Participation of Smad2, Smad3, and Smad4 in Transforming Growth Factor β (TGF-β)-induced Activation of Smad7

THE TGF-β RESPONSE ELEMENT OF THE PROMOTER REQUIRES FUNCTIONAL Smad BINDING ELEMENT AND E-BOX SEQUENCES FOR TRANSCRIPTIONAL REGULATION*

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Smad7 has recently been identified as a player that antagonizes transforming growth factor β (TGF-β) signals by acting downstream of TGF-β receptors. TGF-β rapidly induces expression of Smad7 mRNA in a variety of cell types, suggesting participation in a negative feedback loop to control TGF-β responses. We have previously described the genomic locus of rat Smad7 including the promoter region. Here we report polymerase chain reaction cloning of the corresponding promoter regions of human and murine Smad7 genes and functional characterization of the rat Smad7 promoter. Using transient transfection experiments of HepG2 cells, we identified the TGF-β response element within a strongly conserved region, containing a perfect Smad binding element (SBE; GTCTAGAC). Performing electrophoretic mobility shift assay and cotransfection experiments, we were able to delineate DNA-binding complexes and identified Smad3, Smad4, and Smad2. Mutation of the SBE completely abolished TGF-β inducibility of Smad7 in HepG2 cells, indicating that this sequence is necessary for TGF-β-induced transcription. Furthermore, a 3-base pair adjacent E-box is additionally essential for TGF-β-dependent promoter activation and an overlapping AP1 site is also involved. We conclude that regulation of Smad7 transcription by TGF-β is mediated via a specific constellation of recognition motifs localized around the SBE, which is conserved in human, rat, and murine genes.

Smads are intracellular signaling mediators for members of the transforming growth factor β (TGF-β) family, which signal through transmembrane serine/threonine kinases. Receptor-activated Smads (Smad2 and Smad3 for TGF-β-signal transduction) and the common mediator Smad4 transmit receptor induced signaling (1). After receptor-induced activation, heteromeric Smad complexes translocate into the nucleus, where they act as transcription factors. Smad6 and Smad7 are inhibitors of TGF-β signal transduction. Smad6 seems to preferentially inhibit bone morphogenic protein signaling, and Smad7 obviously is more specific for TGF-β signaling (2–5). mRNA expression of Smad6 and Smad7 is rapidly and transiently induced by TGF-β family members, suggesting the existence of a negative feedback loop, comprising the following steps. Ligation binding to the receptor triggers Smad signaling. Nuclear translocation of activated complexes initiates expression and activity of specific target genes, including inhibitory Smads that interfere with receptor interaction and phosphorylation of receptor-regulated Smads, thus shutting off signal transduction. In contrast to receptor-activated Smads, which are predominantly localized in the cytoplasm and upon activation translocate into the nucleus, Smad7 is mainly found within the nucleus in the absence of ligand and receptor activation induces its transport to the cell membrane (6). We have previously performed a detailed characterization of the rat Smad7 genomic organization including 1,300 bp of the promoter region (7). The gene is composed of 4 exons separated by 3 introns covering a DNA region of about 30 kilobases (kb) in total. Additionally, one potential transcription start site of the gene has been identified.

In the present study, we describe the isolation of corresponding human and mouse Smad7 promoter regions and identified the TGF-β response element of the gene. A fragment of 141 bp, which is strongly conserved during evolution, contains the TGF-β responsive DNA sequence consisting of a Smad binding element (SBE), an E-box in a 3-bp 3′-distance of the SBE and an AP1 site, which is overlapping with the E-box. Disruption of the SBE and/or the adjacent E-box completely abolished TGF-β responsiveness of the Smad7 promoter region in HepG2 cells. Furthermore, we investigated DNA-protein and protein-protein interactions at the TGF-β response element and identified Smad2, Smad3, and Smad4 participating in TGF-β response element binding.

EXPERIMENTAL PROCEDURES

Cells—Human hepatoma HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium, 4 mM l-glutamine, including 10% FCS. Additionally, all culture media were supplemented with penicillin (100 IU/ml)/streptomycin (100 µg/ml), and cells were maintained at 37 °C, 5% CO₂ in a humidified atmosphere. Cells were starved in medium containing 0.5% FCS overnight prior to TGF-β treatment.
PCR Amplification of Genomic DNA—Amplification of human and murine Smad7 promoter regions was performed using primers 5'-CCG-TATCCACGAGTTACATC-3' and 5'-TCCAGGCTTCTAGGAAACC-3' with Expand Long Template PCR System (Roche Molecular Biochemicals, Mannheim, Germany) and 40 ng of genomic DNA as template. The cycling conditions were as follows: 30 cycles, annealing at 60 °C for 15 s, elongation at 68 °C for 2 min, 35 cycles. Resulting PCR products were subcloned into pGEM-T easy vector (Promega Corp., Madison, WI) and sequenced.

