To understand smooth muscle-specific gene expression, we have focused our studies on the smooth muscle myosin heavy chain (SMHC) gene, a smooth muscle-specific marker. In this study, we demonstrate that the SMHC promoter region (−1594 to −1462 base pairs) containing the A/T-rich element can activate the heterologous thymidine kinase promoter in smooth muscle cells, but not in fibroblasts. Mutations of this A/T-rich element decreased SMHC promoter activity significantly. Both gel mobility shift assays and DNase I footprinting revealed that this region binds to specific protein complexes from smooth muscle nuclear extracts, whereas nuclear extracts from skeletal muscle and fibroblasts produced a different binding pattern. We also demonstrate that the protein complex obtained from smooth muscle nuclear extract reacts with MEF2B-specific antibody, but not with antibodies specific to MEF2A, MEF2C, or MEF2D, suggesting that only MEF2B protein binds to the A/T-rich element. Furthermore, MEF2B overexpression in smooth muscle cells up-regulated the SMHC promoter, suggesting that MEF2B is important for SMHC gene regulation. This is the first report demonstrating a role for MEF2 factors in smooth muscle-specific gene expression.

Smooth muscle cells have been the subject of intense study because abnormal growth and proliferation of smooth muscle cells are involved in the pathogenesis of both atherosclerosis (1) and restenosis following percutaneous transluminal coronary angioplasty or atherectomy. The hallmark of restenosis following percutaneous transluminal coronary angioplasty or coronary atherectomy is vascular smooth muscle cell proliferation and migration causing obstructive lesions (2). Numerous observations suggest that smooth muscle cells in vascular lesions undergo phenotypic modulations from a contractile to a synthetic phenotype. These proliferating smooth muscle cells secrete increased extracellular matrix and express several embryonic markers, but lose SM-2 myosin present in mature smooth muscle cells, more closely resembling immature fetal smooth muscle cells. These studies suggest that upon injury, adult smooth muscle cells recapitulate many aspects of embryonic development during their remodeling. Therefore, a better understanding of smooth muscle development and differentiation is important to define the vascular disease process. From this standpoint, a central question in vascular biology relates to understanding the transcriptional control mechanisms underlying smooth muscle growth and differentiation.

To date, transcription factors that are responsible for smooth muscle commitment and differentiation or modulation of the smooth muscle cell phenotype have not been identified (3). The transcription factors that regulate skeletal muscle development and differentiation are better understood and may provide useful paradigms for elucidating control of gene expression in smooth muscle. In recent years, skeletal muscle has become the paradigm for understanding tissue-specific gene activation and cell differentiation due to the discovery of a set of master regulatory genes, namely the MyleD family, which includes MyoD, myogenin, myf-5, and MRF-4/herculin/myf-6 (4). The myogenic factors are unique in their abilities to orchestrate an entire program of skeletal muscle-specific gene activation when introduced into diverse cell types. However, the members of the MyleD gene family are not expressed in smooth muscle, and related helix-loop-helix transcription factors controlling smooth muscle cell differentiation have not yet been identified. This raises the possibility that there are other types of transcription factors involved in smooth muscle myogenesis.

A second class of transcription factors, namely MEF2 (myocyte-specific enhancer factor 2)/RSRF (related to serum response factor), has been implicated in striated muscle differentiation and transcriptional control (5). The MEF2 family includes four genes, MEF2A (6), MEF2B (7), MEF2C (8), and MEF2D (9–11). Each of the MEF2 proteins cloned thus far appears to be subject to complex forms of regulation at different levels. Members of this gene family are transcribed in a wide range of cell types including skeletal, cardiac, and smooth muscles as well as brain and spleen. MEF2 was originally described as a DNA-binding activity present in differentiating myotubes (12). The MEF2-binding site, CTA(A/T)4TA(G/A), has been shown to be important for transcriptional regulation of many cardiac and skeletal muscle-specific genes (12–16). It has also been demonstrated that MEF2 transcription factors can interact with myogenic bHLH1 proteins (17) to synergistically activate muscle-specific genes. Recent studies in the fruit fly Drosophila melanogaster showed that MEF2 is indispensable for muscle development since disruption of the single MEF2 gene, D-MEF2, causes lethality with abnormalities in cardiac.

