The Role of Sp1 Family Members, the Proximal GC-Rich Motifs, and the Upstream Enhancer Region in the Regulation of the Human Cell Cycle Inhibitor p21$^{WAF-1/Cip1}$ Gene Promoter†

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ABSTRACT: In the present study we establish that specific members of the Sp1 family of transcription factors (Sp1 and Sp3) bind to all six GC-rich motifs (elements 1−6) present in the proximal promoter of the human cell cycle inhibitor p21$^{WAF-1/Cip1}$ gene. Competition analysis showed that Sp1 and Sp3 bound with high affinity to elements 1, 3, 4, and 5/6 and with lower affinity to element 2. Transfection experiments in the Sp1-deficient *Drosophila* SL2 cells established that Sp1 and Sp3 but not Sp2 are potent transactivators of the p21 promoter. Transactivation by Sp1 was compromised either by deletion of element 1 (−119/−114) or by using a truncated Sp1 form lacking the C-terminal regulatory domain D. Point mutagenesis of the −2325/+8 p21 promoter, targeting individual elements 1−6, showed that mutations in element 3 (−82/−77) caused a dramatic reduction (90%) in p21 promoter activity whereas mutations in other elements had a less severe effect. The mutations in element 3 abolished p21 promoter induction by upstream enhancer elements in HepG2 cells. Sp1, but not Sp3, mediated the transactivation of the p21 promoter by the TGFβ signaling mediator Smad3 and Smad4 proteins whereas none of the individual mutations in elements 1−6 affected the transactivation of the p21 promoter by Smad proteins in HepG2 cells. Our results suggest that functional interactions between Sp1 family members bound to specific elements of the proximal promoter and factors bound to distal enhancer elements govern the hepatic activity of the human p21 promoter under basal or inducible conditions.

p21$^{WAF-1/Cip1/SDI1}$ (p21) belongs to a family of cell cycle dependent kinase (cdk) inhibitors and modulates various biological processes such as cell growth, differentiation, and apoptosis (*1, 2*). In mammalian cells, p21 is found in complexes that consist of cyclins (D and E), cdks, and Proliferating Cell Nuclear Antigen (PCNA), a subunit of DNA polymerase δ (*3*). Changes in p21 stoichiometry, relative to the other components of these complexes, result in suppression of cdk activity, allowing the accumulation of hypophosphorylated Rb, inhibition of E2F-dependent transcriptional processes, and cell cycle arrest in G1 (*2, 4*). By interacting directly with Proliferating Cell Nuclear Antigen, p21 prevents DNA synthesis and regulates DNA methylation (*5*).

A key regulator of p21 gene expression is the product of the tumor suppressor gene p53 (*6*). Agents that cause DNA damage such as UV radiation or certain carcinogens induce the phosphorylation of p53 at specific Ser residues (*7*). Phosphorylated p53 binds to regulatory elements present in distal regions of the p21 promoter and transactivates this promoter via physical and functional interactions with the ubiquitous transcription factor Sp1 bound to the proximal region (*8*).

In addition to p53, other inducers of differentiation such as steroid hormones, nerve growth factor (NGF), platelet-derived growth factor, tumor necrosis factor-α, phorbol esters, phosphatase inhibitors, interferon γ, progesterone, and transforming growth factor-β (TGF-β) and its signaling effectors, the Smad proteins, enhance p21 gene transcription in different cell systems (*9*−*22*).

Similar to p53, the majority of the above regulatory modulators affect p21 gene expression via Sp1 or Sp3 proteins bound to the regulatory elements present in the proximal p21 promoter. The −125/−45 region of the p21 promoter is especially rich in nucleotide sequences which are identical or resemble the consensus binding sites for members of the Sp1 family of proteins (5′ GGGCGG 3′) (*23*). These regions have been designated by us and other groups as element 1 (−119/−114), element 2 (−109/−104), element 3 (−82/−77), element 4 (−69/−64), element 5 (−60/−55), and element 6 (−55/−50) (*8*, *23*). We have shown previously that deletion of elements 1−4 (p21 promoter region −125/−60) reduced drastically the basal p21

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promoter activity in human hepatoma HepG2 cells (95% reduction), suggesting that the factors that bind to these sites play a very important role in p21 gene regulation in hepatocytes (*22*). Binding of Sp1 and Sp3 has been demonstrated for some of the above elements present on the p21 promoter in different systems (*23*).

We have shown previously that Smad3 and Smad4 proteins, which are key effectors in the TGFβ signaling pathway and AP-1 family members (c-Jun, JunB, JunD, ATF-2), which are activated in response to a variety of extracellular stimuli, transactivate the p21 promoter by interacting physically and functionally with Sp1 bound to the proximal region (*22*, *24*, *25*). In both cases, no direct binding of Smad or AP-1 proteins to DNA occurred, but rather an enhanced affinity of Sp1 for its cognate sites on the p21 promoter was observed in the presence of the activators.

Sp1 is the prototype member in a family of structurally and functionally related transcription factors that binds to GC-rich or GT-rich DNA sequences present in the promoters of a large number of genes (*26*). Currently, this family includes four members: Sp1, Sp2, Sp3 (SPR-2), and Sp4 (SPR-1) (*26*). Sp1 family members are modular in structure and are composed of functionally distinct domains (*26*). The N-terminal half consists of two homologous regions, designated A and B, which are rich in Gln and Ser/Thr residues and have strong transcriptional activation functions. Region C is rich in charged amino acids and seems to play a regulatory role in various Sp1 functions. In Sp3, domain C is acetylated at specific lysine residues, leading to the transcriptional inactivation of the protein (*27*). The DNA binding domain is the most highly conserved domain among the members of the Sp1 family. Alignment of the amino acid sequences in the DNA binding domain revealed that specific amino acids critical for contacting DNA are conserved in Sp1, Sp3, and Sp4 but not in Sp2 (*26*). In agreement with the above observation, Sp1, Sp3, and Sp4 all recognize preferentially the "classical" GC-rich motif 5′ GGGCGG 3′ or slight variations in this consensus sequence whereas Sp2 shows preference for GT-rich motifs (*28*, *29*). Finally, the C-terminal domain D is important for cooperative interactions among Sp1 molecules when bound to promoters containing multiple GC boxes (*30*).

Sp1 has been shown to interact directly with proteins of the basal transcription machinery such as TFIID components (*31*). On the other hand, Sp1 interacts physically and cooperates functionally with several sequence-specific activators including NF-κB, GATA, YY1, E2F1, Rb, and SREBP-1 (*32*−*35*). Thus, although Sp1 has been considered traditionally as a ubiquitous factor associated closely with core promoter activities, it has been shown recently that it participates in several cases of regulated gene transcription triggered by multiple signaling pathways and metabolic or differentiation conditions.

In the present study we have attempted a detailed characterization of the proximal p21 promoter region in hepatocytes. Specifically, we investigated the regulatory role of specific members of the Sp1 family of transcription factors in p21 gene transcription and the contribution of individual Sp1 binding sites in both the constitutive and the inducible p21 promoter activity.