**Plasmid Constructs and Reporter Assays**—The 1321-bp rat Smad7 promoter region (−1276 to +41) was PCR-amplified from a pBluescript II containing the rat Smad7 genomic DNA fragment using Expand Long Template PCR System (Roche). A PCR with primers 5'-tagctgtagtctggctcatc-3' and 5'-tggcctgctgcccttgct-3' containing MluI and XhoI restriction sites, respectively, resulted in one specific DNA fragment at the following cycling conditions: 95 °C, 2 min, 1 cycle; 95 °C for 20 s, annealing at 60 °C for 15 s, elongation at 68 °C for 2 min, 35 cycles. The product was asymmetrically cloned into MluI and XhoI sites of pGL3-Basic Vector resulting in p(−1280)-Smad7-prom-Luc. We used SacI and KpnI to construct two deletion mutants, p(−625 SacI)-Smad7-prom-Luc and p(−469 KpnI)-Smad7-prom-Luc. The SBE of the Luciferase construct p(−1280)-Smad7-prom-Luc was replaced with an EcoRI site by a PCR-based, site-directed mutagenesis using primers 5'-ACCGAAGAT-TCGCCACCTGACAGCGCCCAGC-3' and 5'-ACGTCGACTCAGCAAGAAATG-3'. The KpnI site was used to disrupt the adjacent E-box. Furthermore, one of the E-box containing the promoter sequences from −1280 to −335 (E-box and 3'-flanking promoter sequences deleted) was generated. A second series of reporter plasmids was constructed by ligating various double-stranded wild type (wt) and mutated oligonucleotides covering the Smad7-TGF-β response element into the MluI site of MLP-Luc (8). Therefore, the vector was restricted with MluI, dephosphorylated, and gel-purified. The different oligonucleotides (indicated in detail in Table 1) were synthesized 5’-phosphorylated and were annealed and ligated according to standard procedures. All recombinants were confirmed by sequence analysis. (CAGA)3MLP-Luc has been described elsewhere (8). For reporter gene assays, HepG2 cells were transiently transfected by the calcium phosphate coprecipitation method as described previously (9). Cell lysis and luciferase assays were carried out using the luciferase kit (Promega Corp.) as described by the manufacturer’s instructions. All transient expression experiments were done in triplicate. Luciferase activity values were normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector (pCRIIαZ, Invitrogen, Leek, Netherlands).

**Ethylmethane Sulfonate Shift Assays (EMSAs)**—Nuclear extracts were prepared from control and TGF-β1-treated HepG2. The medium of monolayers was changed from 10% to 0.5% FCS overnight, and subsequently the cells were treated with 5 ng/ml TGF-β. Cells were harvested 30 min after treatment and processed according to the protocol of Dennonier et al. (8) with minor modifications. Briefly, confluent cells from 4 dishes (100 mm²) were washed with phosphate-buffered saline (PBS) and then treated with either washing cells were suspended in 1 ml/dish of ice-cold buffer A (10 mM HEPES, pH 7.9, 20 mM NaF, 1 mM dithiothreitol, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride). The cells were allowed to swell on ice for 10 min and then lysed by 30 strokes of a Dounce all-glass homogenizer. Nuclei were pelleted by centrifugation and resuspended in 50 μl/dish of ice-cold buffer C (20 mM HEPES, pH 7.9, 20 mM NaF, 1 mM Na₂VO₄, 1 μM Na₃P₂O₁₀, 0.13 μM okadacid acid, 1 mM EDTA, 1 mM EGTA, 0.4 mM ammonium molybdate, 420 mM NaCl, 20% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each leupeptin, aprotinin, and pepstatin). The nucleus membrane was lysed by 15 strokes of a Dounce all-glass homogenizer. The resulting suspension was stirred for 30 min at 4 °C. The clear supernatant was aliquoted and frozen at −80 °C. Oligonucleotides were end-labeled with [α-32P]CTP using T7 DNA polymerase. Binding reactions containing 10 μg of nuclear extracts and 2 ng of labeled oligonucleotides were performed for 20 min at 4 °C in 18 μl of binding buffer (20 mM HEPES, pH 7.9, 30 mM KCl, 4 mM MgCl₂, 0.1 mM EDTA, 0.8 mM NaF, 20% glycerol, 4 mM spermidine, 3 μg of poly(dI-dC). Protein DNA complexes were resolved in 5% polyacrylamide gels containing 0.5% TPE. For supershift analysis, SMAD2-specific sera against Smad2, phosphorylated Smad2, Smad3, and Smad4, kindly provided by P. ten Dijke and D. Dunaeva, were added to the nuclear extracts in binding buffer and incubated for 10 min at 4 °C, before the labeled oligonucleotide. Flag-tagged expression constructs for Smad2, Smad3, and Smad4 were kindly provided by P. ten Dijke and are described, e.g., in Ref. 10. Transfection of HepG2 cells with Flag-tagged Smad expression vectors was performed with FUGENE (Roche) according to manufacturer's instructions in order to achieve higher transfection efficiencies. After transfection, cells were serum-starved overnight and subsequently treated with 5 ng/ml TGF-β for 30 min before nuclear extracts were prepared.