* This work was supported by National Institutes of Health Grant R01-HL-38355. 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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The abbreviations used are: bHLH, basic helix-loop-helix; SMHC, smooth muscle myosin heavy chain; bp, base pair(s); TK, thymidine kinase; PCR, polymerase chain reaction; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase; MCK, muscle creatine kinase; SRF, serum response factor.
Role of MEF2B in SMHC Gene Expression

somatic, and visceral muscle development. This demonstrates that MEF2 plays an important role in all three muscle lineages (18, 19). MEF2A, MEF2B, and MEF2D are also expressed (mRNA and protein) in cultured smooth muscle cells and in the adult rat aorta, and their expression is increased in neointimal smooth muscle cells during vascular remodeling (20). However, the exact role of MEF2 transcription factors in regulating smooth muscle gene expression remains to be determined.

Toward understanding smooth muscle-specific gene expression, we have recently isolated and characterized the rabbit smooth muscle myosin heavy chain (SMHC) gene promoter (21), whose expression is highly tissue-specific. We have shown that the promoter region extending to −2266 bp is highly active in cultured rat aortic smooth muscle cells, but not in other cell types. Promoter deletion analyses identified a region between −1548 and −1392 bp as important for high level promoter activity. This region includes a MEF2-like A/T-rich element located at −1540 bp that binds to a specific protein complex in nuclear extracts from vascular smooth muscle cells (21). The goal of this study was to examine the nature of protein binding to this element and its functional relevance to SMHC promoter activity. In this study, we demonstrate that an antibody specific to MEF2B protein supershifted the protein complexes formed on the A/T-rich region from smooth muscle nuclear extracts. However, MEF2B does not appear to bind this A/T-rich element directly. We further show that overexpression of MEF2B protein in smooth muscle cells up-regulates SMHC gene promoter activity. This is the first report demonstrating a role for MEF2B in smooth muscle-specific gene expression.

MATERIALS AND METHODS

Cell Culture—Smooth muscle cells from rat thoracic aorta were isolated and cultured by a modification of the procedures described by Owens et al. (22). Male Sprague-Dawley rats (4–6 weeks old) were anesthetized with an intraperitoneal injection of sodium pentobarbital, and the descending thoracic aorta was excised aseptically. Two vessels were placed in Hanks’ balanced salt solution (Life Technologies, Inc.) with 1% antibiotic/antimycotic (Life Technologies, Inc.), cleaned free of adhering fat and connective tissue by blunt dissection, and opened longitudinally. After preincubation of the vessels for 15–25 min at 37 °C in a 5% CO2 and 95% air atmosphere in Hanks’ balanced salt solution in the presence of 1 mg/ml collagenase (2219 units/mg; Worthington) and 0.16–0.2 mg/ml elastase (4.2 units/mg; Worthington) with 1% antibiotic/antimycotic, the adventitia was carefully stripped off under a dissecting microscope, and the luminal surface was scraped with the convex side of curved forceps to remove endothelial cells. The resulting aortic rings were then placed into fresh collagenase/ elastase solution, rinsed twice with cold Hanks’ balanced salt solution with 1% antibiotic/antimycotic (Life Technologies, Inc.), cleaned free of adhering fat and connective tissue by blunt dissection, and opened longitudinally. After preincubation of the vessels for 15–25 min at 37 °C in a 5% CO2 and 95% air atmosphere, the cells were collected by sedimentation at 1500 rpm for 6 min and resuspended in Medium 199 (Life Technologies, Inc.) containing 10% fetal calf serum and the above antibiotics. Cells were seeded into plastic tissue culture dishes (Falcon) and cultured at 37 °C in a humidified atmosphere of 5% CO2 and 95% air with medium changes three times weekly. The confluent cultures were usually obtained after 4–8 days. The confluent cultures were washed with 1× phosphate-buffered saline, harvested with 0.05% trypsin and 0.53 mM EDTA, and replated at 1.5 or 1.6 split area ratio into 90-mm tissue culture plates. Rat aortic smooth muscle cells after the first passage were used for in vitro transfection studies and for isolation of nuclear extracts. For C57/C12 and Sol-8 myotubes, cells were allowed to propagate in growth medium for 8–10 h, and then growth medium was added and removed with differentiation medium. C6/C12 and Sol-8 myotubes were harvested 36–48 h after the fusion medium change.

