Regulation of Smad7 Promoter by Direct Association with Smad3 and Smad4*

(Received for publication, July 15, 1999, and in revised form, September 3, 1999)

Raman P. Nagarajan, Jingming Zhang, Wei Li, and Yan Chen‡

From the Department of Medical and Molecular Genetics and the Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202

Smad7 is a regulatory Smad protein that is able to antagonize signal transduction by transforming growth factor-β (TGF-β) and activin receptors. To characterize the regulation of Smad7 at the transcriptional level, we isolated the promoter region of the mouse Smad7 gene. When the Smad7 promoter luciferase reporter gene (−408 and +112 bp) was expressed in human hepatoma (HepG2) cells, its transcriptional activity was increased following TGF-β or activin treatment. In addition, this region of the Smad7 promoter was stimulated by ectopic expression of Smad3 as well as constitutively active TGF-β and activin receptors, indicating that Smad7 transcription was modulated by the signaling downstream those two receptors. A gel mobility shift assay indicated that a DNA fragment spanning −408 to −126 bp was able to directly bind purified Smad4. Furthermore, a consensus Smad3-Smad4 binding element (SBE) was discovered in this region of the promoter with a palindromic sequence of GTCTAGAC. A 33-bp Smad7 promoter fragment containing this SBE was able to bind Smad3 and Smad4. In human embryonic kidney 293 cells, the expression of constitutively active TGF-β and activin receptors, indicating that Smad7 transcription was modulated by the signaling downstream those two receptors. A gel mobility shift assay indicated that a DNA fragment spanning −408 to −126 bp was able to directly bind purified Smad4. Furthermore, a consensus Smad3-Smad4 binding element (SBE) was discovered in this region of the promoter with a palindromic sequence of GTCTAGAC. A 33-bp Smad7 promoter fragment containing this SBE was able to bind Smad3 and Smad4. In human embryonic kidney 293 cells, the expression of constitutively active TGF-β type I receptor was able to induce the formation of a Smad3- and Smad4-containing nuclear protein complex that bound the SBE. In HepG2 cells, TGF-β1 treatment could induce the formation of an endogenous SBE-binding complex. Taken together, these data provided the first evidence that Smad7 transcription is regulated by TGF-β and activin signaling through direct binding of Smad3 and Smad4 to the Smad7 promoter.

Smad proteins are a group of recently identified molecules that function as intracellular signaling mediators and modulators of transforming growth factor-β (TGF-β) family members (1, 2). Functional characterization of Smad proteins has allowed their subdivision into three subfamilies: pathway-specific, common mediator, and inhibitory Smads. Pathway-specific Smads are activated by the type I receptor serine kinases through phosphorylation at the C-terminal end in a ligand- and type II receptor-dependent manner (3, 4). This subfamily includes Smads 1, 2, 3, 5, and 8 (2). Smad1 and -5 mediate signaling by bone morphogenetic proteins (BMP) 2 and 4 (5–8), Smad2 and -3 mediate signaling by TGF-β and activins (9–12), and Smad8 mediates signaling by the receptor serine kinase ALK-2 (13). The second Smad subfamily is represented by Smad4 (14), which serves as a common signaling mediator. Activation of the pathway-specific Smads by their individual receptors induces an association of these Smads with Smad4, which is critical for the proper downstream signaling (15). Smad6 and Smad7 comprise the third Smad subfamily and have been reported to function as negative regulators of receptor serine kinases mediating TGF-β and BMP responses (16–18). Smad7 has been shown to inhibit signal transduction by the TGF-β and activin receptors (16, 17, 19), whereas Smad6 was reported to inhibit BMP signaling (18). A Xenopus Smad7 homologue was also reported recently to antagonize BMP and activin signaling in the frog embryo (20, 21).

