the two guanine residues (nucleotides −1101 and −1102) located immediately adjacent to CarG-box1, showed a pattern of hypermethylation and protection similar to the paired guanines located adjacent to CarG-box2. These similarities in guanine methylation reactivities between the two CarGs suggests that these two elements were bound by the same factor within aortic SMCs, most likely SRF.

Several of the guanine residues contained within the GC-rich repressor element were protected from DMS reactivity (Fig. 4, B and C). Protection occurred at nucleotide positions −1212 (sense strand), −1215, −1214, −1213, −1211, and −1209 (antisense strand). No band was evident at guanine nucleotide position −1210. The protection of multiple guanine residues contained within the Sp1 site is consistent with the in vivo methylation pattern previously described for Sp1 binding to its recognition sequence within the murine TK promoter and the growth hormone gene in pituitary tumor cells (42, 43). The detection of protein-DNA interaction at this site within the intact aorta suggests that factor binding to the GC-rich repressor element is important for the normal regulation of SM-MHC expression in the fully differentiated vascular SMC.

The enhancer region previously identified by Kallmeier et al. (21) contained protected and hypermethylated sites almost throughout the entire region (Fig. 4, B and C). This high level of reactivity appears to abruptly end at the distal edge of this region around nucleotide position −1330. Notably, this position also marks the 5′ end of the 227-bp conserved domain (1). Although it was not the focus of this study, these in vivo data are consistent with the in vitro data that suggests that this region contains multiple cis elements that are recognized by a variety of nuclear factors. Other regions that exhibited evidence of significant protein-DNA interaction (e.g. nucleotides −1188 to −1204) may also represent important cis elements that warrant further study.

Increased Levels of Sp1 Factor in Myointimal SMCs—Immunohistochemical characterization of atherosclerotic lesions in human (12), and injury induced vascular lesions in dog (15), and rat (4), demonstrated a marked contrast in the expression of major cytoskeletal proteins between intimal and normal medial SMCs. In particular, SM-MHC protein presence was shown to be greatly reduced in SMCs located in both the atherosclerotic and injury-induced vascular lesion. Although results of our in vivo footprinting assays showing factor binding to the GC-rich element provides evidence for involvement of this element in the normal regulation of SM-MHC expression, we hypothesized that increases in the putative Sp1 repressor protein within the myointimal SMC may also contribute to decreases in SM-MHC expression following vascular injury. As a first step toward testing this hypothesis we attempted in vivo
FIG. 4. *In vivo* footprint analysis of protein interaction with CARG-box1, CARG-box2, and GC-rich elements within the intact rat aorta. A, schematic depicting the location of the oligonucleotides utilized for *in vivo* footprint analysis of the 227-bp conserved domain of the rat...
footprinting of intimal SMC (mechanically harvested) following balloon catheter-induced injury of the thoracic rat aorta. Unfortunately, despite the pooling of multiple injured vessels, DNA yields were far too low for analysis. As an alternative, we performed immunohistochemical analysis to determine if there were qualitative changes in expression of Sp1 protein, which we have shown binds the SM-MHC GC-rich repressor element, following balloon-catheter induced injury. As shown in Fig. 5, whereas only a few isolated positively stained nuclei were found in cells located within the medial and adventitial layers, nearly all nuclei in myointimal cells were strongly positive for the Sp1 presence. Similar to the injured vessel, only few positive cells were found in the media and adventitia of the uninjured control vessel. It should be made clear that these data do not provide any direct causal link, however, this finding provides correlative evidence suggesting that increased expression of Sp1 may contribute to decreased SM MHC protein levels in SMCs found in vascular lesions.

**DISCUSSION**

Relatively little is understood regarding the transcriptional mechanisms that determine vascular SMC phenotype either during normal growth or under pathological conditions. In the present study we demonstrated that SRF-dependent activation of the SM-MHC gene, a highly specific marker of the SMC, was greatly modified by the presence of a GC-rich repressor element that was specifically recognized by Sp1. The fact that Sp1 binding to the GC-rich element did not interfere with SRF-CArG interaction, indicates that the mechanism of Sp1 repression is likely to be different from other negative-acting factors that directly compete with SRF for cis-element binding. Moreover, our transcriptional data together with the immunohistochemical data that showed increased levels of Sp1 in intimal SMCs, suggests that this putative repressor protein may contribute to phenotypic modulation of the SMC in vascular lesions.

