The Smooth Muscle Myosin Heavy Chain Gene Exhibits Smooth Muscle Subtype-selective Modular Regulation in Vivo*

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Received for publication, June 12, 2001
Published, JBC Papers in Press, August 6, 2001, DOI 10.1074/jbc.M105402200

Previous studies in our laboratory demonstrated that the transgene consisting of the −4.2 to +11.6 kilobase (kb) region of the smooth muscle (SM) myosin heavy chain (MHC) gene was expressed in virtually all SM tissue types in vivo in transgenic mice and that the multiple CArG elements within this region were differentially required in SM subtypes, implying that the SM-MHC gene was controlled by multiple transcriptional regulatory modules. To investigate this hypothesis, we analyzed specific regulatory regions within the SM-MHC −4.2 to +11.6 kb region by a combination of deletion analyses of various SM-MHC transgenes as well as by DNaseI hypersensitivity assays and in vivo footprinting in intact SM tissues. The results showed that SM-MHC transgene expression depended on a large number of required regulatory modules that were widely spread over the −4.2 to +11.6 region. Moreover, the results revealed several unexpected novel features of regulation of the SM-MHC gene including: 1) unique combinations of regulatory modules were required for SM-MHC expression in different SM-subtypes; 2) repressor modules as well as activator modules were both critical for SMC specificity of the gene; 3) certain modules were required in certain contexts but were dispensable in others within a given SMC-subtype (i.e. the net activity of the module was determined by interaction between modules not simply by the sum of module activities); and 4) we identified a highly conserved 200-base pair transcriptional regulatory module at +8 kb that was required in the large arteries but dispensable in the coronary arteries and airways in transgenic mice and contained multiple potential cis-elements that were occupied by nuclear proteins in the intact aorta based on in vivo footprinting. Taken together, the results suggest a model of complex modular control of expression of the SM-MHC gene that varies between SMC subtypes. Moreover, the studies establish the possibility of designing derivatives of the SM-MHC promoter that might be used for targeting gene expression to specific SMC subtypes in vivo.

Smooth muscle (SM) myosin heavy chain (MHC) is one of the best markers for smooth muscle cell (SMC) lineages (1). Its expression is regulated precisely and dynamically during SMC differentiation and also during the formation and development of vascular diseases such as atherosclerosis (2, 3). Therefore, studies on the mechanisms that regulate SM-MHC gene expression would be of great importance not only for understanding of transcriptional regulatory mechanisms in SMC differentiation but also for the understanding of the mechanisms of phenotypic modulation of SMCs in vascular diseases.

We previously demonstrated that the SM-MHC genomic region from −4.2 to +11.6 kb was capable of driving LacZ transgene expression in virtually all SMC subtypes in transgenic mice in vivo (4). In contrast, a LacZ transgene containing the −4.2 kb to +88 bp of the SM-MHC gene was not expressed in any SMCs in multiple transgenic lines, indicating an absolute requirement of the intronic sequence from +88 bp to +11.6 kb for SM-MHC expression in vivo. We have confirmed recently the rigorous SMC specificity of the SM-MHC −4.2 to +11.6 region in vivo using a Cre recombinase-activating reporter system in which transgenic mice carrying an SM-MHC −4.2 to +11.6 region Cre transgene were crossed to a floxed indicator line that showed Cre-induced activation of LacZ (5). Because this system provides an integral of promoter activity throughout development and maturation, it provides a very sensitive means of detecting even transient expression in non-SMCs. Transgene expression was tightly confined within the SMCs in this system, thus further demonstrating the SMC specificity of the SM-MHC −4.2 to +11.6 kb sequence (5).

As a first step to elucidate the transcriptional regulatory mechanisms of the SM-MHC gene, we previously analyzed the function of three conserved CArG elements within the 5′-flanking and first intronic sequences in transgenic mice (6). Results of these studies demonstrated that the three CArG elements were differentially required in SMC subtypes in vivo. For example, although CArG1 in the 5′-flanking sequence was required for all SMCs, the intronic CArG element was dispensable in all SMC subtypes other than the large arteries. These results implied that expression of the SM-MHC gene was regulated by multiple transcriptional regulatory regions including the 5′-flanking and intronic regions containing the CArG elements. However, these studies examined the function of individual cis-elements and did not define the precise regulatory regions within the −4.2 to +11.6 SM-MHC region required for SMC-specific gene expression in vivo. Similarly, the

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*This work was supported by National Institutes of Health Grants RO1HL75353 and RO1HL38854 (to G. K. O.) and U54HD28934 (to the University of Virginia Research Histology Core) and Fellowship Grant VA/F99255Y from the Virginia Affiliate of the American Heart Association (to I. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: SM, smooth muscle, MHC, myosin heavy chain; SMC, smooth muscle cell; kb, kilobase(s); bp, base pair(s); TK, thymidine kinase; DMS, dimethyl sulfate; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; GI, gastrointestinal; HS, DNaseI hypersensitive site; SRF, serum response factor; USF, upstream stimulatory factor.
previous studies showing that the proximal SM22α promoter was capable of driving transgene expression in arterial SMCs but not in venous or visceral SMCs suggest that this gene is controlled by multiple regulatory modules (7–9), the specific regulatory modules required for expression within SMC subtypes have not been identified.

To examine the hypothetical modular control mechanisms of the SMC-specific genes in vivo, we analyzed regions within −4.2 to +11.6 kb of the SM-MHC gene that were required for expression of the gene in vivo in transgenic mice. The results support a complex model in which the SM-MHC is controlled by multiple positive- and negative-acting regulatory modules that are widely distributed within the −4.2 to +11.6 kb sequence. Moreover, we present novel evidence showing that these regulatory modules function differentially in SMC subtypes. These results imply that the SM-MHC gene regulatory system consists of multiple modular regulatory domains that confer the capability for SMCs to respond to vastly divergent environmental cues in developmental space and time and under pathophysiological conditions.

MATERIALS AND METHODS

Plasmid Construction and Transfection—Deletion constructs of the −4.2/+11.6 LacZ were generated by restriction digestion and ligation. The integrity of the constructs was determined by restriction enzyme mapping and sequencing. The structure of the constructs is indicated schematically in Fig. 1. To generate the −1.3/−11.6 LacZ construct, a part of the 5′-flanking sequence was taken from the pCAT-1346 (10). For construction of the 3xHS7-TK LacZ plasmid, three copies of the Styl/StuI fragment (+8308 to +8327) containing the highly homologous sequence was subcloned in tandem 5′ of the minimal TK promoter in pTK LacZ (6).

The culture methods for rat aortic SMCs were described previously (10). For transfection, SMCs were plated at 20,000 cells/cm² in 6-well plates. DNA was transfected using Superfect (Qiagen) according to manufacturer recommendations on the next day of plating. The cells were harvested 72 h after transfection. β-Galactosidase assays were performed as described previously (6). To eliminate errors in the production of plasmids and to eliminate variability of plasmid quality, at least two independently prepared plasmid DNA samples of independent clones were transfected in duplicate.

Transgenic Mice—Transgenic mice were produced using standard procedures (4). These mice were used to establish breeding founder lines (F₀) or sacrificed for the analysis of reporter expression (transient transgenic lines). The analysis of transgenic mice was performed as described previously (4). All animal procedures used in these studies were approved by the University of Virginia Animal Use and Care Committee. Transient transgenic lines were harvested at embryonic day 18.5. Because of the relatively weak activity of the wild-type SM-MHC −4.2/+11.6 LacZ construct in many SMC tissues including large blood vessels within the embryo (4), LacZ expression patterns of various transgene constructs were mainly compared using 4–8-week-old F₁ mice from founder lines except as noted otherwise. Because of inherent variations in transgene expression among transgenic lines and minor variations in the preparations from the given line, multiple independent transgenic lines of each construct were examined for transgene expression (see Table I). A similar pattern of transgene expression was observed between independent founder transgenic lines.

