Vascular Smooth Muscle α-Actin Gene Transcription during Myofibroblast Differentiation Requires Sp1/3 Protein Binding Proximal to the MCAT Enhancer*

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The conversion of stromal fibroblasts into contractile myofibroblasts is an essential feature of the wound-healing response that is mediated by transforming growth factor β1 (TGF-β1) and accompanied by transient activation of the vascular smooth muscle α-actin (SmaA) gene. Multiple positive-regulatory elements were identified as essential mediators of basal SmaA enhancer activity in mouse AKR-2B stromal fibroblasts. Three of these elements bind transcriptional activating proteins of known identity in fibroblasts. A fourth site, shown previously to be susceptible to single-strand modifying agents in myofibroblasts, was additionally required for enhancer response to TGF-β1. However, TGF-β1 activation was not accompanied by a stoichiometric increase in protein binding to any known positive element in the SmaA enhancer. By using oligonucleotide affinity isolation, DNA-binding site competition, gel mobility shift assays, and protein overexpression in SL2 and COS7 cells, we demonstrate that the transcription factors Sp1 and Sp3 can stimulate SmaA enhancer activity. One of the sites that bind Sp1/3 corresponds to the region of the SmaA enhancer required for TGF-β1 amplification. Additionally, the TGF-β1 receptor-regulated Smad proteins, in particular Smad3, are rate-limiting for SmaA enhancer activation. Whereas Smad proteins collaborate with Sp1 in activating several stromal cell-associated promoters, they appear to operate independently from the Sp1/3 proteins in activating the SmaA enhancer. The identification of Sp and Smad proteins as essential, independent activators of the SmaA enhancer provides new insight into the poorly understood process of myofibroblast differentiation.

Myofibroblasts have phenotypic properties intermediate between fibroblasts and smooth muscle cells and are thought to provide mechanical force necessary for wound contraction and closure (1–4). Believed to arise from stromal fibroblasts in response to growth factors and inflammatory cytokines released during tissue injury, myofibroblasts contain abundant contractile microfilaments composed of vascular smooth muscle α-actin (SmaA)1 (1, 5–9), smooth muscle myosin (10), the calponin-like, smooth muscle microfilament-binding protein SM22α (11), and caldesmon, a smooth muscle-specific calcium-regulatory protein (12). Normally transient cellular participants in wound-healing processes, chronic accumulation of α-actin-positive myofibroblasts has been associated with pathologic tissue remodeling processes such as hypertrophic scarring, stromal responses to certain neoplasias, and interstitial fibrosis associated with repair of myocardial infarction (13, 14).

Although the molecular control of myofibroblast differentiation is largely unknown, several studies have shown that transforming growth factor β1 (TGF-β1) may be particularly important in their recruitment to sites of tissue inflammatory damage. TGF-β1 is a potent SmaA gene transcriptional activator in both granulation tissue and isolated fibroblasts (2, 5, 7, 15). TGF-β1 also induces expression of SmaA in cultured rat aortic smooth muscle cells, bovine aortic endothelial cells, and rat fibroblasts, where specific transcriptional control elements contained within the rat SmaA gene promoter were shown to mediate TGF-β1-dependent activation (16, 17). However, the sequence context and position of DNA-regulatory elements identified in studies on the rat SmaA promoter and aortic smooth muscle cells differed from those in the mouse SmaA enhancer that undergoes TGF-β1-dependent chromatin conformational changes in AKR-2B stromal fibroblasts (15). TGF-β1 elicits both tissue-specific and cell culture context-specific responses (18, 19), yet studies performed to date have not resolved whether sequence elements required for activation from TGF-β1-dependent promoters are distinctly different or evolutionarily conserved.

Previous studies from our laboratories have indicated that the mouse SmaA gene is regulated by an array of both positive and negative cis-acting elements that behaved differently in fibroblasts compared with muscle cells (20–27). These studies resulted in the identification of a minimal enhancer element that was constitutively active in fibroblasts, immature myoblasts, and cultured aortic smooth muscle cells, but not in quiescent, differentiated skeletal muscle myocytes. The aim of the present work was to determine whether the mouse SmaA enhancer also conveyed the TGF-β1 response in fibroblasts and whether sequence elements and transcription factors previously identified as responsible for TGF-β1 inducibility in rat aortic smooth muscle cells also directed this effect in mouse

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1 The abbreviations used are: SmaA, smooth muscle α-actin; TGF-β1, transforming growth factor β1; TCE, TGF-β1 control element; THR, TGF-β1 hypersensitivity region; FBS, fetal bovine serum; CAT, chloramphenicol acetyltransferase; VSM, vascular smooth muscle; EMSA, electrophoretic mobility shift assay; DE, downstream element.
Vascular Smooth Muscle α-Actin Gene Transcription

Our results indicate that TGF-β1 amplifies basal expression of the mouse SmoA enhancer by concerted action at five positive regulatory sites. Interestingly, stoichiometric changes in nuclear protein binding activity at these specific sites were not observed in TGF-β1-activated mouse fibroblasts. Whereas three of these sites bind activating proteins of known identity, proteins interacting with the previously identified TGF-β1 control element (TCE) plus a newly identified TGF-β1 hypersensitivity region (THR) in fibroblasts were determined to be the Sp1 and Sp3 transcriptional regulatory proteins. Our data indicate that both Sp1 and Sp3 can mediate basal expression from both the TCE and THR but convey TGF-β1-inducible expression only at the THR in the mouse SmoA enhancer. A rate-limiting role in the activation of the mouse SmoA enhancer is indicated for the TGF-β1 receptor-regulated Smad proteins previously shown to govern transcription of TGF-β1-dependent genes associated with wound healing and extracellular matrix remodeling in fibroblasts.