Plasmid Construction—Unidirectional deletions of the SMHC promoter were produced using the Erase-a-base system (Promega) in the pJRCATX vector (21). Heterologous SMHC-TK promoter constructs were made by ligating a 133-bp fragment generated by PCR corresponding to the −1594 to −1462 bp region to pBLCAT5. This produced constructs containing both sense and antisense orientations. Orinta-

The SMHC fragment was confirmed by SnaBI digestion, which is unique to this fragment. The MEF2 expression vectors for MEF2C (pCDNA1-MEF2C) (11) and MEF2B (pCGNR2-MEF2B) were described earlier (23). Constructs pJRCATX, pBLCAT5, pBLcat6, and pSV5_CAT were used as positive and negative controls in transfection analyses (14–15).

Site-directed Mutagenesis of the A/T-rich (MEF2-like) Element in the SMHC Promoter—A single base substitution of the A/T-rich element was performed by a two-step symmetrical PCR procedure using a pair of complementary template-mismatched central primers containing the desired sequence (25). Briefly, the first PCR was done with a primer (5'-GATCCTCCGATCCGGGA-3') that hybridizes to a region encompassing the BamHI site at −1584 bp and a primer (5'-GTATCTCTATATTACGTAGGAAACGCA-3') that includes the mutation of the A/T-rich sequence. A second reaction was performed with a primer (5'-TTCCTGAGGAATTCTCGC-3') that hybridizes to a region encompassing the EcoRI site at −1224 bp and a primer (5'-GGGTCTTCTATTAGAATAC-3') that is the complementary sequence of the above-mentioned MEF2-like (A/T-rich) mutated sequence. The products of both reactions were mixed together, and a third reaction was carried out with the underlined primers. PCR products corresponding to the full-length product was cloned once into plasmid pCR1 (Invitrogen) and digested with BamHI and EcoRI, and then the BamHI-EcoRI and EcoRI-HindIII (which contains the proximal promoter region including the TATA box) fragments were subcloned into the pJRCATX vector. The presence of the correct mutations was verified by sequence analysis before cloning into pJRCATX.

Western Blot Analyses—For Western blots, equivalent quantities of nuclear extracts from (second passage) primary rat aortic smooth muscle cells were separated through a 10% SDS-polyacrylamide gel as described previously (20). Proteins were then transferred to nitrocellulose, incubated with polyclonal antibodies at 1:1000 dilutions, and detected with an enhanced chemiluminescence kit (ECL, Amersham Corp.). Antibodies to MEF2A (GST-MEF2A-(129–263)) and MEF2B (GST-MEF2B-(88–365)) were obtained from Dr. Yie-Teh Yu. MEF2A and MEF2C were from the laboratory of Olson and co-workers (20). The rabbit antisera to MEF2B and MEF2D were provided by Dr. Ron Prywes and have been described previously (23).

DNA Transfections—Transient transfections of the SMHC promoter constructs were performed using the calcium phosphate co precipitation method (21). Briefly, duplicate dishes of cells (5 × 105 cells/dish) were transfected with 15 μg of the SMHC constructs plus 5 μg of pMSV-bgl into cultures of rat aortic smooth muscle cells and NIH3T3 fibroblasts. To determine the role of MEF2 proteins, cotransfections were performed using an expression plasmid pCGNR2-MEF2B (23) and pCDNA1-MEF2C (11). DNA used for transfections was purified by two successive CsCl gradient density centrifugations. After 4–14 h, cells were washed twice with phosphate-buffered saline (137 mM NaCl, 27 mM KCl, 8 mM NaH2PO4, and 1.5 mM KH2PO4), and fresh growth medium (10% fetal calf serum) was added. Rat aortic smooth muscle cells and NIH3T3 fibroblasts were harvested 48 h post transfection.