**Isolation of Total RNA and Northern Blot Analysis**—Total RNA was purified from HepG2 cells with RNA Easy mini kit according to the manufacturer's instructions (Qiagen). Samples of 15 μg of total RNA were resolved by electrophoresis on 1% agarose-formaldehyde gels and transferred to GeneScreen nylon membranes according to the instructions of the manufacturer (NEN Life Science Products). Smad7 and glyceraldehyde-3-phosphate dehydrogenase probes were isolated from plasmid E1/P-Prok (ref. 11) and pKSS6 (11), respectively, and labeled with [α-32P]CTP, using a Random Primer DNA Labeling system (Life Technologies, Inc., Eggenstein, Germany). Hybridization probes for Smad2, Smad3, and Smad4 were generated as purified restriction fragments from the above described Flag-tagged expression vectors. Hybridization and washing were performed using standard procedures.

**Immunoblot Analysis**—Proteins from HepG2 lysates were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to a 0.45 μm nitrocellulose membrane (Protran BA 85; Schleicher & Schuell, Dassel, Germany). Non-specific binding was blocked by 5% nonfat milk powder in PBS overnight at 4 °C followed by incubation with the primary antibody PS2 diluted 1:2,000 in 2.5% nonfat milk powder in PBS for 1 h at room temperature. Blots were washed twice in PBS, 0.05% Tween 20 (Bio-Rad, München, Germany) and subsequently three times in PBS for 10–15 min each. The secondary antibody anti-rabbit horseradish peroxidase (stock solution: 400 μg/ml; Santa Cruz Biotechnology Inc., Heidelberg, Germany) was incubated at a dilution of 1:50,000 for an additional 1 h at room temperature, followed by five washes as described above. Bound antibodies were detected by developing the membrane in Supersignal Ultra (Pierce) for 5 min and subsequent washing with a Lumif Imager (Roche). Specific antibodies against Smad2, Smad3, and Smad4 were kindly provided by P. ten Dijke and C. H. Heldin or purchased by Santa Cruz Biotechnology Inc. and Zymed Laboratories Inc. (San Francisco, CA), respectively. Further antibodies were anti-Flag (Sigma), anti-USF (kindly provided by B. Lüscher), and anti-NFκB p65, anti-Stat3, anti-TjRI, and anti-TjRII (all from Santa Cruz Biotechnology Inc.).

**RESULTS**

**TGF-β Responsiveness in HepG2 Cells**—Members of the TGF-β family rapidly induce the expression of Smad7 in different cell types (5, 12–13). To confirm TGF-β sensitivity of HepG2 cells, we investigated different steps of TGF-β signal transduction. To determine whether endogenously expressed Smad2 becomes phosphorylated upon TGF-β stimulation, HepG2 cell extracts were subjected to immunoblot analysis using an antisera, termed PS2, raised against the phosphorylated carboxyl-terminal SSMS motif of Smad2, which was kindly provided by P. ten Dijke. Upon TGF-β stimulation, the amount of phosphorylated Smad2 was strongly increased, whereas only a weak signal was detected in absence of TGF-β (Fig. 1A). Specificity of PS2 for phosphorylated Smad2 was shown by comparing Smad2 and Smad3-transfected HepG2 cells (Fig. 1D) and was further confirmed by peptide competition (data not shown). These results strengthen the finding that PS2 antisera is specific for phosphorylated Smad2 and does not cross-react with the phosphorylated SSVS motif of Smad3. Furthermore, we performed EMSA with HepG2 nuclear extracts and an oligonucleotide, termed CAGA-box, which was described previously by Dennonier et al. (8). We were able to identify a DNA-binding complex present in serum-starved HepG2 cells, indicating basal, TGF-β-independent binding activity. However, complex formation was significantly enhanced by TGF-β treatment (Fig. 1C). Finally, we purified total RNA from TGF-β-treated HepG2 cells and untreated controls and performed an expression analysis with a human Smad7-specific probe applying Northern blot (Fig. 1D) and real time quantitative reverse transcription-PCR in parallel (data not shown). Similar results were obtained with both techniques. Control cells displayed basal expression of Smad7 mRNA, which was up-regulated in cells stimulated with 5 ng/ml TGF-β.
for 1 h. After 4 h, mRNA expression declined to basal levels, indicating a transient activation of the Smad7 gene by TGF-β in HepG2.

