Identification of Promoter Elements Involved in Cell-specific Regulation of Rat Smooth Muscle Myosin Heavy Chain Gene Transcription*

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In order to identify cis-acting regulatory elements involved in smooth muscle cell-specific gene regulation, we have cloned a 4.7-kilobase pair fragment of the promoter for the rat smooth muscle myosin heavy chain, a protein expressed in differentiated smooth muscle cells. Sequence analysis of a 1.7-kilobase pair portion of this clone reveals potential binding sites for known transcription factors. A comparison of the primary sequence between the rat and rabbit smooth muscle myosin heavy chain promoters reveals numerous conserved consensus binding sites. Transient transfection analysis of promoter deletion constructs in rat aorta and tracheal smooth muscle cells, L8 myoblast cells, and rat pulmonary aorta endothelial cells suggests that a region of the promoter located between –1,249 and –1,317 base pairs is important for the restriction of gene expression to smooth muscle cells. Electrophoretic mobility shift analysis of a highly conserved region located between –1,317 and –1,085 base pairs reveals specific DNA-protein complexes formed in smooth muscle cell extracts, which can be competed with an oligonucleotide containing a nuclear factor 1 binding site.

Smooth muscle cells (SMCs) express a repertoire of specialized proteins important for the cell’s contractile function. Smooth muscle myosin is the primary type-II myosin present in fully differentiated SMCs. Four isoforms of smooth muscle myosin heavy chain (smMHC) exist, produced by alternative splicing from a single gene (1–4). smMHC isoform composition is tissue-restricted and developmentally restricted and is also modulated in response to changes in SMC differentiation state (2, 5, 6). The physiological function of the different isoforms remains unclear; however, it is probable that shifts in isoform composition allow cellular adaptation to physiological stresses, as is the case in skeletal muscle (7–9).

Vascular diseases such as atherosclerosis, hypertension, and restenosis following angioplasty involve smooth muscle cell dedifferentiation and proliferation (5, 10–13). smMHC is an extensively studied marker, which is specific for differentiated SMCs (2, 5, 14, 15). The expression of smMHC is decreased or absent in proliferating, dedifferentiated SMCs (6, 14, 16–20).

Once SMCs in the neointima of atherosclerotic vessels cease proliferating, they reexpress the SM1 smMHC isoform (6, 14). Therefore, the smMHC gene provides a potentially useful promoter for studying the regulation of SMC differentiation state and the factors involved in specifying the proliferative or quiescent/dedifferentiated SMC phenotype.

Relatively little is known regarding the transcription factors involved in the coordinate regulation of smooth muscle-specific genes in differentiated SMCs. Recent studies of the most extensively studied promoter, that for smooth muscle α-actin, have shown that there is a complex regulation involving both positive and negative cis-acting elements, as well as important CArG box elements required for smooth muscle-specific expression (21–23). However, the regulation of this promoter may be somewhat complicated by elements involved in regulating its expression in non-smooth muscle cells such as myofibroblasts and developing cardiac and skeletal muscle (24–26). Furthermore, the expression of α-actin during development and in cell culture suggests that it is an earlier marker on the smooth muscle differentiation pathway than smMHC, which appears to be one of the final markers expressed in fully differentiated smooth muscle cells (5). Studies of additional SMC-specific genes are therefore essential, as it is the coordinate regulation of these genes that is important for establishing the fully differentiated SMC phenotype.

Recently, characterizations of the rabbit smMHC promoter have been reported. Katoh et al. reported a region between –1,223 and –1,548 bp, which appears to be important for cell-specific promoter activity in transiently transfected rat SMCs (27). Kallmeir et al. reported the presence of an enhancer element located between –1,225 and –1,332 bp, which appears to contribute to SMC-specific expression in rabbit vascular SMCs (42).