Smad proteins, when translocated into the nucleus, function as transcriptional regulators that control the expression of target genes (2, 22, 23). Smad exerts its transcriptional regulatory activity by interacting with either a specific transcription factor or a specific DNA element. In Xenopus, Smad2 and Smad4 participate in the activin-mediated transcriptional induction of the Mix.2 promoter through an interaction with a specific DNA-binding transcription factor, forkhead activin signal transducer-1 (FAST-1), a member of the winged-helix forkhead transcription factor family (24, 25). FAST-1 has two functional domains that mediate DNA binding and Smad association respectively. The DNA binding motif of FAST-1 mediates the interaction of FAST-1 with an activin-responsive element on the Xenopus Mix.2 promoter, and the Smad interaction domain is involved in the association with the Smad2-Smad4 complex (25). Smad proteins have been found to interact functionally with other transcription factors including human FAST-1 and mouse FAST-2 (26–28), AP1 (29), Sp1 (30), and TFE3 (31). In addition to the interaction of Smad with other transcription factors, recent studies have suggested that Smad can directly bind DNA. In Drosophila, Mad protein is able to directly bind a GC-rich region of various enhancers (32). A PCR-based screening with random sequences has led to the discovery of specific binding of Smad3 and Smad4 with a palindromic DNA sequence (33). Smad3 and Smad4 were also found to bind a CAGA motif in the promoter of plasminogen S-transferase; JAK, Janus kinase; STAT, signal transducers and activators of transcription.

* This work was supported by the Foundation for Medical Research, Inc., Indiana University School of Medicine and by Grant-in-aid 9951372Z from the American Heart Association Midwest Affiliate (to Y. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF167314.

† To whom correspondence should be addressed: Dept. of Medical and Molecular Genetics, Indiana University School of Medicine, 1000 West Walnut St., Indianapolis, IN 46202. Tel.: 317-278-0275; Fax: 317-274-2387; E-mail: ychen3@iupui.edu.

‡ The abbreviations used are: TGF-β, transforming growth factor-β; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; FAST, forkhead activin signal transducer; HEK293, human embryonic kidney 293 cells; HepG2 cells, human hepatoma cells; Smad, Smad-related protein; SBE, Smad binding element; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); GST, glutathione S-transferase; JAK, Janus kinase; STAT, signal transducers and activators of transcription.
Smad Regulates Smad7 Transcription

activator inhibitor-1 (34). Once Smad protein associates with either a transcription factor or a DNA element, its C-terminal MH2 domain may exert a transactivation function (6). This function of the MH2 domain was supported by the recent discovery that Smad may interact with the general transcription coactivators CBP (CREB-binding protein) and p300 (35–38). CBP/p300 may bridge the general transcription machinery and Smad proteins or the Smad-associated transcription factors, e.g., FAST-1 or FAST-2, and enable the transcriptional regulation of target genes.

Recent studies have indicated that the inhibitory Smad proteins may play a role in a negative feedback loop that modulates the signaling by TGF-β, activin, and BMP. Treatment of Mv1Lu mink lung cells and HaCaT keratinocytes with TGF-β led to a transient increase of the Smad7 mRNA steady state level (17). In mouse B cell hybridoma HS-72 cells, activin is able to induce an increase of the steady state level of Smad7 mRNA (19). In addition, treatment with BMP2 or BMP7 induces an increase of the Smad6 mRNA level in a number of mouse cell lines (39). Those studies have suggested that the inhibitory Smad proteins could be up-regulated by signaling of the TGF-β superfamily; such a regulation might be involved in desensitization of the cells to the continued exposure of ligand. To understand the mechanism underlying the regulation of the inhibitory Smad message, we isolated a mouse Smad7 promoter and characterized the regulation of the promoter by TGF-β and activin signaling at the transcriptional level.

MATERIALS AND METHODS

Cell Culture and Cell Transfection—Human embryonic kidney 293 (HEK293) cells and human hepatoma (HepG2) cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cell transfection was performed by a calcium phosphate method for HEK293 cells and human hepatoma (HepG2) cells were cultured in a 124-bp promoter construct was generated by digestion of the Smad7 genomic clone with KpnI and Stul digestion and subcloned into KS+ pBluescript (Stratagene). This insert was then cut away from the major initiation site, indicating that the binding site is a transcription factor IIId (TFIID) binding site about 200 bp away from the major initiation site, indicating that the binding site is a transcription factor IIId (TFIID) binding site about 200 bp away from the major initiation site.

