Binding of Serum Response Factor to CArG Box Sequences Is Necessary but Not Sufficient to Restrict Gene Expression to Arterial Smooth Muscle Cells*

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Serum response factor (SRF) plays an important role in regulating smooth muscle cell (SMC) development and differentiation. To understand the molecular mechanisms underlying the activity of SRF in SMCs, the two CArG box-containing elements in the arterial SMC-specific SM22α promoter, SME-1 and SME-4, were functionally and biochemically characterized. Mutations that abolish binding of SRF to the SM22α promoter totally abolish promoter activity in transgenic mice. Moreover, a multimerized copy of either SME-1 or SME-4 subcloned 5’ of the minimal SM22α promoter (base pairs −90 to +41) is necessary and sufficient to restrict transgene expression to arterial SMCs in transgenic mice. In contrast, a multimerized copy of the c-fos SRE is totally inactive in arterial SMCs and substitution of the c-fos SRE for the CArG motifs within the SM22α promoter inactivates the 441-base pair SM22α promoter in transgenic mice. Deletion analysis revealed that the SME-4 CArG box alone is insufficient to activate transcription in SMCs and additional 5’-flanking nucleotides are required. Nuclear protein binding assays revealed that SME-4 binds SRF, YY1, and four additional SMC nuclear proteins. Taken together, these data demonstrate that binding of SRF to specific CArG boxes is necessary, but not sufficient, to restrict transgene expression to SMCs in vivo.

The unique contractile properties of smooth muscle cells (SMCs) distinguish this muscle cell lineage from cardiac and skeletal myocytes (for review, see Refs. 1–3). A distinguishing feature of the SMC lineage is its capacity to proliferate and modulate its phenotype during postnatal development (4, 5). This presumably evolved to facilitate adaptive and reparative processes such as those that occur in response to arterial injury. However, the capacity of SMCs to modulate their phenotype has also been implicated in the pathogenesis of atherosclerosis and bronchial asthma (6, 7). Ultimately, SMC phenotype is dependent upon the expression of genes encoding SMC-specific contractile proteins, cell surface receptors, and intracellular enzymes. In cardiac and skeletal myocytes, gene expression is regulated primarily at the level of transcription and skeletal and cardiac muscle-specific and -restricted transcription factors have been identified (8–10). In contrast, relatively little is currently understood about the transcriptional programs that regulate SMC development and differentiation and SMC-specific transcription factors have not as yet been identified.

Accumulating evidence suggests that the MADS box transcription factor SRF plays an important role in regulating SMC specification and differentiation (for review, see Ref. 3). SRF is a 508-amino acid protein that binds to the serum response element (SRE), or CArG box, corresponding to the consensus nucleotide sequence CC(AT)6GG (11). Five alternatively spliced isoforms of SRF have been identified; at least one of which acts in a dominant negative fashion and represses SRF-dependent transcription (12, 13). SRF binds DNA and physically associates with other transcription factors through its conserved MADS box domain (14, 15). Functionally important SRF-binding sites were identified originally in cis-acting regulatory elements controlling expression of growth responsive (11, 16) and striated muscle-specific genes (17–20). Subsequently, functionally important CArG boxes have been identified in multiple SMC-restricted transcriptional regulatory elements including the SM22α promoter (21–23), the smooth muscle myosin heavy chain (SM-MyHC) promoter and intragenic enhancer (24), the SM α-actin promoter and intragenic enhancer (25), the telokin promoter (26), and the γ-entric actin promoter (27). In addition, SRF has been shown to play an important role in specification of SMCs from undifferentiated mesenchyme (28).

Because of its SMC lineage-restricted pattern of expression, our group, and others, have utilized the murine SM22α promoter as a model system to examine the transcriptional programs that regulate SMC development and differentiation (21–23, 29–31). SM22α is a single copy gene encoding a 22-kDa protein with two potential EF-hand calcium-binding domains, an actin-binding domain, and one calponin-repeat homology domain (29, 32). Preliminary characterization of the SM22α promoter revealed six nuclear protein-binding sites, designated smooth muscle elements (SME) 1–6, respectively (21). Two of these cis-acting elements, SME-1 and SME-4, contain consensus CArG boxes and bind specifically to SRF (21). In transgenic mice, the SM22α promoter restricts expression of a transgene encoding LacZ to arterial smooth muscle cells, the myotomal component of the somites, and the bulbocordis region of the
embryonic heart (21–23). However, in contrast to the endogenous SM22α protein, SM22α promoter-driven transgene expression is not observed in visceral or venous SMCs (21–23).

