Efficient TGF-β Induction of the Smad7 Gene Requires Cooperation between AP-1, Sp1, and Smad Proteins on the Mouse Smad7 Promoter

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Sma- and Mad-related protein 7 (Smad7) is an antagonist of transforming growth factor-β (TGF-β) signaling, which has been shown to be induced by TGF-β itself and also by other stimuli. In an effort to understand the molecular mechanisms underlying the transcriptional regulation of the Smad7 gene by TGF-β, we cloned and functionally characterized a mouse genomic DNA fragment encompassing the mouse Smad7 proximal promoter. This region was found to contain a CpG island and to be devoid of a classical TATA box. Cloned upstream of a promoter-lacking luciferase reporter gene, this region conferred robust TGF-β-induced transcription. Point mutations in a palindromic Smad binding element, abolished TGF-β inducibility completely. Through the use of electrophoretic mobility shift assays, we showed the presence of Smad2, Smad3, and Smad4 in complexes binding to the Smad binding element. Interestingly, we also found that point mutation and/or deletion of binding sites for the transcription factors activator protein-1 and Sp1 led to an attenuation of the basal promoter activity, as well as of the TGF-β-mediated induction of Smad7. Taken together, our data imply that Smads, together with activator protein-1 and Sp1 transcription factors, are essential for efficient Smad7 promoter activity.

Members of the transforming growth factor-β (TGF-β) superfamily of peptide growth factors, which include TGF-β isoforms, activin, and bone morphogenetic proteins, play important roles in a broad range of cellular functions, such as proliferation, apoptosis, terminal differentiation, and specification of developmental fate (1–3). They exert their cellular effect by binding to type I and type II serine-threonine kinase receptors, whereby particular intracellular signaling molecules of the Smad family are activated (reviewed in Refs. 4–6). Smads occur as three distinct subclasses: receptor-regulated (R-Smads; Smad2 and Smad3 are TGF-β receptor-regulated Smads, whereas Smad1, -5, and -8 are activated by the bone morphogenetic proteins receptors), common partner (Co-Smads; Smad4) and inhibitory Smads (I-Smads; Smad6 and Smad7). R-Smads are activated by phosphorylation at their C-terminal SS(M/V)S-motifs, whereas they hetero-oligomerize with the common partner Smad4. The Smad complexes are then translocated to the nucleus where the transcriptional activities of different genes are affected. Smad proteins have in general two large conserved domains, the N-terminal mad homology 1 domain and the C-terminal mad homology 2 domain, which are separated by a less conserved linker region. Within the target gene promoters, Smad3 and/or Smad4 can bind with their respective mad homology 1 domain to specific DNA sequences, called Smad binding elements (SBEs) (4–7). The mad homology 2 domain of Smads displays transcriptional activation activity. To date several transcription factors and transcriptional co-activators have been identified to cooperate with Smad molecules in transcriptional activation, such as cAMP-responsive element binding (CREB)-binding protein/p300, forkhead activin signal transducer-1/-2, transcription factor μE3 (TFE-3), PEBP2/core-binding factor A (CBFA), activating transcription factor-2, AP-1, and Sp1 (reviewed in Refs. 8 and 9). The mechanisms whereby proteins participate in TGF-β-induced transcription are believed to involve stabilizing transcription factor complexes on the DNA, bridging Smads with the basal transcription machinery and targeting acetyltransferase activity to the promoter regions (reviewed in Refs. 8 and 9).

Smad6 and Smad7 antagonize ligand-initiated signaling by competing with the receptor-regulated Smads for binding to the receptor (10–12). In the case of Smad6, an additional level of inhibition exists, i.e. competition for complex formation with Smad4 (13). Smad7 was found to be rapidly induced by TGF-β in several cell types, as well as in endothelial cells experiencing fluid shear stress (14, 15). The level of transcription is also up-regulated by other signaling pathways as those of the Janus kinase 1/signal transducers and activators of transcription 1 or nuclear factor-κB/RelA, after stimulation with interferon-γ or tumor necrosis factor-α, respectively (16, 17). Thus, Smad7 is not only a TGF-β-induced antagonist but is also involved in fine tuning the cellular response to TGF-β, by integrating different signaling pathways.

