Smad7 is an inducible intracellular inhibitor of transforming growth factor-β (TGF-β) signaling that is regulated by diverse stimuli including members of the TGF-β superfamily. To define the molecular mechanisms of negative control of TGF-β signaling, we have isolated the human SMAD7 gene and characterized its promoter region. A −303 to +672 SMAD7 region contained a palindromic GTCTAGAC Smad binding element (SBE) between nucleotides −179 and −172 that was necessary for the induction of a Smad7 promoter luciferase reporter gene by TGF-β. Electrophoretic mobility shift assays using oligonucleotide probes demonstrated that TGF-β rapidly induced the binding of an endogenous SBE-binding complex (SBC) containing Smad2, Smad3, and Smad4. Transfection assays in mouse embryonic fibroblasts (MEFs), with targeted deletions of either Smad2 or Smad3, and the Smad4-deficient cell line MD-MBA-468 revealed that both Smad3 and Smad4, but not Smad2, were absolutely required for induction of the Smad7 promoter reporter gene by TGF-β. Furthermore, the TGF-β-inducible SBE-binding complex was diminished in Smad2-deficient MEFs when compared with wild type MEFs and not detectable in Smad3-deficient MEFs and MD-MBA-468 cells. Taken together, our data demonstrate that TGF-β induces transcription of the human SMAD7 gene through activation of Smad3 and Smad4 transcription factor binding to its proximal promoter.

Transforming growth factor-β (TGF-β) is the prototype of a cytokine superfamily with important roles in cell cycle control, differentiation, and apoptosis. TGF-β initiates signaling through the ligand-dependent activation of a complex of heteromeric transmembrane serine/threonine kinases, consisting of type I and type II receptors (1, 2). Upon activation, type I receptor associates with and activates Smad2 and/or Smad3, two signaling mediators of the SMAD protein family (3–6). Activated Smad2 and/or Smad3 associate with the shared partner Smad4 and translocate to the nucleus, where Smad protein complexes participate in transcriptional activation of target genes (7–9).

The TGF-β/Smad signaling system is notable for an autoinhibitory feedback loop which involves Smad7, a structurally and functionally divergent Smad protein of the subfamily of “inhibitory Smads” (10–12). Smad7 interacts stable with ligand-activated type 1 receptor and interferes with receptor binding and phosphorylation of substrate Smads (10). Thus, Smad7 may have an essential role in the regulation of the TGF-β/Smad signaling system by controlling the accessibility of ligand-activated type 1 receptor for substrate Smad2 and/or Smad3. Several reports indicate that Smad7 expression is strongly and rapidly induced by TGF-β itself (12, 13) by the Jak1/Stat1 pathway following stimulation with IFN-γ (14), by activated NF-κB, and by fluid shear stress acting on endothelial cells (11). Together, these observations point to a broad role for Smad7 in trans-modulation of signaling pathways.

Because of the potentially central roles of Smad7 as an effector in an autoregulatory feedback loop in TGF-β/Smad signaling and as a mediator of inhibitory signaling cross-talk between opposing pathways and the TGF-β/Smad pathway, we reasoned that knowledge of the molecular mechanisms that control the expression of Smad7 would advance the understanding of the regulation of the TGF-β/Smad pathway. Here we report a molecular mechanism by which TGF-β induces transcription of the human Smad7 promoter. We have identified a palindromic Smad binding element that binds a protein complex containing Smad2, Smad3, and Smad4 and shown that this is necessary for the transcriptional activation of the Smad7 promoter by TGF-β. In cells that lack Smad3 or Smad4, TGF-β is unable to induce Smad7 promoter activity.