MATERIALS AND METHODS

Materials. Materials used in this study were purchased from the following sources: restriction enzymes and modifying enzymes (T4 DNA ligase, T4 polynucleotide kinase, Klenow fragment of DNA polymerase I, calf intestinal alkaline phosphatase) from Minotech (Heraklion, Greece), New England Biolabs, or GIBCO-BRL; vent DNA polymerase from New England Biolabs; Sequenase v2 kit from Amersham/USB; poly(dI/dC) and dNTPs from Pharmacia; [γ-^{32}P]ATP and [α-^{32}P]dCTP from Amersham or New England Nuclear; all reagents for cell culture (DMEM, Schneider's insect medium, fetal bovine serum, trypsin-EDTA, and PBS) from Life Technologies, Inc.; *o*-nitrophenyl-β-galactopyranoside (ONPG) from Sigma/Aldrich. The luciferase assay system and the Gene Editor site-directed mutagenesis system were from Promega Corp. The polyclonal anti-Sp1 (PEP-2), anti-Sp2 (K-20), and anti-Sp3 (D-20) antibodies were purchased from Santa Cruz Biotechnology. All oligonucleotides were synthesized at the microchemical facility of the IMBB (Heraklion, Crete, Greece). All other chemicals were obtained from the usual commercial sources at the purest grade available.

Plasmid Constructions. The p21 promoter plasmids −2325/+8 luc, −215/+8 luc, −143/+8 luc, −2325/+8 Δ−122/−60 p21 luc have been described previously (*22*). The −2325/+8 p21 luciferase reporter plasmids containing point mutations in elements 1−6 (Mut1−Mut6) were generated using the Gene Editor site-directed mutagenesis system following the instructions of the manufacturer (Promega Corp.). The sequence of the oligonucleotides used for the mutagenesis of the p21 promoter is shown in Table 1. The incorporation of mutations was verified by restriction digestion (all mutations introduced diagnostic restriction sites) and DNA sequencing. The (−119/+8) p21 luciferase reporter plasmid was generated by excising the region of the p21 promoter between nucleotides −119 and −2325 from the (−2325/+8) Mut1 p21 luciferase plasmid with *Xho*I and religation. The (−103/+8) p21 luciferase reporter plasmid was generated by excising the region of the p21 promoter between nucleotides −103 and −2325 from the (−2325/+8) Mut2 p21 luciferase plasmid with *Kpn*I and religation. The (−83/+8) p21 luciferase reporter plasmid was generated by excising the (−83/+8) region of the p21 promoter from the (−2325/+8) Mut3 p21 luciferase plasmid with *Sal*I and *Hin*dIII and subcloning into the *Xho*I and *Hin*dIII sites of vector pGL3-basic. The (−215/+8) Mut1 and (−215/+8) Mut3 p21 luciferase reporter plasmids were constructed by excising the (−215/+8) region of the p21 promoter from the (−2325/+8) Mut1 and (−2325/+8) Mut3 p21 luciferase plasmids with *Pst*I (−215) and *Hin*dIII (+8) and subcloning them into the *Sma*I site of vector pGL3-basic. The *Drosophila* expression vectors pPac-Sp1, pPac-Sp2, and pPac-Sp3 were the generous gift of Dr. J. Noti (Guthrie Research Institute, Sayre, PA). The hsp-lacZ expression vector used for normalization of transfections in *Drosophila* SL2 cells was the generous gift of Dr. C. Delidakis (University of Crete and IMBB, Heraklion). The expression vectors encoding the FLAG-tagged human Smad3 and Smad4 proteins were the generous gift of Dr. R. Derynck (University of California at San Francisco) and Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York), respectively.

Table 1: Oligonucleotides Used as Primers in Site-Directed Mutagenesis of the Human p21 Promoter

oligonucleotide name	sequence	purpose
Sp1-5/6	5′ TTG AGG CGG GCC CGG **TCT AGA** CGG TTG TAT ATC AGG 3′	used as primer for mutagenesis of the −59/−50 region of the p21 promoter (element 5/6); sense orientation; point mutations are shown in bold; *Xba*I site is underlined
Sp1-4	5′ CCC GCC TCC TTG AGG **AAT TC**C CGG GCG GGG CGG T 3′	used as primer for mutagenesis of the −69/−64 region of the p21 promoter (element 4); sense orientation; point mutations are shown in bold; *Eco*RI site is underlined
Sp1-3	5′ GGC CGA GCG CGG GTC **GAG** CCT CCT TGA GGC GGG 3′	used as primer for mutagenesis of the −82/−77 region of the p21 promoter (element 3); sense orientation; point mutations are shown in bold; *Sal*I site is underlined
Sp1-2	5′ GAG GGC GGT CCC GGG **TAC** CGC GGT GGG CCG AGC 3′	used as primer for mutagenesis of the −109/−104 region of the p21 promoter (element 2); sense orientation; point mutations are shown in bold; *Kpn*I site is underlined
Sp1-1	5′ GCC TGG GCC CCG GGG **CTC** GAG GTC CCG GGC GGC GC 3′	used as primer for mutagenesis of the −119/−144 region of the p21 promoter (element 1); sense orientation; point mutations are shown in bold; *Xho*I site is underlined

Cell Cultures, Transient Transfections, CAT, Luciferase, and β-Galactosidase Assays. Human hepatoma HepG2 cells and COS-7 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin at 37 °C, in a 5% CO_2 atmosphere. *Drosophila* Schneider's SL2 cells were cultured in Schneider's insect medium supplemented with 10% insect culture-tested fetal bovine serum and penicillin/streptomycin at 25 °C. Transient transfections were performed using the $Ca_3(PO_4)_2$ coprecipitation method (*36*). β-Galactosidase assays were performed as described previously (*25*). Luciferase assays were performed using the luciferase assay kit from Promega Corp. according to the manufacturer's instructions.

Preparation of Extracts. Rat liver nuclear extracts were prepared as described previously (*37*). For the expression of proteins in SL2 cells, expression vectors bearing the cDNAs for Sp1, Sp2, and Sp3 proteins under the control of the *Drosophila* actin promoter (17 μg of each) were transfected into SL2 cells (10^7 cells in 100 mm dishes) by $Ca_3(PO_4)_2$ coprecipitation. Forty hours later, cells were harvested, washed 2 times in PBS, resuspended in 400 μL of WCE buffer (20 mM Tris-HCl, pH 7.4, 400 mM KCl, 2 mM DTT, 10% glycerol plus antibiotics), and lysed by three freeze−thaw cycles. Cell debris was cleared by centrifugation at 14 000 rpm for 5 min at 4 °C, and the supernatants were aliquoted and stored at −70 °C until use.

Gel Electrophoretic Mobility Shift Assays. Gel electrophoretic mobility shift assays (EMSAs) were performed as described previously (*38*). Sense and anti-sense oligonucleotides were annealed to generate the double-stranded oligonucleotide probe and labeled with Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP. The sequences of the oligonucleotide used as probes are shown in Table 2. Competition assays were performed by the addition of 50-, 100-, 200-, or 400-fold molar excess of unlabeled oligonucleotides in the reaction mix, prior to the addition of the labeled probe. For supershift assays, 1 μL of antibody was included in the reaction mix along with the extract.

