Substitution of the Degenerate Smooth Muscle (SM) α-Actin CC(A/T-rich)₆GG Elements with c-fos Serum Response Elements Results in Increased Basal Expression but Relaxed SM Cell Specificity and Reduced Angiotensin II Inducibility*

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We have previously demonstrated that both CC(A/T-rich)₆GG (CArG) elements A and B of the smooth muscle (SM) α-actin promoter are required for smooth muscle cell (SMC)-specific expression and angiotensin II (AII)-induced stimulation. Moreover, results provided evidence that AII responsiveness of SM α-actin was at least partially dependent on modulation of serum response factor (SRF) binding to the SM α-actin CArGs by the homeodomain containing protein, MHox. The goal of the present study was to investigate whether the degeneracy of the SM α-actin CArGs (both contain a Gua or Cyt substitution in their A/T-rich center) and their reduced SRF binding activity as compared with c-fos serum response element (SRE) is important for conferring cell type-specific expression and AII responsiveness. Transient transfection assays using SM α-actin reporter gene constructs in which the endogenous SM α-actin CArGs were replaced by c-fos SREs demonstrated the following: 1) relaxation of cell-specific expression, 2) a 50% reduction in AII responsiveness, and 3) reduced ability to be transactivated by MHox. In addition, we also showed that the position of the SM α-actin CArGs was important in that interchanging them abolished both basal and AII-induced activities. Taken together, these results suggest that the reduced SRF binding activities of the SM α-actin CArGs and CArG positional context contribute to SMC-specific expression of SM α-actin as well as maximal AII responsiveness.

The CArG motif, characterized by the consensus sequence CC(A/T-rich)₆GG, is found in the promoters of several immediate-early response genes (1–5) including c-fos and has been shown to confer serum- and growth factor-induced transcriptional activation of these genes (reviewed in Ref. 6). CArG boxes are also present in the promoters of many skeletal and cardiac muscle-specific genes and are required for developmental and tissue-specific expression (Refs. 7–15, reviewed in Ref. 16). Although CArG elements bind the ubiquitously expressed transcription factor SRF, it is unlikely that SRF alone is sufficient to confer the functional diversity of CArG elements. A large body of evidence has accumulated suggesting that CArG-dependent gene regulation is modulated by post-translational modification of SRF (17), interaction of SRF with SRF accessory proteins (reviewed in Refs. 6 and 18), and combinatorial interaction with other trans-factors in a promoter-specific fashion (15, 19, 20). For example, Sartorelli et al. (21) demonstrated that muscle-specific expression of cardiac α-actin required the presence of CArG boxes, binding sites for SP 1, and the muscle-specific factor MyoD. Previous results from our laboratory also provided evidence for involvement of the SM α-actin CArGs in cell type-specific expression of SM α-actin in concert with other regulatory elements (20). Moreover, CArG elements have also been shown to play a role in regulation of virtually all SMC differentiation marker genes characterized to date including SM-22α (22, 23), SM MHC (24, 25), telokin (26), and h-caldesmon (27).