MATERIALS AND METHODS

Cell Culture and RNA Analysis—A spontaneously immortalized human keratinocyte cell line (HaCaT), and SV40-transformed mouse mesangial cells were obtained from Dr. Norbert Fusenig and Dr. Fuad Ziyadeh, respectively. NIH3T3 murine fibroblasts and the Smad4-deficient human mammary adenocarcinoma cell line MDA-MB 468 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Wild type and Smad2-deficient (Smad2Δ12Δ12/Δ12Δ12) mouse embryonic fibroblasts were derived from d10.5 embryos as described (15). Wild type and Smad3-deficient MEFs were derived from day 12.5 embryos (16). All cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics. Recombinant human TGF-β1, TGF-β2, and TGF-β3 were purchased from R & D Systems, and interferon-γ (IFN-γ) was obtained from Genzyme. Recombinant murine TNF-α was was from Roche Molecular Biochemicals, and human epidermal growth factor (EGF) was obtained from Promega. Actinomycin

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The abbreviations used are: TGF-β, transforming growth factor-β; MEF, mouse embryonic fibroblast; IFN, interferon; EGF, epidermal growth factor; bp, base pair(s); EMSA, electrophoretic mobility shift assay; SBE, Smad binding element; S7SBE, Smad7 SBE; SBC, SBE-binding complex.

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cin D and cycloheximide were purchased from Sigma and used in concentrations recommended by the supplier. RNA was isolated using Trizol Reagent (Life Technologies, Inc.) following the manufacturer’s protocol. For Northern blot analysis, RNA was electrophoresed on 1% agarose gels and transferred to a filter. Filters were then hybridized in QuickHyb solution (Stratagene) with 32P-labeled cDNA probes for murine Smad7 and analyzed by phosphor imagery.

Isolation of the Human Smad7 Promoter—A 5′-down-to-the-well human genomic PAC library screening system (Genome Systems) was screened with a polymerase chain reaction probe generated by a primer pair (primers E5 (5′-GCTCTCCGGGAGACTGG) and E6 (5′-GAGAAAAAGTTCTTGCCCTG)) located in the 5′-untranslated region of human Smad7 cDNA to give clones 806N1 and 529P14. DNA prepared from both PAC clones was digested with restriction endonucleases (BanHI, EcoRI) and electrophoresed on 1% agarose gel in 1× Tris/acetate/EDTA (TAE). DNA fragments were transferred to Hybond N+ membrane (Amerssharm Pharma Biotech) and hybridized with the 170-bp polymerase chain reaction probe. A 4.6-kilobase pair EcoRI fragment was identified, and gel-purified with pBluescript KS±/vector DNA (Stratagene). This fragment contained mostly 5′-flanking sequence of the Smad7 gene and was used for promoter analyses.

Deletion Constructs, Transfections and Transcriptional Reporter Assays—5′- and 3′- deletions were generated by endonuclease digestions from distinct EcoRI fragments. Seven distinct fragments were ligated into the promoterless luciferase reporter vector pGL3-basic (Promega) (see Fig. 2A). For transcriptional reporter assays, cells (2.5×10^4/well) were seeded in 24-well or six-well dishes and transfected with the indicated luciferase reporter constructs and pRSV-Gal (Promega), using the Superfect Reagent (Qiagen) according to the manufacturer’s protocol. Transfected cells were incubated in 0.2% fetal bovine serum starvation medium for 16 h and then either left untreated or treated with TGF-β1 (1 ng/ml) for 4 h. Luciferase and galactosidase activities in transfected cells were determined using assay kits from Promega. Luciferase activity was measured using an AutoLumat LB953 (EG & G Berthold) luminometer. Galactosidase activities were measured with a Laysystems Multiscan MCC/340 plate reader at 405 nm. To correct for differences in transfection efficiencies, luciferase units were normalized for galactosidase activities in the same cell lysate. Corrected luciferase units were then expressed as ratio (-fold induction) compared with the luciferase readings mediated by the empty vector pGL3 basic in the same experiment. Experiments were performed in triplicate.

Primer Extension Analysis—An oligonucleotide (AAGCCGCCGCTGCCTTGCCGACAC) complementary to the human SMAD7 DNA was annealed with [γ-32P]ATP (Amersham Pharmacia Biotech), hybridized to human kidney mRNA (CLONTECH) and reverse transcribed into cDNA using the avian myeloblastosis virus Reverse Transcriptase System (Promega) following the manufacturer’s protocol. Sequencing of genomic SMAD7 DNA contained in the pS7-5 plasmid was performed using a Sequenase version 2.0 sequencing kit (U.S. Biochemical Corp.) following the manufacturer’s protocol. The sequencing primer was GTGCCGCCGACACAGGCAG. The radiolabeled cDNA primer extension products were analyzed in parallel with the sequencing reactions using an 8 m urea denaturing polyacrylamide gel.