The goals of the current study were to clone, sequence, and identify key regulatory elements in the rat smMHC promoter; determine important cis-acting DNA elements governing SMC-specific transcriptional expression; and examine conserved promoter regions for evidence of SMC DNA-binding proteins. The results of this study provide important new evidence for: 1) conservation of smMHC promoter elements in rat to rabbit sequence comparison, including a set of three CArG-like elements; 2) differential gene regulation in vascular versus airway SMCs; 3) the presence of a DNA element located between –1,249 and –1,317 bp, which is involved in repressing promoter activity in non-smooth muscle cell types; and 4) the existence of a DNA-binding protein, which interacts with a nuclear factor 1-like site that is present in SMC extracts.
**EXPERIMENTAL PROCEDURES**

Screening of the Rat Genomic Library—A rat genomic λDASH II library (Stratagene) containing 2.0 × 10^6 independent clones was screened using a 32P-radiolabeled fragment containing the 5′-untranslated region of a rat smMHC cDNA. Approximately 5.0 × 10^5 independent clones were screened under stringent hybridization and wash conditions, as described previously (3). Eleven positive clones were obtained and further examined by Southern blot analysis of the phage DNA with the 5′-untranslated fragment of the rat smMHC cDNA as probe. A positive clone, RtG-8, was then selected for further analysis. Restriction endonuclease digestion of this clone with Bgl II site of pGL2-Basic. Two deletion constructs (p1621LUC and p1317LUC) were generated via PCR using this fragment as a template. All other promoter constructs used the 1.2-kb EcoRI-Sac I fragment as a PCR template. Due to the presence of Sac I sites in the promoter upstream of −1.2 kb, the p1621LUC and p1317LUC constructs were cloned into the pGL2 vector using oligonucleotides with HindIII restriction sites, and these clones extend to +88 bp in the 5′-untranslated exon. Promoter-luciferase expression constructs plasmid DNAs used for transient transfections were prepared by alkaline lysis and affinity column-purified (Wizard Mega-prep, Promega) followed by phenol-chloroform extraction, ethanol precipitation, and CsCl density gradient centrifugation. Plasmid preparations were examined by gel electrophoresis for purity and the presence of linearized DNA prior to transfection.

**Sequence Comparisons and Analysis**—The MacVector DNA analysis program (IBI) was used to compare rat and rabbit smMHC promoter sequences and to identify transcription factor binding sites.

**Cell Culture, DNA Transfections, and Reporter Assay**—Smooth muscle cells were obtained from adult rat aorta and tracheal tissues by a modification of procedures described by Bodnatou-Piallat et al. (29). Adult Sprague-Dawley rats were anesthetized with sodium pentobarbital, and the aorta and trachea were aseptically removed. Following a 15-min digestion in collagenase solution (400 units/ml collagenase, 0.5 mg/ml elastase, 0.5 mg/ml trypsin inhibitor in Hank's balanced salt
solution containing 300 units/ml penicillin, 300 μg/ml streptomycin sulfate, and 0.75 μg/ml amphotericin B), the trachea were scraped and minced for further digestion. The adventitial layer of the aorta was removed and endothelial cells were removed by scraping with blunt forceps, and the aorta was minced prior to enzymatic digestion. The tissues were digested in fresh collagenase solution for 1–3 h at 37°C, until the cells were completely dissociated. The cells were collected by centrifugation at 3,000 rpm for 5 min, resuspended in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.), and then re-centrifuged to remove all collagenase. The cells were then resuspended in DMEM containing 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B, and seeded onto 100-mm² tissue culture dishes. Fetal bovine serum (FBS) was added to 10% after allowing the cells to attach for 1–2 h. These cell cultures typically reached confluence after 3–7 days and were then passaged onto 12-well plates for transfection. At the time of transfection, at approximately 60–80% confluence, many cells expressed detectable levels of smMHC and smooth muscle α-actin as determined by indirect immunofluorescence analysis (data not shown).

