MOLECULAR AND CELLULAR BIOLOGY, Nov. 2005, p. 9874–9885 Vol. 25, No. 22
0270-7306/05/$08.00+0 doi:10.1128/MCB.25.22.9874–9885.2005

Smooth Muscle-Specific Genes Are Differentially Sensitive to Inhibition by Elk-1

Jiliang Zhou, Guoqing Hu, and B. Paul Herring*

Department of Cellular and Integrative Physiology, Indiana University School of Medicine, 635 Barnhill Drive, Indianapolis, Indiana 46202-5120

Received 14 June 2005/Returned for modification 1 July 2005/Accepted 8 September 2005

Understanding the mechanism of smooth muscle cell (SMC) differentiation will provide the foundation for elucidating SMC-related diseases, such as atherosclerosis, restenosis, and asthma. In the current study, overexpression of Elk-1 in SMCs down-regulated expression of several endogenous smooth muscle-restricted proteins, including telokin, SM22α, and smooth muscle α-actin. In contrast, down-regulation of endogenous Elk-1 in smooth muscle cells increased the expression of only telokin and SM22α, suggesting that smooth muscle-specific promoters are differentially sensitive to the inhibitory effects of Elk-1. Consistent with this, overexpression of the DNA binding domain of Elk-1, which acts as a dominant-negative protein by displacing endogenous Elk-1, enhanced the expression of telokin and SM22α without affecting expression of smooth muscle α-actin. Elk-1 suppressed the activity of smooth muscle-restricted promoters, including the telokin promoter that does not contain a consensus Elk-1 binding site, through its ability to block myocardin-induced activation of the promoters. Gel mobility shift and chromatin immunoprecipitation assays revealed that Elk-1 binds to a nonconsensus binding site in the telokin promoter and Elk-1 binding is dependent on serum response factor (SRF) binding to a nearby CArG box. Although overexpression of the SRF-binding B-box domain of Elk-1 is sufficient to repress the myocardin activation of the telokin promoter, this repression is not as complete as that seen with an Elk-1 fragment that includes the DNA binding domain. In addition, reporter gene assays demonstrate that an intact Elk-1 binding site in the telokin promoter is required for Elk-1 to maximally inhibit promoter activity. Together, these data suggest that the differential sensitivity of smooth muscle-specific genes to inhibition by Elk-1 may play a role in the complex changes in smooth muscle-specific protein expression that are observed under pathological conditions.

There is extensive evidence showing that altered control of the differentiated state of smooth muscle cells (SMCs) contributes to the development and/or progression of a variety of diseases, including atherosclerosis, hypertension, and asthma. These diseases are all associated with decreased expression of proteins required for the differentiated function of smooth muscle cells (28). An understanding of the mechanisms that control smooth muscle cell differentiation is required before it will be possible to determine how these control processes are altered in pathological conditions.

The binding of serum response factor (SRF) to CArG elements within smooth muscle-specific genes is critical for the activity of many of these genes (26). As SRF is widely expressed, the interactions of SRF with other cell-restricted proteins are important for mediating its cell- and promoter-specific functions. Recent studies have demonstrated that the interaction of SRF with the coactivator myocardin is a critical determinant of vascular smooth muscle development (21). Myocardin is expressed in visceral and vascular SMCs, physically associates with SRF, and greatly potentiates SRF-dependent transcription of multiple SMC marker genes (2, 4, 34, 40), including genes such as the telokin gene, that contain only a single CArG element (38, 39, 41). How myocardin specifically activates smooth muscle-specific genes compared to other SRF-dependent genes, such as c-*fos*, is currently unclear. It is likely that complex interactions between myocardin and molecules that either augment or repress its activity are involved in mediating the promoter-specific effects of myocardin (41). For example, ETS proteins (1, 35) have been shown to repress the ability of myocardin to activate smooth muscle-specific promoters (1, 3, 35).

It has recently been reported that growth signals can repress the smooth muscle-specific SM22α gene by triggering the displacement of myocardin from SRF by Elk-1 (1, 35). Elk-1 is an ETS family member that competes with myocardin for a common docking site on SRF. These studies also showed that a consensus ETS binding site adjacent to the distal CArG box in the SM22α promoter is required to facilitate ternary complex formation between Elk-1 and SRF. The N-terminal A domain of Elk-1 contains the ETS DNA binding domain, which binds specifically to ETS binding sites composed of a purine-rich central core (5′-GGA[A/T]-3′). The B domain of Elk-1 interacts with the MADS box transcription factor family member SRF and allows ternary complex formation. The C-terminal C domain of Elk-1 is a mitogen-regulated activation domain; phosphorylation of this domain leads to enhanced DNA binding and transcriptional activation (25). Elk-1 functions as a nuclear transcriptional activator via its association with SRF on serum response elements present in the promoters of many immediate-early genes, such as c-*fos*, *egr1*, *egr2*, *pip92*, and *nurr77* (36). In addition to its regulation of growth-responsive genes, Elk-1 has been shown to play a role in regulating differentiation of smooth muscle, skeletal muscle, and neuronal

* Corresponding author. Mailing address: Department of Cellular and Integrative Physiology, Indiana University School of Medicine, 635 Barnhill Drive, Indianapolis, IN 46202-5120. Phone: (317) 278-1785. Fax: (317) 274-3318. E-mail: pherring@iupui.edu.

cells (17, 33, 35). Recently the SRF gene has also been identified as a target for Elk-1, thereby providing a positive-feedback loop where Elk-1 activation leads to enhanced expression of its partner protein, SRF (16).

Telokin is a smooth muscle-restricted protein with an amino acid sequence that is identical to the carboxyl-terminal domain of myosin light-chain kinase (MLCK) (5). Transcription of telokin is restricted to smooth muscle cells throughout mouse development, which makes this gene a good marker for studying the control of smooth muscle cell lineage (11). In vitro reporter gene assays have shown that 310-bp (−163 to +147) and 370-bp (−190 to +181) fragments of the rabbit and mouse telokin promoters, respectively, are sufficient to mediate cell-specific expression (13, 15). Both of these telokin promoters are also sufficient to direct transgene expression to smooth muscle tissues in adult and embryonic mice with much higher levels of expression observed in visceral as opposed to vascular smooth muscle tissues (15, 32). Several conserved positive-acting elements within the minimal telokin promoter, including an E box (−82 to −93), AT-rich region (−66 to −82), and CArG box (−56 to −65), have been shown to be important for promoter activity (12, 14, 42). Although the telokin promoter contains only a single CArG box, myocardin strongly activates this promoter and induces telokin protein expression in 10T1/2 fibroblast and rat aortic smooth muscle cells (38, 39, 41).

In the current study, we examined the role of Elk-1 in regulating the activity of smooth muscle-restricted genes, including the telokin gene that does not contain a consensus ETS binding site. We found that SRF recruited Elk-1 to bind to a nonconsensus site within the smooth muscle-restricted telokin promoter and inhibited telokin gene expression by competing with myocardin for binding to SRF. Moreover, we show that down-regulation of Elk-1 in smooth muscle cells leads to increased expression of telokin and SM22α without affecting expression of calponin or smooth muscle α-actin (SMα-actin). These studies suggest that Elk-1 may play a general role in regulating SRF-dependent smooth muscle-restricted genes and that these genes are differentially sensitive to the inhibitory effects of Elk-1.

MATERIALS AND METHODS

Mammalian expression and reporter gene assays. The mouse myocardin pcDNA3.1-myc/his vector was kindly provided by Eric N. Olson (Southwestern Medical Center, Dallas, Texas). The mouse full-length Elk-1 (encoding amino acids 1 to 428) cDNA was amplified from Image clone (clone identification, 6506942) (Invitrogen, Carlsbad, CA) by PCR and ligated to pcDNA3.1 His vector. The A-box (amino acids 1 to 99) and B-box (amino acids 134 to 184) domains of Elk-1 were amplified by PCR and fused to the simian virus 40 nuclear localization sequence LYPKKKRKGVEDQYK at the 3′ terminus of the coding sequence (A box-NLS). The C-terminal truncation mutant of Elk-1 comprised of the A- and B-box domains (amino acids 1 to 184) was amplified by PCR and cloned into the pcDNA3.1 His expression vector resulting in the expression of a fusion protein with amino-terminal six-histidine and omni epitope tags. All promoter reporter genes were constructed by cloning fragments of promoters into the pGL_2B luciferase vector (Promega, Madison, WI). The mouse telokin promoter-luciferase reporter gene used includes nucleotides −190 to +81 (T370) of the telokin gene as described previously (13, 15). The SM22α-luciferase reporter gene includes nucleotides −475 to +61 of mouse SM22α (18, 20). The SM α-actin promoter fragment extends from nucleotide −2555 to +2813 (22), and the SM myosin heavy-chain promoter from −4,200 to +11,600 (23), these plasmids were generously provided by Gary Owens. All mutant reporter gene constructs were initially generated in pCR pBlunt vector (Invitrogen, Carlsbad, CA) by QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA) and then transferred to pGL_2B vector. Plasmids were sequenced to verify the integrity of the insert. Transfection was carried out as previously described (42). The level of promoter activity was evaluated by measurement of the firefly luciferase activity relative to the internal control renilla luciferase activity using the dual luciferase assay system essentially as described by the manufacturer (Promega, Madison, WI). A minimum of six independent transfections were performed, and all assays were replicated at least twice. Results are reported as the means ± standard errors of the means (SEMs).

siRNA. A plasmid-based system for production of small interfering RNA (siRNA) was generated by inserting oligonucleotides specific to Elk-1 (5′-CCT CTATTCTACCTTCACAAT-3′) downstream of an H1 promoter in the adenovirus shuttle vector pRNAT-H1.1/Shuttle (GenScript, Piscataway, NJ) that is compatible with the adeno-X system (Clontech, Palo Alto, CA). Plasmids were transferred to the adeno-X adenoviral vector, and adenovirus was generated as described below. An siRNA control was generated by using a similar approach. The adenoviral vector contains the H1 promoter driving the siRNA cassette together with a cytomegalovirus-driven coral green fluorescent protein (GFP) cDNA.