Site-directed Mutagenesis—Site-directed mutagenesis was carried out in the pS7-5 construct using a QuickChange kit (Stratagene) following the manufacturer’s instructions. Thymidine at position −176 and adenine at position −175 in the center of the Smad7 binding element were replaced with adenosine and thymidine, respectively (lowercase italic type), using complementary oligonucleotides (SBE5F, 5′-AAGCCGCCGTTCGAGAAGAGGCAACCCCTG-3′; SBE5R, 5′-TGCTGAGCTGACCCGAC-3′) to generate the mutant construct pS7-5SmBE. Sequence fidelity was confirmed by sequencing.

Preparation of Nuclear Protein Extracts and Electrophoretic Mobility Shift Assays—Nuclear protein extracts were prepared from subconfluent cell cultures on 100-mm dishes. Cells were washed twice in cold phosphate-buffered saline and lysed in 1 ml of ice-cold hypotonic lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, pH 8.0, 0.1 mM EGTA, 1 mM dithiothreitol, 0.6% Nonidet P-40 containing aequorin, leupeptin, aprotinin, pepstatin A, antipain, sodium vanadate, sodium fluoride, and okadaic acid at concentrations recommended by the manufacturer. The nuclei were allowed to swell for 15 min and then sonicated, collected, and washed with hypotonic lysis buffer without detergent. Nuclei were pelleted by centrifugation at 13,000 rpm for 20 s in a microcentrifuge and resuspended in 20 μl of nuclear extraction buffer (lysis buffer with 20 mM Hepes, pH 7.9, and 420 mM NaCl). Nuclear lysates were incubated for 20 min on a shaker and cleared of debris by centrifugation.

Electrophoretic mobility shift assays were performed as described previously (17), using nuclear extracts prepared from either untreated cells or cells treated with TGF-β1 (1 ng/ml) for 1 h. Complementary oligonucleotides “S7BE” containing the Smad binding element (SBE) sequence were γ-32P-end-labeled by T4 polynucleotide kinase reaction and annealed. S7BE probe (50,000 cpm) was incubated with 1 μg of nuclear extract in binding buffer (15% glycerol, 20 mM Hepes, pH 7.9, 0.1 mM EDTA, 30 mM KCl, 3 μg of poly(dI-dC), 0.8 mM NaF, pH 7.8, 4 mM spermidine, 4 mM MgCl2) with or without preincubation for 10 min with a 50- or 100-fold molar excess of cold annealed competitor at 4°C for 30 min. For antibody interference studies (supershift analysis), nuclear extracts were incubated overnight at 4°C with 2 μg of the following antibodies prior to or following the addition of radiolabeled probe as indicated: mouse monoclonal anti-Smad2 (S66220; Transduction Laboratories) or goat polyclonal anti-Smad2 (sc-6200 X), anti-Smad3 (sc-6202 X), and anti-Smad4 (sc-1909 X) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA). DNA-binding protein complexes were separated by nondenaturing 4% polyacrylamide gel electrophoresis at 4°C and visualized by autoradiography.

Western Blotting—Aliquots (100 μg) from wild type, Smad2-deficient (Smad2−/−/−/−), and Smad3-deficient (Smad3−/−/−/−) MEFs were loaded on a SDS-polyacrylamide gel electrophoresis (10% acrylamide). After transfer to nitrocellulose membranes, were probed with antibodies against Smad2 (monoclonal anti-Smad2; Transduction Laboratories) and Smad3 (polyclonal anti-Smad3; Zymed Laboratories Inc.), and GDP dissociation inhibitor (kind gift from Dr. Philipp Scherer) to control for protein loading, as indicated. Bound primary antibodies were detected with horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies, respectively, and developed with enhanced chemiluminescence reagents purchased from Pierce.

