Characterization of an E-box–Dependent cis Element in the Smooth Muscle α-Actin Promoter

Frank Jung, A. Daniel Johnson, Meenakshi S. Kumar, Beiyang Wei, Martina Hautmann, Gary K. Owens, Coleen McNamara

Abstract—Identification of the regulators of smooth muscle specific gene expression is critical for understanding smooth muscle cell (SMC) differentiation and the alterations in SMC phenotype seen in vascular diseases. Previous studies have identified that a 2-bp mutation in a conserved cis-acting element (TGTATTATC) in the promoter of the chicken smooth muscle (SM) α-actin gene abolished nuclear factor binding and decreased transcriptional activity of a 271-bp SM α-actin promoter fragment when transfected into rat aortic SMC. However, the promoter region containing this conserved sequence has negative cis regulatory activity when studied in homologous systems. The goal of the present studies was to further characterize the transcriptional activity of the rat SM α-actin promoter region between –224 and –236 that is conserved across mammals. DNase I analysis and electrophoretic mobility shift assays demonstrated that SMC nuclear proteins bound an extended sequence (TGTATTATCCCCATAA). Transient transfection experiments of wild-type and mutant rat SM α-actin promoter-luciferase constructs into rat aortic SMC revealed that promoter activity was enhanced by mutations of specific nucleotides in the TGTATTATCCCCA region. Interestingly, the TGTATTATCCCCA element in the rat SM α-actin promoter is centered between 2 canonical E-boxes. Mutations of the flanking E-boxes abolished the enhancement in promoter activity seen with mutation of the TGTATTATCCCCA element alone. Thus studies provide evidence for a regulatory cassette in the rat SM α-actin promoter that regulates gene expression via combinatorial interactions between 2 E-boxes and a newly described TGTATTATCCCCA element. (Arterioscler Thromb Vasc Biol. 1999;19:2591-2599.)

Key Words: SM α-actin  ■  E-box  ■  smooth muscle cell  ■  differentiation  ■  transcription

The differentiated state of smooth muscle cells (SMC) within atherosclerotic and restenotic lesions in man as well as lesions generated after vascular injury in animals is altered compared with normal medial SMC. This alteration in the differentiated state, collectively referred to as “phenotypic modulation,” is characterized by a decrease in expression of contractile proteins such as smooth muscle myosin heavy chain and smooth muscle α-actin (SM α-actin), an increase in extracellular matrix production, cell migration into the neointima, and proliferation. The molecular mechanisms regulating this process of phenotypic modulation are poorly understood.

A key to understanding SMC differentiation and phenotypic modulation in response to injury is to identify the mechanisms that regulate SMC specific or selective gene expression. The principle function of mature, fully-differentiated SMC is contraction. Therefore, understanding the mechanisms that regulate transcription of SMC contractile genes may provide important insight into the molecular regulation of SMC differentiation and phenotypic modulation. SM α-actin and smooth muscle myosin heavy chain are logical candidates for studying transcriptional regulatory systems in SMC. SM α-actin is the most abundant contractile protein in differentiated SMC. Although it is also expressed in other cell types such as myofibroblasts, tumor cells, and transiently in cardiac and skeletal muscle cells during development, it is almost exclusively expressed in SMC in the adult. Additionally, it has been shown that its expression is decreased in proliferating SMC found within human atherosclerotic lesions.

There is clear evidence that there are differences in the promoter sequences required for SM α-actin gene expression depending on cell type. Previous studies have demonstrated that 2 highly conserved CArG boxes in the SM α-actin promoter are required for high level expression of a 125-bp rat SM α-actin promoter/reporter construct in rat aortic SMC. Interestingly, this 125-bp promoter region lacked activity in L6 myotubes, a cell type that endogenously expresses the SM α-actin gene. Additionally, this 125-bp promoter/reporter construct exhibited high activity in endothelial cells, a cell type that does not endogenously express the SM α-actin gene. Moreover, this 125-bp promoter fragment was inac-
tive when tested in transgenic mice. It is also clear that the SM α-actin promoter is regulated through combinatorial interactions between different factors. Transforming growth factor-β induced activity of the 125-bp rat SM α-actin promoter is dependent on both an intact transforming growth factor-β control element and intact CArG boxes. Additionally, the homeodomain factor Mhox activates SM α-actin transcription via a CArG/serum response factor–dependent mechanism. Thus the molecular mechanisms regulating SM α-actin gene expression are complex and dependent on combinatorial interactions of both positive and negative acting transcription factors that may differ between cell types.