MATERIALS AND METHODS

Cell Culture and Transfection Methods—AKR-2B embryonic fibroblasts were maintained in McCoy’s 5A medium (Invitrogen) and penicillin-streptomycin (20, 26, 27). Nonhuman primate COS7 kidney fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (4.5 g/liter n-glucose) supplemented with penicillin-streptomycin and 10% FBS. Primary cultures of mouse embryonic fibroblasts derived from wild-type and Smad3 knockout mice were rendered quiescent by a 48-h exposure to HEPES-buffered Dulbecco’s modified Eagle’s medium (1.0 g/liter D-glucose), 0.5% FBS, and penicillin-streptomycin. All fibroblast preparations were rendered quiescent by a 48-h exposure to HEPES-buffered Dulbecco’s modified Eagle’s medium (1.0 g/liter n-glucose), 0.5% FBS, and penicillin-streptomycin. Recombinant human TGF-β1 (2–5 ng/ml, final concentration; R&D Systems, Minneapolis, MN) or an equivalent volume of vehicle (1 mg/ml bovine serum albumin, 4 mM HCl) was added to fibroblast cultures for varying periods before preparation of cell extracts for either reporter gene determinations or electrophoretic mobility shift assays. Mithramycin A (0.1–0.8 μM, final concentration; Sigma) was used in some experiments as specified in the figure legends to specifically block Sp1 binding at GC-rich motifs within the SmoA enhancer (30). Plasmid DNA was prepared by a Qiagen Prep Kit (Qiagen, Chatsworth, CA). In studies using AKR-2B and mouse embryonic fibroblasts, SmoA enhancer-CAT reporter gene fusion plasmids were combined with 5 μg of either pSV-β-galactosidase (Promega, Madison, WI) or pXGH5 (28) reporter gene constructs to normalize CAT expression for variation in transfection efficiency. pXGH5 encodes secreted human growth hormone and pSV-β-galactosidase, which is used as a transfection efficiency control. CAT, β-galactosidase, and human growth hormone assays were performed in triplicate using commercial enzyme-linked immunosorbent assay kits (Roche Molecular Biochemicals) in accordance with the manufacturer’s instructions. For Sp and Smad protein overexpression studies using high transfection efficiency SL2 and COS7 cells (see below), SmoA/CAT fusion gene output was normalized to total protein rather than output from a secondary β-galactosidase or human growth hormone reporter gene. This was done because direct trans-regulation of the pSV-β-galactosidase and pXGH5 promoters by the overexpressed proteins could compromise their role in monitoring transfection efficiency. For transfection, cells at 50% confluence in 6-well plates first were washed with serum- and antibiotic-free medium. Optimized mixtures of SmoA promoter/reporter fusion plasmids (pC3VSM4 and pC3VSM5) and plasmids encoding various transcriptional regulatory proteins (see below) were combined (total plasmid payloads were between 0.5 and 2.5 μg) along with 15 μl of LipofectAMINE reagent (Invitrogen) and incubated for 30 min at room temperature before applying them to cell monolayer for a 5-h period. After washing out unincorporated DNA, the cells were maintained in complete medium for a further 48 h before harvesting the cells as described above. Transfections were routinely performed in triplicate, and each experiment was repeated three to five times. Mean values for normalized CAT activity (±S.E.) were evaluated by analysis of variance with statistical significance set at p ≤ 0.05.

Schneider Drosophila 2 (SL2; kindly provided by Dr. M. Seeger; Ohio State University) cells were grown in Schneider’s medium (Invitrogen) supplemented with penicillin, streptomycin, and 10% FBS. For transfection, replicate preparations of SL2 cells were plated at 50% confluence 1 day before DNA delivery and washed briefly with 2 ml of serum-free Schneider’s medium just before transfection. For each transfection, 1 μg of SmoA promoter:reporter fusion plasmid (pC9VSM5 or pC3VSM4) plus 4 μg of either pSV-β-galactosidase (Promega) or a PAC vector, pPACSp1, and pPACSp3 driven by the Drosophila actin promoter and kindly provided by Dr. G. Suske (University of Marburg) were combined with 9 μl of Cellfectin reagent (Invitrogen), incubated for 15 min at room temperature, diluted with serum-free medium, and then distributed to SL2 cells. After 20 h, the DNA-containing medium was removed, and the cells were washed briefly with 1 ml of complete growth medium for 48 h before harvesting cells for extract assays.

