

Smooth Muscle α -Actin CARG Elements Coordinate Formation of a Smooth Muscle Cell-Selective, Serum Response Factor-Containing Activation Complex

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Abstract—Previous studies have shown that multiple serum response factor (SRF)-binding CARG elements were required for smooth muscle cell (SMC)-specific regulation of smooth muscle (SM) α -actin expression. However, a critical question remains as to the mechanisms whereby a ubiquitously expressed transcription factor such as SRF might contribute to SMC-specific expression. The goal of the present study was to investigate the hypothesis that SMC-selective expression of SM α -actin is due at least in part to (1) unique CARG flanking sequences that distinguish the SM α -actin CARGs from other ubiquitously expressed CARG-dependent genes such as *c-fos*, (2) cooperative interactions between CARG elements, and (3) SRF-dependent binding of SMC-selective proteins to the CARG-containing regions of the promoter. Results demonstrated that specific sequences flanking CARG B were important for promoter activity in SMCs but not in bovine aortic endothelial cells. We also provided evidence indicating that the structural orientation between CARGs A and B was an important determinant of promoter function. Electrophoretic mobility shift assays and methylation interference footprinting demonstrated that a unique SRF-containing complex formed that was selective for SMCs and, furthermore, that this complex was probably stabilized by protein-protein interactions and not by specific interactions with CARG flanking sequences. Taken together, the results of these studies provide evidence that SM α -actin expression in SMCs is complex and may involve the formation of a unique multiprotein initiation complex that is coordinated by SRF complexes bound to multiple CARG elements. (*Circ Res.* 2000;86:221-232.)

Key Words: serum response factor ■ smooth muscle α -actin ■ CARG element

Intimal migration and subsequent proliferation of vascular smooth muscle cells (SMCs) has been shown to be important in the development of atherosclerosis and in restenosis after balloon angioplasty.^{1,2} Medial SMCs are normally highly specialized for contraction and express cell type-specific contractile proteins, surface receptors, and second-messenger pathways that are critical for this function (reviewed in Reference³). However, unlike myocardial and skeletal muscle cells that terminally differentiate, vascular SMCs are highly plastic and retain the ability to modulate their phenotype to a more proliferative synthetic state, even in adult animals.⁴⁻⁸ Retention of this plasticity is important in that it permits processes such as vessel repair after injury, but it may also be detrimental in that it increases susceptibility to atherogenic risk factors.

It is clear that understanding the molecular control of SMC differentiation will be dependent on the identification of mechanisms that control the transcription of genes encoding proteins necessary for the differentiated function of SMCs. Several smooth muscle (SM)-specific contractile or contractile-associated proteins, including SM α -actin, myosin heavy chain, h-caldesmon, and SM-22, are useful markers for studying SMC differentiation.³ Of these, SM α -actin has been

the most extensively studied. It is the most abundant SMC protein and is the first known differentiation marker to appear during vessel development.^{9,10} Although it is transiently expressed in the myocardium and skeletal muscle during early stages of development and in fibroblasts found in healing wounds, its expression in adult animals is restricted to SMCs or SM-like cells.^{11,12}

Our laboratory and others have shown that regulation of SM α -actin expression involves a complex interaction of multiple positive and negative *cis* elements that act in a cell-type-specific fashion.¹³⁻¹⁸ The SM α -actin promoter contains 3 CARG elements, designated CARG A at -62, CARG B at -112, and an intronic CARG at +1001, that are completely conserved between all species in which the promoter has been cloned and have been shown to be required for full promoter activity in cultured SMCs.^{17,19} Moreover, we have recently shown by use of transgenic approaches that all 3 CARGs were required for SM α -actin expression *in vivo*. In those experiments, we demonstrated that the SM α -actin promoter regions from -2600 through the first intron were sufficient to drive expression of a LacZ transgene in a pattern virtually identical to that of the endogenous SM α -actin gene and that mutation of CARG A,

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CAR_G B, or the intronic CAR_G inhibited expression in virtually all SMCs.¹⁹

The CAR_G motif was first identified as the core sequence of the serum response element (SRE) within early-response genes such as *c-fos*, and much is known about SRE-mediated regulation on serum stimulation (reviewed in Reference²⁰). The SRE binds the MADS box transcription factor, serum response factor (SRF), but also contains an Ets motif that stabilizes a ternary complex that forms when one of several SRF accessory proteins (Elk-1, Sap-1, or ERP/NET) is recruited to the SRE through SRF-mediated protein-protein interactions.^{21–23} However, CAR_G elements have also been shown to direct developmental and tissue-specific expression of many muscle-specific genes.^{24–28} Interestingly, virtually all of the SM differentiation marker genes characterized to date contain ≥ 2 CAR_G or CAR_G-like elements that are required for their expression in SMCs.^{17,29–33}

Recent studies from this laboratory indicate that CAR_G elements play a critical role in SMC-specific regulation of SM α -actin. For example, when transfected into SMC cultures, a minimal promoter construct containing the first 125 bp of the 5' promoter (p125CAT) had very high activity that was absolutely dependent on CAR_G A and CAR_G B.¹⁷ This same construct had little or no activity in L6 myoblasts or L6 myotubes, and the modest activity of p125CAT observed in endothelial cells was not affected by CAR_G mutations.¹⁷ Moreover, our transgenic model demonstrated that mutation of the intronic CAR_G abolished expression in SMCs but had no effect on the transient expression of SM α -actin that is normally seen in the heart and skeletal muscle during embryonic development.¹⁹ In addition, separate laboratories have shown that the arterial expression of an SM22 promoter/LacZ transgene was absolutely dependent on a conserved CAR_G element.^{32,33} Taken together, these data provide strong evidence that CAR_G elements are of key importance for SMC-specific gene regulation during differentiation/maturation.

Although the SM α -actin CAR_Gs are required for expression in SMCs, a critical unresolved issue is how SRF contributes to SMC-specific transcriptional regulation. SRF binding to CAR_G and CAR_G-like elements is required for cell-type-specific promoter activation, but evidence suggests that SRF binding by itself is not sufficient.³³ Moreover, although recent evidence suggests that muscle tissues contain more SRF than do nonmuscle tissues,^{34,35} SRF is thought to be ubiquitously expressed, indicating that mechanisms in addition to SRF binding are likely to be involved in determining cell-type-specific expression. Several groups have shown that CAR_G flanking sequences are important in skeletal and cardiac α -actin expression,^{36,37} but the specific DNA sequences and *trans*-activating factors involved have not been clearly identified. SMC-specific regulation may also involve *trans*-acting factors other than SRF that are known to bind CAR_G elements. The multifunctional transcription factor, YY1, binds the skeletal actin and SM-22 promoters and can modulate transcription of these genes.^{38,39} However, like SRF, YY1 is expressed in a wide range of tissues, and its role in cell-type-specific regulation is unclear at this time.⁴⁰ Cell-specific CAR_G-dependent regulation may also involve unique combinatorial interactions between multiple *cis*- and

trans-acting factors that, by themselves, are not cell-type specific. Tissue-specific expression of cardiac actin, for example, is mediated by several *cis*- and *trans*-acting factors, including SRF, Sp-1, Nkx-2.5, and Myo D.^{41,42} In short, the mechanisms involved in CAR_G-dependent regulation of SMC-specific genes are undoubtedly complex, and at present, the specific DNA sequences, *trans*-acting factors, and mechanisms that mediate such regulation are poorly understood.

