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Regulation of the human p21/WAF1/Cip1 promoter in hepatic cells by functional interactions between Sp1 and Smad family members

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ABSTRACT **The cell cycle inhibitor p21/WAF1/Cip1 is expressed in many cell types and is regulated by p53-dependent and p53-independent mechanisms. p21 is an important regulator of hepatocyte cell cycle, differentiation, and liver development, but little is known about the regulation of its synthesis in hepatocytes. We report herein that the p21 gene is constitutively expressed in human hepatoma HepG2 cells. Deletion analysis of the p21 promoter showed that it contains a distal (positions −2,300/−210) and a proximal (positions −124 to −61) region that act synergistically to achieve high levels of constitutive expression. The proximal region that consists of multiple Sp1 binding sites is essential for constitutive p21 promoter activity in hepatocytes. This region also mediates the transcriptional activation of the p21 promoter by members of the Smad family of proteins, which play important role in the transduction of extracellular signals such as transforming growth factor β, activin, etc. Constitutive expression of p21 was severely reduced by a C-terminally truncated form of Smad4 that was shown previously to block signaling through Smads. Smad3/4 and to a much lesser extent Smad2/4 caused high levels of transcriptional activation of the p21 promoter. Transactivation was compromised by N- or C-terminally truncated forms of Smad3. By using Gal4-Sp1 fusion proteins, we show that Smad proteins can activate gene transcription via functional interactions with the ubiquitous factor Sp1. These data demonstrate that Smad proteins and Sp1 participate in the constitutive or inducible expression of the p21 gene in hepatic cells.**

p21/WAF1/Cip1 is a cyclin-dependent kinase (CDK) inhibitor that directly interacts with cyclin–CDK complexes and thus arrests cell proliferation (1, 2). In addition, p21 forms complexes with proliferating-cell nuclear antigen, a subunit of DNA polymerase δ, and thus prevents processive DNA synthesis *in vitro* (1).

The gene encoding p21 is regulated by at least three classes of signals that result in arrest of cell growth. (*i*) The tumor suppressor protein p53, which is activated by DNA damage caused by irradiation and toxic agents (1, 3), its relative p73 (4, 5), or the tumor suppressor protein BRCA1 (6). (*ii*) Extracellular growth factors acting in a p53-independent mechanism such as tumor necrosis factor α, transforming growth factor β (TGF-β), phorbol 12-myristate 13-acetate, retinoic acid, and others (7–9). (*iii*) Factors that induce cellular differentiation of many cell types such as myoblasts, keratinocytes, intestinal epithelial cells, and monocytes (9–12).

p21 gene expression is undetectable in a number of primary cultures or immortalized cell lines such as glioblastoma GM, keratinocyte HaCaT, or monocyte U937 but can be induced to considerable levels after p53 activation (3) or treatment of cells with various agents such as TGF-β, phorbol 12-myristate 13-acetate, and okadaic acid (3, 7, 8). On the other hand, p21 is expressed at high levels in almost every human tissue examined and independently of the cell cycle stage (2). The p21 gene promoter contains at least two binding sites for the transcription factor p53 (3, 13) and specific DNA motifs responsible for the response to extracellular growth factors and hormones (8, 9, 14–17). The majority of these studies has provided convincing evidence on the importance of several Sp1-like motifs clustered between positions −150 and +1 with respect to the transcription initiation site (8, 10, 14, 17). In addition, specific binding of Sp1 protein to oligonucleotides corresponding to the Sp1-like motifs of the p21 promoter has been demonstrated by gel electrophoresis mobility shift assays (8, 14). However, binding of Sp1 to these oligonucleotides was demonstrated to be constitutive and not inducible by the factors under study. In one study, the DNA–protein complexes induced by TGF-β were shown to include in addition to Sp1 its relative protein Sp3 (10). Studies concentrating on the effects of retinoic acid, vitamin D, platelet-derived growth factor, epidermal growth factor, or interferons on p21 promoter activation have uncovered the presence of additional regulatory motifs scattered within the distal (positions −2,300/−150 bp) p21 promoter region (9, 12, 15–17).

Transgenic mice overexpressing p21 specifically in the liver (18) have provided further insights on the importance of p21 in the regulation of hepatic cell proliferation. However, no detailed study of transcriptional regulation of the p21 gene has yet been reported in hepatocytes.

In the present study, we have focused on the transcriptional regulation of the p21 gene in human hepatoma HepG2 cells. These tumor-derived cells of hepatic origin exhibit most of the characteristics of the differentiated hepatocyte (19) and respond to a variety of extracellular growth factors such as TGF-β, phorbol 12-myristate 13-acetate, tumor necrosis factor α, steroids, etc. We have also tested the ability of TGF-β and its signal transducers, Smad proteins, to regulate the p21 promoter activity in these cells. Smad family members are intracellular signaling components of the TGF-β superfamily of growth factors (20). These proteins, when phosphorylated by the activated receptors (21–23), propagate the signal through homo- and heterooligomeric interactions (23, 24) to the nucleus where they activate the transcription of target genes (25–27). Smad4/DPC4 plays a central role because it is the shared heterooligomerization partner of the other Smads (25, 28, 29), participates in a TGF-β/activin-induced transcrip-

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Abbreviations: CAT, chloramphenicol acetyltransferase; TGF-β, transforming growth factor β.