Mammalian Protein Overexpression Plasmids and Construction of VSM α-Actin Promoter Mutations—Recombinant forms of human Sp1 and Sp3 (kindly provided by Dr. J. Horowitz; North Carolina State University) and the human Smad proteins (kindly provided by Drs. L. Choy and R. Derynck, UCSF) were subcloned into mammalian expression plasmids under control of the cytomegalovirus promoter. Transcription mutants of the VSM α-actin promoter:CAT reporter fusion constructs pC3VSM3 and pC3VSM4 (abbreviated henceforth in this work as VSM3 or VSM3 and VSM4 or VSM4) were constructed using PCR amplification or site-directed mutagenesis using a commercial mutagenesis kit (Stratagene, La Jolla, CA). Promoters harboring mutations in the 5′- and 3′-flanking regions were used for an additional 36 cycles in a PerkinElmer Life Sciences DNA Thermacycler using PCR kit (PerkinElmer Life Sciences) reagents and reaction times (94 °C × 1 min; 50 °C × 2 min; 72 °C × 3 min). PCR products were purified from 1–2% agarose gel slices using SpinX columns (Costar, Cambridge, MA). The promoter construct harboring the MCAT mutation (MCAT-mut) was created by amplifying the VSM4 insert with a forward primer containing a SalI restriction site and substituting CTTCCGT for the native MCAT site between −182 and −176. The promoter construct harboring the THR mutation (THR-mut) was created by amplifying the VSM4 insert with a forward primer containing a SfII restriction site that substitutes CTGA for AGGC between −160 and −157. Mutation of the putative TGF-β1 control element (TCE-mut) was made using a primer that substituted TT for GG at positions −50 and −49 in VSM4. Promoters harboring the downstream element mutation (DE-mut) were created by amplification with a reverse primer containing a SalI recognition site, 3′ BarnHI recognition site, and substituting CATCAAAACCA for CCGGGCACACCC between −2 and −11 in both VSM3 and VSM4 context. Promoter constructs harboring StuI recognition site substitution mutations in place of CARG-like elements A, B, or D (A-mut, B-mut, and D-mut, respectively) were prepared as described previously (26). All sequence modifications were confirmed by double-stranded dideoxy sequencing.

Preparation of DNA-binding Protein Extracts and Electrophoretic Mobility Shift Assays (EMSAs)—EMSA reactions typically contained 5–10 μg of nuclear protein extract, 1 μg of poly(dI-dC), 10 mM Tris, pH 7.5, 50 mM NaCl, 0.5 mM dithiobitol, 0.5 mM EDTA, 0.12 mM phenylmethylsulfonyl fluoride, 4% glycerol, and 20,000 cpn (5–50 fmol) of 32P-labeled probe in a 10-μl reaction volume. EMSA probes were constructed in native copy number context with the exception of the CARG D oligonucleotide, which contained a dimerized version of the native motif to improve binding affinity. Mutated versions of each EMSA probe contained nucleotide substitutions identical to those contained in the reporter gene counterparts described in the previous section. Oligonucleotide probes were labeled with Klenow enzyme (Invitrogen) and [γ-32P]ATP (ICN Biomedical, Costa Mesa, CA), purified by electrophoresis on 8% polyacrylamide gels, eluted, and ethanol-precipitated before use. EMSA reaction mixtures were incubated for 30 min at room temperature before electrophoretic analysis on 5% nondenaturing polyacrylamide gels in 0.5× TBE buffer (0.045 M Tris borate and 1 mM EDTA). For competition EMSAs, an excess of unlabeled oligonucleotide competitor was added to the reaction before adding probe extract or labeled probe. Antibody supershift EMSAs using commercial Sp1 and Sp3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were performed as described above but included a 20 min preincubation with 2 μl of anti-Sp antibody before the addition of probe.

Purified mouse IgG was used as a negative control in antibody supershift experiments.

Oligonucleotide Affinity Capture, Immuno blot, and Northern Blot Analysis—Reaction mixtures containing nuclear extract (100 μg of pro-
tein) and biotinylated oligonucleotides (100 pmol; Integrated DNA Technologies) corresponding to the Sp1 consensus site, native TCE site, or mutated TCE site (TCE-mut) were incubated under conditions that mimicked an EMSA reaction. Sp1/Sp3-biotin-DNA complexes were captured on streptavidin-immobilized paramagnetic particles (Promega; 0.6 ml/reaction, 30-min incubation). After washing four times with buffer containing 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl, bound protein was eluted using 2× protein denaturation buffer and analyzed by Western blot (22). Eluted protein was processed by SDS-PAGE using 10% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). After overnight blocking at 4 °C in TBS (25 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 3% (w/v) nonfat dry milk, blots were incubated with anti-Sp1 or anti-Sp3 rabbit polyclonal antibodies for a 60–90-min incubation at ambient temperature with gentle mixing. Goat anti-rabbit IgG-HRP (horseradish peroxidase) (Vector Laboratories, Burlingame, CA) diluted 1:2000 in TBST (TBS containing 0.05% Tween 20) was then applied for 30–45 min. After washing in TBST, the blot was incubated with Vectastain™ reagents for 30 min. Blots were washed four times over a 30-min period and processed for chemiluminescence development for 1 min (ECL™, reagents; PerkinElmer Life Sciences), and the immunocomplexes were visualized on x-ray film (X-Omat; Eastman Kodak) after a 5–10-s exposure. Both antibodies recognized multiple Sp1 and Sp3 isoforms. Northern blot analysis was performed using a SmαA-specific 3′ (untranslated region) cDNA probe as described previously (15, 52).