The goal of the present study was to identify key mechanisms that contribute to CAR_G-dependent SMC-specific expression of SM α -actin. We hypothesized that several parameters unique to the SM α -actin gene or to SMCs in general were involved. First, we proposed that the DNA context in which the CAR_Gs are situated may affect the activity and specificity of the SM α -actin promoter. Although the SM α -actin gene lacks a consensus Ets domain, the sequences directly flanking CAR_Gs A and B are nearly completely conserved,¹⁷ which suggests that they play an important regulatory role. Thus, we tested a variety of mutations to the SM α -actin CAR_G flanking sequences to further define regulatory sequences required for SMC-specific expression of this gene. Second, previous evidence indicated that SRF-induced changes in DNA conformation and the structural positioning of multiple CAR_G elements may be important for SRF-dependent activation.^{25,43,44} Moreover, the 40-bp spacing between CAR_Gs A and B is completely conserved across species, with both elements placed on the same side of the DNA. Therefore, we also tested whether the spacing and phasing of these 2 required elements might be important for activity in SMCs. Third, our initial electrophoretic mobility shift assay (EMSA) data demonstrated that an SMC protein, in addition to SRF, was present in a higher order complex with the CAR_G B-containing region of the promoter.¹⁷ For example, when a 95-bp probe that contained CAR_Gs A and B was combined with SMC nuclear extracts, an SRF-containing DNA binding complex formed that had a lower mobility than did a similar complex formed with recombinant SRF (rSRF). In the present study, we further characterized the molecular interactions that are important for the formation of the SRF-containing higher order complex and extended these studies to the intronic CAR_G-containing region of the promoter. The present study provides novel evidence indicating that CAR_G- and SRF-dependent regulation of SM α -actin expression is cell-type specific and is modulated by several parameters including (1) the structural positioning of the CAR_G elements within the first 125 bp of the promoter, (2) the formation of a unique SRF-containing CAR_G-binding complex in SMCs, and (3) the involvement of specific SM α -actin CAR_G flanking sequences in promoter activation.

Materials and Methods

Cell Culture, Transient Transfections, and Gel-Shift Analyses

Rat aortic SMCs,¹⁶ rat L6 myoblasts,¹⁷ rat L6 myotubes,⁴⁵ and bovine aortic endothelial cells (BAECs)¹⁶ were isolated and cultured as previously described. Transfections were performed with 4 μ g of plasmid DNA per well as previously described.¹⁷ Chloramphenicol acetyltransferase (CAT) activity was measured, normalized to the protein concentration of each cell lysate, and expressed relative to the activity of a promoterless CAT construct. Nuclear extracts were

prepared from confluent rat aortic SMCs, BAECs, and rat L6 myoblasts and myotubes by using the methods of Dignam et al.⁴⁶ Human rSRF was synthesized with the TnT-Quick in vitro transcription/translation kit (Promega) from an expression plasmid generously provided by Richard Treisman (Imperial Cancer Research Fund, London, UK). Oligonucleotides used in EMSAs were purchased commercially (Operon Technologies) and include the following: CARG B, 5'-gaggtccctatggttg-3'; SRE, 5'-ttacacagatgcatattaggacat-3'; and intronic CARG, 5'-ttttacctaattaggaatg-3'. Probes were ³²P end-labeled and annealed or generated by polymerase chain reaction (PCR). A Mut95 plasmid that contained transversion mutations (ie, G→T and A→C) to all non-CARG sequences was constructed by subcloning the following fragment (Operon) into an *EcoRI/XbaI* site of the Bluescript KS vector (Promega): 5'aattctgggtgtatcttgcctatatgggatcgcctgaccgttaactaggactagctgaggtgacctgttggctgcactctgtttcttggctagc-3'. Probes used for circular permutation studies were generated with the use of PCR by selecting primers such that the position of the CARG B element varied within the PCR fragment (see Figure 8A for more detail). EMSAs were incubated for 30 minutes at room temperature in 1× binding buffer (10 mmol/L Tris-HCl [pH 7.5], 100 mmol/L KCl, 50 mmol/L NaCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, and 5% glycerol) that included the following: ≈30 pg of labeled probe; either 5 μg of SMC nuclear extract, 15 to 20 μg of BAECs, and myotube and myoblast extracts or 1 μL of rSRF; and 0.2 to 0.6 μg of poly(dI-dC). Antibodies used for supershifts were purchased commercially (Santa Cruz).

Construction of SM α -Actin Promoter Mutations

CARG flanking mutations in the p125CAT context were constructed by PCR amplification using the wild-type SM α -actin promoter and the Mut95 plasmid described above as templates. CARG flanking mutations were made in the p2600Int/LacZ context by using the QuickChange system (Stratagene). CARG spacing and phasing constructs were made in the p125CAT context by ligating different-length linker sequences (5, 10, 15, 20, 45, and 60 bp) into an *HpaI* site created in the wild-type promoter at -102 by an AG to CA mutation. This mutation had no effect on promoter activity.

Methylation Interference Footprinting

A ³²P end-labeled 95-bp probe spanning CARGs A and B was methylated for 3 minutes with 0.05% dimethyl sulfate. The methylated probe was then used in EMSAs under conditions identical to those described above. The low-mobility SRF-containing bands as well as the bands corresponding to free probe were excised from the gel and electrophoresed through 1.0% agarose onto a DEAE membrane. DNA was eluted from the membrane, extracted with an equal volume of phenol/chloroform, and precipitated with EtOH. DNA was cleaved in 100 μL of 1 mol/L piperidine for 30 minutes at 90°C, and equal counts of each band were loaded onto an 8% acrylamide sequencing gel.

An expanded Materials and Methods section is available online at <http://www.circresaha.org>.

Results

Specific CARG Flanking Sequences Were Important for Promoter Activity in SMCs but Not BAECs

The sequences surrounding the SM α -actin CARGs A and B are highly conserved, and several reports have shown that the activity and specificity of CARG-dependent genes are regulated by CARG flanking sequences.^{22,47} Therefore, a series of mutations was made in the CARG flanking regions of the p125CAT reporter construct in order to test the functional significance of these regions on SM α -actin expression in both SMCs and BAECs. The p125CAT construct was chosen for these experiments because this relatively simple promoter

fragment has very high activity in SMCs, is differentially regulated in SMCs versus non-SMCs in a CARG-dependent fashion, and has consistently yielded valuable insight into the regulation of SM α -actin expression.^{17,19,48} Mutations were designed to leave the CARG elements as well as the recently described transforming growth factor- β_1 control element at -48 intact because these elements are known to be required for promoter activity (Figure 1A).

Transfection results shown in Figure 1B indicate that the activity of the wild-type p125CAT construct was 50-fold and 15-fold that of the promoterless construct when transfected into SMCs and BAECs, respectively. Consistent with previous studies from this laboratory, mutation of CARG B completely abolished activity in SMCs but had little effect on activity in BAECs. Mutations to the 3' flanking region of CARG B (pM92-111) significantly reduced promoter activity in SMCs to ≈10% of control, whereas none of the CARG flanking mutants significantly affected promoter activity in BAECs. More defined site-directed mutations to this region demonstrated that the positive activity in the CARG B flanking region was contained within the 6 nucleotides immediately 3' to CARG B (Figure 1C). These data suggest that, in addition to the CARGs themselves, specific CARG flanking sequences are also important for cell-type-specific expression of p125CAT.

To determine whether the CARG B flanking region interacts with other regulatory components within the SM α -actin promoter, we mutated the 6-bp region in a larger promoter context that we previously demonstrated was sufficient to drive expression of SM α -actin in vivo (-2600 through the first intron).¹⁹ The CARG B flanking mutations had nearly identical effects on the activity of the larger promoter construct (Figure 2).