Further studies in SMC suggest that the combinatorial interactions regulating SM α-actin expression may be species dependent. Addition of 5′ promoter sequences to the minimal 125-bp rat SM α-actin promoter resulted in a decrease in promoter activity when transfected in rat aortic SMC, suggesting the presence of 5′ negative cis-acting elements. Consistent with these findings, addition of the region from −257 to −151 of the chicken SM α-actin promoter to the first 151 bp of the promoter resulted in a decrease in activity when transfected into chicken embryonic and adult SMC. In contrast, the 151-bp chicken promoter had low level activity when transfected into rat aortic SMC. This activity was enhanced by the addition of the −257 to −151 upstream fragment. Mutation of the single E-box in this upstream region had no effect on the activity of the chicken SM α-actin promoter in rat aortic SMC, but the positive activity associated with this region was completely abolished by a 2-bp mutation in a conserved TGGTTATC sequence. Of key importance, results from these studies using a heterologous system are in direct contrast with previous results with this same fragment in a homologous system (chicken SM α-actin promoter in chicken SMC) and with a similar fragment of the rat SM α-actin promoter in rat SMC. Unlike in the heterologous system, results in the homologous system defined a negative regulatory activity to this promoter region. Taken together, results suggest the presence of a putative cis-acting element in the SM α-actin promoter that shows species specific differences in its regulation.

Interestingly, one major difference in the promoter sequence in the region from −257 to −151 between the chicken and the rat is that although the chicken promoter contains one E-box consensus binding site close to the TGTTTATC, the rat promoter contains 2 E-boxes flanking the conserved TGTTTATC. The E-box is a consensus sequence (CANNTG) found in the promoter of many cell specific genes, and E-box–binding basic helix-loop-helix (bHLH) factors are known to regulate differentiation in a variety of cell types. In separate studies, we have shown that mutation of either one of the 2 E-boxes in this region abolished activity of the rat SM α-actin promoter in L6 myotubes. Moreover, specific mutations in both E-boxes resulted in a modest decrease in SM α-actin promoter activity when transfected into rat aortic SMC. However, despite attempts using homology based library screening and the yeast 2-hybrid system, no SMC specific HLH factor has as yet been identified (Owens and McNamara, unpublished data, 1999). A number of ubiquitously expressed HLH factors are present in cultured SMC including Id, and the immunoglobulin transcription factor-1 protein, upstream stimulatory factor-1 (USF-1) and USF-2. Additionally, SMC within neointimal lesions formed after experimental vascular injury in animals and within human atherosclerotic plaques have also been shown to express Id. Thus it is appealing to hypothesize that HLH factors may regulate SM α-actin gene expression through combinatorial interactions between ubiquitously expressed HLH factors and other non-HLH transcription factors.

The goals of the present study were the following: 1) to determine whether the TGGTTATC sequence in the rat SM α-actin promoter bound rat aortic nuclear factors; 2) to identify the full sequence involved in nuclear factor binding; 3) to determine whether this cis element has negative transcriptional activity in this context; and 4) to determine whether the flanking E-boxes are involved in regulating activity of the TGGTTATC element. Results demonstrated specific nuclear factor binding to a region that extends 3′ to this element, ie, TGGTTATCCCCA. Specific mutations throughout this region resulted in loss of nuclear factor binding and enhancement of transcriptional activity. Mutation of the flanking E-boxes abolished the enhancement of promoter activity seen with mutation of the TGGTTATCCCCA element, suggesting that this region contains a negative regulatory cassette that is dependent on combinatorial interaction between the TGGTTATCCCCA element and the flanking E-boxes. This report is the first to identify the negative cis-acting element (TGGTTATCCCCA) in the rat SM α-actin promoter and demonstrate a combinatorial interaction between this element and the flanking E-boxes.