RESULTS

Basal transcription of the native SmαA gene in mouse AKR-2B stromal fibroblasts was minimally enhanced 6-fold up to as much as 20-fold in higher cell density preparations within 6 h of treatment with TGF-β1 (Fig. 1a). To localize sequence elements responsible for TGF-β1 activation, we performed deletion analysis of a 3.6-kb segment of the mouse SmαA promoter (VSMP8) that exhibits correct developmental regulation in transgenic mice. At the low cell density required for efficient transfection, VSMP8 transcription was increased ~4-fold by TGF-β1, indicating that elements contained within the 5′-flanking/first intronic region mediated growth factor responsiveness (Fig. 1, b and c). A subfragment of the 5′-flanking region, located between -191 and +46 relative to the start of transcription (Fig. 16), contains a potent core enhancer element previously shown to contain a MCAT motif required for high-level constitutive expression and serum inducibility in stromal fibroblasts (21, 26), aortic smooth muscle cells (25, 29), and undifferentiated striated muscle myoblasts (21). The VSMP4 construct containing this enhancer was induced about 6-fold by TGF-β1. Further 5′ truncation by 41 bp (VSMP5) or 101 bp (VSMP6) completely eliminated basal and TGF-β1-inducible transcriptional activity, indicating that the region of the mouse SmαA promoter between -191 and -150 was minimally required to mediate the TGF-β1 response in fibroblasts (Fig. 1, b and c).

Site-specific mutations were created within VSMP4 context to evaluate the relative importance of the known positive elements in mediating TGF-β1 inducibility in fibroblasts. Altered sites included an inverted MCAT motif (AGGAATG, between -182 and -176) and CARG-like elements B and A (CCCTATAGG, between -120 and -111, and CCGTGTTCGG, between -70 and -61) that bind the TEF1 and serum response factor transcriptional activating proteins, respectively. Both sites were required for basal expression of VSMP4 in mouse fibroblasts (24, 26), neonatal rat aortic A7r5 smooth muscle cells (29), and undifferentiated mouse BC3H1 myoblasts (21). Also included in the analysis was a variant times containing a 4-bp transversion (AGGC to CTAA) within a recently described 20-bp subdomain of VSMP4 that exhibited differential reactivity to DNA-modifying agents in the presence and absence of TGF-β1 (15). This region, located between -170 and -150, is referred to as the THR. A construct containing a mutation within a putative TCE located between -53 and -43, previously identified in the rat SmαA promoter (16), was also included in the analysis. Fig. 2 shows that all mutations exhibited significantly reduced basal expression relative to native VSMP4. However, all VSMP4 mutants retained TGF-β1 inducibility, except for the construct harboring a THR mutation. Whereas fold induction in the presence of TGF-β1 (relative to that exhibited by native VSMP4) was highest for the construct lacking a functional MCAT element, mutations in the CARG, CARG A, or CARG B motifs all showed approximately the same fold increase. The slight induction exhibited by the THR mutation was not statistically significant (Fig. 2). Thus, with the exception of the THR, mutation of all other activating elements (MCAT, CARG B, CARG A, and TCE) did not fully eliminate the mouse SmαA enhancer responsiveness to TGF-β1 in transfected fibroblasts.

EMSA were performed using extracts prepared from TGF-β1-treated fibroblasts to examine nuclear protein binding to
the Smα enhancer. No net increase in binding activity to the MCAT, CARG B, and TCE probes was observed after treatment with TGF-β1 (data not shown). Protein binding specificity was confirmed by competition with unlabeled excess DNA (see below). In the mouse Smα enhancer, CARG B was previously shown to bind serum response factor (26, 29), whereas the MCAT element binds TEF1 (20, 26, 27). Whereas the identity of the mouse fibroblast TCE-binding protein was not known, an identical binding activity was noted when extracts were assayed using a probe containing the rat Smα A promoter TCE element (data not shown). Because the mouse TCE (TGGGAGGGG) shares homology with GC-rich Sp1/3 transcription factor consensus binding sites (GGGGCGGGG or GGTGTGGGG), we compared Sp1/3 consensus and mouse Smα A TCE probes in both native and mutant context for the ability to bind fibroblast nuclear proteins in oligonucleotide affinity capture assays (Fig. 3). Western blot analysis of eluted proteins revealed that authentic Sp1 and Sp3 proteins bound to both probes equally well but not to the probe harboring a TCE mutation.

Interestingly, DNA-protein complexes with electrophoretic mobilities identical to the Sp1/Sp3-TCE complex were also observed when probes corresponding to other partially or substantially GC-rich regions of the mouse Smα enhancer were used in gel shift analysis (Fig. 4). These additional probes (depicted in Fig. 1b) encompassed the following regions in the mouse Smα core promoter: 1) a 13-bp DE located between −2 and +11 in proximity to the transcription start site, 2) an 11-bp portion of the THR described above, located midway between...
the MCAT and CARG B elements (15), and 3) a 10-bp element previously referred to as CARG D located upstream to the MCAT motif and shown to be involved in transcriptional repression in undifferentiated myoblasts but functionally irrelevant in fibroblasts (21). Although only the TCE sequence strictly resembled an authentic Sp1/3 binding site, each of the four mouse SmαA enhancer elements in native, but not mutant, context specifically competed for protein complexes that bound to a Sp1/3 consensus oligonucleotide probe (Fig. 4). The authentic Sp1/3 consensus element was the most effective competitor, followed by the TCE, DE, THR, and CARG D sites. For all probes, mobility supershift analysis using polyclonal antibodies revealed that the slowest-migrating DNA-protein complexes were specifically eliminated/supershifted by the Sp1-specific antibody, whereas the two more rapidly migrating complexes were eliminated/supershifted by the Sp3-specific antibody (Fig. 5). Taken together, the results demonstrated that both Sp1 and Sp3 were capable of binding at least four sites within or proximal to the mouse SmαA enhancer.

