Smooth Muscle α-Actin Gene Requires Two E-Boxes for Proper Expression In Vivo and Is a Target of Class I Basic Helix-Loop-Helix Proteins

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Abstract—Changes in the differentiated state of smooth muscle cells (SMCs) play a key role in vascular diseases, yet the mechanisms controlling SMC differentiation are still largely undefined. We addressed the role of basic helix-loop-helix (bHLH) proteins in SMC differentiation by first determining the role of two E-box (CAAnnTG) motifs, binding sites for bHLH proteins, in the transcriptional regulation of the SMC differentiation marker gene, smooth muscle α-actin (SM α-actin), in vivo. Mutation of one or both E-boxes significantly reduced the expression of a −2560- to 2784-bp SM α-actin promoter/LacZ reporter gene in vivo in transgenic mice. We then determined the potential role of class I bHLH proteins, E12, E47, HEB, and E2-2, in SM α-actin regulation. In cotransfection experiments, E12, HEB, and E2-2 activated the SM α-actin promoter. Activation by HEB and E2-2 was synergistic with serum response factor. Additionally, the dominant-negative/inhibitory HLH proteins, Id2, Id3, and Twist, inhibited both the E12 and serum response factor–induced activations of the SM α-actin promoter. Finally, we demonstrated that E2A proteins (E12/E47) specifically bound the E-box–containing region of the SM α-actin promoter in vivo in the context of intact chromatin in SMCs. Taken together, these results provide the first evidence of E-box–dependent regulation of a SMC differentiation marker gene in vivo in transgenic mice. Moreover, they demonstrate a potential role for class I bHLH factors and their inhibitors, Id and Twist, in SM α-actin regulation and suggest that these factors may play an important role in control of SMC differentiation and phenotypic modulation. (Circ Res. 2003;92:840-847.)

Key Words: smooth muscle α-actin ■ transgenic mice ■ E-box ■ basic helix-loop-helix protein

A key feature of atherosclerosis, restenosis, and hypertension is abnormal growth and proliferation of vascular smooth muscle cells (SMCs) that are normally quiescent and highly specialized for contraction in mature animals (reviewed in Owens1). Despite the importance of such changes in the differentiation state of SMCs in vascular disease, the molecular mechanisms controlling SMC differentiation are still poorly understood.

Smooth muscle (SM) α-actin is a useful gene for studying transcriptional regulation of the SMC differentiation program. SM α-actin is the first SMC differentiation marker to appear during development,1 and although it is transiently expressed in cardiac and skeletal muscle during development and in myofibroblasts during wound healing,1 its expression is primarily restricted to SMCs in adult animals.

Previous studies in our laboratory have identified cis-regulatory elements in the SM α-actin promoter (including CArG or CArG-like motifs and a transforming growth factor-β control element) that are important for expression in SMCs.2 Although we and others have shown that serum response factor (SRF) binds to CArG boxes and plays an important role in SMC marker gene expression,2 a key unresolved issue is whether SMC differentiation is regulated by members of the basic helix-loop-helix (bHLH) family of proteins shown to be important in the control of cell-specific gene expression and differentiation of a wide variety of cell types.3

bHLH proteins bind as homodimers or heterodimers to E-box elements (CAAnnTG motifs) and have been categorized into several “classes” based on tissue distribution, dimerization capabilities, and DNA binding specificities (reviewed in Massari and Murre3). The general paradigm of HLH function is that a ubiquitously expressed class I bHLH factor, which includes E12, E47, HEB, and E2-2, dimerizes with a tissue-specific class II factor, such as MyoD (skeletal muscle) or NeuroD (neurons), to regulate cell-specific gene transcription. Another class of HLH proteins, exemplified by the Id proteins (Id1 through Id4), lacks the basic (DNA binding) domain and functions as naturally occurring dominant-negative inhibitors of, primarily, class I bHLH factors by forming nonfunctional heterodimers with these factors.3 In addition, the class II bHLH protein, Twist, has been shown to
inhibitors, Id and Twist, in the regulation of the SM
specific gene regulation. 