Chloramphenicol Acetyltransferase (CAT) Assays—The transfected cells were rinsed twice with cold Hanks’ balanced salt solution and harvested in release buffer (40 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 150 mM NaCl). The cells were lysed in 250 mM Tris-HCl, pH 7.5, by three cycles of freeze-thawing, and the cell lysate was used for the enzyme assays. β-Galactosidase assays were performed on 30% of the cell extract to provide values of relative transfection efficiency. The resulting values were used to normalize the amount of extract added to the subsequent CAT assays. The reaction mixture contained 20 μl of the extract in a 150-μl assay containing 4 mM acetyl coenzyme A (Sigma) and 0.05 mCi of [14C]chloramphenicol (54 mCi/mmol; Amersham Corp.) in 250 mM Tris-HCl, pH 7.8. The reaction was stopped and extracted with 1 ml of ethyl acetate. The organic phase was dried, suspended in 20 μl of ethyl acetate, and spotted onto a silica gel thin-layer plate. The chromatogram was developed in a chloroform/methanol (95:5) system, dried, and autoradiographed. For quantitating the conversion of chloramphenicol to its acetylated forms, the spots were excised, and the radioactivity was measured in a liquid scintillation counter. CAT activity for each promoter construct was determined by at least three separate transfection experiments using two different DNA concentrations. The construct pSV5_CAT was used as a positive control (26).

Preparation of In Vitro Translated MEF2 Proteins, Vascular Smooth Muscle Nuclear Extracts, and Gel Mobility Shift Assays—In vitro transcriptions and translations were performed together in a coupled rabbit reticuloocyte lysate system (Promega) according to the manufacturer’s recommended conditions. The T7 promoter was used in plasmids pCDNA1-
MEF2A, pCDNA1-MEF2C, pCDNA1-MEF2D1a or pCDNA1-MEF2D1b, and pCI-MEF2B (described in Refs. 8, 11, and 27) to give transcription products followed by translation products of MEF2A, MEF2C, MEF2D, and MEF2B, respectively. Nuclear extracts from cultured rat aortic smooth muscle cells were prepared essentially as described by Dignam et al. (28). The protein concentration was determined by the Bradford assay (29). Gel mobility shift assays were performed as described previously (21). A 133-bp fragment that contains the A/T-rich element (see Fig. 1B) was 3’-end-labeled with [α-32P]dATP using DNA polymerase (Klenow fragment, New England Biolabs Inc). Briefly, 10–20 μg of nuclear extract was incubated in a total volume of 30 μl for 30 min at room temperature in the presence of 1 ng of radiolabeled fragment or double-stranded oligonucleotides and 2 μg of poly(dI-dC) and analyzed by electrophoresis on 4 and 5% polyacrylamide gels. Competition experiments were performed with a 100–500-fold molar excess of specific or nonspecific unlabeled DNA oligomers. The oligonucleotides used as probes or competitors in the gel mobility shift assays were as follows: SMHC-A/T-rich, CTGGGGGTATTAATATAAGACCC; MEF2A, CTGGGGGTATTAATATAAGACCC; MEF2B, CTGGGGGTATTAATATAAGACCC; MEF2C, CTGGGGGTATTAATATAAGACCC; MEF2D, CTGGGGGTATTAATATAAGACCC; E-box (E3), CCACGCACACGTGGTTGCA; MCK-MEF2, GCTCTAAAAATAACCCTG; and αMHC-MEF2, CTTCTGATTAAATAACTAAGG. To determine whether these protein families bind to the A/T-rich element in the 133-bp fragment, antibodies that react specifically to the MEF2A, MEF2B, MEF2C, and MEF2D isoforms were incubated together with the DNA and nuclear extracts in the gel shift reaction. MEF2A, MEF2B, MEF2C, or MEF2D antibodies (1–2 μl) were added to a 30-μl gel shift reaction and incubated at 4 °C for 2 h. Following electrophoresis, the gels were stained in 10% acetic acid and 30% methanol, dried, and autoradiographed.

RESULTS

The Rabbit SMHC Promoter Region (−1594 to −1462 bp) Can Activate the Heterologous TK Promoter in Smooth Muscle Cells—Using promoter deletion mapping analyses, we have previously demonstrated that the region between −1594 and −1392 bp is essential for high level SMHC promoter activity in cultured smooth muscle cells (21). Deletion of this region resulted in a 52% decrease in CAT activity in smooth muscle cells. This region includes an A/T-rich element (5’TATTA-ATATA-3’) located at −1540 bp (Fig. 1A). To determine whether this region contained positive regulatory (enhancer-like) elements, the DNA from the −1594 to −1462 bp region was cloned upstream of the heterologous TK promoter-CAT plasmid (pBLCAT5) both in the sense and antisense orientations. These heterologous promoter constructs together with control vectors were transiently transfected into primary cultures of rat aortic smooth muscle cells and NIH3T3 fibroblast cells. The basal TK promoter pBLCAT5 produced low levels of CAT activity in smooth muscle and NIH3T3 fibroblast cells. The sense orientation produced an 8–10-fold increase in TK-CAT expression, whereas the antisense orientation produced an increase of 3–4-fold compared with the basal TK promoter pBLCAT5 (Fig. 2). However, the same heterologous promoter constructs did not produce elevated CAT expression in NIH3T3 fibroblast cells (Fig. 2). Thus, the SMHC promoter region (−1594 to −1462 bp) appears to function as a positive regulatory element in smooth muscle cells.