In contrast to the c-fos gene which contains a single high affinity binding site for SRF in its promoter, many muscle-specific genes including skeletal, cardiac, SM α-actin, SM MHC, and SM-22α contain two or more CArG elements (7, 20, 24, 28, 29). Based on direct measurements of SRF binding (30, 31) and/or predicted SRF binding affinity based on DNA sequence analysis (see Leung and Miyamoto (32) for criteria) many of these CArG elements bind SRF with relatively low affinity as compared with the c-fos SRE. For example, both SM α-actin CArGs A and B, the two distal skeletal α-actin SREs (31), human cardiac α-actin CArG 2, 3, and 4 (28), as well as the SM MHC CArG 2 (24) contain Gua or Cyt substitutions within their central A/T-rich region that reduces SRF binding activity (32). In addition, the most proximal cardiac α-actin CArG, although not containing a Gua or Cyt substitution, was shown to bind SRF less effectively than the c-fos SRE (30). The preceding observations raise the question as to why relatively low affinity CArG elements have been highly conserved during evolution, especially in the light of strong evidence indicating that increased transcriptional activity of a number of CArG-dependent genes is associated with increased SRF binding activity (33–36). One possible hypothesis is that weaker CArG elements might offer an additional level of control through mechanisms that influence SRF binding. In contrast, strong...
SRF binding CArGs appear to be regulated primarily at post-SRF-CARG binding steps through interaction with SRF accessory proteins whose activity is controlled by kinase/phosphatase regulatory systems (reviewed in Ref. 37). A number of mechanisms have been shown to increase SRF binding to CArG elements including post-translational modification of SRF (17), increased SRF protein expression (34), and interaction of SRF with homeodomain factors that modulate SRF binding or kinetics (38). For example, Croissant et al. (34) have shown that increased SRF protein levels appeared to be obligatory for increased skeletal α-actin expression during myoblast differentiation. In addition, we have previously provided evidence that TGF-β and AII-induced increases of SM α-actin expression in SMC were accompanied by increased SRF binding to CArG A and B (39, 40). Whereas TGF-β increased SRF protein expression, AII treatment did not. Our work suggested that the AII effects on SM α-actin transcription were mediated, at least in part, by modulation of SRF binding to CArG B by the homeodomain containing protein Mhox, which has been shown to increase SRF binding to CArG B in vitro (40). Walsh and co-workers (41) have carried out extensive and eloquent studies of the importance of both the CArG central AT region and flanking sequences of the skeletal α-actin muscle regulatory element for muscle-specific expression. For example, the authors demonstrated that replacement of the muscle regulatory element by a c-fos SRE resulted in loss of muscle-specific expression of skeletal α-actin. However, to our knowledge, no studies have specifically addressed the importance of weakly binding degenerate CArG elements per se in regulation of muscle-specific genes nor have any studies addressed the importance of such elements in control of SMC-specific and agonist-induced transcriptional regulation.

The aim of the present study was to determine whether sequence degeneracy of the SM α-actin CArG elements and their reduced SRF binding activity contributes to cell-specific SM α-actin expression as well as the ability of the gene to be regulated in response to AII or the mesodermally restricted homeodomain containing protein Mhox.

MATERIALS AND METHODS

Construction of Promoter-CAT Expression Plasmids—The generation of various SM α-actin promoter CAT constructs, including the CArG A and B mutants, have been described previously (20). Additional CArG mutations within a 155-bp or 2.8-kb SM α-actin promoter CAT construct (pProm CAT) were generated using the Ex-site mutagenesis kit according to the manufacturer’s instructions (Stratagene). The integrity and accuracy of the mutated constructs were determined by dyeoxy sequencing (42).

All promoter-CAT plasmid DNAs used for transfections were prepared by using an alkaline lysis procedure (43) followed by banding on two successive ethidium bromide cesium chloride gradients. Transfection results were not altered when independent plasmid preparations were tested.

Cell Culture, Transient Transfections, and Reporter Gene Assays—SMC from rat thoracic aorta and bovine endothelial cells (BAEC) were isolated and cultured as described previously (20, 44). The culture conditions for the rat L6 skeletal myoblast were also described previously (20), and fusion into myotubes was induced by reducing fetal bovine serum (FBS) concentrations to 1% when cells reached confluency. AKR-2B mouse embryonic fibroblasts were a gift of Dr. Harold Moses (Vanderbilt University, Nashville, TN) and were cultured in McCoy’s 5A medium (Life Technologies, Inc.) supplemented with 5% bovine serum (FBS) and 10% fetal bovine serum (FBS). Cells were seeded for transient transfection assays into 6-well plates at a density of 1.5 × 10^6 cells/cm^2 and BAEC at a density of 2 × 10^6 cells/cm^2. Transfection of the CAT reporter gene constructs (4 μg of DNA per well) was performed in triplicate 30 h (in case of L6 myoblasts, 48 h) after plating using the transfection reagent DOTAP (Boehringer Mannheim) (6.7 μg/μg DNA) according to the manufacturer’s recommendations. BAEC were transfected using the transfection reagent Transfectam (Promega) according to the manufacturer’s instructions since transfection efficiency in these cells was lower with DOTAP.2 No differences in transfection efficiencies were observed between Transfectam and DOTAP in other cell types. Cells were exposed to the DNA/DOTAP or DNA/Transfetam mixture for 4 h under serum-free conditions. After incubation for 4 days in serum-free medium, the cells were harvested for the reporter assay 72 h later by scraping into ice-cold buffer A (15 mM Tris (pH 8.0), 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.15 mM spermine tetrahydrochloride, 1 mM dithiothreitol) (45). Cell lysates were prepared by four freeze-thaws, followed by 10 min heat inactivation at 65 °C; 95-μl aliquots of each cell extract were assayed for CAT activity by enzymatic cleavage of arylhydrocarbon hydroxylase (NEN Life Science Products) (46). CAT activities were normalized to that of a control promotorless construct set to one as described previously (20). This permits comparison of the activity of the wild-type p155 region versus that of the various mutants including disruption of the CArGs or substitution with the c-fos SRE. Normalization of CAT activity to that of SM α-actin constructs containing mutations of CArG A and B was not performed because CArG mutations have differential effects in SMC versus non-SMC, and such normalization would preclude comparison of the cell-specific functionality of the SM α-actin CArGs versus the c-fos SRE, a major aim of the present studies. Experiments were repeated two to three times, and relative CAT activity data were expressed as the mean ± S.D. unless otherwise noted.