Western Blotting. For Western blotting analysis, protein extracts were resolved by 8.5% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Electrophoresis was performed in 500 mL of 1× TGS (1 L of 10× TGS: 30.3 g of Tris, 144.2 g of glycine, 10 g of SDS, pH 8.3), using the Bio-Rad Protean electroblotting apparatus. Proteins on the membrane were visualized by Ponceau S staining. Nitrocellulose membranes were washed with TBS-T (TBS + 0.05% Tween-20) for 10 min, at room temperature. Nonspecific sites were blocked by soaking the membrane in TBB buffer (1× TBS + 5% nonfat milk, 0.05% Tween-20) for 2 h at 4 °C. Western blotting was performed with a 1:1000 dilution (anti-Sp1, anti-Sp2) or 1:500 dilution (anti-Sp3) of the polyclonal antibodies in TBB overnight at 4 °C. The membranes were washed 3 times with TBS-T, for 10 min, at room temperature. As a secondary antibody, we used anti-goat or anti-rabbit horseradish peroxidase-conjugated (HRP), in a 1:10 000 dilution in TBS-T, for 1 h at room temperature. After 3 washes of 15 min with TBS-T at room temperature, bands were visualized by enhanced chemiluminescent detection.

RESULTS

Sp1 and Sp3 Bind to Multiple Elements of the Proximal p21 Promoter with Different Affinities. Binding of liver nuclear factors to the proximal human p21 promoter was analyzed by gel electrophoretic mobility shift assays. For that purpose, six partially overlapping oligonucleotides covering the −124/−45 region of the p21 promoter were synthesized (Figure 1A and Table 2). These oligonucleotides were the following: p21 (−123/−97) which includes elements 1 and 2; p21 (−124/−109) which includes

Table 2: Oligonucleotides Used as Probes in Gel Mobility Shift and Competition Assays

oligonucleotide name	sequence	location
p21 (−63/−45) sense	5′ TTT CCC GGG CGG GGC GGT TGT A 3′	corresponds to −63/−45 region of the p21 promoter; sense strand
p21 (−63/−45) mut 5/6 sense	5′ TTT CCC GG**T** C**TA** G**A**C GGT TGT A 3′	corresponds to −63/−45 region of the p21 promoter mutated at element 5/6; sense strand; mutations are shown in bold
p21 (−76/−58) sense	5′ TTT TCC TTG AGG CGG GCC CGG G 3′	corresponds to −76/−58 region of the p21 promoter; sense strand
p21 (−76/−58) mut4 sense	5′ TTT TCC TTG AGG **AAT T**CC CGG G 3′	corresponds to −76/−58 region of the p21 promoter mutated at element 4; sense strand; mutations are shown in bold
p21 (−86/−70) sense	5′ GGG GGT CCC GCC TCC TTG A 3′	corresponds to −86/−70 region of the p21 promoter; sense strand
p21 (−86/−70) mut3 sense	5′ GGG GGT C**GA C**CC TCC TTG A 3′	corresponds to −86/−70 region of the p21 promoter mutated at element 3; sense strand; mutations are shown in bold
p21 (−123/−97) sense	5′ GGG AGG GCG GTC CCG GGC GGC GCG GTG 3′	corresponds to −123/−97 region of the p21 promoter; sense strand
p21 (−123/−97) mut2 sense	5′ GGG AGG GCG GTC CCG GG**T AC**C GCG GTG 3′	corresponds to −123/−97 region of the p21 promoter mutated at element 2; sense strand; mutations are shown in bold
p21 (−123/−97) mut1 sense	5′ GGG **CTC** G**A**G GTC CCG GGC GGC GCG GTG 3′	corresponds to −123/−97 region of the p21 promoter mutated at element 1; sense strand; mutations are shown in bold
p21 (−115/−95) sense	5′ TTT GGT CCC GGG CGG CGC GGT GGG 3′	corresponds to −115/−95 region of the p21 promoter; sense strand
p21 (−115/−95) mut2 sense	5′ TTT GGT CCC GGG **TAC** CGC CGC GGT GGG 3′	corresponds to −115/−95 region of the p21 promoter mutated at element 2; sense strand; mutations are shown in bold
p21 (−124/−109) sense	5′ TTT GGG GAG GGC GGT CCC G 3′	corresponds to −124/−109 region of the p21 promoter; sense strand
p21 (−124/−109) mut1 sense	5′ TTT GGG G**CT C**G**A** GGT CCC G 3′	corresponds to −124/−109 region of the p21 promoter mutated at element 1; sense strand; mutations are shown in bold

element 1; p21 (−115/−95) which includes element 2; p21 (−86/−70) which includes element 3; p21 (−76/−58) which includes element 4; and p21 (−63/−45) which includes the overlapping elements 5 and 6 (referred to as element 5/6)

As shown in Figure 1B, incubation of rat liver nuclear extracts with the p21 (−86/−70) probe (element 3) revealed the formation of three protein−DNA complexes. Using antibodies specific for Sp1 or Sp3 proteins, it was shown that the slowest migrating protein−DNA complex corresponded exclusively to Sp1 whereas the two faster migrating complexes corresponded to full-length Sp3 and possibly a truncated form or a splicing isoform of Sp3. Binding of Sp1 and Sp3 proteins to the p21 (−86/−70) probe was further verified using proteins expressed in *Drosophila* SL2 cells. This cell line lacks endogenous Sp1 and related activities, and thus it is a very useful model for studies of Sp1-dependent mechanisms of transcriptional activation of eukaryotic genes (*39*). As shown in Figure 1C, both Sp1 and Sp3 but not Sp2, ectopically expressed in SL2 cells, bound efficiently to the p21 (−86/−70) probe. In control experiments, the anti-Sp1 antibody supershifted specifically the complex formed by Sp1 expressed in SL2 cells and the p21 (−86/−70) probe whereas it could not supershift the complex formed by Sp3. The reverse experiment, i.e., the utilization of the anti-Sp3 antibody with both Sp1 and Sp3 proteins expressed in SL2 cells, confirmed the specificity of this antibody for Sp3 (data not shown). Immunoblotting analysis revealed equal levels of expression of Sp1, Sp2, and Sp3 proteins in SL2 cells (Figure 1D).

We next sought to characterize in detail the binding properties of Sp1 and Sp3 proteins to all GC-rich motifs present in the proximal p21 promoter (elements 1−6). As shown in Figure 1E, incubation of double-stranded oligonucleotides −63/−45 (element 5/6), −76/−58 (element 4), and −124/−109 (element 1) with rat liver nuclear extracts resulted in the formation of identical protein−DNA complexes with the ones formed with the −86/−70 (element 3) probe (Figure 1E, compare lanes 1, 3, and 9 with lane 5) which could be attributed to Sp1 and Sp3. The only difference was the appearance of an additional faster migrating protein−DNA complex with probe −63/−45 (element 5/6) (Figure 1E, lane 1). However, the appearance of this band was not reproducible (data not shown). Interestingly, binding of Sp1 and Sp3 to oligonucleotide −115/−95 (element 2) was barely detectable (Figure 1E, lane 7), suggesting that the two factors have extremely low affinity for this specific site at least under the in vitro experimental conditions utilized.