In the studies described in this report, we show that SRF binding is required for activity of the SM22α promoter in arterial SMCs, but that SME-1 and SME-4 function in a partially redundant fashion in vivo. Most importantly, we demonstrate that a multimerized copy of SME-1 (bp -279 to -256) or SME-4 (bp -171 to -135) is necessary and sufficient to confer arterial SMC-restricted gene expression in transgenic mice, while a multimerized copy the c-fos SRE is not. Consistent with these data, substitution of the c-fos SRE (16) for the two CArG boxes in the SM22α promoter totally inactivates the SM22α promoter in transgenic mice. Deletion analyses revealed that the SME-4 CArG box and additional 5′-flanking nucleotides are required for transcriptional activity in arterial SMCs. Nuclear protein binding assays demonstrated that SRF binds six nuclear proteins, two of which are enriched in SMC nuclear extracts. Taken together, these data demonstrate that binding of SRF to specific CArG boxes is necessary, but not sufficient, to restrict gene expression to tissue-restricted subsets of SMCs.

EXPERIMENTAL PROCEDURES

Plasmids—All transgenic vectors were prepared in the modified BluescriptII KS (Stratagene) plasmid containing the bacterial LacZ gene subcloned in the HindIII site of the AscI-flanked polylinker described previously (21). The p-441SM22.LacZ transgenic vector in which LacZ is placed under the transcriptional control of the 441-bp mouse SM22α promoter was described previously (21). p-441SM22 μSME-1.LacZ is identical to p-441SM22.LacZ except three nucleotides (underlined) in SME-1 (5′-TCTTGCCCCATATGCTTTTTCC3′) were mutated to abolish SRF and Sp1 binding activity (21). The −441SM22α-SME-4.CArG.LacZ transgenic plasmid is identical to p-441SM22α except two nucleotides in SME-4 (5′-GCGACATCTGGTTCTTTCC3′) have been substituted for the c-fos SRE (5′-CCATATTAGG-3′) has been substituted for the c-fos SRE and its 5′- and 3′-flanking sequences (5′-CCCTACACGCCAGATCACTGGTTCTTTCC3′) was mutated to abolish SRF binding activity (21). p-441SM22α.CArG.LacZ is identical to p-441SM22α.LacZ except it contains the mutations in both SME-1 and SME-4 described above. p-441SM22α.c-fos.LacZ is identical to p-441SM22α.LacZ except the nucleotide sequence of the c-fos SRE (5′-CCATATTAGG-3′) has been substituted for the c-fos SRE and its 5′- and 3′-flanking sequences (5′-CCCTACACGCCAGATCACTGGTTCTTTCC3′) subcloned into SME-1 promoter, SME-4 promoter. p-441SM22α.CArG.LacZ contains four copies of SME-4 (bp -171 to -136) subcloned into pSmad-digested pSM22α.LacZ. pSMM2.SME4.LacZ contains three copies of SME-4 (bp -279 to -256) subcloned into pSmad-digested pSM22α.LacZ. pSMM2.c-fos.LacZ contains three copies of the c-fos SRE and its 5′- and 3′-flanking sequences (5′-CCCTACACGCCAGATCACTGGTTCTTTCC3′) was subcloned into pSmad-digested pSM22α.LacZ. pSMM2.SME4.CArG.LacZ contains four copies of the SME-4 CArG box (bp -150 to -141) subcloned into pSmad-digested pSM22α.LacZ. pSMM2.SME4.CArG.LacZ and pSMM2.SME4.CArG.LacZ contain four copies of the SME-4 CArG box including its 5′-flanking sequence (bp -171 to -141) or 3′-flanking sequence (bp -151 to -136), respectively, subcloned into pSmad-digested pSM22α.LacZ.

Transgenic Mice—Transgenic mice harboring the −441SM22α.LacZ, −441SM22α-μSME-1.LacZ, −441SM22α-μSME-4.LacZ, −441SM22α μCarG.LacZ, −441SM22α-μc-fos.LacZ, SM22α.LacZ, SM22α.SME4.LacZ, SM22α.SME1.LacZ, SM22α-μc-fos.LacZ, SM22α.SME4.CArG.LacZ, SM22α.SME4.5′CArG.LacZ, and SM22α.SME4.3′CArG.LacZ were generated according to standard techniques as described previously (21). To identify transgenic embryos, high molecular weight DNA was prepared from the yolk sacs of embryonic day (E)11.5 embryos and Southern blot analyses were performed as described previously (21). The number of copies per cell was quantitated by comparing the hybridization signal intensity to standards corresponding to 1, 10, and 100 copies/cell using a Molecular Dynamics PhosphorImager. E11.5 embryos were fixed, stained for β-galactosidase activity, and cleared as described previously (21). Photography was performed using a Nikon SMZ-U dissecting microscope, Nikon 6006 camera, and Kodak EPT160 film.