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tion complex (26, 30), and binds to specific DNA sequences (31). Finally, we propose a model in which Smad proteins regulate p21 promoter activity via functional interactions with the transcription factor Sp1.

MATERIALS AND METHODS

Materials. Reagents were purchased from the following vendors: Restriction and modifying enzymes and DNA polymerases, from Minotech, New England Biolabs, or GIBCO/BRL; the Sequenase version 2 kit, from Amersham/United States Biochemicals; acetyl-CoA and dNTPs, from Pharmacia; cell culture reagents, from GIBCO/BRL; *o*-nitrophenyl galactoside, from Sigma; TGF-β1, from R & D Systems; rabbit polyclonal anti-TGF-β1 neutralizing antibody, from Celtrix; sheep total IgG, from Jackson ImmunoResearch; fluorescein isothiocyanate-conjugated goat anti-mouse IgG, from Chemicon; mouse monoclonal anti-FLAG M2 antibody, from IBI/Kodak; mouse monoclonal anti-myc (9E10) antibody, a gift from L. LeGallic (Univ. Crete); the ECL Western blotting kit including goat anti-mouse, horseradish peroxidase-conjugated antibody, from Amersham; the luciferase assay system, from Promega; all oligonucleotides, from the Microchemical facility of the Institute of Molecular Biology and Biotechnology; all other chemicals, from commercial sources at the purest grade available.

Cell Culture and Treatments. Human hepatoma HepG2 cells were grown in DMEM supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin. Treatment with TGF-β1 or a neutralizing anti-TGF-β antibody always followed a 24-hr starvation period and lasted for 16–24 hr. The TGF-β1 concentrations tested ranged from 10 to 400 pM, and most experiments were performed with 100 or 200 pM.

Plasmid Constructions. Plasmid pWWP-luc containing the promoter of human p21/WAF1 between positions −2,300 and +8 was a gift from B. Vogelstein (Johns Hopkins Univ., Baltimore, MD) (3). The *Hin*dIII promoter fragment was subcloned to pUC-SH-CAT vector (32) to produce −2,300/+8 p21-CAT. The −2,100/+8 p21-CAT was made from from -2,300/+8 p21-CAT that was digested by *Xba*I and *Sac*I, filled-in with the Klenow fragment of DNA polymerase I, and religated. The −1,600/+8 p21-CAT was made by exonuclease III/mung bean nuclease treatment of the −2,300/+8 p21-CAT and was verified by dideoxynucleotide sequencing. The −210/+8 p21-CAT construct was made by subcloning the *Pst*I–*Hin*dIII fragment from positions −210 to +8 into pUC-SH-CAT. The −143/+8 p21-CAT was made by PCR amplification with oligonucleotides corresponding to positions −150 to −130 of the p21 promoter (5′-GGATCTAGAGCAGC-CAGGAGCCTGGG-3′) and a modified universal primer corresponding to sequences adjacent to the polycloning region of pBluescript. The −63/+8 p21-CAT was made by *Sma*I and *Xba*I digestion of −210/+8 p21-CAT, filled-in with the Klenow fragment of DNA polymerase I, and religated. The internal promoter deletion mutant −2,300/+8 Δ(−124/−61) was a gift from X.-F. Wang (Duke Univ.) (14).

Expression vectors for human Smad2 and Smad3 were gifts from R. Derynck (Univ. of California, San Francisco) (21). Smad3N was constructed by subcloning the *Eco*RI–*Pst*I N-terminal Smad3 fragment to the expression vector pcDNA3xmyc (gift from N. Grammatikakis, Tufts Univ.) in-frame with the C-terminal myc epitope tag. Smad3C was constructed by subcloning the *Pst*I–*Sal*I C-terminal Smad3 fragment to the expression vector pSG5-FLAG (gift from E. Hatzivassiliou, Harvard Univ.) in-frame with the N-terminal FLAG epitope tag. The following expression constructs were generously provided to us: p3TP-lux, pCMV5-DPC4, and pCMV5-DPC4(1–514) by J. Massagué (Sloan-Ketering Institute, New York), pG_5B-CAT by G. Mavrothalassitis (Univ. of Crete), pGal4, pGal4-Sp1, and pGal4-Sp1(A+B) by S. Smale (Univ. of California, Los Angeles), and pCEP-WAF1 by E. Gonos (National Institute for Research, Athens).

Transient Transfections and Reporter Assays. HepG2 cells (5×10^5 cells per well) were transfected by the calcium phosphate coprecipitation protocol (33). Each precipitate contained the reporter plasmid (3 μg), pCMV-β-gal or pRSV-β-gal for normalization of transfection efficiency (1 μg), and either the empty vector pcDNA1-neo or expression vectors for Smad proteins (2 μg). For immunofluorescence analysis, 5×10^5 cells were transfected in 60-mm plates with 5 μg of Smad expression vector and 5 μg of empty vector. β-Galactosidase, chloramphenicol acetyltransferase (CAT), and luciferase assays were performed as described (34).