A review of the promoter activities of all SM-specific genes analyzed thus far suggests that, with the possible exception of h1-calponin (44), the CarG motif is critical for expression of SM-specific genes, and thus may represent an important mechanism for the coordinate expression of SM-specific marker proteins during differentiation. Of note, the presence of two CarG boxes, or a “dual-CarG” motif, has been shown to be important for maximal expression of SM MHC (this study) (1), SM α-actin (22), and SM-22α (23). A detailed analysis of these three promoters suggests that, although highly similar, there exists distinct differences in CarG-dependent activities. For the SM α-actin promoter, mutation of either CarG resulted in complete abrogation of all activity (22). The removal of additional negative elements located further upstream failed to increase activity in vascular SMCs. Hence, for SM α-actin, in all sequence contexts thus far tested, both CarGs were absolutely required for activity in vascular SMCs. Using transgenic mice, Kim et al. (23) demonstrated that mutation of the dual-CarG motif contained within a −441-bp fragment of the SM-22α promoter, which is normally highly expressed in arterial SMCs, virtually eliminated all activity. Additional transient transfection analyses in A10 SMCs demonstrated that the most proximal CarG was absolutely required for activity, and that mutation of a fragment carrying the more distal CarG only reduced activity by 55%. It should be noted that, similar to SM-MHC, the SM-22α promoter also contains an Sp1 site juxtaposed to the CarG motif which is normally highly expressed in arterial SMCs, virtually eliminated all activity. Additional transient transfection analyses in A10 SMCs demonstrated that the most proximal CarG was absolutely required for activity, and that mutation of a fragment carrying the more distal CarG only reduced activity by 55%. It should be noted that, similar to SM-MHC, the SM-22α promoter also contains an Sp1 site juxtaposed to the more distal CarG box. The moderate 55% reduction in activity may be due to the fact that the adjacent Sp1 binding site was simultaneously mutated along with the CarG element. A CarG mutation with the Sp1 site left intact was not tested. Analysis of the data presented in this study suggests that the SM-MHC...
Promoter is similar to both SM \( \alpha \)-actin and SM-22\( \alpha \), in that, like SM \( \alpha \)-actin, mutation of either CA\( G \) led to complete abrogation of all CA\( G \)-dependent activity. However, similar to SM-22\( \alpha \), when the GC-rich repressor element was mutated, the most proximal CA\( G \) was capable of potentiating transcription on its own. The most conspicuous feature common to all three of these promoters, as well as the telokin promoter, is that the most proximal CA\( G \) element was invariably required for CA\( G \)-dependent activity.

Several lines of evidence suggest that SRF is the likely factor mediating CA\( G \)-dependent expression of the SM-MHC gene within the SMC. First, using antibody supershift analyses we demonstrated that both of the SM-MHC CA\( G \)s were specifically recognized by SRF or an antigenically related protein derived from SMC nuclear extract (1). Second, specific nucleotide mutations known to disrupt SRF-CA\( G \) interaction also disrupted expression in SMCs (Fig. 1). Finally, our in \textit{vivo} footprint analysis of protein-DNA interaction within the intact aorta yielded a pattern of altered methylation reactivity that is highly consistent with SRF binding. In particular, although the data is incomplete for CA\( G \)-box1, for CA\( G \)-box2, both pairs of guanine doublets located within the 10-bp element were protected from DMS reactivity. This pattern of methylation protection is virtually identical to methylation reactivity patterns generated from in \textit{vivo} SRF-SRE binding studies (39). Additionally, the hypermethylation of the guanine residues located adjacent to both CA\( G \) boxes 1 and 2, is also consistent with in \textit{vivo} methylation binding studies of SRF to the c-fos SRE (39). Taken together, these data strongly suggest that SRF mediates CA\( G \) dependent expression of the SM-MHC gene.

CA\( G \)-SRF interaction appears to be critical for SM-specific expression, however, SRF binding to a CA\( G \) element and activating transcription is clearly not an event unique to the SMC. Although not as ubiquitous as once thought, a recent analysis of SRF levels in the developing chicken demonstrated that SRF is indeed very highly expressed in all three (cardiac, skeletal, and smooth) types of muscle tissues (45). Thus, it is unlikely that SRF-CA\( G \)-interaction alone is sufficient to drive SM-specific expression. Studies of striated muscle-specific genes (reviewed in Sartorelli et al. (46)), have demonstrated that SRF-CA\( G \)-interaction can function synergistically with other muscle-specific factors (e.g. Myod) to direct muscle-specific expression. In a similar manner, it is possible that tissue-specific expression of the SM-MHC gene is generated through SRF binding to the SM-MHC CA\( G \)s and interacting with an additional SM-specific trans-acting factor. The in \textit{vivo} footprint presented in this report, and data presented in a recent study by Kallmeier et al. (21), suggest that the enhancer region present within the SM-MHC 227-bp conserved domain represents a good candidate region for identifying other potential \textit{cis} elements and \textit{trans} factors that may be important for directing SM-specific expression. Additionally, studies of SRF activation of genes expressed in non-SMCs (47–49) demonstrated that members of the \textit{ets} and homeodomain binding proteins function synergistically with SRF to potentiate transcription. Although no homeodomain sites were identified within the conserved 227-bp domain, it is worth noting that two \textit{ets} consensus binding sites (AGGA), positioned at nucleotides $\sim$1202 and $\sim$1195 ($\sim$20 bp downstream of CA\( G \)-box2), were bound by protein within the intact aorta (Fig. 4, C and E). Although the positioning of these \textit{ets} motifs relative to CA\( G \)-box2 is different from that found in the c-fos promoter, in \textit{vitro} studies on ternary complex formation between SRF-SRE and the \textit{ets} protein Elk-1 have demonstrated that there exists great flexibility in the orientation and spacing of the \textit{ets} motif relative to the SRF binding site (50). As previously postulated based on their presence in the SM-22\( \alpha \) promoter (23), these AGGA elements and binding by an \textit{ets} protein may also play a role in directing tissue-specific transcription of the SM-MHC gene.