The functional effect of Sp1 and Sp3 overexpression on mouse SmαA enhancer activity was examined in both Drosophila SL2 and nonhuman primate COS7 cells. Whereas VSMP4 showed little baseline activity in SL2 cells, which naturally lack authentic Sp1/3 proteins, expression of either Sp1 or Sp3 stimulated VSMP4 transcription to a different extent, with co-expression of both Sp1 and Sp3 showing an intermediate level of activation (Fig. 6). For both SL2 and COS7 cells, Sp3 was a less efficient activator of VSMP4 transcription. Western blot analysis of transfected COS7 cells revealed that reduced efficiency of Sp3 enhancer activation was not due to reduced Sp3 protein synthesis relative to cells transfected with identical mass amounts of Sp1 expression plasmid (data not shown).

Whereas the THR and TCE Sp1/3 binding sites were both required for basal activity of the mouse SmαA promoter in fibroblasts (Fig. 2), the functional significance of the newly identified DE and CARG D Sp1 binding sites was unknown and therefore examined in cell transfections assays using reporter gene constructs containing site-specific mutations (Fig. 7). The DE site was fully contained within the 191-bp VSMP4 promoter, but the CARG D binding site was farther upstream and thus mutated in the context of a slightly larger 224-bp SmαA promoter construct (VSMP3) that is normally repressed in fibroblasts, undifferentiated myoblasts, and proliferating cultured aortic smooth muscle cells (29, 21). The construct containing a THR mutation served as a negative control in this experiment and, as before (refer to Fig. 2), was inactive when compared with the native VSMP4 promoter (Fig. 7). In contrast, disruption of the DE Sp1/3 binding site had no significant effect on VSMP4 activity. Moreover, mutation of the CARG D Sp1/3 binding site in context of the repressed VSMP3 construct had no de-repression/activating effect in fibroblasts when present alone or in combination with a DE mutation. Taken together with the EMSA results, the data suggested that Sp1/3 proteins function as transcriptional activators in fibroblasts, most likely through their essential interaction with the TCE and THR sites.

TGF-β1 appeared to amplify SmαA enhancer output in fibroblasts in the absence of a net increase in Sp1/3 binding to the THR, suggesting that another regulatory protein may be rate-limiting for this response. In this regard, Sp1/3 has been reported by several investigators to mediate transcriptional response to TGF-β1 through both direct and indirect association with members of the Smad family of TGF-β1 receptor-regulated proteins (31–34). Western blot analysis revealed that Smad2 and Smad3 co-activating proteins were rapidly translocated to the nuclear compartment of fibroblasts within 30 min of exposure to TGF-β1 (Fig. 8). Suggestive of Smad independence, VSMP4 was induced by TGF-β1 in transfected normal mouse embryonic fibroblasts but failed to respond in transfected embryonic fibroblasts derived from Smad3-deficient mice (Fig. 8). Either Smad2 or Smad3 was capable of transactivating VSMP4 in transfected COS7 cells, but only when an expression plasmid encoding the collaborating Smad4 protein was included (Fig. 9). Interestingly, co-expression of Smad2/3/4 and Sp1/3 proteins in COS7 cells did not increase VSMP4 output beyond what was observed when Smad or Sp1/3 proteins were overexpressed individually. These observations suggest that either Sp1/3 or Smad proteins may be equally capable of activating the SmαA enhancer via a mechanism not based on transcriptional synergy. This idea was further supported by experiments using the Sp1-selective transcriptional inhibitor, mithramycin A. Mithramycin A is an intercalating agent that preferentially binds GC-rich sequences in both native and synthetic oligonucleotide contexts (30). Basal expression of SmαA mRNA was reduced in mithramycin A-treated fibroblasts, yet in three independent Northern blot studies, typical levels of TGF-β1 inducibility were still fully preserved (compare LS + M and TGF+ M in Fig. 10 showing 6-fold induction). Allowing for more rigorous statistical evaluation, studies using COS7 cells transfected with the SmαA enhancer and Smad protein expression plasmids revealed that enhancer output in the presence of Smad2/3/4 was reduced by about 55% (p < 0.01) in the presence of mithramycin A (Fig. 11, compare SMP4/Smads and SMP4/Smads/MA). However, this residual expression still represented a nearly 17-fold increase (p < 0.006) over baseline expression in cell lacking exogenous Smad proteins (Fig. 11, compare SMP4/MA and SMP4/Smads/MA). Taken together, the results were consistent with a model for SmαA enhancer activation that includes both Sp1-dependent (mithramycin A-sensitive) and Sp1-independent/Smad-mediated (mithramycin A-resistant) components.