DNase I Footprinting Reveals That the A/T-rich Element Located at −1540 bp Binds Nuclear Proteins from Smooth Muscle Cells—The SMHC promoter region (−1594 to −1462 bp) described above includes an A/T-rich element (5’TATTA-ATATA-3’) located at −1540 bp (Fig. 1A). To determine the nature of protein-binding sites within the −1594 to −1462 bp DNA region, in vitro DNase I footprint analysis was carried out using nuclear extracts from cultured rat aortic smooth muscle cells. A 133-bp probe (−1594 to −1462 bp) was generated in which one of the amplification primers was 5’-end-labeled with

Fig. 1. Schematic representation of the SMHC gene promoter. A, the relative positions of various putative cis-elements are shown. A canonical TATA box is present 26 nucleotides upstream of the transcription start site. 5’UTR, 5’-untranslated region. E1–5 indicated E-boxes. The black box represents the A/T-rich element located at −1540 bp. B, the SMHC-CAT reporter constructs with or without the A/T-rich element are shown.
To determine precisely
that Bind to the A/T-rich element—
critical for maximal SMHC promoter activity.

These results indicate that the A/T-rich element is 
produced a 6.5-fold reduction (68% decrease) in reporter activ-
ity (Fig. 4).

Mutations of the A/T-rich Element Produced a Significant Decrease in SMHC Promoter Activity in Vascular Smooth Muscle Cells—To determine whether the A/T-rich element located at 
-1540 bp is critical for SMHC promoter activity, the A/T-
rich element was modified in the p1548-CAT plasmid using 
site-directed mutagenesis. A point mutation was introduced 
into the A/T-rich element (GTATTAATA) where a single nucleotide was modified from ade-
ine to cytidine. The introduction of C in the place of A aboli-
ished MEF2-binding activity in the MCK promoter (31). The 
CAT reporter constructs containing the wild-type SMHC pro-
moter (p1548-CAT) and the mutated A/T-rich element (p1548-
CATmutA/T) were transfected into primary cultures of rat aortic smooth muscle cells, and CAT reporter activity was determined. The wild-type SMHC promoter produced a 20-fold in-
crease in CAT activity over the promoterless CAT vector (pJR-
CATX) (Fig. 4). Conversely, mutation of the A/T-rich element produced a 6.5-fold reduction (68% decrease) in reporter activity (Fig. 4). These results indicate that the A/T-rich element is critical for maximal SMHC promoter activity.

MEF2B-specific Antibody Supershifts the Protein Complexes That Bind to the A/T-rich Element—To determine precisely what protein factors bind to the A/T-rich element, we per-
formed gel shift analyses using nuclear extracts from smooth, 
skeletal, and nonmuscle cells. The 133-bp DNA fragment 
(-1594 to -1462 bp) that contained the A/T-rich element (5'- 
TATTAATAATA3') was used as a probe. As shown in Fig. 5A, 
the gel shift analyses revealed three closely migrating protein complexes with the nuclear extracts from vascular smooth muscle cells (lanes 1 and 2). However, the same probe produced a different protein binding pattern with nuclear extracts from Sol-8 myotubes, C2C12 myotubes, and NIH3T3 fibroblasts, where only a single protein complex was observed in C2C12 and 
NIH3T3 fibroblast extracts (Fig. 5B). Interestingly, a 100-fold 
molar excess of an oligonucleotide containing the A/T-rich element (5'-CTGGGGGTATTAATATAAAGACACC-3') abolished all three protein complexes (Fig. 5A, lane 3). However, an 
A/T-rich oligomer containing three nucleotide substitutions, 
5μT rich (5'-CTGGGGGCATTCTATAGACACCC-3'), did not compete protein binding at a 100-fold excess (lane 4). In addition, we performed competition assays using an oligonucleotide containing a point mutation in the A/T-rich core se-
cquence (which was shown to decrease SMHC promoter activity 
in the transient transfection analyses) (Fig. 4). This oligonucle-
otide also failed to compete protein binding at 10-, 20-, 
and 100-fold molar excesses (data not shown), suggesting that any 
mutation to the A/T-rich core region abolishes protein bind-
ing. An E-box oligonucleotide corresponding to the E-box se-
cquence located at -1516 bp did not act as a competitor (Fig. 5B, 
lane 2). To our surprise, a MEF2 consensus oligonucleotide 
derived from the MCK promoter (5'-GATCCTCGCTCTA-
AAATACCCCTGTC-3') did not compete protein binding 
lanes 5 and 6).