Spacing and Phasing of CARGs A and B Were Important for Promoter Function in SMCs

We have previously shown that all 3 of the CARG elements were required for SM α -actin expression in vivo, which indicated that these elements somehow function interdependently.¹⁹ SRF-induced changes in DNA conformation have been reported in several CARG-containing genes, including *c-fos* and skeletal α -actin, and such changes to DNA structure are likely to be important for their regulation.^{25,43} Of interest, CARGs A and B are located in proximity, and the 40-bp interval that separates them positions them on the same side of the DNA double helix.¹⁷ Moreover, this structural orientation has been completely conserved across all species in which the promoter has been cloned.¹⁷ To determine whether CARG positioning was important for transcriptional activity, we altered the position of CARG B by inserting several different-length linkers between CARGs A and B that not only changed their spacing but also altered their phasing by rotating the elements to different planes along the DNA double helix (Figure 3A).

Results demonstrated (Figure 3B) that as CARG B was moved 5, 10, and 15 bp upstream from CARG A, p125CAT activity dropped significantly. The 15-bp insertion positioned CARGs A and B on opposite sides of the DNA and resulted in an 85% drop in promoter activity. With the insertion of an

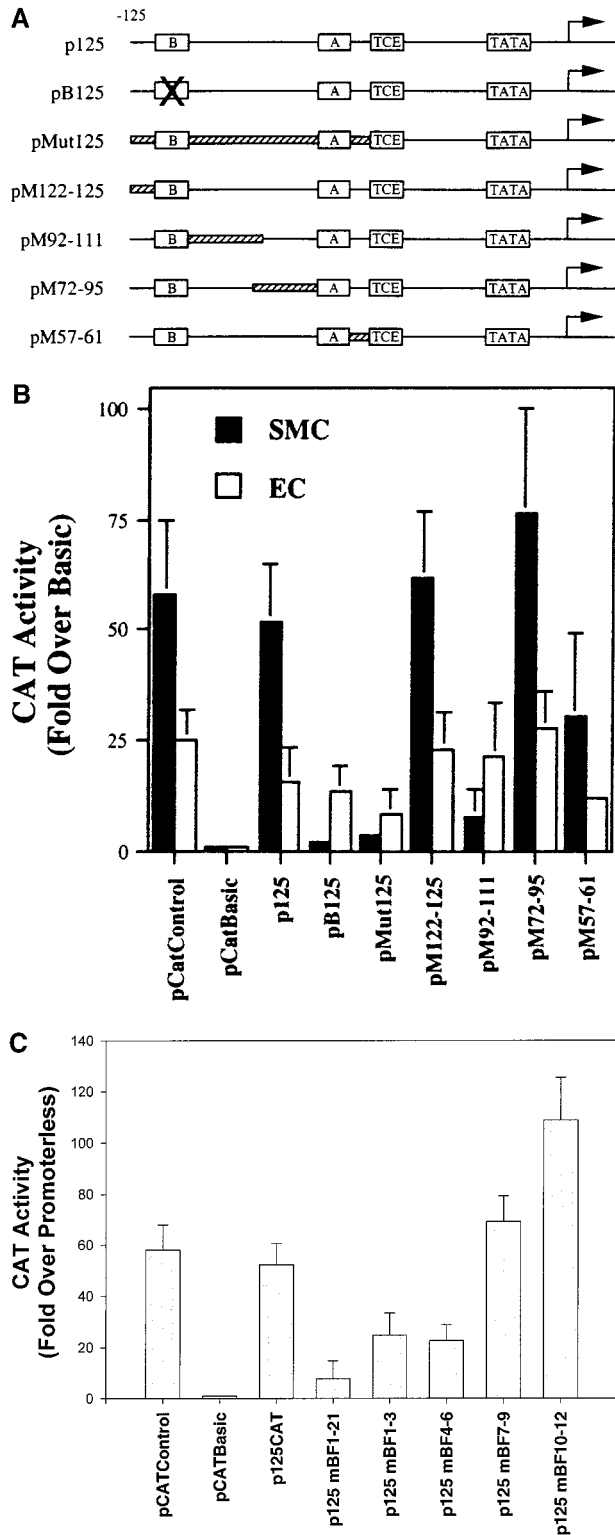


Figure 1. Effects of CARG flanking mutations on p125CAT activity in rat aortic SMCs and bovine aortic endothelial cells (ECs). A, p125CAT mutant constructs were designed with a site-directed mutation to CARG B (indicated by an X) or block mutations to selected CARG flanking regions (indicated by hatching). In all flanking mutations, both CARGs A and B as well as the transcriptionally active transforming growth factor- β 1 control element (TCE) were left intact. B, Constructs were transfected into rat aortic SMCs and ECs. CAT activity (mean \pm SD) is expressed relative to the baseline CAT activity of a promoterless CAT construct set to 1. An SV40-driven CAT construct (pCAT control)

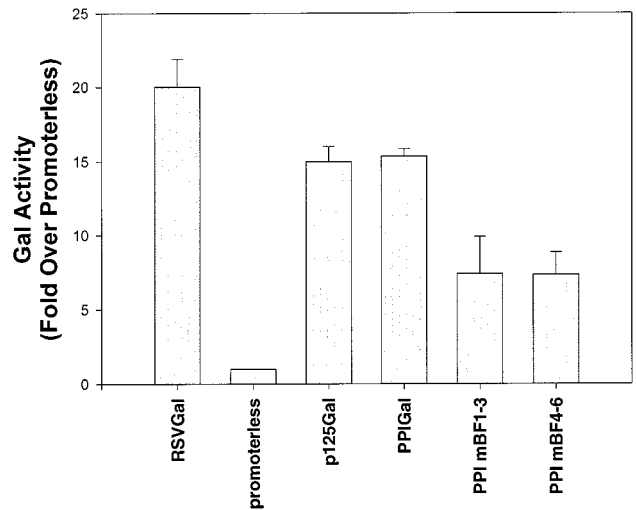


Figure 2. Effects of CARG flanking mutations on the activity of the SM α -actin promoter region from -2600 through the first intron. The 6-bp region on the 3' flank of CARG B was mutated (mBF) in the context of the SM α -actin promoter region from -2600 through the first intron (PPI) that was previously shown to be sufficient to drive expression of SM α -actin in a transgenic mouse model. Constructs were transfected into rat aortic SMCs, and LacZ activity (mean \pm SD) is expressed relative to the baseline activity of a promoterless LacZ construct set to 1. RSV indicates Rous sarcoma virus; Gal, galactosidase.

additional 5 bp (from 15 to 20) and the accompanying half turn of DNA, promoter activity increased significantly to 50% of control. The addition of larger stretches of DNA led to large reductions in promoter activity that could not be relieved by changes in phasing. These results indicate that a higher order interaction that includes SRF bound to both CARGs A and B is critical for transcriptional activity of the SM α -actin promoter. Although we have not directly tested the importance of CARG phasing and spacing in the context of p2600Int/CAT, the fact that the spacing and phasing of CARGs A and B within the endogenous gene are highly conserved provides strong evidence that our results in the p125 context are relevant to regulation of the endogenous gene.