Drosophila melanogaster coding sequence fused to the
heavy chain, SM-22α, SM-22β and SM-22δ actin promoters in an E1-dependent (but not E2-dependent) manner. 9 A
function as an inhibitor by several mechanisms, including titration of class I factors (similar to Id). 3

Although E-boxes have been found in the promoters of many SM-specific or selective genes, including SM myosin heavy chain, SM-22α, aortic preferentially expressed gene-1,6,7 and SM α-actin, 8,9 very little is known about their specific binding factors and their precise role in SMC-specific gene regulation.

We have previously shown that two E-boxes in the SM α-actin promoter at −214 bp (E1) and −252 bp (E2). The mutated base pairs in each E-box are underlined. The reporter consisted of a β-galactosidase (LacZ) coding sequence fused to the Droso phila melanogaster alcohol dehydrogenase translation initiation region (ADH-S AUG).

The goals of the present study were as follows: (1) to determine the role of the SM α-actin E1 and E2 E-boxes in the transcriptional regulation of the −2560- to 2784-bp SM α-actin promoter in vivo in transgenic mice and (2) to determine the potential role of class I bHLH factors and their inhibitors, Id and Twist, in the regulation of the SM α-actin promoter.

Materials and Methods

Construction of Rat SM α-Actin Promoter/LacZ Reporters

E-box mutant constructs were made, by polymerase chain reaction (PCR)-based site-directed mutagenesis strategies, in the context of the −2560- to 2784-bp SM α-actin promoter/intron LacZ transgene (described in a study by Mack and Owens, 10 in which it was referred to as p2600Int/LacZ). Mutated sequences for each construct are shown in Figure 1.

Generation and Analysis of Transgenic Mice

Transgenic mice were generated using standard methods within the Transgenic Core Facility at the University of Virginia, Charlottesville. Transgene presence and LacZ expression were analyzed as described previously. 10

Transient Transfections, Reporter Gene Assays, and DNA Constructs

BALBc 3T3 cells were obtained from the Tissue Culture Core Facility at the University of Virginia, Charlottesville. Cells were seeded at 3 × 10⁴ cells/cm² into 6-well or 12-well plates the day before transfection and were transiently transfected (in triplicate) using Superfect transfection reagent (Qiagen) according to the manufacturer’s protocol. Cell extracts were prepared ~48 hours after transfection using Passive Lysis Buffer (Promega), and β-galactosidase activity was measured using the Galacto-Star System (Applied Biosystems). β-Galactosidase activities were normalized to total protein (Coomassie Plus Protein Assay Reagent, Pierce). Transfection experiments were repeated at least three times, and the relative β-galactosidase activity data are expressed as mean ± SD.

Expression plasmids for human E12 and E47 cloned into pHBAPr-1-neo (pBABP) and human HEB cloned into pXS were a generous gift from Dr C. Murre (Department of Biology, University of California, San Diego). Human E2-2 cDNA was a generous gift from Dr T. Kadesch (Department of Genetics, University of Pennsylvania School of Medicine, Philadelphia) and was subcloned into pXS. Empty pBABP and pXS vectors were used as controls in transfection experiments. Expression plasmids for rat Id2 and Id3 in pKc/RSV (pRSV) were a generous gift from Dr C.A. McNamara (Department of Medicine, Cardiovascular Division, University of Virginia, Charlottesville). Expression plasmid for Twist in pCS2 MT was a generous gift from Dr D. Spicer (MMC Research Institute, Scarborough, Maine). Empty pRSV and pCS2 MT vectors were used as controls in the Id and Twist cotransfection experiments, respectively.

Chromatin Immunoprecipitation

Rat aortic SMCs were plated at 1.0 × 10⁶ cells/cm². Three days after plating, chromatin immunoprecipitation (ChIP) assays were performed as described previously. 11 Chromatin samples were immunoprecipitated with no antibody, anti-SRF antibody (Santa Cruz Biotechnology, Inc), anti–dimethyl histone H3 (K9) antibody (gift from Dr C. David Allis, Department of Biochemistry, University of Virginia, Charlottesville), or anti-E2A antibody (reactive with E12 and E47, Santa Cruz Biotechnology, Inc).

