MOLECULAR AND CELLULAR BIOLOGY, June 1995, p. 3415–3423 Vol. 15, No. 6
0270-7306/95/$04.00+0

Serum Induction of MEF2/RSRF Expression in Vascular Myocytes Is Mediated at the Level of Translation

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Received 27 October 1994/Returned for modification 16 January 1995/Accepted 24 March 1995

Vascular smooth muscle cells (VSMCs) reversibly coordinate the expression of VSMC-specific genes and the genes required for cell cycle progression. Here we demonstrate that isoforms of the MEF2/RSRF transcription factor are expressed in VSMCs and in vascular tissue. The MEF2A DNA-binding activity was upregulated when quiescent VSMCs were stimulated to proliferate with serum mitogens. The serum-induction of MEF2A DNA-binding activity occurred approximately 4 h following serum activation, and this correlated with an increase in the level of MEF2A protein without changes in the level of MEF2A mRNA or protein stability. These results indicate that MEF2A induction by serum is regulated at the level of translation.

In contrast with adult skeletal and cardiac muscle cells which are terminally differentiated, vascular smooth muscle cells (VSMCs) can respond to growth factor stimulation by reentering the cell cycle. Following mitogen activation VSMCs express lower levels of contractile proteins and appear to resemble their fibroblast-like precursor cells that occur during early blood vessel development (28, 34). In adults these phenotypically modified VSMCs contribute to cardiovascular pathologies in which their excessive growth and deposition of extracellular matrix can lead to vessel occlusion. Very little is known about the nuclear processes that determine the state of VSMC differentiation or mechanisms by which these cells reversibly coordinate the expression of VSMC-specific and cell cycle genes.

One well-characterized model of myocyte differentiation is the specification of the skeletal muscle cell lineage by the MyoD family of basic helix-loop-helix transcription factors. These factors bind to a consensus DNA sequence, CANNTG, that is referred to as an E box (10, 24, 52). However, many muscle-specific genes do not contain functional E-box sequences, and the MyoD family of transcription factors have not been identified in the cardiac or the smooth muscle cell lineage, raising the possibility that other nuclear factors are involved in these processes. Recently, the transcription factors dubbed "related to serum response factor" (RSRF) or myocyte-specific enhancer factor 2 (MEF2) have been implicated in striated muscle differentiation and transcriptional control. This group of transcription factors are encoded by the four genes MEF2A, -B, -C, and -D (nomenclature as suggested in reference 3), which are alternatively spliced. All of these factors contain the highly conserved MADS domain that was originally identified in the yeast MCM1 factor, which controls cell type switching in response to pheromone binding to cell surface receptors (18, 39), and in the serum response factor (SRF), which regulates the mitogen-induced expression of the c-*fos* proto-oncogene (37) and the muscle-specific expression of the α-actin genes (16, 50, 51). The MEF2 proteins bind to a consensus AT-rich sequence, (C/T)T(A/T)(A/T)AAATA(A/G), which is widely found in the control regions of muscle-specific and growth factor-induced genes (5, 11, 15, 17, 19, 25, 33, 35, 36); however, these sites are bound by non-MADS domain proteins as well (23, 56, 57). MEF2 regulates the expression of immediate-early genes, such as c-*jun*, that are rapidly activated in quiescent cells by mitogen stimulation (17, 39). In *Drosophila melanogaster*, the single MEF2 gene is indispensable for skeletal, cardiac, and smooth muscles (26). In vertebrates, MEF2 DNA-binding activity is upregulated during myogenesis, and MEF2D expression precedes the expression of myogenin upon C2C12 myoblast differentiation (3). Although the question of whether MEF2 is located upstream or downstream of the myogenic basic helix-loop-helix transcription factors is controversial, evidence suggests that these factors are cross-regulated and that ectopic MEF2 expression can initiate skeletal myogenesis (6, 20, 54).

Since the MEF2 transcription factors control the expression of myocyte-specific and mitogen-activated genes, we initiated experiments to examine the expression of this factor in VSMCs and to test whether its expression is modulated during mitogen-induced dedifferentiation. Here we document the expression of MEF2A in cultured VSMCs and in vascular tissue. Given the purported role of MEF2 in striated muscle differentiation, we expected that its expression might be higher in contractile quiescent cells than in proliferating smooth muscle cells. Instead, surprisingly, MEF2A expression was upregulated when quiescent VSMCs were stimulated to proliferate with mitogens. This activation correlates with an increase in MEF2A protein levels but not mRNA levels, indicating that the upregulation is mediated by a translational control mechanism.

MATERIALS AND METHODS

cDNA library screening and sequence determinations. An adult human aorta cDNA library in λgt11 (Clontech) was screened under conditions of low stringency with the MADS domain of human MEF2A cDNA which was amplified by PCR. Approximately 500,000 phage plaques in *Escherichia coli* were adsorbed in duplicate to nitrocellulose membranes and hybridized with human MEF2A MADS domain cDNA labeled with [α-^{32}P]dCTP in a mixture containing 0.5 M sodium phosphate (pH 7.0), 7% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 1% bovine serum albumin. The filters were successively washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and 0.5× SSC–0.1% SDS at room temperature for 15 min and exposed to X-ray film. A rat

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aorta cDNA library in λZAP (22) was also screened as described above. The MADS-MEF2 domains of mouse MEF2A, -B, and -D (kind gifts from E. N. Olson) were used as probes. Positive signals were isolated and rescreened until they were plaque purified. The inserts were excised from λgt11 phage by digesting with *Eco*RI and subcloned into the Bluescript plasmid vector. The inserts in the λZAP vector were excised by the protocol recommended by the manufacturer (Stratagene). The structure of the double-stranded phagemid insert cDNAs was determined by the cycle sequencing method using fluorescent dideoxy terminator nucleotides with an Applied Biosystems 373A automated DNA sequencer. Sequences were analyzed on Macintosh Quadra computers with MacVector and Sequence Navigator software. Further analyses of derived sequences were performed with the Blast algorithm to search the latest versions of the GenBank and EMBL databases by sending the sequence by electronic mail via the Internet.

Cell culture. The internal mammary artery or saphenous vein was the source of cultured human VSMCs prepared by the explant method (46). In brief, adventitia and endothelium were removed by scraping with a scalpel, and the vessels were cut into small pieces with a blade. The fragments were placed on a 60-mm-diameter culture dish with adventitia on top and incubated at 37°C in Dulbecco's modified Eagle medium (DMEM) containing 15% fetal bovine serum (FBS) for 1 week. The VSMCs migrating from the fragments were trypsinized and subcultured in DMEM containing 10% FBS (high-serum medium). When cultured cells reached 50 to 60% confluence, the medium was replaced with low-serum medium, DMEM with 0.2% FBS, and the cells were incubated at 37°C for 3 to 4 days in a humidified incubator to induce quiescence. Primary cultures of rat smooth muscle cells were prepared from thoracic aortas of adult male Sprague-Dawley rats by the method of Mader et al. (27). A10 and A7r5 cells (American Type Culture Collection), which are rat embryonic smooth muscle cell lines, were also cultured in DMEM with 10% FBS.