Methods

Plasmid Constructs

The initial construction of the p271CAT construct was previously described by Shimizu et al. Site directed mutations in p271CAT were introduced using the Altered Sites in vitro mutagenesis system (Promega). The mutated promoter fragments were then subcloned into the Smal/Xho I restriction site of the luciferase reporter vector (pGL3-basic) and sequenced to verify the presence of the mutation and the proper orientation of the promoter element.

Cell Culture and Transient Transfection Assays

SMCs were plated 24 hours before transfection at a seeding density of 2 × 10⁴ cells/cm² on a 30-mm plate in 2 mL of medium containing equal parts of Dulbecco’s modified Eagle’s medium ( Gibco) and Ham’s F-12 ( Gibco), supplemented with 10% fetal bovine serum ( Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin, designated DMEM-F12. Transfection of plasmid DNA into rat aortic SMC was performed using DOTAP transfection reagent (Boehringer Mannheim). The cells were washed twice with 2 mL of serum free medium before transfection. Five μg of rat SM α-actin promoter luciferase plasmid-DNA was mixed with 15 μL of DOTAP and transfected into rat aortic SMC in serum-free medium supplemented with antibiotics. Cells were incubated for 6 hours, the media was changed to serum containing DMEM-F12 medium and the cells were incubated at 37°C in 4% CO₂ for 48 hours. The cells were subsequently harvested in luciferase cell lysate buffer and prepared by a freeze-thaw cycle, followed by centrifugation to remove cellular debris. Twenty μL of each cell lysate was mixed with 100 μL of luciferase substrate and luciferase activity measured in a luminometer (Promega). An equal volume of cell lysate was used for protein determination to normalize for variation in cell numbers. Relative luciferase promoter activity was expressed as fold increase over promoterless. To control for variations in transfection efficiency, the experiments were performed in triplicate and repeated a minimum of 3 times with a least 2 different preparations of DNA. We have previously found that use of a cotransfected reporter plasmid to control for transfection efficiency confounds data interpretation,
apparently due to competition between viral promoter LacZ constructs and test constructs. This is particularly evident for the smooth muscle α-actin promoter that includes a number of elements, such as CarG boxes that are important for regulation of many viral promoters. Indeed, previous studies have demonstrated that the activity of an internal control plasmid may be suppressed by certain plasmids, overestimating the enhancer/promoter activity of the cotransfected test plasmid. To avoid introducing these undefined variables, we have used the accepted alternative of conducting the experiments several times (n=3, each run performed in triplicate) with at least 2 different preparations of each DNA. As indicated by our data, the variability between replicate samples is very acceptable.

**Nuclear Extracts**

Rat aortic SMC were grown in 225-cm² flasks in DMEM-F12. The cells were harvested and nuclear extracts were prepared by the procedure of Dignam et al.26

**Electrophoretic Mobility Shift Assay**

Twenty-one bp double stranded oligonucleotide probes were obtained by hybridizing single-stranded oligonucleotides (Operon Technologies). The sense sequence for the wild-type oligonucleotide was CAGGATGTTTATCCCCATAAG; for the mutated oligonucleotides CAGGATGTTTATCCCCATAAG, CAGGATGTTTATCCCATAG, CAGGATGTTTATCATTAAAG, and CAGGATGTTTATCCCATAAAG. The probes were labeled with γ^32P-dATP, using the Klenow fragment of DNA polymerase, and subsequently purified on Stratagene push columns (Stratagene). Binding reactions were carried out in 10 mmol/L Tris(hydroxymethyl) aminomethaneHCL pH 7.5, 1 mmol/L ethylenediamine-tetraacetic acid (EDTA), 1 mmol/L diithiothreitol, 5% glycerol, and 50 mmol/L NaCl. Labeled DNA probe (0.5 to 1.0 ng; 1 to 5×10⁶ cpm) was added to each reaction mixture containing 1 μg of double stranded poly dI-dC and 6 μg of protein from crude nuclear extracts. Reaction mixtures were incubated for 30 minutes at room temperature (RT). For the competition experiments, the same conditions were used except that the specific competitor oligonucleotides were added to the reaction mixture 15 minutes before addition of the labeled probe. The sample was loaded on a 5% polyacrylamide gel and incubated for 30 seconds at 170 V until the dye front ran off the gel. The gel was dried and autoradiographed with an intensifying screen.