SMC used for transfection experiments involving AII stimulation were plated at a density of 3 × 10^4/cm^2, grown to confluency in 10% fetal bovine serum containing medium, and then growth-arrested for 4 days (i.e., serum-free medium (SFM) (47) prior to stimulation with AII (Peninsula Laboratories, 10^-6 m) or SFM. Cells used for these experiments were between the 6th and the 12th passage. SMC that are growth-arrested in this fashion express multiple SMC differentiation marker proteins including SM α-actin, SM MHc, h-caldesmon, h1 calponin, SM tropomyosin, and SM myosin light chain (MLC crunch (48–50). Confluent, growth-arrested SMC were then transiently transfected (in triplicate in 6-well plates) with 5 μg of DNA using the transfection reagent DOTAP (Boehringer Mannheim) according to the manufacturer’s recommendations. After an incubation period of 12–14 h, the medium was replaced with fresh serum-free medium, and AII (10^-5 m) or vehicle were added. Cells were harvested 72 h later and processed for the reporter assay as described above.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSA)—Crude nuclear extracts from SMC were prepared by the method of Dignam et al. (51). SMC were either grown to confluency in 10% FBS or growth-arrested in SFM for 4 days when treated with AII. SMC were then exposed to AII (10^-5 m) or SFM for 4 h. Protein concentrations were measured by the Bradford assay (Bio-Rad). Probes for EMSA were obtained by end-labeling 20 μm single-stranded oligonucleotides with 150 μCi of [-32P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase. Labeled single-stranded oligonucleotides were annealed, and unincorporated nucleotides were removed using Nuc Trap columns (Stratagene) as recommended by the manufacturer. The specificity for all probes used was 1.0–1.1 μCi/μmol. The following nucleotides, purchased commercially (Operon Technologies, Inc.), were used as a probe (only sense strand shown): CArG B, 5′ GAGGTC CCTATATGGTTGTC 3′; CArG A, 5′ TTGGTCTTTGGTTGGAGACG 3′; c-fos SRE, 5′ GAGTTCATTTAGGGATCATG 3′.

EMSA were performed with 20-μl binding reactions that contained ~50 pg of [-32P]-labeled annealed oligonucleotides, nuclear extracts (3 or 5 μg in Dignam buffer D), human recombinant SRF (1 or 2 μl), 100 μm KCl, 5 μm HEPES (pH 7.9), 1 mM EDTA, 35 mM Tris (pH 7.5), 1.125 mM dithiothreitol, 10% glycerol, and 0.125 μg of poly(di-l-c). as a nonspecific competitor. Specific antibodies against SRF (Santa Cruz, 2 μg/reaction) were added as indicated. The binding reaction was incubated for 20 min at room temperature before radioabeled probe was added, followed by another 20 min at room temperature incubation. All binding reactions were loaded on a 4.5% polyacrylamide gel and electrophoresed at 170 V in 0.5× TBE. The gels were dried and subjected to autoradiography at ~70 °C.

In Vitro Synthesis of SRF—In vitro synthesis of SRF was performed using a TNT®-coupled reticulocyte lysate translation system (Promega) with the human SRF cDNA clone p T7 AE ATG (52) as a template.