Electrophoretic Mobility Shift Assays (EMSAs)—Primary rat aortic SMCs, A7r5 SMCs, NIH3T3 fibroblasts, HepG2 cells, HeLa cells, C2C12 myoblasts, and myotubes and EL4 T cells were grown in culture, harvested, and nuclear extracts prepared as described previously (21, 29). EMSAs were performed in 0.25 × TBE (1 × TBE is 100 mM Tris, 100 mM boric acid, and 2 mM EDTA) as described previously (21). The following complementary oligonucleotides were synthesized with BioHi and BglII overhanging ends: SME-1 (5′-CAAGGAAGGTTCC- acGCGTTCCATTTCCGC-3′), SME-4 (5′-CCTCAACCGTGTCCTTTCCGC-3′), SME-4 (5′-CTCAACATTGTGTCCTTTCCGC-3′), SME-4 (5′-CTCAACCGTGTCCTTTCCGC-3′). For cold competition experiments, 10 to 200 ng of unlabeled competitor oligonucleotide was included in the binding reaction mixture. For antibody supershift experiments, 1 μl of rabbit preimmune serum (Santa Cruz), α-SRF rabbit polyclonal IgG (Santa Cruz sc-335X), or α-YY1 rabbit polyclonal IgG (Santa Cruz sc-446X), or α-Ets-1 rabbit polyclonal IgG (Santa Cruz sc-350X), or α-EkI rabbit polyclonal IgG (Santa Cruz sc-355X) was incubated with the indicated nuclear extract at 4 °C for 20 min prior to the binding reaction as described previously (21).

UV Cross-Linking Analyses—EMSA reactions performed with 32P-labeled deoxyribonucleotide-substituted SM22 oligonucleotide probes (bp −190 to −110) were scaled up four times as described (33). The binding reactions were exposed to UV light in an UV Stratallinker 2400 (Stratagene Inc.) and treated with 4 μg of DNase I (Workington) and 3 units of micrococcal nuclease (Workington). DNA-bound proteins were fractionated by SDS-polyacrylamide gel electrophoresis and autoradiographically visualized.

RESULTS

An Intact CArG Box Is Required for Activity of the SM22α Promoter in Arterial SMCs in Vivo—The arterial SM-specific 441-bp SM22α promoter contains six nuclear protein-binding sites, two of which (SM22α and SME-4) contain embedded CArG boxes (21, 22). To determine whether binding of SRF to the 441-bp SM22α promoter is required for transcriptional activity in SMCs, the transcriptional activity of the 441-bp SM22α promoter containing site-directed mutations in SME-1 and SME-4 that abolish SRF binding was assayed in F0 transgenic mice (Fig. 1, upper panel). Consistent with our previous report (21), in E11.5–441SM22α.LacZ embryos, the 441-bp SM22α promoter restricted activity of the LacZ gene to arterial SMCs, the bulboconus region (BC) of the heart (future right ventricle), the cardiac outflow tract (OFT), and the myotomal component of the somites (S) (Fig. 1, A–C). LacZ-positive cells (blue-stained cells) were observed in presumptive SMCs underlying the endothelium of major arteries (Fig. 1B). In addition, β-galactosidase activity was reproducibly observed in regions of the cardiac outflow tract including the aortopulmonary septum (Fig. 1C). In contrast, in 11 independent E11.5–441SM22α.CArG.LacZ transgenic embryos, containing mutations in both SME-1 and SME-4 that abolish SRF binding to the SM22α promoter, β-galactosidase activity was not observed (Fig. 1D). These data demonstrate that binding of SRF to the mouse SM22α promoter is required for transcriptional activity in all three embryonic muscle cell lineages.