DNaasel Hypersensitivity Assay—Rat-cultured SMCs were permeabilized and treated with DNaasel as described previously (11). In brief, confluent rat-cultured SMCs in 15-cm dishes were rinsed with PBS. PBS was replaced with 3 ml of permeabilizing buffer (15 mM Tris-HCl, pH 7.5, 60 mM KCl, 15 mM NaCl, 0.5 mM MgCl₂, 0.5 mM EGTA, 300 mM sucrose, and 0.5 mM β-mercaptoethanol) containing 0−3200 units/ml of DNaasel (Life Technologies, Inc.). Cells were permeabilized by adding 3 ml of permeabilizing buffer containing 0.12% lysosolcin (Sigma) and incubated for 6 min at room temperature. The cells were lysed by adding 1.2 ml of lysis buffer (312.5 mM Tris-HCl, pH 8.0, 125 mM EDTA, 6.25% SDS, and 1.25 mg/ml proteinase K). Samples were incubated at 37°C overnight. Genomic DNA was purified by phenol, phenol/chloroform and chloroform extractions followed by ethanol precipitation. DNA was digested with restriction enzymes as indicated. The probes for Southern hybridization were a BamHI/ApaI fragment (probe A, −3140 to −2120), SacI/XmnI fragment (probe B, +3980 to +4580), and KpnI/XbaI fragment (probe C, +5310 to +5880) of the SM-MHC gene.

In Vivo Footprinting—Dimethyl sulfate (DMS) treatment of intact tissues was described previously (12). Fat and matrices were removed from tissues prior to DMS treatment. The intact rat aorta was incubated in 5 ml of PBS containing 8 μl of DMS for 2 min at 37°C with agitation. Aortas and endothelial layers were removed from the aorta after DMS treatment. Genomic DNA was purified from the treated tissues and subjected to piperidine treatment as described previously (12). Ligation-mediated PCR was performed as described previously (12) with minor modifications. In brief, 2 μg of DNA samples was subjected to first primer extension from biotinylated gene-specific primer 1. A second primer containing a LA Taq buffer and a 11.6 kb DNA ligation buffer (New England Biolabs), 0.7 μl of 100 mM ATP, 50 pmol of the double-stranded linker oligonucleotide, and 2 μl of T4 DNA ligase (New England Biolabs), and the reactions were incubated at 16°C overnight. The linker oligonucleotide was produced by annealing 5′-GGCGTGACCCGGGAGATTCTGAATTCT-3′ and 5′-GAAATTCAGATC-3′ (13). A T nucleotide was added to the original sequence of the former oligo (13) to facilitate ligation of Taq amplified products. Primer extension products were purified using Dynabeads M-280 streptavidin (Dynal). Forty microliters of Dynabeads were washed once with 100 μl of 2× binding and washing buffer (20 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) using a magnetic separation stand (Promega). Beads were resuspended in 70 μl of 2× binding and washing buffer and then added to ligation reactions. The samples were mixed for 5 min at room temperature with constant rotation. Beads were washed two times with 100 μl of 2× binding and washing buffer (20 mM NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA). Beads were resuspended in 40 μl of 0.15 M NaOH and incubated for 10 min at 37°C. Beads were captured on a magnetic stand, and the supernatants were ethanol-precipitated using 250 μl of ethanol, 4 μl of 3 M sodium acetate, pH 5.2, and 2 μl of SeeDNA coprecipitant (Amersham Pharmacia Biotech). Precipitated DNA was washed twice with 70% ethanol and dissolved in 10 μl of distilled H₂O. Exponential amplification was done in 90 μl of reaction containing 1× LA Tq buffer, 200 nM of the gene specific primer 2, 200 nM linker primer, and 2.5 units of LA Tq. The PCR conditions were 94°C for 2 min and 15–18 cycles of 94°C for 30 s, Tₙ−5°C for 2 min; 76°C for 3 min and 1 cycle of 76°C for 10 min. Fifteen microliters of PCR products were labeled for visualization by adding 5 μl of labeling mixture containing 1× LA Tq buffer, 20 nt 32P end-labeled gene-specific primer 3, 1.5 μl each of dNTPs, and 1.25 units of LA Tq and incubated at 94°C for 2 min and 5–6 cycles of 94°C for 30 s, Tₙ−5°C for 2 min, 76°C for 3 min, and 1 cycle of 76°C for 10 min. PCR products were ethanol-precipitated and resolved on denaturing 6% Long Ranger (BMA) gels. Sequencing ladders produced from the gene specific primer 3 were run on the same gel as size markers (data not shown). The PCR primers for the antisense strand (forward primers) are: gene-specific primer 1, 5′-CAAAGGATCCGATGAGACGACGCGCACGTTGGCAGACT-3′; primer 2, 5′-CTTCTCAGTGAGGGAAGCTTGGACCCGATTTCTGTCGGG-3′; primer 3, 5′-GAGGAGGATGGCTGGGAGAGGCAGATGATG-3′; and the antisense strand (reverse primers) are: primer 1, 5′-AACATATGGATGAGACGCTTGGACCTGTCGGG-3′; primer 2, 5′-GAGGAGGATGGCTGGGAGAGGCAGATGATG-3′; and primer 3, 5′-CAGGAGGATGGCTGGGAGAGGCAGATGATG-3′. Electrophoretic Mobility Shift Assay (EMSA)—The methods for preparation of nuclear extracts and for performing EMSAs were described previously (6). In brief, 200 kcpm of 32P-labeled probe was incubated with nuclear extracts in 20 μl of binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM dithiothreitol, 10% glycerol, and 0.5% Nonidet P-40) and incubated with 0.25 μg of poly(dI-dC)-poly(dI-dC). Reactions were incubated on ice for 20 min. For supershift assays, 1 μl of specified antibody was added after the 20-min incubation period, and the reactions were incubated for an additional 10 min. The reactions were run on 5% polyacrylamide gels. Sequences of EMSA probes are: rat P3 probe, 5′-CTTCTC- CGTTTCTGAGGCGCAGA-3′; human P3, 5′-GCTTCTCTTTTCTCCAGA-3′; serum response element, 5′-AGAATGCCTAATAGTTGCA- CATCCT-3′; and P4, 5′-GACAACAACTTGTGACCCTGTGAC-3′.
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RESULTS

SM-MHC Expression Requires Additional Regulatory Regions in Addition to Known Regions within the 5’-Flanking Sequence—Results of our previous studies showed that expression of the SM-MHC gene in vivo depended on 1) the region from –4.2 to +11.6 kb and 2) three CARG elements located within two highly conserved regions of –200 bp in the 5’-flanking sequence at –1.3 kb and the first intronic sequence at +1.6 kb (6). On this basis, we hypothesized that the SM-MHC gene might be regulated by multiple transcriptional regulatory subregions including these two CARG-containing regions. The overall goal of the present studies was to test this hypothesis and to identify specific transcriptional regulatory regions required for SM-MHC expression in vivo. Given the very large size of the SM-MHC regulatory region (~16 kb), we employed multiple experimental methods and strategies to characterize regulatory regions including 1) DNaseI hypersensitive assays and in vivo footprinting to identify regions that may contain transcriptionally active elements based on evidence of binding of nuclear proteins, 2) identification of regions that are conserved across species, 3) testing various SM-MHC promoter constructs based on transient transfection studies in cultured SMCs, and 4) testing selected promoter constructs in transgenic mice. However, our major focus was on testing candidate regions in vivo in transgenic mice, because cultured SMCs are known to be phenotypically modulated, and we have shown previously that results obtained in cultured SMCs are often invalid for predicting the activity of promoter constructs in vivo (4, 14). Expression patterns of various SM-MHC transgenic constructs were examined mainly using 4–8-week-old F1 mice because of the late induction of SM-MHC –4.2/+11.6 LacZ transgenes during mouse development (4) and the inherent variability in transgenic expression observed in embryos that would confound direct comparisons of the activity of various test constructs.