To determine whether any members of the MEF2 family bind to 
this A/T-rich region in nuclear extracts from vascular smooth 
muscle cells, antibodies that react specifically to the MEF2A, 
MEF2B, MEF2C, and MEF2D isoforms were incubated to-
gether with the DNA and proteins in the gel shift reaction. As 
shown in Fig. 5A, MEF2B antibody alone supershifted the three protein complexes (lanes 9 and 10), whereas the antibodies to 
MEF2A, MEF2C, and MEF2D did not interfere with protein binding (lanes 7, 8, and 11-14). MEF2B-specific antibody from 
two different sources (obtained from Drs. Yu and Prywes) gave 
the same result, supporting that MEF2B is present in this 
complex. The entire complex is shifted by MEF2B antibody, 
suggesting that other MEF2 proteins (MEF2A, MEF2C, 
and MEF2D) are not part of the protein complexes that bind to the 
A/T-rich element. These results unambiguously demonstrate that 
the MEF2B protein isoform is contained in the A/T-rich binding complex in smooth muscle cells, but not in skeletal 
muscle cells. The pretreatment with MEF2B antibody also 
produced the same supershift pattern (Fig. 5B, lane 3), sug-
gestng that MEF2B antibody does not interfere with protein 
binding to the A/T-rich element. In addition, an oligonucleotide 
corresponding to the E-box element located at -1581 bp did not compete protein binding (Fig. 5B, lane 2).

The MEF2 Family of Transcription Factors (MEF2A, 
MEF2B, MEF2C, and MEF2D) Is Present in Nuclear Extracts

Fig. 2. The SMHC promoter region (-1594 to -1462 bp) up-regulates the TK-CAT reporter in cultured rat aortic smooth muscle 
cells. A, schema showing the -1594 to -1462 bp region linked in both sense and antisense orientations to the TK-CAT reporter. AAUAA 
represents polyadenylation signals in the plasmid. B, SMHC-TK-CAT reporter activity in vascular smooth muscle cells (VSMC) and NIH3T3 
fibroblasts. The CAT data represent the averages of at least three transient transfection experiments in both cells and are represented as the -fold 
activity over the pBLCAT5 vector. Each error bar represents the S.E.
Role of MEF2B in SMHC Gene Expression

To determine whether MEF2B protein can positively regulate the SMHC promoter, we cotransfected the SMHC promoter constructs (p1548-CAT, p1392-CAT, and pJRCATX) together with the MEF2B expression vector pCGNR2 (23) into cultured rat aortic smooth muscle cells and NIH3T3 fibroblasts. In response to MEF2B overexpression, the construct p1548-CAT, which includes the MEF2B-binding site, showed a 61% increase in promoter activity, whereas the promoter construct p1392-CAT, which lacks the A/T-rich element, failed to show an increase in promoter activity in cultured rat aortic smooth muscle cells (Fig. 8). On the other hand, MEF2B cotransfected with p1548-CAT and p1392-CAT into NIH3T3 fibroblasts did not significantly increase SMHC promoter activity, which is very low in fibroblasts. Cotransfection of the MEF2C expression vector (pCDNA1-MEF2C) failed to increase SMHC promoter activity significantly both in smooth muscle cells and in NIH3T3 cells (data not shown). These results demonstrate that MEF2C can up-regulate SMHC promoter activity in smooth muscle cells, but not in NIH3T3 fibroblast cells. These data further suggest that MEF2B function is dependent upon other tissue-specific cofactors.