To characterize further the binding of Sp1 and Sp3 proteins to the Sp1 elements 1 and 2, an additional oligonucleo-

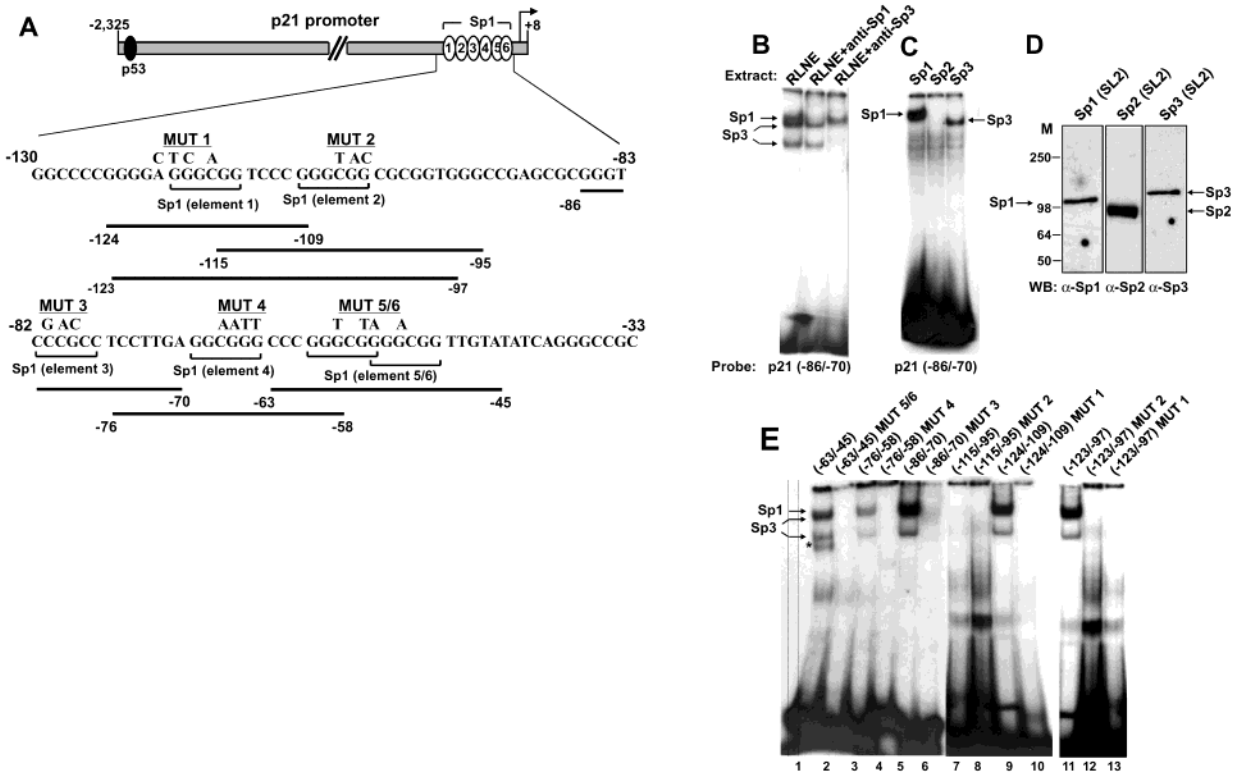

FIGURE 1: (A−E) Sp1 and Sp3 but not Sp2 bind to all GC-rich motifs of the proximal p21 promoter with different affinities. Panel A: Sequence of the human p21 promoter between nucleotides −130 and −33. Nucleotide substitutions that were introduced into the p21 promoter Sp1 elements 1−6 are shown on top of the sequence and are designated MUT 1−MUT 5/6. Heavy lines underneath the sequence indicate oligonucleotides used in the gel electrophoretic mobility shift assays of panels B−E. Panel B: Gel electrophoretic mobility shift assays using the oligonucleotide p21 (−86/−70) as probe and rat liver nuclear extracts (RLNE) in the absence or in the presence of antibodies specific for Sp1 (anti-Sp1) or Sp3 (anti-Sp3) as indicated. Arrows show the position of Sp1 and Sp3 proteins. Panel C: Gel electrophoretic mobility shift assay using the oligonucleotide p21 (−86/−70) as probe and Sp1, Sp2, or Sp3 proteins expressed in the *Drosophila* SL2 cells that lack endogenous Sp1 or related activities. Arrows show the positions of Sp1 and Sp3 proteins. Panel D: Analysis of the levels of expression of Sp1, Sp2, and Sp3 in SL-2 cells by immunoblotting. SL-2 cells were transfected with 17 μg of the vectors pPac-Sp1, pPac-Sp2, and pPac-Sp3. Forty hours following transfection, cells were lysed, and expression of Sp1, Sp2, and Sp3 proteins was monitored by SDS−PAGE and Western blotting (WB) using polyclonal antibodies specific for these proteins as indicated at the bottom of the panel. The positions of Sp1, Sp2, and Sp3 proteins are indicated by arrows. Panel E: The labeled wt or mutated oligonucleotides indicated on top of the autoradiographs were incubated with rat liver nuclear extracts. Free from bound probes were resolved by nondenaturing PAGE and visualized by autoradiography. The arrows show the positions of the Sp1 and Sp3 protein−DNA complexes. The asterisk in lane 1 shows the appearance of a band that was not reproducible in subsequent experiments.

tide was synthesized that contains both of these elements (p21 −123/−97). As shown in Figure 1E, incubation of rat liver nuclear extracts with this probe resulted in the formation of complexes with the same electrophoretic mobility as those formed with the oligonucleotide containing a single element 1 (compare lane 11 with lane 9).

Next, point mutations were introduced into the p21 oligonucleotides utilized in the gel electrophoretic mobility shift assays of Figure 1E. All mutations targeted the consensus Sp1 binding sequences, as shown in Figure 1A. The mutations were designated MUT1 (for element 1), MUT2 (for element 2), MUT3 (for element 3), MUT4 (for element 4), and MUT5/6 (for element 5/6). As shown in Figure 1E, none of the mutant oligonucleotides tested in gel electrophoretic mobility shift assays was able to bind Sp1 and Sp3 proteins (lanes 2, 4, 6, 8, 10). Notably, binding of Sp1 and Sp3 to the −123/−97 oligonucleotide probe (containing both elements 1 and 2) was abolished by mutations in either element (lanes 12 and 13).

The relative affinity of Sp1 and Sp3 proteins for the proximal p21 promoter elements 1−6 was analyzed by gel electrophoretic mobility shift competition experiments. Binding of Sp1 and Sp3 proteins present in rat liver nuclear extracts to the p21 (−86/−70) probe (element 3) was competed by increasing amounts of unlabeled oligonucleotides. As shown in Figure 2A, strong competition was observed by oligonucleotides −63/−45 (element 5/6, lanes 2−5), −76/−58 (element 4, lanes 6−9), −86/−70 (element 3, lanes 10−13), and −124/−109 (element 1, lanes 18−21), whereas weak competition was observed by oligonucleotide −115/−95 (element 2, lanes 14−17). Quantitation of this competition experiment by phosphorimage analysis established that the affinity of the different elements for Sp1 and Sp3 follows the order: element 3 > element 1 = element 4 = element 5/6 ≫ element 2 (Figure 2B).