Isolation of the Promoter Region of Human and Murine Smad7 Genes—By similarity search in the GenBank data base, we identified a 566-bp MseI fragment sequence from Homo sapiens chromosome 18 (accession no. AJ236598) showing 85.5% identity to the region 21,285 to 2719 of the rat Smad7 promoter. This human sequence was originally discovered as a CpG island (CGI), which was isolated by a technique for bulk purification of CGIs from whole genomes (14). Knowing that the human Smad7 gene has previously been localized on chromosome 18 (15), we expected the CGI to be part of the human Smad7 promoter and generated a forward primer, which was 100% homologous comparing rat Smad7 promoter sequences and human chromosome 18 CGI (position 2901 to 2921). A corresponding reverse primer was selected upon investigating murine (accession no. AJ000550) and rat (accession no. AJ236598) Smad7 promoter sequences.

Fig. 1. TGF-β treatment of HepG2 cells results in Smad2 phosphorylation, increased CAGA-box binding activity, and upregulation of Smad7 mRNA. A, analysis of TGF-β-induced Smad2 phosphorylation in HepG2 cells. Cells were grown to confluence, followed by serum starvation for 24 h. Subsequently, cells were directly used for lysate preparation or stimulated with 5 ng/ml TGF-β for 30 min before. Cell lysates were then subjected to SDS-gel electrophoresis and immunoblot, and phosphorylated Smad2 (P-Smad2) was detected with P2 antiserum. Controls with blocking peptide to which the P2 anti-serum was originally raised were performed, resulting in blank blots. B, specificity of the P2 antibody for phospho-Smad2 was determined by testing lysates from TGF-β-treated, Flag-tagged Smad2- or Smad3-transfected HepG2 cells, and untransfected controls (C). The blot was reprobed with α-Flag to confirm exogenously expressed Smads 2 and 3. C, an EMSA was performed using a 32P-labeled double-stranded probe containing an artificial CAGA-sequence (5′-TCGAGAGCCAGACAAGGAGCCAGACAAGGAGCCAGACAC-3′; Ref. 8), as indicated, and nuclear extract from HepG2 cells induced for 30 min by 5 ng/ml TGF-β or not induced. Bands corresponding to TGF-β-induced and constitutive complexes are indicated. D, HepG2 cells were exposed to TGF-β (5 ng/ml) for various times as indicated. 15 μg of total RNA were separated and analyzed for levels of Smad7 mRNA by Northern blotting. As a loading control for the Northern experiment, the membrane was hybridized with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.
AF156727) 5′-untranslated regions, because the human 5′-untranslated region was only partially known. A potential sequence with 100% conservation was found between position +561 and +580 (rat gene). We used these primers in a PCR reaction with human and murine genomic DNA as template and were successful in amplifying both promoter regions (accession nos. AF156731 and AF188834). Sequence analysis of human and murine Smad7 promoters identified a 141-bp region of almost 100% conservation between rat, human, and mouse (position −417 to −276). This region was enclosed within a minimal TGF-β-responsive Smad7 promoter construct and contained a perfect SBE, GTCTAGAC between position −346 and −338, a palindromic sequence with two copies of GTCT and its reverse complement AGAC in the opposite strand. This motif has been described earlier as the optimal distance, an E-box, CACGTG, is connected with the SBE and the remaining 3′-untranslated regions, because the human 5′-untranslated region was only partially known. A potential sequence with 100% conservation was found between position +561 and +580 (rat gene). We used these primers in a PCR reaction with human and murine genomic DNA as template and were successful in amplifying both promoter regions (accession nos. AF156731 and AF188834). Sequence analysis of human and murine Smad7 promoters identified a 141-bp region of almost 100% conservation between rat, human, and mouse (position −417 to −276). This region was enclosed within a minimal TGF-β-responsive Smad7 promoter construct and contained a perfect SBE, GTCTAGAC between position −346 and −338, a palindromic sequence with two copies of GTCT and its reverse complement AGAC in the opposite strand. This motif has been described earlier as the optimal sequence according to the method described above, replacing the C/EBP-binding site (−394 to −377), TGTTGTTCGGACACGAC. The only species-specific differences in this 141-bp homologous region are the length of a poly(A) stretch, consisting of 12 (rat), 15 (human), and 14 (mouse) T, respectively, and a G→A exchange at position −270 within murine DNA.