RNA isolation and quantitative RT-PCR. RNA was isolated from human VSMC cultures by the acid guanidinium thiocyanate-phenol-chloroform extraction method (7). One microgram of total RNA, extracted from quiescent and serum-stimulated human VSMC, was processed by the reverse-transcriptase reaction (RT) and subjected to the PCR procedure according to the directions of the manufacturer (Perkin-Elmer). A glyceraldehyde-3-phosphate dehydrogenase (G3PDH) transcript was amplified as an internal standard. PCR was performed in a 25-μl reaction volume containing 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM deoxyribonucleotide triphosphate mixture, 0.3 μM sense and antisense primers, 5 μCi of [α-^{32}P]dCTP, and 0.5 U of *Taq* DNA polymerase. The amplification conditions were 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 20 cycles for G3PDH and 25 cycles for MEF2A. Primer sequences are as follows: 5′-GCAAAGTCATGCCTACAAAGT-3′ (human MEF2A sense primer), 5′-GGCAAGCTTGGGGTTGTCACA-3′ (human MEF2A antisense primer), 5′-TGAAGGTCGGAGTCAACGGAT-3′ (G3PDH sense primer), and 5′-TGATGGCATGGACTGTGGTCA-3′ (G3PDH antisense primer).

Analysis of alternatively spliced isoforms of MEF2 in the rat aorta. In order to examine alternatively spliced isoforms of MEF2 in smooth muscle cells, RNA was extracted from rat tissue and RT-PCR was carried out by the method described above. Primers were designed to amplify the two alternatively spliced exons in the coding region (see Fig. 2). The amplification conditions were 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min for 40 cycles. The PCR-amplified products of the amino-terminal exon (flanked by P1 and P2 in Fig. 2) were subcloned into the Bluescript plasmid vector and sequenced to determine which exon was included. Primer sequences are as follows: 5′-GAATTCTTCAACAGCTCTAACAAG-3′ (P1), 5′-GGATCCACTCCCTGGGTTAGTGTA-3′ (P2), 5′-AAGTCATGCCTACAAAGTC-3′ (P3) and, 5′-GGCAAGCTTGGAGTTGTCA-3′ (P4).

Electrophoretic mobility shift assay. Whole-cell extracts were prepared from human VSMC and A10 and A7r5 cells as described elsewhere (31). In brief, cells were washed twice in phosphate-buffered saline (PBS), removed from culture dishes by scraping, and collected by centrifugation. The pellet was resuspended in an equal volume of 2× lysis buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 7.8], 0.6 M KCl, 1 mM dithiothreitol, 20% glycerol, 2 mM EDTA, 2 μg of leupeptin per ml) and subjected to three cycles of freezing and thawing. After centrifugation at 16,000 × *g* for 10 min at 4°C, the supernatant was stored at −80°C. Protein concentration was measured by the Bradford method according to the directions of the manufacturer (Bio-Rad).

Electrophoretic mobility shift assays were carried out in reaction mixtures containing 5 to 10 μg of the extract, 20 fmol of the probe, 1 μg of poly(dI-dC), and 200 ng of a single-stranded oligonucleotide as nonspecific DNA competitors. Electrophoresis was carried out on 5% nondenaturing polyacrylamide gels with 0.5× TBE (0.45 M Tris[hydroxymethyl]aminomethane, 0.45 M boric acid, 0.01 M EDTA) in a circulating-water-cooled gel box. Probes and competitor DNAs were double-stranded, synthetic oligonucleotides, and the coding-strand sequences are as follows:

MEF2 (MCK)	5′-CGCT**CTAAAAATAA**CCCT-3′
Gax MEF2	5′-CTCACATTACACT**CTATTTATAA**CCTCTCAGAGCC-3′
MEF2 mutant (Gax)	5′-CACACT**CggTTTATAA**CCTCTC-3′
CArG (skeletal actin)	5′-GCCCGACAC**CCAAATATGG**CGACGGCCG-3′

Probes to MEF2 sites were prepared from the mouse muscle creatine kinase (MCK) or the mouse *Gax* genes, and the MEF2 mutant was derived from the MEF2 site in the *Gax* gene promoter. The CArG probe was prepared from the chicken skeletal actin promoter. The conserved protein binding motifs are boldfaced, and the mutated nucleotides are lowercased. One picomole of each probe was 5′ terminally labeled with [γ-^{32}P]ATP and used at a concentration of 10 fmol/μl.

Dissociation constants for MEF2-DNA complexes were determined by electrophoretic mobility shift assay. A constant amount of cell extracts from quiescent or serum-stimulated human VSMCs was incubated with increasing amounts of the MEF2 probe. The probes used in this assay were purified with Bio-Spin 6 columns according to the directions of the manufacturer (Bio-Rad). After electrophoresis, the bands corresponding to MEF2-probe complexes and free probes were scanned with a Phosphorimager (Molecular Dynamics) and Scatchard analysis was performed.

Immunoprecipitation. After the human VSMC culture was left in low-serum medium for 3 days, medium was replaced with fresh high- or low-serum medium lacking methionine, and 350 μCi of [^{35}S]methionine (1,000 Ci/mmol) was added simultaneously. The cells were harvested 12 h later, and whole-cell extracts were made by the method described above. The extracts were mixed with 0.45 ml of RIPA buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS) plus 2.5 μl of preimmune serum and incubated on ice for 1 h, following which, an aliquot of protein A-agarose (Boehringer Mannheim) was added and the mixtures were rocked for 30 min at 4°C. After centrifugation, the supernatants were transferred to new tubes, and 2.5 μl of anti-MEF2A antibody or preimmune serum was added. Antibodies were raised against a glutathione *S*-transferase–MEF2A fusion protein containing MEF2A amino acids 129 to 253. After rocking at 4°C for 1 h, an aliquot of protein A-agarose was added and the mixtures were rocked at 4°C for 1 h. Proteins associated with the protein A-agarose were precipitated by centrifugation, and applied to an SDS–10% polyacrylamide gel. After electrophoresis, the gel was exposed to Enlightning (DuPont) for 20 min, dried, and subjected to autoradiography.