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Double E-Box Mutation in SM α-Actin Promoter Virtually Abolished Expression In Vivo in Transgenic Mice

To determine the role of the two SM α-actin E-boxes at −214 bp (E1) and −252 bp (E2) in vivo, we used transgenic mouse technology. We have previously shown that −2560 to 2784 bp of the SM α-actin promoter is sufficient to drive the expression of a LacZ reporter in all three muscle subtypes during development and in the SMCs of adult mice in a manner similar to that of the endogenous gene. 10 Aggressive 4-bp mutations of E1 and E2, designed to disrupt binding by bHLH factors, were created in the context of this transgene construct (Figure 1), which was then used to generate transgenic founder lines (E1E2dM/LacZ). Analysis of 13.5-day embryos carrying the E1E2dM/LacZ transgene revealed virtually no staining in all three muscle subtypes in three of three independent founder lines examined at this developmental stage (Figure 2A), demonstrating that these E-boxes are...
critical for the developmental regulation of the SM \( \alpha \)-actin promoter in vivo. Analysis of adult tissues from E1E2dM/LacZ mice revealed a dramatic reduction in LacZ expression, with little or no staining seen in 6 of 7 independent founder lines (Figure 2B). The remaining line showed some heterogeneous expression of LacZ in SMC-rich tissues. Given the low frequency of this staining pattern (1 of 7 lines), we believe that it is likely due to insertional/locus effects. Of major significance, results showing the reduction or absence of LacZ staining in all three muscle subtypes during development and in SMCs of adult mice seen across multiple independent founder lines clearly demonstrate that these E-boxes are required for the activity of the SM \( \alpha \)-actin promoter in vivo.

Mutation of Single E-Box, E1 or E2, in the SM \( \alpha \)-Actin Promoter Reduced but Did Not Abolish Expression In Vivo in Transgenic Mice
To determine whether one or both E-boxes are required for proper regulation of the SM \( \alpha \)-actin promoter in vivo, we generated transgenic mice carrying a 4-bp mutation of E1 alone (E1.4bpM/LacZ) or E2 alone (E2.4bpM/LacZ). Mutated base pairs are shown in Figure 1. In contrast to the double E1/E2 mutation (E1E2dM/LacZ), which resulted in nearly complete abolition of expression, mutation of E1 or E2 alone resulted in variable effects on expression patterns ranging, in general, from nearly complete loss of expression to only modestly reduced expression in these mice compared with mice carrying the wild-type (Wt) transgene in 6 and 8 independent founder lines for E1 and E2, respectively (Figures 2A and 2B). Taken together, these results clearly demonstrate a requirement for the SM \( \alpha \)-actin E-box elements for proper expression in vivo but suggest that E1 and E2 may perform somewhat redundant functions based on the persistence of at least some expression with the single E-box mutations.

Class I bHLH Proteins Differentially Activated SM \( \alpha \)-Actin Promoter
To determine whether class I bHLH proteins could activate the SM \( \alpha \)-actin promoter, we cotransfected the SM \( \alpha \)-actin/LacZ construct used in the transgenic studies into BALBc 3T3 cells with expression plasmids for E12, E47, HEB, or E2-2. Fibroblast cell systems such as 3T3 cells have been used extensively in studies of transcriptional regulation of muscle promoters\(^{12,13}\) because baseline promoter activity is generally low in these cells, and endogenous regulators (which could interfere with or mask the effect of transfected factors) are likely absent or expressed at low levels. In these cells, the SM \( \alpha \)-actin/LacZ construct alone (transfected with empty vector controls, pBAP or pXS) showed little or no activity above background. Interestingly, three of the four factors tested (E12, HEB, and E2-2) induced an \( \approx \)3- to 5-fold activation of SM \( \alpha \)-actin/LacZ above background levels, while failing to activate the promoterless/LacZ construct (Figure 3). These results demonstrate that several, but not all, class I bHLH proteins have the potential to activate the SM \( \alpha \)-actin promoter.
Activation of SM \(\alpha\)-Actin Promoter by E12 Was E-Box Dependent

To determine whether the E1 and/or E2 E-box in the SM \(\alpha\)-actin promoter mediates the activation by class I bHLH factors, we cotransfected the Wt or E-box mutant SM \(\alpha\)-actin reporters used in the transgenic studies with empty vector or E12 expression plasmid. We found that mutation of E1, E2, or both E-boxes significantly reduced the E12-induced activation (Figure 4), demonstrating a requirement for paired E-boxes in the response of the SM \(\alpha\)-actin promoter to E12. Unlike E12, however, HEB and E2-2 activated the Wt and E-box mutant promoters to similar degrees (data not shown), suggesting that these bHLH proteins are capable of being recruited to the promoter through binding to other \(\text{cis}\) elements or through protein-protein interactions.