To determine whether SME-1 and SME-4 mediate distinct or redundant functions, site-directed mutations in SME-1 and SME-4 (Fig. 1, upper panel), respectively, were generated within the context of the intact 441-bp SM22α promoter and the transcriptional activity of these mutant promoters were assessed in F1 transgenic mice. In four independent -441SM22α-μSME-1.LacZ embryos, containing a mutation that abolishes binding of SRF and Sp1 to SME-1, blue staining was observed in arterial SMCs, the myotomal component of the somites (S), the cardiac OFT, and the BC region of the embryonic heart (Fig. 1E). This pattern closely resembled that observed in the control -441SM22α.LacZ embryos although the intensity of staining was somewhat diminished (compare Fig. 1, A and E). In contrast, in four independent -441SM22α-μSME-4.LacZ transgenic embryos, containing a promoter mutation that selectively abolishes binding of SRF to SME-4, β-galactosidase activity was observed in arterial SMCs and the cardiac outflow tract,
of the CArG box-containing transcriptional regulatory elements SME-1, SME-4, and SME-4. The SME-1 and SME-4 nuclear protein-binding sites in the mouse SM22α promoter were identified by DNase I footprint analyses (21). The site-directed mutations generated in SME-1 (μSME-1) and SME-4 (μSME-4) that abolish SRF binding are shown below each respective element. A, E11.5 transgenic embryos were harvested and stained for β-galactosidase (LacZ) activity as described previously (21). In -441SM22.LacZ embryos, LacZ activity was observed in the dorsal aorta (Ao) and smaller branch arteries, the myotomal component of the somites (S), the cardiac OFT, and the BC region of the embryonic heart. B, cross-section through the iliac artery of an E11.5–441SM22.LacZ embryo. β-Galactosidase activity is restricted exclusively to the layer of cells (SMC) underlying the endothelium. Bar, 1 μm. C, cross-section through the chest cavity of an E11.5–441SM22.LacZ embryo at the level of the cardiac outflow tract. β-Galactosidase activity is restricted to cells within the aortopulmonary septum (APS). Bar, 1 μm. D, in E11.5–441SM22.LacZ/CARG embryos, LacZ activity was not observed. E, in E11.5–441SM22.μSME-1 embryos LacZ activity was observed in the dorsal aorta (Ao) and smaller branch arteries, myotomal component of the somites (S), cardiac OFT, and BC region of the heart. F, in four E11.5–441SM22.μSME-4 embryos, LacZ activity was observed in the dorsal aorta (Ao), cardiac OFT, and BC region of the heart but not in the somites.

![Diagram](http://www.jbc.org/)

**Fig. 1. In vivo mutational analyses of CArG boxes in the arterial SMC-restricted SM22α promoter.** Upper panel, nucleotide sequences of the CArG box-containing transcriptional regulatory elements SME-1, μSME-1, SME-4, and μSME-4. The SME-1 and SME-4 nuclear protein-binding sites in the mouse SM22α promoter were identified by DNase I footprint analyses (21). The site-directed mutations generated in SME-1 (μSME-1) and SME-4 (μSME-4) that abolish SRF binding are shown below each respective element. A, E11.5 transgenic embryos were harvested and stained for β-galactosidase (LacZ) activity as described previously (21). In -441SM22.LacZ embryos, LacZ activity was observed in the dorsal aorta (Ao) and smaller branch arteries, the myotomal component of the somites (S), the cardiac OFT, and the BC region of the embryonic heart. B, cross-section through the iliac artery of an E11.5–441SM22.LacZ embryo. β-Galactosidase activity is restricted exclusively to the layer of cells (SMC) underlying the endothelium. Bar, 1 μm. C, cross-section through the chest cavity of an E11.5–441SM22.LacZ embryo at the level of the cardiac outflow tract. β-Galactosidase activity is restricted to cells within the aortopulmonary septum (APS). Bar, 1 μm. D, in E11.5–441SM22.LacZ/CARG embryos, LacZ activity was not observed. E, in E11.5–441SM22.μSME-1 embryos LacZ activity was observed in the dorsal aorta (Ao) and smaller branch arteries, myotomal component of the somites (S), cardiac OFT, and BC region of the heart. F, in four E11.5–441SM22.μSME-4 embryos, LacZ activity was observed in the dorsal aorta (Ao), cardiac OFT, and BC region of the heart but not in the somites.

but not within the myotomal component of the somites (Fig. 1F). Of note, blue staining in the dorsal aorta was consistently less intense than that observed in -441SM22.LacZ and -441SM22.μSME-1.LacZ transgenic embryos (compare Fig. 1, A and F). Taken together, these data demonstrate that mutations that abolish binding of SRF to SME-1 and SME-4, respectively, attenuate, but do not abolish, activity of the mouse SM22α promoter in arterial SMCs. To examine this possibility, SM22.c-fos.LacZ transgenic mice were generated containing the SM22 promoter linked to three copies of SME-1 (bp 164–209) and three copies of SME-4 (bp 279 to 250) and SME-4 (bp –190 to –110), respectively, did not alter the pattern of transgene expression observed (data not shown). Taken together, these data demonstrate that a multimerized copy of either SME-1 or SME-4 is necessary and sufficient to restrict transgene expression to arterial SMCs in F0 transgenic embryos. However, differences in the level of expressed transgene as well as differences in the muscle-restricted pattern of transgene expression were observed between SME-1 and SME-4 promoter-driven constructs.