Western blot (immunoblot) analysis. Whole-cell extracts were prepared from quiescent and serum-stimulated human VSMCs by the method described above. Thirty microgram samples of the extracts were applied to an SDS–10% polyacrylamide gel and transferred to Immobilon-P (Millipore) by semidry blotting. Filters were blocked for 1 h at room temperature in PBS–0.2% Tween 20–5% nonfat dry milk. The filters were then incubated with anti-human MEF2A antibody (Santa Cruz; 1:200 dilution) or anti-human smooth muscle α-actin antibody (DAKO; 1:200 dilution) for 1 h at room temperature in PBS–0.2% Tween 20–2% nonfat dry milk. Washes, incubations with secondary antibodies, and visualization of the immune complexes were carried out as recommended by the manufacturer (Enhanced Chemiluminescence kit; Amersham).

Plasmid transfections. The truncated mouse c-*fos* promoter (47), positions −56 to +109, was subcloned upstream of the luciferase gene (Δ56 luciferase), and a MEF2 binding site from the muscle creatine kinase gene was inserted at a *Sal*I site which is located directly upstream of the promoter (MEF2/Δ56 luciferase). This truncated promoter fragment included the TATA element. A10 cells growing in DMEM–10% FBS were transfected with 1.7 μg of Δ56 luciferase or MEF2/Δ56 luciferase and 0.3 μg of an internal control pSV2-alkaline phosphatase by using DOTAP (Boehringer Mannheim). After 5 h, medium was replaced with low-serum medium, and cells were incubated for 3 days. To examine the effect of serum stimulation on reporter gene expression, some plates were incubated for 12 h in the high-serum medium, and cells were harvested and luciferase and alkaline phosphatase activities were determined.

For anti-MEF2 antibody cross-reactivity studies, 10 μg each of pMT2-human MEF2A, -human MEF2C, and -human MEF2D were transfected in Cos cells and whole-cell extracts were prepared as described above. Those protein extracts were used in electrophoretic mobility-antibody supershift assays and in Western blot analyses to examine the specificity of the anti-human MEF2A antibodies.

RESULTS

MEF2 cDNA structures from VSMCs. To initiate studies on the expression of MEF2 in vascular myocytes, cDNAs for this transcription factor were isolated from human and rat aorta libraries and their structures were determined. Libraries were screened under conditions of low stringency with MEF2 probes from mouse or human genes. Screening of the human aorta library with the human MADS domain probe of MEF2A resulted in the isolation of 14 positive clones, all of which en-

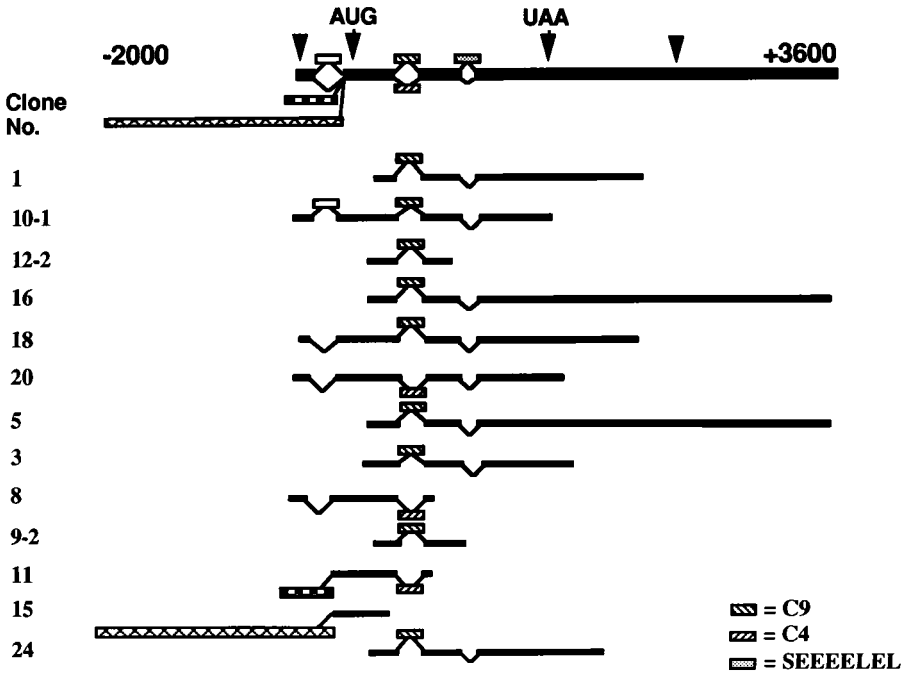

FIG. 1. MEF2A cDNAs isolated from the human aorta. Open, crosshatched, and stippled bars, previously identified exons including those reported for RSRF C4 and RSRF C9. Protein coding sequences occur between the start and stop codons (arrows). Sequences outside unlabeled arrowheads have not been reported previously.

coded isoforms of the MEF2A gene. Some of these cDNA clones had long tracts of 5′ or 3′ untranslated regions (UTRs) that had not been previously identified. These data are summarized in Fig. 1. Of particular note, all MEF2A cDNAs isolated from the human and rat aorta libraries lacked the SEEEELEL miniexon sequence that has been reported for MEF2A transcripts expressed in the brain, heart, and skeletal muscle. Further, cDNA clones 11 and 15 contained novel upstream sequences that have not been reported previously (55).

Rat MEF2 cDNA molecular clones were also isolated and analyzed. MEF2A isoform cDNAs were isolated from an aortic smooth muscle library regardless of whether it was screened with cDNA probes to MEF2A, -B, or -D. These partial rat cDNAs also lacked the SEEEELEL miniexon and corresponded to MEF2A homologs of the human RSRFC9 and RSRFC4 cDNAs (39). The human C9 and C4 isoforms differ because of alternative splicing at the most N-terminal exon within the protein coding region (see Fig. 2). PCR primers, designated P1 and P2, were synthesized to the flanking regions of this alternatively spliced exon and used to amplify cDNA (RT-PCR) prepared from the adult rat aortic smooth muscle. Sequence analysis of 16 cDNA subclones revealed that 10 corresponded to the C9 isoform and 6 corresponded to the C4 isoform. Comparing nucleotide and amino acid sequences from codon 17 to the stop codon, the rat MEF2A isoforms are 93% conserved on the peptide level and 76% conserved on the nucleotide level in relation to the human C9 and C4 genes.