Electrophoretic Mobility Shift Assay—A 282-bp fragment (−408 to −124 bp) of the Smad7 promoter was released by KpnI and Stul digestion and subcloned into KS+ pBluescript. This insert was then cut away from the major initiation site, indicating that the binding site is a transcription factor IIId (TFIID) binding site about 200 bp away from the major initiation site.

RESULTS AND DISCUSSION

Isolation of a Mouse Smad7 Promoter—Smad7 is a newly found regulatory protein that is able to antagonize TGF-β and activin signaling (1). Smad7 has been implicated in a negative feedback loop in which TGF-β and activin treatment in different cells were able to increase the expression of Smad7 (17, 19).

To determine whether the regulation of Smad7 expression occurs at the transcriptional level, we isolated the 5′ region of the mouse Smad7 gene. A 500-bp probe corresponding to the 5′-end of rat Smad7 cDNA was used to screen a mouse genomic library at high stringency. Six independent clones that contain sequences 5′ to the Smad7 cDNA were isolated. Genomic mapping analysis using restriction enzyme digestion and partial sequencing of these clones confirmed that we obtained as much as 4.3 kb of the 5′-end of the Smad7 gene (data not shown). The 512-bp fragment adjacent to the Smad7 cDNA sequence was subjected to full sequencing (Fig. 1A). To identify the putative transcription initiation site, we used a primer extension assay with the total RNA isolated from mouse aorta, mouse whole brain, or the mouse C2C12 myoblast cells. In all of the RNA samples, a major extension product was detected at 4.3 kb of the 5′-end of the Smad7 gene. A 500-bp probe corresponding to the 5′-end of rat Smad7 cDNA was used to screen a mouse genomic library at high stringency. Six independent clones that contain sequences 5′ to the Smad7 cDNA were isolated. Genomic mapping analysis using restriction enzyme digestion and partial sequencing of these clones confirmed that we obtained as much as 4.3 kb of the 5′-end of the Smad7 gene (data not shown). The 512-bp fragment adjacent to the Smad7 cDNA sequence was subjected to full sequencing (Fig. 1A). To identify the putative transcription initiation site, we used a primer extension assay with the total RNA isolated from mouse aorta, mouse whole brain, or the mouse C2C12 myoblast cells. In all of the RNA samples, a major extension product was detected at 4.3 kb of the 5′-end of the Smad7 gene (Fig. 1B). The clarity of data presented here, this major initiation site was designated as the +1 position. Sequence information suggests that there was no TATA box in the promoter region. However, the promoter region has multiple Sp1 sites, which are common to most TATA-less promoters (42).

**Primer Extension Assay**—An oligonucleotide (5′-GCTCGAGGTGCCGTACAGGGTGTCACGCGGGC-3′) corresponding to the very 5′-end of the known mouse Smad7 cDNA sequence was labeled at its 5′-end by T4 polynucleotide kinase (Promega) in the presence of [γ-32P]ATP. The labeled probe was annealed with total RNA (2–4 μg) isolated from mouse aorta, mouse whole brain, and C2C12 mouse myoblast cells. The extension reaction was performed in the presence of actinomycin D (20 ng/ml), the four dNTPs (0.5 mM each), 4 mM dithiothreitol, and 20 units of SuperScript reverse transcriptase (Life Technologies, Inc.) at 50 °C for 30 min. The reaction was then extracted with phenol, precipitated with isopropanol, and loaded on a 6% denaturing polyacrylamide gel. An unrelated plasmid was sequenced and used as a marker to determine the relative position of the promoter.

**Promil Construction and GST Fusion Proteins**—The wild type Smad2, Smad3, and Smad4 and the constitutively active activin type I receptor have been described previously (12, 13). The Smad3 (G5) mutant was generated by a PCR strategy that specifically changed glycine 379 to serine. The C-terminal truncation of Smad3 was generated by removing the region that spans a Hpal site and the end of the cDNA clone. These mutations were confirmed by DNA sequencing. All of the GST fusion proteins were generated by in-frame fusion of the full-length mouse Smad7 cDNA with pGEX-tt2 (Amersham Pharmacia Biotech). The constructs were transferred into Escherichia coli BL21 strain (Amersham Pharmacia Biotech), and the GST fusion proteins were purified according to the manufacturer’s protocol.