RESULTS

TGF-β Regulates SMAD7 by Transcriptional Activation—Smad7 is a member of the Smad protein family that has been shown to antagonize TGF-β receptor signaling. Several TGF-β family members including activin and BMP7 were found to increase Smad7 expression and induce its interaction with activated TGF-β type I receptor (10, 12, 13). Thus, it has been proposed that Smad7 mediates an autoregulatory negative feedback loop in TGF-β signaling (18). To determine whether the regulation of Smad7 by TGF-β is mediated at the level of gene transcription, we examined Smad7 mRNA levels in response to TGF-β in the absence or presence of actinomycin D, an inhibitor of transcription (Fig. 1). TGF-β-mediated up-regulation of Smad7 mRNA was completely blocked by pretreatment of cells with actinomycin D (Fig. 1, lanes 3 and 4), indi-
cating that TGF-β activates transcription of the SMAD7 gene. Pretreatment with cycloheximide had no effect on the transcrip
tional activation of SMAD7 by TGF-β, suggesting that de novo protein synthesis was not required for this activity (data
not shown).

Characterization of the Human Smad7 Promoter—We iso
lated the human SMAD7 gene by screening a human P1-arti
ficial chromosome library with a 170-bp polymerase chain re
action probe corresponding to 5'-untranslated sequence of the
human Smad7 cDNA. 3 An EcoRI–SpHI genomic fragment
spanning 4.6 kilobase pairs of 5'-flanking sequence of SMAD7
was isolated and subcloned into the promoterless luciferase
reporter vector pGL3basic. In order to define the basal and
TGF-β-inducible Smad7 promoter elements in this region, we
generated a total of seven 5' and 3' deletion constructs (pS7-1
to pS7-7) using convenient restriction sites (Fig. 2A). Transfections
of these constructs into NIH3T3 fibroblasts revealed that
the constructs pS7-1 to pS7-5 mediated both TGF-β-inducible
and basal promoter activity (Fig. 2B). Further 5' deletion of the
Smad7 promoter (pS7-6) between a Kpn I (-303) and a BssHI
(-146) site resulted in a complete loss of TGF-β-inducibility of
the Smad7 promoter without affecting basal promoter activity
(Fig. 2B). In contrast, the region between a Hin IIII and a SpHI
site (pS7-7) mediated luciferase activities that were not dif
ferent from empty control vector, indicating that the basal
Smad7 promoter region was located upstream of this fragment
(Fig. 2B).

Smad7 mRNA expression is inducible by a number of cyto
kines and growth factors other than TGF-β, including activin,
BMP7, and EGF (13) as well as IFN-γ (14) and TNF-α. 2 To
determine whether the ~303 to +672 Smad7 fragment was inducible by these extracellular signals, we transfected the
reporter construct pS7-5 (~303 to +672) into NIH3T3 fibro
blasts and stimulated the cells with cytokines as indicated (Fig.
2C). TGF-β isoforms β1, β2, and β3 stimulated luciferase ac
tivity by 4.1-, 3.4-, and 3.1-fold, respectively (Fig. 2C). Both
 activin and BMP-7 induced the pS7-5 promoter activity 1.5-
and 1.7-fold, respectively, whereas no induction was observed
with TNF-α, IFN-γ, and EGF (Fig. 2C). These data suggest that
TGF-β family members may activate a common element in the
Smad7 promoter, albeit with different levels of stimulation. In
contrast, up-regulation of Smad7 gene expression by TNF-α,
IFN-γ, and EGF is not mediated through activation of the ~303
to +672 Smad7 promoter region.

To examine whether the ~303 to +672 SMAD7 fragment
was TGF-β-inducible in different cell types, we transfected the
pS7-5 plasmid into murine mesangial cells and HaCaT cells in
addition to NIH3T3 fibroblasts. The pS7-5 plasmid gave rise to
comparable basal and TGF-β-inducible luciferase activities in
all three cell lines, indicating that the TGF-β-responsive ele
ment is activated in a cell-type-independent manner (Fig. 2D).