The MEF2A isoform expressed in vascular muscle tissue lacks the SEEEELEL miniexon. The nucleotide sequence information from the rat MEF2A gene allowed the construction of specific PCR primers, designated P3 and P4, that flanked the splice site of the SEEEELEL exon. These primers were then used for the RT-PCR analysis of tissue transcripts to compare MEF2A splice patterns in the rat aortic smooth muscle, skeletal muscle, heart, brain, and liver (Fig. 2). Amplification products containing the miniexon give rise to a 186-bp amplification product, while a 162-bp amplification product is obtained from cDNAs that lack the miniexon. This analysis revealed that the SEEEELEL miniexon was excluded from the MEF2A transcripts of the aortic smooth muscle, giving rise to an amplification product identical in size to that of liver. This is in contrast to the 186-bp fragment produced from cDNAs from terminally differentiated skeletal muscle, cardiac muscle, and brain tissues that express the MEF2 protein and DNA-binding activities (55). These data are significant for two reasons. First, this splice pattern is a feature that distinguishes the MEF2A isoforms in vascular myocytes from the MEF2A isoforms expressed in terminally differentiated striated muscle

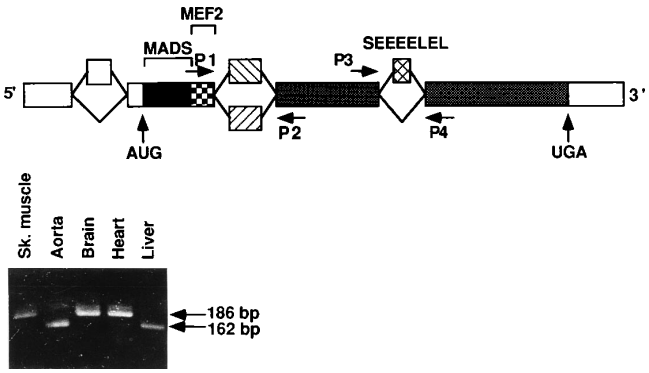

FIG. 2. RT-PCR analysis of alternatively spliced MEF2A isoforms in different rat tissues. Primers were designed to amplify the alternative coding exons (P1 to P4) in the coding region. RNA was extracted from the rat skeletal (Sk.) muscle, aorta, brain, heart, and liver. The PCR-amplified products from P3-P4 primer combinations were electrophoresed on a 2% agarose gel and stained with ethidium bromide. The positions of the 162-bp fragment (excluding the SEEEELEL-encoding miniexon) and the 186-bp fragment (including the miniexon) are indicated. The PCR-amplified products from P1-P2 primer combinations were subcloned into the Bluescript vector and sequenced.