**Electrophoretic Mobility Shift Assay**—A 282-bp fragment (−408 to −124 bp) of the Smad7 promoter was released by KpnI and Stul digestion and subcloned into KS+ pBluescript. This insert was then cut away from the major initiation site, indicating that the binding site is a transcription factor IIId (TFIID) binding site about 200 bp away from the major initiation site. The extension reaction was performed in the presence of actinomycin D (20 ng/ml), the four dNTPs (0.5 mM each), 4 mM dithiothreitol, and 20 units of SuperScript reverse transcriptase (Life Technologies, Inc.) at 50 °C for 30 min. The reaction was then extracted with phenol, precipitated with isopropanol, and loaded on a 6% denaturing polyacrylamide gel. An unrelated plasmid was sequenced and used as a marker to determine the relative position of the promoter.

**Electrophoretic Mobility Shift Assay**—A 282-bp fragment (−408 to −124 bp) of the Smad7 promoter was released by KpnI and Stul digestion and subcloned into KS+ pBluescript. This insert was then cut away from the major initiation site, indicating that the binding site is a transcription factor IIId (TFIID) binding site about 200 bp away from the major initiation site. The extension reaction was performed in the presence of actinomycin D (20 ng/ml), the four dNTPs (0.5 mM each), 4 mM dithiothreitol, and 20 units of SuperScript reverse transcriptase (Life Technologies, Inc.) at 50 °C for 30 min. The reaction was then extracted with phenol, precipitated with isopropanol, and loaded on a 6% denaturing polyacrylamide gel. An unrelated plasmid was sequenced and used as a marker to determine the relative position of the promoter.
of TFIID at this site might contribute to the initiation of Smad7 transcription.

Smad7 Promoter Is Stimulated by TGF-β and Activin Signaling—We next determined whether this putative Smad7 promoter was regulated by TGF-β and activin signaling. Different lengths of Smad7 promoter sequence were fused with a basic luciferase reporter that did not contain any promoter sequence or TATA box. These reporter constructs spanned from −408 to +112 bp relative to the major transcription initiation site. When these three luciferase fusion plasmids were transfected in HEK293 cells, all of them gave rise to very high luciferase activity, over 100-fold higher than the value from the cells transfected with the parental luciferase vector (data not shown). These data suggested that the Smad7 genomic clone that we isolated, especially the −2408 to +1112-bp region, did contain an endogenous transcription initiation site controlled by the basic transcriptional machinery. In addition, we have made another luciferase fusion plasmid that contains the sequence from the −2408 to −1112-bp region, and when transfected in HEK293 cells, this construct gave no appreciable promoter activity (data not shown), further indicating that the transcription of the Smad7 gene starts from a region within the −408 to +112-bp area.

We next transfected these luciferase constructs into human HepG2 cells that contain functional TGF-β and activin receptors (30, 43). Interestingly, treatment of these cells with activin A or TGF-β1 was able to stimulate the activity of these promoter constructs (Fig. 2A). Activin or TGF-β treatment was able to induce the activity of all three promoter constructs 5–7-fold. Both of the longer constructs had a slightly higher basal activity when compared with the −408 to +112-bp construct. However, the fold inductions by either activin or TGF-β treatment were very similar among the three promoter constructs. These data, therefore, provided the first piece of evidence that the Smad7 promoter could be regulated by TGF-β and activin at the transcriptional level. Furthermore, they suggested that the putative regulatory element(s) that mediates the TGF-β and activin effect is localized within the −2408 to +112-bp region.