Adenovirus construction and cell infection. Adenovirus constructs were made using the adeno-X vectors essentially following the manufacturer's instructions (BD Biosciences, Palo Alto, CA) as previously described (42). The recombinant adenovirus was packaged in HEK293 cells and amplified to obtain high-titer stocks. For adenoviral transduction, A10 cells or 10T1/2 cells were seeded in six-well plates at a density of 2×10^5 cells/well and grown overnight to near confluence. These cells were washed with phosphate-buffered saline to remove serum and transduced with adenovirus encoding LacZ, Elk-1, A box-NLS, YFP-NLS (nuclear localized yellow fluorescent protein), myocardin, Elk-1 siRNA, or control siRNA in phosphate-buffered saline for 4 h at 37°C. These conditions resulted in close to 100% infection of cells. Seventy-two hours following transduction, cell protein extracts were prepared using radioimmunoprecipitation assay buffer, containing a cocktail of protease inhibitors. and protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL).

Western blotting. Western blotting analysis was carried out essentially as described previously (6). Fifteen micrograms of protein was fractionated on a 7.5% or 15% sodium dodecyl sulfate-polyacrylamide gel, electrophoretically transferred to a polyvinylidene difluoride membrane, and then verified by Ponceau S staining. The membrane was then probed with a series of antibodies. Antibodies used in this study were directed against calponin (Sigma, clone hCP; diluted 1:10,000), GFP (Clontech; 1:400), hemagglutinin (HA) tag (BabCO; 1:1,000), MLCK (Sigma, clone K36; 1:10,000), SM22α (a gift from Len Adam; 1:6,000), SM α-actin (Sigma, clone 3A1; 1:10,000), β-actin (Sigma; 1:10,000), SRF (Santa Cruz; 1:10,000), and telokin (1:6,000) (6).

RT-PCR. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA). Primer sets unique to telokin were designed as sense (5′-GACACCGCC TGAGTCCAACCTCCG-3′) and antisense (5′-GACCCTGTTGAAGATTTC CTGCCACTG-3′) and yielded a 127-bp product. Other sequences of PCR primers are available upon request. Two hundred nanograms of RNA was utilized as a template for reverse transcription coupled to PCR (RT-PCR) with respective gene-specific primers using SuperScript one-step RT-PCR system (Invitrogen, Carlsbad, CA).

Immunocytochemistry. Rat aortic A10 cells were grown on coverslips at 3×10^5 cells per 60-mm dish overnight. Cells were transfected with the Elk-1 AB-box expression plasmid for 24 h and then fixed, permeabilized, and incubated with monoclonal anti-SM α-actin (Sigma Chemical Co.; diluted 1:80) and polyclonal anti-omni (Invitrogen; 1:500), followed by Texas Red-conjugated anti-mouse immunoglobulin G (IgG) (1:400) and fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:80) secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.).

Mammalian two-hybrid assay. Mammalian two-hybrid assays were performed according to the instructions of the manufacturer (Promega, Madison, WI). Expression plasmids of fusion proteins for GAL4-SRF (pBIND-SRF) and VP16-myocardin (pACT-myocardin) were constructed and confirmed by DNA sequencing. Expression plasmids for GAL4 and VP16 fusion proteins were co-transfected into 10T1/2 cells together with pG5 luciferase reporter plasmid, and luciferase activity was measured according to the manufacturer's directions (Promega, Madison, WI).

Gel mobility shift analysis of DNA binding. Nuclear protein extraction and mobility shift assays were performed as described previously (42). The sequences of the sense strand of each probe from telokin and SM22α promoters are described in figure legends. Annealed oligonucleotides were labeled using [α-^{32}P]dCTP and Klenow DNA polymerase (Promega). All binding reaction mixtures were incubated for 15 min at room temperature. For supershift assays, 1 μl of anti-omni antibody (Invitrogen) was added to the binding assay mix after this initial incubation and then incubated on ice for an additional hour. The

DNA-protein complexes were resolved by electrophoresis through 4% polyacrylamide gels.

ChIP assay. Rat A10 aortic smooth muscle cells were cultured in Dulbecco modified Eagle medium supplemented with 20% fetal bovine serum (FBS) on 100-mm dishes. For some experiments, A10 cells were seeded in medium containing 0.2% FBS, grown overnight, and then switched to 20% FBS medium for 2 h prior to harvesting for chromatin immunoprecipitation (ChIP) assays. A10 cells infected with adenovirus encoding HA-tagged Elk-1, HA-tagged nuclear localized Elk-1 A box, HA-tagged myocardin, and HA-tagged nuclear localized YFP were cultured in 20% FBS medium for 3 days. Cells (1×10^6) in 9 ml of medium were fixed with 243 μl of 37% formaldehyde and then incubated at 37°C for 10 min. Fixed cells were harvested for immunoprecipitation essentially as described by the manufacturer (Upstate Biotechnology Inc., Lake Placid, New York) with minor modifications. Cross-linked chromatin was immunoprecipitated with anti-HA antibody (Covance) or anti-Elk-1 antibody (Santa Cruz) and bound to protein G or protein A agarose beads, respectively. Healthy mouse and rabbit IgG (Santa Cruz) were used for control experiments. The precipitated chromatin DNA bound to the protein of interest was then purified and amplified by PCR for enrichment of the target sequences. Primers for amplifying the telokin promoter CArG-containing region, which produced a 357-bp product, were designed as sense 5′-(GTCAGCAAT AAGTCTTTGGAGCTGTCTCAG-3′) and antisense (5′-ATGGCTCTCACAGCA TTGCCCGTTTTCTGG-3′). For amplifying the SRE region from the c-*fos* promoter, the specific primers were designed as sense (5′-CATTGAATCAGG TGCGAATGTTCGC-3′) and antisense (5′-GCCGTGGAAACCTGCTGA CGCA-3′), which yielded a 193-bp product. After 35 cycles of amplification, PCR products were run on 3% agarose gels.

RESULTS

Full-length Elk-1 decreases smooth muscle-specific promoter activity and protein expression. We and others have shown that myocardin can induce telokin expression despite the fact that the telokin promoter contains only one CArG box (38, 39, 41). Recently it has been reported that Elk-1, a member of the ETS family of transcription factors, can negatively regulate SM22α gene expression by competing with myocardin for binding to SRF (35). To examine whether Elk-1 acts similarly on other smooth muscle-restricted promoters, myocardin was cotransfected together with telokin, SM22α, and SM α-actin promoter-luciferase reporter genes with or without Elk-1 expression plasmid in 10T1/2 cells. Elk-1 was able to reduce the myocardin activation of each of these promoters in a dose-dependent manner (Fig. 1A). Elk-1 alone had no effect on the basal activity of a telokin reporter gene in these nonmuscle cells (data not shown). In contrast, in A10 smooth muscle cells, Elk-1 alone caused a dose-dependent decrease in telokin and SM22α promoter activity (Fig. 1B). To determine whether overexpression of Elk-1 in SMCs can regulate endogenous smooth muscle-specific gene expression, A10 SMCs were infected with adenovirus encoding wild-type Elk-1 and endogenous protein expression was examined by Western blotting. Results from this analysis revealed that overexpression of Elk-1 significantly down-regulated expression of telokin, SM22α, and SM α-actin but had little effect on expression of the 220-kDa and 130-kDa isoforms of MLCK, glyceraldehyde-3-phosphate dehydrogenase, or β-actin (Fig. 1C).

A C-terminal truncation mutant of Elk-1, comprised of only the A- and B-box domains, inhibits the activity of SMC-specific promoters and blocks myocardin induction of SMC-specific genes. To determine the role of the carboxy-terminal transcription activation domain of Elk-1 on its ability to inhibit expression of SMC-specific genes, an Elk-1 deletion mutant lacking this domain (AB box) was generated and characterized. The AB-box fragment of Elk-1, like full-length Elk-1, was

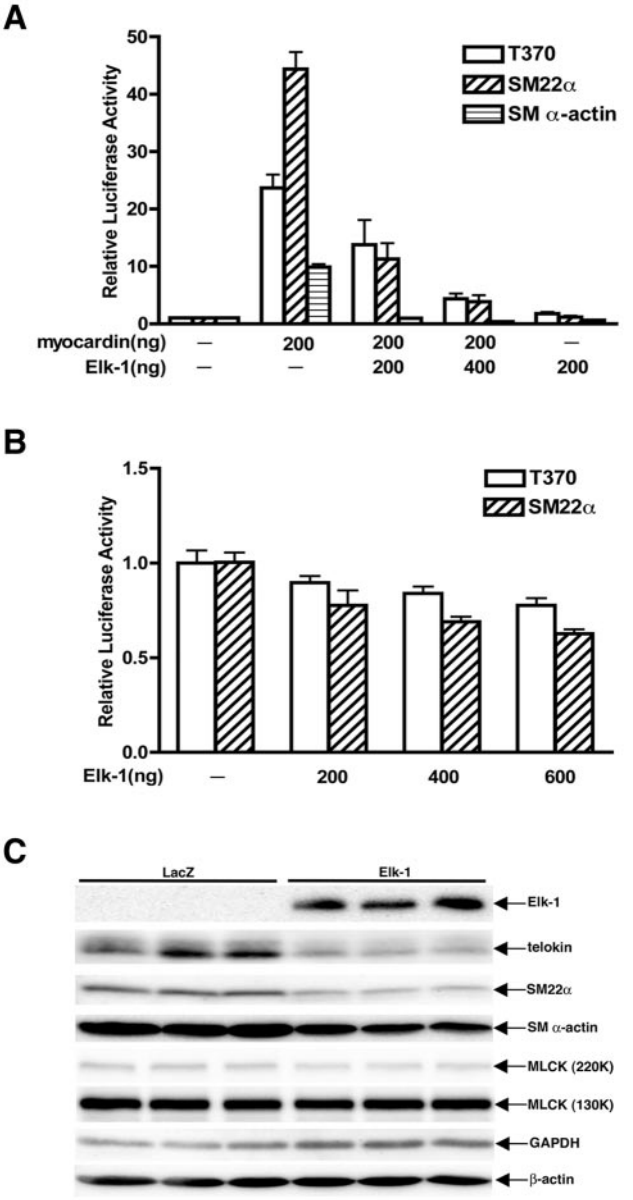


FIG. 1. Effects of full-length Elk-1 on telokin promoter activity and expression. (A) 10T1/2 cells were transiently transfected with expression vectors for mouse myocardin, full-length Elk-1, and telokin promoter T370 or with SM22α and SM α-actin promoter-luciferase reporter plasmids. The amount of each expression plasmid used (in nanograms) is indicated (−, none). Values are presented as relative luciferase activity compared to empty expression vector and represent the means ± SEMs (error bars) for six samples. (B) A10 cells were transiently cotransfected with increasing amounts of expression vector for Elk-1 and T370 or SM22α promoter-luciferase reporter genes, as indicated. For each promoter, activity is normalized to a renilla luciferase internal control and expressed relative to vector control transfections. (C) A10 cells were seeded in six-well plates overnight and then infected with adenovirus encoding LacZ or Elk-1 as indicated above the lanes for 4 h. Seventy-two hours following infection, protein extracts were prepared from infected cells and analyzed by Western blotting to detect endogenous protein expression. The positions of proteins, such as the 220-kDa (220K) and 130-kDa (130K) isoforms of MLCK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are shown to the right of the blots.