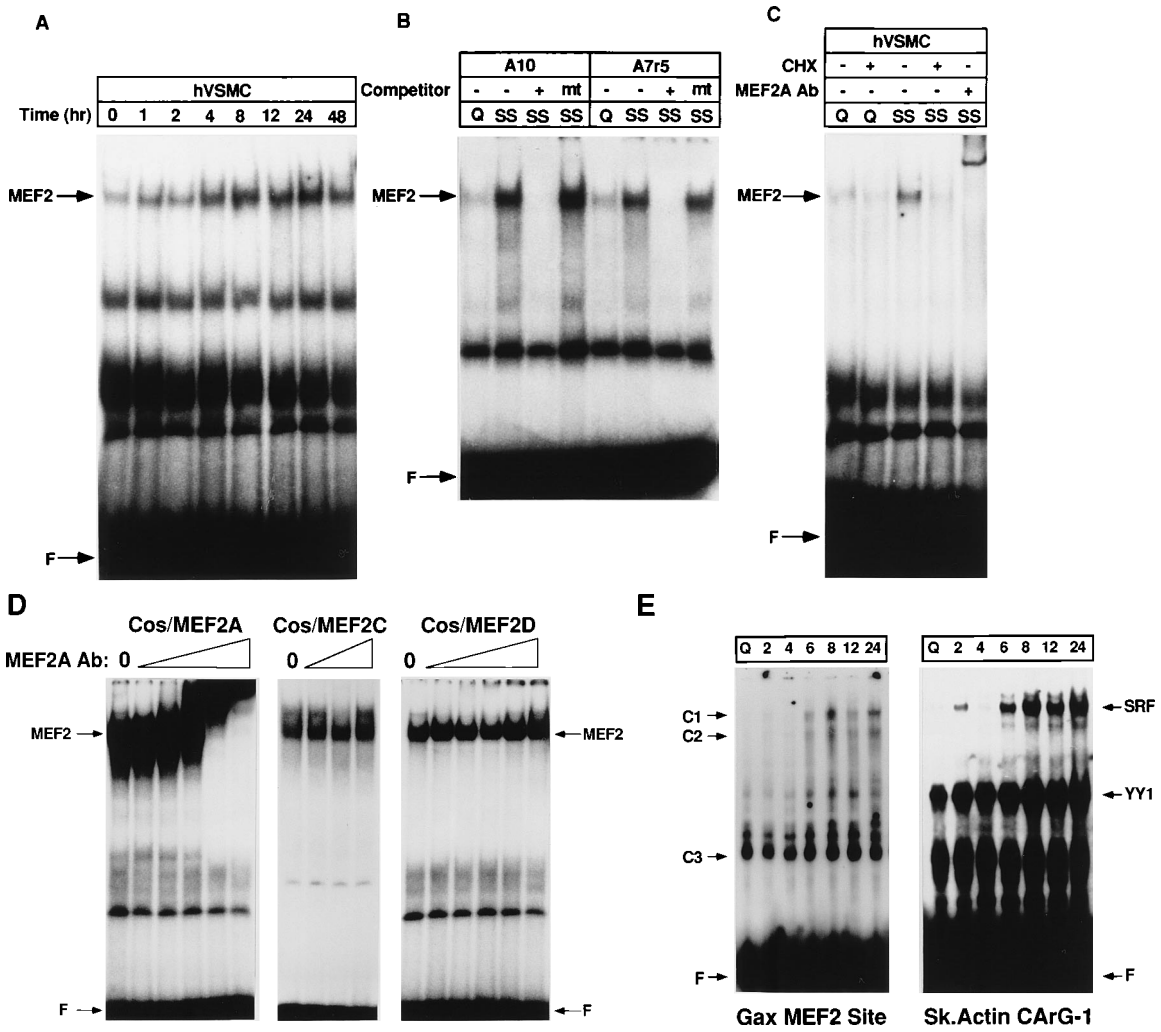

FIG. 3. Serum induction of MEF2 DNA-binding activity in VSMCs. (A) Time course of the serum induction of the MEF2 DNA-binding activity in human VSMCs (hVSMC). Human VSMCs were incubated in low-serum media for 3 days and were exposed to high-serum media for the indicated periods. (B) Serum induction of the MEF2 DNA-binding activity in A10 and A7r5 cells. The cultured cells were incubated in low-serum media for 3 days (quiescent [Q]) and stimulated with high-serum media for 12 h (serum stimulated [SS]). Electrophoretic mobility shift assays were also performed in the presence of a 100-fold molar excess of an unlabeled probe or mutant probe (mt). (C) Cycloheximide (CHX) blocks MEF2 induction. Cultured hVSMC were incubated in low-serum media for 3 days and stimulated with high-serum media for 12 h in the presence or absence of 10 μg of cycloheximide per ml. Electrophoretic mobility shift assay was also performed in the presence of anti-human MEF2A antibody (Ab). The positions of the MEF2-probe complexes and free probes (F) are indicated. (D) The anti-human MEF2A antibody does not cross-react with human MEF2C or MEF2D. Whole-cell extracts were made from Cos cells transfected with 10 μg each of pMT2-human MEF2A, -human MEF2C, and -human MEF2D. Five micrograms of the extract was preincubated with serial dilutions of the anti-MEF2A antibody, and electrophoretic mobility shift assay was performed. Binding reaction mixtures with MEF2A and MEF2D contained 0, 1/16, 1/8, 1/4, 1/2, or 1 μl of the immune serum. Binding reaction mixtures with MEF2C contained 0, 1/4, 1/2, or 1 μl of the immune serum. (E) Time course of the serum induction of MEF2, SRF, and YY1 in primary cultures of rat aortic smooth muscle cells. Primary cultures of rat VSMCs were incubated in low-serum media for 3 days and were exposed to high-serum media for the indicated periods. For each experiment in panels A through D, a constant mass of extracted protein was used in the binding reactions, but for the experiment in panel E the volumes of the extracts were adjusted to represent a constant number of cells harvested from each culture plate.

cell types. Second, it has been proposed that the SEEEELEL peptide participates in the tissue specificity of MEF2 expression on the basis of an exact correlation between the occurrence of the miniexon and the detection of endogenous MEF2 DNA-binding activity and transactivation of gene expression in the brain, skeletal muscle, and heart (55). However, since VSMCs lack the SEEEELEL peptide, but contain MEF2 protein and DNA-binding activity (see below), it appears that this mechanism cannot solely account for the tissue specificity of MEF2 expression. Finally, a faint PCR amplification product that was larger than 186 bp was also detected with the aortic smooth muscle cDNA. Nucleotide sequence analyses of this product revealed no MEF2 sequence homology outside the P3 and P4 primer sequences, indicating that this minor band arises from the artifactual amplification of a non-MEF2 cDNA.

Mitogens induce MEF2A DNA-binding activity in VSMCs. MEF2 DNA-binding activities could be detected in electrophoretic mobility shift assays using whole-cell extracts prepared from VSMCs by using a DNA probe to the MCK gene MEF2 site (Fig. 3). This DNA-binding activity was expressed at low levels in extracts prepared from quiescent cells, but higher levels of this activity could be detected in extracts prepared from serum-activated VSMCs. In passaged human VSMCs, a marked upregulation of the MEF2 DNA-binding activity occurred approximately 4 h after stimulation and this induction was maintained at 24 h poststimulation (Fig. 3A).

This upregulation of MEF2 activity was also detected in the rat smooth muscle cell lines A10 and A7r5 and in cultures of rat VSMCs (Fig. 3B and E). The relative binding of MEF2 to DNA in quiescent human VSMC extracts was compared with that in serum-stimulated extracts by measuring the quantity of the specific MEF2 nucleoprotein complex as a function of the input DNA concentration in the electrophoretic mobility shift assay. At approximately saturating levels of the DNA probe, more complex was formed with extracts from the serum-stimulated cells than from quiescent cells, but no significant difference in the relative affinity for DNA was revealed by Scatchard plot analysis (data not shown), indicating that the serum induction does not result from an increase in the affinity for DNA. Overall, MEF2 DNA-binding activity was induced between 2- and 10-fold; however, the induction was usually most notable in the extracts prepared from passaged human VSMC cultures.

The specificity of this serum-induced MEF2 DNA-binding activity was demonstrated by competition assays using whole-cell extracts from A7r5 and A10 cells (Fig. 3B) and passaged human and rat VSMCs (data not shown). A 100-fold excess of a nonlabeled probe could effectively compete for the formation of the MEF2 nucleoprotein complex, but an oligonucleotide with a mutated MEF2 site was unable to compete. Further, specific anti-MEF2A antibodies could quantitatively supershift the nucleoprotein complex formed with extracts prepared from passaged or immortalized VSMCs (Fig. 3C and data not shown). Preimmune serum did not supershift this nucleoprotein complex (data not shown). Because this anti-MEF2A antibody did not cross-react with human MEF2C and MEF2D under these assay conditions (Fig. 3D), these results suggest that the A isoforms of MEF2 contribute significantly to the nucleoprotein complex formed with VSMC extracts. Further, the induction of MEF2 DNA-binding activity was blocked by treating the cells with cycloheximide (Fig. 3C), suggesting that translation is required for the upregulation.

Serum induction of MEF2 DNA-binding activity was also examined in cultures of passaged rat VSMCs (Fig. 3E). By using the Gax MEF2 site probe, three specific nucleoprotein complexes were identified. The nucleoprotein complexes C1 and C2 appeared to contain MEF2, as indicated by their susceptibility to competition by a molar excess of a nonlabeled MCK MEF2 probe (data not shown). The C3 nucleoprotein complex was sensitive to competition with the Gax, but not the MCK, gene probe, indicating that it arose from the specific binding of a factor other than MEF2. Further, the C1 and C2 complexes, but not C3, were detected in a parallel experiment using the same extracts and a radiolabeled MCK MEF2 probe (data not shown). The time course of serum induction of MEF2 DNA-binding activity (C1 and C2) in rat VSMCs was comparable to the time course of MEF2 induction in human VSMCs. In contrast, no induction in the intensity of the C3 nucleoprotein complex was detected. Further, by using these extracts and the skeletal actin CArG motif probe, little or no induction of YY1 DNA binding was observed, but the SRF DNA-binding activity increased significantly. Similarly to MEF2, SRF DNA-binding activity increased approximately 6 h after serum stimulation, and this expression persisted for at least 24 h. The serum induction of SRF has been noted previously in other cell types (32, 37, 40), and this observation was not pursued further in this study.

