Serum Response Factor-dependent Regulation of the Smooth Muscle Calponin Gene

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Smooth muscle cell calponin is a multifunctional, thin filament-associated protein whose expression is restricted to smooth muscle cell lineages in developing and postnatal tissues. Although the physiology of smooth muscle calponin has been studied extensively, the cis-elements governing its restricted pattern of expression have yet to be identified. Here we report on smooth muscle-specific enhancer activity within the first intron of smooth muscle calponin. Sequence analysis revealed a proximal consensus intronic CArG box and two distal intronic CArG-like elements, each of which bound recombinant serum response factor (SRF) as well as immunoreactive SRF from smooth muscle nuclear extracts. Site-directed mutagenesis studies suggested that the consensus CArG box mediates much of the intronic enhancer activity; mutating all three CArG elements abolished the ability of SRF to confer enhancer activity on the smooth muscle calponin promoter. Cotransferring a dominant-negative SRF construct attenuated smooth muscle-specific enhancer activity, and transducing smooth muscle cells with adenovirus harboring the dominant-negative SRF construct selectively reduced steady-state expression of endogenous smooth muscle calponin. These results demonstrate an important role for intronic CArG boxes and the SRF protein in the transcriptional control of smooth muscle calponin in vitro.

Smooth muscle cell (SMC)† lineages are defined by a battery of cell-restricted differentiation genes whose encoded proteins facilitate the unique contractile activity of these cell types (1, 2). In pathophysiological states, the contractile phenotype of SMCs may be subverted to one of growth, migration, and matrix secretion with a coincident reduction in SMC-restricted gene expression (3–5). Although the mature phenotype of SMCs can be compromised in a variety of natural and experimental settings, there is evidence for reacquisition of a differentiated phenotype (6). This process of phenotypic modulation (7) implies that the genetic program of SMC differentiation is not fixed. In an effort to begin understanding the transcriptional programs underlying phenotypic modulation, several SMC-restricted promoters have been studied, including telokin (8); SM22 (9); and the smooth muscle isoforms of α-actin (10), γ-actin (11), and myosin heavy chain (12). Analyzing these and other SMC-restricted gene promoters provides a necessary foundation to identify cis-elements that mediate SMC-restricted gene expression and to ascertain whether such elements and/or their transacting factors are the targets of signals that lead to gene repression in SMC-associated diseases.

The CArG box was originally defined as an evolutionarily conserved element (CC(A/T)6GG) found in the 5′-promoter region of the cardiac α-actin gene (13). It also forms the core binding sequence of the serum response element, which was first defined in the proximal promoter of the c-fos gene (14). Homodimers of the immediate-early transcription factor, serum response factor (SRF) (15), bind CArG boxes in the regulatory region of viral genes, early growth response genes, and muscle differentiation genes (16). Although SRF is often stated to be a widely expressed transcription factor, it is particularly enriched in skeletal, cardiac, and smooth muscle lineages during embryonic and postnatal development (17–19). Many of the highly restricted genes defining SMCs harbor one or more CArG boxes in their 5′-promoter region, and in at least a few cases, the in vivo expression of these genes is absolutely dependent upon a functional SRF-binding CArG box (20–22). A major effort therefore has been directed toward understanding how a widely expressed transcription factor confers SMC-specific gene activation in vivo.

Smooth muscle calponin (SM-Calp) gene expression is highly restricted to SMC lineages in developing and postnatal tissues, making it an ideal model gene to further define the transcriptional program of SMC differentiation (23–26). Although SM-Calp expression is tightly restricted to SMCs, its 5′-promoter region displays promiscuous activity in several non-SMC lineages in vitro (26). Paradoxically, the same promoter constructs either are inactive or show ectopic (position effect-mediated) expression in transgenic mice.² These divergent data have led us to hypothesize that cell-specific regulatory elements governing SM-Calp expression lie within the transcription unit itself and/or reside great distances from the core promoter, as has been described for other muscle-restricted genes (27–29). To begin to distinguish between these possibilities, we have analyzed nucleotide sequences 3′ from the preinitiation complex (PIC) of SM-Calp. In this report, we describe SMC-specific enhancer activity within the first intron of SM-Calp that appears to be mediated entirely by several evolutionarily conserved SRF-binding CArG boxes. Using an adenovirus-mediated gene transfer approach, we show that endogenous expression of SM-Calp is dependent upon a functional SRF-CArG box axis. The results are discussed within the

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¶ The abbreviations used are: SMC, smooth muscle cell; SRF, serum response factor; DNSRF, dominant-negative serum response factor; SM-Calp, smooth muscle calponin; PIC, preinitiation complex; IC, intronic CArG box; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; m.o.i., multiplicity of infection.