In the present report, we have analyzed the mechanism of the Smad7 gene promoter regulation. We show that the promoter contains a methylation-free CpG island and that binding of Smads, as well as AP-1 and Sp1, to specific sequence ele-
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**Fig. 1. Sequence of the mouse Smad7 promoter region.** DNA sequence of 725 nucleotides from nucleotide –613 to +112 of the BamHI-XhoI (B-X) fragment is shown. This fragment was most frequently used in the luciferase reporter assays. Relevant endonuclease restriction sites are indicated with *vertical arrows*. The palindromic SBE, gtctagac, is marked in *bold* type. The capitalized G and C in the SBE are mutated to T and A, respectively, in the SBE mutant reporter construct as well as in the mutant EMSA probe. *Heavy lines* above the sequence denote the SBE, AP-1, Sp1 B, and Sp1 A probes used in the EMSAs. The AP-1 core binding motif, tgac, is marked in *bold* type. The bases “AC” are mutated to “TG” in the AP-1 mutant EMSA probe and in the respective reporter construct. GC-boxes/Sp1 binding sites are marked with *bent arrow*, (18) is marked with a *bent arrow*, and the following transcribed sequence is shown in capital letters.

ments is necessary for efficient basal promoter activity and induction by TGF-β.

**EXPERIMENTAL PROCEDURES**

**Isolation of the Mouse Smad7 Promoter**—A genomic mouse DNA library (strain 129/SV) was screened with a 800-bp Smad7 cDNA as a probe (cDNA nucleotide 883–1614 according to Ref. 12). Inserts of positive phages were subcloned into plBluescript II SK+ (Stratagene). A genomic 4.3-kilobase XhoI fragment containing the major transcriptional start site (18) at its 3'-end, and deletions thereof were used for promoter analysis.

**Computer Programs**—The following computer programs were used for sequence analyses: TSSG, TSSW, and Grail 1.3.

**DNA Methylation Analysis**—Genomic DNA was prepared from mouse tail biopsies according to Laird et al. (19). One to three μg of genomic DNA was incubated in a total volume of 30 μl containing no enzyme, 10 units of HpaII, or 10 units ofMspI (New England Biolabs) according to the manufacturer’s recommendations, at 37 °C over night. One μl of DNA was used for polymerase chain reaction (PCR) reactions according to Ref. 20 using BioTaq DNA polymerase (Bioline). The primers used and cycling conditions were as follows: proximal promoter, 5'-CTTCTCGGATTTCCCTGTC-3' and 5'-CCCCACCGTCCCTCTGTGTCC-3'; initial denaturation at 93 °C for 3 min followed by 32 cycles of denaturation at 93 °C for 30 s, annealing at 60 °C for 30 s, extension at 68 °C for 30 s; exon I to exon II, 5'- GCCGAGCTCGGGGAGGAGAA-3', 5'-GGAGTTAAGGAGGGGGAGGACT-3', initial denaturation 93 °C for 3 min, followed by 38 cycles of denaturation at 93 °C for 30 s, annealing at 65 °C for 30 s, extension at 65 °C for 2 min; exon IV, 5'-GGCTGAGGGGCTGTGTTAATTT-3', 5'-TGAGGGCGTGGTTTCTTCA-3'; initial denaturation 93 °C for 3 min, followed by 27 cycles denaturation at 93 °C for 30 s, annealing at 53 °C for 30 s, extension at 65 °C for 15 s. PCR reactions were analyzed on 1 or 3% agarose gels containing ethidium bromide.

**Cell Culture**—NIH 3T3 murine fibroblasts and human hepatoma HepG2 cells were obtained from American Type Culture Collection (ATCC). NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal calf serum (Bio-tech Line AS), l-glutamine (2 mM), and penicillin-streptomycin (100 IU/ml; Life Technologies, Inc.). HepG2 cells were maintained under the same conditions, except for the addition of modified Eagle’s medium nonessential amino acid solution (Sigma).