Indirect Immunofluorescence of Epitope-Tagged Smad Proteins. Transfected cells (5×10^4 cells per cm^2) were seeded on glass coverslips, 22 × 22 mm, covered with 0.1% gelatin and cultured as described above. Indirect immunofluorescence was performed as described (35). An Olympus BH-2 microscope equipped with epifluorescent illumination and a 35-mm Olympus (C-35AD-4) camera were used for visualization and photography.

Northern Blot Analysis of p21/WAF1 mRNA in HepG2 Cells. Total RNA was isolated from subconfluent HepG2 cell cultures by the guanidinium isothiocyanate method (36); 10 μg of total RNA was electrophoresed in 1% formaldehyde/agarose gels (36), blotted on nitrocellulose, and hybridized at 42°C to a ^{32}P randomly labeled 900-bp *Hin*dIII–*Kpn*I p21 cDNA isolated from pCEP-WAF1 (3). Autoradiography was performed on Kodak X-Omat film at −80°C for 2 days.

Western Blot Analysis of Epitope-Tagged Smad Proteins. Equal amounts of transfected cell lysate protein were subjected to SDS/PAGE and transferred to nitrocellulose membranes with a Bio-Rad Protean electroblot apparatus. Nitrocellulose blots were incubated with a mouse anti-FLAG monoclonal antibody, followed by incubation with anti-mouse horseradish peroxidase-conjugated secondary antibody, using the ECL Western blotting kit. Nitrocellulose blots were exposed to ECL-hyperfilm (Amersham) for various lengths of time.

RESULTS AND DISCUSSION

High Levels of Constitutive Activity of the p21 Promoter in HepG2 Cells: Synergistic Interactions Between a Proximal Region and a Distal Region. Northern blot analysis of total RNA using a p21 cDNA probe detected a single band with the correct size of human p21 mRNA (2.1 kb) (2), indicating that the p21 gene is constitutively expressed in HepG2 cells (Fig. 1*A*). To identify promoter elements required for p21 gene expression, transient transfections of HepG2 cells with p21 promoter/CAT fusion constructs were used. In accordance with the Northern blot analysis, the p21 promoter extending from positions −2,300 to +8 relative to the transcription initiation site (+1) is very active in HepG2 cells, and its activity is comparable to that of the simian virus 40 promoter/enhancer (Fig. 1*B*). Deletion analysis of the promoter defined the regions required for high levels of activity. Deletion of the region between positions −2,300 and −2,100 resulted in a 15% reduction of promoter activity that could be attributed to the removal of the previously characterized binding site (positions −2,235 to −2, 216) for the tumor suppressor p53 (13), a protein that is constitutively expressed in HepG2 cells (37). Further deletion to position −1,600 markedly decreased promoter activity to 30% of control, suggesting the presence of strong positive regulatory elements within this 500-bp-long distal region from positions −2,100 to −1,600. Deletions extending to positions −210 and −143 resulted in a further reduction of activity to 20% and 10% of the control promoter, respectively. Deletion to position −63 reduced the promoter activity to 3% of the control. Finally, a −2,300/+8 p21 promoter containing an internal deletion between positions −124 and −61 has only a 5% of activity compared with the control. The proximal region from positions −124 to −61 contains four G+C-rich motifs, two of which have been