3 M. Hautmann and G. Owens, unpublished observations.

4 M. Thompson and G. K. Owens, unpublished observations.

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CArG Box Dependent Regulation of SM α-Actin

RESULTS

The SM α-Actin CArG Elements A And B Showed Reduced SRF Binding as Compared with the c-fos SRE—There is extensive evidence showing that the internal A/T-rich center of CArG elements affects SRF binding affinities (24, 31, 32, 53). Leung and Miyamoto (32) demonstrated that substitution of A/Ts in the core of the c-fos SRE by guanidines or cytosines resulted in marked reduction of SRF binding affinity, especially when the substitution was made in the middle of the A/T-rich center. In contrast to the c-fos SRE, the A/T-rich center of CArG B of the SM α-actin promoter contains a cytosine substitution (see Fig. 1), and CArG A contains a guanidine substitution in the middle of the A/T-rich center (see Fig. 1). Thus, we would predict that binding activity of the SM α-actin CArGs for SRF would be reduced as compared with the c-fos SRE. To test this directly, recombinant, in vitro translated SRF was incubated at different concentrations with radiolabeled CArG A, B, and c-fos SRE oligonucleotides. EMSA demonstrated that SRF binding activity was lowest with the CArG A probe, intermediate with the CArG B probe, and highest with the c-fos SRE probe (Fig. 2A, lanes 1–6). To determine whether the pattern of SRF binding activities to the different CArG elements was similar with SMC nuclear extracts as compared with those observed with recombinant SRF, we performed gel shift assays using nuclear extracts derived from SMC grown in 10% serum. Similar to the results obtained with recombinant SRF, SRF binding activity derived from SMC was lowest with CArG A, intermediate with CArG B, and highest with the c-fos SRE (Fig. 2B, lanes 1–3). These identical binding patterns suggest that SRF obtained from the SMC extract was not modified in such a manner as to significantly affect binding activity to each of these three probes and that SRF binding activity was not altered by any additional SMC proteins present in the extract, at least under the conditions of our gel shift assays.

Transcriptional Activity of SM α-Actin Varied as a Function of SRF Binding Activities of Its CArG Motifs—Previous studies by Lee et al. (54) provided evidence that the three skeletal α-actin CArGs are bound by SRF in a specific order as determined by their relative SRF binding affinities and that the position of the individual CArGs to each other plays an important role in formation of transcriptionally active complexes. To test whether CArG positioning was also critical for activation of the SM α-actin promoter, we tested three sets of paired constructs in which the SRF binding elements (c-fos SRE, CArG A, and CArG B) were replaced at position 1 (proximal) and position 2 (distal) in reversed combinations (see Fig. 1). As shown in Fig. 4, differences in activities between these constructs clearly demonstrate that positions 1 and 2 are not functional equivalents of each other. In all instances, when the lesser affinity CArG was located at position 2 activity was decreased. Particularly striking was the significant decrease in activity when CArG A and B were switched (pBL 155 CAT A-B). This loss was not due to a negative effect of placing CArG B at position 1 since pBL 155 CAT A-A also had essentially the same low activity (see Fig. 3). No significant changes in transcriptional activity were observed when the positions of the two CArG elements with relatively high SRF binding capacities (CArG B and c-fos SRE)
were interchanged. These results demonstrate that positioning of the CArG elements is critical in the regulation of the SMα-actin gene and that maximal levels of activity appeared to require the presence of an intermediate or high affinity SRF binding motif at position 2.

Polyclonal SRF antibodies (Santa Cruz) raised against the COOH terminus of human SRF was added at a concentration of 2 μg/reaction (lanes 7 and 9). Unprogrammed lysate (UP) was incubated with a CArG B probe (lane 10). Nuclear extracts (5 μg) from rat SMC growing in 10% FBS were incubated with radiolabeled CArG A, B, and c-fos SRE oligonucleotides (lanes 1–3). An SRF antibody (Santa Cruz) was added to the binding reaction in lanes 4–6. Based on cold competition experiments (lanes 7–9, and Ref. 20) and the SRF supershift analyses (Ref. 20 and this figure) only the band labeled SRF represents specific SRF binding. The faint lower mobility bands seen in virtually all lanes of this figure and in Fig. 7, lanes 5, 6, and 9, were not consistently observed and appeared to represent nonspecific binding based on competition experiments with wild-type and mutant oligonucleotides (Ref. 20 and data not shown).