**CArG Box Specificity Restricts Gene Expression to Arterial SMCs**—Functionally important CArG boxes have been identified in transcriptional regulatory elements controlling expression of growth-related genes, most notably c-fos (16). To determine whether the c-fos SRE (Fig. 2, upper panel) could be substituted for the two CArG boxes in the SM22α promoter, -441SM22Δc-fos.LacZ transgenic mice were generated containing a transgene encoding LacZ under the transcriptional control of the 441-bp SM22α promoter in which the c-fos SRE has been substituted for the CArG boxes within SME-1 and SME-4, respectively. Surprisingly, in five independent -441SM22Δc-fos.LacZ embryos, β-galactosidase activity was not observed (data not shown). In theory, nucleotides flanking the c-fos SRE may also be required for activity of this element in arterial SMCs. To examine this possibility, SM22.c-fos.LacZ transgenic embryos were generated containing the LacZ reporter gene
FIG. 2. SME-1 or SME-4 is necessary and sufficient to restrict transgene expression to arterial SMCs. Upper panel, the nucleotide sequences of SME-1, SME-4, and the c-fos SRE. The respective CArG box sequences are indicated. A, SM22.SME1.LacZ embryos demonstrated intense LacZ activity restricted to the dorsal aorta (Ao) and smaller branch arteries, the myotomal component of the somites, the cardiac outflow tract and the bulbocordis region of the embryonic heart. B, in four of seven SM22.SME4.LacZ embryos LacZ activity was restricted to arterial SMCs and the cardiac outflow tract. In three of seven SM22.SME4.LacZ embryos staining was also observed in the somites (see Fig. 3B). C, LacZ activity was not observed in any SM22.c-fos.LacZ embryo.

FIG. 3. The SME-4 CA rG box alone is not sufficient to restrict gene expression to arterial SMCs in transgenic mice. Upper panel, the nucleotide sequence of SME-4 and SME-4 deletion mutants SME-4.CArG, SME-4.5’CArG, and SME-4.3’CArG are shown. Four copies of each oligonucleotide were subcloned immediately 5’ of the 90-bp SM22α promoter and the activities of each respective promoter were analyzed in F0 transgenic mice. A, three of seven SM22.SME4.LacZ embryos demonstrated LacZ activity in the dorsal aorta (Ao), the myotomal component of the somites (S), and the cardiac OFT. In four of seven SM22.SME4.LacZ embryos LacZ activity was restricted to arterial SMCs and the cardiac outflow tract (see Fig. 2B). B, SM22.SME4.CArG.LacZ embryos did not express the LacZ transgene. C, SM22.SME4.5’CArG embryos demonstrated LacZ activity restricted to arterial SMCs, the myotomal component of the somites and the cardiac outflow tract.

under the transcriptional control of the minimal SM22α promoter to arterial SMCs, the myotomal component of the somites, the cardiac outflow tract and the bulbocordis region of the embryonic heart. SM22.SME4.LacZ embryos demonstrated intense LacZ activity restricted to the dorsal aorta (Ao) and smaller branch arteries, the myotomal component of the somites, the cardiac outflow tract and the bulbocordis region of the embryonic heart. SM22.SME4.5’CArG, SME-4.3’CArG and SME-4.1’CArG embryos demonstrated LacZ activity restricted to arterial SMCs, the myotomal component of the somites and the cardiac outflow tract. In three of seven SM22.SME4.LacZ embryos staining was also observed in the somites (see Fig. 3B). C, LacZ activity was not observed in any SM22.c-fos.LacZ embryo.

Nucleotides Flanking the SME-4 CArG Box Are Required for Transcriptional Activity in Arterial SMCs—To determine whether the SME-4 CArG box alone is necessary and sufficient to restrict transgene expression to arterial SMCs, SM22.SME4.CArG[LacZ transgenic mice were generated containing a transgene encoding LacZ under the transcriptional control of four copies of the SME-4 CArG box linked to the minimal SM22α promoter (Fig. 3, upper panel). Of note, this 10-bp sequence (CCAAATATGG) binds SRF in EMSAs (data not shown). However, in six independent SM22.SME4.CArG[LacZ embryos, β-galactosidase activity was not observed (Fig. 3B). Therefore, to determine whether 5’ or 3’ sequences flanking the SME-4 CArG box are required for transcriptional activity in arterial SMCs, transgenic mice were generated in which the LacZ gene was placed under the transcriptional control of the minimal SM22α promoter linked to four copies of SME-4 deletion mutants (Fig. 3, upper panel). In two independent E11.5 SM22.SME4.5’CArG.LacZ embryos, containing four copies of the SME-4 CArG box and its 5’-flanking sequences (bp −171 to −142), β-galactosidase activity was observed in a pattern recapitulating that observed in the SM22.SME4.LacZ embryos (compare Fig. 3, A and C). In contrast, LacZ activity was not observed in four independent SM22.SME4.3’CArG.LacZ embryos, containing 4 copies of the SME-4 CArG box and its 3’-flanking sequence (bp −151 to −136) (data not shown). These data demonstrate that the SME-4 CArG box sequence alone is not sufficient to restrict gene expression to arterial SMCs in transgenic embryos and that additional 5’-flanking sequence in SME-4 (bp −171 to −152) is required to activate transcription in arterial SMCs.