**DNase I Footprinting Analysis**

A radiolabeled 386-bp polymerase chain reaction product containing the TGGTTATCCCCA element was synthesized from a p271Luc template using ^32P-end-labeled forward primer. After gel purification, double stranded probe was diluted to 1 to 2×10⁶ cpm/μL in water. For footprint assays, 20 μL of Dignam buffer D (controls) or 20 μL of SMC nuclear extract in Dignam buffer D (60 to 70 μg of protein) was subsequently diluted to 49 μL with 0.5×Tris-Boric-EDTA buffer at RT for 30 minutes. Electrophoresis was carried out at 170 V until the dye front ran off the gel. The gel was dried and autoradiographed with an intensifying screen.

The Conserved TGGTTATCCCCA Sequence in the Rat SM α-Actin Promoter is a Binding Site for Nuclear Factors in Rat Aortic SMC

In the SM α-actin promoter, there is complete conservation of the nucleotide sequence TGGTTATCCC in the region from −228 to −236 across all species whose SM α-actin promoters have been cloned (Figure 1). The sequence conservation extends further 3′ in the rat, mouse, and human (TGGTTATCCCCC) but not in the chicken promoter (TGGTTATCC). To determine the essential nucleotides for nuclear protein binding to this region, electrophoretic mobility shift assay (EMSA) was performed using ^32P-labeled wild-type and mutant oligonucleotides (Figure 2A). Results demonstrated that the wild-type TGGTTATCCCCC sequence binds several nuclear factor(s) with a prominent shift band in the midmolecular weight range (arrow Figure 2B, lane 1). EMSA using radiolabeled oligonucleotides containing specific mutations introduced into the conserved TGGTTATCCCCC sequence (Figure 2B) showed a variable pattern of nuclear factor binding (Figure 2B). All 4 mutations abolished nuclear factor binding in the midmolecular weight range when incubated with rat aortic SMC nuclear extract. Except for Mut-1, all mutations also caused a loss of some of the shifted bands in the higher molecular weight range. However, none of the mutations caused a loss of all of the shifted bands in the higher molecular weight range (Figure 2B, lanes 2 to 5). To confirm specific interactions between nuclear proteins and specific nucleotides in the conserved TGGTTATCCCCC sequence, competition experiments were performed using an excess of unlabeled wild-type oligonucleotide and oligonucleotides containing the above mutations (Figure 2A). The unlabeled WT oligonucleotide competed for all nuclear factor-binding complexes (Figure 3A, lanes 2 to 4 and 3B lanes, 2 to 4). A variable pattern of nuclear factor binding competition was observed when oligonucleotides containing the mutations were used as cold competitors. Unlike the wild-type competitor, the mutated oligonucleotides (Mut-1 to -4) were not able to completely compete for the strong midmolecular weight nuclear factor-binding complex even at the highest concentration (Figure 3A, lane 4 versus lanes 7 and 10). However, Mut-1 and -2 did demonstrate partial competition at the higher concentrations. Interestingly, stronger binding of this

![Figure 1. Comparison of the sequence of the SM α-actin gene promoter regions from −189 to −257 in rat, mouse, human, and chicken. The TGGTTATCCCCCA element (TGGTT) and the E-boxes are highlighted with a box.](http://atvb.ahajournals.org/)

![Figure 2A. Electrophoretic mobility shift assay (EMSA) was performed using ^32P-labeled wild-type and mutant oligonucleotides.](http://atvb.ahajournals.org/)

![Figure 2B. All 4 mutations abolished nuclear factor binding in the midmolecular weight range when incubated with rat aortic SMC nuclear extract.](http://atvb.ahajournals.org/)