Transfections were performed in triplicate using Lipofectamine reagent (Life Technologies, Inc.) with each well receiving 1.5 μg of test plasmid and 5 μl of Lipofectamine in a total volume of 500 μl of Opti-MEM reduced serum media (Life Technologies, Inc.). Plasmid DNA and Lipofectamine were incubated for 0.5–1 h prior to transfection to allow the formation of DNA-liposome complexes. Cells were washed once with Opti-MEM, and then the DNA-liposome mix was layered over the cells. At 16 h post-transfection, the DNA-liposome mix was removed, the cells were washed with 1 ml of DMEM, 10% FBS, and then fresh medium (DMEM, 10% FBS) was added to each well and the cells grown for 24 h, during which time they usually reached confluence.

Cell extracts were obtained by washing the cells three times with phosphate-buffered saline (137 mM NaCl, 27 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4), followed by incubation for 15 min in 100 μl of 1× reporter lysis buffer (Promega). Cells were scraped into microcentrifuge tubes and centrifuged at 14,000 rpm for 5 min to remove cell debris. A 20-μl aliquot of the supernatant was removed for the determination of luciferase activity. The luciferase activity was determined using a luciferase reporter assay kit (Promega) with signal detected via luminometer (Berthold).

Early transfection experiments utilized a secreted alkaline phosphatase plasmid (p-SEAP, Tropix) as a co-transfection plasmid to measure transfection efficiency. However, the secreted alkaline phosphatase measurements did not result in qualitative changes in the data and also had no effect on experimental variability. The use of a co-transfection plasmid was discontinued, primarily to allow the use of lower levels of test DNA in the transfection, as higher levels of DNA per well (≥5 μg) were observed to have toxic effects on the cells, and because the p-SEAP plasmid might compete for transcription factors with the smMHC promoter plasmids. Instead, for each experiment, pGL2-Basic, a promot-

![Fig. 2. Nucleotide sequence of the rat smMHC gene promoter.](image-url)
RESULTS

Isolation of the Rat smMHC Gene Promoter and Sequence Analysis—The rat smMHC gene promoter was isolated from a rat genomic DASH II library by screening with a 1.2-kb EcoRI fragment of a smMHC cDNA containing 80 bp of the 5'-untranslated region. The strongest positive of 16 positive clones examined by Southern blot, A1TGA8, was further characterized by restriction enzyme and Southern blot analysis and determined to contain the rat smMHC gene promoter. This clone was found to contain approximately 4.7 kb of upstream promoter (Fig. 1).

The sequence of the promoter extending to −1,699 bp is shown in Fig. 2. A TATA box (TATAA; Ref. 31) was located at position −24 bp; however, no typical CAAT box motif could be found in this promoter. The transcription start site, as well as the 5'-untranslated exon's intron/exon boundaries, were determined by sequencing several cDNA clones obtained by a rapid amplification of cDNA ends procedure conducted on rat aorta mRNA (data not shown). An examination of the sequence for DNA-binding protein/transcription factor consensus binding sites revealed numerous sites for known factors. Three CArG box-like motifs (32, 33) are located at positions −1317 to −1085 bp; SMHC 1275, 5'-gctgtatgcggacag-3'; SMHC 1143, 5'-tggtatgagcactac-3'; and A1TGA8, 5'-attggatacagcagctattg-3', which are completely conserved (Fig. 3A). This region also contains two NF1-like sites, which are not precisely conserved between the rat and rabbit primary sequences. The −118 to +60 bp region contains the TATA box and flanking sequences (Fig. 3B). The TATA box is completely conserved, as are several regions adjacent to it. The E box present in the rat

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

The promoter segment used in the electrophoretic mobility shift assay (EMSA) analysis was generated by PCR using complementary primers and the 4.2-kb BglII promoter construct (p4.2LUC) as a template. Following PCR, the promoter segment was purified by gel purification on a 10% agarose gel. This fragment was end-labeled using [γ-32P]ATP and T4 kinase, followed by removal of unincorporated nucleotides on a NucTrap push column (Stratagene).