**Functional Characterization of TGF-β-responsive Regions in the Rat Smad7 Promoter**—To determine DNA elements, which confer TGF-β responsiveness, we transfected HepG2 cells with a pGL3-Basic luciferase construct, p(CAGA)9-MLP-Luc, containing 1,321 bp of the rat Smad7-gene promoter and 5′-untranslated region (−1280 to +41; Fig. 3A). Two deletion constructs thereof, p(CAGA)9-MLP-Luc and p(469-Smad7prom-Luc) were generated based on restriction enzyme recognition sites at positions −825 (SacI) and −469 (KpnI). As a control system, we used a vector containing nine copies of the CAGA sequence cloned upstream of a minimal adenovirus MLP, (CAGA)9 MLP-Luc, previously shown to be highly TGF-β-responsive (8). Transfection efficiency was determined by cotransfection of a β-galactosidase construct. Minimal luciferase activity was detected in HepG2 cells transfected with (CAGA)9 MLP-Luc without TGF-β treatment and independently of TGF-β treatment in cells transfected with empty vectors (pGL3-Basic or MLP-Luc). Stimulation of (CAGA)9 MLP-Luc-transfected HepG2 with 5 ng/ml TGF-β induced luciferase activity about 80-fold. In HepG2 cells transfected with Smad7 promoter constructs, luciferase activity was more than 100-fold increased compared with the (CAGA)9 MLP-Luc-transfected cells, independent of TGF-β stimulation, indicating high basal Smad7 promoter activity. TGF-β treatment of these cells further increased luciferase activity to a level more than 230-fold above basal measurement. Both TGF-β stimulation and basal promoter activity were still present in the two deletion constructs, indicating that TGF-β responsiveness and basal promoter activity are localized downstream of position −470 of the Smad7 promoter.

**Mutation of the SBE in the Rat Smad7 Promoter Completely Abolishes TGF-β Responsiveness**—To confirm that the SBE is responsible for TGF-β-dependent transcriptional response of Smad7, we replaced TCTAGA within the SBE with an EcoRI recognition site by PCR-based site-directed mutagenesis of the complete rat Smad7 promoter/luciferase reporter construct. With this construct, termed p(−1280)-Smad7prom-SBE-Luc, we transfected HepG2 cells and performed luciferase reporter assays upon TGF-β treatment. TGF-β responsiveness was completely abrogated, whereas basal promoter activity remained unaffected (Fig. 3A). This indicates that the SBE is not involved in basal Smad7 promoter activity in HepG2 cells; however, its presence is essential for TGF-β-dependent stimulation of the gene.

**A Functional SBE-adjacent E-box Is Essential for TGF-β Inducibility**—Corresponding to the plasminogen activator inhibitor-1 (PAI-1) promoter TGF-β response element (17), an E-box, located 3 bp downstream of the SBE was identified within the Smad7 promoter. We have mutated the E-box sequence according to the method described above, replacing CAGTGTG by GAATTC. Transfection experiments of HepG2 cells displayed that, despite an intact SBE, TGF-β inducibility was completely suppressed by this E-box mutation. Furthermore, basal transcriptional activity was slightly decreased (Fig. 3B). Finally, we generated a deletion construct containing the 5′-part of the promoter including the SBE, lacking the E-box and the remaining 3′-promoter sequences. With this construct, both TGF-β-dependent and basal transcriptional activity were shut off (Fig. 3A).
Functional characterization of rat Smad7 promoter revealed basal promoter activity in HepG2 cells, and SBE as well as E-box-dependent TGF-β-inducible transcriptional activity. HepG2 cells were transiently transfected with 2 μg/5 ml medium of reporter constructs and control vectors. Cells were starved for 8 h in medium containing 0.5% FCS before overnight stimulation with 5 ng/ml TGF-β as indicated. Thereafter, cells were harvested and luciferase activities were quantified. Luciferase activity values were normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector pCR3lacZ (1.5 μg/5 ml medium) and are indicated as arbitrary units. One representative of three independent experiments (mean values ± standard deviation) is shown. A, luciferase reporter constructs containing 1280 bp of rat Smad7 promoter, p(-1280)-Smad7prom-Luc, three deletion mutants thereof (p(-625)-Smad7prom-Luc, p(-469)-Smad7prom-Luc, and p(-1280 to -335))-Smad7prom-E-boxdel), and two constructs (p(-1280)-Smad7prom-SBE*-Luc with a disrupted SBE and p(-1280)-Smad7prom-E-box*-Luc with a disrupted E-box). (CAGA)9MLP-Luc (8) as a TGF-β-inducible positive control and the pGL3-Basic vector as a negative control were included. B, a luciferase reporter construct containing a wild type 33-bp Smad7-TGF-β response element (S7-wt-MLP-Luc) and its variations with mutated SBE (S7-SBE*-MLP-Luc), mutated E-box (S7-Ebox*-MLP-Luc), inverted TGF-β response element (tw-7S-MLP-Luc), and a 5 times repeated TGF-β response element ((S7-wt)5-MLP-Luc) were used to analyze TGF-β responsivity. For comparison and as controls, p(-1280)-Smad7prom-Luc, (CAGA)9MLP-Luc, and MLP-Luc (8) were included.
TGF-β Responsiveness Is Independent of Smad7-TGF-β Response Element Orientation and Can Be Additively Increased by Repetition—Luciferase reporter genes driven by isolated wild type and different mutant TGF-β response elements as indicated in Table I were generated, and the resulting constructs were transfected into HepG2 cells for luciferase assays. With one exception, the obtained results mirrored the data from transfection experiments with reporter constructs containing surrounding rat Smad7 promoter sequences.(Fig. 3B). TGF-β-dependent stimulation of both pGL3 basic containing one copy of the wild type TGF-β response element (pS7-wt-MLP-Luc) and the complete rat Smad7 promoter fragment, p(−1280)-Smad7prom-Luc, is about 2.7-fold above basal luciferase activity in HepG2 cells. This is independent from orientation of the response element as shown with the construct ptw-7S-MLP-Luc, which contains the wild type sequence recombined in opposite direction. Furthermore, a construct containing five copies of S7-wt in original direction and two copies in the reversed orientation (pS7-wt5-MLP-Luc) displayed a 9-fold increased promoter activity after treatment with TGF-β, indicating an additive effect of multiple response elements. In contrast, basal luciferase activity remained unaffected. A disrupted SBE within pS7-wt-MLP-Luc, designated pS7-SBE*-MLP-Luc, did not respond on TGF-β treatment but still displayed basal transcriptional activity. A corresponding plasmid containing an intact SBE but a mutated E-box, described as pS7-E-box*-MLP-Luc, also had lost TGF-β responsiveness. However, in contrast to the disrupted E-box within p(−1280)-Smad7prom-E-box*-Luc, which still showed high basal promoter activity, no basal transcriptional activity was detectable using pS7-E-box*-MLP-Luc.