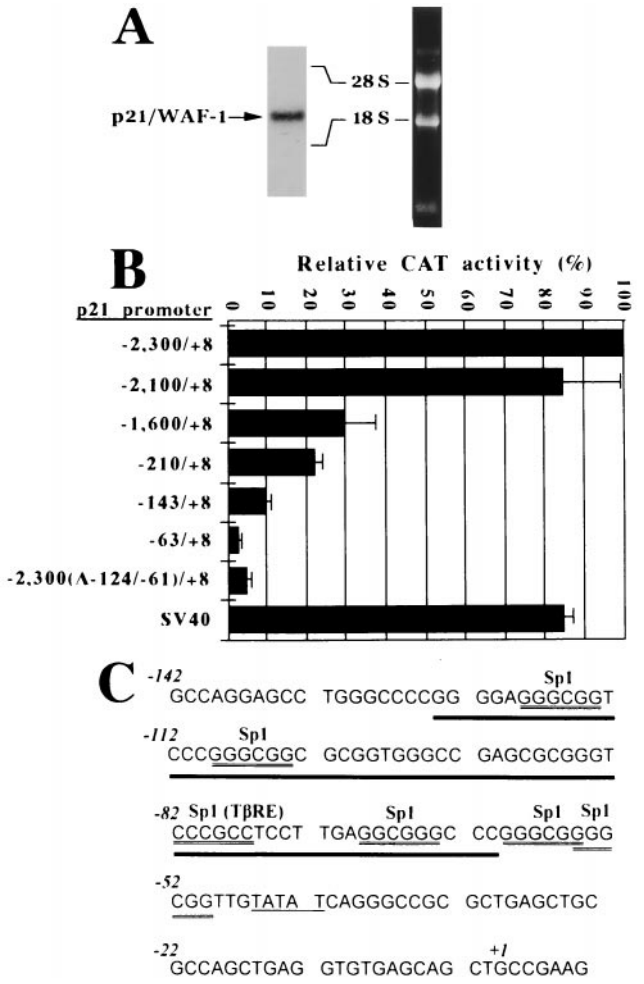

FIG. 1. p21 gene expression and promoter activity in HepG2 cells. (*A*) Analysis of p21 mRNA expression in HepG2 cells. Total HepG2 RNA was electrophoresed on 1% formaldehyde/agarose gel, blotted onto nitrocellulose filters, and hybridized to a ^{32}P-labeled 900-bp 5′ p21 cDNA probe. The resulting autoradiogram and the ethidium bromide staining of the gel before blotting are shown. The positions of the p21 mRNA (arrow) and the 18S and 28S rRNA bands are indicated. (*B*) Constitutive expression and deletion analysis of the p21 promoter in HepG2 cells. Cells were transfected with the indicated p21 promoter-CAT constructs and CAT activity was determined. In this and the subsequent figures, relative CAT activity (mean ± SEM) of at least two experiments performed in duplicate are shown in the form of a bar graph. The activity of the −2,300/+8 p21 promoter was set arbitrarily to 100% (control). (*C*) Summary of the regulatory elements present in the proximal −142/+7 p21 promoter. The nucleotide sequence of the proximal −142/+7 region of the human p21 promoter is shown. The −124/−61 promoter region shown to be essential for p21 promoter activity in hepatocytes is heavily underlined. Numbers are relative to the transcription initiation site (+1). The TATA box (single underline), the predicted Sp1-like motifs (double underline), and the TGF-β-responsive element (TβRE) (14) are shown.

identified as the binding sites for the transcription factor Sp1 or related proteins (Fig. 1*C*) (8, 10, 14).

The deletion analysis suggests that the p21 promoter is organized in distinct functional regulatory regions: a strong distal region between positions −2,100 and −1,600 and a strong proximal region between positions −124 and −61. Maximal p21 promoter activity in HepG2 cells requires both strong distal and proximal regions, suggesting synergistic interactions between transcription factors recognizing these sites.

Regulation of the p21 Promoter by Smad Proteins. TGF-β1 activates the p21 promoter in HaCaT keratinocytes through a proximal DNA sequence between positions −81 and −69 termed the TGF-β-responsive element (Fig. 1*C*) (14). Treatment of HepG2 cells with TGF-β1 resulted in the up-regulation of the p21 promoter by 1.3-fold (Fig. 2*A*). This moderate up-regulation is probably due to the high constitutive levels of the promoter activity or the autocrine production of TGF-β by HepG2 cells. To examine the latter possibility, HepG2 cells were transfected with the −2,300/+8 p21 promoter and cultivated in the presence or absence of a neutralizing anti-TGF-β antibody or a nonspecific sheep IgG (Fig. 2*A*). The neutralizing anti-TGF-β antibody reduced the constitutive p21 promoter activity by 25%, whereas the nonspecific antiserum had no effect. To verify the efficiency of the neutralizing anti-TGF-β antiserum, HepG2 cells were transfected with the reporter p3TP-lux and cultivated with added TGF-β1, in the presence or absence of the neutralizing antibody (Fig. 2*B*). This antibody caused a 3.5-fold reduction in the TGF-β1-induced activity of the p3TP-lux promoter in HepG2 cells. To further assess the contribution of TGF-β, as well as other members of the TGF-β family, to the activity of the p21 promoter, HepG2 cells were cotransfected with the −2,300/+8 p21 reporter construct along with an expression vector carrying the cDNA of a truncated form of Smad4 (amino acids 1–514) (Fig. 2*C*) (25). This Smad4 mutant has been shown to block the signal transduction pathway stimulated by members of the TGF-β family, possibly acting in a dominant negative fashion (25, 28, 29). Immunofluorescence analysis confirmed the cytoplasmic localization of this truncated Smad4 protein in the transfected HepG2 cells as opposed to its normal counterpart that was localized in both the cytoplasm and the nucleus (Fig. 3*C*). Smad4(1–514) caused a dose-dependent repression in the −2,300/+8 p21 promoter activity either in the presence or in the absence of added TGF-β1 (Fig. 2*C*). Thus the data in Fig. 2 *A–C* suggest that the hepatic activity of the p21 promoter is regulated by TGF-β family member signaling pathways and that Smad proteins present in HepG2 cells contribute significantly to the high constitutive levels of the p21 promoter. However, the possibility that Smad4(1–514) acts as a transcriptional repressor by sequestering other positive regulators of p21 expression cannot be excluded.

To further investigate the involvement of Smad proteins in the regulation of the p21 promoter, HepG2 cells were cotransfected with the −2,300/+8 p21 promoter along with expression vectors for human Smad3 and Smad4 (Fig. 2*D*). This cotransfection resulted in a 2.3-fold increase in p21 promoter activity. To define the p21 promoter region that is required for activation by Smads, HepG2 cells were cotransfected with the promoter deletion constructs shown in Fig. 1*B* along with Smad3 and Smad4 (Fig. 2*D*). This analysis showed that coexpressed Smad3 and Smad4 strongly transactivated the −210/+8 and −143/+8 p21 promoters by 10- and 100-fold, respectively, but failed to transactivate the −63/+8 p21 promoter and the −2,300/+8 p21 promoter containing the internal deletion between positions −124 and −61. These data indicate that the proximal region between positions −124 and −61 (Fig. 1*C*) is essential for mediating activation of the p21 promoter by Smad proteins. In agreement with these findings, the activity of the −143/+8 p21 promoter was repressed by Smad4(1–514) (data not shown). The fact that transactivation of various p21 promoters by Smad3/4 was higher than the transactivation achieved by TGF-β1 (Figs. 2*A* and 3*B*) implies that endogenous Smad3 and Smad4 are not expressed at saturating amounts in HepG2 cells. Finally, it is of interest that the level of transactivation of the −143/+8 p21 promoter by Smad3 and Smad4 was consistently 4- to 5-fold higher than the level of transactivation of the −2,300/+8 p21 promoter by the same proteins. These results imply the existence of other regulatory regions between positions −2,300 and −143 that could play a modulating role in Smad function.