To begin identification of specific regulatory regions required for SM-MHC expression in vivo, we first investigated whether subfragments containing known transcriptional regulatory regions were sufficient for transcription in vivo. We initially tested the sequence from –1342 bp to +11.6 kb (~1.3+/+11.6 LacZ, Construct 2 in Fig. 1), because the 1346 bp of the 5’-flanking sequence contains the entire conserved 227-bp domain (~1321 to ~1095) containing the CARG1 and CARG2 elements that we previously showed were required for expression within the context of the SM-MHC –4.2+/+11.6 LacZ transgene (6). Moreover, in reporter assays using cultured SMCs, the –1.3/+11.6 LacZ construct showed activity equivalent to that of the –4.2+/+11.6 LacZ (data not shown). In contrast, –1.3+/+11.6 LacZ transgenic mice clearly showed weaker LacZ expression in many SMC subtypes as compared with –4.2+/+11.6 LacZ transgenic mice (Fig. 2 and Table I). For example, the –1.3+/+11.6 LacZ construct showed no expression in vascular SMCs except very weak expression in several small arteries (Fig. 2, compare panels 2a with 1a). Relatively strong transgene expression was observed in the trachea and main trunks of bronchi (Figs. 2, 2b, and 3e), but no expression was detected in airway SMCs within the smaller branches of the bronchial tree. The transgene was expressed strongly in the GI tract and bladder (Fig. 2, 2c–2e), although expression was somewhat uneven with some cells staining very intensely, whereas others were very weakly stained. Of particular significance, unlike the wild-type –4.2/+11.6 LacZ transgene, which is highly SMC-specific, the expression of the –1.3+/+11.6 LacZ transgene was observed consistently in a fraction of cardiac muscle cells and various non-SMC mesenchymal cells (data not shown). These results thus clearly demonstrate that the –1.3 to +11.6 region was not sufficient for SMC-specific expression in vivo and the region from –4.2 to –1.3 contains regulatory sequences important for the expression in vascular and airway SMCs as well as elements required for restricting SM-MHC expression to SMCs.

The First 2.5-kb of the Intronic Sequence Containing the Intronic CARG Region Was Not Sufficient for SM-MHC Expression in Vivo—We next tested whether the sequence from –4200 to +2500 bp (~4.2+/+2.5 LacZ, Construct 3 in Fig. 1) was sufficient to drive SMC-specific expression in vivo. The 2500 bp of the first intronic sequence was chosen based on the fact that it contains the intronic CARG region (6) and on results of tran-
sient transfection assays in cultured SMCs showing that the activity of the −4.2/+11.6 LacZ construct was equivalent to that of the −4.2/+11.6 LacZ construct (6). Despite the strong reporter activity in cultured SMCs, the −4.2/+2.5 LacZ construct was not fully active in SMCs in vivo and showed differential reductions in transgene expression in several SMC subtypes in transgenic mice (Fig. 2, compare row 3 with row 1). For example, very weak expression was observed in vascular SMCs.

Fig. 2. Transgene expression in the SM-MHC deletion mutant transgenic mice. Transgene expression in transgenic mice carrying various SM-MHC LacZ transgene constructs. SM tissues were harvested from 4–8-week-old transgenic mice and stained for β-galactosidase activity. The heart and lung were cleared using benzyl benzoate/benzyl alcohol. Column a, frontal view of the heart; column b, the lung; column c, the stomach, esophagus, and duodenum; column d, the ileum; column e, the bladder. Representative tissues samples of multiple transgenic lines are shown (see “Materials and Methods”). Arrowheads indicate the aortas (column a).

Table I

Summary of reporter gene expression in SM-MHC LacZ transgenic mice

| No. | Construct | LacZ-positive lines/total lines (transient) | Aorta | Coronary artery | Mesenteric artery | Vena cava | Airways | Stomach | Intestine | Bladder | Expression in non-SMCs/positive lines |
|-----|-----------|-------------------------------------------|-------|----------------|-------------------|-----------|---------|---------|----------|---------|-------------------------------------|
| 1   | −4.2/+11.6 | 4/4 (1)                                   | ++    | ++             | ++                | ++        | ++      | ++      | ++       | ++      | 1/4                                 |
| 2   | −1.3/+11.6 | 2/4 (0)                                   | −      | −              | −                 | −         | ++      | ++      | ++       | ++      | 2/2                                 |
| 3   | −4.2/+2.5  | 3/8 (3)                                   | +/−   | −              | −                 | −         | −       | +       | +        | +       | 2/3                                 |
| 4   | −4.2/+5.3  | 8/10 (5)                                  | −      | +              | −                 | +/−      | −       | ++      | +        | +       | 0/8                                 |
| 5   | −4.2/+5.3;+7.5/+9 | 10/11 (8) | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | 0/10 |
| 6   | −4.2/+11.6 ΔHS7 | 3/3 (0) | − | + | ++ | ++ | ++| ++ | ++ | ++ | 0/3 |
| 7   | −4.2/+9    | 3/3 (1)                                   | +/−   | +/−          | +/−               | +/−      | ++      | ++      | ++       | ++      | 0/3                                 |
| 8   | −4.2/+2.5;+5.3/+11.6 | 4/4 (0) | ++ | +/− | ++ | ++ | ++ | ++ | ++ | ++ | 1/4 |
| 9   | 3xHS7 − TK LacZ | 0/3 (0) | − | − | − | − | − | − | − | − | 0/0 |
within the aorta (Figs. 2, 3a, and 3a). However, transgene expression in the airways was very strong and equivalent to that of the full-length transgenic lines (Fig. 2, compare 3b with 1b). The −4.2/+2.5 LacZ transgene was also expressed strongly in the GI tract. However, transgene expression in the GI tract differed among parts of the tissues (Figs. 2, 3d, and 3g) and did not show consistently strong staining as observed in −4.2/+11.6 LacZ transgenic mice. Strong transgene expression was also observed in cardiac and skeletal muscles (Figs. 2, 3a, and 3i). These data indicate that the SM-MHC −4.2 to +2.5 kb region is not sufficient for expression of the SM-MHC gene in vivo and that the region between +2.5 and +11.6 kb is required for expression in subsets of SMCs including vascular and bladder SMCs. Results also show that the region from +2.5 to +11.6 contains regulatory regions that suppress transgene expression in non-SMCs. Taken together, the results of our initial experiments show that SM-MHC expression in vivo requires regulatory modules located outside of the −1.3 to +2.5 kb region.