**DISCUSSION**

TGF-β1 transiently induces expression of SmαA in stromal fibroblasts and therefore may represent an important mediator

![Figure 5: EMSA antibody supershift analysis of protein-DNA-antibody complexes in fibroblasts.](http://www.jbc.org/)

**Fig. 5.** EMSA antibody supershift analysis of protein-DNA-antibody complexes in fibroblasts. Probes encompassed the four Sp1/3 binding sites within the SmαA enhancer (labeled below each panel). The first lane in each series (−) contains no antibody, the second lane contains a mouse IgG control (ns), and the third lane contains an equivalent amount of either a Sp1 (top panel)- or Sp3 (bottom panel)-specific antibody. The positions of Sp1, Sp3, and their respective antibody-supershifted complexes (ssSp1 and ssSp3) are marked with arrows. In some cases, treatments with Sp-specific antibodies resulted in the disappearance of specific Sp protein bands rather than the formation of discrete supershifted complexes (e.g. CARG D in both the top and bottom panels).
of myofibroblast differentiation (15) and short-term contractile function during the wound-healing process (35). Tightly regulated activation of the SmαA gene and consequent assembly of smooth muscle actin stress fibers are likely to be crucial features of myofibroblast differentiation that contribute to the generation of contractile force, rapid wound closure, and effi-

![Graph showing CAT activity](image1)

**FIG. 6.** Effect of overexpression of Sp1 and Sp3 proteins on SmαA enhancer activity in *Drosophila* SL2 and nonhuman primate COS7 cells. Sp expression plasmids contained either the insect β-actin promoter (left panel, SL2 cells) or the cytomegalovirus promoter (right panel, COS7 cells), and SMP4 activity was assayed between 36 and 48 h after transfection. All transfections were performed in triplicate, and each experiment was repeated five times. Data are presented as mean normalized units of CAT expression ± S.E. For both SL2 and COS7 cell studies, all Sp protein overexpression data were statistically significant (*p* < 0.05) relative to the level of expression observed in cells transfected with empty expression plasmids (pPAC for SL2 cells and pCMV for COS7 cells).

![Graph showing CAT activity](image2)

**FIG. 7.** Effect of mutations in novel Sp1/3 binding sites on SmαA enhancer output in fibroblasts. Mutations in the DE, CARG D, and THR Sp1/3 binding sites were compared in the context of either fully active (SMP4) or fully repressed (SMP3) SmαA enhancer contexts. Whereas the THR mutation (SMP4/THR) eliminated enhancer activity compared with expression exhibited by native SMP4 (*p* < 0.05), neither the DE nor CARG D mutations had any statistically significant functional effects on enhancer activity, regardless of whether these mutations were placed in constitutively active (SMP4/DE) or constitutively repressed (SMP3/DE, SMP3/CARGD, and SMP3/DE/CARGD) contexts in AKR-2B fibroblasts. All transfections were performed in triplicate, and each experiment was repeated three times.

![Western blot analysis](image3)

**FIG. 8.** Top panel, Western blot analysis of nuclear and cytoplasmic Smad proteins in AKR-2B fibroblasts. A pair of 60–65-kDa polypeptides was detected with a Smad2/3-specific antibody that appeared to preferentially accumulate in the nuclear compartment within 30 min after treatment with 5 ng/ml TGF-β1. The numbers refer to the number of minutes of treatment with TGF-β1. Bottom panel, the SMP4 SmαA enhancer construct was active and significantly TGF-β1-inducible (*p* < 0.05) in stromal fibroblasts prepared from wild-type mouse embryos (SMP4-WT) but not in embryonic fibroblasts prepared from the Smad3 knockout mouse (SMP4-NULL). Transfections were performed in triplicate, and each experiment was repeated three times. ■, control; ■, TGF-β1.
cient tissue healing. Tissue fibrosis and scar formation may result if myofibroblast differentiation is poorly controlled as in chronic inflammatory diseases such as liver cirrhosis, scleroderma, cardiac allograft dysfunction, and pulmonary fibrosis (2, 3, 14, 36). In this regard, we have identified transcriptional regulatory elements and cognate repressor protein complexes that may be important not only in preventing unscheduled activation of this gene in quiescent stromal fibroblasts but also in limiting the magnitude and duration of Sm/A transcription in injury-activated myofibroblasts (15, 20, 22, 27, 29).

Deletion mutation analysis of the smooth muscle-specific VSMP8 promoter construct revealed that an amplified transcriptional response to TGF-β1 in AKR-2B fibroblasts minimally required sequence elements located within the first 191 bp of the 5′-flanking region generally referred to as the MCAT enhancer. Basal activity required multiple positive elements within the MCAT enhancer that were also required for transcriptional amplification after exposure of stromal fibroblasts to TGF-β1. A recent analysis of native mouse fibroblast chromatin encompassing the SmA enhancer region (15) indicated

![Graph](image1)

**Fig. 9.** Effect of individual or combined Sp and Smad protein overexpression on SmA enhancer activity in transfected COS7 cells. All cells minimally contained the SMP4 enhancer-CAT reporter gene plasmid plus one or more expression plasmids encoding Sp or Smad proteins. The DNA payloads used for the various transfections were balanced to the same molar concentration. Combined expression of Smad4 with either Smad2 or Smad3 or both (SMP4/Smad2,4, SMP4/Smad3,4, and SMP4/Smad2,3,4) effectively activated (p < 0.05) SMP4 (left panel). However, Smads appear to act independently from the Sp1 and Sp3 proteins (right panel) because combined overexpression of all three Smads plus either the Sp1 or Sp3 proteins (SMP4/Sp1/Smads and SMP4/Sp3/Smads) did not activate SMP4 to levels greater than that observed with individual Sp proteins (SMP4/Sp1 and SMP4/Sp3) or Smad2/3/4 (SMP4/Smads) alone. pCMV, empty expression vector; Smads, combination of the Smad2/3/4 expression plasmids. Transfections were performed in triplicate, and each experiment was repeated five times.