EMSAs were performed in a 30-μl binding reaction containing 2–5 ng of probe, 50–200 μg of whole cell extract, 1 × binding buffer (10 mM Tris, pH 7.5, 50 mM sodium chloride, 1 mM EDTA, 1% Ficoll) and 2.5 μg of d/dIC (Pharmacia Biotech Inc.). Whole cell extracts were preincubated on ice in the binding reaction mix without probe for 15 min. The probe was then added, and incubation was continued on ice for an additional 30 min. In experiments involving competitive EMSAs, the cold competing oligonucleotides or promoter fragments were included in the preincubation. The following double-stranded (only one strand shown) oligonucleotides were used for competitive EMSA: SMHC 1317, promoter fragment spanning from −1317 to −1085 bp; SMHC 1275, 5'-gctgtatgcggacag-3'; SMHC 1143, 5'-tggtatgagcactac-3'; SMHC 1182, 5'-attggatacagcagctattg-3'; AP1, 5'-cgtgataagacaggagac-3'; AP2, 5'-gatgagaagacaggagac-3'; CTFF1, 5'-ccgtgataagacaggagac-3'; and NF1, 5'-attggatacagcagctattg-3'.

Binding reactions were loaded and electrophoresed on a 5% polyacrylamide gel, which had been pre-run at 150 V for 1 h. Electrophoresis was performed at 150 V in Tris-glycine buffer (200 mM glycine, 25 mM Tris, 1 mM EDTA, pH 8.3). Gels were dried and exposed to film for 24–72 h with an intensifying screen at −70 °C.
Fig. 4. A, transfection analysis of smMHC promoter deletion constructs in adult rat aorta smooth muscle cells. Subconfluent cultures were transiently transfected with the constructs shown in Fig. 1B. Luciferase activity is expressed relative to the base-line luciferase activity of a promoterless-luciferase construct (pGL-Basic) set to equal 1. An SV40 enhancer/promoter-luciferase construct (pGL-SV40) was transfected as a positive control. Data shown are from 4 independent experiments performed in triplicate. Data are presented ± S.E. B, transfection analysis of smMHC promoter deletion constructs in adult rat tracheal smooth muscle cells. Subconfluent cultures were transiently transfected with the
sequence, adjacent to the TATA box, does not appear to be conserved in the rabbit sequence.

Negative-acting cis-Elements Are Involved in Cell-type Specific Regulation of the Smooth Muscle Myosin Heavy Chain Promoter—As a first step toward defining the regulatory elements required for promoter activity in smooth muscle cells, SMCs derived from adult rat aortas and trachea were transiently transfected with fusion plasmids containing portions of the rat smMHC promoter fused to a luciferase reporter gene (Fig. 1B). The promoter construct p1249 was found to be the most active in adult aorta cells with a relative luciferase activity approximately 30-fold over background (Fig. 4A). Addition of upstream sequences led to decreases in relative luciferase activity, as did deletion of sequences downstream of −1249 bp. The p48 construct was found to be completely inactive, indicating that elements located between −138 and −48 bp are required for basal promoter activity. The two larger constructs, p4.2 and p1621, were found to have relatively low levels of luciferase activity, suggesting the presence of negative regulatory elements within these regions.

Transient transfection of promoter constructs into adult rat tracheal SMCs produced a somewhat different pattern of luciferase activity (Fig. 4B). The p1249 was again one of the most active plasmids; however, deletions of downstream elements appear to have less of an effect on luciferase activity in tracheal SMCs, compared to aorta cells. As observed for the adult aorta SMCs, the p48 construct is inactive. Addition of regions upstream of −1249 bp also leads to decreased luciferase activities, as observed for the rat aorta SMCs.

In order to identify cis-elements involved in cell-specific gene regulation, the smMHC promoter deletion constructs were transiently transfected into non-SMC types (Fig. 5). Neither rat pulmonary aorta endothelial cells (rPAECs) nor rat L8 myoblast cells express endogenous smMHC protein, based upon immunofluorescence staining with smMHC-specific antibodies (data not shown). In the rPAECs, the p48 construct was inactive, as it was in SMCs. The three shortest constructs had relatively high luciferase activities, ranging from 18- to 50-fold over background. The luciferase activity was somewhat depressed in p825 and p1249 constructs, but was still 8- to 15-fold higher than background. Addition of regions upstream of −1249 bp led to abrupt decreases in luciferase activity. The activity of the p4.2, p1621, and p1317 constructs was reduced to background or near background levels.