These experiments identified a 975-bp fragment (Kpn I to
HindIII) of the SMAD7 gene that contained both the TGF-β-
responsive and basal promoter elements. This DNA fragment
was sequenced in its entirety. Sequence analysis using the
MatInspector version 2.2 program (19) did reveal several pu
tative binding sites for transcription factors (Fig. 3A). The
absence of a TATA-box and the presence of multiple Sp1 sites
in this region suggested that the SMAD7 gene has a TATA-less
promoter (20). To identify putative transcription initiation sites
in the Smad7 promoter, we performed a series of primer exten
sion analyses with poly(A) RNA from human kidney (CLON
TECH). A major extension product of 49 bp was obtained in
multiple experiments and compared with sequence analysis of

\[ \text{ genomic SMAD7 DNA, using the same primer (Fig. 3B). This } \]

\[ \text{ major initiation site was designated as the +1-position (Fig. } \]

\[ \text{3A). } \]

Smad Protein Complexes Bind a Palindromic Consensus Se
quence That Is Essential for Induction of the Smad7 Promoter
by TGF-β—Inspection of the sequence of the ~303 to +672
DNA fragment revealed an 8-bp palindromic Smad3/Smad4

3 K. Susztak and E. P. Bottinger, unpublished observations.
binding sequence (the SBE) at positions −179 to −172 (Fig. 3A). This sequence has been shown to interact with recombinant Smad3 and Smad4 and was sufficient to confer transcriptional activation by TGF-β upon a heterologous promoter reporter construct (21). To determine whether the SBE in the Smad7 promoter was necessary to confer TGF-β-inducibility, we used site-directed mutagenesis to change thymidine to adenine and adenine to thymidine in pS7-5, resulting in pS7-5mSBE (Fig. 4A). These point mutations were expected to abolish binding of Smad3 and/or Smad4 to the SBE completely (21). When transfected into NIH3T3 cells, wild type pS7-5 conferred 2.7-fold induction of luciferase activity by TGF-β (Fig. 4B). In contrast, pS7-5mSBE was able to mediate basal promoter activity but did not confer induction by TGF-β (Fig. 4B), demonstrating that the SBE at −179 to −172 was necessary for induction of the Smad7 promoter by TGF-β. Next, we used radiolabeled oligonucleotide probes spanning positions −185 to −166 in electrophoretic mobility shift assays (EMSAs) to examine whether the Smad7 promoter (S7SBE) was able to interact with nuclear protein complexes. Nuclear protein extracts were prepared from untreated and TGF-β-treated NIH3T3 fibroblasts. DNA binding of a protein complex labeled the SBE-binding complex (SBC) was specific and strongly increased in nuclear extracts from TGF-β-treated NIH3T3 when compared with untreated NIH3T3 cells (Fig. 4C, lanes 3 and 2, respectively). Time course experiments indicated that the induction of SBC was detectable as early as 10 min and strongest after 40 min of TGF-β treatment. Preincubation of nuclear extracts from TGF-β-treated NIH3T3 cells with anti-Smad2, anti-Smad3, and anti-Smad4 antibodies revealed significantly reduced SBC binding in the presence of anti-Smad2 antibodies (Fig. 4C, lane 6) or supershifted SBC complexes in the presence of anti-Smad3 (lane 7) or anti-Smad4 (lane 8), suggesting that SBC contained Smad2, Smad3, and Smad4 antigens. We obtained similar results when nuclear extracts from untreated NIH3T3 cells were used, albeit the intensity of the SBC signal was much weaker throughout the experiment (Fig. 4C, lanes 9–14). These results indicated that a nuclear protein complex containing Smad2, Smad3, and Smad4 formed at the SBE in the Smad7 promoter at baseline and that TGF-β treatment strongly increased the amount of bound Smad protein complexes, resulting in transcriptional activation of the Smad7 promoter. The SBE probe specifically interacted with a higher molecular weight complex in most experiments (labeled with an asterisk, Fig. 4C). The binding characteristics of this complex were not significantly altered by TGF-β or anti-Smad antibodies. Since it has been reported that the GTCTAGAC sequence does not interact with recombinant Smad2 (21), we further investigated whether Smad2 antigens participated in SBC on the Smad7 promoter. We used, in addition to the polyclonal anti-Smad2 antibody, a monoclonal anti-Smad2 antibody that specifically detected Smad2 but not Smad3 or Smad4 (see Fig. 5A). This antibody supershifted the SBC irrespective of whether it was added to the binding reaction before or after the addition of the SBE probe (Fig. 4D), suggesting that Smad2 participates in the SBC.
extracts (Fig. 5B, lane 8), and neither basal nor TGF-β-inducible SBC were detectable in nuclear extracts derived from Smad4-deficient MD-MBA468 (Fig. 5B, lanes 10 and 11). The high molecular weight DNA-protein complex (*) was detectable irrespective of the presence or absence of Smad2 and Smad3 but was not observed in Smad4-deficient cells (Fig. 5B). Our data suggest that Smad4 is absolutely required for the formation of basal and TGF-β-inducible SBC and that Smad3 is a major component of the TGF-β-inducible SBC. In contrast, Smad2 is not required for binding of SBC to the SBE probe.