**Reporter Constructs, Transfections, and Luciferase Assays**—Using the 4.3-kilobase genomic XhoI fragment as source, several 5'- and 3'-deletion constructs were generated (see Figs. 3 and 5). All fragments were cloned into the pGL-3 basic vector (lacking promoter and enhancer sequences; Promega) by conventional molecular biology techniques. All constructs were confirmed by sequencing using an ABI Prism 310 Genetic Analyzer (Perkin-Elmer). Using the calcium phosphate method (21), HepG2 cells (2.5 × 10^5 cells/one 6-well) were cotransfected with 0.1 μg of the internal control vector pCH110 (SV40 ß-gal reporter; Amer sham Pharmacia Biotech) and 0.5 μg of the indicated luciferase reporter constructs. In parts of the study, an Sp1 expression vector, pCMV4 Sp1flu (22), was used (see Fig. 5B). After transfection, the cells were incubated in Dulbecco’s modified Eagle’s medium containing 0.3% fetal calf serum, either treated or not treated with 10 ng/ml TGF-ß1 (kind gift of Napoleon Ferrara, Genentech) for 16 h. The ß-galactosidase and luciferase activities of the transfected cells were determined using reagents from Promega and a Victor+1420 multilabel counter (Wallac). Variation in transfection efficiency was corrected by normalizing luciferase units with galactosidase activity from the same cell lysates. Luciferase assays were carried out in triplicates, indicated by error bars for standard deviation in the figures. In each case, one representative of three or more experiments is shown.

**Site-directed Mutagenesis**—Point mutations in the SBE and AP-1 sites of the luciferase reporters were generated using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s recommendations. The SBE “gtctaTaA”, whereas the “tgAC” sequence was replaced by tgTG in the AP-1 site (see Fig. 1). Altered sequences were confirmed by sequencing.

**Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays**—Nuclear extracts were prepared according to Schreiber et al. (23). Briefly, subconfluent cultures of NIH 3T3 cells were washed and collected in cold phosphate-buffered saline, resuspended in ice-cold hypotonic homogenization buffer (10 mM Hepes, pH 7.9, 10 mM KC1, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.1 μg/ml Pefabloc SC (Fluka)). After 15 min swelling on ice, 25 μl of 10% (w/v) Nonidet P-40

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Detergent was added to the cells followed by vigorous vortexing for 10 s. Nuclei were pelleted through centrifugation at 13,000 rpm for 20 s and resuspended in nuclear extraction buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mg/ml Pefabloc SC). Following incubation on a shaker at 4 °C for 15 min, the nuclear lysates were centrifuged for 5 min, and the supernatant was collected.

Electrophoretic mobility shift assays were performed using nuclear extracts from NIH 3T3 cells either untreated or treated with TGF-β1 (10 ng/ml) for 70 min. Complementary oligonucleotides, constituting the different binding motifs (see Fig. 1), were annealed followed by end labeling using [γ-32P]ATP and T4 polynucleotide kinase. Twenty thousand cpm of the respective probe (2.5 fmols) was incubated with 3 mg of nuclear protein extract in binding buffer (16% 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 for SBE and Sp1 probes, 15 mM Hepes, pH 7.9, 4 mM Tris-HCl, pH 8.0, 100 mM KCl, 5 mM MgCl2, 0.2 mM EGTA, 0.2 mM EDTA, pH 8.0, 1.0 mM dithiothreitol, 12% glycerol, 3 µg of poly(dI-dC) for AP-1 probe) for 30 min at 4 °C. In competition experiments the extract was preincubated for 30 min with a 200-fold molar excess of cold competitor. Oligonucleotides used were as shown in Fig. 1, except where the Sp1 consensus oligo (5'-ATT CGA TCG GGG CGG GAG C-3') was used. For supershift analysis, nuclear extracts were preincubated for 30 min at 4 °C with 2 µg of antibody (except anti-c-Jun, 0.1 µg) followed by the addition of radiolabeled probe. Mouse monoclonal anti-Smad2 was purchased from Transduction Laboratories, rabbit polyclonal anti-CREB from New England BioLabs, goat polyclonal anti-Smad3, anti-Smad4, rabbit polyclonal anti-c-Jun, goat polyclonal anti-JunD, goat polyclonal anti-c-Fos, and goat polyclonal anti-Sp1 were from Santa Cruz Biotechnology. Rabbit polyclonal anti-Smad7 was obtained by purifying antisera raised against a peptide...
FIG. 4. Smads, AP-1, and Sp1 are physically interacting with distinct regions in the mouse Smad7 promoter. Gel EMSAs were performed, identifying R-Smads, AP-1, and Sp1 proteins interacting with specific sequences of the Smad7 promoter. Nuclear extracts were prepared from NIH 3T3 cells that were either unstimulated or stimulated with 10 ng of TGF-β1/ml for 70 min. EMSAs were performed according to "Experimental Procedures" (see Fig. 1 for individual probe sequences). A 200-fold excess of unlabeled competitor oligonucleotides or supershift-
Coding region in exon IV is methylated.