![Figure 3A. The unlabeled WT oligonucleotide competed for all nuclear factor-binding complexes.](http://atvb.ahajournals.org/)

![Figure 3B. A variable pattern of nuclear factor binding competition was observed when oligonucleotides containing the mutations were used as cold competitors.](http://atvb.ahajournals.org/)
complex was observed in competition studies using Mut-3 and -4 (Figure 3A, lanes 5 to 10 and 3B, lanes 5 to 10). Only Mut-4 completely competed for all nuclear factor complexes in the higher molecular weight range (Figure 3B, lanes 8 to 10). Mut-4 had mutations introduced at the 5′ and 3′ end of the conserved TGGTTATCCCCA-sequence. Taken together these data suggest that there are multiple nuclear proteins binding to the conserved sequence TGGTTATC-

CCA and that this specific binding activity is dependent on the interaction of multiple proteins.

The TGGTTATCCCCA Sequence in the Rat SM α-Actin Promoter Functions as a Negative cis Element in Rat Aortic SMC

Previous studies in the chicken SM α-actin promoter demonstrated that the conserved sequence TGGTTATC was transcriptionally active in rat aortic SMC. Results of the preceding studies demonstrate specific rat aortic nuclear factor binding to the conserved TGGTTATCCCCA sequence in the rat SM α-actin promoter. To determine whether this conserved element is transcriptionally active in rat aortic SMC, transient transfection studies were performed. Various mutations were introduced into this region of the rat SM α-actin promoter by site directed mutagenesis. The 271-bp fragments of the SM α-actin promoter with wild-type sequence and specific mutations in the TGGTTATCCCCA element was subcloned into a luciferase reporter vector. The specific mutations tested were as follows: TacTTATCCCCA=pGL271 Mut-1, TGGTTgggCCCCA=pGL271 Mut-2, TGGTTATCtagA=pGL271 Mut-3, and TacTTATC-tagA=pGL271 Mut-4 (Figure 4A). These plasmid constructs were transiently transfected in parallel with the wild-type plasmid (pGL271WT) into rat aortic SMC. As shown in Figure 4B, all mutations of this conserved region caused an increase in transcriptional promoter activity relative to the wild-type promoter when transfected into rat aortic SMC, suggesting that this cis-acting element functions as a repressor of SM α-actin transcription.

The Repressor Function of the TGGTTATCCCCA Element in the SM α-Actin Promoter is E-Box–Dependent

Mutation of the TGGTTATCCCCA element in the SM α-actin promoter results in enhancement of SM α-actin transcription. Interestingly, this element is flanked by 2 E-boxes in the rat SM α-actin promoter. E-boxes, which contain a consensus sequence (CANNTG), are found in regulatory regions of many skeletal muscle specific genes and bind a family of helix loop helix proteins that are involved in skeletal muscle differentiation. However, no SMC specific helix-loop-helix proteins have been identified. Previous studies have shown that mutation of essential nucleotides in the E-box flanking the TGGTTATC element at position −215 in the chicken SM α-actin promoter had no effect on promoter activity. Additionally, in separate studies, we have shown that mutation of either 1 of the 2 E-boxes in the rat SM α-actin promoter alone did not affect promoter activity. However, specific mutations in both E-boxes modestly reduced SM α-actin promoter activity when transfected into rat aortic SMC. To determine whether the E-boxes at positions −214 to −219 and −252 to −257 in the rat SM α-actin promoter were involved in combinatorial interactions with the TGGTTATCCCCA element, transient transfection experiments were performed. Mutations were made in both E-boxes (CAGTTG to gtacTG at position −252 and CAGCTG to CAggtC at position −214 (see Figure 5A) flanking the wild-type (pGL271EbmWT) and mutated (pLG271EbmMut-2) TGGTTATCCCCA element.