The Region from +2.5 to +5.3 Exhibited Repressor Activity in Transgenic Mice—Our initial results clearly demonstrated that the SM-MHC region from +2.5 to +11.6 contains regulatory regions required for SMC-specific expression of the SM-MHC gene in transgenic mice. Given the large size of this region and the relative lack of large regions that are highly conserved across species, we analyzed various S−deletion mutants of this region in cultured SMCs to identify possible regulatory regions that could be subsequently tested in transgenic mice. The results of transient transfection studies in cultured rat aortic SMCs showed that the construct containing the SM-MHC sequence from −4.2 to +5.3 kb (−4.2/+5.3 LacZ, Construct 4 in Fig. 1) had the strongest activity (65-fold activity over promot-erless pAUG LacZ versus 57-fold activity of the −4.2/+11.6 LacZ), suggesting that the region from +2.5 to +5.3 might contain additional positive regulatory regions required for SM-MHC expression. However, in contrast to observations in cultured SMCs, addition of the region from +2.5 to +5.3 resulted in a marked reduction in expression of the transgene as compared with that of the full-length −4.2/+11.6 LacZ transgenic lines or the shorter −4.2/+2.5 LacZ transgenic lines (Fig. 2, compares row 4 with rows 1 and 3). For example, multiple −4.2/+5.3 LacZ lines showed no transgene expression in vascular SMCs or airway SMCs (Figs. 2, 4a and 4b, and 3b). Although some transgene expression was detected in the GI tract and bladder, LacZ staining was limited to a small fraction of SMCs (Figs. 2, 4c–4e). As such, transgene expression in the SMCs of the −4.2/+5.3 lines was markedly weaker than that of −4.2/+11.6 LacZ and −4.2/+2.5 LacZ transgenic mouse lines. Interestingly, although expression of the −4.2/+2.5 LacZ transgene was observed in non-SM tissues, this was not the case with multiple −4.2/+5.3 kb LacZ lines. These results suggest that the +2.5 to +5.3 region contains a negative-acting regulatory region necessary for limiting SM-MHC expression within SMCs. However, expression in −4.2/+5.3 lines was not equivalent to that of −4.2/+11.6 LacZ transgenic mice, indicating that further regulatory regions are also located within the sequence from +5.3 to +11.6.

To further test the function of the +2.5/+5.3 region we tested a construct, −4.2/+2.5;+5.3/+11.6 LacZ transgene (Construct 8 in Fig. 1), in which the +2.5/+5.3 region was deleted. Paradoxically, although the results observed with the −4.2/+5.3 LacZ transgenic lines indicated that the +2.5 to +5.3 region had repressor activity, deletion of this region from the −4.2/+11.6 LacZ did not result in a detectable increase in expression of transgenes as compared with the −4.2/+11.6 LacZ transgene (Fig. 2, compare row 1 with row 8). Transgene expression in the large arteries and airways was strong and indistinguishable from that of the −4.2/+11.6 LacZ transgenic mice (Figs. 2, 8a and 8b, and 3f). However, transgene expression of the −4.2/+2.5;+5.3/+11.6 LacZ was much weaker in the coronary arteries (Fig. 2, column 8a versus 1a). Expression of the −4.2/+2.5;+5.3/+11.6 LacZ transgene was strong but somewhat variable in the GI tract and bladder. Within the intestine and bladder, some SMCs showed very strong bandlike staining (Fig. 2, 8c–8e), and histological examination of the stomach clearly demonstrated clusters of SMCs that were stained similarly and much higher than that of adjacent cells (Fig. 3h). Similar heterogeneous LacZ staining patterns were

FIG. 3. Histological and macroscopic examination of LacZ expression in various SM-MHC deletion mutant transgenic mice. β-Galactosidase staining of various transgenic mouse tissues. a–d, histological examination of the aorta of the −4.2/+2.5 LacZ (a), −4.2/+5.3 LacZ (b), −4.2/+5.3;+7.5/+9 LacZ (c), and −4.2/+9 LacZ (d) transgenic mice. e and f, histological sections of bronchus of the −1.3/+11.6 LacZ (e) and −4.2/+2.5;+5.3/+11.6 LacZ (f) transgenic mice. g and h, histological examination of the stomach of the −4.2/+2.5 LacZ (g) and −4.2/+2.5;+5.3/+11.6 LacZ (h) transgenic mice. Note the clear difference in staining between parts of SMC layers. i and j, sections of the left ventricle of the −4.2/+2.5 LacZ (i) and −4.2/+11.6 ΔHST LacZ (j) transgenic mice. Cardiomyocytes were stained positively, but no coronary SMCs were stained in the −4.2/+2.5 LacZ transgenic mouse (i), whereas coronary SMCs were stained positively in the −4.2/+11.6 ΔHST LacZ transgenic mouse (j). k and l, frontal view of the kidneys and abdominal blood vessels of the −4.2/+11.6 LacZ (k) and −4.2/+11.6 ΔHST LacZ (l) transgenic mice. Tissues were cleared. Arrowheads indicate the position of the abdominal aorta (l). Ao, aorta; CA, celiac artery; SMA, supramesenteric artery; IVC, inferior vena cava.

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observed also in the \(-4.2/+2.5\) LacZ transgenic mice (Fig. 3g). Taken together, these results showed that the region from \(-2.5\) to \(+5.3\) did not exhibit repressor activity when deleted from the \(-4.2/+11.6\) LacZ transgene but did repress transgene expression when it was added to the \(-4.2/+2.5\) LacZ construct. The results suggest that the repressor activity of this region may be suppressed by dominant positive-acting regulatory regions located in the \(SM-MHC\) region from \(+2.5\) to \(+11.6\). In addition, observations that expression of the \(-4.2/+11.6\) LacZ transgene was weaker in intestinal, coronary, and bladder SMCs than that of the \(-4.2/+11.6\) LacZ transgene suggest that the \(+2.5/+5.3\) region may also contain a positive-acting regulatory region necessary in these SMC subtypes.

The Region from \(+5.3/+11.6\) kb Contained a DNasel Hypersensitive Site That Was Required for SM-MHC Expression in Vivo—Because results of the preceding analyses of transgenic mice indicated that additional transcriptional regulatory regions were located within the \(+5.3/+11.6\) kb region, we sought to identify these possible regulatory regions. We first attempted to identify regulatory regions in cultured SMCs. However, the addition of various fragments of the region from \(+5.3/+11.6\) kb to the \(-4.2/\) LacZ did not alter the reporter activity in cultured SMCs. The lack of an effect may be the result of phenotypic modulation of SMCs. Alternatively, it is possible that regulatory regions within the \(+5.3/+11.6\) sequence might function only in the context of intact chromatin, as has been demonstrated for a number of transcriptional regulatory modules (15). In reporter assays using transient transfection of plasmid DNAs, the vast majority of plasmid DNA stays as episomal DNA and is not organized properly into a nucleosomal structure (16). Therefore, we elected to identify candidate transcriptional regulatory regions within the context of the endogenous \(SM-MHC\) gene in intact chromatin using DNasel hypersensitivity assays. Because the opened chromatin structure frequently associated with transcriptionally active regions allows access of large DNasel molecules, opened active regions are digested by DNasel much more strongly than other silent regions and thus often show DNasel hypersensitivity. Intact cultured SMCs were permeabilized and treated directly with DNasel. Genomic DNA was purified from DNasel-treated SMCs, digested with either \(Bam\) HI or \(Sac\) I, and subjected to Southern analyses using probes \(A\), \(B\), and \(C\) as indicated in \(b\). \(a–p\) indicate DNA bands formed by preferential DNasel digestion (DNasel hypersensitivity) in \(a\). The breaking points corresponding to these bands are shown schematically in \(b\). The regions that show DNasel hypersensitivity were designated DNasel hypersensitive sites \(HS1–7\) (panel \(b\)). Known cis-elements including the \(C\) ArG, GC repressor, CCTCCC, and TATAA elements (see Fig. 1 legend) are indicated (panel \(b\)).