To further characterize the regulation of the Smad7 promoter by TGF-β and activin signaling, we analyzed the ability of different Smad proteins and serine receptor kinases of the TGF-β superfamily to transactivate the Smad7 promoter (Fig. 2B). Transfection of Smad3, but not Smad1, Smad2, or Smad4 alone, into HepG2 cells was able to stimulate the promoter activity of the −408 to +112-bp construct. This is consistent with previous findings that Smad3, when overexpressed, is able by itself to up-regulate target genes downstream of TGF-β and activin signaling (12). In addition, Smad3 appeared to synergize with Smad4 to stimulate the Smad7 promoter, as co-expression of Smad3 and Smad4 was able to strongly increase the promoter activity (Fig. 2B). As expected, the constitutively active activin type I receptor (ALK-4) and TGF-β type I receptor (ALK-5), but not the type I receptor for BMP2/4...
Regulation of Smad7 promoter by different Smad proteins and TGF-beta signaling. co-expression of Smad7 (Fig. 2B). In addition, we used dominant negative Smad3 to further determine whether Smad proteins were involved in the activation of the Smad7 promoter by TGF-beta signaling. Both the Smad3 G/S point mutation and C-terminal deletion constructs were transfected into HepG2 cells together with the −408 to +112-bp Smad7 promoter construct (Fig. 2C). The G/S mutation was originally discovered in Drosophila Mad in which it led to a compromised development characteristic of a defective decapentaplegic (dpp) pathway (44), indicating that this mutation is able to disrupt the signaling by TGF-beta family members. The C terminus or MH2 domain of Smads has been implicated in the transactivating activity of these proteins (6). We found that both G/S point mutation and C-terminal deletion of Smad3 was able to significantly ablate the TGF-beta-mediated activation of Smad7 promoter. Taken together, these data would support a model in which Smad7 is involved in a mutual feedback regulation in TGF-beta and activin signaling. Activation of TGF-beta or activin signaling by ligand binding may initiate Smad7 transcription, which may desensitize the further signaling by TGF-beta or activin. Meanwhile, the shut-off of this signaling by up-regulated Smad7 also blocks its own transcription, making the cells available to respond to new stimuli after a certain delay that would be dependent on the turnover rate of Smad7.

Binding of Smad3 and Smad4 to Smad7 Promoter—Transcriptional regulation by TGF-beta or activin signaling is achieved mainly by two pathways. One of them is the interaction of Smad proteins with other transcription factors that bind specific sequences of TGF-beta/activin-responsive promoters. The classical example of this category is the FAST-2-mediated transcriptional regulation of the Mix2 promoter in which Smad2 and Smad4 need to complex with FAST-2 in order to mediate TGF-beta or activin response (24). The second mode of transcriptional regulation by TGF-beta or activin is through direct binding of Smad3 and Smad4 with specific DNA sequences. This pathway is exemplified by the finding that these two Smad proteins are able to associate with the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene (34). To explore the possible mechanism underlying the TGF-beta- and activin-mediated transcriptional regulation of the Smad7 promoter, we used a gel mobility shift assay to determine whether Smad proteins directly bind Smad7 promoter. A 282-bp DNA fragment that spans from −408 to −126 bp was labeled with 32P and used in a gel shift assay with Smad2, Smad3, and Smad4 GST fusion proteins (Fig. 3A). Compared with GST alone (Fig. 3A, lane 1), the full-length Smad2 protein could not cause any appreciable shift of the probe (lane 2). The full-length Smad3 protein led to a slightly detectable shift of the probe (lane 3). Furthermore, this 282-bp probe was significantly shifted by the full-length Smad4 fusion protein (lane 7), and this shift could be competed off by the excess of cold 282-fragment (lane 8), supporting the specificity of the binding of Smad4 to the sequence.