A similar competition analysis was performed using Sp1 and Sp3 proteins ectopically expressed in SL2 cells. As shown in Figure 3, binding of Sp1 (panel A) or Sp3 (panel B) to the −86/−70 p21 probe (element 3) was efficiently competed by a 100-fold molar excess of the unlabeled oligonucleotides −63/−45 (element 5/6), −76/−58 (element 4), −86/−70 (element 3), and −124/−109 (element 1) whereas a weaker competition was observed when the oligonucleotide −115/−95 (element 2) was used, in agree-

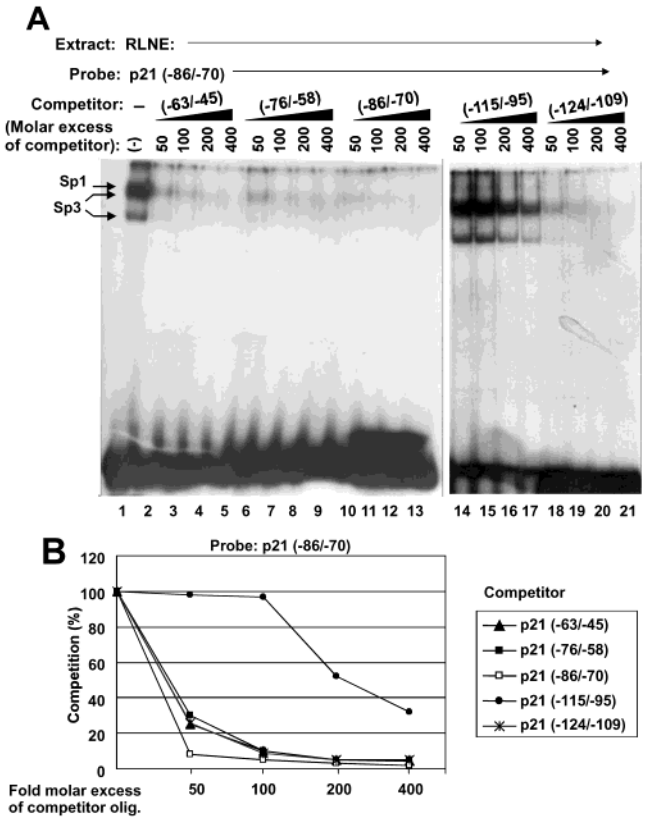

FIGURE 2: (A, B) Competition analysis of Sp1 and Sp3 factors present in rat liver nuclear extracts using oligonucleotides corresponding to elements 1−6 of the p21 promoter. Panel A: Gel electrophoretic mobility shift assays using labeled p21 (−86/−70) oligonucleotide and rat liver nuclear extracts (RLNE) in the absence (−) or in the presence of increasing molar excess (50-, 100-, 200-, and 400-fold) of the unlabeled p21 promoter oligonucleotides indicated on top of the autoradiographs. Free from bound probes were resolved by nondenaturing PAGE and visualized by autoradiography. The arrows show the positions of the Sp1 and Sp3 protein−DNA complexes. Panel B: Quantitation of the gel electrophoretic mobility shift competition experiment of panel A by phosphorimage analysis. This experiment was repeated 3 times with similar results. The mean values for each point are shown in the graph.

ment with the findings of Figure 2. Again, no binding of Sp2 was observed in these experiments (data not shown).

In summary, the data presented in Figures 1−3 indicate that the six GC-rich motifs (elements 1−6) present in the proximal p21 promoter are occupied by two members of the Sp1 family, Sp1 and Sp3, with different affinities. Under the in vitro conditions used in this study, no other factors present in rat liver nuclear extracts or SL2 cells were found to occupy these sites.

Contribution of the Proximal GC-Rich Motifs in the Transactivation of the Proximal p21 Promoter by Sp1 and Sp3. To evaluate the relative contribution of individual GC-rich motifs of the proximal p21 promoter in hepatic p21 gene transcription, a series of truncated proximal p21 promoter fragments were placed upstream of the luciferase reporter gene and were utilized in transient transfection experiments. These constructs included p21 promoter fragments truncated to nucleotides −143, −115, −103, and −78 (Figure 4A). All p21 promoter fragments extended to nucleotide position +8 relative to the transcription start site of the gene. The −143/+8 p21 fragment included all 6 GC-rich motifs

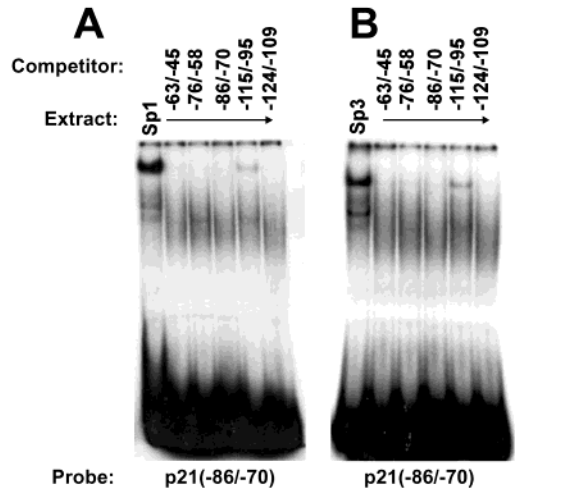

FIGURE 3: (A, B) Competition analysis of Sp1 and Sp3 factors expressed in SL2 cells using oligonucleotides corresponding to elements 1−6 of the p21 promoter. Panels A and B: Gel electrophoretic mobility shift assays using the oligonucleotide p21 (−86/−70) as probe and Sp1 (panel A) or Sp3 (panel B) proteins expressed in SL2 cells. The assays were performed in the absence or in the presence of 100-fold molar excess of the competitor oligonucleotides p21 (−63/−45), p21 (−76/−58), p21 (−86/−70), p21 (−115/−95), and p21 (−124/−109) as indicated on top of the autoradiographs.

(elements 1−6) whereas the −115/+8, −103/+8, and −78/+8 p21 promoter fragments were lacking elements 1, 2, and 3, respectively. As shown in Figure 4B, truncation of the (−2,325/+8) p21 promoter to nucleotide −143 caused a 90% reduction in hepatic p21 promoter activity, in agreement with our previous findings (*22*). Deletion of element 1 caused an additional 3-fold reduction in p21 promoter activity (3.3% relative to the full-length p21 promoter), indicating that element 1 is important for the hepatic activity of the proximal p21 promoter. Further deletion of elements 2 and 3 did not affect significantly p21 promoter activity. The findings of Figure 4B indicated that binding of Sp1 or Sp3 to element 1 contributes significantly to the basal activity of the proximal p21 promoter in HepG2 cells.

The contribution of the proximal GC-rich motifs in the Sp1- and Sp3-mediated transactivation of the proximal p21 promoter was studied further in SL2 cells. In a first approach, the proximal p21 promoter constructs shown in Figure 4C were utilized in transient transfections of SL2 cells along with expression vectors for human Sp1 and Sp3 proteins. As shown in Figure 4D, both Sp1 and Sp3 strongly transactivated the −143/+8 p21 promoter 160- and 216-fold, respectively. Deletion of element 1 caused a marked decrease in the Sp1- and Sp3-mediated transactivation of the p21 promoter to 25- and 21-fold, respectively, in agreement with the findings in HepG2 cells presented in Figure 4B. Further deletion of elements 2 and 3 decreased the Sp1- and Sp3-mediated transactivation of the p21 promoter to 12- and 8.5-fold, respectively.