Perhaps one of the most perplexing aspects of SM-MHC expression is the identification of an Sp1 binding site that functions as a repressor, and the presence of two other Sp1 binding sites within the same promoter that function as activators (20). In general, Sp1 binding to its recognition element leads to activation of transcription. However, Sp1 has also been shown to function as a negative regulator. For example, it was recently demonstrated that Egr-1, the immediate-early growth response gene product, displaces Sp1 from a GC-rich composite element to activate expression of the platelet-derived growth factor A chain (51). Additionally, it was recently demonstrated that Sp1 activates and inhibits transcription from separate elements in the promoter of the human adenine nucleotide translocase 2 gene (52). Thus, these studies provide precedence for Sp1-mediated inhibition and activation of the SM-MHC gene. However, the possibility remains that another factor that also recognizes the GC-rich repressor is responsible for the observed negative activity associated with this element. One candidate is Sp3, a member of the Sp1 family, that we previously demonstrated will also bind to the GC-rich element (1). In contrast to Sp1, it is possible EMSA would reveal that Sp3 will directly inhibit binding of SRF to CA\( G \)-box2. However, the noted long range effect of the GC-rich element on CA\( G \)-box1 activity strongly suggests that the mechanism of repression must be more than steric hindrance. To this extent, we are currently focusing our efforts on experiments that identify which factors are indeed important within the cell, and have begun testing models that incorporate the noted long range repressor effects of the GC-rich element. As previously mentioned, factor binding to this element in the fully differentiated SMCs found in the intact aorta, suggests that it contributes to the normal regulation of this gene. In the absence of the GC-rich repressor, activity of the SM-MHC promoter in cultured vascular SMCs was essentially identical to that of the powerful SV40-enhancer promoter (1). Thus, given the fact that SM-MHC protein is very stable and high levels of SM-MHC gene transcription are not likely to be required for normal cell function, it is conceivable that a repressor activity, like many other eukaryotic genes (53), is necessary for proper SM-MHC expression. The fact that guanine residues contained within the GC-rich element were not fully protected in the in \textit{vivo} footprinting analysis indicates that binding/repression was potentially incomplete within the medial SMC. Thus, it is conceivable that additional increases of Sp1 protein could lead to enhanced binding to the GC-rich element and further repression of SM-MHC transcription within the myointimal SMC. If SM-MHC mRNA levels are already at critical concentrations then even slight decreases in SM-MHC gene expression could have significant effects.

The combinatorial mutation and deletion data indicated that the GC-rich element negatively regulated CA\( G \)-box1 activity. The required presence of CA\( G \)-box1 precluded us from directly testing GC-rich repressor effects on CA\( G \)-box2. Nevertheless, EMSA data indicated that the mechanism by which Sp1 inhibited CA\( G \)-dependent activation of the SM-MHC gene was likely to be different from other factors, such as YY1 (33) and glucocorticoid receptor (54), that function through antagonization of SRF binding. Sp1 repression of SRF-CA\( G \) activation is also different from that of Net, a member of the \textit{ets} domain family that forms a ternary complex with SRF-SRE and negatively regulates transcription (55). The data presented here indicated that SRF and Sp1 do not interact directly (Fig. 2, lanes 7 and 8), but rather are capable of binding to their...
respective sites simultaneously (Fig. 2, lane 9). The greater than 100-bp spacing between CARG-box1 and the GC-rich element further suggests that repression is not mediated through binding disruption, but rather via some other mechanism, possibly disruption of SRF interaction with other proteins or complexes that are part of the transcription initiation complex.

Several studies have documented enhanced expression of immediate-early response genes (e.g. c-fos and c-jun) in SMCs as a result of vascular injury (56). Additionally, increases in transcriptional activators such the MEF2 family members has also been observed for myointimal SMCs (57). To our knowledge, the enhanced abundance of Sp1 described in this study, represents the first report of up-regulation of a putative repressor factor in myointimal SMCs. Whether the noted differences in Sp1 abundance directly, or even indirectly, affect SM-MHC gene expression cannot be determined at this time. It is quite possible that the increased presence of Sp1 is the result of the proliferative state of the intimal cells and is only correlative related with the absence of SM-MHC protein. Moreover, as a cautionary note, although there is clear evidence of decreased SM-MHC protein expression in vessels with lesions, there is a lack of evidence that this is due to decreased transcription as opposed to post-transcriptional mechanisms. Consistent with the latter possibility, we have previously presented evidence showing that a factor present in injured vessels, platelet derived growth factor (PDGF-BB) induces selective destabilization of SM α-actin (58) and SM-MHC (59) messenger RNAs. Thus, additional studies that discriminate between transcriptional and post-transcriptional processes are needed. In addition, it will be of future interest to determine if Sp1 negatively regulates other SM-specific promoters where GC-rich elements are found, such as SM-22α, and to what extent does up-regulation of Sp1 affect expression of other genes that play a role in SMC phenotypic modulation.

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