Transient transfections into L8 myoblasts revealed a pattern of luciferase activity similar in some respects to the rPAEC pattern. The p48 construct again showed minimal levels of activity, whereas the p825, p602, and p291, and p136 constructs all showed high levels of activity. However, unlike the rPAECs, L8 cells transfected with p1249 demonstrated very high activity levels, approximately 50- to 100-fold over background. Addition of sequences upstream of −1249 bp, however, led to greatly decreased levels of luciferase activity similar to that observed in the rPAECs. The composite data strongly suggest the presence of a negative-acting cis element, located between −1317 and −1249 bp, which functions to restrict expression of the smMHC promoter to SMCs.

The p4.2, p1621, and p1317 constructs contain an extra 43 bp of 5′-untranslated exon, due to the use of the BglI site for cloning rather than the adjacent SacI site. In order to eliminate the possibility that this 43-bp region was responsible for the cell-specific expression of the p4.2, p1621, and p1317 constructs, two additional constructs (−1249 to +88 and −1249 to +600 bp) were made and transiently transfected into rat aorta and tracheal SMCs, L8 myoblasts, and rat pulmonary endothelial cells. The pattern of luciferase activity observed for these constructs did not differ from that of the p1249 construct (data not shown). Therefore, the additional 43 bp of 5′-untranslated exon present in the p4.2, p1621, and p1317 constructs is not sufficient to cause the cell-specific expression observed for these promoter constructs.

Factors Present in Smooth and Non-smooth Muscle Cell Types Bind to the −1317 to −1085 bp Fragment of the smMHC Promoter—To investigate the relevance of the observed conservation of sequence between the rat and rabbit promoters in the −1.3 to −1.1 kb region, EMSAs were performed on cell extracts using a probe which extended from −1,317 to −1,085 bp. This region contains the three CArG box-like elements, as well as two NF1-like elements.

EMSA using whole cell extracts from primary rat tracheal and aortic SMCs, rat pulmonary aorta endothelial cells, and rat L8 myoblast cells reveal a large shifted complex, which appears similar in all cell extracts (Fig. 6). This DNA-protein complex can be completely abolished by addition of excess unlabeled probe or by the addition of 100-fold excess unlabeled oligonucleotide containing an NF1 binding site, when tested with rat tracheal SMC extract (Fig. 7). Whole cell extracts made from intact aorta and tracheal tissues form a pattern of shifted complexes which is different from that observed in cultured cells (Fig. 8). These shifted bands can also be abolished by addition of excess unlabeled probe or NF1 oligonucleotide to a binding reaction containing rat aorta extract (Fig. 9). In order to determine which of the NF1-like sites located in the −1,317 to −1,085 probe is involved in the formation of this DNA-protein complex, two oligonucleotides, corresponding to the two NF1-like sites were used in a competitive EMSA (Fig. 10). Addition of cold double-stranded oligonucleotide spanning from −1147 to −1124 was found to abolish formation of the DNA-protein complexes in extracts derived from cultured primary SMCs and in tracheal and aorta tissue cell extracts (Fig. 10A). Competitive EMSA using an oligonucleotide spanning the −1275 NF1 site did not affect formation of the DNA-protein complex (Fig. 10B). However, an oligonucleotide containing the NF1 site from an adenovirus sequence is capable of effectively competing for the proteins present in this DNA-protein complex. These data suggest that factors present in cultured cell extracts or extracts derived from smooth muscle tissues bind specifically to the NF1-like element located at −1143 bp.