Figure 2. Gel shift analysis of nuclear factors binding the TGGTTATCCCCA sequence. 6 μg of nuclear extracts from rat aortic SMCs were incubated with a radiolabeled 21-bp wild-type oligonucleotide containing the TGGTTATCCCCA sequence (WT) or an oligonucleotide with specific mutations in the TGGTTATC-CCCCA sequence (Mut-1 to -4) (A). Incubation of nuclear extracts with oligonucleotides containing the wild-type TGGTTATCCCCA sequence demonstrated several shift bands with a prominent band in the midmolecular weight range (B, arrow in lane 1). EMSA using radiolabeled oligonucleotides containing specific mutations of the TGGTTATCCCCA-sequence (Mut-1 to -4) showed a variable pattern of loss of shift bands, as depicted in lanes 2 to 5.
were transiently transfected into rat aortic SMC. Results demonstrated that the specific 4-bp mutations of both E-boxes in the context of an intact core TGTTTATCCCCA region only minimally affected promoter activity of the rat SMα-actin promoter in this rat aortic SMC line. However, mutation of both E-boxes abolished the increase of transcriptional promoter activity seen with mutation of the TGTTTATCCCCA in the wild-type E-box context (pGL271 Mut-2) (Figure 5B). These data suggest that this promoter region functions as a regulatory cassette where the activity of the TGTTTATCCCCA element is E-box dependent.

DNAse I Footprint Analysis Provided Evidence of Protein Interaction With the TGTTTATCCCCCATAA Element That Was Modified but not Abolished by Mutations That Disrupted Functional Activity of This Element

To further elucidate the nature of SMC nuclear protein interaction with the TGTTTATCCCCCA-containing region, DNAse I footprint analysis was performed with a 386-bp radiolabeled polymerase chain reaction product containing the TGTTTATCCCCCA sequence incubated with SMC nuclear extract. Results demonstrated protection of the TGTTTATCCCCCATAA-containing region from –221 to –236 (Figure 6, compare lanes 2 and 3 to lane 4), as well as changes in DNAse hypersensitivity (see upper and lower arrows). No footprint was seen over E1 or E2 with the wild-type probe. To determine whether mutation of the ATC sequence resulted in an alteration in DNAse I footprinting of this region, we performed the same experiment using a 386-bp promoter fragment containing the ATC to GGG (Mut-2) mutation shown to abolish the repressor function of this element (Figure 5). Interestingly, the ATC mutation resulted in alteration of the DNAse digestion pattern even in the absence of SMC nuclear extract (Figure 6, lane 6 versus lanes 2 and 3). For example, there was diminution of the upper hypersensitive site and modification of the digestion pattern over the TCCCC region. Addition of SMC nuclear extract resulted in a lack of protection at the site of the mutation but not complete loss of protein binding to the full TGTTTATCCCCCATAA region as there was some protection of the region 5′ and 3′ to the GGG mutation, and the hypersensitive region 5′ to the TGTTTATCCCCCATAA region was again seen. Interestingly, as observed with the wild-type probe, no footprint was seen over E1 or E2 with the mutated probe.