² J. M. Miano, unpublished observations.

9814 This paper is available on line at http://www.jbc.org
context of other SRF-dependent SMC genes and how SRF may mediate such SMC-specific gene expression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**All cell lines were grown in Dulbecco’s modified Eagle’s medium containing high glucose, 10% fetal bovine serum (Life Technologies, Inc.), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and were maintained in a humidified incubator (37 °C, 5% CO₂). Rat aortic SMCs were isolated from the thoracic aortas of adult male Harlan Sprague-Dawley rats as described previously (26) and were used between passages 15 and 25. The A7r5 SMC line (30), Balb/c 3T3 fibroblasts, L6 skeletal myoblasts, COS-7, and HeLa cells were purchased from American Type Culture Collection (Manassas, VA) and grown according to the supplier’s specifications. The PAC1 (31) and pup aortic cell lines were kindly provided by Dr. Stephen M. Schwartz (University of Washington). All SMC lines (rat aortic SMC, A7r5, and PAC1) have been characterized previously and shown to express most of the major markers for SMC identity (32).

**Sequence Analysis of the First Intron of SM-Calp—**Dideoxynucleotide sequencing of the first intron of mouse (26) and human (33) SM-Calp was performed with an ABI 377 automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA). Sequences were analyzed with the FASTA and FINDPATTERNS algorithms provided in the GENETYX Wisconsin Package (Version 10.0, Genetics Computer Group, Inc., Madison, WI).

**Construction of Reporter Plasmids—**All plasmids were amplified in Escherichia coli (University of Washington) and Dr. Warren Zimmer (University of South Florida) and purchased commercially (Operon Technologies, Inc., Alameda, CA) and dissolved in sterile water. Sense (shown in boldface in Fig. 4) and antisense oligonucleotides encompassing each of the ICs (underlined in Fig. 4) or their mutant counterparts (mutant bases are shown in boldface italics below the 5'-nucleotides of each IC in Fig. 4) were individually labeled with [γ-32P]dATP (3000 Ci/mmol) in the presence of T4 polynucleotide kinase (New England Biolabs Inc., Beverly, MA). Each pair of labeled oligonucleotide probes was then mixed, heated to 65 °C for 5 min, and then slowly cooled at room temperature to facilitate annealing. Labeled double-stranded oligonucleotide probes were then placed on a nondenaturing 6% polyacrylamide gel and eluted at 50 °C for 4 h in 0.5 M sodium acetate (pH 5.2), 1 mM EDTA, and 0.1% SDS. Probes were then centrifuged briefly to pellet the small pieces of acrylamide. The supernatant was passed over a filter column (Whatman) and precipitated at −80 °C with 1 μl of glycerol (20 mg/ml) and 4 volumes of 100% ethanol. The purified probes were then centrifuged, washed with 70% ethanol, recentrifuged, and resuspended in 100 μl of Tris/EDTA (pH 8.0).

For EMSAs, −5 μg of nuclear extract or 1 μl of in vitro translated SRF was incubated for 10 min on ice in 1× binding buffer (40 mM KCl, 0.4 mM MgCl₂, 4% glycerol, 5 mM HEPES, and 2.4 mM EDTA), 16 μg of bovine serum albumin, 0.125 μM of poly(dI-dC), 2 mM spermidine, and 0.2 mM dithiothreitol in the absence or presence of unlabeled competitor oligonucleotide (−100×). Approximately 50,000 cpm of labeled probe (−0.2–0.5 pm) was then added to the mixture and incubated at room temperature for 20 min. In some samples, 1 μl of antisense to SRF (sc-335, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), transcription factor YY1 (sc-281, Santa Cruz Biotechnology, Inc.), or SM-Calp (clone hCP, Sigma) was included following probe addition, and the mixture incubated for an additional 15 min at room temperature. A second aliquot of SRF raised against the NH₂-terminus was used to further confirm the identity of nucleoprotein complexes. Samples were resolved on a nondenaturing 6% polyacrylamide gel (19:1 acrylamide/bisacrylamide) that pre-run for 30 min at 150 V prior to loading. Gels were vacuum-dried and then exposed to x-ray film for varying periods of time at −80 °C.