Characterization of Smad7-TGF-β Response Element Binding Activity by EMSA Competition and Using Specifically Disrupted Recognition Motifs—We performed EMSA using HepG2 nuclear extracts in an attempt to characterize DNA binding activity of the Smad7-TGF-β response element. We identified a complex, which was already present in untreated control cells (C1) and a TGF-β-inducible complex (C2). The TGF-β-inducible complex is rapidly formed within 15 min after addition of TGF-β into the medium, suggesting preexisting factors, which were posttranslationally activated. We produced a set of double-stranded oligonucleotides, which contain altered recognition motifs (see Table II), in order to characterize DNA binding specificity of complexes formed across the Smad7-specific TGF-β response element. These oligonucleotides were labeled and used as EMSA probes or used as cold probes for binding competition with a double-stranded labeled oligonucleotide probe corresponding to the wt Smad7-TGF-β response element. C2 was identified as TGF-β-inducible complex (Fig. 4, A (lane 2) and C (lane 3)). C1, which is detected in HepG2 cells with or without TGF-β treatment, is strongly dependent on the presence of an intact E-box, which was shown by competition experiments (Fig. 4, A (lane 4) and B (lane 3)) and by measuring binding activity with probes containing a disrupted E-box motif (Fig. 4C, lane 7). Both the SBE and the E-box overlapping AP1 site are involved in formation of a complex that can be detected in the gel as one common signal designated C2. EMSA competition experiments and using labeled oligonucleotide probes containing mutated binding sites showed, that the majority of C2 depends on a functional SBE (Fig. 4, A (lanes 6 and 7), B (lane 5), and C (lane 4)). A smaller part of the complex is dependent on the presence of the AP1 site (Fig. 4, A (lane 9), B (lane 6), and C (lanes 6 and 10)). Further experimentation showed that the immunoprecipitatively present complex C1 is reduced after formation of the TGF-β inducible complex C2 and can be increased, if C2 complex formation is diminished or avoided in competition experiments (data not shown). Treatment of HepG2 nuclear extracts with an antiseraum against upstream stimulatory factor (USF) resulted in reduced complexes C1 and C2 and a new supershifted complex with a low mobility (Fig. 5A, lane 14). These observations combined with both, SBE and E-box mutational luciferase assays indicate a cross-talk of the different motifs within the TGF-β response element (SBE, E-box, and AP1 site), which is essential for Smad7-specific TGF-β responsiveness.

Smad3, Smad4, and Smad2 Participate in Complex Formation at the TGF-β Response Element—To examine the composition of the Smad7-TGF-β response element-binding complexes, nuclear extracts were incubated with specific antisera to Smad3, Smad4, Smad2, and phosphorylated Smad2 (PS2) as indicated. We were able to detect supershifted bands (Fig. 5, A (lanes 4–6) and B (lane 3)) or a diminished complex C2 (Fig. 5A, lane 3) with various Smad2 antisera. A strongly reduced TGF-β-induced complex formation (C2), in one case accompanied by a supershifted band, was obtained with anti-Smad4 antiserox (Fig. 5A, lane 13). Comparable results were obtained when a Smad3-specific antisera was used (Fig. 5A, lane 12). Several control antisera were used to confirm specificity of the results obtained with Smad3 antisera, and no effects on complex formation were identified (Fig. 5, A (lanes 7–10) and C (lanes 2 and 7)). Where available, preincubation of antisera with corresponding peptides was used to further improve specificity of the effects (data not shown). Moreover, we overexpressed Flag-tagged Smad2, Smad3, and Smad4 in HepG2 cells and verified participation of the different Smad proteins by supershifting the complexes with an anti-Flag antibody. In addition to a supershifted complex, a significant reduction of C2 complex formation is obtained in the case of Flag-Smad3- and Flag-Smad4-transfected cells (Fig. 5B, lanes 3–5).