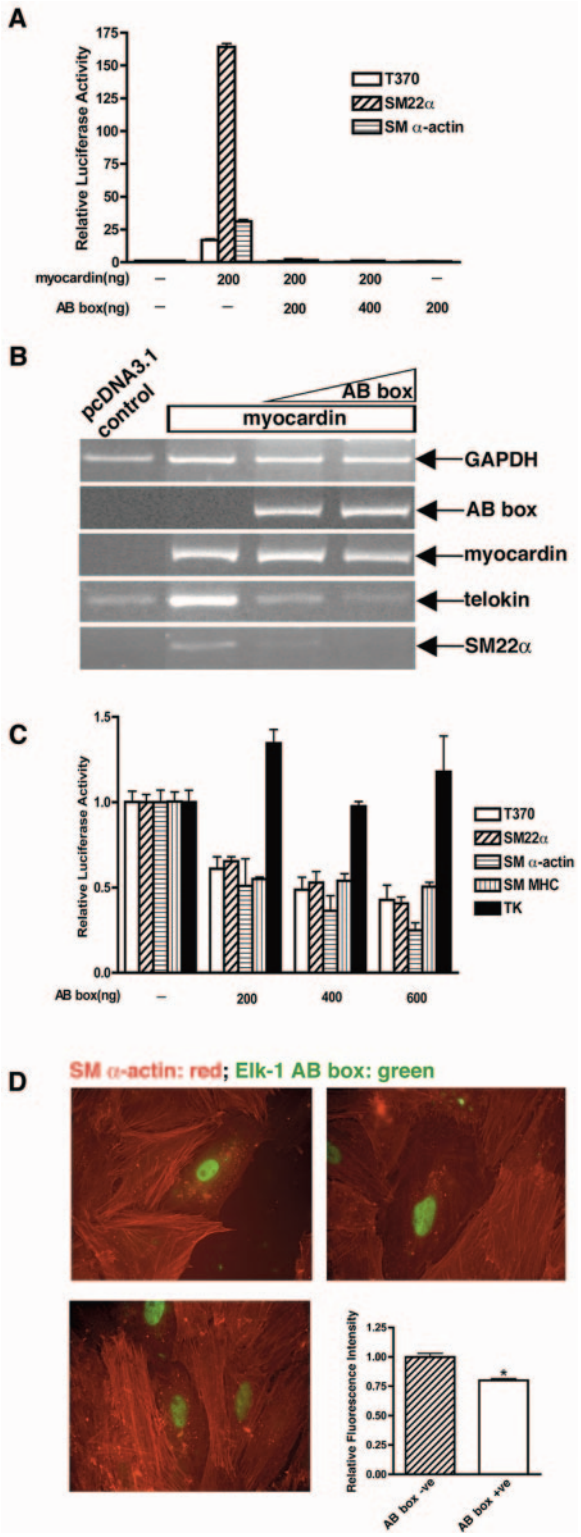


FIG. 2. Effects of an Elk-1 C-terminal truncation mutant, comprised of only the A and B box, on telokin expression. (A) 10T1/2 cells were transiently transfected with expression vectors for mouse myocardin, the AB-box domains of Ek-1, and a mouse telokin promoter T370 or with SM22α or SM α-actin promoter-luciferase plasmid, and then the luciferase activity was measured. The amount of each expression plasmid (in nanograms) is indicated (−, none). Values are presented as relative luciferase activity compared to empty expression vector. (B) Mouse myocardin expression vector, together with mouse Elk-1 AB box or empty plasmid pcDNA3.1, was transfected into 10T1/2 cells. Twenty-four hours after transfection, total RNA was harvested from cells using TRIzol reagent, and RT-PCR was performed to detect endogenous telokin and SM22α expression with SuperScript one-step RT-PCR system (Invitrogen). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene served as an internal control showing equal RNA input and RT-PCR efficiency. (C) A10 cells were transiently cotransfected with increasing amounts of expression vectors for the Elk-1 AB box and either T370, SM α-actin, SM22α, SM myosin heavy-chain (SM MHC), or minimal thymidine kinase (TK) promoter-luciferase reporter genes. For each, promoter activity is normalized to a renilla luciferase internal control and expressed relative to vector control transfections. Data are presented as means ± SEMs (error bars) for six samples. (D) A10 cells were plated on coverslips and transiently transfected with the Elk-1 AB box for 24 h and then stained with monoclonal anti-SM α-actin (red) and polyclonal anti-omni epitope tag (green) to detect Elk-1 AB-box expression. Three representative overlay images are presented. The graph shows the quantitation of changes in SM α-actin expression seen in Elk-1 AB-box-transfected cells. The SM α-actin fluorescence intensity was measured using ImageTool 3.0 software, and the intensity measured in AB-box-expressing cells (AB box +ve) was expressed relative to the intensity observed in nontransfected cells (AB box −ve; assigned an arbitrary value of 1). Data are presented as means ± SEMs from 14 cells. The intensities of the transfected and nontransfected cells were statistically significantly different ($P < 0.05$) (indicated by the asterisk).

able to dramatically suppress myocardin activation of SMC-specific promoters (Fig. 2A). In addition, overexpression of the AB-box fragment of Elk-1 blocked the myocardin-induced activation of endogenous telokin and SM22α expression in 10T1/2 cells (Fig. 2B). In A10 vascular smooth muscle cells, overexpression of the AB-box fragment of Elk-1 alone also resulted in a dose-dependent inhibition of several SMC-restricted promoters without affecting a thymidine kinase promoter (Fig. 2C). Immunocytochemistry also revealed that the AB-box fragment of Elk-1 decreased expression of endogenous SM α-actin to 75% of control levels in A10 smooth muscle cells (Fig. 2D). This is consistent with effects of the full-length Elk1 on SM α-actin expression seen in Fig. 1C. Taken together, these data suggest that the AB-box fragment of Elk-1 is able to mimic the effects of the full-length molecule and inhibit SMC-specific gene expression.

Elk-1 B-box domain interferes with the binding of myocardin to SRF. It has been reported that the B box of Elk-1 is required for Elk-1 to block the myocardin-induced activation of SMC-specific genes (35). A series of experiments were performed to determine whether the B box alone is sufficient for this inhibitory activity. A mammalian two-hybrid assay was carried out to determine whether the B box of Elk-1 alone can compete with myocardin for binding to SRF. In 10T1/2 cells this assay revealed an interaction between SRF and myocardin that could be disrupted by the AB box or B box of Elk-1, but not by the A box alone (Fig. 3A). To determine whether the Elk-1 B box alone is sufficient to suppress myocardin activation of the telokin promoter, reporter gene assays were performed in which myocardin and a telokin promoter reporter gene were cotransfected into A10 cells in the presence or absence of the B-box fragment of Elk-1. This analysis revealed that the Elk-1 B box alone is able to significantly decrease myocardin activation of the telokin promoter (Fig. 3B). This inhibition is, how-

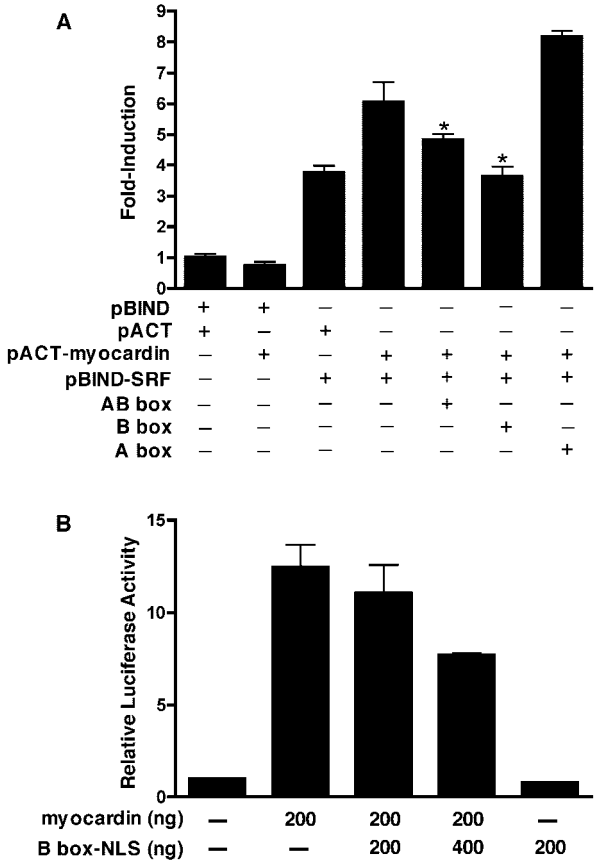


FIG. 3. Mapping the Elk-1 domain that competes with myocardin for binding to SRF. (A) Mammalian two-hybrid assays were performed in 10T1/2 cells. 10T1/2 cells were transfected (+) with a pG5Luc reporter plasmid and expression plasmids encoding fusion proteins of GAL4 DNA binding domain-SRF (pBIND-SRF) or VP16 activation domain-myocardin (pACT-myocardin) in the presence (+) or absence (−) of the Elk-1 AB box, A box-NLS, or B box-NLS. Promoter activity is expressed as luciferase activity normalized by renilla luciferase control (mean ± SEM [error bar]; $n = 6$). Data obtained from cotransfection of pBIND and pACT plasmids was assigned a value of 1, as indicated by the leftmost bar. Increased luciferase activity indicative of protein-protein interactions was seen when pBIND-SRF and pACT-myocardin were cotransfected. Expression of either the Elk-1 AB box or the B domain alone, but not the A domain alone, significantly inhibited the interaction of myocardin and SRF. Values that were significantly different from the values for the pBIND-SRF and pACT-Myocardin cotransfection group ($P < 0.01$) are indicated by an asterisk. (B) Two hundred nanograms of mouse myocardin and increasing amounts of the Elk-1 B box-NLS or an empty expression vector plasmid were transiently cotransfected together with a mouse telokin promoter-luciferase construct (T370) and a minimal thymidine kinase promoter-driven renilla luciferase internal control plasmid into 10T1/2 fibroblast cells, and luciferase activity was measured. An arbitrary value of 1 was assigned to the activity of T370 only. Data presented are means ± SEMs (error bars) for six samples.