Class I bHLH Proteins and SRF Synergistically Activated SM \(\alpha\)-Actin Promoter

Previous studies in skeletal muscle gene regulation have demonstrated the ability of myogenic bHLH factors to activate transcription synergistically with MEF2, a MADS box transcription factor like SRF.\(^{12}\) In addition, myogenic bHLH proteins have been shown to physically interact with SRF.\(^{14}\) Given these findings, combined with results of previous studies from our laboratory demonstrating that SM \(\alpha\)-actin transcription is CArG-SRF dependent,\(^{10,15,16}\) we hypothesized that class I bHLH factors may activate SM \(\alpha\)-actin transcription cooperatively with SRF. To test this, we cotransfected SM \(\alpha\)-actin/LacZ into BALBc 3T3 cells with expression plasmids for E12, E47, HEB, or E2-2 along with pCDNA3 (control) or an SRF expression plasmid. As shown previously, there was a modest activation of the promoter by E12, HEB, and E2-2 alone and a greater activation by SRF alone (Figure 5, lanes 1 to 8). Coexpression of E12 or E47 with SRF had little effect on the SRF-induced activation (Figure 5; compare lanes 9 and 10 with lane 7), whereas, interestingly, coexpression of HEB or E2-2 with SRF synergistically activated the promoter (Figure 5; compare lanes 11 and 12 with lanes 5, 6, and 8). Because of the relatively low transfection efficiencies in these experiments, it was not possible to quantify the relative level of expression of each class I bHLH protein. As such, it is possible that at least some of the differential effects seen are partly due to differences in the expression levels of the bHLH factors.

Because of the functional synergy observed between HEB or E2-2 and SRF, we investigated the possibility that HEB or E2-2 could enhance SRF binding or form a ternary complex with SRF. However, despite extensive electrophoretic mobility shift assays (EMSAs), we were unable to detect enhanced SRF binding or ternary complex formation on either a CArG box probe (see online Figure 1, available at http://www.circresaha.org) or an E-box probe (data not shown). Thus, although we cannot rule out the possibility that a physical interaction (perhaps weak or transient) exists between these factors, our results suggest that the mechanism of this synergy may be more complex or require additional cofactors/conditions that cannot be adequately recapitulated in in vitro assays.

Inhibitory HLH Proteins, Id and Twist, Negatively Regulated SM \(\alpha\)-Actin Promoter

To further explore the possible role of HLH proteins in the regulation of SM \(\alpha\)-actin transcription, we used Id proteins (naturally occurring dominant-negative inhibitors of class I
bHLH factors) and Twist, which has been shown to inhibit the activity of bHLH proteins through multiple mechanisms. The E12-induced activation of SM α-actin/LacZ was inhibitable by the coexpression of Id2 or Id3 (Figure 6A) and by Twist (Figure 6B). Similar results were seen when Id and Twist proteins were coexpressed with E2-2 (data not shown).

More interesting, however, was the effect of these proteins on the SRF-induced activation of SM α-actin/LacZ. Id2, Id3, and Twist inhibited the SRF-induced activation by ~60% (Figure 7). These results suggest that the activation of the SM α-actin promoter by SRF may be due, at least in part, to recruitment and interaction (direct or indirect) with endogenous class I bHLH factors and lend further support for a functional cooperation between SRF and class I bHLH factors in the regulation of SM α-actin transcription.

**E2A Proteins (E12/E47) and SRF Specifically Bound E-Box–Containing Region of SM α-Actin Promoter Within Intact Chromatin in Cultured SMCs**

The preceding transient transfection studies demonstrate the potential of class I bHLH proteins and SRF to activate the SM α-actin promoter and suggest that, at least for E12, this activation is dependent on binding to the E1 and E2 E-boxes. However, inasmuch as these studies were performed in 3T3 fibroblasts, it is possible that the results are more reflective of regulation in myofibroblasts than in SMCs. For this reason and because several studies have shown differences in transcriptional regulation between episomal reporter genes and chromosomal genes and also because we and others have demonstrated that protein binding to oligonucleotide probes in EMSAs does not necessarily correlate with binding in vivo in the context of intact chromatin, we wanted to determine whether E2A proteins and SRF were specifically bound to the endogenous SM α-actin promoter in SMCs. We addressed this question using ChIP assays.