SMC Extracts Contained Unique CARG-Binding Activities

Previous studies have demonstrated that CARG elements are critical for SMC-specific regulation of SM α -actin expression, and the results from the present study indicated that specific CARG flanking sequences and CARG spacing and phasing were important for SMC-selective expression of SM α -actin. To further explore the mechanisms that control SMC-specific regulation, we performed extensive gel-shift analyses to determine whether the CARG-containing regions of the SM α -actin promoter exhibited DNA binding activities that were specific to SMCs. Nuclear extracts from SMCs,

served as a control for transfection efficiency and CAT activity. C, To further characterize the positive activity on the 3' flank of CARG B, 3-bp site-directed mutations were made to this region (mBF). Mutant constructs were transfected into rat aortic SMCs, and CAT activity was measured as above.

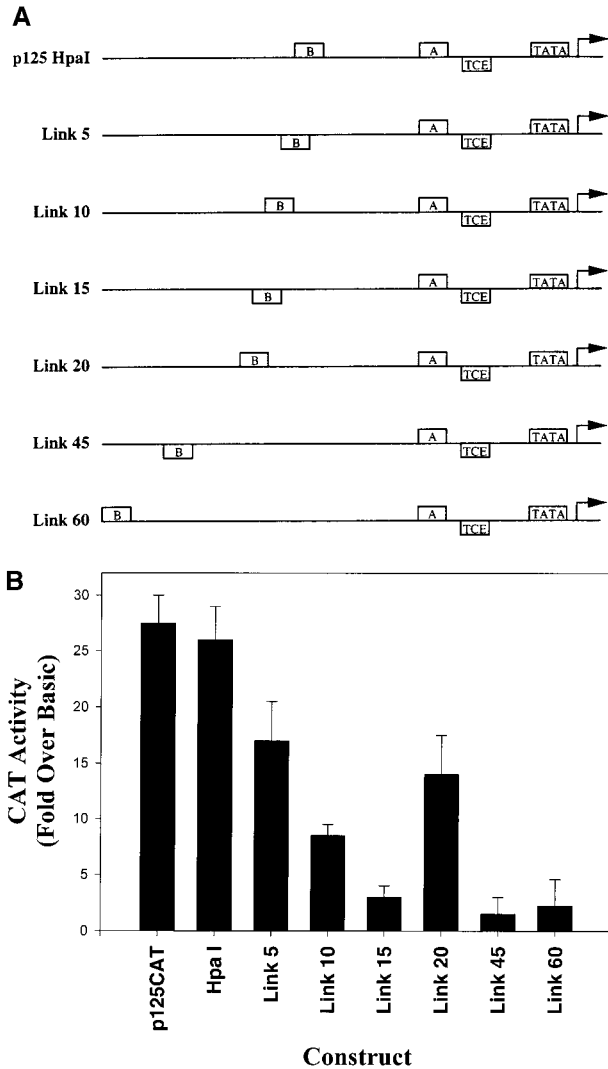


Figure 3. The effects of CARg spacing and phasing on the transcriptional activity of the p125CAT construct. A, CARg spacing and phasing constructs were altered by ligating different-length linker sequences (5, 10, 15, 20, 45, and 60 bp) into an *HpaI* site created between CARg A and B in the wild-type promoter at -102. This mutation had no significant effect on promoter activity. In both the wild-type SM α -actin promoter and the *HpaI* construct, CARg A and B are on the same side of the DNA helix. B, Each construct (4 μ g) was transfected into rat aortic SMCs, and CAT activity (mean \pm SD) is expressed relative to the baseline CAT activity of a promoterless CAT construct set to 1.

BAECs, L6 myoblasts, and L6 myotubes were combined with the 20-bp CARg B oligonucleotide or with a 95-bp probe that contained CARg A and B, because both are required for promoter activity. As previously observed, Figure 4 demonstrates that SMC extracts formed 2 SRF-containing shift bands when reacted with CARg B (lanes 3 and 4, bands A and B) and 2 lower mobility SRF-containing complexes when reacted with the 95-bp probe (lanes 1 and 2, bands 1 and 2). Of particular interest, these results also showed that bands 1 and 2 were present only when SMC extracts were used, whereas shift bands formed with non-SMC types had electrophoretic mobilities identical to SMC complexes A and B formed with the 20-bp CARg B oligo.

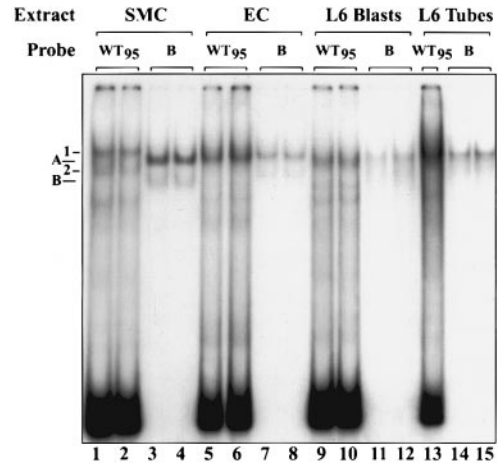


Figure 4. Binding of nuclear factor(s) from SMCs, bovine aortic ECs, L6 myoblasts, and L6 myotubes to the 95-bp and CARg B oligonucleotide probes. A radiolabeled 20-bp CARg B oligonucleotide probe or a 95-bp probe from -42 to -137 of the SM α -actin promoter (WT₉₅) was incubated with 10 μ g of nuclear extracts prepared from SMCs, bovine aortic ECs, L6 myoblasts, and L6 myotubes. The positions of the 2 SRF-containing shift complexes that formed when SMC extract was combined with the WT₉₅ probe are labeled 1 and 2. Shift complexes that formed when SMC extract was reacted with the CARg B probe are labeled A and B.

Results from Figure 4 indicate that the SRF-containing shift complexes that were formed when the 95-bp probe was reacted with SMC nuclear extracts were distinct from those that were formed when a 20-bp CARg B probe was used. We previously reported results based on supershift and cross-linking analyses demonstrating that band 1 contained SRF or an SRF-like protein.¹⁷ As a first step in determining the DNA regions required for the formation of the higher order SRF-containing complex in SMCs, we compared the shift complexes that form with the 95-bp probe, a 20-bp CARg B probe, an SRE CARg probe, and a 40-bp probe containing CARg A and B oligonucleotides linked together (A+B). The results (Figure 5A) demonstrated that the 2 SRF-containing complexes that formed when the 95-bp probe was reacted with SMC nuclear extracts had lower mobilities than those formed when rSRF was used (compare lane 2, bands A and B, with lane 7, bands 1 and 2). However, when 20-bp double-stranded oligonucleotides containing SRE or CARg B were used as probes, the SMC complexes had mobilities equal to those formed with rSRF (compare lanes 3 and 4 with lane 1). Shift complexes formed with the A+B probe had mobilities similar to those of bands A and B. These results are consistent with our earlier observations and suggest that a higher order SRF-containing complex forms with the 95-bp probe when SMC nuclear extracts are used but that 20-bp CARg-containing oligonucleotides alone are not sufficient for its formation. Importantly, the reduced mobility of the SRF-containing SMC complexes cannot be accounted for by differences between SRF in SMC extracts and rSRF because SRF-dependent shift mobilities were identical when SMC extracts or rSRF was reacted with all other CARg-containing probes tested. Moreover, the reduced mobility of the complex was not due to differences in probe size per se, in view of the fact