**DISCUSSION**

The goal of the present study was to isolate the rat smMHC promoter and begin the characterization of cis-acting elements and trans-acting factors that regulate the promoter. A comparison of the rat to the rabbit promoter sequence revealed two regions of relatively high identity, with highly divergent intervening sequence. The TATA box and flanking sequences are somewhat conserved, as is a region located over 1 kb from the start of transcription. The region, −1232 to −1098 bp, contains three CArG box-like elements, which are completely conserved, as well as two NF1-like elements that are not completely conserved between rat and rabbit sequences. The conservation of regions of the promoter between the two species suggests that they may be important for regulation of gene expression.
FIG. 5. A, transfection analysis of smMHC promoter deletion constructs in rat pulmonary aorta endothelial cells. Subconfluent cultures were transiently transfected with the constructs shown in Fig. 1B. Luciferase activity is expressed relative to the base-line luciferase activity of a promoterless-luciferase construct (pGL-Basic) set to equal 1. An SV40 enhancer/promoter-luciferase construct (pGL-SV40) was transfected as a positive control. Data shown are from 3 independent experiments performed in triplicate. Data are presented ± S.E. B, transfection analysis of smMHC promoter deletion constructs in rat L8 myoblast cells. Subconfluent cultures were transiently transfected with the constructs shown in
Sequence analysis of the promoter for known transcription factor binding sites revealed numerous binding motifs. The three CArG box-like motifs clustered at 21297, 21223, and 21106 bp were most striking. Serum response factor (SRF), a member of the MADS box transcription factor family, recognizes and binds to CArG box elements (45). CArG box elements may confer tissue-specificity to gene expression by competition for the site between different factors, or by utilization of tissue-restricted SRFs or SRF-associated proteins (46). The triplet set of CArG boxes may potentially play a role in the coordinate regulation of SMC contractile proteins, as three CArG box motifs have also been located in the smooth muscle a-actin promoter (21–23). These CArG box elements have been shown to be important for tissue-specific positive activation of the smooth muscle a-actin promoter, which may involve cell-specific CArG box-binding proteins (21).

Two nuclear factor 1-like sites were identified, located at 21275 and 21143 bp. NF1 exists as a family of proteins which were originally isolated as adenovirus replication factors (38). NF1, which is ubiquitously expressed, has been shown to act as either a positive or a negative regulator of transcription (39, 40). The importance of these elements in smMHC gene regulation is currently unknown.

Eight E box motifs were found scattered throughout the smMHC promoter. The smooth muscle a-actin promoter has also been shown to contain E boxes, known to be important for muscle-specific transcription (41), as do many promoters that are active in striated muscle. E box elements are bound by heterodimers of E proteins and helix-loop-helix proteins. Their importance with regard to smooth muscle-specific transcription is unknown; however, the presence of helix-loop-helix proteins in SMCs suggests that they may potentially play some role in

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**Fig. 6.** Electrophoretic mobility shift analysis of proteins binding to the −1317 to −1085 bp fragment of the smMHC promoter. Whole cell extracts (50 and 150 μg) from cultured rat aorta SMCs (rASMC), cultured rat tracheal SMCs (rTSMC), cultured rat pulmonary aorta endothelial cells (rPAEC), and cultured rat L8 myoblast cells (L8 Mb) were incubated with the 32P-labeled promoter fragment in lanes 2–9. The probe alone is shown in lane 1.

**Fig. 7.** Competitive electrophoretic mobility shift analysis of proteins binding to the −1317 to −1085 bp fragment of the smMHC promoter in the presence of competing DNA and oligonucleotides. Cell extracts from adult rat tracheal SMCs (rTSMC) were incubated with the probe with and without addition of competing oligonucleotides. Lane 1 shows the probe alone. Lane 2 shows extract without competing DNA. Lane 3 shows the results of addition of 100-fold molar excess of unlabeled −1317 to −1085 DNA fragment. Lanes 4–7 show the results of addition of 100-fold molar excess of oligonucleotides containing the indicated binding motifs.