Intact cultured SMCs were fixed with formaldehyde to cross-link protein/DNA complexes. The cross-linked chromatin was sheared and immunoprecipitated with antibodies to SRF, E2A (E12/E47), or (as a control) dimethyl histone H3 (H3K9), a ubiquitous histone H3 modification that has been shown to be enriched in silenced chromatin. The precipitated chromosomal DNA was purified and subjected to PCR for detection of target sequences. Although there was some association of H3K9 with the SM α-actin 5′ E-box–containing region, of major significance, this region was highly enriched in the SRF and E2A immunoprecipitation samples (Figure 8). The association of SRF with the E-box–containing region may be due to direct or indirect interaction of SRF with E-box–bound bHLH factors or to the close proximity of CArG elements (SRF binding sites) to the E-boxes combined with the inherent limited resolution of ChIP assays (~200 to 800 bp). The association of SRF and E2A with the SM α-actin promoter was highly specific, inasmuch as the skeletal α-actin and insulin 5′ E-box–containing regions were enriched only in the H3K9 samples and not in the SRF and E2A samples (Figure 8), consistent with these genes being silenced in SMCs. The association of SRF and E2A with the SM α-actin, but not skeletal α-actin or insulin, promoter in multiple independent experiments provides powerful evidence of the specificity with which these factors can discriminate between SMC-specific and non–SMC-specific genes in vivo.

**Discussion**

Since the discovery in 1989 that MyoD, a bHLH protein, functions as a master regulatory gene for skeletal myogenesis, there has been intense interest in identifying similar master regulatory bHLH proteins in other cells. Indeed, class II bHLH proteins critical for the differentiation of numerous cell types, including pancreatic beta cells and neurons (BAT2/NeuroD), hematopoietic cells (SCL/Tal1), and cardiomyocytes (dHAND/eHAND), have been described. Yet, despite more than a decade of extensive search by our laboratory and others, no SMC-specific master regulatory bHLH protein has been identified. Although three class II bHLH factors (dHAND/Thing2, eHAND/Thing1, and epicardin) have each been found in at least a subset of SMCs in vivo, their role in SMC differentiation is still

**Figure 5.** Effect of the class I bHLH proteins, E12, E47, HEB, and E2-2, alone or in the presence of SRF, on the activity of the SM α-actin promoter in transient transfection assays in BALBc 3T3 cells. Galactosidase activity (normalized to total protein) is expressed as mean ± SD. The baseline activity of SM α-actin/LacZ cotransfected with empty vectors, pCDNA3 and pBAP or pXS (lanes 1 and 2), was set to 1.
In the present study, we addressed the question of whether bHLH proteins are involved in SM differentiation by first determining the role of two highly conserved E-boxes (binding sites for bHLH proteins) in the transcriptional regulation of the SMC differentiation marker gene, SMα/α-H9251-actin, in vivo. We then determined the potential role of class I bHLH proteins (the dimerization partners for factors such as MyoD and BETA2/NeuroD) in SMα/α-H9251-actin regulation.

Of major significance, our results are the first to demonstrate E-box–mediated regulation of a SMC differentiation marker gene in vivo in transgenic mice. Moreover, the following results of our studies provide strong evidence in support of a role for class I bHLH factors in this regulation:

1. E12, HEB, and E2-2 were sufficient to activate the SMα/α-H9251-actin promoter in 3T3 cells, and the activation by E12 was E-box dependent;
2. HEB and E2-2 activated the SMα/α-H9251-actin promoter synergistically with SRF;
3. The dominant-negative HLH proteins, Id2 and Id3, as well as the inhibitory bHLH protein, Twist, inhibited the E12 and SRF-induced activations of the SMα/α-H9251-actin promoter; and

The inhibition of the SMα/α-actin promoter by Id and Twist proteins is particularly interesting. Id2 has been found to be upregulated in the neointima after vascular injury,26 at a time when SMC differentiation markers such as SMα/α-actin are downregulated.27–29 In addition, preliminary results suggest that Twist expression is also upregulated in the neointima after vascular injury (D. Spicer, unpublished data, 2000). Although Id and Twist proteins have been implicated in cellular proliferation,26,30,31 this is the first report, to our knowledge, that these inhibitory HLH factors could negatively regulate a SMC differentiation marker gene and thus possibly provide a link between SMC proliferation and dedifferentiation in conditions such as vascular injury.