Discussion

Identification of the factors that regulate expression of genes coding for SMC contractile proteins such as SMα-actin is important for understanding the molecular mechanisms that regulate SMC differentiation and the phenotypic modulation observed in vascular disease. Results from DNAse I footprint analysis and EMSA demonstrated that rat aortic nuclear
factors bind to the specific nucleotides TGTTATCCCCA within the proximal 271 bp of the SM α-actin promoter. Multiple mutations along the length of the TGTTATCCCCA element resulted in loss of nuclear factor binding and increased activity of the rat SM α-actin promoter/reporter when transfected into rat aortic SMC. Thus our results confirm and extend previous observations indicating that the TGTTATCCCCA element in the –224- to –236-bp fragment of the SM α-actin promoter indeed repressed expression of the rat SM α-actin reporter construct in rat aortic SMC. Recent studies by Kimura et al provide evidence in support of our hypothesis that the TGTTATCCCCA element within the –222- to –234-bp fragment of the SM α-actin promoter is a negative regulatory element and also help clarify some significant differences in regulation that may be a function of SMC tissue type or species. 27 Kimura et al demonstrated that the TATCTTA sequence from –222 to –228 of the chicken SM α-actin promoter was essential for the negative regulation of the chicken SM α-actin promoter in gizzard SMC. Recent studies by Kimura et al provide evidence in support of our hypothesis that the TGTTATCCCCA element within the –222- to –234-bp fragment of the SM α-actin promoter is a negative regulatory element and also help clarify some significant differences in regulation that may be a function of SMC tissue type or species. 27 Kimura et al demonstrated that the TATCTTA sequence from –222 to –228 of the chicken SM α-actin promoter was essential for the negative regulation of the chicken SM α-actin promoter in gizzard SMC. Consistent with our data, mutation of this region resulted in a 3-fold increase in chicken SM α-actin promoter activity. 27 However, the sequence from –222 to –228 in the chicken SM α-actin promoter is only 57% homologous with the sequence in this region of the mammalian SM α-actin promoters. The sequence of this region in the rat SM α-actin promoter (TGTTATCCCCA) is a consensus binding site for MSSP-1. There is some overlap in this region of the rat promoter (TATC) with that of the chicken SM α-actin promoter (TATCTTA). However, Takai et al demonstrated that it is the TCTT sequence in the MSSP consensus site that is important for DNA binding of the MSSP proteins. 28 Mutation of either the TC or TT sequences in this cis element resulted in loss of ability to compete the MSSP proteins off of the promoter fragment containing the wild-type consensus sequence in a gel shift assay. The rat SM α-actin promoter does not contain the TT portion of this element. Although we cannot rule out the fact that MSSP may be involved in the regulation of the rat SM α-actin promoter, these data suggest that it is not mediated by the TGTTATCCCCA element. MSSP may be an important factor accounting for the differences in our data when we tested the chicken SM α-actin promoter in rat SMC 21 versus the rat SM α-actin promoter in rat SMC (Figure 4). Taken together, these data provide evidence that the cis- and trans-acting factors that regulate the negative activity of the SM α-actin promoter are tissue type or species specific.

Further evidence for tissue-type or species-specific differences in the cis- and trans-acting factors that regulate SM α-actin promoter activity comes from additional transient transfection studies with promoter fragments containing mu-
tations of the TGTT 5' to the MSSP-1 consensus site in the chicken SM α-actin promoter. Kimura et al demonstrated that mutation of this TGTT sequence in the chicken promoter had no effect on promoter activity. In contrast, our transient transfection data demonstrated that a 2-bp mutation (TGTT-TATCCCCA to TacTTATCCCCA) in the rat SM α-actin promoter enhanced promoter activity suggesting that the TGTT sequence is important in regulating the expression of the SM α-actin promoter in vascular but not gizzard SMC. A TGTTT (TGT3) promoter element has been identified by DNAse I footprinting in the Hepatitis B Virus enhancer 29; however, beyond the TGTTT, there is no sequence homology in the flanking nucleotides of the element in the Hepatitis B virus with that of the full protein-binding, transcriptionally-active SM α-actin promoter TGTTTATCCCCA element. The TGTTT-site in the Hepatitis B Virus enhancer does overlap with the recognition sequences of at least 2 other DNA binding factors. Analysis of subcomponents of the cis element containing the TGTT revealed that neither of the cis elements alone exhibited enhancer activity, strongly suggesting transcriptional regulation through combinatorial interaction of nuclear proteins.29