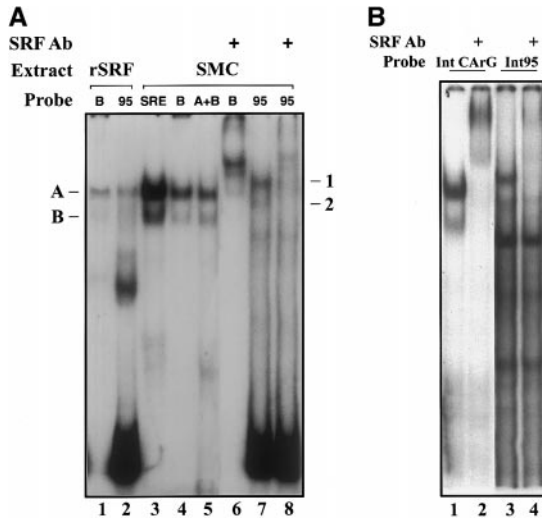


Figure 5. Comparison of SMC nuclear extract and rSRF binding to SRE, CArG B, WT₉₅ (95), and 40-bp A+B probes. A, SRE, CArG B, 95, and a 40-bp probe containing CArGs A and B (A+B) were radiolabeled and incubated with rSRF (lanes 1 and 2) or SMC nuclear extract (lanes 3 through 8). A polyclonal antibody to SRF (SRF Ab) was added to lanes 6 and 8. The positions of the SRF-containing SMC shift complexes that formed when SMC extract was combined with the 95 probe are labeled 1 and 2. SRF-containing shift complexes that formed with SMC extracts and the CArG B probe are labeled bands A and B. B, A 20-bp intronic CArG oligonucleotide (Int) and a 95-bp probe from +968 to +1063 (Int95) were reacted with SMC nuclear extracts. A polyclonal antibody to SRF was added to lanes 2 and 4.

that shift complexes formed with rSRF were identical when the 95- or 20-bp probes were used.

Because we have shown recently that the intronic CArG binds SRF and was required for *in vivo* expression of SM α -actin in SMCs,¹⁹ we also performed gel-shift analyses to determine whether SRF can bind this element as part of a higher order complex. As shown in Figure 5B, the SRF-containing binding complex that forms when the intronic CArG is contained within a 95-bp intronic fragment had lower mobility than the SRF-containing complex that binds to a 20-bp intronic CArG oligonucleotide.

Methylation Interference Footprinting Provided Evidence That SMCs May Contain an SRF-Associated DNA Binding Protein

The preceding gel-shift data provide strong evidence that the higher order SRF-containing complex in SMCs contains a protein (or proteins) in addition to SRF. To further test this possibility and to define promoter regions involved in binding, we performed methylation interference footprinting on the lowest mobility SRF complex that bound to the 95-bp probe that contained CArGs A and B. Consistent with previously published reports involving DNA binding by rSRF,²⁰ results shown in Figure 6 demonstrated that the interference footprint of rSRF covered only the guanine residues of CArG B (compare lane 5 with lane 3). In contrast, the footprint obtained with SMC extracts covered CArG B but was also extended to several guanine residues on the 5' flank of CArG B (compare lane 4 with lane 3). CArG A did

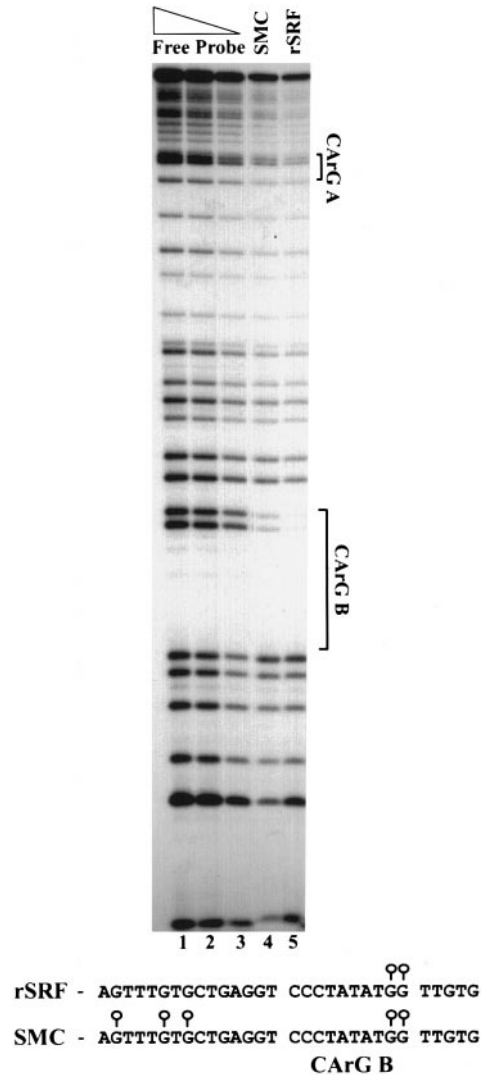


Figure 6. Methylation interference footprinting of protein-DNA interactions within the 95-bp probe. The WT₉₅ probe was generated by PCR such that the sense strand was labeled. The gel-purified probe was incubated with either 5 μ L of *in vitro*-synthesized rSRF or 15 μ g of SMC nuclear extract. The lowest mobility shift band from each lane and the band corresponding to free probe were cut out, and DNA was purified and treated with piperidine. Products were resolved on a 6% denaturing polyacrylamide gel. At the bottom is a schematic summary of these results.

not exhibit footprinting with either rSRF or SMC extracts (compare lanes 4 and 5 with lane 3). These data indicate that methylation of guanine residues in the region 5' to CArG B interferes with the formation of the low-mobility SMC-specific/selective SRF-containing complex.

Formation of the Higher Order Complex Required CArG B and Was Based on SRF-Mediated Protein-Protein Interactions

It has been shown that the DNA sequences flanking *c-fos* SRE CArG contain a critical *cis* binding site and play an important role in ternary complex formation.^{22,23} Although the SM α -actin gene does not contain an Ets or Ets-like domain in proximity to CArG B, results of our gel-shift