Our gel shift assay with the 282-bp probe provided the clue that Smad3 and Smad4 may directly bind the Smad7 promoter and mediate the transcriptional response by TGF-beta and activin signaling. Careful examination of the Smad7 promoter sequence has led our attention to a putative Smad binding element inside the −408 to +112-bp region, in which there is indeed a consensus Smad binding sequence previously identified through a PCR-based oligonucleotide screening with the MH1 domain of Smad3 and Smad4 (33). It has been found that both Smad3 and Smad4 could preferentially bind an octamer sequence GTCTAGGTCTAG (X stands for any one of the four nucleotides). The Smad7 promoter contains a palindromic sequence of GTCTAGXGC in the area spanning −285 to −278 bp (Fig. 1A). To determine whether this putative Smad binding element (SBE) confers the binding of Smad7 promoter to Smad3 and Smad4, we used a synthetic oligonucleotide probe that covered this consensus binding element. As shown in Fig. 3B, this 33-bp probe was not able to bind GST or the Smad2 fusion protein (lanes 1 and 2). However, Smad3 was able to associate with this probe (lane 4), and this binding could be competed off by increasing concentrations of the cold oligonucleotide (lanes 5-7). Furthermore, the binding of Smad3 with the 33-bp probe could be supershifted by an anti-Myc antibody that recognizes the Myc epitope tag of Smad3 (lanes 8-10). In addition to Smad3 binding, the probe could strongly bind the Smad4 (lane 11) that was competed off by the cold probe (lanes 12-14), and it appeared that Smad4 might bind this sequence as a monomer, dimer, or trimer. Taken together, these data strongly suggested that Smad3 and Smad4 were able to directly associate with the consensus Smad binding motif of the Smad7 promoter. To further determine whether the binding of Smad3 or Smad4 with this consensus sequence is involved in the binding of these
Smad proteins with the longer Smad7 promoter (i.e. the 282-bp probe), we examined the ability of the 33-bp cold probe to compete with the binding of Smad proteins with the 282-bp fragment. Inclusion of this 33-bp cold oligonucleotide was able to completely compete off the binding of Smad4 with the 282-bp probe (Fig. 3A, lane 9), further indicating that the binding of these Smad proteins to the Smad7 promoter is conferred by the SBE.

We next examined whether an intact SBE was required for the responsiveness of the Smad7 promoter to activin and TGF-β treatment. We used two Smad7 promoter constructs, −408 to −124 bp and −283 to −124 bp. The −283 to −124-bp construct had a partial truncation in the 5′-end of the SBE, and this truncation changed the putative SBE of the Smad7 promoter from GTCTAGAC to CTCTAGAC (i.e. G to C at the first position). When the −408 to −124-bp construct containing the intact SBE sequence was transfected into HepG2 cells, it was stimulated by both activin and TGF-β treatment (Fig. 4). However, the −283 to −124-bp construct was no longer responsive to activin or TGF-β, further indicating that this consensus Smad binding motif in the Smad7 promoter was involved in activin- and TGF-β-mediated transcriptional regulation.

**FIG. 3. Association of Smad3 and Smad4 with Smad7 promoter**. A, binding of Smad3 and Smad4 to a 282-bp Smad7 promoter fragment by gel mobility shift assay. The GST, GST-Smad2, GST-Smad3 (tagged with a Myc epitope), and GST-Smad4 (about 0.2 μg/reaction) purified from bacteria were incubated with 32P-labeled Smad7 promoter. Different cold competitor DNA (282 or 33 bp) or an anti-Myc antibody (about 1 μg) were included in the binding reaction as indicated. The Smad3-mediated shift is marked by an arrow. B, association of Smad3 and Smad4 with the Smad binding element of the Smad7 promoter. Different GST fusion proteins were incubated with a 33-bp probe that contains the putative SBE of Smad7 promoter in a gel shift assay. Different amount of cold 33-bp competitor (10–200 ng) or an anti-Myc antibody (0.05–1 μg) were used as indicated.

**FIG. 4. An intact Smad binding motif is required for TGF-β stimulation of the Smad7 promoter**. HepG2 cells were transfected with Smad7 promoter/luciferase constructs −408 to −124 bp or −283 to −124 bp (0.25 μg) and treated with or without activin A (10 ng/ml) or TGF-β1 (1 ng/ml). The −124 to −283-bp construct changed the putative consensus Smad binding site GTCTAGAC of Smad7 promoter to CTCTAGAC. The fold change of luciferase activity was shown as the mean ± S.D.
supershift with anti-Myc antibody) and Smad4 (lane 16, supershift with anti-FLAG antibody). These data clearly indicate that TGF-β signaling is able to induce the formation of Smad2-Smad4 and Smad3-Smad4 complexes in the nucleus and that these complexes are implicated in the regulation of Smad7 promoter through the direct interaction of Smad3 or Smad4 with the SBE of the Smad7 promoter. It is also interesting to note that the putative nuclear Smad2-Smad4 complex may behave differently from the Smad3-Smad4 complex in its interaction with the Smad7 promoter. We could not detect a convincing presence of Smad2 in the hypothetical SBE-binding Smad2-Smad4 complex by the gel shift assay (Fig. 5A, lane 11), although Smad2 expression was confirmed by anti-Myc Western blotting analysis with the same nuclear extracts (data not shown). This observation indicates that Smad4 may dissociate from Smad2 after the Smad2-Smad4 complex is translocated into the nucleus.