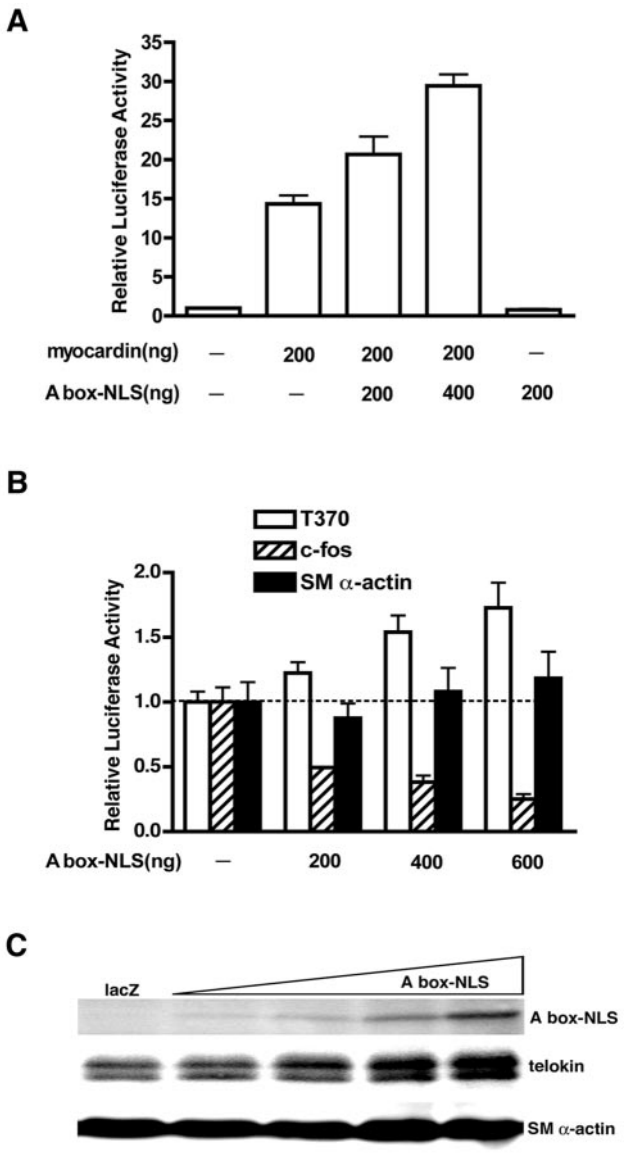


FIG. 4. Effects of the A domain of Elk-1 on telokin promoter activity. (A) Two hundred nanograms of mouse myocardin and increasing amounts of Elk-1 A box-NLS expression plasmid or an empty expression vector plasmid were transiently cotransfected into 10T1/2 fibroblast cells together with a mouse telokin promoter-luciferase construct (T370 [200 ng]) and a minimal thymidine kinase promoter-driven renilla luciferase internal control plasmid. Luciferase assays were performed as described in Materials and Methods. Data presented are means ± SEMs (error bars) for six samples relative to the activity of the telokin promoter alone. (B) A telokin (T370), SM α-actin, or c-*fos* luciferase reporter gene was cotransfected with increasing amounts of the A box-NLS of Elk-1 into A10 SMCs, and then luciferase activity was determined. (C) A10 cells were infected with increasing amounts of adenovirus encoding the A box-NLS of Elk-1, as indicated, for 4 h. Seventy-two hours following infection, protein extracts were prepared from transduced cells and analyzed by Western blotting to detect endogenous protein expression.

ever, not as dramatic as the inhibition caused by an Elk-1 fragment that also includes the A-box DNA binding domain (Fig. 2A).

An Elk-1 A-box fragment competes with endogenous Elk-1 and increases telokin promoter activity while decreasing c-*fos* promoter activity. As the B box of Elk-1 is the region of the molecule that interacts with SRF and this domain is required to block the myocardin activation of smooth muscle genes, we examined the possibility that overexpression of the Elk-1 DNA binding domain (A box) alone may displace endogenous Elk-1 from ETS binding sites in SMC-specific genes and thereby

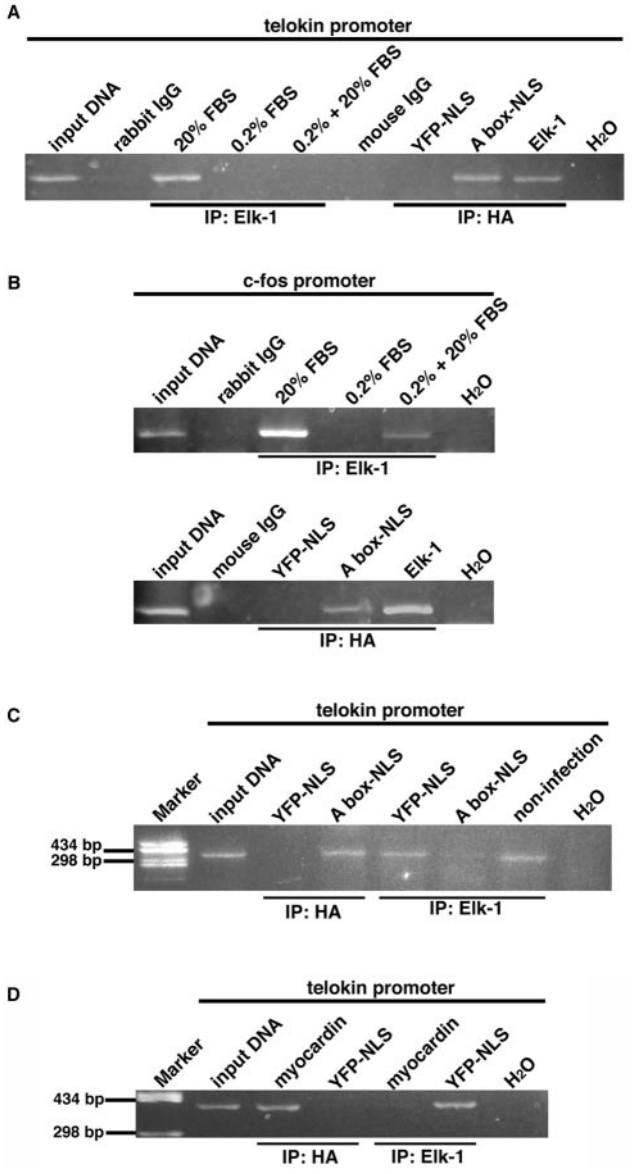
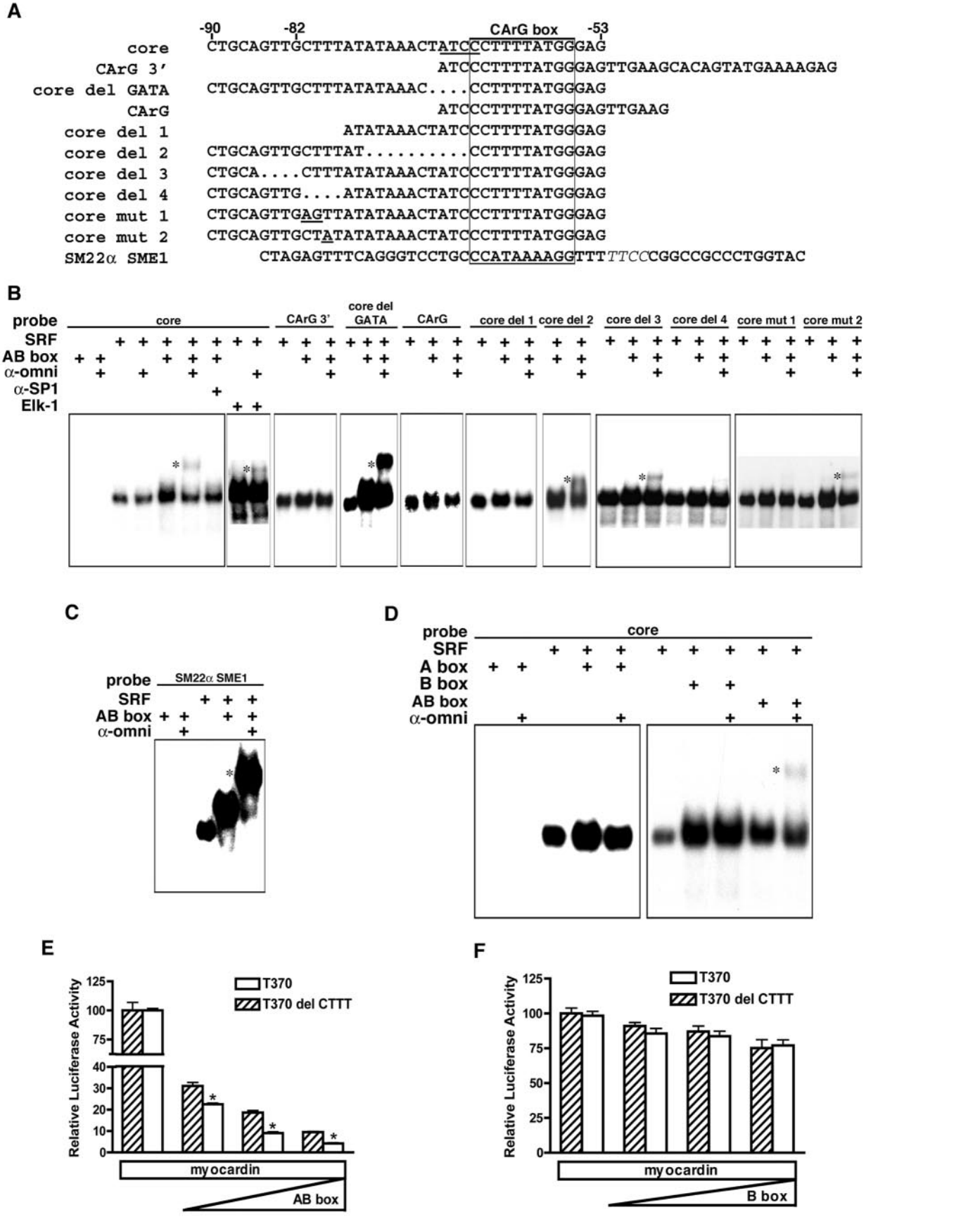