![Graph](image2)

**Fig. 10.** Effect of mithramycin A on actin mRNA expression in fibroblasts. Mithramycin A specifically intercalates at GC-rich Sp1/3 DNA-binding sites and had a marked inhibitory effect on basal expression of SmA mRNA, but not TGF-β1 inducibility per se. There were no discernable effects of mithramycin A on the level of nonmuscle β,γ-actin or glyceraldehyde-3-phosphate dehydrogenase mRNA expression. LS, cells receiving 0.5% FBS alone; TGF, cells receiving 0.5% FBS and then exposed to 5 ng/ml TGF-β1 for 12 h; M, cells receiving a 6-h dose of mithramycin A (0.8 μM) in 0.5% FBS alone or before the 12-h TGF-β1 treatment. The Northern blot depicted is representative of three independent experiments that essentially showed the same trends.

![Graph](image3)

**Fig. 11.** The effect of mithramycin A on Smad protein-dependent SmA enhancer activity in COS7 cells. COS7 cells were transfected with either SMP4 alone (SMP4) or SMP4 plus Smad2/3/4 (SMP4/Smads) and subsequently exposed to mithramycin A (0.8 μM) for 24 h before cell extraction and CAT assays. The data are presented as the normalized means (units of CAT activity/μg protein) ± S.E. Only 55% of the total Smad-dependent enhancer output was inhibited by mithramycin A (compare SMP4/Smads and SMP4/Smads/MA, p < 0.006). Transfections were performed in triplicate, and each trial was repeated five times.
that TGF-β1 markedly affected chemical reactivity of nucleotides located between −170 and −150 that fully encompassed the THR identified by functional cell transfection studies reported in this current work. SmoA promoter constructs harboring mutations in five sequence elements (MCAT, THR, CARG A, CARG B, and TCE) all exhibited impaired basal transcriptional activity in mouse fibroblasts, yet only the THR was required for an amplified response to TGF-β1. Mechanisms governing TGF-β1 inducibility of the 191-bp mouse Smo enhancer in fibroblasts apparently differ from those in rat aortic smooth muscle cells, bovine aortic endothelial cells, and rat fibroblasts, where elements required for basal expression of a 125-bp segment of the rat SmoA promoter were shown to be necessary and sufficient for TGF-β1 inducibility (16, 17). Moreover, those investigators noted increases in the amount and/or DNA binding activity of nuclear protein factors that bound to transcriptional activating elements in the rat Smo core enhancer (CARG A, CARG B, and TCE) after exposure of quiescent rat aortic smooth muscle cells to TGF-β1. However, we noted that although TGF-β1-amplified output from mutant SmoA enhancers in mouse AKR-2B fibroblasts was markedly diminished, each mutation nonetheless retained significant inducibility. These observations thus preclude strict classification of the mouse SmoA CARG A, CARG B, and TCE sites as TGF-β1 response elements, although each was required for maximum enhancer activity in mouse fibroblasts. This interpretation is more than a semantic argument because transcriptional activity from each mutant enhancer was quite substantial in TGF-β1-treated fibroblasts and comparable with the basal activity elicited from the native VSMP4 enhancer in the absence of growth factor. The observation that net protein binding to known SmoA enhancer elements was not affected by TGF-β1 further suggests that none of the known enhancer-binding proteins are rate-limiting. Rather TGF-β1 amplification of the mouse VSM α-actin promoter in fibroblasts appears to require participation of all sequence elements that normally contribute to basal transcriptional activity. TGF-β1 may stabilize higher order protein complexes between these multiple SmoA enhancer sites or permit unique protein-DNA conformational arrangements that do not depend on increased stoichiometric binding of any one individual class of enhancer-binding protein.

Differences in TGF-β1 responsiveness between the mouse and rat SmoA enhancers may be further explained by the existence of additional positive regulatory elements in the mouse promoter located between −191 and −125 (THR and MCAT) that, as shown here and elsewhere, are required for high level constitutive expression of the mouse SmoA core promoter in both muscle and nonmuscle cells from the mouse (21) but appear to be dispensable in the rat gene (16, 17). Shorter mouse SmoA promoter constructs consisting of sequence elements between −143 and −1 were inactive in mouse muscle cells and fibroblasts as well as primary rabbit (25) and A7r5 neonatal rat (29) aortic smooth muscle cells. Positive regulatory elements between −191 and −143 may facilitate the formation of more complex DNA structures or assembly of novel DNA-protein complexes that could diminish the importance of any one site while enhancing cooperative interactions that maximize the transcriptional response to TGF-β1. Currently, we are analyzing dynamic structural changes within the closely positioned transcriptional silencing and activating sequences located in the SmoA enhancer as well as evaluating the higher order composition of enhancer-protein complexes that form in this region.