region and at least parts of exon I, whereas the 3'-end of the methylation-free CpG island spanning the promoter proximal

cause the methylation-insensitive preparations, however, resulted in an amplification product that cannot be amplified by PCR.

PCR product, whereas unmethylated DNA is cleaved and thus methylated DNA cannot be digested with the methylation-sensitive

I restriction endonucleases, both recognizing the sequence for the SBE.

An antibody directed against Jun-D did not lead to a supershift, but it clearly interfered with the protein DNA interaction, resulting in a smear toward lower molecular weights (Fig. 4D, lane 6). Because AP-1 sites have been shown to also bind members of the CREB proteins, we tested an antibody against CREB (26). No shift or interference was detectable, even after longer exposure (Fig. 4D, lane 7, data not shown). The Sp1 probe A derived from the Smad7 promoter, specifically interacted with a protein complex that was competed with an excess of unlabeled probe or a Sp1 consensus oligonucleotide, but not with an unrelated oligonucleotide (Fig. 4E, compare lanes 2 and 3 with 4–6). An antibody against Sp1 was able to supershift this protein DNA complex indicating the presence of Sp1 (Fig. 4E, lanes 9 and 11). Repeating the same experiment using the Sp1 probe B showed interactions of the same specificity and identity, however slightly weaker (data not shown). Taken together, the EMSA results indicate that all putative DNA binding sequences bound to their respective transcription factors in vitro.

SBE, AP-1, and Sp1 Are All Required for Proper Function of the Smad7 Promoter—Because deletion of the SBE abolished TGF-β inducibility, without affecting the basal promoter activity (Fig. 3), we were interested to see which role the AP-1 and Sp1 DNA binding sequences might play in the Smad7 promoter. For that purpose, we generated a series of reporter constructs that contained mutations and/or deletions in the SBE, AP-1, and Sp1 binding sequences (Fig. 5A). As expected, point mutation or removal of the SBE rendered the promoter insensitive to TGF-β stimulation (Fig. 5A, compare 1 with 3 and 6). Removal of both the AP-1 and Sp1 sites, however, led to a collapse of both basal and TGF-β-induced promoter activity, although the major start site was still present (Fig. 5A, comparing antibody were added as indicated in the figure. For antibody amounts, see “Experimental Procedures.” Arrows indicate the positions of relevant protein-DNA complexes as well as the positions of supershifted DNA-protein complexes. A, EMSA using a radioactive labeled probe with the sequence for the SBE. B, supershift of TGF-β-induced SBE-protein complexes, using antibodies against different Smad proteins. C, EMSA using a radioactive labeled probe containing the putative AP-1 recognition sequence. D, testing for supershift of the AP-1 probe-protein complex, using antibodies against c-Fos, c-Jun, Jun-D, and CREB. E, EMSA using the radiolabeled Sp1 A probe and supershift experiment using an antibody specific for Sp1 (right half).
Smad7 has been characterized as an antagonist of TGF-β-initiated signaling (10, 12). The ability of the Smad7 promoter to respond to TGF-β is therefore believed to play a central role in the negative autoregulation of TGF-β signaling. To investigate the molecular mechanisms underlying the TGF-β-induced expression of the Smad7 gene, we cloned and functionally characterized the mouse Smad7 promoter.