In a second approach, the proximal −215/+8 p21 promoter-luciferase reporters bearing nucleotide substitutions in elements 1 and 3 (Figure 4E) were utilized in transient transfections of SL2 cells along with expression vectors for Sp1 and Sp3. These mutations abolished binding of Sp1 and Sp3 proteins to elements 1 and 3, respectively (Figure 1E). As shown in Figure 4F, Sp1 and Sp3 transactivated the wild-

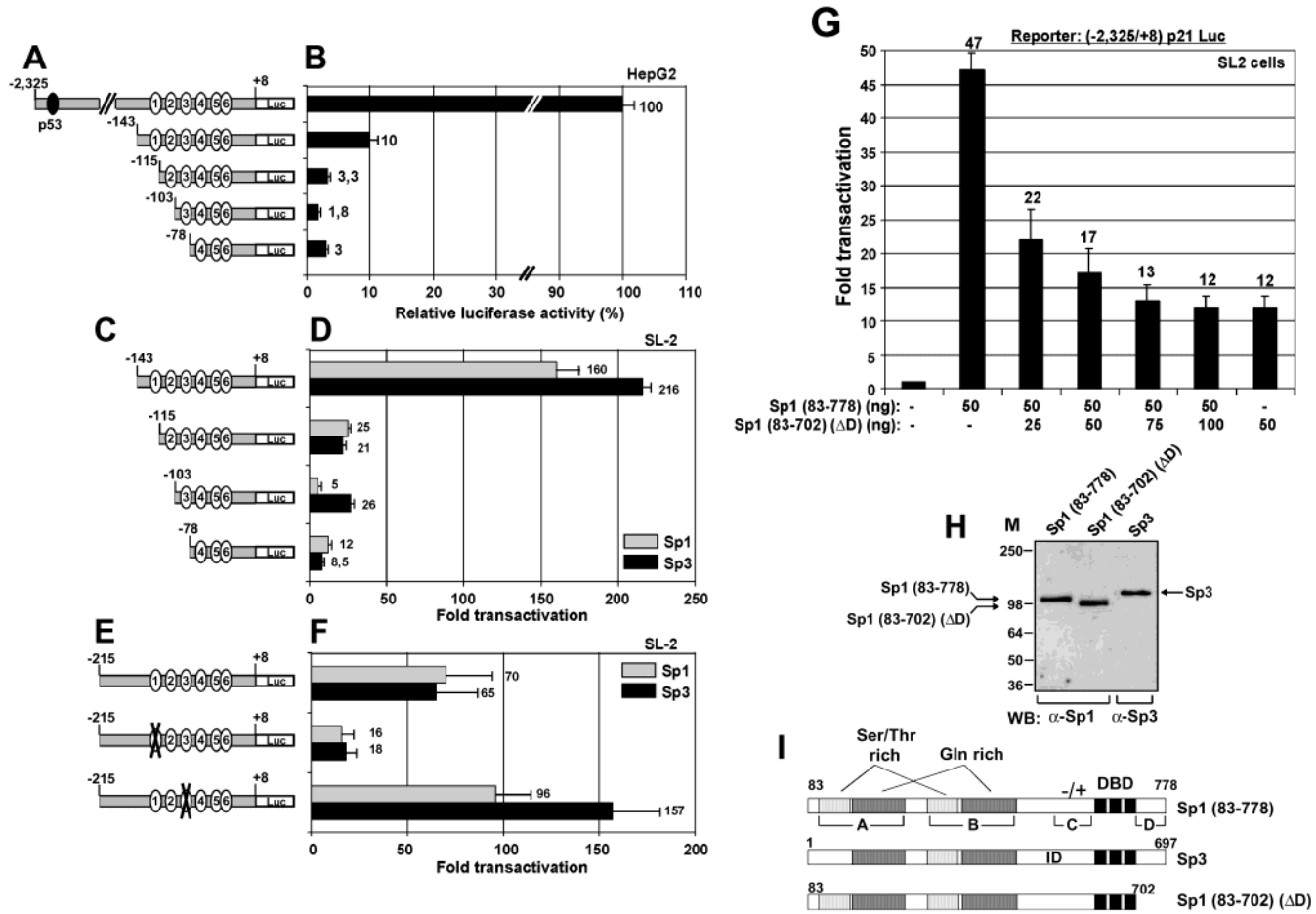

FIGURE 4: (A−I) Importance of Sp1 binding elements for the activity of the proximal p21 promoter in HepG2 cells and in SL2 cells expressing Sp1 and Sp3. Panel A: Schematic representation of the p21 promoter−luciferase reporter vectors used in the transactivation experiments of panel B. Proximal Sp1 elements 1−6 are shown with white ovals numbered 1−6. The distal p53 binding site is shown with a black oval. Panel B: HepG2 cells were transfected with the p21 promoter−luciferase reporter vectors shown in panel A (2 μg). The CMV-β galactosidase plasmid (1 μg) was included in each sample for normalization of transfection variability. The normalized, relative luciferase activities (mean ± SEM) of at least two independent experiments performed in duplicate are shown in the form of a bar-graph. Actual values are shown next to each bar. Panel C: Schematic representation of the p21 promoter−luciferase reporter vectors used in the transactivation experiments of panel D. Panel D: *Drosophila* SL2 cells were cotransfected with the p21 promoter−luciferase constructs shown in panel C (2 μg) in the absence or in the presence of expression vectors pPac-Sp1 (83−778) or pPac-Sp3 (100 ng of each). The fold-transactivation of the various p21 promoter constructs by Sp1 (gray bars) and Sp3 (black bars) is presented in the form of a bar graph and is shown next to each bar. Panel E: Schematic representation of the p21 promoter−luciferase reporter vectors used in the transactivation experiments of panel F. Mutations in elements 1 and 3 are shown with an X. Panel F: *Drosophila* SL2 cells were cotransfected with the p21 promoter−luciferase constructs shown in panel E (2 μg) in the absence or in the presence of expression vectors pPac-Sp1 (83−778) or pPac-Sp3 (100 ng of each). The fold-transactivation of the various p21 promoter constructs by Sp1 (gray bars) and Sp3 (black bars) is presented in the form of a bar-graph and is shown next to each bar. Panel G: SL2 cells were cotransfected with the −2325/+8 p21 luciferase reporter construct (2 μg) in the absence or in the presence of expression vectors pPac-Sp1 (83−778) and pPac-Sp1 (83−702) (ΔD) as indicated at the bottom of the graph. The fold-transactivation of the p21 reporter construct by Sp1 (83−778), Sp1 (83−702) (ΔD), or the combination of the two is shown on top of each bar and is graphed versus the amount of the transfected plasmid DNA for each expression vector. Panel H: Analysis of the levels of expression of Sp1 (83−778), Sp1 (83−702) (ΔD), and Sp3 in SL-2 cells by immunoblotting. SL-2 cells were cotransfected with 17 μg of the vectors pPac-Sp1 (83−778), pPac-Sp1 (83−702) (ΔD), and pPac-Sp3. Forty hours following transfection, cells were lysed, and protein expression was monitored by SDS−PAGE and Western blotting (WB) using polyclonal antibodies specific for these proteins as indicated at the bottom of the panel. The positions of Sp1 (83−778), Sp1 (83−702) (ΔD), and Sp3 proteins are indicated by arrows. Panel I: Schematic representation of the Sp1 (83−778), Sp1 (83−702) (ΔD), and Sp3 proteins used in the transactivation experiments of panels B, D, F, and G. The Ser/Thr-rich and the Gln-rich domains are shown as light gray and dark gray boxes, respectively. The zinc-fingers are shown as three small black squares, and −/+ indicates sequences that modulate the transcriptional activity of Sp1. ID: Inhibitory domain present in Sp3.

type −215/+8 p21 promoter 70- and 65-fold, respectively, whereas mutations in element 1 markedly repressed Sp1- and Sp3-mediated transactivation of the p21 promoter by 77% and 72%, respectively. In contrast, mutations in element 3 slightly enhanced the Sp1- and Sp3-mediated transactivation of the p21 promoter (1.4- and 2.4-fold, respectively) (Figure 1F). Immunoblotting analysis revealed equal levels of expression of Sp1 and Sp3 proteins in SL2 cells (Figure 4H).