Our functional analysis with Smad7 promoter constructs has indicated that signaling with TGF-β and activin was able to activate the promoter activity in HepG2 cells (Fig. 2). To further confirm that this transactivation was directly related to the regulation of the SBE present in Smad7 promoter, we asked whether or not TGF-β1 treatment in HepG2 cells was able to induce the formation of an endogenous nuclear protein complex that binds the SBE. HepG2 cells were treated with TGF-β1 for 30, 60, and 120 min, and the nuclear extracts were used in a gel shift experiment with the 33-bp SBE probe. As shown in Fig. 5B, TGF-β1 did induce the formation of a nuclear complex that associated with this SBE probe, and the association appeared to reach a maximum at 60 min after the treatment. Because this SBE was able to associate specifically with Smad3 and Smad4 (Figs. 3 and 5A), these data strongly suggest that TGF-β was able to induce the formation of an endogenous Smad complex that binds the SBE of Smad7 promoter.

In conclusion, our initial characterization of the Smad7 promoter provides one of the molecular mechanisms underlying the regulation of this inhibitory Smad by TGF-β and activin signaling at the transcriptional level. Treatment with TGF-β and activin in HepG2 cells was able to stimulate the activity of the Smad7 promoter. Furthermore, the Smad7 promoter contains a SBE binding element that is able to directly associate with Smad3 and Smad4. Activation of TGF-β by signaling by the activated type I receptor was able to mediate the formation of a nuclear Smad3-Smad4 complex that bound the Smad7 promoter. These data clearly indicated that TGF-β and activin signaling may activate their cognate Smad proteins to trans-activate the promoter of Smad7, which in turn blocks the signaling by these two extracellular factors. In addition to the function in a negative feedback loop that modulates TGF-β and activin signaling, regulation of the Smad7 message has been identified in other biological processes. The Smad7 mRNA level is up-regulated by laminar shear stress in vascular endothelial cells (45). Interferon-γ is also able to stimulate the expression of Smad7 through JAK1 and STAT1 to block TGF-β signaling (46). With the cloned Smad7 promoter, it is now more feasible to address the question of how this inhibitory Smad is regulated by multiple signals at the transcriptional level.

Acknowledgments—We thank R. Harland for the mouse Smad2 clone and M. Schütte for the human Smad4 clone. We also thank L. Carr for careful reading and constructive comments on the manuscript.