Fig. 5. Distinct sequence elements for Smad, AP-1, and Sp1 are required for efficient TGF-β response. A, HepG2 cells were transiently transfected with Smad7 B-X promoter reporter constructs containing different combinations of either deletions or mutations in the binding regions for Smads, AP-1, or Sp1. The experimental procedures were the same as described in Fig. 3. Mutations in the SBE and AP-1 regions are indicated by an asterisk (*) on top of the schematic representation for the respective promoter construct. The mutated sequences are identical to those used for the EMSA (Figs. 1 and 4). The transcriptional start site (see Fig. 1) is indicated by a bent arrow. B, an expression vector for Sp1 (0.4 μg) was cotransfected along with different luciferase vectors to test if the Smad7 promoter responds to Sp1 overexpression.

Deletion or point mutation of the AP-1 element resulted in a significant decrease in TGF-β responsiveness and only a small, but reproducible negative effect on basal promoter activity (Fig. 5; compare 1 with 4 and 7). Deletion of the Sp1 region had a reducing effect on the TGF-β-inducible activity (Fig. 5A, compare 5 with 1 and 7) and an even more pronounced negative effect on the basal activity than mutation or deletion of the AP-1 site (Fig. 5A, compare 5 with 4 and 7). Deletion of the major transcriptional start site, lead to a general reduction in promoter activity, whereas a significant TGF-β inducibility of the promoter was still retained (Fig. 5A, 8). A promoter construct that lacked the activating sequences upstream of the major transcriptional start site showed, not unexpectedly, the lowest promoter activity of all constructs tested (Fig. 5; 9). To test the functionality of the GC-boxes, which bound Sp1 in vitro (Fig. 4E), we cotransfected an expression vector for Sp1 together with a small series of reporter constructs. The overexpression of Sp1 increased the basal transcriptional activity only if the cotransfected luciferase vector contained Sp1 binding sites in its promoter (compare Fig. 5A, 1, 7 and 5, with Fig. 5B, 1, 3, and 2).

DISCUSSION

Smad7 has been characterized as an antagonist of TGF-β-initiated signaling (10, 12). The ability of the Smad7 promoter to respond to TGF-β is therefore believed to play a central role in the negative autoregulation of TGF-β signaling. To investigate the molecular mechanisms underlying the TGF-β-induced expression of the Smad7 gene, we cloned and functionally characterized the mouse Smad7 promoter.

Sequencing of a 725-bp region containing the major transcriptional start site (Fig. 1), as defined by Nagarajan and co-workers (18), revealed a very high G+C content, reminiscent of CpG islands. These CpG islands are 0.5–5 kilobase in size with a characteristically high G+C content (60–70%) and no suppression of CpG dinucleotide frequency, in sharp contrast to bulk DNA. Initially, CpG islands had been detected as short stretches of DNA, which lack the methyl group in the 5-position of cytosine within the CpG dinucleotide, a fact that is also reflected in their alternative name, methylation-free islands (27). CpG islands contain a nucleosome-free region allowing for easy access to transcription factors. They are also devoid of histone H1, whereas the other histones are heavily acetylated. Approximately half of all genes in mouse and humans (40,000 to 50,000 genes) contain CpG islands, comprising mainly housekeeping genes with a broad expression pattern (28). However, there are also ~40% of genes with a tissue-restricted pattern of expression containing CpG islands. Computer analysis of the 725-base pair Smad7 upstream gene sequence indeed predicted a CpG island starting at the DraI site (Fig. 1). Because CpG islands are normally hypomethylated compared with bulk DNA, we tested the methylation status of the Smad7 gene using a method that combines digestion of genomic DNA with the methylation-sensitive restriction enzyme HpaII and PCR (29). The results suggest that the promoter region containing the SBE/AP-1 sites, as well as parts of exon I, qualify as a CpG island (Fig. 2; compare C with A and B). The cluster of multiple Sp1 sites, which we found upstream of the transcriptional start site, is also a characteristic of the CpG islands. In addition to their known function as transcription factors, there is evidence that Sp1 blocks incorrect methylation of CpG islands (30). Methylation of CpG islands, which goes in hand with gene silencing, occurs naturally only on the inactive X chromosome and in imprinting of one of the parental alleles. Interestingly, a number of tumor suppressor genes have been found to be transcriptionally inactive in cancer because of aberrant CpG island hypermethylation (reviewed in Ref. 31). To predict if Smad7 would be a likely candidate for gene silencing by this type of epigenetic change is not straightforward.
In an effort to elucidate the molecular mechanisms of TGF-β activation of the Smad7 gene, we subcloned a series of 5′-deleted Smad7 upstream promoter regions into the pGL3-Basic luciferase reporter vector (Fig. 3). Transient transfection of these constructs lead to the identification of a 27-base pair fragment, which contained a palindromic SBE that was essential for TGF-β response of the Smad7 promoter (Fig. 3 and Fig. 5A; compare 1 with 3 and 6). The GTCTAGAC motif of the SBE was initially identified by Zawel et al. (7) in a PCR-based screen in search of DNA sequences optimal for Smad binding. The functional relevance of this element in the Smad7 promoter has recently also been investigated by Nagarajan et al. (18). They showed that overexpressed, tagged versions of Smad3 and Smad4, in nuclear extracts from cells cotransfected with constitutive active TGF-β type I receptor, are able to associate with the SBE. They also found that mere overexpression of Smad3 in HepG2 cells was able to activate transcription from a Smad7 promoter fragment (18). Here we demonstrate the presence of endogenous Smad2, -3, and -4 in two SBE-protein complexes, which are specifically induced in NIH 3T3 cells, following stimulation with TGF-β (Fig. 4B). These complexes can be competed by an oligonucleotide corresponding to the wild type SBE sequence but not with one containing a point mutation or by an unrelated probe (Fig. 4A), indicating that the protein-DNA interaction is specific and sequence dependent.