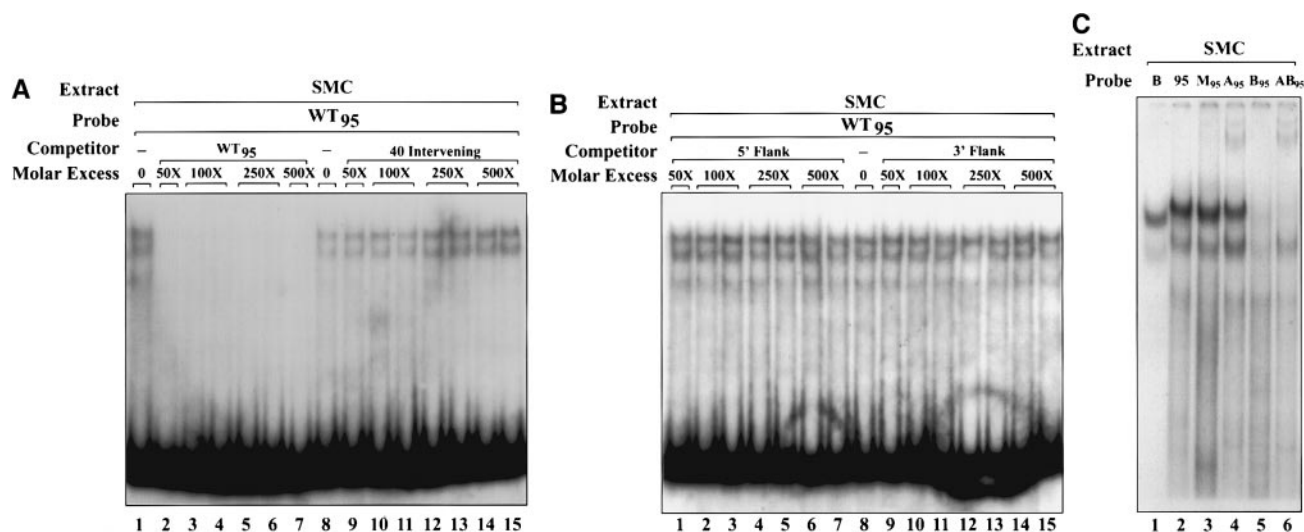


Figure 7. Competition analysis of the higher order SRF containing SMC shift complex. Radiolabeled WT₉₅ probe was incubated with 5 μ g of SMC nuclear extract. Competitions were performed with concentrations of cold double-stranded probe ranging from 50 \times to 500 \times molar excess. A, Competition with cold WT₉₅ probe (lanes 2 through 7) or a cold probe consisting of the 40-bp intervening CARGs A and B (lanes 9 through 14). B, Competition with a cold probe from the 5' flank of CARG B (lanes 1 through 7) or from the 3' flank of CARG A (lanes 9 through 15). C, SMC nuclear extract (5 μ g) incubated with radiolabeled 95-bp probes containing mutations of CARG A (lane 4, A₉₅), CARG B (lane 5, B₉₅), or CARGs A+B (lane 6, AB₉₅) and with a CARG flanking mutant shift probe that had transversion mutations to all of the non-CARG regions (lane 3, M₉₅). For comparison, SMC extracts were also incubated with CARG B and WT₉₅ probes (lanes 1 and 2, respectively).

analyses (Figures 3 and 4) and methylation interference footprinting studies (Figure 6) provided evidence for the presence of an additional protein in the shift complex. Moreover, our functional data have demonstrated the importance of the 3' flank of CARG B for p125CAT activity in SMCs. To define more precisely the DNA sequences involved in the formation of the higher order complex at CARG B, we performed additional EMSA analyses. Figures 7A and 7B show that the higher order complex was not affected by competition with oligonucleotides containing the 40-bp region between the CARGs (Figure 6A, lanes 9 through 15), the region 5' to CARG B (Figure 6B, lanes 1 through 7), or the region 3' to CARG A (Figure 6B, lanes 9 through 16).

We also tested the effects of mutations to the 95-bp gel-shift probe itself, including the CARGs as well as the CARG-flanking regions. One such probe, M₉₅, left CARGs A and B intact but contained mutations to the remaining non-CARG regions, a mutational strategy similar to that used for the pMut125CAT transfection construct. When the M₉₅ probe was reacted with SMC nuclear extracts, an SRF-containing higher order complex formed that was nearly identical to the complex formed with the wild-type probe (Figure 7C, compare lanes 2 and 3). A mutation in CARG A that had previously been shown to inhibit SRF binding and the functional activity of the p125CAT construct had no effect on complex formation (lane 4), whereas a similar mutation in CARG B caused nearly complete disruption of all protein interactions with the 95-bp probe (lanes 5 and 6). These data indicate that formation of the higher order SRF-containing shift complex was primarily due to SRF mediated protein-protein interactions but also required that the CARG B element be contained within a minimal-length (ie, 95-bp) probe that may have provided a structural backbone to stabilize the shift complex. In an attempt to charac-

terize the protein-protein and protein-DNA interactions within the SRF-containing shift complex, we systematically tested a large number of gel-shift variables, including ionic strength, ionic composition, pH, DNA template concentrations, extract concentrations, competitor rSRF protein, and detergent addition. Interestingly, none of these treatments disrupted the formation of the SRF-containing complex without disrupting SRF binding to CARG B.

Importantly, although we showed that mutations to the CARG B 3' flanking region significantly decreased the functional activity of both the p125 and 2600Int constructs in SMCs, these mutations failed to affect formation of the CARG B-SRF higher order complex in *in vitro* gel-shift assays. Thus, the formation of the higher order CARG B complex *in vitro* is clearly not dependent on these flanking regions. However, we cannot rule out the possibility that the CARG B flanking region interacts with *trans*-acting factors within (or separate from) the higher order complex but that such interactions are simply not detectable under *in vitro* gel-shift conditions.

Reduced Mobilities of the SMC Shift Complexes Were Not due to SRF-Induced DNA Bending

SRF binding is known to cause changes in DNA conformation that can be responsible for significant differences in complex mobility when long shift probes are used.⁴³ We directly tested this possibility by performing circular permutation assays in which the position of the CARG B element was moved incrementally from one end of a 95-bp probe to the other. The shift probes generated for these experiments are shown in Figure 8A, and the EMSA results obtained are depicted in Figure 8B. Changing the position of CARG B within a 95-bp probe had little effect on the mobility of complexes formed with SMC extracts or rSRF, suggesting

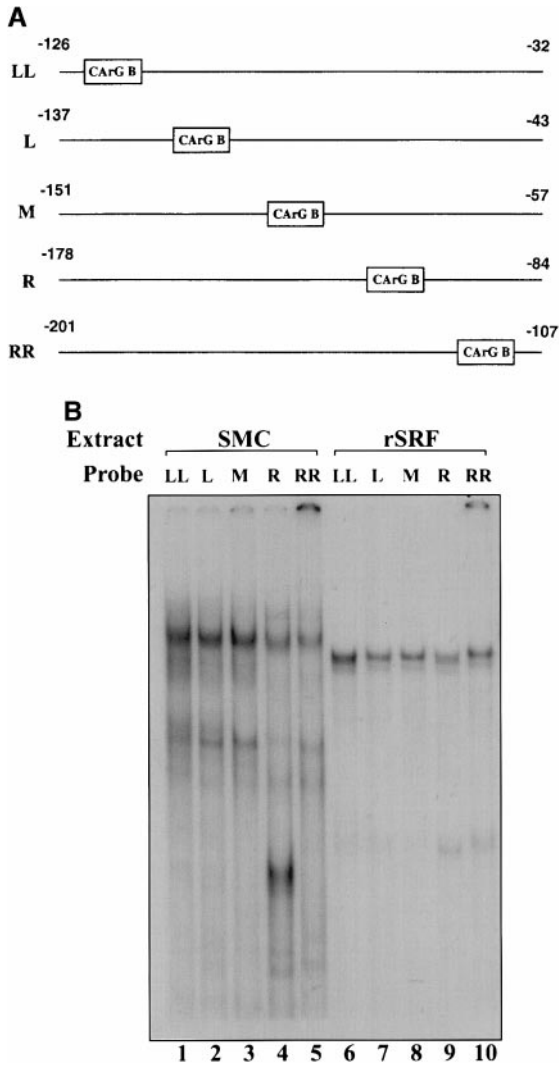


Figure 8. Circular permutation assay for SRF-induced bending of the WT₉₅ shift probe. A, Radiolabeled WT₉₅ probes were generated in which the position of the CARG B element was moved incrementally from the 5' end (L indicates left) to the 3' end (R indicates right) of the probe. PCR primers were chosen such that the 95-bp probes spanned the promoter sequences indicated (+1 equals transcription start site). B, Probes were incubated for 30 minutes with 5 μ g of SMC nuclear extracts (lanes 1 through 5) or 1 μ L of in vitro-synthesized rSRF (lanes 6 through 10).

that SRF-induced DNA bending did not contribute significantly to the mobility differences observed in these in vitro experiments.