FIG. 5. Elk-1 binds to the telokin promoter in vivo and can be replaced by overexpression of A box in A10 SMCs. A10 cells were cultured in medium containing 20% FBS or 0.2% FBS overnight and then stimulated with or without medium containing 20% FBS for 2 h, as indicated. Protein-DNA complexes were then immunoprecipitated from these cells using anti-Elk-1 (for detecting endogenous Elk-1). Alternatively, A10 cells grown in 20% FBS medium were infected with adenovirus encoding HA-tagged YFP-NLS, A box-NLS, or Elk-1. Three days following infection, chromatin immunoprecipitation assays were performed using anti-HA antibody to detect the epitope-tagged exogenous proteins. A rabbit or mouse IgG was used as a negative control for each immunoprecipitation (IP). The precipitated DNA was purified and used as a template for PCR, utilizing primers that flank the CArG box and Elk-1 binding site of the telokin promoter (A) or the SRE of the c-*fos* promoter (B). (C) Chromatin was immunoprecipitated from A10 cells grown in 20% FBS and infected with adenovirus encoding the Elk-1 A box-NLS or YFP-NLS using either anti-HA or anti-Elk-1 antibody (the Elk-1 antibody recognizes an epitope in the C domain of Elk-1 and therefore does not cross-react with the A-box fragment). The 375-bp fragment encompassing the core of the telokin promoter was amplified from Elk-1 immunoprecipitates of cells expressing YFP-NLS and uninfected cells, but not from cells expressing the A-box domain of Elk-1. In contrast, when chromatin was immunoprecipitated with an HA epitope tag antibody, the telokin promoter could be amplified from cells infected with the Elk-1 A-box domain. This indicates that the Elk-1 A box is able to bind to the endogenous telokin promoter and that this binding can displace endogenous Elk-1 from the promoter. (D) A10 cells were infected with myocardin or YFP-NLS adenovirus (both proteins include an HA epitope tag) and then prepared for chromatin immunoprecipitation assays using HA or Elk-1 antibodies as described above. The precipitated DNA was used as a template for PCR, utilizing primers that flank the CArG box and Elk-1 binding site of the telokin promoter. These data demonstrate that overexpressed myocardin binds to the telokin promoter and that its binding displaces endogenous Elk-1 from the promoter.

increase the activity of these promoters. Myocardin was cotransfected with a telokin promoter reporter gene into 10T1/2 cells in the presence or absence of the Elk-1 A box alone, and luciferase activity was measured. As the A box of Elk-1 does not contain a nuclear localization sequence, the nuclear localization sequence from simian virus 40 was added to the A box to facilitate its nuclear entry. Results from analysis of this construct showed that the A-box fragment of Elk-1 augmented myocardin activation of the telokin promoter in 10T1/2 cells (Fig. 4A). In A10 SMCs, the A-box fragment of Elk-1 also significantly increased basal telokin promoter activity in a dose-dependent manner, without affecting SM α-actin promoter activity (Fig. 4B). Furthermore, overexpression of the A-box fragment in A10 cells, mediated by adenoviral transduction, increased endogenous telokin expression without significantly changing SM α-actin protein expression (Fig. 4C). In contrast, the c-*fos* promoter, which is normally activated by Elk-1, exhibited a dose-dependent inhibition by the A-box fragment of Elk-1 (Fig. 4B). This would suggest that in A10 SMCs grown under normal high-serum conditions, overexpression of the Elk-1 A-box fragment displaces endogenous ETS factors from the telokin promoter, releasing their inhibitory effects and thus increasing promoter activity. Whereas in the c-*fos* promoter where Elk-1 acts an activator, displacement of Elk-1 by overexpression of the Elk-1 A box decreases promoter activity.

In support of this model, we demonstrated that the A-box fragment of Elk-1 can bind to the telokin and c-*fos* promoters in vivo and displace endogenous Elk-1. Chromatin immunoprecipitation assays demonstrated that endogenous Elk-1 is bound to the telokin and c-*fos* promoters in A10 cells grown in 20% but not 0.2% fetal bovine serum-containing medium (Fig. 5A and B). Interestingly, stimulation with 20% FBS medium for 2 h following serum starvation resulted in detectable levels of Elk-1 binding to the c-*fos* but not telokin promoter. This would suggest that Elk-1 likely binds to the telokin promoter with an affinity lower than that of the c-*fos* promoter. In A10 cells infected with adenovirus encoding HA-tagged full-length Elk-1 or the A-box fragment, chromatin immunoprecipitation assays using anti-HA antibodies demonstrated binding of both these molecules to the telokin and c-*fos* promoters (Fig. 5A and B). In contrast, cells infected with adenovirus encoding HA-tagged YFP displayed no binding of the YFP to the telokin promoter. Chromatin immunoprecipitation assays using an Elk-1 antibody that is directed against the C domain of Elk-1 showed that overexpression of the Elk-1 A domain abolished the binding of endogenous Elk-1 to the telokin pro-


A
-90
-82
CArG box
-53
core CTGCAGTTGCTTTATATAAACTATCCCTTTTATGGGAG
CArG 3' ATCCCTTTTATGGGAGTTGAAGCACAGTATGAAAAGAG
core del GATA CTGCAGTTGCTTTATATAAAC....CCTTTTATGGGAG
CArG ATCCCTTTTATGGGAGTTGAAG
core del 1 ATATAAACTATCCCTTTTATGGGAG
core del 2 CTGCAGTTGCTTTAT.....CCTTTTATGGGAG
core del 3 CTGCA....CTTTATATAAACTATCCCTTTTATGGGAG
core del 4 CTGCAGTTG....ATATAAACTATCCCTTTTATGGGAG
core mut 1 CTGCAGTTGAGTTATATAAACTATCCCTTTTATGGGAG
core mut 2 CTGCAGTTGCTATATATAAACTATCCCTTTTATGGGAG
SM22α SME1 CTAGAGTTTCAGGGTCCTGCCCATAAAAGGTTTTTCCCGGCCGCCCTGGTAC
B
probe
core
CArG 3'
core del GATA
CArG
core del 1
core del 2
core del 3
core del 4
core mut 1
core mut 2
SRF
AB box
α-omni
α-SP1
Elk-1
C
probe
SM22α SME1
SRF
AB box
α-omni
D
probe
core
SRF
A box
B box
AB box
α-omni
E
Relative Luciferase Activity
125
100
75
40
30
20
10
0
T370
T370 del CTTT
myocardin
AB box
F
Relative Luciferase Activity
125
100
75
50
25
0
T370
T370 del CTTT
myocardin
B box

moter (Fig. 5C). Similarly, overexpression of myocardin, by adenoviral transduction, showed that myocardin binds to the telokin promoter and displaces endogenous Elk-1 (Fig. 5D). Together, these data demonstrate that myocardin and Elk-1 compete with each other for binding to SRF on the telokin promoter.

SRF is required for Elk-1 binding to the telokin promoter. The presence of an ETS binding site adjacent to a critical CArG box in the SM22α promoter is important for the ability of Elk-1 to block the activation of this promoter by myocardin (35). Similar ETS binding sites are not readily identifiable on some of the other SMC-restricted promoters, such as the telokin promoter, that are also regulated by Elk-1. However, chromatin immunoprecipitation assays have demonstrated that full-length Elk-1 and the A box of Elk-1 bind to the telokin promoter in vivo in intact chromatin. To identify the Elk-1 binding site in the telokin promoter, gel mobility shift analysis was carried out using probes from the telokin gene that contain the CArG box and flanking sequences (Fig. 6A). Using this approach, we were unable to demonstrate binding of full-length Elk-1 alone or the AB-box fragment of Elk-1 alone to any of these probes or to a probe from the SM22α promoter (Fig. 6B, leftmost two panels, and Fig. 6C). However, binding of Elk-1 or the AB fragment of Elk-1 could be detected using a probe that included the CArG box and 5′-flanking sequence of the telokin promoter when SRF was also added to the gel mobility shift assays. This is most clearly indicated by the supershifted band specific to the omni epitope tag on Elk-1 that is observed in the presence but not the absence of SRF (Fig. 6B). This is consistent with previous data indicating that Elk-1 DNA binding is stabilized by the presence of SRF bound to an adjacent CArG box (30). To further refine the Elk-1 binding site, a series of mutant or truncated probes were generated as shown in Fig. 6A. Even in the presence of SRF, no Elk-1 binding could be detected to a probe that included the CArG box and 3′-flanking region (Fig. 6B), indicating that sequences 5′ of the CArG box are important for Elk-1 binding. A potential ETS binding site, GGAT, on the opposite DNA strand that overlaps a GATA site was found within the telokin core probe. To examine the possibility that Elk-1 binds through this site, a probe containing a deletion of the GATA/ETS site was generated and then incubated with the AB-box fragment of Elk-1. The AB-box fragment of Elk-1 was still able to bind to this mutant probe (Fig. 6B). In contrast, a series of probes containing 5′ deletions or internal deletions within the −90 to −66 region demonstrated that a CTTT sequence between −81 and −78 is important for Elk-1 binding to the telokin promoter, as deletion of these four nucleotides diminished binding of the AB-box fragment of Elk-1 (core del 4 [Fig. 6B]). Deletion of nucleotides −82 to −85 or −74 to −66 which flank these four nucleotides did not attenuate Elk-1 binding (core del 3 and core del 2 [Fig. 6B]). Within the −81 to −78 sequence, mutation of CT (−81, −80) to AG abolished binding of the AB box, whereas mutation of T (−79) to A had no effect on binding, indicating that nucleotides −81 to −80 are critical for Elk-1 binding in vitro.