Interestingly, the TGTTTATCCCCA site in the rat SM α-actin promoter exhibits combinatorial interactions with the flanking E-boxes. Expression of the rat SM α-actin promoter/reporter construct is enhanced with mutation of the TGTTTATCCCCA element only in the presence of intact E-boxes. The established paradigm for E-box–mediated regulation of muscle specific gene expression involves E-box binding of heterodimers of a cell specific HLH (such as MyoD) and a ubiquitously expressed HLH (E-protein), resulting in transcriptional activation of cell specific genes. However, recent data suggests that HLH factors can control gene expression through combinatorial interactions with other non-HLH transcription factors. For example, muscle enhancer factor 2 potentiates the transcriptional activity of the myogenic HLH factors,30 and the intermediate Ets-1 binding site in the immunoglobulin µ Heavy-Chain enhancer is required for transcriptional synergy of the flanking E-boxes.31 Our data demonstrating E-box–dependent activity of the TGTTTATC-CCCA further extends this observation. Moreover, these data provide the first evidence for E-box–dependent combinatorial interactions regulating a SM selective gene in SMC, although the specific mechanisms whereby the TGTTTATCCCA sequence interacts with the E-boxes are unclear. Of interest, in the present studies, the activity of the E-boxes was only “unveiled” in the context of mutation of the repressor element. In contrast, in separate studies in separate SMC lines at earlier passage number, we observed a modest decreases in promoter activity with specific E-box mutations in the absence of mutation of the TGTTTATCCCCA element.23 Although the exact reasons for these modest differences are unclear, one possibility is that alteration in the stoichiometry of the factors that interact with the E-boxes and TGTTTATCCCCA change with phenotypic modulation in culture. Consistent with this, cells at high passage showed greater repressor activity. However, it is interesting to speculate that such differences may be important in modifying the level of expression of SM α-actin in vivo under conditions that lead to SMC phenotypic modulation such as in response to vascular injury.

Results of our DNAse I footprint analysis failed to detect nuclear proteins binding to the E-boxes flanking the TGTTTATCCCCA in either the wild-type or mutated fragment.

![Figure 5. Transient transfection analysis of the pGL271 constructs containing either the wild-type SM α-actin promoter (pGL271WT) or mutations of the E-boxes flanking the TGTTTATCCCCA sequence with (pGL271EbmMut-2) or without (pGL271EbmWT) mutations of the TGTTTATCCCCA core region (A). Constructs were transiently transfected into rat aortic SMC. Cells were harvested on day 3 and assayed for luciferase activity (B). Protein determination was used to normalize for protein harvest variations. Results are representative of three independent experiments. Differences in luciferase activity between the wild-type pGL271 and plasmid construct pGL271 Mut-2, pGL271 Mut-2 and pGL271EbmWT, and pGL271EbmWT and pGL271EbmMut-2 were all statistically significant using a 2-way variance analysis (P<0.05).](http://atvb.ahajournals.org/content/full/9/10/2597/F5.large.jpg)
However, we cannot rule out the possibility that bHLH factors bind these E-boxes under other conditions. Indeed, separate studies by Johnson and Owens using EMSA demonstrated that 2 cMyc-related bHLH-leucine zipper proteins (USF-1 and USF-2) bound to the E-box 3′ to the TGGTTATCCCCA element. Additionally, overexpression of USF-1 and USF-2 enhanced SM α-actin transcription of p271CAT 2.5- to 3-fold. Johnson and Owens further demonstrated that factors from rat aortic SMC nuclear extract also bound the E-box 5′ to the TGGTTATCCCCA; however, the identity of this binding factor is unknown. Interestingly, these EMSA studies were performed using oligonucleotides containing the specific E-box sequences with 6 bp on the 5′ end and 9 bp on the 3′ end of both E-boxes. Of note, these promoter fragments do not contain the TGGTTATCCCCA element. It is possible that differences in E-box binding observed in our DNase footprint assay and Johnson and Owen’s EMSA could be secondary to differences in experimental conditions. Alternatively, SMC nuclear proteins may bind both cis elements in the cellular context and the interaction may be regulated by chromatin structure, phosphorylation, or other posttranslational modifications. It is also possible that mutation of the E-boxes alters the secondary structure of the DNA affecting the stability of the adjacent TGGTTATCCCCA protein complex.

In summary, results of the present study provide evidence for a novel cis-acting element in the rat SM α-actin promoter (TGGTTATCCCCA). Additionally, we demonstrate that this novel element regulates rat SM α-actin gene transcription through combinatorial interaction with the adjacent E-boxes. It is clear that different cis- and trans-acting factors regulate SM α-actin promoter activity in different species. As rat models are commonly used to study the role of SMC in vascular lesion formation and SM α-actin expression is decreased during vascular lesion formation, identification of the TGGTTATCCCCA-binding factors and their specific interaction with the adjacent E-boxes may provide important insight into the molecular regulation of the SMC phenotypic modulation in vascular disease.

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References


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