The Higher Order SRF-Containing SMC Shift Complex Did Not Contain YY1 or the Known SRF Accessory Proteins SAP-1 or Elk-1

YY1 is a multifunctional transcription factor that has been shown to bind CARG elements through contacts with both the internal A/T-rich region as well as the 2 guanine residues on the end of the element.^{38,39} Kim et al³² have shown that a YY1-containing shift complex forms when SMC extracts are reacted with a probe containing an SM-22 CARG that is identical to the CARG B sequence at 8 of 10 nucleotide positions. Therefore, to determine whether YY1 was present

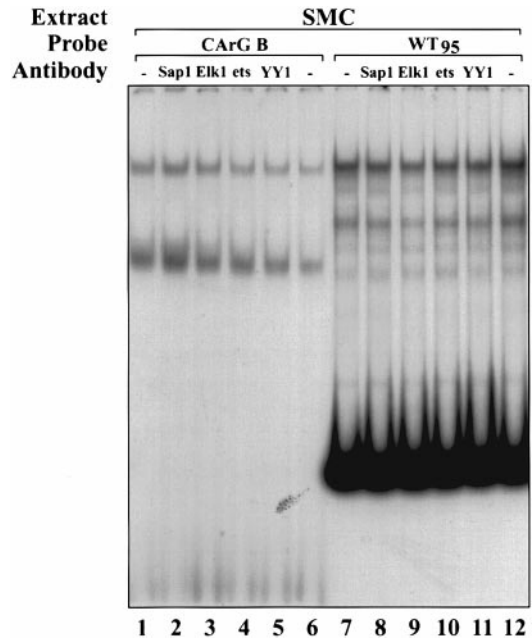


Figure 9. Supershift analysis of SMC nuclear protein-DNA complexes. SMC nuclear extract (5 μ g) was incubated for 20 minutes with radiolabeled CARG B (lanes 1 through 6) and WT₉₅ (lanes 7 through 12) probes. Antibodies to the following transcription factors were added as follows: Sap-1, lanes 2 and 8; Elk-1, lanes 3 and 9; Ets family consensus DNA binding domain, lanes 4 and 10; and YY1, lanes 5 and 11. Incubation was continued for 15 minutes, and then samples were loaded on a 5% nondenaturing polyacrylamide gel and electrophoresed at 170 V for 2 hours.

in any of the SMC DNA binding complexes, we performed supershift analysis using both the CARG B and 95-bp probes. Figure 9 shows that an anti-YY1 antibody, which we have previously used to supershift YY1-containing complexes in EMSA, failed to affect our gel-shift results. Thus, although YY1 may well bind the CARG regions of the SM α -actin promoter, its presence cannot account for the SMC shift complexes observed in the present study.

We also tested whether the presence of any of the known SRF accessory factors was responsible for the decreased mobility of the SRF-containing higher order complex. In these experiments, we used antibodies specific for Sap-1 and Elk-1 and a third antibody specific for the highly conserved DNA binding domain of the Ets family of transcription factors. Results (Figure 9) showed that none of these antibodies had an effect on the binding complexes formed when SMC extracts were combined with either the CARG B or 95-bp probes, although they did react with ternary complexes formed with a *c-fos* SRE probe (data not shown). Taken together, these results indicate that none of the known Ets family members were contained within the unique SMC SRF-containing shift complexes.

Discussion

The central focus of the present study was to identify mechanisms whereby binding of the ubiquitously expressed transcription factor, SRF, to the SM α -actin CARGs contributes to SMC-specific regulation of this gene. Studies of CARG-mediated transcriptional control in promoters of other

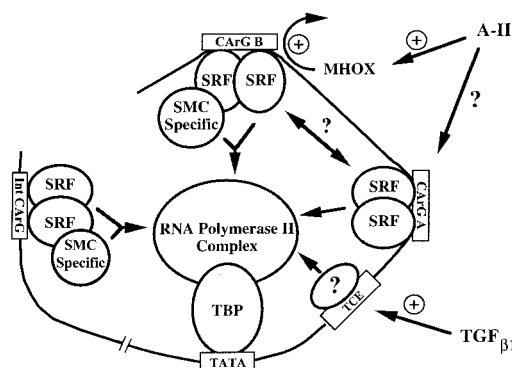


Figure 10. Schematic model depicting mechanisms that contribute to SMC-specific regulation of SM α -actin. In this model, formation of the TATA binding protein (TBP)-containing DNA polymerase II transcription initiation complex is dependent on cooperative interaction of the 3 SM α -actin CarG elements. Changes to CarG spacing and/or phasing between CarGs A and B would disrupt the formation of this larger complex by changing the physical positions of these components such that interactions necessary for complex formation or activation could not occur. SRF binding to CarG B and the intronic CarG, perhaps facilitated by the presence of the muscle-specific homeodomain protein, MhoX, aids in the recruitment of an additional SMC-specific factor that may interact with CarG flanking sequences. SRF binding to all 3 CarGs coordinates the formation of a larger complex through protein-protein interactions and possibly SRF-induced changes in DNA conformation. Binding of an as-yet-unknown transforming growth factor- β_1 (TGF β_1)-inducible factor to the transforming growth factor- β_1 control element (TCE) is also required for activity and may help to recruit or position additional general or SMC-specific transcription components. A-II indicates angiotensin II.

genes suggest that cell-specific regulation via this element may be conferred by a combination of differences in SRF expression, by the DNA sequences within and flanking the CarG element, and by additional regulatory motifs and *trans* factors that regulate the function of SRF in a cell-type and promoter-specific fashion.^{34,35,37,49,50} Results from the present study provide evidence indicating that SMC-specific CarG-dependent expression of SM α -actin in vascular SMCs is likewise complex and involves several unique interactive mechanisms including (1) the structural positioning of the CarG A and B elements within the promoter, (2) the formation of SRF-containing CarG binding complexes that were unique to SMCs, and (3) the involvement of specific SM α -actin CarG flanking sequences.

The data from the present study demonstrate that spacing and phasing of CarG B in relation to CarG A and possibly other core promoter components are critical determinants of SM α -actin promoter activity. We have previously shown that specific CarG sequences are required at each position, because switching CarGs A and B also resulted in loss of promoter function in SMCs.⁴⁸ Of interest, virtually all SMC-specific promoters characterized to date, including SM α -actin, SM myosin heavy chain, and SM-22, contain multiple conserved CarG elements that have been shown to be required for maximal expression.^{17,29,30} Moreover, paired CarG elements have also been shown to be required for expression of many skeletal- and cardiac-specific genes.²⁴⁻²⁸ Thus, it is possible that the specific structural requirements described herein may apply to other SMC differentiation

marker genes as well as to CarG-dependent genes in other cell types. Indeed, Chow et al⁴⁴ have shown that the activity of the skeletal α -actin promoter was dependent on CarG spacing and phasing. It was postulated by these authors that promoter activation occurred when protein-induced changes in DNA conformation aligned the CarGs and other required *cis* elements on the same face of the DNA, which allowed recruitment of a transcription initiation complex. Interestingly, the intronic CarG, which is located ≈ 1000 bp 3' to CarGs A and B, is also required for *in vivo* expression of SM α -actin,¹⁹ but the importance of its positioning within the promoter is unknown at this time. We do have preliminary evidence from cell culture experiments demonstrating that the activity of the intronic CarG region was not significantly affected by changes in positioning (C.P. Mack and G.K. Owens, unpublished results, 1999). However, this region functioned as an enhancer in only one orientation, which argues that it does have at least some structural requirements. The mechanisms by which SRF recruits components of the basal transcriptional machinery are largely unknown. SRF has been shown to directly bind the general transcription factor, TFIIF,^{51,52} and SRF and Sap-1 have been shown to interact with the transcription coactivators, p300 and CREB-binding protein.⁵³⁻⁵⁵