Mitogens increase MEF2A protein levels but do not affect MEF2A protein stability. To demonstrate further the expression of MEF2A protein in human VSMCs and to test whether the serum induction of the MEF2 DNA-binding activity correlated with an increase in MEF2 protein, immunoprecipitation analyses were performed with ^{35}S-labeled cells and anti-MEF2A antibodies. A single protein band with a molecular mass of 67 kDa, in agreement with the reported molecular weight for MEF2A expressed in skeletal muscle (55), was detected in both quiescent and serum-stimulated human VSMC, while no band of this mass was detected in immunoprecipitates with preimmune serum (Fig. 4). The level of radiolabeled MEF2A protein increased ninefold following serum stimulation for 12 h on the basis of a constant mass of extract protein. This increase in radiolabeled MEF2A protein did not result solely from a general increase in translation, because the incorporation of the ^{35}S label into total protein increased only by a factor of 1.7 under these conditions.

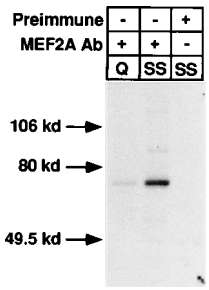

FIG. 4. Serum induction of MEF2 protein in human VSMCs. Cultured human VSMCs were incubated in low-serum media for 3 days and labeled by [^{35}S]methionine for 12 h in the presence of 0.2% FBS (quiescent [Q]) or 10% FBS (serum stimulated [SS]). A constant amount of protein extract (30 μg) was immunoprecipitated with anti-human MEF2A antibody (Ab) or a preimmune serum. The precipitated materials were separated on an SDS–10% polyacrylamide gel with molecular weight markers and detected by autoradiography. The positions of molecular weight markers are indicated.

Western blot analyses of human MEF2A were performed to corroborate the immunoprecipitation data and to demonstrate that the increase in the ^{35}S label was not the result of an increase in MEF2A protein turnover (Fig. 5). Immunoblots with anti-MEF2A antibody (Santa Cruz) detected increased MEF2 protein expression when quiescent human VSMCs were stimulated for 12 h with serum. Little or no change in smooth muscle actin protein levels was detected in these extracts, though a platelet-derived growth factor-induced decrease in ^{35}S-labeled actin protein has been reported at later time points (8). Surprisingly, two bands corresponding to MEF2 were detected in the human VSMC extracts and in Cos cells transfected with a MEF2A expression vector (Fig. 5) but not in Cos cells transfected with a MEF2C or MEF2D expression vector (data not shown). By using two independent sources of anti-MEF2A antibodies, two protein bands were reproducibly detected in Western blot analyses, but a single band corresponding to the faster-migrating protein was detected in the corresponding immunoprecipitation analyses. The nature of the more slowly migrating band in the Western blots is un-

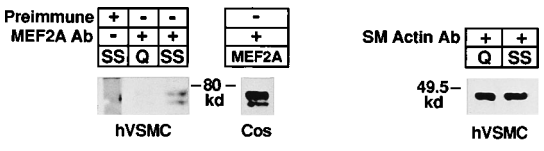

FIG. 5. Western blot analyses of human MEF2A and smooth muscle (SM) α-actin. Cultured human VSMCs (hVSMC) were incubated in low-serum media for 3 days (quiescent [Q]) and stimulated in high-serum media for 12 h (serum stimulated [SS]). Constant amounts of total protein (30 μg) were loaded in each lane. The extract from Cos cells transfected with pMT2-human MEF2A was used as a positive control. Ab, antibody.

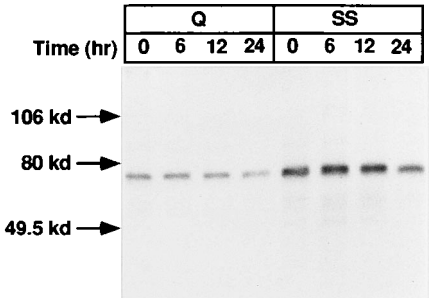

FIG. 6. Serum induction does not significantly alter the stability of MEF2A protein. Cultured human VSMCs were incubated in low-serum media for 3 days and labeled with [^{35}S]methionine for 12 h in the presence of 0.2% FBS (quiescent [Q]) or 10% FBS (serum stimulated [SS]). The labeling media were then replaced with low- or high-serum media containing nonlabeled methionine, and the cells were incubated for the indicated periods of time. A constant amount of protein extract was immunoprecipitated with anti-human MEF2A antibody and analyzed by SDS–10% polyacrylamide gel electrophoresis and autoradiography. The positions of molecular weight markers are indicated.

known, but it may represent a posttranslationally modified form of MEF2A that is not recognized by the antibody under native conditions.

To test whether serum changes the stability of MEF2 protein, human VSMCs were labeled with [^{35}S]methionine for 12 h and cold-chased with nonlabeled methionine for different lengths of time prior to the immunoprecipitation. With both the quiescent and the serum-stimulated cells, MEF2A protein levels did not change measurably at time points up to 12 h following serum stimulation, but a decrease could be detected at the 24-h time point (Fig. 6). These data reveal that the MEF2A protein is stable relative to the time course of the serum induction experiments (typically 12 h) and that serum does not significantly alter the half-life of this protein. Collectively, these data indicate that the induction of MEF2 DNA-binding activity by serum is due, at least in part, to an increase in the rate of MEF2A protein synthesis.

Mitogens do not alter steady-state MEF2A mRNA levels. Northern (RNA) blot analyses were performed to investigate whether serum-induced upregulation of MEF2A also occurred at the level of transcription or mRNA stability. Northern blot analysis with the MEF2A cDNA probe revealed a set of bands, some greater than 7 kb, that was similar to the band pattern previously reported for skeletal muscle (55). Though the band pattern or the relative intensities of the bands did not differ between the quiescent and serum-stimulated VSMCs (data not shown), the complexity of the band pattern led us to seek an alternative approach to quantify the relative levels of transcripts that encode the MEF2A protein. Quantitative RT-PCR was performed on RNA extracted from quiescent and serum-stimulated human VSMCs with primers that span the alternatively spliced site for the miniexon. RT-PCR was also performed on the same samples with primers to the human G3PDH gene. Conditions for the PCR were chosen such that the level of the amplification product was linear with the level of input cDNA (Fig. 7A). The PCR conditions were determined empirically and involved 25 cycles for MEF2A and 20 cycles for G3PDH. Under these conditions, similar intensities of MEF2A PCR products were obtained with input cDNAs prepared from quiescent and serum-stimulated VSMCs. Likewise, the PCR products corresponding to the G3PDH internal control also gave rise to similar band intensities under the two sets of growth conditions. The density of the bands was determined by densitometry, and the ratio of MEF2 transcripts to