In addition to the observation that Smad protein binding to the intact SBE motif seems to be critical for the TGF-β inducibility of the Smad7 promoter, we provide strong evidence that AP-1 and Sp1 transcription factors are not only essential for basal transcription but also for the efficient TGF-β inducibility of the Smad7 promoter. We found that an AP-1 site, just a few nucleotides downstream of the SBE, bound protein in a sequence-specific manner (Fig. 4C). Using antibodies specifically directed against different AP-1 family members in EMSAs indicated that this protein complex contained c-Fos and c-Jun; possibly the complex also contained Jun-D, because the antibody against Jun-D interfered with the integrity of the DNA protein interaction (Fig. 4D, lanes 4–6). It is well known that members of the CREB family of transcription factors are able to associate with AP-1 sites (26). In addition it had been found that TGF-β induces the phosphorylation of Ser-133 within CREB, which is necessary for its interaction with the transcriptional coactivator CREB-binding protein (32). We therefore tested if CREB could be a component of the AP-1 probe-protein complex, but we did not detect any effect of the CREB antibody, even after longer exposure (Fig. 4D, lane 7 and data not shown). We cannot, however, exclude the possibility that other members of the CREB family are involved in the binding of this sequence. We furthermore demonstrate Sp1 interaction to a region containing multiple GC-boxes/Sp1 sites upstream of the major transcriptional start (Fig. 4E).

These in vitro data are functionally supported by experiments showing that mutating and/or deleting elements containing binding sites for AP-1 or Sp1 drastically reduce the ability of the Smad7 promoter to respond to TGF-β in luciferase reporter assays (Fig. 5A). Not only the relative response to TGF-β is affected a significant decrease in basal promoter activity in unstimulated cells is also observed (Fig. 5A).

What could explain the ability of Sp1 and AP-1 to support the TGF-β response? Studies conducted on several different promoters have identified Sp1 sites as major TGF-β-responsive promoter elements (22, 33–36). In addition it has been shown that c-Jun can superactivate Sp1 on the p21Waf1/Cip1 promoter (37). Our findings suggest that neither the binding sites for Sp1 alone (Fig. 5A; 10) nor the binding sites for AP-1 and Sp1 together are able to respond to TGF-β stimulation, the latter of which is indicated by point mutation or deletion of the SBE (Fig. 5A; 3 and 6). Significant TGF-β inducibility over basal promoter activity requires the combination of the SBE with at least either AP-1 or Sp1. Overexpression of Sp1 resulted in a more than 6-fold increase of the basal Smad7 promoter (compare Fig. 5B; 1 and Fig. 5A; 1), whereas deletion of the Sp1 region drastically reduced the promoter’s response to the Sp1 overexpression (Fig. 5B; 2). Interestingly, overexpression of Sp1 cannot compensate for the mutation of the AP-1 binding site in an otherwise wild type promoter configuration (Fig. 5B; compare 1 and 3), underlining the necessity for both DNA binding elements in the transcriptional regulation of the Smad7 promoter.