The ability of different Smad proteins to transactivate the p21 promoter was examined by cotransfection of HepG2 cells with the −143/+8 p21 reporter construct along with expression vectors for FLAG-tagged versions of Smad2, Smad3, and

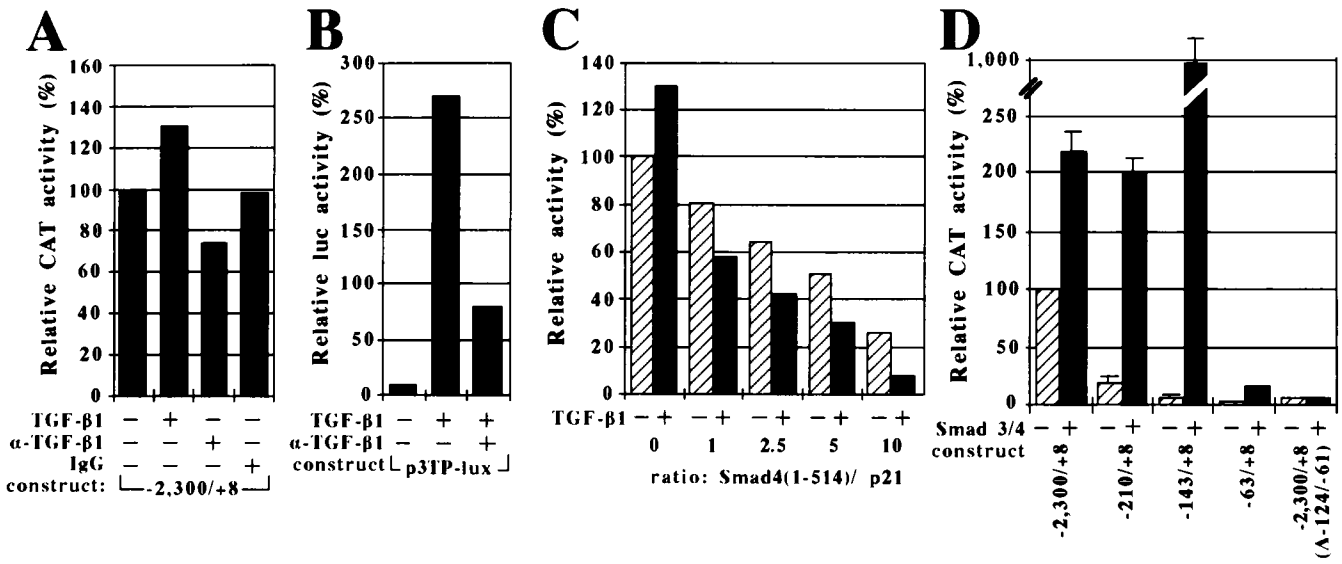

FIG. 2. Regulation of the p21 promoter by TGF-β1 and Smad proteins. (*A* and *B*) Effect of TGF-β1 on p21 and 3TP promoter activity. HepG2 cells were cotransfected with the −2,300/+8 p21 (*A*) or the p3TP-lux (*B*) reporter constructs alone (−) or in the presence (+) of TGF-β1 with (+) or without (−) a neutralizing anti-TGF-β1 antibody or a sheep IgG. Relative CAT (*A*) or luciferase (*B*) activity is reported. The activity of the −2,300/+8 p21 promoter in the absence of TGF-β1 and antibodies was set arbitrarily to 100%. (*C*) Dose-dependent repression of the −2,300/+8 p21 promoter activity by Smad4(1–514). HepG2 cells were cotransfected with the −2,300/+8 p21 reporter plasmid and increasing amounts of Smad4(1–514) in the absence (−, striped bars) or presence (+, solid bars) of TGF-β1. The ratio of Smad4(1–514) to −2,300/+8 p21 reporter plasmid input is indicated. (*D*) Mapping of the p21 promoter region that mediates transactivation by Smad3/4. HepG2 cells were cotransfected with the indicated p21 reporter plasmids without (−, striped bars) or with (+, solid bars) expression vectors for Smad3 and Smad4. The activity of the −2,300/+8 p21 promoter in the absence of Smads was set arbitrarily to 100%.