FIG. 3. Effects of strong and weak CArGs on reporter activity. Wild-type SM α-actin p155 CAT (subcloned into a promoterless pBL CAT vector) and constructs containing a c-fos SRE ("strong CArG") substituting CArG B (pBL 155 CAT SRE-A) or CArG A (pBL 155 CAT B-SRE) or both (pBL 155 CAT SRE-SRE) as well as a construct containing two CArG A ("weak CArGs") were transiently transfected into subconfluent SMC, growing in 10% FBS. CAT activities were expressed relative to the base-line CAT activity of a promoterless CAT construct. Data represent means ± S.E. of three independent experiments.

FIG. 4. Analysis of the effects of CArG positions on reporter activity. Three pairs of constructs containing the CArG elements in a reversed position were transfected into subconfluent SMC growing in 10% FBS. CAT activities were expressed relative to the base-line CAT activity of a promoterless CAT construct. Data represent means ± S.E. of three independent experiments.
demonstrated that the region of the SM construct in BAEC. Results of our earlier studies (20) also required for the high transcriptional activity of the p125 CAT. These results suggested that factors other than SRF were either CArG A or B completely abolished activity of a p125 bp.

Previously, we have demonstrated that the SM construct. Data represent means ± S.E. of three independent experiments. ■, SMC; □, EC.

Replacement of the SM α-Actin CArGs by c-fos SREs Resulted in Relaxation of Cell Specificity—Previously, we have demonstrated that the SM α-actin CArG elements A and B contribute to cell-specific regulation of this gene (20) in that mutation of either CArG A or B completely abolished activity of a p125 bp SM α-actin promoter construct in SMC but not in BAEC. These results suggested that factors other than SRF were required for the high transcriptional activity of the p125 CAT construct in BAEC. Results of our earlier studies (20) also demonstrated that the region of the SM α-actin promoter upstream from −125 bp to −2.8 kb contained negative regulatory elements that completely suppressed the transcriptional activity of the −125-bp region in BAEC. To test whether CArG elements with strong SRF affinities like the c-fos SREs would alter the activity of the SM α-actin promoter in BAEC, we transfected a pBL 155 CAT wild-type (Wt) construct and a pBL 155 CAT construct containing two c-fos SREs (pBL 155 CAT SRE-SRE) into BAEC (Fig. 5). For comparison, constructs were also transfected in parallel into SMC. Transfection data demonstrated that activity of the pBL 155 CAT Wt construct was ~6-fold higher in SMC as compared with BAEC. Consistent with our earlier studies, mutations of the CArG elements A and B alone or in combination completely abolished activity in SMC but had little effect in BAEC. However, replacement of the SM α-actin CArGs with c-fos SREs (i.e. pBL 155 CAT SRE-SRE) resulted in a marked increase in CAT activity in BAEC (~600-fold over promoterless controls). This activity was CArG-dependent, since mutation of the SRE at either position 1 or 2 reduced CAT activity in BAEC to that of the pBL 155 CAT Wt construct. Importantly, single SRE mutations reduced but did not abolish CAT activities in SMC. Taken together, these results suggest the following: 1) replacement of the relatively weak SRF binding sites of the SM α-actin promoter with strong CArG elements resulted in high level SM α-actin expression in BAEC; 2) the gain in transcriptional activity obtained with the pBL 155 CAT SRE-SRE construct in BAEC was mediated through a CArG-dependent mechanism whereas CArG-independent factors regulated the activity of the pBL 155 CAT Wt construct; and 3) the presence of a c-fos SRE at either position 1 or 2 reduced the dependence of transcriptional activity on a second CArG element in SMC.

Since we previously have shown that upstream sequences in the SM α-actin promoter selectively repressed transcriptional activity of SM α-actin in BAEC, we also tested whether c-fos SRE substitutions in the context of a 2.8-kb SM α-actin promoter (designated pProm CAT SRE-SRE) could overcome the effects of these negative acting elements. Transfection results demonstrated that the activity of the pProm CAT SRE-SRE construct in BAEC was increased as compared with the wild-type (pProm CAT) construct (Fig. 6). However, activity of pProm CAT SRE-SRE in BAEC was much less than the activity of the same construct in SMC. Taken together, these results indicate that strong CArG elements could only partially overcome negative regulatory elements between −125 bp to −2.8 kb that suppress activity in BAEC.