**Oligonucleotide Adenoviral Vectors and Cellular Transduction—**A nuclear locZ reporter gene (kindly provided by Dr. Yassemi Capetanaki, Baylor College of Medicine) or the SRFpm1 dominant-negative mutant (DNSR) cDNA was cloned into the pCA3 shuttle plasmid (Microbix Biosystems, Inc., Toronto, Ontario, Canada). Each transgene was placed under the control of the mouse SM22 promoter (−445 nucleotides), which was cloned into a BglII site of pCA3 in place of the cytomegalovirus promoter. Recombinant shuttle plasmids were integrated into a serotype 5 replication-defective adenovirus (strain dl327) by direct ligation or homologous recombination in human embryonic kidney 293 cells as described (39). Crude viral lysates were analyzed for proper integration of the shuttle plasmid by restriction digestion and PCR analysis of the transgene. High titer viral stocks were prepared in human embryonic kidney 293 cells and purified as described (39).

For viral transductions, cells were plated in 100-mm dishes and allowed to grow until −80–90% confluent, at which time, the cells were washed and incubated with adenovirus at a multiplicity of infection (50 μl) over 1 h incubation period to facilitate uniform adsorption of the virus to the cell monolayer. After 1 h, complete medium was added, and the cells were allowed to grow for periods up to 4 days post-transduction. Cells were then harvested for mRNA and protein expression assays as described below.

**RNA Expression Analyses—**Total RNA was isolated from cell monolayers by the guanidinium isothiocyanate/acid phenol method (40). For reverse transcription-PCR, samples of total RNA (−5 μg) were subjected to reverse transcription (first strand cDNA synthesis kit, Amer...
were carried out with radiolabeled riboprobes to SM-Calp, (Gen Inc.) for dideoxynucleotide sequencing. RNase protection assays were sheared through a 23-gauge needle and then boiled for 5 min. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

RT-PCR was performed with cDNA templates using reverse transcriptase 2 (VERATRIUM, Palo Alto, CA). PCR products were resolved on a 1% agarose gel and then either blotted for Southern hybridization to an internal oligonucleotide or excised and purified (Qiaquick column, QIAGEN Inc.) for dideoxynucleotide sequencing. RNAse protection assays were carried out with radiolabeled riboprobes to SM-Calp, a-tubulin, and 18 S as described (32). Northern blotting was performed with cDNA probes to rat SM22 and smooth muscle a-actin (32) as well as mouse retinoid acid receptor-a (gift from Dr. Pierre Chambon).

Western Blotting—Cell monolayers were washed twice with ice-cold phosphate-buffered saline and then scraped in extraction buffer containing 55 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 10 mM dithiothreitol, 0.5 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A, 0.1 μg/ml leupeptin, and 1 μg/ml aprotinin. Samples were sheared through a 22-gage needle and then boiled for 5 min. Protein concentration was determined by the BCA assay. Initially, samples of protein extracts (25–50 μg) were resolved on a 10% polyacrylamide gel and stained with Coomassie Blue to confirm sample integrity and equal loading. Parallel samples were then electrophoresed to nitrocellulose (Bio-Rad) and processed for Western blotting as described (26). The SRF (1:2000) and SM-Calp (1:5000) antisera are described above. As a control, we incubated blots with an antibody to the SH2 adaptor protein Grb-2 (-7, Santa Cruz Biotechnology, Inc.). Specific immunoreactive proteins were revealed on x-ray film with ECL reagents (Amersham Pharmacia Biotech.).

Statistical Analyses—Transfection data are expressed as the mean ± S.E. Paired comparisons were made with a t test. Where more than two groups of data occur, an analysis of variance was performed, followed by Tukey’s post-hoc test for intergroup comparisons. All graphical data and statistical analyses were generated with GraphPad Prism software (Version 2.0). Data were considered statistically significant at p < 0.05.