The role of AP-1 in TGF-β-regulated gene expression was investigated by Zhang and co-workers (38), who demonstrated that Smad3 directly associated to 12-O-tetradecanoyl-13-acetate-responsive gene promoter elements. These 12-O-tetradecanoyl-13-acetate-responsive gene promoter elements are in effect AP-1 binding sites, because they could bind to and mediate transcription through c-Jun and c-Fos. It was also shown that c-Jun and Smad3 simultaneously could associate to overlapping sequences in the 12-O-tetradecanoyl-13-acetate-responsive gene promoter element. This was supported by the observation that following activation by TGF-β, Smad3, and Smad4 can physically interact with c-Jun, Jun-B, Jun-D, and c-Fos (38, 39). Other examples showing primary importance of AP-1 conferring TGF-β-induced transcription are the promoters for the TGF-β1 gene itself and the promoter for the plasminogen activator inhibitor-I (40, 41). In the Smad7 promoter juxtaposed to the SBE we detected an AP-1 site, which was able to bind AP-1 family members. Mutation of the AP-1 site completely abolished any protein binding in vitro (Fig. 4C) and led to a drastic reduction in Smad7 promoter activity (Fig. 5) compare lanes 1 and 7) underscoring the importance of AP-1 for the Smad7 promoter. In summary, our data strongly support a molecular mechanism demanding the cooperation between Smads, Sp1, and AP-1, in mediating a full TGF-β response in the mouse Smad7 promoter.

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REFERENCES
1. Roberts, A. B., and Sporn, M. B. (1990) in Peptide Growth Factors and Their Receptors, Part I (Sporn, M. B., and Roberts, A. B., eds) Vol. 95, pp 419–472, Springer-Verlag, Berlin
2. Kingsley, D. M. (1994) Genes Dev. 10, 133–146
3. Attisano, L., and Wraha, J. L. (1998) Curr. Opin. Cell Biol. 10, 188–194
4. Denuener, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J.-M. (1998) EMBO J. 17, 3091–3100
5. Jonk, L. J., Itoh, S., Heldin, C. H., ten Dijke, P., and Krujiver, W. (1998) J. Biol. Chem. 273, 21145–21152
6. Yingling, J. M., Datto, M. B., Wong, C., Frederick, J. P., Liberati, N. T., and Wang, X. F. (1997) Mol. Cell. Biol. 17, 7019–7028
7. Zawel, L., Liu, J. L., Buckland, P., Zhou, S., Kinard, K. W., Vogeletsing, B., and Kern, S. E. (1998) Mol. Cell 1, 611–617
8. Attisano, L., and Wraha, J. L. (2000) Curr. Opin. Cell Biol. 12, 235–243
9. ten Dijke, P., Miyazono, K., and Heldin, C.-H. (2000) Trends Biochem. Sci. 25, 64–70
10. Hayashi, H., Abdollah, S., Qiu, Y. B., Cai, J. X., Xu, Y. Y., Grinnell, B. W., Richardson, M. A., Topper, J. N., Gimbrone, M. A., Jr., Wraha, J. L., and Falb, D. (1997) Cell 89, 1165–1173
11. Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M., and Miyazono, K. (1997) Nature 389, 622–626
12. Nakao, A., Afrakhite, M., Moren, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N.-E., Heldin, C.-H., and ten Dijke, P. (1997) Nature 389, 631–635
13. Hata, A., Lagna, G., Massague, J., and Hemmati-Brivanlou, A. (1998) Genes Dev. 12, 186–197
14. Afrakhite, M., Moren, A., Jossan, S., Itoh, S., Samphath, K., Westermark, B., Heldin, C. H., Heldin, N. E., and ten Dijke, P. (1998) Biochem. Biophys. Res. Commun. 249, 505–511
15. Topper, J. N., Cai, J., Qiu, Y., Anderson, K. R., Xu, Y.-Y., Deedes, J. D., Feeley, R., Gimmor, C. J., Woolf, E. A., Tayber, O., Mays, G. G., Sampson, B. A., Schoon, P. J., Gimbrone, Jr., M. A., and Falb, D. (1997) Proc. Natl. Acad. Sci. USA 94, 13334–13339.