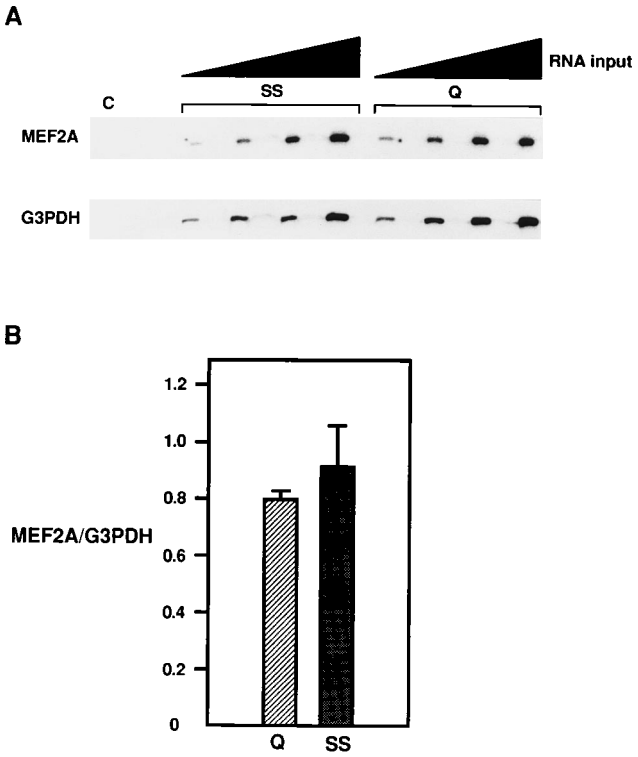

FIG. 7. Serum induction does not significantly alter MEF2A mRNA levels. MEF2A transcripts in human VSMCs were analyzed by quantitative RT-PCR. (A) Cultured human VSMCs were incubated in low-serum media for 3 days (quiescent [Q]) and stimulated by serum for 12 h (serum stimulated [SS]). One microgram of total RNA, extracted from quiescent or serum-stimulated human VSMCs, was converted to cDNA with reverse transcriptase. Dilution of cDNAs was amplified by the PCR procedure. Primers were designed to amplify the SEEEELEL-encoding miniexon. Both MEF2A and G3PDH were amplified simultaneously. After electrophoresis, the gels were dried and subjected to autoradiography. C, no-template control. (B) Statistical analysis of the amounts of MEF2A transcripts in human VSMCs. The amounts of MEF2A transcripts in quiescent and serum-stimulated human VSMCs were determined by densitometry, and the ratios of the MEF2A transcript to the G3PDH transcript were compared. The data are means with standard errors of the means ($n = 4$).

G3PDH transcripts was calculated. The ratio of the transcripts did not change significantly between the two conditions (0.80 $\pm$ 0.03 versus 0.91 $\pm$ 0.15 [Fig. 7B]). These data demonstrate that MEF2A transcript levels do not change significantly between the conditions of quiescence and serum stimulation. Furthermore, the MEF2A PCR product obtained from these reactions had a size that corresponds to transcripts that lack the SEEEELEL miniexon, indicating that this exon is excluded in both the quiescent and the serum-stimulated state.

A single MEF2 site is sufficient to confer mitogen-inducible gene expression in VSMCs. To test whether the serum induction of MEF2A could have functional consequences on MEF2-mediated gene transcription in VSMCs, expression from a heterologous promoter construct containing the AT-rich MEF2 binding site was analyzed in quiescent and serum-stimulated cultures. A single MEF2 binding site from the MCK enhancer was subcloned into the Δ56 minimal heterologous promoter construct (Fig. 8A) that had been used previously to analyze *cis*-regulatory elements from muscle-specific and mitogen-responsive genes (47). The MEF2/Δ56 luciferase construct was active in transfected quiescent A10 cells, giving levels of reporter gene expression that were 12-fold above that of the control plasmid, Δ56 luciferase, which lacks the MEF2

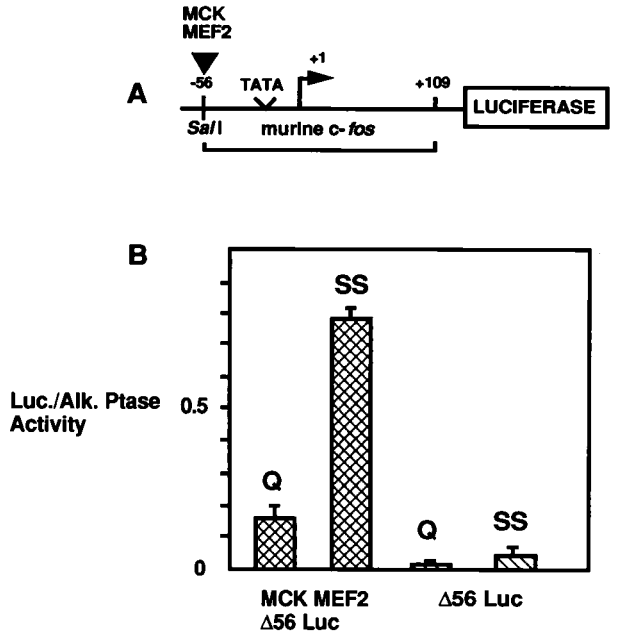

FIG. 8. A single MEF2 site is sufficient to confer serum-inducible reporter gene expression in A10 cells. (A) MEF2/Δ56 luciferase and Δ56 luciferase. The truncated murine c-*fos* promoter, positions −56 to +109, was subcloned upstream of the luciferase gene. A single MEF2 binding site from the muscle creatine kinase gene was inserted at the *Sal*I site. (B) Transient transfection analysis of MEF2/Δ56 and Δ56 in quiescent and serum-stimulated A10 cells. A10 cells in the high-serum media were transfected with MEF2/Δ56 luciferase (Luc) or Δ56 luciferase. The media were then replaced with low-serum media and incubated for 3 days (quiescent [Q]). Some cultures were restimulated with high-serum media for 12 h (serum stimulated [SS]) and harvested for luciferase assay. Alkaline phosphatase (Alk. Ptase) activity from a cotransfected internal control plasmid was used to correct the differences in transfection efficiency. The relative values are presented as means with standard errors of the means ($n = 3$).

binding site (Fig. 8B). Expression from the MEF2/Δ56 luciferase construct was activated more than fivefold by serum stimulation for 12 h, whereas expression from the control plasmid did not change significantly between the state of serum starvation and the state of serum stimulation. A similar extent of MEF2/Δ56 reporter activation was also achieved in cotransfection experiments with a MEF2A expression vector (data not shown). Others have reported mitogen activation of transcription via MEF2 heterologous promoter constructs in NIH 3T3 cells (39), and this site has also been implicated in the mitogen activation of the c-*jun* proto-oncogene (17). Collectively, these transfection data are consistent with the hypothesis that MEF2 levels may be limiting in VSMCs and other cell types and that the mitogen-induction of MEF2 expression can have functional consequences on the transcription of downstream genes.