We present several lines of evidence suggesting that SMC-specific regulation of SM α -actin expression may also involve binding of a novel SRF accessory protein that is selectively expressed in SMCs. First, extensive EMSA analysis showed that the mobility of the complexes formed when SMC nuclear extracts were reacted with the 95-bp probes that contained CarGs A and B or the intronic CarG could not be attributed to the presence of SRF alone, because complexes formed with rSRF alone ran significantly faster. Differences in probe length could not account for these results, and Western analysis (data not shown) confirmed that SRF in SMC extracts and rSRF were nearly identical in size. However, we cannot rule out the possibility that SMC-selective posttranscriptional modifications to SRF may have affected SRF tertiary structure, dimerization, or DNA binding under the non-denaturing conditions of gel-shift analyses and that these differences were only detected when SRF was bound to the longer 95-bp probe. Second, the results of methylation interference footprinting studies on the SMC low-mobility shift complex showed extension of the SRF footprint. When taken together with the results of our gel-shift analyses, these data are consistent with the presence of an additional protein in the SMC SRF-containing complex. Additional EMSA analyses demonstrated that formation of this complex was most likely the result of protein-protein interactions between the unknown factor and SRF and not by specific interactions between the unknown protein and specific CarG flanking sequences. However, a significant stretch of nonspecific DNA flanking sequence was required for complex formation, perhaps to stabilize the entire complex through nonspecific protein-DNA interactions or through interactions based on DNA structure. Third, the low-mobility SRF-containing complexes formed with SMC extracts were distinct from those formed with the other cell types tested, which ran with different mobilities than did the SMC complexes. Fourth,

based on supershift analyses, the higher order complex did not appear to be due to binding of the known SRF accessory proteins, since we could not supershift the low-mobility complex with antibodies specific to Elk-1 or Sap-1 or with an antibody that reacts with the conserved DNA binding domain of Ets protein family members. However, results do not rule out the involvement of an as-yet-unidentified member of the Ets protein family that does not react with the antibodies tested.

Results from the present study provided clear evidence indicating that the highly conserved CARG B 3' flanking region plays a key role in SMC-specific expression of SM α -actin, in that mutations to the CARG B 3' flanking region abolished promoter activity in SMCs but had no effect in BAECs. The requirement for specific flanking sequences for SM α -actin promoter activity is analogous to similar requirements in the regulation of skeletal actin expression by the muscle response element.³⁶ However, these are the first studies to show that such flanking sequences are involved in SMC-specific regulation of an SMC differentiation marker such as SM α -actin. Interestingly, although mutations to the CARG B 3' flanking region abolished the transcriptional activity of the P125 construct in SMCs, we were unable to detect protein binding to this region. It is important to note that formation of the unique SRF higher order complex in SMCs was not dependent on specific DNA sequences in the 3' region because complete mutation of this region had no effect on formation of that complex. As such, we have no direct evidence that the functional activity of the CARG B 3' flanking region and formation of the unique SRF complex are related. If the 3' flanking region of CARG B does interact with the higher order complex, it must be in a more subtle way that affects complex conformation and/or activation instead of complex binding. Consistent with this idea, studies by Acton et al⁵⁶ on the SRF-related yeast protein, MCM1, have shown that mutations in CARG flanking sequences of the consensus MCM1 element did not affect MCM1 binding but did affect MCM1-induced DNA bending and promoter activation. It is also possible that formation of the higher order complex might prevent binding to the CARG B 3' flanking region because of its proximity and possible steric effects.

There is now extensive evidence demonstrating that muscle-specific gene regulation is dependent on a complex interaction between multiple independent *cis* elements or modules and their corresponding *trans* factors (see References³⁷ and ⁵⁷ for reviews). The nature of the protein interactions within the higher order SRF-containing complex is of particular interest because of recent evidence demonstrating that several muscle-specific factors have been shown to interact with SRF to regulate muscle-specific genes. For example, Chen and Schwartz⁵⁸ have shown that the heart-specific homeobox factor, Nkx2.5, binds SRF and can *trans*-activate the cardiac α -actin promoter even in the absence of Nkx2.5 DNA binding. Muscle-specific basic helix-loop-helix proteins, such as Myo D and myogenin, have also been shown to interact with not only SRF but also with other members of the MADS box family (including MEF2) that contain conserved DNA binding domains.⁵⁹ Interestingly, the known SRF-associated transcription factors have relatively

low affinities for the Ets binding site, but protein-protein interactions with SRF aid in their binding to the *c-fos* SRE.^{22,60}

Previous studies from this laboratory have demonstrated that regulation of SRF binding to the SM α -actin CARGs may be a critical pathway for controlling SM α -actin expression. Most CARG elements, although highly conserved among species, show considerable differences in their internal A/T-rich sequences when they are compared between promoters, and such differences can have effects on SRF binding. The SM α -actin CARGs A and B, for example, bind SRF with low affinity because of G/C substitutions in the A/T-rich core that are completely conserved across all species in which that promoter has been cloned.⁶¹ Interestingly, in previous studies, we found that when the "weak" SM α -actin CARGs were replaced with the much stronger SRE CARG, promoter activity increased dramatically and was no longer limited to SMCs.⁴⁸ In addition, we presented evidence indicating that angiotensin II-induced upregulation of SM α -actin expression was mediated by increased expression of the homeodomain protein, Mhox, which increased SRF binding to CARG B.⁶¹ Importantly, Mhox itself was not found within the SRF-containing gel-shift complexes and could not account for the SMC SRF-containing higher order complex. Taken together, our results suggest that cooperative interactions between multiple CARG elements, G/C substitutions within the internal A/T-rich region, and the accompanying regulation of SRF binding provide still other mechanisms for CARG-dependent regulation of SMC gene expression. Such regulatory mechanisms may also be shared by CARG-dependent genes in other muscle cell types. For example, Lee et al²⁸ have demonstrated that 3 CARG elements are essential for skeletal α -actin expression and that SRF binding to 2 strong CARGs facilitates binding of SRF to the third relatively weak CARG element. Cooperative SRF binding to the 95-bp probe was not detected in the present study, but perhaps other factors within SMC may mediate the SMC-specific increase in SRF binding and thus the activation of CARG-dependent SMC genes.

In conclusion, the data in the present study and in others suggest that SMC-specific regulation of SM α -actin expression is CARG dependent and that several mechanisms are involved, including (1) increased SRF expression in SMCs, (2) SRF binding to CARG elements that have precise structural requirements relative to one another, (3) unique combinatorial interactions with other regulatory elements including the region flanking CARG B, (4) a unique SMC-selective SRF-containing complex bound to the CARG B and intronic CARG regions of the promoter, and (5) homeodomain protein-mediated regulation of SRF binding to CARG elements that exhibit reduced SRF binding affinity. Taken together, results suggest that SRF complexes that are bound to the SM α -actin CARGs, perhaps aided by SRF-induced changes in DNA conformation, coordinate the assembly of a higher order transcription complex that activates SM α -actin gene expression (Figure 10). In any case, given the requirement of CARG elements for the expression of multiple SMC differentiation marker genes,^{19,29-31} it is clear that further studies to identify ubiquitous and/or SMC-specific transcription factors that

interact with SRF to control SM α -actin gene expression may provide key insight regarding the molecular mechanisms that control SMC differentiation.

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