RESULTS

SM-Calp Intronic Sequences Confer Specific Enhancer Activity in SMCs—SM-Calp expression is highly restricted to SMC lineages (Fig. 1) (26); however, 5-primer sequences do not direct SMC-specific activation of a luciferase reporter gene (26). To determine the role of 3-prime sequences in the activity of the SM-Calp promoter, we cloned the entire first intron of SM-Calp downstream of a −549CalpLuc reporter gene. The data depicted in Fig. 2 show that the presence of the first intron results in a 2-3-fold increase in luciferase activity over the −549CalpLuc construct in PAC1 and rat aortic SMCs. A smaller incremental increase in activity was noted in the A7r5 SMC line. Cloning the intron in the opposite orientation resulted in essentially the same elevation in luciferase activity. In contrast to the enhancer-like activity contributed by the first intron of SM-Calp in SMCs, every non-SMC line tested showed reduced luciferase activity (Fig.2). Comparable cell-specific activation of the SM-Calp promoter was observed when the first intron was studied in its proper sequence context (i.e. the first intron follows exon 1 of SM-Calp).2 The data in Fig. 3 document SMC-specific enhancer activity conferred by the first intron of SM-Calp in a heterologous promoter context. Taken together, these results suggest that the first intron of SM-Calp contains a SMC-specific enhancer(s) as well as repressive sequences that mediate the activity of the SM-Calp promoter in non-SMC lineages. Because PAC1 SMCs consistently yielded the highest enhancer activity, we restricted subsequent studies to this SMC line.

The First Intron of SM-Calp Contains SRF-binding CArG Boxes—Fig. 4 shows the nucleotide sequence of the first intron of SM-Calp. A consensus CArG box is present +852 nucleotides from the transcription start site (IC1, in Fig. 4). In addition to the consensus CArG box, two CArG-like elements (IC2 and IC3 in Fig. 4) are present at the 3-prime end of the first intron (+1530 and +1745 nucleotides downstream from the transcription start site). To determine whether SRF binds the individual CArG elements within the first intron of SM-Calp, we performed EMSA as described under “Experimental Procedures.” The data in Fig. 5A show that recombinant SRF binds the consensus IC1 element. A nucleoprotein complex of comparable mobility was observed with nuclear extracts from the PAC1 SMC line (Fig. 5A) as well as from several non-SMC lines that do not express SM-Calp (Fig. 5B). The data presented in Fig. 6 show comparatively weak binding of SRF to IC2 and IC3. Denaturation analysis indicated that the binding of SRF to IC1 was >200 times greater than that observed with IC2 and IC3. Indeed, unlabeled IC1 oligonucleotide was a more effective competitive inhibitor of SRF binding than either IC2 or IC3 (Fig. 6). These EMSA studies establish SRF-binding CArG boxes within the first intron of the murine SM-Calp gene. All three intronic CArG boxes are completely conserved in the human SM-Calp locus.

SM-Calp Intronic Enhancer Activity Is Conferred Largely by the Consensus SRF-binding CArG Box IC1—To determine the functional importance of the SRF-binding CArG boxes in mediating intronic enhancer activity in SMCs, each CArG box was mutated and tested in a transient transfection assay. The mutations introduced into each CArG box (see Fig. 4) prevented detectable SRF binding (Figs. 5 and 6; data not shown). The

![Fig. 1](http://www.jbc.org/) Reverse transcription-PCR analysis of SM-Calp mRNA expression. Total RNA was harvested from the indicated cell lines, incubated in the absence (−) or presence (+) of reverse transcriptase (RT), and subjected to PCR as described under “Experimental Procedures.” The PAC1 SMC line consistently yielded the highest levels of SM-Calp. In contrast, no detectable signal was observed in non-SMCs even after high cycle PCR (see Footnote 2). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