Gel mobility shift assays utilizing either the A-box or B-box fragment of Elk-1 alone failed to demonstrate ternary complex formation with either fragment (Fig. 6D). Taken together, these data suggest that similar to previous reports on growth factor-responsive genes, such as c-*fos*, both the DNA binding domain and SRF interaction domain of Elk-1 are required for the Elk-1 to interact with the telokin promoter through a nonconsensus ETS site in vitro. To confirm the functional importance of the nonconsensus ETS site in the telokin promoter, reporter gene assays were performed using a promoter that contained a deletion of the −81 to −78 region shown to be important for ternary complex formation in vitro (Fig. 6B). Deletion of the −81 to −78 region (CTTT) in the telokin promoter reporter gene did not affect myocardin activation of the promoter (41), but it significantly impaired the ability of the AB-box fragment of Elk-1 to inhibit myocardin activation (Fig. 6E). However, deletion of the Elk-1 binding site in the telokin promoter did not affect the ability of the B-box domain of Elk-1 alone to partially inhibit the myocardin activation of the promoter. The B-box domain of Elk-1 inhibited the myocardin activation of both the wild-type and mutant telokin promoters equally (Fig. 6F). Conversely, introduction of a consensus ETS binding site into the telokin promoter promoted the ability of Elk-1 to inhibit the myocardin activation of the promoter in 10T1/2 cells and promoted the ability of Elk-1 to inhibit basal telokin promoter activity in A10 smooth muscle cells (Fig. 7).

FIG. 6. Mapping the Elk-1 binding site on the telokin promoter. (A) Schematic summary of the sequences of the telokin promoter and SM22α promoter probes that were used for gel mobility shift assays. A potential Elk-1 core binding site (ATCC) adjacent to the CArG box in the telokin promoter is underlined, and the Elk-1 core binding site in SM22α SME1 is italicized. In mutant probes (core mut 1 and 2), nucleotides that are different from the nucleotides in the wild type are underlined. The CArG element is boxed. del, deletion. (B) Gel mobility shift assays were performed using each of the probes indicated as described in Materials and Methods. Two micrograms of COS-7 cell extract from cells transfected with the Elk-1 AB box (AB box) or full-length Elk-1 were incubated with probe from telokin in the presence (+) or absence (−) of 0.5 μg of SRF-containing nuclear extract as indicated. Following incubation for 15 min at room temperature, an anti-omni antibody (α-omni) was added to detect the omni epitope tag of Elk-1; reaction mixtures were then incubated on ice for an additional hour. Following incubation, samples were run on a 4% polyacrylamide gel, and mobility-shifted complexes were visualized by autoradiography. An asterisk indicates a supershifted band. (C) Gel mobility shift assays were performed as described above for panel B except that the probe was derived from the SM22α SME1 sequence. (D) Two micrograms of nuclear protein extracted from COS cells transfected with expression plasmids for either the Elk-1 AB box, A box-NLS, or B box-NLS was incubated with core probe in the presence (+) or absence of 0.5 μg SRF nuclear extract as indicated. An anti-omni antibody (α-omni) was used to detect the omni epitope tag on the Elk-1 fusion proteins. (E) Either a wild-type telokin promoter (T370) or a mutant telokin promoter containing a deletion of the −81 to −78 region (T370 del CTTT) shown to be required for binding to Elk-1 in panel B was cotransfected into 10T1/2 cells together with myocardin or the Elk-1 AB box, and 24 h later, luciferase activity was measured. The promoter activity relative to myocardin-stimulated levels (assigned arbitrary value of 100) is presented. The values are means ± SEMs (error bars) for six samples. Statistical differences between the activity of the two reporter genes ($P < 0.05$) are indicated by an asterisk. (F) Luciferase reporter gene assays identical to those described above for panel D were performed except that the Elk-1 B box was used in place of the AB box.

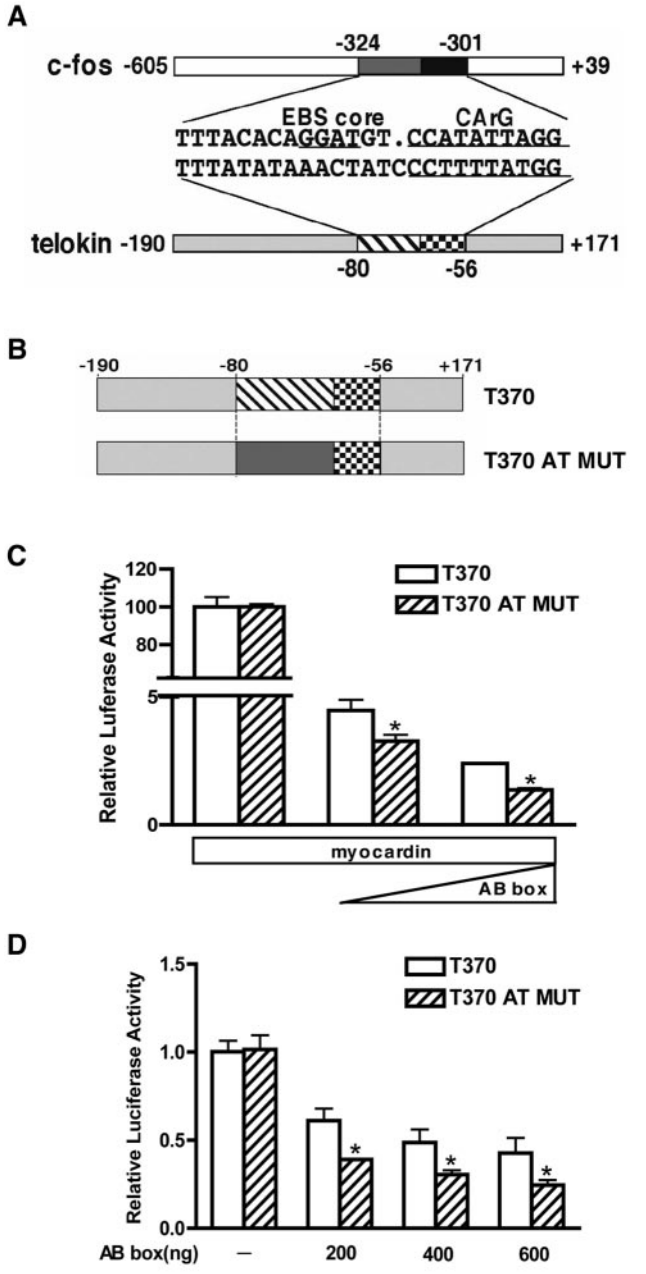


FIG. 7. Effects of the Elk-1 AB box on a mutant telokin promoter containing a consensus Elk-1 binding site. (A) Schematic diagram showing the sequences of the CArG box and adjacent 5′ sequence from c-*fos* and telokin promoters. The c-*fos* promoter (open bar) ETS binding site (EBS) within the c-*fos* 5′-flanking region (dark gray) and CArG box (black) and the telokin promoter (light gray) AT-rich region (hatched) and CArG box (checkered) are indicated. The ETS core binding site and CArG box are underlined within these sequences. (B) Schematic diagram showing a chimeric telokin promoter in which the AT-rich region was mutated to the c-*fos* promoter ETS binding sequence (T370 AT MUT) by employing QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA). (C) Mouse myocardin and increasing amounts of the Elk-1 AB box or an empty expression vector plasmid were transiently cotransfected with a wild-type mouse telokin promoter-luciferase construct (T370) or a mutant construct containing the c-*fos* ETS binding site sequence (T370 AT MUT) and a minimal thymidine kinase promoter-driven renilla luciferase internal control plasmid into 10T1/2 fibroblast cells, and luciferase activity was measured. A value of 100 was arbitrarily assigned to the activation of the promoters by myocardin alone. Data presented are means ± SEMs (error bars) for six samples. Statistical differences in the activity of the two reporter genes ($P < 0.001$) are indicated by an asterisk. (D) Wild-type (T370) or mutant (T370 AT MUT) luciferase reporter gene constructs were cotransfected with increasing amounts of the Elk-1 AB-box expression plasmid into A10 SMCs, and the resultant changes in luciferase activity were determined. Data are expressed relative to the activity of the reporter genes cotransfected with empty expression vectors and presented as means ± SEMs (error bars) for six samples. Statistical differences in the activity of the two reporter genes ($P < 0.001$) are indicated by an asterisk.

Elk-1 negatively regulates SM gene expression in vivo. Thus far, the data presented suggest that Elk-1 can inhibit smooth muscle-specific gene expression when Elk-1 is expressed at high levels. To determine the role of endogenous Elk-1 in regulating SMC-specific gene expression, endogenous Elk-1 levels were decreased using siRNA. siRNA specific for Elk-1 was able to decrease expression of exogenous Elk-1 (Fig. 8A) and endogenous Elk-1 (Fig. 8B) following adenovirus-mediated transduction of A10 cells. Adenovirus-mediated transduction of A10 smooth muscle cells with Elk-1 siRNA resulted in an increase in SM22α and telokin mRNA and protein without any significant changes in the levels of expression of SM α-actin or calponin (Fig. 8B, C, and D); conversely, expression of c-*fos* mRNA decreased (Fig. 8B). Transduction with a control siRNA had no significant effects on the expression of any of these proteins (Fig. 8C and D).

DISCUSSION

In this study we show that Elk-1 can suppress telokin, SM22α, and SM α-actin expression in A10 smooth muscle cells through a mechanism in which Elk-1 interferes with the ability of myocardin to activate the promoters of these genes. Knocking down endogenous Elk-1 levels in A10 SMCs resulted in increased expression of telokin and SM22α, demonstrating that in these partially dedifferentiated smooth muscle cells, Elk-1 is playing an important role in attenuating smooth muscle-specific gene expression.