Characterization of Nuclear Protein Complexes That Bind to SME-4 and c-fos SRE—To determine whether SME-4 and c-fos SRE differentially bind nuclear protein complexes expressed in SMCs, a series of EMSAs was performed. SME-4 binds four specific nuclear protein complexes, designated A-D, each of which is competed by unlabeled SME-4 competitor oligonucleo-
to EMSAs with 10 ng of the indicated unlabeled competitor oligonucleotides or 1 ng prepared from A7r5 SMCs. Some binding reaction mixtures included supershifted with serum. Left nuclear protein complexes, designated A to D, are identified to the c-fos SRE. EMSAs were performed as described above. Four specific nuclear protein complexes were detected and data not shown. Antibody supershift experiments demonstrate that the low mobility complex A contains YY1 (Fig. 4B, lane 11, arrow), while complex B contains YY1 (Fig. 4B, lane 11, arrow). Despite the fact that the c-fos SRE is flanked by a consensus TCF-binding site (CAGGA) (see Fig. 2, upper panel), none of the nuclear protein complexes were supershifted or abolished when the binding reactions were preincubated with SAP-1A, Elk-1, and Ets-1 antisera (Fig. 4B, lanes 11–14). Taken together, these data confirm that SME-4 and the c-fos SRE oligonucleotide more efficiently competes for SRF binding activity (data not shown). Antibody supershift experiments revealed that complex A contains SRF or an antigenically-related protein (Fig. 4A, lane 10, arrow), while complexes C and D contain YY1 (Fig. 4A, lane 11, arrows). To determine whether Ets family members and/or ternary complex factors (TCFs) expressed in SMCs bind to the radiolabeled SME-4 oligonucleotide (and/or SRF), the binding reactions were preincubated with polyclonal antibodies that recognize SAP-1A, Elk-1, and Ets-1, respectively. However, none of the nuclear protein complexes, including SRF-containing complex A, were supershifted or abolished (Fig. 4A, lanes 12–14).

To characterize SMC nuclear protein complexes that bind to the c-fos SRE, an EMSA was performed with a radiolabeled oligonucleotide corresponding to the c-fos SRE and nuclear extracts prepared from A7r5 SMCs. The c-fos SRE also bound specifically to four nuclear protein complexes, designated A-D, each of which is competed with increasing concentrations of unlabeled competitor c-fos SRE oligonucleotide (Fig. 4B, lanes 2–5). Once again, several nonspecific binding activities were identified that were not cross-competed by unlabeled c-fos SRE oligonucleotide. In addition, complexes A-C were competed with unlabeled SME-4 (Fig. 4B, lanes 6–8, and data not shown). EMSAs performed with the radiolabeled c-fos SRE probe and increasing concentrations of unlabeled competitor c-fos SRE and SME-4 oligonucleotide (5, 10, 25, 50, and 100 ng) revealed that the c-fos oligonucleotide more efficiently competes for SRF binding activity (data not shown). Antibody supershift experiments demonstrated that the low mobility complex A contains SRF (Fig. 4B, lane 10, arrow), while complex B contains YY1 (Fig. 4B, lane 11, arrow). That the c-fos SRE is bound by at least two unidentified nuclear protein complexes that are also expressed in other cell lineages.

Biochemical Characterization of Nuclear Protein Complexes That Bind Directly to SME-4—As discussed above, EMSAs failed to reveal an SMC-specific nuclear protein complex that binds to SME-4. However, an inherent limitation of EMSAs is that the band shifts observed may contain a single nuclear protein or a multiprotein complex. Therefore, to biochemically characterize each nuclear protein that binds directly to SME-4, with particular emphasis on identification of SMC-specific nuclear proteins, UV cross-linking analyses were performed with a deoxybromouridine-substituted SME-4 oligonucleotide and nuclear extracts prepared from primary rat aortic SMCs, A7r5 SMCs, and multiple other cell lines. As shown in Fig. 5, SME-4 bound six nuclear proteins ranging in size from 40 to 130 kDa that are expressed in arterial SMCs. As expected, a 68-kDa band (arrow), corresponding to the size of SRF was observed in all lanes. In addition, a 44.5-kDa band (arrow) corresponding to the expected size YY1 was identified. Moreover, 130-, 80-, 56-, and 40-kDa proteins were also identified. p80 and p56 (open arrowheads) were enriched in SME-4 nuclear extracts (data not shown). Antibody supershift experiments revealed that complex A contains SRF or an antigenically-related protein (Fig. 4A, lanes 6–8). Each of these nuclear protein complexes was also observed in EMSAs performed with nuclear extracts prepared from primary rat aortic SMCs, NIH3T3 cells, C2C12 myoblasts, and myotubes, EL4 T cells, HepG2 cells, HeLa cells, and C3H10T1/2 fibroblasts (data not shown). Antibody supershift experiments revealed that complex A contains SRF or an antigenically-related protein (Fig. 4A, lane 10, arrow), while complexes C and D contain YY1 (Fig. 4A, lane 11, arrows). To determine whether Ets family members and/or ternary complex factors (TCFs) expressed in SMCs bind to the radiolabeled SME-4 oligonucleotide (and/or SRF), the binding reactions were preincubated with polyclonal antibodies that recognize SAP-1A, Elk-1, and Ets-1, respectively. However, none of the nuclear protein complexes, including SRF-containing complex A, were supershifted or abolished (Fig. 4A, lanes 12–14).