Basal and TGF-β1-inducible promoter activity necessarily relies on transcription factor availability within the cell nucleus. Mouse AKR-2B fibroblasts do not appear to contain novel, potentially rate-limiting transcription factors that were reported to bind the TCE site in rat aortic smooth muscle cells (16, 17). However, given the high homology of the GC-rich TCE motif to binding sites for the ubiquitous Sp1/Sp3 DNA-binding proteins, we examined whether these proteins could contribute to basal SmoA transcriptional activity in mouse fibroblasts, even though they do not appear to mediate promoter activity in the rat. The Sp1/Sp3 proteins are well-characterized members of a multigene family (37–39) that bind to widely distributed GC-rich promoter elements such as the GC box (GGGCGG-GG) and the related GT/CACCC box (GGTTGGGGG). We analyzed protein factors that bound to four GC semi-rich elements contained within truncated portions of the VSM α-actin enhancer that were either constitutively active (VSMP4) or fully repressed (VSMP3) in mouse fibroblasts. Whereas all four sites bound Sp1/Sp3, mutation of only two of these sites (THR and TCE) had functional consequences in transfection assays, and only mutations in the THR significantly affected TGF-β1 inducibility of the SmoA enhancer. Whereas Sp1 has been shown to function exclusively as a transcriptional activator, Sp3 can either activate or repress transcription, depending on the promoter context (40–45). In general, Sp3 behaves as an activator when targeted to a single binding site within a promoter but acts as a repressor when bound to multiple sites within a promoter (46), although exceptions to this scheme have been reported (45). One such exception may pertain to stromal fibroblasts because we showed that either Sp1 or Sp3 alone can activate SmoA enhancer constructs containing multiple Sp1/Sp3 binding sites when overexpressed in Sp factor-deficient Drosophila SL2 cells. When expressed in combination using equimolar concentrations of plasmid DNA, activation was intermediate to that observed in the presence of equivalent amounts of Sp1 or Sp3 alone, implying that their effects are simply additive rather than antagonistic or synergistic. However, our inability to detect a change in the level of Sp1/Sp3 binding, or any other activator for that matter, at any one site in the mouse SmoA enhancer after exposure of fibroblasts to TGF-β1 leads us to speculate that growth factor activation of this gene may require more than individual protein loading events at functionally insulated cis-acting elements. For example, Li et al. (43) reported that phosphorylation of the B activation domains of Sp1 rather than overt changes in Sp1 binding activity to DNA may be an important molecular feature of TGF-β1-activated transcription.

Rate-limiting co-regulatory factors may enhance dynamic interactions between individual transcriptional activating proteins that bind to closely positioned sites within the SmoA enhancer. Relevant to control of the SmoA enhancer response to TGF-β1 are members of the Smad family of co-regulatory proteins (47–50), which are known to modulate transcription through direct or indirect interaction with Sp1 (31–34). Results presented here show that both Smad2 and Smad3 rapidly accumulated in the fibroblast nucleus within 30 min after exposure to TGF-β1. Consistent with a possible rate-limiting role, TGF-β1 treatment failed to up-regulate the SmoA enhancer in transfected embryonic fibroblasts prepared from Smad3 knockout mice, and combined overexpression of all three Smad proteins in COS7 cells substantially activated the SmoA enhancer. However, unlike the results reported for the p15ink4B (32) and α2Icollagen promoters (33, 34), co-expression experiments that included all the Smad proteins plus either Sp1 or Sp3 showed no further SmoA enhancer transcription than that obtained when either Smads or Sp proteins were expressed alone. One idea emerging from these results and currently under investigation is that Smad proteins di-
rectly activate the SmoA enhancer by a mithramycin-resistant mechanism that does not require physical interaction of Sp1 or Sp3. In this regard, all activation sites in the SmoA enhancer may be physically occupied by latent transcriptional regulatory proteins/repressors awaiting coordinate activation/neutralization by TGF-β1 during a wound-healing event. Within the vicinity of the MCAT and THR motifs, the single-strand-specific DNA-binding proteins MSY1 and Pur α/β are believed to block access of the TEF1 activating protein to the VSM α-actin enhancer via a conformational switch that disrupts duplex DNA structure. In vivo evidence for this switch (15) showed that TGF-β1 promoted accumulation of both hyper-reactive and protected DNA, particularly in regions encompassing both the TEF1 and Sp1/Sp3 binding sites (the MCAT and THR sites, respectively). TGF-β1 acting through the rate-limiting Smad pathway does not appear to enhance binding of positive regulatory proteins to probes spanning this conformational switch. However, it remains a distinct possibility that changes in chromatin conformation are associated not with a gain in activating factor binding at these sites but rather with depletion of the single-strand-specific transcriptional repressors Pur α/β and MSY1 from the enhancer. Release of SmoA transcriptional repression through net loss of Pur and MSY1 repressor proteins from their corresponding single-stranded enhancer binding sites could be achieved via higher order interactions between activator proteins that permanently reside at the MCAT, THR, CARG, and/or TCE sites. The Smad proteins, which may possess intrinsic kinase activity, could facilitate these hypothetical protein-protein or protein-DNA interactions within the enhancer (32). In this regard, there are two Smad protein-binding CAGA motifs embedded in the TGF-β1 hyper-reactivity region of the enhancer between −170 and −150. Identification of higher order complexes containing multiple transcriptional activators such as TEF1, Sp1/3, serum response factor, and Smad factors, which may possess intrinsic kinase activity, could facilitate these hypothetical protein-protein or protein-DNA interactions within the enhancer (32). In this regard, there are two Smad protein-binding CAGA motifs embedded in the TGF-β1 hyper-reactivity region of the enhancer between −170 and −150. Identification of higher order complexes containing multiple transcriptional activators such as TEF1, Sp1/3, serum response factor, and Smad factors, which may possess intrinsic kinase activity, could facilitate these hypothetical protein-protein or protein-DNA interactions within the enhancer (32).
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Vascular Smooth Muscle α-Actin Gene Transcription during Myofibroblast Differentiation Requires Sp1/3 Protein Binding Proximal to the MCAT Enhancer

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