REFERENCES

1. Massague, J. (1998) Annu. Rev. Biochem. 67, 753–791
2. Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997) Nature 390, 465–471
3. Souchelnytskyi, S., Tamaki, K., Engstrom, U., Wernstedt, C., ten Dijke, P., and Heldin, C. H. (1997) J. Biol. Chem. 272, 28107–28115
4. Abdollah, S., Macias-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L., and Wrana, J. L. (1997) J. Biol. Chem. 272, 7678–7685
5. Hoodless, P. A., Haerry, T., Abdollah, S., Stapleton, M., O’Connor, M. N., Attisano, L., and Wrana, J. L. (1996) Cell 85, 489–500
6. Liu, P., Hata, A., Baker, J. C., Doody, J., Carrasco, J., Harland, R. M., and Massague, J. (1996) Nature 381, 620–623
7. Graff, J. M., Bansal, A., and Melton, D. A. (1996) Cell 85, 479–487
8. Yamamoto, N., Akiyama, S., Katagiri, T., Namiki, M., Kurokawa, T., and Suda, T. (1997) Biochem. Biophys. Res. Commun. 236, 574–580
9. Baker, J. C., and Harland, R. M. (1996) Genes Dev. 10, 1880–1889
10. Macias-Silva, M., Abdollah, S., Hoodless, P. A., Pirone, R., Attisano, L., and Wrana, J. L. (1996) Cell 87, 1215–1224
11. Wang, Y., Feng, W., Xu, R., and Melton, D. A. (1996) Nature 383, 168–172
12. Chen, Y., Lebrun, J. J., and Vale, W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12982–12987
13. Chen, Y., Bhushan, A., and Vale, W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12938–12943
14. Hahn, S. A., Schütte, M., Koeber, A. T., Moskaluk, C. A., da Costa, L. T., Rosenthal, E., Weisberg, E., Fridmacher, V., Watanabe, M., and Heldin, C. H. (1997) Curr. Opin. Genet. Dev. 8, 103–111
15. Derynck, R., Zhong, Y., and Fang, X. H. (1998) Cell 95(6), 737–740
16. Chen, X., Rubeck, M. J., and Whitman, R. M. (1996) Nature 383, 691–696
17. Chen, X., Weisberg, E., Fridmacher, V., Watanabe, M., Naco, G., and Derynck, R. (1997) Cell 89, 85–89
18. Zhou, S., Zavel, L., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1998) Mol. Cell 2, 121–127
19. Labbé, E., Silvestri, C., Hoodless, P. A., Wrana, J. L., and Attisano, L. (1998) Cell 87, 1215–1224
Smad Regulates Smad7 Transcription

Mol. Cell 2, 109–120
28. Liu, B., Dou, C. L., Prabhu, L., and Lai, E. (1999) Mol. Cell. Biol. 19, 424–430
29. Zhang, Y., Feng, X. H., and Derynck, R. (1998) Nature 394, 909–913
30. Moustakas, A., and Kardassis, D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6733–6738
31. Hua, X., Liu, X., Ansari, D. O., and Lodish, H. F. (1998) Genes Dev. 12, 3884–3985
32. Kim, J., Johnson, K., Chen, H. J., Carroll, S., and Laughon, A. (1997) Nature 388, 304–308
33. Zawel, L., Dai, J. L., Buckhaults, P., Zhou, S., Kinzler, K. W., Vogelstein, B., and Kern, S. E. (1998) Mol. Cell 1, 611–617
34. Dennler, S., Itui, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (1998) EMBO J. 17, 3091–3100
35. Topper, J. N., DiChiara, M. R., Brown, J. D., Williams, A. J., Falb, D., Collins, T., and Gimbrone, M. A., Jr. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9506–9511
36. Feng, X. H., Zhang, Y., Wu, R. Y., and Derynck, R. (1998) Genes Dev. 12, 2153–2163
37. Janknecht, R., Wells, N. J., and Hunter, T. (1998) Genes Dev. 12, 2114–2119
38. Pouponnot, C., Jayaraman, L., and Massague, J. (1998) J. Biol. Chem. 273, 22865–22868
39. Takase, M., Imamura, T., Sampath, T. K., Takeda, K., Ichijo, H., Miyazono, K., and Kawahata, M. (1998) Biochem. Biophys. Res. Commun. 244, 26–29
40. Attisano, L., Wrana, J. L., Montalvo, E., and Massague, J. (1996) Mol. Cell. Biol. 16, 1066–1073
41. Andrews, N. C., and Faller, D. V. (1991) Nucleic Acids Res. 19, 4999
42. Smale, S. T. (1997) Biochim. Biophys. Acta 1351, 73–88
43. Zauberman, A., Oren, M., and Zipori, D. (1997) Oncogene 15, 1705–1711
44. Sekelsky, J. J., Newfeld, S. J., Rafferty, L. A., Chartoff, E. H., and Gelbart, W. M. (1995) Genetics 139, 1347–1358
45. Topper, J. N., Cai, J., Qiu, Y., Anderson, K. R., Xu, Y. Y., Deeds, J. D., Feeley, R., Gimbrone, C. J., Woolf, E. A., Tayber, O., Mays, G. G., Sampson, B. A., Schoen, F. J., Gimbrone, M. A., Jr., and Falb, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9314–9319
46. Ullea, L., Doody, J., and Massague, J. (1999) Nature 397, 710–713