To test further the importance of the SM α-actin CArGs for SMC-specific regulation of this gene, we tested the activity of SRE containing SM α-actin constructs in L6 skeletal myotubes. Skeletal myotubes express SM α-actin transiently during development (55), but expression is differentially regulated as compared with SMC (20). For example, high transcriptional activity in skeletal myotubes was shown to be dependent on the upstream region from −125 bp to −271 bp, whereas little or no activity was seen with the p125 CAT construct (20). Moreover, we have shown that this gene is a target of the skeletal muscle-specific HLFs whose effects are mediated by two E boxes located at −214 bp and −254 bp (56). Transfection results demonstrated that the pBL 155 CAT SRE-SRE construct had markedly higher activity in skeletal myotubes as compared with the wild-type construct (Fig. 7A). Indeed, activities exceeded that of the pProm CAT construct indicating that the presence of two strong CArG elements supplants the normal requirement for E boxes for expression of SM α-actin in skeletal myotubes.

Cell specificity of the SM α-actin CArGs was also tested in
AKR-2B fibroblasts that do not express their endogenous SM α-actin gene under normal circumstances, although they express mouse SM α-actin promoter/reporter constructs containing disruption or deletion of the promoter region from −191 bp to −221 bp which acts as a repressor in these cells (57). Results of our studies demonstrated significant activity of the p155 CAT construct in AKR-2B cells that was increased to extremely high levels by replacement of the CArGs with c-fos SREs (i.e. p155 CAT SRE-SRE was −600-fold over control) (Fig. 7B). However in marked contrast to observations in SMC, inclusion of the upstream region from −155 bp to −2.8 kb completely abolished activity of the wild-type p155 CAT construct in AKR-2B cells, and also greatly reduced the activity of the pProm CAT SRE-SRE construct. These results suggest that enhancement of SRF binding to CArG A and B may be important in SMC, whereas they can override negative regulatory elements upstream from −155 bp in SMC.

**AII Increased SRF Binding to the SM α-Actin CArGs as Well as to the c-fos SRE**—The preceding studies provide clear evidence that the relatively weak SRF binding sites of the SM α-actin promoter are important for cell type-specific expression of SM α-actin. Results of recent studies from our laboratory demonstrated that AII inducibility of SM α-actin was dependent on both CArG boxes A and B and partially dependent on a MHox binding site (ATTA) situated 5′ to CArG B (40). Moreover, AII-induced stimulation of SM α-actin was associated with markedly increased SRF binding to both CArG elements, and MHox was shown to enhance SRF binding to CArG B and overexpression of MHox transactivated SM α-actin expression. These results suggest that enhancement of SRF binding to lesser affinity SRF binding sites may represent an important mechanism to maximally up-regulate SM α-actin expression. If so, then replacement of low SRF binding sites by high SRF binding sites should result in higher constitutive activity and reduced responsiveness to AII.

To test this hypothesis, we first addressed whether SRF binding activity was increased in nuclear extracts from SMC treated with AII as compared with SFM vehicle and whether similar differences in binding activity of probes for CArG A, CArG B, and the c-fos SRE were seen with SMC extracts as observed with recombinant SRF (Fig. 2). This is important to rule out possible post-translational modifications of SRF that might differentially affect binding to these probes. Consistent with our previous findings, results showed that AII treatment was associated with increased SRF binding activity, with binding activity being greatest with the c-fos SRE, intermediate with CArG A, and lowest with CArG B in a manner similar to that seen with recombinant SRF (Fig. 8). Interestingly, the fact that AII was capable of increasing SRF binding to the c-fos SRE suggests that some degree of AII-mediated stimulation of constructs containing c-fos SREs might be expected.

**AII Responsiveness of SM α-Actin Was Reduced When CArG A and B Were Replaced by c-fos SREs and Was Abolished by the Presence of a Weak SRF Binding Site at Position 2**—To address whether the reduced SRF binding activities of the SM α-actin CArGs and their position were important for AII responsive-
SRE-A) was reduced approximately by half as compared with CAT B-SRE, pBL 155 CAT SRE-SRE, and pBL 155 CAT, the extent of AII inducibility of these constructs (pBL 155 B9 pBL 155 CAT constructs (Fig. 9). One or two c-fos SRE substitutions and SM CArG positions on AII-induced stimulation of reporter activity. SMC cultures were grown to confluency and growth-arrested in SFM for 4–5 days. Cells were then transiently transfected with constructs described earlier (Figs. 2 and 3) and stimulated with AII (10^{-6} M) or SFM (20% FBS) for 72 h. CAT activities of AII- or SFM-treated groups were expressed relative to the base-line CAT activity of a promoterless CAT construct (A) or expressed as percent of SFM controls (B). Data represent means ± S.E. of three independent experiments.