**DISCUSSION**

The goal of this study was to characterize cis-regulatory elements important for SMHC gene expression. In this study, we demonstrated that the SMHC promoter region (−1594 to −1462 bp), which contains the A/T-rich element, is capable of activating a heterologous (TK) promoter in smooth muscle cells, but not in fibroblasts. Point mutation of this A/T-rich element reduces SMHC promoter activity significantly in vascular smooth muscle cells. Gel mobility shift analyses demonstrated that the probe (133 bp) containing the A/T-rich element binds to three closely migrating protein complexes from smooth muscle cell nuclear extracts, whereas nuclear extracts from skeletal muscle showed a different protein binding pattern. We further demonstrated that the protein complexes were super-shifted by an antibody specific to MEF2B protein, indicating that MEF2B is a component of a smooth muscle-specific complex that binds the A/T-rich element in the SMHC promoter. By Western blot analyses, we demonstrated that all four MEF2 isoforms (A, B, C, and D) were present in nuclear extracts from rat aortic smooth muscle cells. Therefore, the preferential binding of MEF2B over the other MEF2 factors. Furthermore, our data suggest that MEF2B overexpression can activate the SMHC promoter in smooth muscle cells, but not in NIH3T3 fibroblast cells. These findings taken together suggest that MEF2B protein may play an important role in regulating SMHC gene expression in smooth muscle cells.

In this study, we have shown that the antibody specific to MEF2B protein can recognize the protein complexes formed on the A/T-rich element. This is a novel finding and suggests that...
MEF2 transcription factors might be important in the regulation of smooth muscle-specific gene expression. MEF2B mRNA is expressed in brain, heart, and skeletal muscle (7) and in smooth muscle (20). However, the precise role of MEF2B protein in transcriptional regulation is not well understood. Initial studies showed that MEF2B protein did not bind DNA efficiently (6, 7). Nevertheless, forced expression of MEF2B resulted in expression of a reporter gene containing multimers of the MEF2 recognition sequence. These studies suggested that MEF2B may be heterodimerizing with other MEF2 isoforms to

**FIG. 4.** Mutation of the A/T-rich element decreases SMHC promoter activity in rat aortic smooth muscle cells. 15 µg of the SMHC-CAT reporter plasmids (schematically represented on the left) and 5 µg of the pMSV-βgal reference plasmid were transfected into cultures of primary vascular smooth muscle cells, and CAT and β-galactosidase activities were determined. CAT activities, corrected for differences in transfection efficiencies, were normalized to the CAT activity observed following transfection of the promoterless control plasmid, pJRCATX. A representative CAT assay is shown in the middle, and relative CAT activities ± S.E. are shown on the right.

**FIG. 5.** Gel mobility shift analyses of the 133-bp SMHC promoter fragment (−1594 to −1462 bp) containing the A/T-rich element. A: lanes 1 and 2, 20 and 10 µg of nuclear extracts from rat aortic smooth muscle cells, respectively; lane 3, a 100-fold molar excess of unlabeled oligonucleotides for the A/T-rich element; lane 4, a 100-fold molar of mutant oligonucleotides for the A/T-rich element; lanes 5 and 6, 100- and 500-fold molar excess of the MCK-MEF2 oligonucleotides, respectively; lanes 7–14, antibodies specific to MEF2A, MEF2B, MEF2C, and MEF2D as shown. B: lanes 1–4, 10 µg of vascular smooth muscle cell (VSMC) nuclear extracts (lane 3 was preincubated with MEF2B antibody); lane 5, 10 µg of Sol-8 myotube nuclear extracts; lane 6, 10 µg of C2C12 myotube nuclear extracts; lane 7, 10 µg of NIH3T3 fibroblast nuclear extracts. The following oligonucleotides were used as unlabeled competitors in the gel mobility shift assay: SMHC-A/T-rich, 5′-CTGGGGAATTAATACAACCC-3′; μA/T-rich, 5′-CTGGGGCATTCCATAAGACCC-3′ (mutated bases in the A/T-rich sequence are underlined); E-box (E3), 5′-CCACGCACACGTGGTTGCAG-3′; and MCK-MEF2, 5′-GATTCGCTCTAAAATAACCCCTGTCG-3′.
become a transactivator. Recently, Molkentin et al. (27) demonstrated that MEF2B protein can bind directly to the MCK-MEF2 consensus sequence and that MEF2 transcription factors can interact with the MyoD-E12 protein complex and cooperate in activating muscle-specific gene expression. In this study, we observed that the probe containing the A/T-rich region revealed three closely migrating protein complexes only in smooth muscle nuclear extracts. Interestingly, all three protein complexes were supershifted by MEF2B antibody. This raises the possibility that MEF2B protein may be complexing with other proteins to produce these complexes. Alternatively, we should also consider the possibility that these multiple protein complexes are due to phosphorylated forms of MEF2B protein (27). Although MEF2B protein is known to be expressed in Sol-8 and C2C12 skeletal muscle cells, the nuclear extracts from these cells did not produce the same protein binding pattern. This finding also supports the idea that MEF2B binding may depend upon cell type-specific interacting proteins. DNase I footprinting with smooth muscle nuclear extracts revealed extended protection including the A/T-rich element and its flanking nucleotides, further supporting multiple protein binding in the A/T-rich region.