A mammalian two-hybrid assay demonstrated that the B-box domain of Elk-1 is sufficient to compete with myocardin for binding to SRF (Fig. 3A). These data are consistent with previous studies that demonstrated that SRF and Elk-1 can interact directly through protein-protein interactions in the absence of DNA (31). However, the B-box domain alone does not inhibit the myocardin-induced activation of the telokin promoter as well as either wild-type Elk-1 or truncated Elk-1 comprised of the A box and B box domain (Fig. 2 and 3). This is consistent with data obtained from gel mobility shift assays that showed that neither the Elk-1 A box alone nor the Elk-1 B box alone could form a ternary complex with SRF on the telokin promoter in vitro (Fig. 6C). In contrast, a ternary complex was formed using a fragment of Elk-1 that included both the A box and B box or with the full-length Elk-1. In addition, mutation of the Elk-1 binding site in the telokin promoter partially attenuated the ability of Elk-1 to inhibit the myocardin-induced activation of the promoter, whereas addition of a higher-affinity ETS binding site from the c-*fos* promoter enhanced the

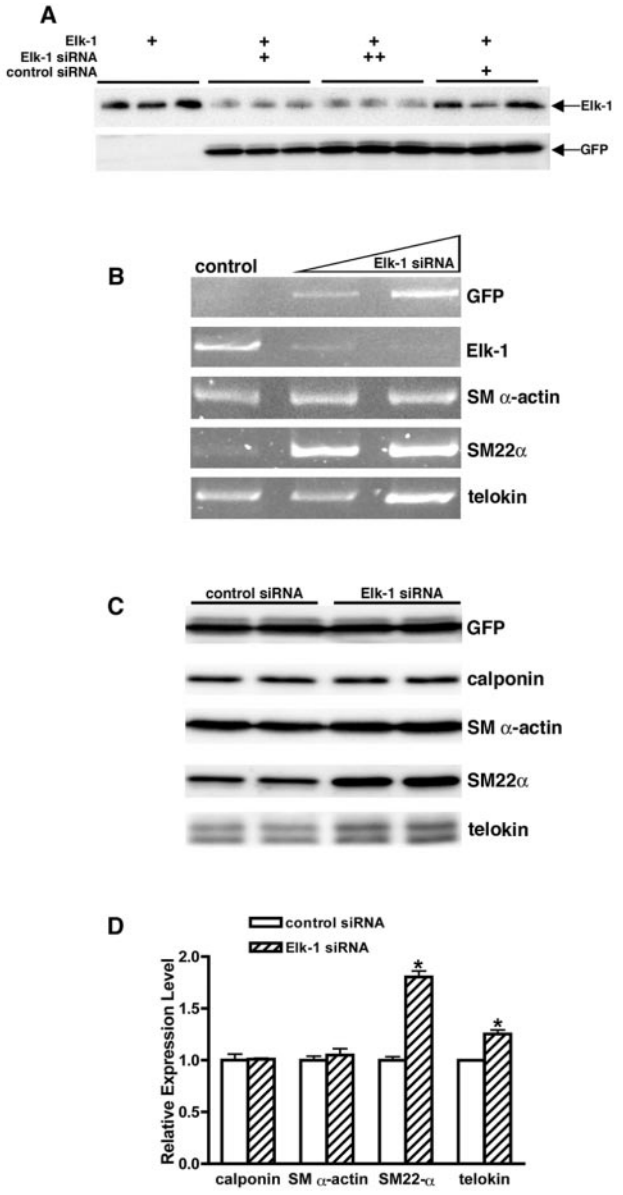


FIG. 8. Effects of knocking down Elk-1 levels on smooth muscle-specific gene expression. (A) A10 cells were coinfected with adenovirus encoding Elk-1, Elk-1 siRNA, or a scrambled siRNA control as indicated above the lanes. Seventy-two hours following infection, protein extracts were prepared from infected cells and analyzed by Western blotting. The level of Elk-1 siRNA or control siRNA transduction is indicated by the expression of GFP, which is encoded on the same virus. (B) A10 cells were infected with increasing amounts of Elk-1 siRNA adenovirus. An uninfected sample served as a control. Seventy-two hours following infection, total RNA was harvested from cells using TRIzol reagent, and RT-PCR was performed to detect GFP expression and endogenous Elk-1, SM α-actin, SM22α, telokin, and c-*fos* mRNA expression. (C) An equal amount of Elk-1 siRNA adenovirus or a control siRNA adenovirus was used to transduce A10 cells, and the levels of endogenous protein expressed were examined by Western blotting. Equal levels of GFP expression indicate similar levels of transduction of the two adenoviruses. (D) Quantification of data presented in panel C. The level of protein expressed was determined by measurement of the protein absorbance units relative to the respective GFP expression as an internal control. A value of 1 was arbitrarily assigned to the protein expression level in cells infected with control siRNA. Changes in expression that were statistically significantly different from the values for the siRNA control ($P < 0.05$ by Student nonpaired *t* test) are indicated by an asterisk.

ability of Elk-1 to block the activation of the telokin promoter by myocardin. Together, these data suggest that the in vivo binding of Elk-1 to smooth muscle-restricted promoters may play an important role in increasing the local effective concentration of endogenous Elk-1, thus allowing it to more effectively compete with myocardin for binding to SRF. In overexpression experiments, the concentration of exogenous proteins may be sufficient to make the ability of Elk-1 to bind to DNA unnecessary for Elk-1 to compete with myocardin for binding to SRF.

Gel mobility assays demonstrated that a nonconsensus Elk-1 binding site was required for ternary complex formation on the telokin promoter (Fig. 6B). Deletion of this nonconsensus Elk-1 binding site in the telokin promoter abrogated ternary complex formation and rendered the promoter less susceptible to repression by Elk-1 (Fig. 6E). The presence of the B-box domain of Elk-1 was also required for ternary complex formation on the telokin promoter in vitro (Fig. 6D). These data support a model in which the interaction of Elk-1 with SRF and with DNA is stabilized in the ternary complex and suggest that a direct interaction of SRF and Elk-1 is required to stabilize Elk-1 binding to the low-affinity *cis* element in the telokin promoter. Interactions between SRF and Elk-1 allow each of these molecules to bind to a much broader range of *cis*-acting regulatory sequences than they can otherwise bind individually. In addition to SRF recruiting Elk-1 to low-affinity ETS binding sites, Elk-1 binding to high-affinity ETS sites in the *pip92* and *nur77* genes can recruit SRF into a ternary complex on these promoters in the absence of high-affinity SRF binding sites (19). Together, these data suggest that the sequence variability of distinct ETS *cis*-acting elements and closely opposed CArG boxes contributes to the diversity of responses of different genes to ETS proteins and SRF.

Although overexpression studies demonstrated that Elk-1 can inhibit the expression of several smooth muscle-specific proteins (Fig. 1C) (3, 35), siRNA-mediated knockdown experiments and expression of a dominant-negative Elk-1 (the A box alone) revealed that endogenous levels of Elk-1 in A10 cells repress some smooth muscle genes more than others. For example, although Elk-1 overexpression was able to decrease SM α-actin expression in A10 cells (Fig. 1), SM α-actin expression was refractory to the effects of siRNA-mediated knockdown of Elk-1 (Fig. 8) or to the effects of a dominant-negative Elk-1 (Fig. 4C), while under the same conditions telokin expression was increased by both of these approaches. Although the reason for these differences is unclear, it may reflect either different binding affinities of these genes for Elk-1 or it may reflect a differential myocardin dependence of the expression of each gene. As SM α-actin is expressed in many cell types, such as myofibroblasts, some fibroblast cells, and skeletal muscle myoblasts that have not been reported to express myocardin, it is likely that regulation of expression of this gene can occur by both myocardin-dependent and -independent mechanisms. Elk-1 appears to act by blocking the interaction between myocardin and SRF; hence, if the activity of a promoter is independent of myocardin, then Elk-1 is unlikely to repress promoter activity. In support of this model, although Elk-1 blocked the myocardin-induced expression of myosin light chain kinase in 10T1/2 cells (35), neither Elk-1 overexpression nor myocardin knockdown decreased MLCK expression in

A10 smooth muscle cells (Fig. 1C) (41). Similar to SM α-actin, MLCK is expressed in many cell types in addition to smooth muscle cells (10); hence, its expression is also likely to be regulated by both myocardin-dependent and myocardin-independent pathways. These observations together with those of Yoshida et al. (39) highlight important differences in the regulation of smooth muscle-restricted genes by myocardin family members.

Previous studies would suggest that the up-regulation of ETS family members, such as Ets-1, together with growth factor-mediated phosphorylation of these factors is likely to play an important role in the down-regulation of contractile proteins that occurs during phenotypic modulation of smooth muscle (3, 8). For example, the DNA binding affinity of Elk-1 is enhanced when serine 383 and serine 389 in the C-terminal domain are phosphorylated by mitogen-activated protein kinase (7). This allows Elk-1 to displace myocardin from the SM22α promoter and inhibit SM22α expression (35). The activity of Elk-1 can also be regulated by sumoylation of the Elk-1 R domain, which alters the binding of extracellular signal-regulated kinase 1 and can also regulate the nuclear retention of Elk-1 (29, 37). Growth factor-mediated changes in the phosphorylation or sumoylation of Elk-1 are thus likely to play an important role in the down-regulation of contractile protein expression that is observed following vascular injury. The differential responses of smooth muscle contractile proteins to phenotypic modulation may be due in part to their differential sensitivity to Elk-1-mediated repression. In addition to elevated activity of inhibitory factors, such as Elk-1 and Sp1, the down-regulation of positive-acting factors, such as myocardin and GATA-6, may also differentially affect the down-regulation of smooth muscle-specific proteins seen under pathological conditions. Myocardin expression is down-regulated in all cultured SMC lines examined and following vascular injury coinciding with attenuated expression of several SMC-restricted genes (2, 9). However, myocardin is not sufficient to direct the expression of all smooth muscle-restricted proteins, suggesting that it acts in concert with other factors to activate the full complement of genes characteristic of a differentiated smooth muscle cell (39). Another factor that has been shown to play a role in smooth muscle-specific gene expression is GATA-6. Following vascular injury, GATA-6 is rapidly down-regulated, and forced expression of GATA-6 following vascular injury helps to prevent the down-regulation of smooth muscle contractile proteins (24). GATA-6 can either positively or negatively regulate the expression of smooth muscle-restricted genes (27, 38). The specific effects of different activators on individual smooth muscle-specific genes together with the different sensitivities of these genes to repression by ETS family members likely account for the complex changes in the expression of smooth muscle-specific proteins that are observed under pathological conditions following different types of smooth muscle injury.