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16. Ulloa, L., Doodly, J., and Massagué, J. (1999) Nature 397, 710–713
17. Bitzer, M., von Gerdsdorff, G., Liang, D., Dominguez-Rosales, A., Beg, A. A., Rojkind, M., and Bottinger, E. P. (2000) Genes Dev. 14, 187–197
18. Nagarajan, R. P., Zhang, J., Li, W., and Chen, Y. (1999) J. Biol. Chem. 274, 33412–33418
19. Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenisch, R., and Berns, A. (1991) Nucleic Acids Res. 19, 4293
20. Kogan, S. C., Doherty, M., and Gitschier, J. (1987) N. Engl. J. Med. 317, 985–990
21. Graham, F. L., and Eb, A. J. van der (1973) Virology 52, 456–467
22. Moustakas, A., and Kardassis, D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6733–6738
23. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419
24. Brodin, G., ten Dijke, P., Funa, K., Heldin, C.-H., and Landström, M. (1999) Cancer Res. 59, 2731–2738
25. Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaki, A., Oda, E., Tamaki, K., Hanai, J.-I., Heldin, C.-H., Miyazono, K., and ten Dijke, P. (1997) EMBO J. 16, 5353–5362
26. Masquiler, D., and Sassone-Corsi, P. (1992) J. Biol. Chem. 267, 22460–22466
27. Bird, A. P. (1986) Nature 321, 209–213
28. Antequera, F., and Bird, A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11995–11999
29. Singer-Sam, J., Grant, M., LeBon, J. M., Okuyama, K., Chapman, V., Monk, M., and Riggs, A. D. (1999) Mol. Cell. Biol. 19, 4987–4989
30. Siegfried, Z., Eden, S., Mendelsohn, M., Peng, X., Tsuberi, B. Z., and Cedar, H. (1999) Nat. Genet. 22, 203–206
31. Herman, J. G. (1999) Semin. Cancer Biol. 9, 359–367
32. Potchinsky, M. B., Weston, W. M., Lloyd, M. R., and Greene, R. M. (1997) Exp. Cell Res. 231, 96–103
33. Datto, M. B., Yu, Y., and Wang, X.-F. (1995) J. Biol. Chem. 270, 28623–28628
34. Greenwell, P., Imagaki, Y., Hu, W., Walsh, M., and Ramirez, F. (1997) J. Biol. Chem. 272, 19738–19745
35. Li, J. M., Datto, M. B., Shen, X., Hu, P. P. C., Yu, Y., and Wang, X. F. (1998) Nucleic Acids Res. 26, 2449–2456
36. Li, J.-M., Nicholas, M. A., Chandrasekharan, S., Xiong, Y., and Wang, X.-F. (1995) J. Biol. Chem. 270, 26750–26753
37. Kardassis, D., Papakosta, P., Pardali, K., and Moustakas, A. (1999) J. Biol. Chem. 274, 29572–29581
38. Zhang, X., Peng, X. H., and Derynck, R. (1998) Nature 394, 909–913
39. Liberati, N. T., Datto, M. B., Frederick, J. P., Shen, X., Wang, C., Rougier-Chapman, E. M., and Wang, X.-F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4844–4849
40. Keeton, M. R., Curriden, S. A., van Zonneveld, A. J., and Loskutoff, D. J. (1991) J. Biol. Chem. 266, 23048–23052
41. Kim, S. J., Denhez, F., Kim, K. Y., Holt, J. T., Sporn, M. B., and Roberts, A. B. (1989) J. Biol. Chem. 264, 18373–18378