![Image](image.png)

**Fig. 4.** DNasel hypersensitivity assays for identification of potential transcriptional regulatory regions. panel \(a\), DNasel hypersensitive assays of the \(SM-MHC\) gene in cultured SMCs. To detect possible transcriptional regulatory regions of the endogenous \(SM-MHC\) gene, intact cultured SMCs were permeabilized and treated directly with DNasel. Genomic DNA was purified from DNasel-treated SMCs, digested with either \(Bam\) HI or \(Sac\) I, and subjected to Southern analyses using probes \(A\), \(B\), and \(C\) as indicated in \(b\). \(a–p\) indicate DNA bands formed by preferential DNasel digestion (DNasel hypersensitivity) in \(a\). The breaking points corresponding to these bands are shown schematically in \(b\). The regions that show DNasel hypersensitivity were designated DNasel hypersensitive sites \(HS1–7\) (panel \(b\)). Known cis-elements including the \(C\) ArG, GC repressor, CCTCCC, and TATAA elements (see Fig. 1 legend) are indicated (panel \(b\)).
These data show the presence of multiple DNaseI hypersensitive sites within the region from −3.1 to +12 and identified a novel candidate transcriptional regulatory region at +8 kb (HS7) within the region from +5.3 to +11.6 for further studies.

To determine the function of the HS7 hypersensitive site in vivo, a 1.5-kb restriction fragment containing HS7 (+7.5 to +9 kb) was added to the −4.2/+5.3 LacZ (−4.2/+5.3;+7.5/+9 LacZ, Construct 5 in Fig. 1). Although the inclusion of the fragment did not significantly increase reporter activity in cultured SMCs in transient transfection experiments (data not shown), transgenic mice carrying the −4.2/+5.3;+7.5/+9 LacZ transgene showed marked increases in transgene expression as compared with the −4.2/+5.3 lines. Indeed, expression patterns were very similar to that of −4.2/+11.6 LacZ transgenic mice (Fig. 2, compare row 5 with row 1). The −4.2/+5.3;+7.5/+9 LacZ transgene was expressed in virtually all SMCs including vascular and airway SMCs, in which the −4.2/+5.3 transgene was not expressed (Figs. 2, row 5 versus row 4, and 3, c versus b). Visceral SMCs also showed strong LacZ staining (Fig. 2, 5c–5e). These data clearly demonstrated that the 1.5-kb sequence contained a positive-acting regulatory region that markedly increased transgene expression in all SMC subtypes in vivo in transgenic mice when added to the −4.2/+5.3 LacZ construct.

The HS7 DNaseI Hypersensitive Site at +8 kb Contained Multiple cis-Regulatory Elements—Results of analyses of the −4.2/+5.3;+7.5/+9 LacZ transgenic lines clearly indicated that the 1.5-kb from +7.5 to +9 kb contained a positive-acting regulatory region. We next characterized this putative transcriptional regulatory region. Sequence analyses of the rat and human genes revealed a highly conserved 200-bp domain at 5′ of HS7 (Fig. 6). The 200-bp sequence contained a positive-acting regulatory region that markedly increased transgene expression in all SMC subtypes in vivo in transgenic mice when added to the −4.2/+5.3 LacZ construct.

To examine further if these protected regions were also bound by proteins in SMCs in intact aortic SMCs, the rat intact aorta was subjected to in vivo methylation footprinting. Because the intact aorta does not permit penetration of large DNaseI molecules, we employed an alternative method in which the intact rat aortas were treated directly with DMS that preferentially methylates G residues (12). DNA was purified from DMS-treated aortas, and purified DNA was cleaved specifically at methylated G residues by piperidine treatment (13). The cleavage points of DNA were visualized using ligation-mediated PCR. As shown in Fig. 5a, multiple G nucleotides in DNaseI digestion were protected from DNaseI digestion (Fig. 5a–5f), indicating that protein binding to these sequences at HS7 of the endogenous SM-MHC gene in intact cultured SMCs.

Because the data of DNaseI hypersensitivity assays and in vivo footprinting suggest that HS7 may be transcriptionally active, we tested whether the HS7 region might exhibit enhancer activity in cultured SMCs. Despite observations of protein binding to this region of the endogenous SM-MHC gene within chromatin, neither 257 bp of highly conserved sequence (+8038 to +8294) nor the 1.5 kb from +7.5 to 9 kb significantly increased reporter activity when subcloned into the minimal TK-LacZ construct in transient transfection in cultured SMCs (data not shown). Enhancer activity was also not detected in Chinese hamster ovary (CHO), 10T1/2, and NIH3T3 cells (data not shown).

**HS7 at +8 kb Was Required for Transcription in Vivo**—Although the DNaseI hypersensitive and in vivo footprinting data indicated that HS7 was opened and bound by nuclear protein in the context of the endogenous SM-MHC gene within chromatin, transient transfection experiments using cultured SMCs did not detect the transcriptional activity of this region. To test whether HS7 might function in vivo in transgenic mice, the 200-bp sequence (+8095 to +8294) at HS7 was deleted from the −4.2/+11.6 LacZ construct (−4.2/+11.6 ΔHS7 footprinting of cultured SMCs were protected (Fig. 6), suggesting that at least some of these footprinting regions detected in cultured SMCs were also bound by proteins in the intact rat aorta.

To further characterize protein binding to Ft1–Ft9 footprint regions, these regions were examined by EMSAs using nuclear extracts prepared from cultured SMCs. All probes for Ft1–Ft9 regions formed specific DNA-protein complexes with SMNuc extracts in EMSAs (Fig. 7 and data not shown). Of particular interest, Ft3 contained a CARG-like element at +8132 bp, and Ft4 contained an E-box at +8159 bp. The CARG-like sequence within the Ft3 region contains a mismatch nucleotide (CCGTTTTTGG) compared with the CARG consensus, CCA/TGGGG, and is well conserved between the rat and human genes. In EMSAs, both rat and human CARG sequences formed a major shift band that had a mobility identical to that of the major shift band of the well defined CARG element of the c-fos promoter, c-fos SRE (Fig. 7a). These shift bands were super-shifted by the addition of anti-serum response factor (SRF) antibody, indicating that SRF was present in the DNA-protein complexes. However, a 50-fold molar excess of cold probes of the rat and human Ft3 CARG elements did not compete efficiently with SRF binding to the c-fos SRE probe as compared with c-fos SRE itself (Fig. 7a, lanes 8–10), suggesting a relatively low affinity to SRF of these CARG elements in EMSAs in vitro.

E-boxes (CANNTG) are target sites for basic helix-loop-helix factors such as the MyoD family myogenic regulatory factors and are important for control of a number of skeletal muscle-specific genes (18). The E-box and its flanking sequence within the Ft4 footprint region are identical between the rat and human genes. In EMSAs, the probe for Ft4 formed a major shift band that was competed by a 50-fold molar excess of cold self-probe and an oligonucleotide containing the muscle creatine kinase E-box sequence (Fig. 7b, lanes 2 and 4). Although the sequence of Ft4 (5′-TCAAGTG-3′ in the antisense strand) also contains the consensus sequence of homeobox proteins, Nkx2 factors (5′-TNNAGTG-3′) (19), the major band was not competed by a consensus Nkx2-1 binding site (lane 5) (19). Formation of the major shift band was inhibited by anti-upstream stimulatory factor (USF)-1 and USF-2 antibodies but not by antibodies against other basic helix-loop-helix transcription factors including E12 and E2A (lanes 6 and 7). Taken together, data of EMSAs demonstrate that the CARG element and E-box within HS7 can bind SRF and USF in vitro, respectively.
amplified using forward and reverse primer sets, respectively. The antisense (lanes 3 and 4) strands were amplified using forward and reverse primer sets, respectively. C and T indicate lanes of control naked DNA and cultured SMC DNA, respectively. Footprint domains are indicated by bars. 1–9 refers to the footprint regions Ft1–Ft9. b, DMS in vivo footprint analyses using the intact rat aorta. To examine whether footprinting regions identified above were also bound by proteins in the rat aorta, intact rat aorta was treated directly with DMS that preferentially methylated guanine residues. Methylated G residues were specifically cleaved by piperidine treatment. The levels of cleavage (i.e., the levels of methylation) were visualized using ligation-mediated PCR. The antisense (lanes 1 and 2) and sense (lanes 3 and 4) strands were amplified using forward and reverse primer sets, respectively. Protected guanine nucleotides are indicated by circles. These G nucleotides consistently showed protection in multiple ligation-mediated PCR analyses of multiple independently prepared DNA samples. Representative gel images are shown. A hypermethylated guanine nucleotide is indicated by an arrowhead.