A double-stranded oligonucleotide containing the A/T-rich element effectively competed all three protein complexes, whereas the oligonucleotides containing mutations of the A/T-rich core sequence (TATTAAAT) failed to act as competitors. These results suggest that direct protein interaction with the A/T-rich element is critical for the formation of the three protein complexes. In this study, we have shown that in vitro translated MEF2 family members (MEF2A, MEF2B, MEF2C, and MEF2D) can directly bind to the A/T-rich element. However, the binding affinity for the SMHC-A/T-rich element is much lower compared with the MCK-MEF2 consensus element, but comparable to the αMHC-MEF2 site. Although other MEF2 family members (MEF2A, MEF2C, and MEF2D) are also expressed in smooth muscle cells, they do not bind to this A/T-rich element in smooth muscle nuclear extracts, which suggests that MEF2B binding specificity may depend upon the interacting proteins present in smooth muscle cells.

MADS box proteins have been shown to be highly interactive molecules in both yeast and higher organisms (reviewed in Ref. 32). Recently, it has been shown that the MADS box protein MEF2C physically interacts with the myogenic bHLH proteins as a heterodimer with E-box proteins (17). The MADS box protein SRF has also been shown to physically interact with this same heterodimer formed between the myogenic bHLH proteins and E-box proteins (33). Furthermore, MEF2 proteins have also been shown to interact with the bHLH protein Twist (34) as well as with the bHLH protein MASH1 (35). Together, these results suggest that MADS box proteins are interactive molecules that complex with other cell type-specific transcript-
tion factors to direct tissue-restricted gene expression.

To date, transcription factors that are unique to smooth muscle cells have not been identified, and smooth muscle-specific gene expression remains underexplored. However, the recent isolation and characterization of the smooth muscle-specific gene promoters for smooth muscle α-actin (36–38), smooth muscle γ-actin (39), SM22α (40, 41), calponin (42, 43), and SMHC provide unique opportunities for dissecting the various cis- and trans-regulatory factors involved in smooth muscle cell-specific gene expression. Studies using the above-mentioned promoters have identified a number of known cis-elements including the CArG box, E-box, GATA-binding site, AP-2, SP1, and A/T-rich element. In particular, CArG elements that bind SRF have been shown to be important for smooth muscle α- and γ-actin gene expression (36, 37, 44). Similarly, CArG box elements are found in the SM22α proximal promoter and may be important for its regulation in myogenic lineages (41). On the other hand, there are no CArG elements in the human calponin gene, which is expressed exclusively in smooth muscle-containing tissues in adult stages.

The SMHC promoter region (−1281 to −1038 bp) contains three CArG box-like elements (Fig. 1) and is conserved between mouse, rat, and rabbit promoters. This region is found to be necessary for maximal SMHC promoter activity (45, 46).2 The CArG box element is known to bind SRF or related factors; however, the precise role of SRF transcription factors in smooth muscle-specific gene expression remains to be explored. Future experiments will be focused toward identifying cis/trans-factors responsible for smooth muscle cell-specific gene expression.

In summary, the data presented here suggest that MEF2B protein may play an important role in regulating SMHC gene expression in smooth muscle cells. We propose that MEF2B protein may interact with other transcription factors in controlling the smooth muscle-specific expression of this gene. Future experiments will attempt to identify and characterize the protein factors that interact with MEF2B protein.

Acknowledgments—We thank Dr. Ron Prywes for MEF2 expression vectors (pCGNR2) and antibodies to MEF2B and MEF2D and Dr. Yie-Teh Yu for antibodies to MEF2A and MEF2B. We thank Alla Zilberman for expert technical assistance.

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