Smad4 independently or in combinations. As shown in Fig. 3*A*, moderate transactivation of the p21 promoter was observed by Smad2 (3-fold) or Smad2/4 (12-fold). In contrast, Smad3, which has a 92% amino acid sequence similarity with Smad2 (21), transactivated the proximal p21 promoter 25-fold when transfected independently and 40-fold and 110-fold when cotransfected along with Smad2 and Smad4, respectively. The high levels of transactivation achieved by Smad3 as opposed to Smad2 is not due to a difference in expression levels of the two proteins as judged by Western blot analysis (Fig. 3*D*). In addition, both proteins were strictly nuclear in the absence of added TGF-β1 (Fig. 3*C*). Smad4 had a minor (1.3-fold) effect on p21 promoter activity when transfected alone. The highest transactivation was observed when all three Smad proteins were cotransfected in the same cells (140-fold). This transactivation was 35-fold higher than the −143/+8 p21 promoter activity measured in the presence of exogenous TGF-β1 (Fig. 3*B*). These results indicate that activation of the p21 promoter is favored by the combination of Smad3 and Smad4.

To investigate whether added TGF-β1 could enhance the transactivating potential of the transfected Smad proteins, HepG2 cells were cotransfected with the −143/+8 p21 reporter construct along with Smad3 and Smad4 in the presence and absence of added TGF-β1. As shown in Fig. 3*B*, TGF-β1 raised the level of transactivation of the p21 promoter by Smad3 and Smad4.

To investigate the involvement of MH1 and MH2 domains of Smad3 in p21 transactivation, two truncated forms of Smad3 were constructed: a C-terminally truncated form (Smad3N) lacking amino acids 223–424 (MH2) and an N-terminally truncated form (Smad3C) lacking amino acids 1–222 (MH1 and linker region) (see Fig. 4*A*). Smad3N transactivated the −143/+8 p21 promoter by 3-fold (see Fig. 4*B*), whereas it repressed the activity of the −2,300/+8 p21 (data not shown). This difference in Smad3 behavior could be attributed to the presence of other regulatory regions within the −2,300/−143 p21 promoter that play a modulating role for Smad function as discussed above. Smad3C was unable to transactivate the −143/+8 p21 promoter when transfected alone (Fig. 4*B*). Both Smad3 truncated proteins were found to be strictly nuclear in the absence of added TGF-β1 as judged by immunofluorescence analysis (Fig. 3*C*) and were expressed at comparable levels (data not shown). These two truncated Smad3 proteins were tested for their ability to transactivate the −143/+8 p21 promoter synergistically with coexpressed Smad4. As shown in Fig. 4*B*, Smad3N was unable to synergize with Smad4. In contrast, Smad3C acted synergistically with Smad4 and transactivated the p21 promoter by 7-fold. These results suggest that the conserved C-terminal domain of Smad3 (MH2) has an effector function similar to the corresponding domain of Smad2 (30). Finally, the possibility that Smad3 function depends on the presence of an intact protein domain in the vicinity of amino acid 222 cannot be excluded.

Thus, our data support the contribution of Smad proteins to the regulation of the p21 promoter in HepG2 cells. p21 promoter activity can depend on the state of activation and the relative concentration of Smad proteins. Although the state of Smad activation is regulated by receptor serine/threonine kinases of the TGF-β superfamily, the levels of Smad expression could be regulated by as yet unidentified factors acting at either the transcriptional or posttranscriptional level.

Smad Proteins Act as Transcriptional Activators via Functional Interactions with the Transcription Factor Sp1. Our finding that Smad proteins could transactivate the p21 promoter via the proximal −124/−61 region that contains multiple Sp1 binding sites, prompted experiments to test the involvement of Smad–Sp1 interactions in p21 promoter activation. For this purpose, chimeric proteins consisting of the full-length (wild type, wt) or the N-terminal half (A+B) of Sp1 fused to the DNA binding domain (amino acids 1–147) of the yeast regulatory protein Gal4 (Fig. 5*A*) (38) were used. These proteins were tested for their ability to transactivate a synthetic promoter consisting of five tandem copies of a Gal4 DNA binding sequence in front of the CAT reporter (Fig. 5*A*) (39). As expected, the synthetic 5×Gal4 promoter had no activity in HepG2 cells (Fig. 5*B*). The Gal4 DNA-binding domain by itself is not sufficient to induce promoter activity, whereas the chimeric Gal4-Sp1(wt) protein is capable of activating the Gal4