![Fig. 2](http://www.jbc.org/) Effect of intron 1 sequences on SM-Calp promoter activity in cultured cells. Cells were transfected with either the −549CalpLuc reporter (black bars) or the same reporter containing the entire first intron of SM-Calp (hatched bars). A Renilla reporter plasmid was cotransfected to correct for varying transfection efficiencies. Normalized luciferase data are shown as -fold changes from the −549CalpLuc reporter (set to 1). Each column of data represents the mean ± S.E. from at least eight samples derived from two independent studies. A two-tailed paired t test revealed significant differences between −549CalpLuc and −549CalpLuc-I for every cell line (p < 0.05). RASMC, rat aortic SMCs; RLU, relative light units.
results in Fig. 7 show a significant inhibition of enhancer activity when IC1 was mutated. A small inhibition (not statistically significant) in enhancer activity was noted when either IC2 or IC3 was mutated (Fig. 7). To further establish a role for SRF in mediating intronic enhancer activity, we performed cotransfection studies with SRF-VP16 and reporter genes with or without mutated CArG boxes. SRF-VP16 had only a small effect on −549CalpLuc (Fig. 8, bar 1 versus bar 2), which is consistent with the absence of SRF-binding CArG elements within this promoter context (26, 41). When SRF-VP16 was cotransfected with the −549CalpLuc-I reporter, an ~10-fold increase in intronic enhancer activity was observed (Fig. 8, bar 3 versus bar 4). This augmented enhancer activity was reduced by >50% when IC1 was mutated (Fig. 8, bar 4 versus bar 6). Mutation of all three intronic CArG elements abolished the ability of SRF-VP16 to augment intronic enhancer activity (Fig. 8, bar 4 versus bar 8). Collectively, the studies presented in Figs. 7 and 8 verify the functionality of SRF in binding its cognate elements within the first intron of SM-Calp and in activating reporter gene activity.

A Dominant-negative SRF Construct Attenuates Enhancer Activity and Endogenous SM-Calp Expression—As a further means of establishing a role for SRF-binding CArG boxes in mediating the SMC-specific enhancer activity observed with the first intron of SM-Calp, we cotransfected reporter genes with varying amounts of a DNSRF cDNA that is defective for DNA binding (17, 36). Pilot studies established a dose-dependent effect of DNSRF on blocking activation of the c-fos serum response element. Consistent with the SRF-VP16 data (Fig. 8), very little effect was observed when the DNSRF construct was cotransfected with the −549CalpLuc reporter (Fig. 9, black bars). In contrast, a significant decrease in intronic enhancer activity was noted when DNSRF was cotransfected with the −549CalpLuc-I reporter (Fig. 9, hatched bars). This attenuation in SM-Calp enhancer activity could be rescued with the

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**Fig. 3.** Effect of SM-Calp intron 1 sequences on a heterologous promoter construct. The first intron of SM-Calp was cloned downstream of an SV40 early promoter-driven luciferase reporter and co-transfected with a Renilla plasmid into PAC1 or L6 cells. The same SMC-specific enhancer activity documented in Fig. 2 is shown in this heterologous promoter context. A two-tailed paired t test revealed significant differences between SV40Luc and SV40Luc-I in both PAC1 and L6 cells (p < 0.01). Similar results were seen in an independent experiment with the first intron cloned in the opposite orientation (see Footnote 2). RLU, relative light units.

**Fig. 4.** Sequence analysis of the first intron of SM-Calp. Nucleotide sequence comprising the first exon, the first intron, and a portion of the second exon of mouse SM-Calp is shown with the transcription start sites (arrows), the initiating methionine (overlined ATG), and several features relating to the oligonucleotide probes used for mutagenesis and EMSAs (boldface sequences within intron 1). The consensus intronic CArG box (IC1) and the intronic CArG-like boxes (IC2 and IC3) are underlined at the center of each oligonucleotide. The italicized boldface gtc sequences depicted below each of the CArG elements represent the base substitutions used for creating the mutant oligonucleotides for EMSAs and mutagenesis studies. Upper- and lowercase letters correspond to exonic and intronic sequences, respectively. The sequence shown corresponds to nucleotides 1–1923 of the mouse SM-Calp transcription unit (GenBank TM/EBI Data Bank accession number U28932).
simultaneous introduction of a wild-type SRF expression plasmid.2

To assess the biological activity of DNSRF more directly, we introduced the DNSRF construct into adenovirus and transduced cells in culture. Adenovirus-mediated gene transfer of the SM22-DNSRF construct resulted in a dose-dependent increase in DNSRF protein levels in PAC1 SMCs (Fig. 10). No DNSRF expression was observed when non-SMC lineages were transduced with the virus since the SM22 promoter is inactive in such cell types.2 At m.o.i. 30, the DNSRF construct caused
DISCUSSION