**FIG. 4.** An inducible complex consisting of Smad2, Smad3, and Smad4 interacts with a Smad binding element that is required for activation of the Smad7 promoter by TGF-β. A, oligonucleotide sequence containing wild type (S7SBE) and mutant (S7mSBE) Smad binding elements. B, corrected luciferase activities (relative luciferase units (RLU)) in untreated (black bars) and TGF-β-treated (gray bars) NIH3T3 fibroblasts after transfection with wild type pS7-5 (pS7-5wt) or mutant pS7-5 (pS7-5mSBE) Smad7 promoter luciferase reporter gene constructs. C, EMSA demonstrating interaction of a double-stranded oligonucleotide probe (S7SBE) with a TGF-β-inducible SBC in nuclear protein extracts from untreated (--) or TGF-β-treated (+) NIH3T3 fibroblasts (2.5 ng/ml TGF-β1 for 40 min). Open and filled arrowheads denote supershifted complexes when nuclear extracts were incubated with anti-Smad3 (α-Smad3) or anti-Smad4 (α-Smad4) antibodies before the addition of the probe, respectively. The asterisk denotes a complex of constitutive SBE-binding proteins (see “Results”). A 50-fold molar excess of unlabeled, annealed SBE oligonucleotides ablates SBC binding (lane 4). Nonimmune goat IgG and anti-Smad2 (α-Smad2) antibody are shown. D, EMSA comparing the effect on the SBC of a polyclonal goat anti-Smad2 (α-Smad2 SC) or a monoclonal anti-Smad2 (α-Smad2 TL) added to the binding reaction either before or after the S7SBE probe, respectively.

**FIG. 5.** Smad3 and Smad4 but not Smad2 are necessary for SBC binding. A, Western blot analysis of cell lysates from untreated (--) or TGF-β-treated (+) wild type (wt), Smad2-deficient (Smad2^−/−) and Smad3-deficient (Smad3^−/−) MEFs. The membrane was probed with a monoclonal anti-Smad2 antibody (Transduction Laboratories) and a polyclonal anti-Smad3 antibody (Zymed Laboratories Inc.). The same blot was probed for GDP dissociation inhibitor (GDI) to control for equal protein loading. B, EMSA using the S7SBE oligonucleotide probe and nuclear protein extracts from wild type (wt) (lanes 1–3), Smad2-deficient (Smad2^−/−) (lanes 4–6), Smad3-deficient (Smad3^−/−) (lanes 7–9) MEFs and MDA-MB-468 cells (lanes 10–12). SBC and an asterisk denote TGF-β-inducible and constitutive S7SBE-binding protein complexes, respectively.
Transcriptional Activation of Smad7 by TGF-β

We report a molecular mechanism that may have a central role in negative autoregulation of TGF-β/Smad signaling. Our results demonstrate that ligand-dependent activation of TGF-β receptor complexes induces the interaction of Smad2, Smad3, and Smad4 transcription factor complexes with a palindromic consensus Smad binding element in the human Smad7 promoter. Mutations in this cis-acting element or deletion of either Smad3 or Smad4, but not Smad2, ablate the ability of TGF-β to induce the human Smad7 promoter. Thus, the transcriptional regulation of Smad7, an intracellular inhibitor of the TGF-β type I receptor (10, 12), by TGF-β itself is mediated through a rapid and direct Smad3- and Smad4-dependent signaling mechanism.