The aim of the present study was to determine whether the reduced SRF binding activities of CArG A (−62) and B (−112) within the SM α-actin promoter contribute to cell type-specific expression of SM α-actin and responsiveness to AII, a contract-
ile agonist shown to mediate hypertrophic growth responses in SMC. We demonstrated that replacement of CarG A and CarG B by the strong SRF binding site c-fos SRE resulted in increased basal activity. However, it also resulted in relaxed cell-specific expression, reduced inducibility by AII, and markedly reduced transactivation by MHOX. Moreover, we provided evidence that the position of the CarG elements was also important for basal and AII-mediated stimulation of SM α-actin expression. These results suggest that CarG elements with reduced SRF binding activities contribute to cell type-specific expression of SM α-actin and are required for maximal AII responsiveness.

CarG elements are present in the promoters of many genes that are independently regulated including immediate-early genes (Refs. 1–5, reviewed in Ref. 6) and muscle-specific genes (Refs. 7–15, reviewed in Ref. 16). Gene-specific differences are due at least in part to sequence variations within the internal AT-rich center of the CarGs as well as by changes in flanking sequences that provide additional binding sites for factors that interact with SRF and modulate its function (reviewed in Refs. 6, 37, 38, 40, 53, 54, 59). For example, Walsh and co-workers (41, 60) demonstrated that CarG elements are not functionally interchangeable. Replacement of the most proximal CarG box of the skeletal (SK) α-actin promoter (muscle regulatory element) by a c-fos SRE led to constitutive expression of SK α-actin in non-muscle cells. Consistent with the observations by Walsh et al. (41, 60), we also found that replacement of the SM α-actin CarGs by c-fos SREs was associated with relaxed cell type-specific expression of SM α-actin. However, our studies also revealed a fundamental difference between cells that express their endogenous SM α-actin gene (SMC and skeletal myoblasts) and those that do not (e.g. BAEC and AKR-2B fibroblasts) with respect to the effects of c-fos SRE substitutions when examined in a short (155 bp) versus a longer (2.8 kb) SM α-actin promoter context. In SMC, strong CarGs completely overcame the effects of negative acting elements located between −155 bp and −2.8 kb. However, whereas the presence of strong CarGs within a −155-bp context resulted in high transcriptional activity in BAEC and AKR-2B, strong CarG elements were only modestly effective in overcoming the repressor effects of negative regulatory elements upstream of −155 bp in the longer promoter context. In L6 skeletal myoblasts, expression of wild-type SM α-actin promoter constructs was dependent on the combinatorial interaction of the CarG boxes and E boxes (20). However, substitution of the SM α-actin CarGs with c-fos SREs resulted in high transcriptional activity of the p155 CAT construct that lacks the E box elements that are normally required for expression in this cell type. Taken together, these results indicate that cell type-specific expression is not only dependent on the reduced SRF binding activity of the degenerate SM α-actin CarGs but also on powerful cell type-specific repressor elements that limit expression even in the presence of c-fos CarG elements that bind SRF with very high affinity.

Based on genomic footprint analysis showing that the c-fos SRE is constitutively occupied, many models of CarG-dependent regulation of c-fos have assumed that SRF-CarG interaction was not rate-limiting or regulated (61). Rather, serum and growth factor responsiveness of the c-fos gene was shown to be mediated at least in part by the interaction of SRF with members of the ets domain containing transcription factors including ELK-1 and SAP-1 (reviewed in Refs. 18, and 37) that formed ternary complexes with SRF as well as non-ternary complex-dependent pathways that signal via Rhô family GTPases (62). A growing body of recent evidence, however, suggests that CarG-dependent regulation of the c-fos as well as other cell type-specific genes is also regulated at the level of SRF-CarG interactions (33–36, 39, 40). This includes post-translational modifications of SRF that modify its binding (17, 63), alterations in the level of SRF expression (34, 39), and interaction of SRF with factors including YY1 (54, 64, 65) and homeodomain factors (38, 40, 53). Studies by Grueneberg et al. (53) have shown that Phox1, the human counterpart of MHOX, enhanced SRF binding to the c-fos SRE and that overexpression of Phox1 transactivated a test construct consisting of a single copy of the c-fos SRE coupled to a minimal c-fos promoter. The present studies are consistent with those of Grueneberg et al. (53) and for the first time identify a gene and a cell context in which regulation of SRF binding to highly conserved degenerate CarG motifs by a homeodomain factor are critical for both SMC-specific and agonist inducibility. Our results in this and previous studies (40) suggest that the presence of relatively weak SRF binding CarG motifs within the SM α-actin promoter permits a greater extent of regulation of this gene than would be possible with strong CarGs that are likely to constitutively bind SRF even under basal conditions. Several lines of evidence support this hypothesis. First, AII responsiveness was reduced by half in the presence of c-fos SREs. Second, overexpression of MHOX transactivated the wild-type promoter by ~400%, whereas transactivation of a construct containing two c-fos SREs was only ~40% (Fig. 10).