Results of the present study showed that simultaneous mutation of E1 and E2 resulted in nearly complete loss of SMα/α-actin promoter activity in vivo, whereas mutation of E1 or E2 alone resulted in only partial loss of activity/variable effects. Interestingly, comparison of the SMα/α-actin promoter across species reveals that the E2 E-box is 100% conserved across mammalian rat, mouse, and human promoters but does not
not exist in the chicken. The E1 E-box, on the other hand, is conserved in the rat and chicken but contains a 1-bp mismatch in the 5′ half site of the mouse and human promoters. From an evolutionary perspective, this suggests that the E1 E-box can function as an “imperfect” E-box (ie, it can tolerate some degeneracy in the 5′ half site). Alternatively, perhaps only one intact E-box (E1 in the case of the chicken or E2 in the case of the mouse and human) is required for proper regulation of the SM α-actin promoter; ie, the rat promoter used in these studies may be somewhat unique in that it contains two intact E-boxes, such that mutation of one E-box is partially compensated by the presence of the other. Further analysis of the role of these E-boxes in the expression of SM α-actin in various species will help distinguish between these possibilities.

The present has implicated a role for class I bHLH proteins in the regulation of the SM α-actin promoter. Given the general paradigm of tissue-specific gene regulation by bHLH proteins, it is logical to speculate that a SMC-specific/selective class II bHLH protein (or proteins) exists that would be capable of activating the SM α-actin promoter through heterodimerization with class I bHLH proteins. However, when we tested the three class II bHLH proteins shown to be expressed in SMCs to date (dHAND, eHAND, and capsulin), either alone or in the presence of E12, these factors did not activate the promoter (data not shown). However, because cell culture studies cannot recapitulate the environmental cues that occur in vivo, it is quite possible that these or other class II bHLH factors do positively regulate SM α-actin expression in vivo. Future studies using SMC-specific knock-outs of dHAND, eHAND, or capsulin in vivo will be necessary to determine the precise role of these factors in SMC differentiation. In addition, novel methods and screens are needed to determine whether additional tissue-specific class II bHLH proteins exist in SMCs. Finally, one must consider the alternative possibility that the role of class I bHLH proteins in SMC gene expression is similar to the paradigm observed in B lymphocytes in which B-cell–specific gene expression is thought to be regulated in large part by homodimers/heterodimers of various class I bHLH proteins.

Given the central role that SRF has been shown to play in the regulation of most (if not all) SM-specific promoters and the extensive evidence that myogenic bHLH proteins syner-gize with MEF2 (a MADS box transcription factor like SRF) to activate skeletal muscle–specific genes, it is interesting that we found synergistic activation of the SM α-actin promoter with HEB or E2-2 and SRF. In addition, Id proteins (naturally occurring dominant-negative inhibitors of class I bHLH factors) inhibited the SRF-induced activation of the SM α-actin promoter. The possibility that Id directly inhibits SRF was considered unlikely, inasmuch as Groisman et al showed that SRF does not interact with Id in a GST pull-down assay. However, of interest, these authors showed that SRF did interact with heterodimers of E12 and the class II bHLH protein, myogenin, but not isolated E12 or myo-genin in a GST pull-down assay and demonstrated a physical interaction between myogenin, E12, and SRF using a triple-hybrid approach in yeast. Using EMSAs, we investigated the possibility that HEB or E2-2 interacted with SRF (through the formation of a stable ternary complex on a CArG probe or E-box probe) or enhanced SRF binding to a CArG probe. We could not detect enhanced SRF binding or ternary complex formation by HEB or E2-2 (online Figure 1 and data not shown). Although we cannot rule out that a physical interaction (perhaps weak or transient) exists between these factors, it is possible that the mechanism of the synergy is more complex and may involve factors such as modulation of SRF binding in the context of chromatin and/or modulation of SRF expression/activity. Alternatively, simultaneous binding of HEB or E2-2 and SRF to the promoter may require a class II bHLH (as observed with E12/myogenin12) and/or induce a conformational change in the DNA that allows for binding of additional factors or for the formation of specific higher-order protein complexes.