Detailed molecular studies of a number of TGF-β-responsive promoters suggest that the TGF-β/Smad pathway regulates transcription by at least two distinct mechanisms. The first mechanism involves the interaction of Smad proteins with other transcription factors at their specific binding sequences in TGF-β-responsive promoters. For example, the TGF-β or activin response of the Mix.2 promoter is mediated by a Fast-2-dependent transcriptional activator complex consisting of Fast-2 and Smad2-Smad4 complexes (9). In contrast, a number of examples support a distinct mechanism of regulation in which Smad3 and Smad4 activate transcription through direct interaction with specific DNA sequences (i.e. CAGA) or so-called Smad binding elements (22–24). Our observations that the Smad7 promoter contains a palindromic GTCTAGAC sequence that mediates binding of and transcriptional activation by Smad3 and Smad4 now provide an additional important example for direct Smad3/Smad4-dependent transcriptional regulation by TGF-β.

However, our observation that Smad2 is associated with Smad3 and Smad4 in the TGF-β-inducible SBE-binding complex that interacts with the GTCTAGAC element in the Smad7 promoter has not previously been reported and raises additional questions. First, we show that Smad2 is associated with the basal and TGF-β-inducible SBE-binding complex, but in contrast with Smad3 and Smad4, Smad2 is not required for induction of the Smad7 promoter by TGF-β. A functional role for Smad2 in the SBC therefore remains to be established. In addition, the GTCTAGAC sequence has been identified in a oligonucleotide-based screening by its binding to the major homology-1 domain of recombinant Smad3 and Smad4 (21). The major homology-1 domain of Smad2 was unable to bind to this artificial binding sequence directly. However, the human Smad7 promoter represents the first naturally occurring gene that contains the GTCTAGAC sequence as a functional SBE. Since our binding studies were performed using whole nuclear protein extracts, instead of recombinant proteins, it is likely that Smad2 does not bind DNA directly but participates in a heterotrimeric Smad-binding complex containing at least Smad2, Smad3, and Smad4 (25). Additional studies will be needed to clarify this issue.

Several reports indicate that the expression of Smad7 is induced by independent pathways including TGF-β, activin, BMP-7, IFN-γ, shear stress, and NF-κB pathways (11, 13, 14).2 Our results provide evidence that signaling by the TGF-β superfamily members TGF-β, activin, and BMP-7 may converge on the SBE in the Smad7 promoter. Whereas both activin and BMP-7 stimulation resulted in a small but significant increase of the Smad7 promoter reporter gene activity, induction mediated by the three TGF-β isoforms β1, β2, and β3 was considerably stronger (see Fig. 2C). These results are consistent with a previous report demonstrating strong induction of Smad7 mRNA expression by TGF-β1, compared with moderate induc-
Transcriptional Activation of Smad7 by TGF-β

In contrast, our results indicate that TNF-α, IFN-γ, and EGF may regulate the Smad7 gene through cis- and trans-acting elements independent of the SBE and its binding complex. These data support a model in which the overall degree of Smad7 gene expression under physiological or pathophysiological conditions may be determined through combinatorial activation of distinct regulatory elements at the level of the Smad7 promoter.

Our findings provide new insights into the regulation of the major TGF-β/Smad signaling pathway that support an oscillating rather than a static mode of feedback regulation involving inhibitory Smad7. In such a model, activation of TGF-β receptor complexes by its ligands results in the activation of Smad3 and Smad4 and leads to the rapid and transient transcriptional activation and/or repression of a set of target genes that can be characterized as immediate early gene responses and include activation and/or repression of a set of target genes that can be characterized as immediate early gene responses and include activin and BMP-7 (13). The molecular mechanisms of regulation (this report) and inhibitor function (10, 12) of Smad7 are consistent with an emerging theme in feedback during signaling.

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