In the last 10 years, significant progress has been made with respect to identifying SMC-restricted genes and characterizing their promoter regions. Such studies are of essential importance in understanding SMC identity and, by extension, vessel wall development and pathobiology. A recurring theme derived from SMC promoter studies has been the role of SRF as a key transcription factor for optimal expression of SMC-restricted genes. As with many viral and immediate-early genes, most SMC-restricted genes harbor at least one CArG box in their immediate 5’-flanking promoter region (42). A notable exception is, however, the SM-Calp gene, whose proximal (up to −1300 nucleotides) promoter region lacks a CArG box as well as other cis-elements involved in muscle-restricted gene expression (e.g. MEF-2 and MCAT) (26, 41). The absence of signature muscle elements in the 5’-promoter region of SM-Calp, which may explain its promiscuous activity in vitro (26, 41), prompted us to evaluate 3’-sequences within the transcription unit itself. This analysis revealed three SRF-binding CArG boxes within the first intron of SM-Calp that appear to mediate SMC-specific enhancer activity in vitro. Adenovirus-mediated gene transfer of a DNSRF construct attenuated endogenous SM-Calp expression, providing in vivo evidence for SRF-dependent SM-Calp expression. The latter finding supports the work of Landerholm et al. (43), who showed that two DNSRF constructs reduced SM-Calp protein levels within coronary artery SMCs from the chick proepicardial organ. Collectively, these studies demonstrate that SM-Calp is an SRF-dependent gene and suggest that CArG boxes within the first intron play an important role in SM-Calp transcription, at least in vitro. Table I summarizes the known SRF-dependent SMC differentiation genes and the positions of their SRF-binding CArG box elements.

Evidence has accumulated implicating proximal CArG elements in the regulation of SM-Calp and other SMC differentiation genes (19, 43–45); however, the role of more distal CArG boxes in gene regulation has yet to be assessed. For example, in addition to the CArG boxes defined in the first intron of SM-Calp, there are six CArG-like elements located at −2429, −2382, −1297, +6129, +6802, and +7213 nucleotides from the PIC (see GenBank™/EBI Data Bank accession numbers U37071 and U28932). Although these CArG-like elements probably bind SRF, we do not believe they play a critical role in mediating SMC-specific enhancer activity for the following reasons. First, deletion studies in which the three 5’-flanking CArG-like elements (−2429, −2382, and −1297 nucleotides) were removed failed to reveal a significant decrement in promoter activity. In fact, −549CalpLuc, which contains no 5’-CArG boxes, displays higher activity than longer constructs harboring all of the 5’-CArG boxes (26). Second, all of the >50 SRF-dependent genes defined to date contain functional SRF-binding CArG boxes within <3 kilobases (and in most cases, 1 kilobase) of the PIC. Finally, the carboxyl-terminal transactivation domain of SRF has been shown to interact directly with the Rap74 subunit of transcription factor IIF, suggesting that the activity of SRF may require a strict spatial dependence of CArG box elements relative to the PIC (46). Thus, the available data support an important role for proximal intronic CArG boxes in SM-Calp-specific gene expression. It must be emphasized, however, that the in vitro data presented in this report will require necessary corroboration from transgenic mouse studies such as those that have been performed with other SRF-dependent SMC-restricted markers (20–22).

The enhancer activity conferred by the consensus CArG box in the first intron of SM-Calp is strikingly similar to that reported for the smooth muscle α-actin gene (22, 47). The latter reports showed −3-fold enhancement of smooth muscle α-actin promoter activity in vitro when the first intron of smooth muscle α-actin (containing a consensus CArG box located at +1001 nucleotides) was included; mutation of the consensus intronic CArG box abolished enhancer activity (22). Interestingly, when the intronic CArG box of smooth muscle α-actin was mutated in transgenic mice, reporter gene activity was selectively abolished in SMC lineages, providing the first in vivo evidence for a SMC-specific regulatory element (22). In addition to SM-Calp and smooth muscle α-actin, other SRF-dependent genes containing intronic CArG boxes include Elk-1 (48), β-actin (49), and smooth muscle γ-actin (GenBank™/EBI Data Bank accession number U19488 (50)). Future studies are necessary to determine whether the intronic CArG boxes in these genes as well as SM-Calp are critical for in vivo transcription.