Involvement of the different Smads was further confirmed by cotransfection experiments using Flag-tagged expression vectors for Smad proteins in combination with p(−1280)-Smad7prom-Luc. Subsequent to transfection, HepG2 cells were serum-starved for 8 h and 5 ng/ml TGF-β was added. Lysates were prepared the next day, and luciferase activity was measured and related to β-galactosidase activity (Fig. 5C). There is an increase in basal as well as TGF-β-dependent promoter activity after transfection of the different Smad constructs. The highest rating was detected by cotransfecting both Smad2 and Smad4. Only basal activity was increased after transfection of Smad3 and Smad4. In total, the effects of transfected Smad proteins were not very strong, suggesting that the physiologically expressed Smad proteins are constitutively present in amounts sufficient to mediate TGF-β signaling and are not
rate-limiting for Smad7 promoter activation. We conclude from these data that the Smad7 TGF-β response element binding activity, which is induced by TGF-β, contains all TGF-β signaling Smads, including Smad2, which is in contrast to several earlier investigations, describing only Smad3 and Smad4 participating in SBE complex formation (8, 18–23).

**DISCUSSION**

TGF-β is the prototype of a family of signaling molecules that exhibit pleiotropic effects on cell proliferation, differentiation, as well as on pattern formation during early vertebrate development (24). The recent discovery of Smad proteins as downstream effectors of signaling from activated receptors activated by members of the TGF-β superfamily has opened new ways for investigating regulation of target gene expression. Inhibitory Smad6 and Smad7 are able to block TGF-β superfamily signaling in a negative feedback loop upon transcriptional activation. In the present study, we describe the isolation of Smad7 promoter regions from rat, human, and murine genes. A region of 141 bp (position −417 to −275) is completely conserved in all three organisms, with the exceptions that a poly(T) stretch varies between 12 and 15 bp and a G-A exchange occurs at position −270 within murine DNA. It has previously been shown by several investigators that the expression of Smad7 can be rapidly and transiently induced by TGF-β with kinetics similar to early response gene activation after treatment of quiescent cells with, e.g., FCS (5, 12–13). Transient transfection experiments with promoter reporter constructs localized the TGF-β-responsive region downstream to −470 of the Smad7 promoter. A potential TGF-β-responsive element consisting of a perfect 8-bp SBE, GTCTAGAC, at position −346 to −338, previously identified by a PCR-based selection as optimal DNA sequence for Smad3 and Smad4 binding (16), was identified within the 141-bp region. Structurally, this sequence represents a palindromic motif containing two copies of GTCT and its reverse complement AGAC in the opposite strand. It has been shown that tandem repeats of this sequence element confer TGF-β-inducible transcriptional activation and that CAGA-like sequences in, e.g., the PAI-1 and JunB promoters are able to directly bind Smad3 and Smad4 (8, 20).

**TABLE II**

Oligonucleotides used in EMSA competition experiments

| Oligonucleotides | wt: | SBE*: | rS7-M1 | rS7-M2 | E-box*: | rS7-M3 | rS7-M4: | rS7-M5: | rS7-M6: | rS7-M7: |
|------------------|-----|-------|--------|--------|---------|--------|---------|--------|---------|---------|
|                  | GCG ACA GGG TGT CTA GAC CAC GTG AGC AGG CC | GCG TGT CCC ACA GAT CTC CGG GTG CAC TGC GCC GG | GCG ACA GGG TCA TTA GAC GCC CAC GTG AGC AGG CC | GCG TGT CCC AGT CAT CTC CGG GTG CAC TGC GCC GG | GCG ACA GGG TGT CTA GAC GTG AGC AGG CC | GCG TGT CCC ACA GAT CTC CGG GTG CAC TGC GCC GG | GCG ACA GGG TCA TTA GAC GCC CAC GTG AGC AGG CC | GCG TGT CCC AGT CAT CTC CGG GTG CAC TGC GCC GG | GCG ACA GGG TCA TTA GAC GCC CAC GTG AGC AGG CC | GCG TGT CCC AGT CAT CTC CGG GTG CAC TGC GCC GG |