In summary, these results provide the first evidence of E-box–dependent regulation of an SMC differentiation marker gene in vivo in transgenic mice. Moreover, they demonstrate a potential role for class I bHLH factors and their inhibitors, Id and Twist, in SM α-actin regulation. Although we focused on SM α-actin in the present study, most SMC-specific promoters contain closely spaced CArG boxes and E-boxes. Thus, it is interesting to postulate that interactions between class I bHLH proteins, their inhibitors, and SRF may play a general role in control of expression of multiple SMC genes during differentiation and phenotypic modulation.

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EXPANDED MATERIALS AND METHODS SECTION

Construction of Rat SM α−actin promoter/LacZ Reporters

E-box mutant constructs were made in the context of the -2560 to +2784 bp SM α−actin promoter/intron LacZ transgene (described in Mack and Owens 1999 where it was referred to as p2600Int/LacZ). This construct is often referred to as Wt (for wild type) or simply, SM α−actin/LacZ in this article. E-box mutations were generated using PCR based site directed mutagenesis strategies on small fragments of the promoter which were then subcloned back into the parental SM α−actin/LacZ plasmid. The subcloned fragments were sequenced to verify integrity. Mutated sequences for each construct are shown in Figure 1.

Generation and Analysis of Transgenic Mice

All constructs were prepared for transgenic injection as described previously. Transgenic mice were generated using standard methods within the Transgenic Core Facility at the University of Virginia (Charlottesville, VA). Transgenic mice were used to establish breeding founder lines. Transgene presence and LacZ expression were analyzed as described previously. Mice were euthanized by IP injection of pentobarbital (100 mg/kg) or CO2 asphyxiation. All animal procedures used in these studies were reviewed and approved by the University of Virginia Animal Use and Care Committee.

Transient Transfections, Reporter Gene Assays, and DNA constructs

BALBc 3T3 cells were obtained from the Tissue Culture Core Facility at the University of Virginia (Charlottesville, VA) and were maintained in Dulbecco’s Modified Eagle’s Medium with high glucose (Life Technologies) supplemented with 10% fetal
bovine serum (Life Technologies), 0.075% sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 200 µg/ml L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies). Cells were seeded at 3 x 10^4 cells/cm^2 into 6-well or 12-well plates the day before transfection and were transiently transfected (in triplicate) using Superfect transfection reagent (QIAGEN) according to the manufacturer's protocol. Cell extracts were prepared ~48 hrs after transfection using Passive Lysis Buffer (Promega), and β-galactosidase activity was measured using the Galacto-Star System (Applied Biosystems). β-galactosidase activities were normalized to total protein (Coomassie Plus Protein Assay Reagent, Pierce). Transfection experiments were repeated at least three times, and the relative β-galactosidase activity data are expressed as mean ± S.D.

Expression plasmids for human E12 and E47 cloned into pHBAPr-1-neo (pBAP) and human HEB cloned into pXS were a generous gift from Dr. C. Murre (Dept. of Biology, University of California, San Diego, CA). Human E2-2 cDNA was a generous gift from Dr. T. Kadesch (Dept. of Genetics, U. Penn School of Medicine, Philadelphia, PA) and was subcloned into pXS. Empty pBAP and pXS vectors were used as controls in transfection experiments. Expression plasmids for rat Id2 and Id3 in pRc/RSV (pRSV) were a generous gift from Dr. C.A. McNamara (Dept. of Medicine, Cardiovascular Division, Univ. of Virginia, Charlottesville, VA). Expression plasmid for Twist in pCS2MT was a generous gift from Dr. D. Spicer (MMC Research Institute, Scarborough, ME). Empty pRSV and pCS2MT vectors were used as controls in the Id and Twist co-transfection experiments, respectively.
**Chromatin Immunoprecipitation**