**FIG. 5.** *In vivo* footprinting analyses of the HS7 region +8 kb. a, DNasel *in vivo* footprinting analyses using cultured SMCs. To specify protein binding sequences within the HS7 region of the endogenous SM-MHC gene in intact chromatin, intact cultured SMCs were treated directly with DNasel. Protection from DNasel digestion (footprints), which indicates protein binding to DNA sequences, was visualized using ligation-mediated PCR. The antisense (lanes 1 and 2) and sense (lanes 3 and 4) strands were amplified using forward and reverse primer sets, respectively. Pro-tection of guanine nucleotides is indicated by circles. These G nucleotides consistently showed protection in multiple ligation-mediated PCR analyses of multiple independently prepared DNA samples. Representative gel images are shown. A hypermethylated guanine nucleotide is indicated by an arrowhead.

LacZ, Construct 6 in Fig. 1. The deletion did not significantly change reporter activity in cultured SMCs (data not shown). However, the deletion resulted in differential reduction in reporter activity in SMC subtypes *in vivo* in transgenic mice (Fig. 2, row 6 versus row 1). Of particular note, reporter expression in large arteries was very weak (Figs. 2, 6a versus 1a, and 3, l versus k), although the expression in veins and smaller arteries was equivalent to that in the −4.2/+11.6 LacZ transgenic mice (Fig. 3, l versus k). Transgene expression was also detected easily in the coronary arteries in the −4.2/+11.6 ΔHS7 LacZ transgenic mice (Fig. 2, 6a, and 3j). Transgene expression in the airways was strong, whereas no expression was detected in pulmonary blood vessels (Fig. 2, 6b). Expression in the GI tract and bladder was strong but showed some uneven staining among SMCs (Fig. 2, 6c–6e). These results indicate that the conserved 200-bp region containing HS7 is absolutely required for expression in large arteries but dispensable in small arteries and airways. The region may also be required for maximal expression in the GI tract and bladder. However, compared with the expression patterns of the −4.2/+5.3 LacZ lines, the −4.2/+11.6 ΔHS7 LacZ transgenic mice clearly showed a higher level of LacZ expression in subsets of SMCs including visceral and small arterial SMCs. These data suggest that there may be additional regulatory regions within the +5.3 to +11.6 sequence.

We next tested whether the HS7 region was not only necessary but also sufficient to drive transcription *in vivo*. Three copies of the conserved region were subcloned 5’ of a minimal TK promoter (3xHS7-TK LacZ), and transgenic mice were generated. Three independent founder lines showed no transgene expression in any cells. Taken together, the HS7 region is differentially required for SM-MHC expression *in vivo* but is not sufficient to drive transcription when coupled with a minimal TK promoter.

The Sequence from +5.3 to +11.6 kb Contained Additional Negative- and Positive-acting Regulatory Regions That Played Crucial Roles in SM-MHC Expression in Vascular SMCs—The results of analyses of transgenic mice carrying the HS7 deletion mutant (−4.2/+11.6 ΔHS7 LacZ) suggested that there might be additional regulatory regions within the sequences from +5.3 to +7.5 and +9 to +11.6. As such, we next tested the function of the sequence from +5.3 to +7.5 in transgenic mice. A construct containing the SM-MHC genomic sequence from −4.2 to +9 kb (−4.2/+9 LacZ, Construct 7 in Fig. 1) was generated and used to produce transgenic mice. Although we expected positive activity, transgenic mice carrying the −4.2/+9 LacZ transgene showed clearly weaker transgene expression in vascular SMCs as compared with that seen in −4.2/+5.3;+7.5/+9 LacZ transgenic mice (Fig. 2, 7a and 7b versus 5a and 5b, and Fig. 3, d versus c). Only a minor fraction of vascular SMCs were stained positively for LacZ in large and small arteries (Figs. 2, 7a and 7b, and 3d). In contrast, transgene expression in the GI tract and bladder was very strong and equivalent to that of −4.2/+11.6 LacZ and 4.2/+5.3;+7.5/+9 LacZ transgenic mice (Fig. 2, 7c–7e versus 1c–1e and 5c–5e). Transgene expression in the airways was also strong (Fig. 2, 7b). The weaker transgene expression observed in vascular SMCs of −4.2/+9 LacZ transgenic mice as compared with −4.2/+5.3;+7.5/+9 LacZ mice suggests that the sequence from +5.3 to +7.5 may contain a regulatory module that inhibits transgene expression at least in vascular SMCs. In addition, the fact that the −4.2/+9 transgene was expressed at lower levels than the −4.2/+11.6 LacZ in vascular SMCs also suggests that the sequence between +9 and +11.6 may contain a positive-acting regulatory module that is required for SM-MHC expression in vascular SMCs.
DISCUSSION

Expression of the SM-MHC Gene Is Regulated by Multiple Modular Control Regions That Exhibit SMC Subtype-selective Activity—Our previous studies of the function of multiple CArG elements in the SM-MHC transcriptional control (6) and studies of others of SM22α promoter regions in transgenic mice (7–9) implied that transcriptional control depended on multiple control regions that varied between SMC subtypes. However, these studies failed to identify specific modules required for expression in diverse SMC subtypes. Results of the present studies provide clear identification of the specific genomic regions required for controlling the SM-MHC gene in different SMC subtypes. Furthermore, the present studies revealed novel and unexpected features of the SM-MHC regulatory system.
First, unique combinations of transcriptional regulatory modules that are spread widely over a very large genomic region are required for expression of the SM-MHC gene in different SMC subtypes. Indeed, deletion of any one of seven restriction fragments of the SM-MHC −4.2 to +11.6 kb region resulted in differential changes in transgene expression in SMC subtypes in vivo (Fig. 2), indicating very different roles of each region in different SMC subtypes. That is, expression of the SM-MHC gene in SMC subtypes is clearly not controlled by single or a few SMC subtype-specific enhancer regions but rather by complex combinations of an unexpectedly large number of regulatory modules, the activity of which varies in different SMC subtypes.