In summary, the findings of Figure 4A−F indicated that element 1 (−119/−114) seems to play the most important role in the transactivation of the p21 promoter by Sp1 and Sp3 proteins in the absence of upstream enhancer elements. In the absence of element 1, binding of Sp1 and Sp3 to the

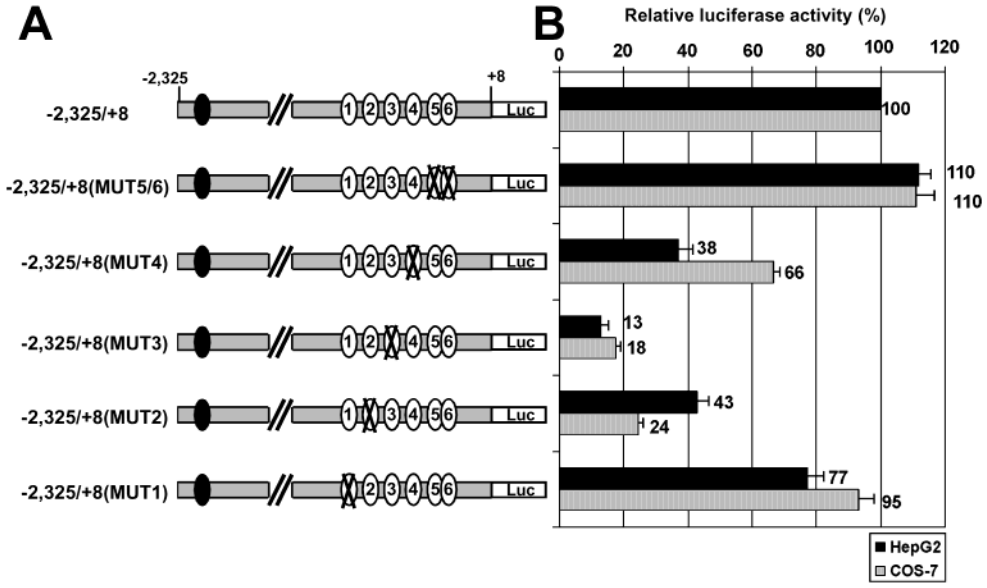

FIGURE 5: (A, B) Role of individual elements 1−6 in the −2325/+8 p21 promoter activity. Panel A: Schematic representation of the wild type −2325/+8 p21 promoter or p21 promoter mutated at individual elements 1−6. Symbols are as in Figure 4A. Mutations in individual elements are shown with an X. Panel B: HepG2 or COS-7 cells were cotransfected with the wild-type or mutant −2325/+8 p21 promoter−luciferase reporter vectors shown in panel A (2 μg). The CMV-β galactosidase plasmid (1 μg) was included in each transfection for normalization of transfection variability. The normalized, relative luciferase activities (mean ± SEM) of each reporter construct in HepG2 (black bars) or COS-7 (gray bars) cells are shown in the form of a bar-graph and were derived from at least three independent experiments performed in duplicate. Actual values are shown next to each bar.

other GC-rich motifs present in the proximal p21 promoter does not seem to contribute significantly to p21 promoter transactivation.

A Mutant Sp1 Protein Lacking Domain D (aa 703−778) Is Less Effective in Transactivating the p21 Promoter. It was shown previously that a mutant Sp1 protein lacking the C-terminal domain D (aa 703−778) (Figure 4I) is able to bind to GC-rich motifs and transactivate promoters containing single Sp1 binding sites as efficiently as the wild-type Sp1 but is unable to transactivate cooperatively promoters that contain multiple Sp1 binding sites (*30*). As shown in Figure 4G, deletion of domain D of Sp1 repressed the transactivation of the −2325/+8 p21 promoter by 75% (12-fold compared to 47-fold transactivation achieved by wt Sp1). This finding suggested that domain D of Sp1, which is involved in cooperativity between different Sp1 molecules bound to adjacent elements, is required for the high levels of transactivation of the p21 promoter by Sp1. Furthermore, coexpression of the (83−702) Sp1 mutant lacking domain D along with wild-type Sp1 in SL2 cells repressed the Sp1-mediated transactivation of the −2325/+8 p21 promoter in a dose-dependent manner (Figure 4G). The Sp1 (83−702) mutant bound to the p21 (−86/−70) probe as efficiently as wild-type Sp1 when expressed in SL2 cells, in agreement with previous findings (*30*) (data not shown). Immunoblotting analysis revealed equal levels of expression of Sp1 and Sp1 (83−702) proteins in SL2 cells (Figure 4H). The findings of Figure 4G suggested that the Sp1 (83−702) form, which lacks domain D, acts as a dominant negative mutant and represses p21 promoter activity possibly due to the competition with wild-type Sp1 for the same binding elements of the p21 promoter.

Sp1 Elements 1−6 Are Not Functionally Equivalent in Mammalian Cells. The experiments of Figure 4 were designed in order to evaluate the contribution of individual Sp1 binding sites in the Sp1- and Sp3-mediated transactivation of the p21 promoter in the absence of upstream enhancer elements. However, the proximal −143/+8 region of the p21 promoter that contains elements 1−6 contributes only by 10% to the hepatic activity of this promoter, the next 90% being contributed by upstream enhancer elements (*22* and Figure 4B). To determine the effect of mutations at individual GC-rich motifs of the proximal p21 promoter activity in the presence of upstream enhancer elements, the mutations in elements 1−6 shown in Figure 1A were introduced into the (−2325/+8) p21-Luc reporter construct (Figure 5A). The activity of each mutated promoter in HepG2 cells and COS-7 fibroblasts was evaluated by transient transfections and luciferase assays. As shown in Figure 5B, mutations in element 5/6 (MUT 5/6) did not affect basal p21 promoter activity. In fact, this mutation caused a slight increase in p21 promoter activity (110% relative to 100% of the wt promoter). This finding suggests that Sp1 and Sp3 factors bound to the very proximal element 5/6 either may not contribute to p21 promoter activity or may play a minor inhibitory role. In contrast, mutations in elements 4, 3, 2, and 1 caused a significant reduction in p21 promoter activity in both cell lines tested. Specifically, mutations in element 4 reduced p21 promoter activity to 38% and 66% relative to the control in HepG2 and COS-7 cells, whereas mutations in element 3 had the most dramatic phenotype, reducing p21 promoter activity to 13% and 18% in HepG2 and COS-7 cells, respectively. Mutations in element 2 reduced p21 promoter activity to 43% and 28% relative to the control in HepG2 and COS-7 cells, respectively. Finally, mutations in the more distal element 1 had the less severe effect, causing a 23% and 5% reduction in p21 promoter activity in HepG2 and COS-7 cells, respectively (Figure 5B).