Rat aortic SMC, cultured in DMEM + F12 (Life Technologies) supplemented with 10% fetal bovine serum (Hyclone), 200 µg/ml L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies), were plated at 1.0 X 10⁴ cells/cm². Three days after plating, chromatin immunoprecipitation (ChIP) assays were performed as described previously². Chromatin samples were immunoprecipitated with no antibody, anti-SRF antibody (Santa Cruz Biotechnology, Inc.), anti-dimethyl histone H3 (K9) antibody (gift from Dr. C. David Allis, Dept. of Biochemistry, Univ. of Virginia, Charlottesville, VA), or anti-E2A antibody (reactive with E12 and E47; Santa Cruz Biotechnology, Inc.). Immunoprecipitated chromatin samples were reverse-crosslinked, purified, and subjected to PCR amplification using primers specific to E-box containing promoter regions of SM α-actin, skeletal α-actin, and insulin. The supernatant of the no antibody immunoprecipitation reaction was used as total input DNA. The sequences of the PCR primers were as follows: *SM α-actin E-box*: 5’-GCATCTTCTGAGGAATGTG-3’, 5’-GGTCTCTTCCACTGCATTC-3’; *skeletal α-actin E-box*: 5’-AGCAGCCTGGGAGCCAGTTG-3’, 5’-TCCCTTCGGCTGCTTCTCAG-3’; *insulin E-box*: 5’-GCCAAAAACTCTAGGGACTTTAGGAAGGATG-3’, 5’-GCCGGGCAACCTCCAGTGCCAAGGTCTGAAGATC-3’. PCR cycle numbers were as follows: *SM α-actin E-box*: 30 cycles, *skeletal α-actin E-box*: 31 cycles, and *insulin E-box*: 35 cycles.

**Electrophoretic Mobility Shift Assays**

Electrophoretic mobility shift assays were performed as previously described with minor modifications³. Briefly, oligonucleotides were purchased commercially (MWG
Biotech) and included the following (only the sense strand is shown): \(C\text{ArGB}, 5\'-G\text{AGGTCCCTATATGGTTGTG-3'}; C\text{ArGBmut, 5\'-GAGGTA}\text{ACTATATAATTGTG-3'}; E2, 5\'-A\text{T}\text{T}\text{TGAGCAGTTGTTCTGAG-3'}\). Complimentary oligonucleotides were annealed and the double-stranded duplexes were end-labeled with T4 polynucleotide kinase (New England Biolabs) and \([\gamma^{32}\text{P}]\text{ATP}\) (Perkin Elmer Life Sciences, Inc). Unincorporated nucleotides were removed using the QIAquick Nucleotide Removal Kit (Qiagen). SRF, E2-2, and HEB were synthesized using the TNT Coupled Reticulocyte Lysate System (Promega Corporation). EMSAs were performed with 20 \(\mu\)l of binding reaction that contained double-stranded, end-labeled CArGB probe (~50,000cpm), double-stranded, cold E2 oligonucleotides (~0.5pmol), 10% glycerol, 10 mM HEPES (pH 7.9), 4 mM Tris-HCl (pH7.9), 80 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 300 \(\mu\text{g/ml bovine serum albumin, 4 \(\mu\text{g poly(dI-dC) \cdot poly(dI-dC)}\)}, 2 \(\mu\text{l of in vitro synthesized SRF and/or 2 \(\mu\text{l of E2-2 or HEB, and cold competitor (~100x molar excess) or antibody (2 \(\mu\text{g}) where indicated. Antibodies for supershifts were purchased commercially (SRF, HEB, and Sp1 (control Ab), Santa Cruz Biotechnology, Inc; E12/E2-2, PharMingen). Plasmids for in vitro synthesis of E2-2 and HEB were a generous gift of Dr. S. Tapscott (Fred Hutchinson Caner Research Center, Seattle, WA).} \)
Supplemental Figure 1 Legend:

Radiolabeled CArGB probe was incubated with 2 µl of in vitro synthesized SRF and/or 2 µl of E2-2 or HEB. In addition, cold E2 probe was included in the binding reaction in the case that both DNA binding sites were needed for ternary complex formation. Specificity of SRF binding was determined by the addition of cold wild type CArGB oligonucleotide competitor (WT CArG, ~100x molar excess) and cold mutant CArGB competitor (Mut. CArG, ~100x molar excess). SRF bands were supershifted by the addition of 2 µg of anti-SRF rabbit polyclonal antibody. A rabbit polyclonal SP1 control antibody (Cont.) failed to supershift the SRF-containing complex. There was no evidence of enhanced SRF binding or ternary complex formation with either of the Class I bHLH factors tested.
References