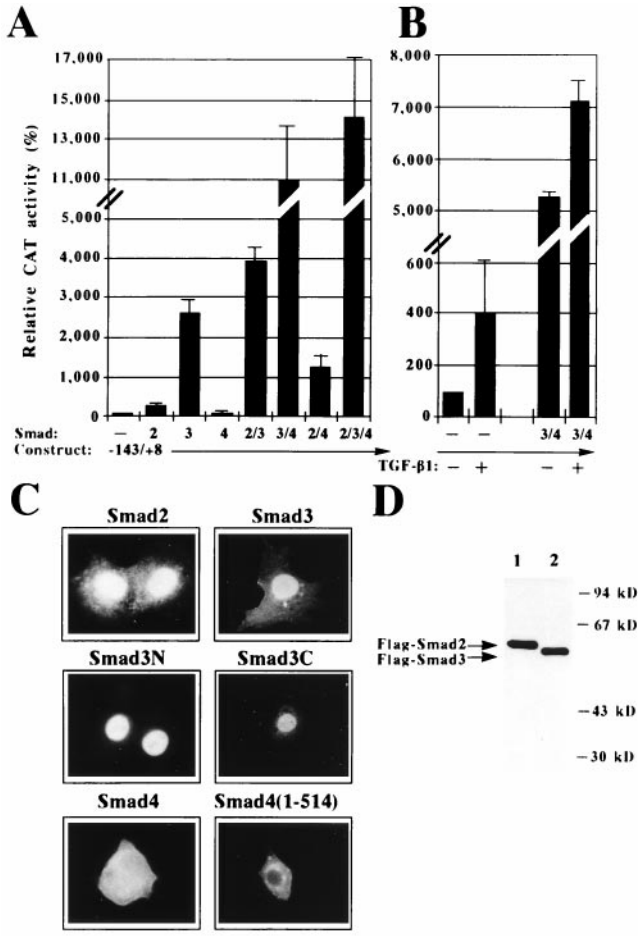

FIG. 3. Transactivation of the proximal p21 promoter by Smad family members. (*A* and *B*) Transactivation of the −143/+8 p21 promoter by Smad family members. HepG2 cells were cotransfected with the −143/+8 p21 promoter construct in the absence (−) or presence of the indicated Smad proteins. Cells were grown in the absence (*A* and *B*, as indicated by −) or in the presence (*B*, as indicated by +) of TGF-β1. The activity of the −143/+8 p21 promoter in the absence of Smads or TGF-β1 was set arbitrarily to 100%. (*C*) Subcellular localization of Smad proteins in transfected HepG2 cells. Human Smad proteins were transfected into HepG2 cells and their localization was monitored by indirect immunofluorescence with an antibody against their unique epitope tag [C-terminal FLAG, Smad2 and Smad3; N-terminal FLAG: Smad3C, Smad4, and Smad4(1–514); C-terminal myc, Smad3N]. (*D*) Western blot analysis of Smad proteins in transfected HepG2 cells. Human Smad proteins 2 (lane 1) and 3 (lane 2) were transfected into HepG2 cells and cell extracts were subjected to SDS/PAGE and Western blot analysis. The resulting chemiluminogram is shown. Arrows indicate the relative migration of the two Smad proteins. Molecular mass markers are in kDa.

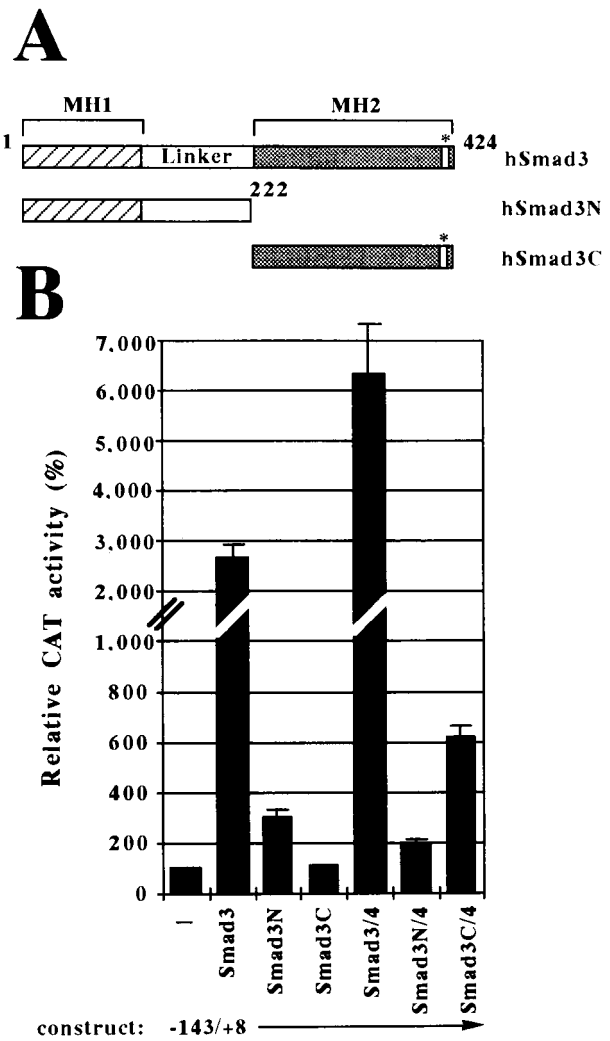

FIG. 4. Truncated Smad3 proteins only minimally transactivate the proximal p21 promoter. (*A*) Schematic representation of human Smad3 and the two truncated forms (Smad3N and Smad3C) used in *B*. Amino acid numbers, the N-terminal Mad-homology domain 1 (MH1, striped box), the linker domain and the C-terminal Mad-homology domain 2 (MH2, grey box) of these proteins are shown. Asterisks in hSmad3 and 3C denote the conserved C-terminal phosphorylation motif SSXS. (*B*) Transactivation of the −143/+8 p21 promoter by truncated Smad3 mutants. HepG2 cells were cotransfected with the −143/+8 p21 promoter construct in the absence (−) or presence of the indicated Smad proteins. The activity of the −143/+8 p21 promoter in the absence of Smads was set arbitrarily to 100%.