Second, the activity of certain modules varies not only in different SMC subtypes but also depending on genomic contexts. For example, the region +2.5 to +5.3 appeared to contain both positive- and negative-acting modules but exhibited predominantly repressor activity when added to the −4.2/+2.5 region (Fig. 2, compare −4.2/+5.3 LacZ with −4.2/+2.5 LacZ). However, this same region exhibited positive activity in vascular SMCs, particularly in coronary vessels, in the context of additional promoter-intronic regions (e.g., Fig. 2, compare −4.2/+2.5;+5.3/+11.6 LacZ with −4.2/+11.6 LacZ). Thus, the positive and negative activity of this region seemed dispensable in certain SMC subtypes in certain contexts but were required in other contexts. Likewise, although the 1.5-kb region from +7.5 to +9 clearly increased transgene expression in all types of SMCs when added to the −4.2/+5.3 kb LacZ construct (Fig. 2, compare row 4 with row 5), high expression in airway SMCs was observed despite the complete omission of this region in the case of the −4.2/+5.5 LacZ transgene construct (Fig. 2, compare row 3 with row 4). These results indicate that expression of the SM-MHC gene is controlled by complex interplay between regulatory modules. That is, the net effect of a given module on overall transcription of the SM-MHC gene is not determined by the isolated activity of this module but by interaction with other modules. In other words, the activity of a given module seems to be processed in reference to the activity of other modules, and activity is not simply the linear sum of all modules present.

Third, negative-acting modules as well as positive-acting modules are both required for SMC specificity of expression of the SM-MHC gene. For example, addition of the fragment +2.5/+5.3 eliminated the strong transgene expression observed in cardiac and skeletal muscle cells in the −4.2/+2.5 LacZ transgenic mice (Fig. 2, compare 3a with 4a), whereas elimination of the −4.2 to −1.3 region also resulted in expression non-SMCs. These data suggest that negative-acting regulatory modules play a critical role in confining SM-MHC expression within SMCs.

Taken together, results of the present studies revealed the extremely complex modular structure of the SM-MHC cis-regulatory system in vivo. This multiplicity of the modular regulatory system of the SM-MHC gene is likely to enable the system to respond to vastly divergent local environmental cues in vivo between large and small arterial SMCs, vascular and intestinal SMCs, etc., and thus may contribute to the marked heterogeneity of SMCs (20). The modular system may be required also to respond to changing environmental cues during development and also in pathophysiological conditions. The results are consistent with a growing body of evidence indicating that a multiple modular structure is a common feature of transcriptional regulatory systems of developmentally regulated genes (21, 22). For example, several skeletal and cardiac-specific genes including MyoD, myogenin, Myf5, and Nkx2-5/Csx have been demonstrated to be regulated by multiple modules in vivo (21, 23, 24). However, the present studies are the first to provide clear evidence showing the complex modular regulatory nature of an SMC differentiation marker gene in vivo. Fig. 8 summarizes the structure and function of transcriptional regulatory regions within the −4.2 to +11.6 kb of the SM-MHC gene and illustrates the differential roles of SM-MHC fragments or regulatory modules that we observed in SMC subtypes in vivo. However, we want to emphasize that it is possible, and indeed likely, that some modules might function differently in different contexts (i.e., different combinations of modules) (25) and that functions of some modules might have been (a) masked by other modules, (b) not detected because of redundancy in function or by chance because of the location of restriction sites, and/or (c) not have been functional under conditions of our experiments. That is, certain modules may only be active at specific stages of SMC development, during modulation of SMC after vascular injury, and other pathophysiological circumstances. In any case, it is clear that much further work will be necessary to identify specific functions of
transcriptional regulatory modules. Nevertheless, the data presented in the present studies provide a strong foundation for extensive further characterization of mechanisms that control the SM-MHC gene in vivo.

HST at +8 kb Is Evolutionarily Conserved and Required for SM-MHC Transcription in Vivo—Transgenic mouse lines carrying the −4.2/+11.6 ΔHST LacZ transgene clearly demonstrated the requirement of HST in expression of the SM-MHC gene in vivo. Furthermore, in vivo footprint assays demonstrated that this region contained multiple potential cis-elements occupied by proteins in both intact cultured SMCs and the rat aorta. However, this region did not exhibit enhancer activity in cultured SMCs when coupled with several promoter constructs including a minimal TK promoter and the −4.2/+5.3 LacZ. These data add to a growing body of evidence clearly showing that results of promoter activity obtained by transient transfection of reporter plasmids into cultured SMCs may not provide a valid index of regulation of endogenous SMC-specific genes. There are at least two possible explanations for these contradictory results about the function of HST. First, because of the highly modulated phenotype of SMCs in culture, HST may not exhibit its optimum activity in cultured SMCs. It is well known that SMCs are highly modulated in culture, and virtually all SMC marker genes including the SM-MHC gene are down-regulated in cultured SMCs (1). Therefore, it is conceivable that the activity or expression of transcription factors necessary for optimum enhancer activity of HST7 might be suppressed. Second, because plasmid DNA largely stays as episomal DNA when transiently transfected into cultured cells and is not organized properly into the nucleosomal structure (16), reporter assays may not provide information regarding transcriptional control mechanisms that operate in the context of intact chromatin. Indeed, it has been demonstrated that transcriptional regulatory modules of several genes including the two DNaseI hypersensitive sites of the β-globin locus control region do not work as classical enhancers in conventional reporter assays, whereas they do exhibit enhancer activity when integrated into chromatin (15, 16). As such, contradictory results obtained from studies of the endogenous SM-MHC gene (DNaseI hypersensitive assays, in vitro footprinting, and transgenic mice) and transient transfection experiments suggest that HST7 may function only when integrated into chromatin. Indeed, the successful identification of HST7, which was not detected by conventional transient transfection experiments in cultured SMCs, using methods that detect transcription factor binding to target sites in the context of intact chromatin exemplifies the necessity and strength of these novel methods in studies of complex transcriptional regulatory systems in vivo.

Although the results of −4.2/+11.6 ΔHST LacZ and −4.2/+5.3; +7.5/+9 LacZ transgenic mice clearly established an important role of HST7 in control of SM-MHC expression in vivo, the 3xHS7-TK LacZ transgene was not expressed in three independent transgenic mouse lines. Because of the small number of transgenic lines we have analyzed, we cannot clearly conclude that the 3xHS7-TK LacZ transgene is not functional in vivo. However, because of significant inherent limitations involved in minimal promoter transgene experiments, we decided not to produce more transgenic lines carrying the 3xHS7-TK LacZ construct. For example, the data in the present studies as well as previous studies in other cell systems (15, 25, 26) demonstrated that the functions of regulatory modules are highly dependent on the context in which they are tested including chromatin structure, the presence of other regulatory modules, and the proper orientation, spacing, and order of modules (15, 25, 26). Most of these factors cannot be fully reconstituted in small synthetic transgenes using foreign minimal promoters. As such, although transgenic mice carrying minimal promoter constructs have provided valuable information regarding functions of isolated regulatory modules, the functions of modules observed in this type of experiment need to be interpreted very cautiously.

HST contained an E-box that was found by USF in EMSA experiments using nuclear extracts prepared from cultured SMCs. We also detected USF binding activity to this E-box in nuclear extracts prepared from intact rat SM tissues.2 Previous studies in our laboratory provided evidence for a role of USF in SM α-actin expression through E-boxes in the 5′-flanking sequences (27). Importantly, these E-boxes were required for transcription in vivo in transgenic mice (28). Recently, the osteopontin and APEG-1 genes have also been shown to depend on E-boxes in cultured SMCs (29, 30). These studies support important roles of E-boxes in transcriptional regulation of SMC-specific genes. However, analysis of E-box-binding proteins using in vitro assays such as EMSAs and in vitro footprinting may be inconclusive, because binding activity of some basic helix-loop-helix factors including c-Myc cannot be detected using these in vitro assays even when these factors do bind target E-boxes in chromatin (31). In contrast, USF is known to be detected easily in nuclear extracts prepared from various types of cells, and thus in vitro binding assays are biased toward detecting USF (31). In fact, the uncertainty regarding functional roles of transcription factors detected by in vitro binding assays in the control of endogenous target genes in chromatin is an inherent problem of this type of assay. Many studies have shown major differences in transcription factor binding to the target sites between in vitro assays and endogenous genes in chromatin (31–33), and the results of in vitro binding assays alone by no means can be used as evidence for functionality. As such, we cannot conclude that USF binds the E-box of the endogenous SM-MHC gene. Nevertheless, results of in vivo footprinting assays showing occupancy of the endogenous SM-MHC E-box provide novel evidence for the possible function of E-boxes in control of expression of SMC-specific genes. Whereas the function of E-boxes and myogenic regulatory factors in control of skeletal muscle-specific genes and skeletal muscle differentiation has been extensively studied, little is known regarding the function of E-boxes in control of SMC-specific genes. Thus, it would be of great importance to identify binding proteins to E-boxes of the SMC-specific genes.