**Fig. 8.** Electrophoretic mobility shift analysis of proteins in extract from smooth muscle tissue binding to the −1317 to −1085 bp fragment of the smMHC promoter. Figure shows whole cell extract of the proteins in cultured smooth muscle cells compared to extract obtained from intact aorta and trachea tissues. Lane 1 shows the probe alone. Lane 2 shows the shift observed for cultured tracheal SMCs. Lanes 3–5 show increasing amounts (50–250 μg) of aorta tissue extract. Lanes 6 and 7 show 100 and 250 μg of trachea tissue extract.
SMC-specific gene regulation (41).

The transient transfections clearly demonstrate the complexity of the smMHC promoter. Multiple positive and negative regulatory regions exist, and it appears that they are differentially utilized in different smooth muscle cell types. In both the rat aorta and tracheal SMCs, the −1,249 bp promoter construct produced high luciferase activity. However, whereas shorter constructs produced decreased luciferase activity in aorta SMCs, this decrease was not observed in tracheal SMCs. The −1249 bp construct appears to contain a positive regulatory element important for gene expression in aorta SMCs, but this element appears to be less important for promoter activity in tracheal SMCs. This result also further emphasizes the importance of cell context with respect to studies of the smMHC promoter, as these two SMC types appear to be able to utilize different regulatory strategies.

A surprising result of the transient transfection experiments was the high activity of smMHC promoter constructs p1249, p825, p602, p291, and p138 in rat L8 myoblast and rat pulmonary aorta endothelial cells. This activity was, however, nearly abolished by the addition of 68 bp of 5′-flanking sequence to the −1249 bp construct. This result strongly suggests the presence of a negative regulatory element, which represses promoter activity in non-SMC types. Addition of this region also reduced luciferase activity in the rat aorta and tracheal SMCs. This result may be a reflection of the heterogeneous population of cells present in primary smooth muscle cell cultures representing varying degrees of differentiation, some of which are no longer able to express smMHC (16–21). For example, an ele-
ment contained in this 68-bp region may be important for restricting gene expression in non-SMCs and in silencing SMC-specific gene expression in SMCs that have modulated their phenotype and are no longer able to express differentiated SMC-specific genes.

Studies of the rabbit smMHC promoter also demonstrate that a region of the promoter located between −1332 bp and −1225 bp is important for high activity specific to SMCs (42). In another study, transfections of the rabbit promoter fragments into SMCs, fibroblasts (NIH3T3), and myoblasts (C2C12) also showed a dramatic SMC-specific increase in activity when a −1548 bp construct was used, whereas a −1223 bp construct’s CAT activity was not significantly different from that observed for the fibroblasts and myoblast cells (27). These results are consistent with our findings, and we are able to refine the location of an important regulatory element to a region lying between −1249 and −1317 bp. However, unlike the enhancer element recently described in the rabbit promoter, this study has revealed an element that acts as a repressor of gene activity in non-SMCs. This combination of positive and negative regulatory elements contribute to the cell specificity of smMHC expression.

Electrophoretic mobility shift assays of a region that is highly conserved between the rat and the rabbit smMHC promoter sequence (−1317 to −1085 bp) demonstrated a specific DNA-protein complex formed in extracts from cultured smooth muscle cells. The DNA-binding proteins were found to interact with an NF1-like site in the promoter located at −1143 bp. Cell extracts derived from aorta or tracheal smooth muscle tissues show two smaller complexes formed in EMSA analysis using the −1317 to −1085 bp probe. Both complexes are also due to binding to the NF1-like site at −1143 bp. The importance of this NF1-like site in terms of promoter regulation and the precise identity of the DNA-binding proteins that interact with it remains to be determined, as does the role of the different complexes formed in extracts from cultured SMCs versus SMCs from smooth muscle tissue.

In summary, we have presented new evidence for a regulatory element that acts to repress activity of the smMHC gene in non-SMCs. We have also identified a nuclear factor 1-like site that is recognized and bound by proteins present in SMC and non-SMC extracts. Transfection analysis of vascular and airway SMCs provides the first evidence for cell-specific regulation of the smMHC gene. Future studies of this promoter will be required to further define the repressor element, to identify the factors that bind to this region and to ascertain the role of the nuclear factor 1-like site in the regulation of gene activity.

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