Results of the present studies also provide evidence that the positional context of the SM α-actin CarGs was critical for transcriptional activity in that switching of CarG A and B resulted in almost complete loss of both basal and AII-induced activity. Whereas results of our previous studies (20) have clearly shown that both CarG A (position 1) and CarG B (position 2) are required for transcriptional activity in SMC, there appears to be a requirement for the stronger of the two CarGs to be located in the more distal position (Figs. 3 and 4). This may be important because of proximity to the homeodomain binding site or alternatively may relate to structural requirements for formation of a higher order transcription initiation complex (66). Of interest, however, our data show that the SRF binding activity of CarG A appears to be too low to function effectively at position 2, although it is essential for activity in its normal context (20). Consistent with these observations, Grueneberg et al. (53) has shown that Phox1, the human homologue of MHOX, failed to impart serum responsiveness to very poor SRF-binding sites, and we found that MHOX did not transactivate a construct containing two CarG A elements. In addition, MHOX did not increase SRF binding to CarG A in gel shift assays using recombinant proteins (40). Together these results suggest that binding of SRF to CarG B, promoted by MHOX, may be a crucial initial step in transcriptional activation. Subsequently, SRF-CarG B interaction may induce DNA bending (67), allowing the weaker SRF-binding site CarG A to be occupied, thereby forming an energetically favorable multiprotein-DNA complex. Consistent with this model, Lee et al. (54) demonstrated that the two high affinity proximal and distal skeletal α-actin SREs are first bound cooperatively by SRF with concurrent DNA bending, which then
facilitates SRF interaction with the weak central site CArG. Taken together, these data suggest that cooperative interaction of strong and weak CARGs contribute to CARG-dependent regulation of multiple muscle-specific genes including SM-α-Actin.

Although our studies focused on a single SMC differentiation marker, SM-α-actin, the observation that SRF binding is modulated by homeodomain proteins may represent a general regulatory paradigm that may contribute to control of other CARG-dependent muscle genes. Consistent with this, Chen and Schwartz (59), have demonstrated that the homeodomain protein Nkx-2.5, in concert with SRF, was required for expression of Nkx-2.5 in conferring cell type-specific expression of cardiac α-actin. In addition, it is interesting to speculate that the interaction of homeodomain proteins and SRF may also contribute to the coordinate expression of multiple CARG-dependent genes that are characteristic of mature differentiated smooth muscle, including SM MHC and SM-22α. However, there is also evidence that CARG-dependent expression of these SMC genes is regulated in a gene-specific manner. For example, both CARG boxes A and B were required for high level expression of SM-α-actin (20). In contrast, a single proximal CARG element with relatively high SRF binding activity (predicted based on the lack of Gua or Cyt substitution in the A/T-rich CArG center) was sufficient for high level expression of SM MHC and SM-22α in SMC (22, 24) suggesting that a single strong CARG might be sufficient to drive significant transcription of these genes. Consistent with this, our results demonstrated that SM-α-actin promoter constructs containing a single c-fos SRE were sufficient to drive transcription of SM-α-actin (Fig. 5), whereas a single wild-type SM-α-actin CARG failed to do so. Finally, it is critical to emphasize that MHox-dependent regulation of SRF binding to the SM-α-actin promoter constructs containing MHox alone is unlikely to be sufficient to control cell type-specific expression of SM-α-actin, since MHox expression is clearly not restricted exclusively to SMC, although it does show mesodermally restricted activity (68). Rather, cell type-specific expression of SM-α-actin appears to depend on the combinatorial interaction of multiple cis-elements and trans-factors in a manner analogous to many cardiac specific genes (reviewed in Ref. 16).

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