promoter to relatively low levels. However, coexpression of this chimeric protein with Smad3 resulted in a further 7.5-fold transactivation of the Gal4 promoter (Fig. 5*B*). This transactivation was strictly dependent on the Sp1 part of the chimeric molecule because Smad3 had no effect on the DNA binding domain of Gal4. The truncated Smad3N and Smad3C proteins (Fig. 4*A*) elicited a 2.5-fold activation compared with the 7.5-fold activation exhibited by the full-length protein. Smad3 expression had the same effect on the Gal4-Sp1(A+B) protein, suggesting functional interactions between Smad3 and the N-terminal half of Sp1. This N-terminal Sp1 region consists of glutamine and serine/threonine-rich domains (Fig. 5*A*) (38). Smad2 and Smad4 transfected independently caused a minor 1- to 2-fold transactivation by interacting with the Sp1(A+B) protein, whereas cotransfection of the two proteins had a synergistic effect. Finally, the strongest transactivation (30-fold) was elicited by the Smad3/4 combination (Fig. 5*B*).

These results suggest that Smad proteins can transactivate Sp1-dependent promoters possibly by functionally interacting with the ubiquitous transcription factor Sp1. The present work demonstrates mammalian Smad cooperation with a mammalian ubiquitous transcription factor such as Sp1. This mode of action of Smad proteins is similar to the one proposed for *Xenopus* gene regulation by TGF-β family members where Smad proteins have been shown to participate in the formation of complexes with transcription factor Fast-1 (26, 30). The present data favor a model in which Smad proteins interact with Sp1 either directly or through intermediary factors. Which Smad proteins participate in such a complex is the subject of further investigation. The observation that Smad3/4 coexpression exhibited much higher transactivation than expression of Smad3 alone implies that the formation of functional transcriptional complexes with Sp1 must involve the participation of both Smad3 and Smad4. This is in

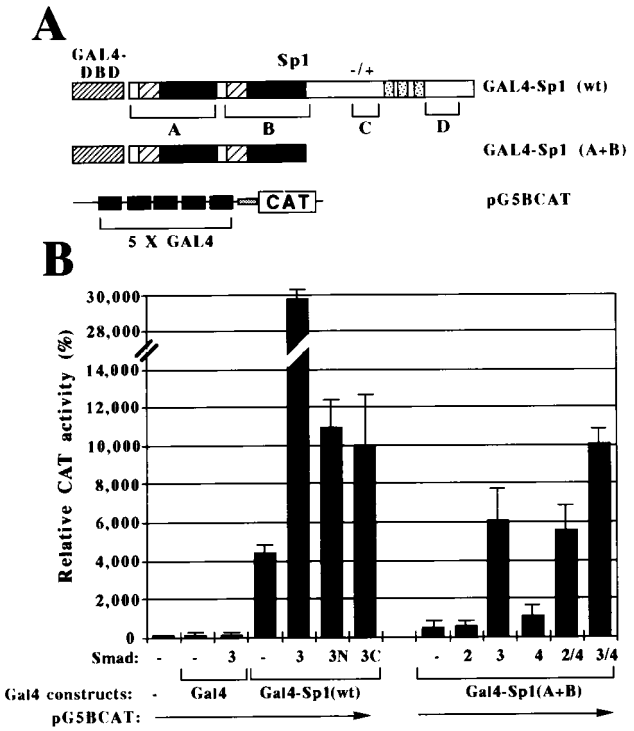

FIG. 5. Smad proteins cooperate with Sp1 to induce gene transcription. (*A*) Schematic representation of the two GAL4-Sp1 fusion constructs (wild type, wt, Sp1 amino acids 83–778; (A+B), N-terminal transactivation domains, amino acids 83–621) and the pG_5B-CAT reporter construct. The Gal4 DNA binding domain (DBD, densely striped box), the glutamine (solid box), and the serine/threonine (striped box)-rich domains, the three zinc finger motifs (small stippled boxes), the transactivation domains (brackets A, B, and D), and the highly charged domain (−/+, bracket C) (38) are shown. (*B*) Transactivation of the pG_5B-CAT reporter by Gal4-Sp1 fusion proteins and Smad family members. HepG2 cells were cotransfected with pG_5B-CAT in the absence (−) or in the presence of the indicated GAL4 constructs and Smad proteins. The relative activity of the pG_5B promoter alone was set arbitrarily to 100%.

agreement with recent findings (26, 30) about the stabilizing and activating roles of Smad4 in the formation of TGF-β or activin-inducible transcriptional complexes. The possibility that Smad proteins transactivate the p21 promoter by direct association with the DNA, as it has been suggested for *Drosophila* Mad (27) and human Smad4 proteins (31), although it cannot be excluded, is not supported by gel electrophoresis mobility shift experiments (data not shown). Finally, Smads could directly or indirectly activate Sp1 by a posttranslational modification. This would assume that Smads either have enzymatic properties or activate specific Sp1-modifying enzymes. Such modification(s) could enhance the transactivation potential or the DNA binding properties of Sp1. The latter possibility is not supported by our preliminary studies (data not shown) or by published data (14).

In conclusion, the TGF-β family signal transducers Smads and the ubiquitous transcription factor Sp1 are important regulators of p21 promoter activity in hepatic cells. The fact that several mammalian genes regulated by TGF-β contain Sp1 motifs (20) suggests that Smad–Sp1 interactions may represent a common mechanism of gene regulation by TGF-β.

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