To characterize SMC nuclear protein complexes that bind to the c-fos SRE, an EMSA was performed with a radiolabeled oligonucleotide corresponding to the c-fos SRE and nuclear extracts prepared from A7r5 SMCs. The c-fos SRE also bound specifically to four nuclear protein complexes, designated A-D, each of which is competed with increasing concentrations of unlabeled competitor c-fos SRE oligonucleotide (Fig. 4B, lanes 2–5). Once again, several nonspecific binding activities were identified that were not cross-competed by unlabeled c-fos SRE oligonucleotide. In addition, complexes A-C were competed with unlabeled SME-4 (Fig. 4B, lanes 6–8, and data not shown). EMSAs performed with the radiolabeled c-fos SRE probe and increasing concentrations of unlabeled competitor c-fos SRE and SME-4 oligonucleotide (5, 10, 25, 50, and 100 ng) revealed that the c-fos oligonucleotide more efficiently competes for SRF binding activity (data not shown). Antibody supershift experiments demonstrated that the low mobility complex A contains SRF (Fig. 4B, lane 10, arrow), while complex B contains YY1 (Fig. 4B, lane 11, arrow). Despite the fact that the c-fos SRE is flanked by a consensus TCF-binding site (CAGGA) (see Fig. 2, upper panel), none of the nuclear protein complexes were supershifted or abolished when the binding reactions were preincubated with SAP-1A, Elk-1, and Ets-1 antisera (Fig. 4B, lanes 11–14). Taken together, these data confirm that SME-4 and the c-fos SRE cross-compete for binding of SRF and YY1, although their relative affinity SRF binding to the c-fos SRE and SME-4 vary. In addition, SME-4 and the c-fos SRE bind at least two unidentified nuclear protein complexes that are also expressed in other cell lineages.
were enriched in VSMC and A7r5 cell extracts. open and YY1 were identified. Two unidentified proteins, p80 and p56 (diagnostically. 68- and 44.5-kDa proteins corresponding in size to SRF were incubated with radiolabeled deoxybromouridine-substituted SME-4 oligonucleotides (bp – 190 to –110), exposed to UV light, and treated with DNase I and micrococcal nuclease. DNA-bound proteins were fractioned by SDS-polyacrylamide gel electrophoresis and visualized autoradiographically. EMSAs performed with nuclear extracts prepared from primary rat aortic SMCs (VSMC), A7r5 SMCs, NIH 3T3 cells, HepG2 cells, C2C12 myoblasts and myotubes (C2B and C2T), and EL4 T cells demonstrated that in addition to SRF and YY1 at least four additional nuclear proteins, two of which are enriched in SMCs, bind to SME-4.

**DISCUSSION**

Accumulating evidence suggests that SRF plays a critical role in regulating SMC specification, differentiation, and phenotype (21–28). However, the molecular basis underlying activity of SRF in SMCs remains to be elucidated. Mouse harboring targeted mutations in SRF die at the onset of gastrulation (E7.5) and do not form detectable mesoderm precluding assessment of the function of SRF in smooth muscle cells (34). SRF is a member of the MADS box family of transcription factors which have evolved to control cell fate decisions and regulate cellular differentiation (for review, see Ref. 35). The pattern of SRF expression in the developing embryo remains controversial. Although originally characterized as a ubiquitously expressed gene product (36), detailed analyses of avian and murine embryos revealed that SRF is preferentially expressed in the developing neural tube, heart, skeletal muscle, and smooth muscle (37–39). Consistent with this finding, the SRF promoter activity is activated in a muscle-restricted fashion (39). However, the pattern of SRF expression alone cannot explain how SMC-restricted genes are activated and repressed in the developing embryo and in response to injury. SMC-restricted genes are expressed in a temporally coordinated pattern in the developing embryo that is distinct from the pattern of SRF expression (for review, see Ref. 2). Epicardial cells derived from the proepicardial organ expressing SRF do not differentiate into SMCs and express genes encoding SMC markers until they undergo mesenchymal transformation (28). Given the plasticity that distinguishes the SMC lineage, it is not surprising that molecular mechanisms(s) have evolved to precisely modulate activity of SRF in SMCs.