ACKNOWLEDGMENTS

We thank Eric Olson and Gary Owens for reagents. We are grateful to April Hoggatt and Ketrija Touw for expert technical assistance and to members of the Herring lab for helpful discussions.

This work was supported by NIH grants HL58571, DK61130, and DK65644 to B.P.H. and by an American Heart Association fellowship to J.Z.

REFERENCES

1. **Buchwalter, G., C. Gross, and B. Wasylyk.** 2004. Ets ternary complex transcription factors. Gene **324:**1–14.
2. **Chen, J., C. M. Kitchen, J. W. Streb, and J. M. Miano.** 2002. Myocardin: a component of a molecular switch for smooth muscle differentiation. J. Mol. Cell. Cardiol. **34:**1345–1356.
3. **Dandre, F., and G. K. Owens.** 2004. Platelet-derived growth factor-BB and Ets-1 transcription factor negatively regulate transcription of multiple smooth muscle cell differentiation marker genes. Am. J. Physiol. Heart Circ. Physiol. **286:**H2042–H2051.
4. **Du, K. L., H. S. Ip, J. Li, M. Chen, F. Dandre, W. Yu, M. M. Lu, G. K. Owens, and M. S. Parmacek.** 2003. Myocardin is a critical serum response factor cofactor in the transcriptional program regulating smooth muscle cell differentiation. Mol. Cell. Biol. **23:**2425–2437.
5. **Gallagher, P. J., and B. P. Herring.** 1991. The carboxyl terminus of the smooth muscle myosin light chain kinase is expressed as an independent protein, telokin. J. Biol. Chem. **266:**23945–23952.
6. **Gallagher, P. J., B. P. Herring, S. A. Griffin, and J. T. Stull.** 1991. Molecular characterization of a mammalian smooth muscle myosin light chain kinase. J. Biol. Chem. **266:**23936–23944.
7. **Gille, H., M. Kortenjann, O. Thomae, C. Moomaw, C. Slaughter, M. H. Cobb, and P. E. Shaw.** 1995. ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. EMBO J. **14:**951–962.
8. **Goetze, S., U. Kintscher, K. Kaneshiro, W. P. Meehan, A. Collins, E. Fleck, W. A. Hsueh, and R. E. Law.** 2001. TNFα induces expression of transcription factors c-fos, Egr-1, and Ets-1 in vascular lesions through extracellular signal-regulated kinases 1/2. Atherosclerosis **159:**93–101.
9. **Hendrix, J. A., B. R. Wamhoff, O. G. McDonald, S. Sinha, T. Yoshida, and G. K. Owens.** 2005. 5′ CArG degeneracy in smooth muscle alpha-actin is required for injury-induced gene suppression in vivo. J. Clin. Investig. **115:** 418–427.
10. **Herring, B. P., S. Dixon, and P. J. Gallagher.** 2000. Smooth muscle myosin light chain kinase expression in cardiac and skeletal muscle. Am. J. Physiol. **279:**C1656–C1664.
11. **Herring, B. P., G. E. Lyons, A. M. Hoggatt, and P. J. Gallagher.** 2001. Telokin expression is restricted to smooth muscle tissues during mouse development. Am. J. Physiol. Cell Physiol. **280:**C12–C21.
12. **Herring, B. P., and A. F. Smith.** 1997. Telokin expression in A10 smooth muscle cells requires serum response factor. Am. J. Physiol. **272:**C1394–C1404.
13. **Herring, B. P., and A. F. Smith.** 1996. Telokin expression is mediated by a smooth muscle cell-specific promoter. Am. J. Physiol. **270:**C1656–C1665.
14. **Hoggatt, A. M., A. M. Kriegel, A. F. Smith, and B. P. Herring.** 2000. Hepatocyte nuclear factor-3 homologue 1 (HFH-1) represses transcription of smooth muscle-specific genes. J. Biol. Chem. **275:**31162–31170.
15. **Hoggatt, A. M., G. M. Simon, and B. P. Herring.** 2002. Cell-specific regulatory modules control expression of genes in vascular and visceral smooth muscle tissues. Circ. Res. **91:**1151–1159.
16. **Kasza, A., A. O'Donnell, K. Gascoigne, L. A. Zeef, A. Hayes, and A. D. Sharrocks.** 2005. The ETS-domain transcription factor Elk-1 regulates the expression of its partner protein, SRF. J. Biol. Chem. **280:**1149–1155.
17. **Khurana, A., and C. S. Dey.** 2002. Involvement of Elk-1 in L6E9 skeletal muscle differentiation. FEBS Lett. **527:**119–124.
18. **Kim, S., H. S. Ip, M. M. Lu, C. Clendenin, and M. S. Parmacek.** 1997. A serum response factor-dependent transcriptional regulatory program identifies distinct smooth muscle cell sublineages. Mol. Cell. Biol. **17:**2266–2278.
19. **Latinkic, B. V., M. Zeremski, and L. F. Lau.** 1996. Elk-1 can recruit SRF to form a ternary complex upon the serum response element. Nucleic Acids Res. **24:**1345–1351.
20. **Li, L., J. M. Miano, B. Mercer, and E. N. Olson.** 1996. Expression of the SM22α promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. J. Cell Biol. **132:**849–859.
21. **Li, S., D. Z. Wang, Z. Wang, J. A. Richardson, and E. N. Olson.** 2003. The serum response factor coactivator myocardin is required for vascular smooth muscle development. Proc. Natl. Acad. Sci. USA **100:**9366–9370.
22. **Mack, C. P., and G. K. Owens.** 1999. Regulation of smooth muscle alpha-actin expression in vivo is dependent on CArG elements within the 5′ and first intron promoter regions. Circ. Res. **84:**852–861.
23. **Madsen, C. S., C. P. Regan, J. E. Hungerford, S. L. White, I. Manabe, and G. K. Owens.** 1998. Smooth muscle-specific expression of the smooth muscle myosin heavy chain gene in transgenic mice requires 5′-flanking and first intronic DNA sequence. Circ. Res. **82:**908–917.
24. **Mano, T., Z. Luo, S. L. Malendowicz, T. Evans, and K. Walsh.** 1999. Reversal of GATA-6 downregulation promotes smooth muscle differentiation and inhibits intimal hyperplasia in balloon-injured rat carotid artery. Circ. Res. **84:**647–654.
25. **Marais, R., J. Wynne, and R. Treisman.** 1993. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell **73:**381–393.
26. **Miano, J. M.** 2003. Serum response factor: toggling between disparate programs of gene expression. J. Mol. Cell. Cardiol. **35:**577–593.

27. **Oh, J., Z. Wang, D. Z. Wang, C. L. Lien, W. Xing, and E. N. Olson.** 2004. Target gene-specific modulation of myocardin activity by GATA transcription factors. Mol. Cell. Biol. **24:**8519–8528.
28. **Owens, G. K., M. S. Kumar, and B. R. Wamhoff.** 2004. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol. Rev. **84:**767–801.
29. **Salinas, S., A. Briancon-Marjollet, G. Bossis, M. A. Lopez, M. Piechaczyk, I. Jariel-Encontre, A. Debant, and R. A. Hipskind.** 2004. SUMOylation regulates nucleo-cytoplasmic shuttling of Elk-1. J. Cell Biol. **165:**767–773.
30. **Shaw, P. E., H. Schroter, and A. Nordheim.** 1989. The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-fos promoter. Cell **56:**563–572.
31. **Shore, P., and A. D. Sharrocks.** 1994. The transcription factors Elk-1 and serum response factor interact by direct protein-protein contacts mediated by a short region of Elk-1. Mol. Cell. Biol. **14:**3283–3291.
32. **Smith, A. F., R. M. Bigsby, R. A. Word, and B. P. Herring.** 1998. A 310-bp minimal promoter mediates smooth muscle cell-specific expression of telokin. Am. J. Physiol. **274:**C1188–C1195.
33. **Vanhoutte, P., J. L. Nissen, B. Brugg, B. D. Gaspera, M. J. Besson, R. A. Hipskind, and J. Caboche.** 2001. Opposing roles of Elk-1 and its brain-specific isoform, short Elk-1, in nerve growth factor-induced PC12 differentiation. J. Biol. Chem. **276:**5189–5196.
34. **Wang, D., P. S. Chang, Z. Wang, L. Sutherland, J. A. Richardson, E. Small, P. A. Krieg, and E. N. Olson.** 2001. Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. Cell **105:** 851–862.
35. **Wang, Z., D. Z. Wang, D. Hockemeyer, J. McAnally, A. Nordheim, and E. N. Olson.** 2004. Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. Nature **428:**185–189.
36. **Wasylyk, B., J. Hagman, and A. Gutierrez-Hartmann.** 1998. Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. Trends Biochem. Sci. **23:**213–216.
37. **Yang, S. H., E. Jaffray, R. T. Hay, and A. D. Sharrocks.** 2003. Dynamic interplay of the SUMO and ERK pathways in regulating Elk-1 transcriptional activity. Mol. Cell **12:**63–74.
38. **Yin, F., and B. P. Herring.** 2005. GATA-6 can act as a positive or negative regulator of smooth muscle-specific gene expression. J. Biol. Chem. **280:** 4745–4752.
39. **Yoshida, T., K. Kawai-Kowase, and G. K. Owens.** 2004. Forced expression of myocardin is not sufficient for induction of smooth muscle differentiation in multipotential embryonic cells. Arterioscler. Thromb. Vasc. Biol. **24:**1596–1601.
40. **Yoshida, T., S. Sinha, F. Dandre, B. R. Wamhoff, M. H. Hoofnagle, B. E. Kremer, D. Z. Wang, E. N. Olson, and G. K. Owens.** 2003. Myocardin is a key regulator of CArG-dependent transcription of multiple smooth muscle marker genes. Circ. Res. **92:**856–864.
41. **Zhou, J., and B. P. Herring.** 2005. Mechanisms responsible for the promoter-specific effects of myocardin. J. Biol. Chem. **280:**10861–10869.
42. **Zhou, J., A. M. Hoggatt, and B. P. Herring.** 2004. Activation of the smooth muscle-specific telokin gene by thyrotroph embryonic factor (TEF). J. Biol. Chem. **279:**15929–15937.