DISCUSSION

The MEF2 transcription factors regulate cardiac- and skeletal muscle-specific gene transcription, and the expression of specific MEF2 isoforms can be correlated with the terminal differentiation of these cell types (3, 31, 55). The MEF2 factors also regulate the expression of mitogen-inducible genes that are critical for the initiation of cell proliferation (17). Little is known, however, about the role of the MEF2 transcription factors in VSMCs which do not terminally differentiate and must reversibly coordinate the expression of VSMC-specific genes and genes required for cell cycle progression. Here we provide evidence that the MEF2 transcription factor is expressed in VSMCs and that its expression is upregulated when these cells are stimulated to proliferate with mitogens. The evidence for MEF2 expression in vascular myocytes includes cDNA sequence information, detection of MEF2 transcripts by RT-PCR and Northern blot analyses, detection of a DNA-binding activity that specifically forms complexes with a MEF2 DNA probe, immunological data that indicate that MEF2A is a component of the nucleoprotein complex, and the demonstration that anti-MEF2A antibodies, but not preimmune serum, can immunoprecipitate a 67-kDa protein from VSMC extracts. Further, a MEF2 DNA-binding site, in the context of a minimal heterologous promoter, was sufficient for transcriptional activation in transfected VSMCs.

MEF2 is a member of a growing list of transcription factors that are regulated by mitogens in VSMCs. The induction of MEF2 was detected approximately 4 h after mitogen stimulation of quiescent VSMCs, and this increased expression was maintained for at least 24 h. The induced nucleoprotein complex formed with these extracts was quantitatively supershifted with antibodies specific for the products of the MEF2A gene, suggesting that the A isoform is significantly expressed in VSMCs. To elucidate the mechanism of the MEF2A serum induction, we examined the transcript levels and protein stability in the state of serum starvation and the state of serum stimulation. The MEF2A transcript level did not change significantly between the two states, suggesting that the regulation occurred at a posttranscriptional level. Cold-chase experiments revealed that the MEF2A protein was stable relative to the time course of the serum induction, and no significant difference in MEF2A protein stability could be detected in the quiescent and serum-induced cells. Furthermore, cycloheximide suppressed the induction of the DNA-binding activity. Collectively, these data indicate that the induction of MEF2A by serum is regulated at the level of translation in VSMCs. MEF2 expression may also be under translational control during development, as is suggested by observations of discordant expression between MEF2 protein and mRNA distribution. For example, the expression of MEF2A, -B, and -D proteins displays a high degree of tissue specificity, but their corresponding mRNAs are expressed ubiquitously (3, 30, 31, 55).

An unusual feature of the MEF2A transcript is its length, which may exceed 7 kb. One of the cDNAs isolated in this study (no. 15 in Fig. 1) potentially encodes a 5′ UTR of 1,853 nucleotides. Long 5′ UTRs are typically found in many proto-oncogenes and other growth-regulatory genes, including the transforming growth factor β gene, c-*myc*, c-*sis*, etc., that are subject to translational regulation (2, 9, 29, 38, 41, 44, 48). Long 5′ UTRs can dramatically inhibit translation because they inhibit ribosomes from traversing from the 5′ end of the mRNA to the bona fide start of translation. It is possible that MEF2A translation is controlled through this type of mechanism. Alternatively, the mitogen induction of MEF2A expression may be controlled by elements in its long 3′ UTR. Many examples of 3′ UTR-mediated translational regulation have been reported, including c-*mos* and cyclin A1, B1, and B2 (49), and 3′ UTR elements are also important in the temporal and spatial regulation of the maternal mRNAs in embryos (1, 12–14, 53). Furthermore, the 3′ UTRs of skeletal muscle-specific genes have been implicated in the regulation of growth and differentiation (42, 43).

The MEF2 transcription factors are similar to SRF in structure and in function. MEF2 and SRF both contain MADS box DNA-binding domains, they bind similar AT-rich DNA sites, and they regulate the expression of muscle-specific and immediate-early genes. Serum-inducible expression is another feature that MEF2 and SRF have in common. The SRF DNA-

binding activity is induced by mitogens in some cell types, such as A431 cells (40), but the activity appears to be constitutive in other cell types, including HeLa. However, even in the cell types in which the DNA binding is constitutive, the SRF mRNA levels can be induced 5- to 10-fold by the serum activation of quiescent cells (32, 37). Collectively, these data indicate that mitogen-inducible expression may be a general property of MADS box transcription factors, but the mechanism of the mitogen activation may differ between the family members.

Studies with the myocyte lines have demonstrated that the processes of proliferation and myogenesis are antagonistic in that growth factors will suppress differentiation while serum deprivation will induce myogenesis. Thus, it is not readily apparent from these cell culture studies why the MEF2 factors, or SRF, should activate the expression of immediate-early as well as contractile protein genes or why these transcription factors should be induced by mitogens. It is possible that low levels of MEF2 activate the expression of contractile protein genes and participate in the maintenance of the contractile phenotype in VSMCs whereas higher levels contribute to the activation of the genetic program that leads to the phenotypic modulation of this cell type. It is also possible that the mitogen induction of MEF2 and SRF in cell culture may mimic processes in early development that may contribute to the initiation of the myogenic program. For example, the initiation of myogenesis in the presomitic mesoderm depends upon the presence of inductive signals from the neural tube and notochord (4) in a process that appears very similar to the induction of mesoderm by FGF and activin in the *Xenopus* embryo (21). In *Xenopus* embryos, the mesoderm-inducing factors stimulate the phosphorylation and activation of eIF-4E in mesodermal precursor cells, which selectively facilitates the translation of the inducing-factor mRNAs because they have long 5′ UTRs with inhibitory secondary structures (45). By analogy, the as-yet unidentified inductive factors required for myogenesis may be partly responsible for the upregulation of MEF2 and SRF expression in the newly formed somites leading to the transcriptional activation of the basic helix-loop-helix and α-actin genes.

In conclusion, we report that specific isoforms of the MEF2A transcription factor are expressed in VSMCs. MEF2A expression was induced when quiescent VSMCs were stimulated by serum mitogens, and this induction was mediated by an activation of MEF2A translation. Future studies will elucidate the mechanism of the translational control and the role of MEF2 in VSMC differentiation.

ACKNOWLEDGMENTS

This work was supported by grants AR40197 and HL50692 from the NIH and by a grant from the American Heart Association. K.W. is an Established Investigator of the AHA.

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