Our group and others reported that CArG box-containing transcriptional regulatory elements restrict gene expression to tissue-restricted subsets of smooth muscle cells (21–25, 40). In fact, every SMC-specific transcriptional regulatory element characterized to date contains one, and usually several, CArG boxes (21–27). Most SMC-specific genes, including SM22α, are also transiently expressed in the embryonic heartbeat and skeletal muscle (2). In skeletal and cardiac myocytes, SRF functions in concert with muscle-restricted transcription factors, including myogenic basic helix loop helix factors, Nkx-2.5 and GATA-4, to activate cell lineage-restricted transcription (37–39). Despite the fact that the 441-bp SM22α promoter activates transgene expression in the somites and embryonic heart, it does not bind directly to any previously described muscle-restricted transcription factors. Moreover, a multimerized copy of SME-1 or SME-4 is necessary and sufficient to restrict gene expression to arterial SMCs as well as the myotomal component of the somites, the bulbocordis region of the heart, and the cardiac outflow tract. These data demonstrate that the minimal transcriptional regulatory program that restricts gene expression to arterial SMCs includes those factors that bind directly or indirectly to the CArG box-containing elements in the SM22α promoter, SME-1 and SME-4. Moreover, these data suggest that this transcriptional regulatory program shares common features with the regulatory program(s) that restricts gene expression to embryonic skeletal muscle and the embryonic heart (at least the bulbocordis and outflow tract).

Mutational and deletion analyses of the SM22α promoter demonstrate conclusively that nucleotides within, and flanking, CArG boxes distinguish the activity and specificity of SRF in arterial SMCs. These data suggest that a common nucleotide sequence within and/or flanking CArG boxes distinguish their activity in arterial SMCs. However, a computer-assisted homology search failed to reveal conserved nucleotides within or flanking SMC-specific CArG boxes.3 In this regard it is noteworthy that SRF-1 and SME-4, as well as all other previously identified SMC-specific CArG box-containing transcriptional regulatory elements (21–26), are not flanked by TCF-binding sites. Consistent with this observation, antibodies that recognize Ets factors including Ets-1, Elk-1, and SAP-1A, failed to supershift SMC-derived nuclear protein complexes that bind to SME-1, SME-4, or the c-fos SRE. Association of ternary complex factors with SRF increases the DNA binding affinity of SRF (41). Thus, it is possible that differences in DNA binding affinity of SRF mediated through its association with TCFs distinguishes the activities of SMC-specific CArG boxes (i.e. low affinity SRF-binding sites) from CArG boxes that regulate expression of growth-responsive and immediate early genes (i.e. high affinity SRF-binding sites).

How then does a transcription factor, such as SRF, restrict gene expression to SMCs? In the developing embryo, SMC-restricted genes are expressed in distinct temporal and spatial patterns (for review, see Ref. 2). In the mouse embryo, SM22α is expressed as early E8.0 in the cells surrounding the dorsal aorta (42), while the CArG box-dependent smooth muscle myosin heavy chain (SM-MyHC) gene is not expressed until much later in embryonic development (24, 43). One possibility is that

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2 S. Kim and M. Parmacek, unpublished observation.
3 M. Parmacek, unpublished observation.
activity of SRF is modulated by its capacity to differentially heterodimerize with other transcriptional activators and/or repressors. However, despite intense investigation, to date no SMC-specific transcription factor(s) have been identified. Consistent with these data, EMSAs failed to reveal an SMC-specific nuclear protein complex that binds specifically to SME-1 or SME-4. However, UV cross-linking analyses revealed four nuclear proteins that bind to SME-4 in addition to SRF and YY1. While none of these proteins was expressed exclusively in SMCs, at least two proteins appeared to be enriched in SMC nuclear extracts. Thus it remains possible that SRF may function in a combinatorial fashion with other ubiquitously expressed factors. However, despite intense investigation, to date no SMC lineage-restricted transcription factors have been identified. Conversely, SRF-dependent gene expression could be regulated in a combinatorial fashion to repress transcription (48). Finally, it should be noted that these molecular mechanisms (intracellular signaling and alternative splicing) are not mutually exclusive and, in theory, explain how a single transcription factor could serve as a nuclear sensor integrating multiple signals from the cell surface and translating these signals into a gradient of SMC phenotypes.

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