Development of SMC Subtype-selective Promoters—One of the most significant implications of the present studies is that studies establish the feasibility of engineering derivatives of the SM-MHC genomic sequence that function only in subsets of SMCs (34). Such derivatives could be of major utility for targeting expression of therapeutic agents to specific SMC subtypes and/or for purposes of studying the function of candidate genes in SMC subtypes in vivo using targeted knockout or overexpression systems. For example, on the basis of results of the present studies and our previous studies showing selective functional activity of the CARG elements in subsets of SMCs, we would predict that a derivative SM-MHC promoter construct containing the sequence −4.2/+2.5 plus +7.5/+9 and mutation of the intronic CARG should be selectively expressed in bronchial SMCs with little activity in large arteries and coronary arteries. However, as discussed above, because of the functional redundancy and interplay between modules, some regulatory modules might exhibit functions that were not detected in the present studies. Thus, SM-MHC promoter derivatives need to be tested empirically for their functions under the situations they would be used (adults versus embryos and in--

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2 I. Manabe and G. K. Owens, unpublished observations.
Integrated versus episomal genes). Nevertheless, results of the present studies provide the framework for future studies of SM-MHC regulatory modules.

In summary, we have mapped transcriptional regulatory regions within the SM-MHC −4.2 to +11.6 kb sequence and revealed the complex modular cis-regulatory program of the SM-MHC gene in vivo. SMC-specific expression of the SM-MHC gene is not controlled by a single regulatory region but by the complex interplay of multiple positive- and negative-acting regulatory modules in SMC subtypes in vivo. Because of its unique degree of SMC specificity and tight regulation during SMC differentiation, results are likely to be broadly applicable to defining the transcriptional circuitry that controls cell type-specific gene expression during SMC differentiation.

Acknowledgments—We gratefully acknowledge the expert technical assistance of Diane Raines, Douglas Mullineux, Margaret Ober, and Angela Miller.

REFERENCES
1. Owens, G. K. (1995) Physiol. Rev. 75, 487–517
2. Aikawa, M., Sakomura, Y., Ueda, M., Kimura, K., Manabe, I., Ishiwata, S., Komiyama, N., Yamaguchi, H., Yazaki, Y., and Nagai, R. (1997) Circulation 96, 82–90
3. Aikawa, M., Kim, H. S., Kuro-o, M., Manabe, I., Watanabe, M., Yamaguchi, H., Yazaki, Y., and Nagai, R. (1995) Ann. N. Y. Acad. Sci. 748, 578–585
4. Madsen, C. S., Regan, C. P., Hungerford, J. E., White, S. L., Manabe, I., and Owens, G. K. (1998) Circ. Res. 82, 908–917
5. Regan, C. P., Manabe, I., and Owens, G. K. (2000) Circ. Res. 87, 363–369
6. Manabe, I., and Owens, G. K. (2001) J. Clin. Invest. 107, 823–834
7. Kim, S., Ip, H. S., Lu, M. M., Clendenin, C., and Parmacek, M. S. (1997) Mol. Cell. Biol. 17, 2266–2278
8. Li, L., Liu, Z., Mercer, B., Overbeek, P., and Olsen, E. N. (1997) Dev. Biol. 187, 311–321
9. Moesler, H., Meriksky, M., Li, Z., Nagl, S., Paulin, D., and Small, J. V. (1996) Development 122, 2415–2425
10. Madsen, C. S., Hershey, J. C., Hautmann, M. B., White, S. L., and Owens, G. K. (1997) J. Biol. Chem. 272, 6332–6340
11. Ymer, S., and Jans, D. A. (1996) BioTechniques 20, 834–840
12. Madsen, C. S., Regan, C. P., and Owens, G. K. (1997) J. Biol. Chem. 272, 29842–29851
13. Mueller, P. R., and Wold, B. (1989) Science 246, 780–786
14. Mack, C. P., and Owens, G. K. (1999) Circ. Res. 84, 852–861
15. Li, Q., Harjo, S., and Peterson, K. R. (1999) Trends Genet. 15, 403–408
16. Smith, C. L., and Hager, G. L. (1997) J. Biol. Chem. 272, 27493–27496
17. Grange, T., Bertrand, E., Espinas, M. L., Frumont-Racine, M., Rigaud, G., Roux, J., and Pictet, R. (1997) Methods 11, 151–163
18. Ludolph, D. C., and Konieczny, S. F. (1995) FASEB J. 9, 1595–1604
19. Chen, C. Y., and Schwartz, R. J. (1995) J. Biol. Chem. 270, 15628–15633
20. Schwartz, S. M., Heimark, R. L., and Majesky, M. W. (1990) Physiol. Rev. 70, 1177–1209
21. Firulli, A. B., and Olson, E. N. (1997) Trends Genet. 13, 364–369
22. Yuh, C.-H., Bolouri, H., and Davidson, E. H. (1998) Science 279, 1896–1902
23. Summerbell, D., Ashby, P. R., Coutelle, O., Cox, D., Yee, S.-P., and Rigby, P. W. J. (2000) Development 127, 3745–3757
24. Schwartz, R. J., and Olson, E. N. (1999) Development 126, 4187–4192
25. Yuh, C.-H., Bolouri, H., and Davidson, E. H. (2001) Development 128, 617–629
26. Fry, C. J., and Farnham, P. J. (1999) J. Biol. Chem. 274, 28583–28586
27. Johnson, A. D., and Owens, G. K. (1999) Am. J. Physiol. 276, C1420–C1431
28. Kumar, M., Johnson, A., Mack, C., and Owens, G. (1999) FASEB J. 13, 531 (abstr.)
29. Malyankar, U. M., Hanson, B., Schwartz, S. M., Ridall, A. L., and Giachelli, C. M. (1999) Exp. Cell Res. 250, 535–547
30. Hsieh, C. M., Yet, S. F., Layne, M. D., Watanabe, M., Hong, A. M., Perrella, M. A., and Lee, M. E. (1999) J. Biol. Chem. 274, 14344–14351
31. Harris, V. K., Cotecha, C. M., List, H.-J., Wellestein, A., and Riegel, A. T. (2000) J. Biol. Chem. 275, 28539–28548
32. Boyd, K. E., and Farnham, P. J. (1999) Mol. Cell. Biol. 19, 8393–8399
33. Bennett, M. K., and Osborne, T. F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6340–6344
34. Li, X., Eastman, E. M., Schwartz, R. J., and Draghia-Akli, R. (1999) Nat. Biotechnol. 17, 241–245
35. Watanabe, M., Sakomura, Y., Kurabayashi, M., Manabe, I., Aikawa, M., Kuro-o, M., Suzuki, T., Yazaki, Y., and Nagai, R. (1996) Circ. Res. 78, 978–989