Optimal transcription of virtually all known SMC-restricted
genes requires SRF interacting with its cognate CArG box elements (Table I). Similar SRF-binding CArG boxes govern cell-specific gene expression in both cardiac and skeletal muscle lineages. A critical question therefore is how SRF mediates cell-specific expression in each muscle type. A popular hypothesis is that SRF recruits other transcription factors that either bind adjacent cis-elements surrounding the CArG box or bind SRF itself without contacting the DNA template. In this model, SRF is viewed as an essential platform for the generation of a multiprotein complex that may be unique to any given muscle promoter. For example, optimal activity of the cardiac α-actin gene requires the cooperative interplay between SRF, MyoD, and Sp1 (51). In some instances, the interaction between SRF and a neighboring transcription factor is a consequence of a specific signaling event. In this context, angiotensin II was shown to stimulate smooth muscle α-actin promoter activity through an interaction between SRF and the muscle-restricted homeobox gene, MHox (52). These studies suggest that the flanking sequences surrounding a CArG box, as well as signaling pathways that may be unique to a given muscle type, play an important role in SRF-dependent cell-specific gene activation. Whether SRF, bound to CArG boxes in the first intron of SM-Calp, is the target of unique signaling cues or interacts with adjacent transacting factors remains to be determined. Although there is great interest in uncovering SRF-associated proteins that could mediate SMC-restricted gene expression, other mechanisms should be considered. For example, a very recent report showed that SRF activation was closely linked to the process of actin treadmilling (53). In this model,
decreases in G-actin led to SRF activation through a LIM kinase-dependent pathway. This mechanism of SRF activation may be well suited for SMCs in which levels of G-actin are likely to vary considerably during developmental and disease states. Another possibility that has yet to be formally tested is the accessibility of critical CArG boxes in different cell types. For example, it is possible that key CArG boxes within SMC differentiation genes are “closed” in non-SMC types through the action of chromatin. In this context, sequences encompassing a CArG box in the first intron of Elk-1 were shown recently to reside within a DNase I hypersensitivity site (48). Interestingly, this region of “open” chromatin was confined to premonocytc cells and not other myeloid cell types. Whether CArG elements within SMC-restricted gene loci are differentially chromatinized between cell types is presently unknown. Whatever mechanisms are involved in conferring SRF-mediated SMC-restricted gene expression, a critical question will be whether the SRF-CArG axis is perturbed in SMC-associated diseases.

| SMC-restricted Gene             | SRF-binding CArG boxes | Ref. |
|--------------------------------|-------------------------|-----|
| Telokin                        | −56                     | 8   |
| SM* α-actin                    | −112, −62, +1001         | 10, 22|
| SM γ-actin                     | −2088, −1877, −455, −306, −125, −88 | 19, 306, 125, 88 |
| SM22                           | −273, −150              | 20, 21|
| α1-Integrin                    | −156                    | 56   |
| Caldesmon                      | −309                    | 44, 57|
| SM myosin heavy chain          | −1307, −1231, −1112     | 45, 58|
| SM calponin                    | +852, +1530, +1745      | This study|

*SM, smooth muscle.
In summary, we have shown that the first intron of SM-Calp confers SMC-specific enhancer activity in vitro and that most of this activity is attributable to a consensus CArG box located within 1 kilobase of the PIC. On the other hand, intronic sequences result in a significant decrease in SM-Calp promoter activity in non-SMCs, suggesting the presence of one or more repressor elements. We also have shown that a DNSRF construct can selectively reduce the steady-state expression of SM-Calp, possibly by limiting the interaction of endogenous SRF with one or more of the intronic CArG elements. Future studies will determine if the first intron of SM-Calp confers SMC-specific expression in vivo and whether the accessibility of SRF to these intronic CArG elements is impaired in non-SMC lineages. The use of adenovirus containing a cell-specific promoter (SM22) to target transgenes (e.g., DNSRF) to SMC lineages represents a useful approach for studying gene function in the context of normal and diseased blood vessels.

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