The promoter of Smad7 displayed already significant basal activity in HepG2 cells, which was even higher than the activity of TGF-β-induced (CAGA)<sub>8</sub>-MLP-Luc, a very sensitive reporter of TGF-β signal transduction. This basal activity is also based on regulatory elements located downstream from the −470 position. Using a site directed mutational assay, we have exchanged 6 of 8 bp from the SBE with an EcoRI restriction site within the Smad7 promoter/reporter construct, thereby completely abolishing TGF-β inducibility without interfering with basal promoter activity of the gene. In gel shift experiments using nuclear extracts of serum-starved and TGF-β-treated cells, two complexes were found. C1, displaying slower mobility, was present in serum-starved cells representing TGF-β-independent DNA binding activities. TGF-β treatment of the cells strongly induced a faster migrating complex C2, which could be supershifted or avoided, respectively, by antiserum raised against Smad2, Smad3, and Smad4, indicating participation of all three Smad proteins in TGF-β inducibility of Smad7 in HepG2 cells. Participation of Smad2 in DNA binding of an SBE is in contrast to several previous investigations (8, 18–23). Based on structural differences, Smad2, in contrast to Smad3 and Smad4, is not able to interact with DNA directly (25). However, it has been shown that Smad2 is able to act as a transcription factor after binding to nuclear proteins like, for example, Fast2 (26, 27). Therefore, protein–protein interactions of SBE binding Smads and factors binding to adjacent DNA sequence elements may be necessary to recruit Smad2 stably in the complex. In a recent paper, Hua and colleagues (28) described a synergistic cooperation of an E-box-binding protein and Smad3/Smad4 in TGF-β-induced transcription of the PAI-1 gene. Interestingly, mutation of the E-box sequence abolished TGF-β inducibility without preventing DNA binding of Smad3/Smad4 to the neighboring SBE. Furthermore, any insertion or deletion of nucleotides in the spacer region between the SBE and the E-box also completely abrogated TGF-β-induced transcription. Hua et al. suggested a model in which phosphorylated Smad3 and likely Smad4 bind to TFE3, a transcription factor, which is specific for E-boxes. This complex binds to the DNA and subsequently recruits coactivators such as p300 and CREB-binding protein, leading to enhanced transcription of the PAI-1 gene. Exactly the same constellation is found in the case of the investigated Smad7 promoters. In a downstream distance of 3 bp to the SBE, the promoters contain an E-box (CACCCTG) and an overlapping AP1 site (TGACCA). AP1 sites have also been described as target elements involved in TGF-β signaling before (10, 21, 29). We show the necessity of an intact E-box within the Smad7-specific TGF-β response element for TGF-β-dependent inducibility. Mutation of this motif completely abrogated the stimulatory effect of TGF-β. Within the Smad7 promoter, the situation is even more complex than in the PAI-1 gene promoter. In addition to the adjacent E-box, an E-box overlapping AP1 site was identified, which also participates in TGF-β-dependent complex formation.
and therefore may be involved in determining specificity of TGF-β signal transduction to the Smad7 target. Using EMSAs, a constitutive binding activity C1 was detected in serum-starved HepG2 cells, which is independent of TGF-β treatment and which contains (an) E-box-binding protein(s). Furthermore, this binding activity may be involved in, but seems not to be necessary for, basal promoter activity of Smad7 in HepG2 cells. If this motif was mutated in the wild type rat Smad7 promoter, basal activity could not be diminished. However, basal promoter activity was down-regulated after disruption of this E-box within a Smad7-TGF-β response element containing oligonucleotide, which lacks flanking promoter sequences. We conclude from these findings that several SP1 sites, which are located further downstream of the E-box within the Smad7
promoters (−274 to −190), additionally are able to confer basal transcriptional activity; therefore, disruption of the E-box in presence of functional SP1 sites has only a minor effect. Whether Smad proteins are involved in basal promoter activity is presently not clear. Based on our EMSA results with a CAGA-box lacking E-box sequences and nuclear extracts of
HepG2, significant Smad binding activity is already present in serum-starved cells (Fig. 1). Moreover, transfections with Smad-expressing plasmids increase basal promoter activity, indicating basal pathway restricted Smad activation. Further confirmation of a “cross-talk” of the SBE, E-box, and AP1 motifs within the Smad7 promoter TGF-β response element was shown by supershift experiments with an antisemum against an E-box-binding protein (USF), which resulted in a strong supershifted band and reduced C1 and C2 complexes. We have not yet investigated the role of the overlapping AP1 site in more detail, and we do not know if TFE3, as described for the PAI 1 TGF-β response element, is part of the E-box-binding complex.

In summary, our data functionally identify the immediate early TGF-β response element of the rat Smad7 gene promoter. This motif and flanking sequences are strongly conserved in human and murine genes. We show that Smad3, Smad4, and Smad2 are involved in TGF-β-induced Smad7 promoter binding activity. An SBE and an adjacent E-box, located 3 bp downstream of the SBE, are essential for TGF-β responsiveness of the promoter. Reciprocal interactions of DNA-binding proteins, which recognize neighboring motifs, determine Smad7 specificity of TGF-β signal transduction.

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Addendum—Since submission of this article, two similar reports have been published (Nagarajan, R. P., Zhang, J., Wei, L., and Chen, Y. (1997) Nature 389, 622–626; Nakao, A., Afraekhte, M., Moren, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, N., Heldin, N. E., Heldin, C. H., and Tendjike, P. (1997) Nature 389, 631–635).

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