Collectively, the data presented in Figure 5B indicate that the Sp1/Sp3 elements 1−6 of the p21 promoter are not

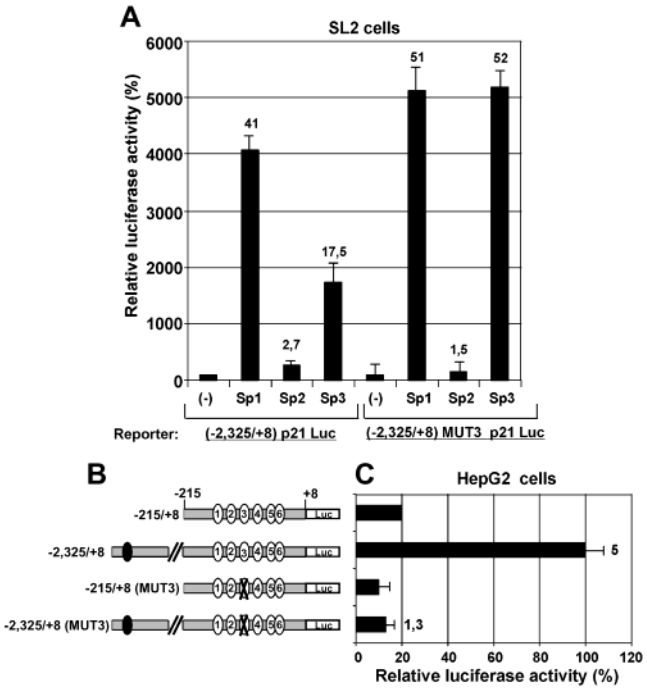

FIGURE 6: (A−C) Role of element 3 in the transactivation of the p21 promoter by Sp1 and Sp3 or by upstream enhancer sequences. Panel A: SL2 cells were cotransfected with the −2325/+8 p21 or −2325/+8 MUT3 luciferase reporter construct (2 μg) in the absence or in the presence of expression vectors pPac-Sp1, pPac-Sp2, or pPac-Sp3 (100 ng) as indicated at the bottom of the graph. The fold-transactivation of the p21 reporter construct by Sp1, Sp2, or Sp3 is shown on top of each bar. Panel B: Schematic representation of the wild-type or mutated p21 promoter constructs used in the transfections of panel C. Panel C: HepG2 cells were transfected with the wild-type or mutant p21 promoter−luciferase reporter vectors shown in panel B (2 μg). The fold-transactivation of the wild-type or mutant proximal p21 promoters by upstream enhancer regions is shown next to the corresponding bars.

functionally equivalent in two different cellular environments, hepatocytes and fibroblasts.

Element 3 of the Proximal p21 Promoter Is Critical for the Functional Synergism between Sp1 and Sp3 and Factors Bound to Upstream Enhancer Sequences. The mutagenesis analysis of the p21 promoter presented in Figure 5B clearly indicated that element 3, located at position −82/−77 of the p21 promoter, is functionally the most important element for hepatic p21 promoter activity. The dramatic (87%) reduction in p21 promoter activity caused by mutations at element 3 could be attributed either to the inability of Sp1 and Sp3 proteins to transactivate the (−2325/+8) p21 promoter or to the inability of Sp1 and Sp3 proteins to functionally synergize with factors bound to upstream −2325/−215 p21 enhancer regions. To distinguish between the two possibilities, transient transfections were performed in SL2 and HepG2 cells. First, the ability of the different Sp family members to transactivate the p21 promoter in SL2 cells was investigated. As shown in Figure 6A, ectopic expression of Sp1 and Sp3 proteins in SL2 cells caused a 41-fold and 17.5-fold transactivation of the (−2325/+8) p21 promoter whereas ectopic expression of Sp2 caused a minor 2.7-fold transactivation. The inability of Sp2 to transactivate the p21 promoter is in agreement with the findings of Figure 1 showing that Sp2 could not bind to p21 promoter sequences. Thus, mutations in element 3 do not appear to affect the ability of Sp1 and Sp3 to transactivate the p21 promoter when these proteins are overexpressed in SL2 cells.

On the other hand, the strong negative effect of mutation in element 3 on the hepatic activity of the −2325/+8 p21 promoter could be due to the loss of synergism between Sp1 and Sp3 with other factors expressed in HepG2 cells bound to distal p21 enhancer sequences. To test this hypothesis, p21 promoter constructs bearing the mutations in element 3 in the presence or in the absence of the upstream enhancer region (Figure 6B) were utilized in transient transfection experiments in HepG2 cells. As shown in Figure 6C, the mutation in element 3 caused a 50% reduction in the −215/+8 p21 promoter activity in the absence of the distal region. However, the activity of the proximal −215/+8 p21 promoter mutated at element 3 could not be enhanced further by the upstream −2325/−215 p21 region. In contrast, the wild-type −215/+8 p21 promoter was enhanced 5-fold by the upstream enhancer region. The findings of Figure 6C suggested that mutations in element 3 disrupted the synergistic interactions between Sp1 and Sp3 factors bound to the proximal p21 promoter element 3 and factors bound to the upstream −2325/-215 region.

Smad Proteins Transactivate the p21 Promoter by Functionally Cooperating with Sp1 but Not with Sp3. We have shown previously that the proximal p21 promoter mediated the transactivation of this promoter by TGFβ and Smad proteins in HepG2 cells (*22*). We sought here to investigate the relative contribution of Sp1 and Sp3 factors as well as of elements 1−6 in the transactivation of the p21 promoter by Smad3 and Smad4 proteins. As shown in Figure 7A, Smad3 and Smad4 proteins expressed in HepG2 cells transactivated the (−2325/+8) p21 promoter 2.5-fold whereas deletion of the −125/−60 region abolished the Smad-mediated transactivation of the p21 promoter, in agreement with previous findings (*22*). To investigate the role of Sp1 and Sp3 proteins in the Smad-mediated transactivation of the p21 promoter, transient transfections were performed in SL2 cells. As shown in Figure 7B, ectopic expression of Smad3 and Smad4 proteins in SL2 cells did not have any effect on the activity of the −2325/+8 p21 promoter. In contrast, Smad3 and Smad4 proteins strongly transactivated the p21 promoter in the presence of Sp1 (125-fold transactivation in contrast to 35-fold transactivation caused by Sp1 alone). Furthermore, deletion of the −125/−60 p21 promoter region abolished the synergistic transactivation of the p21 promoter by Sp1 and Smad proteins (Figure 7B).

Synergism between Sp1 and Smad3/Smad4 proteins was also observed using the proximal −143/+8 p21 promoter lacking the upstream region. As shown in Figure 7C, coexpression of Sp1 and Smad3/Smad4 caused a potent 1575-fold transactivation of the p21 promoter, in contrast to the 150-fold and 2-fold transactivation achieved when Sp1 and Smad3/Smad4 proteins were expressed separately. However, in contrast to Sp1, the Sp3-mediated transactivation of the p21 promoter could not be significantly enhanced by Smad3/Smad4 proteins in SL2 cells (Figure 7C). These findings indicate that the transactivation of the p21 promoter by Smad3/Smad4 proteins in mammalian cells is mediated exclusively by Sp1.

Transactivation of the p21 Promoter by Smad Proteins Is Not Affected by Mutations at Individual Elements 1−6. We

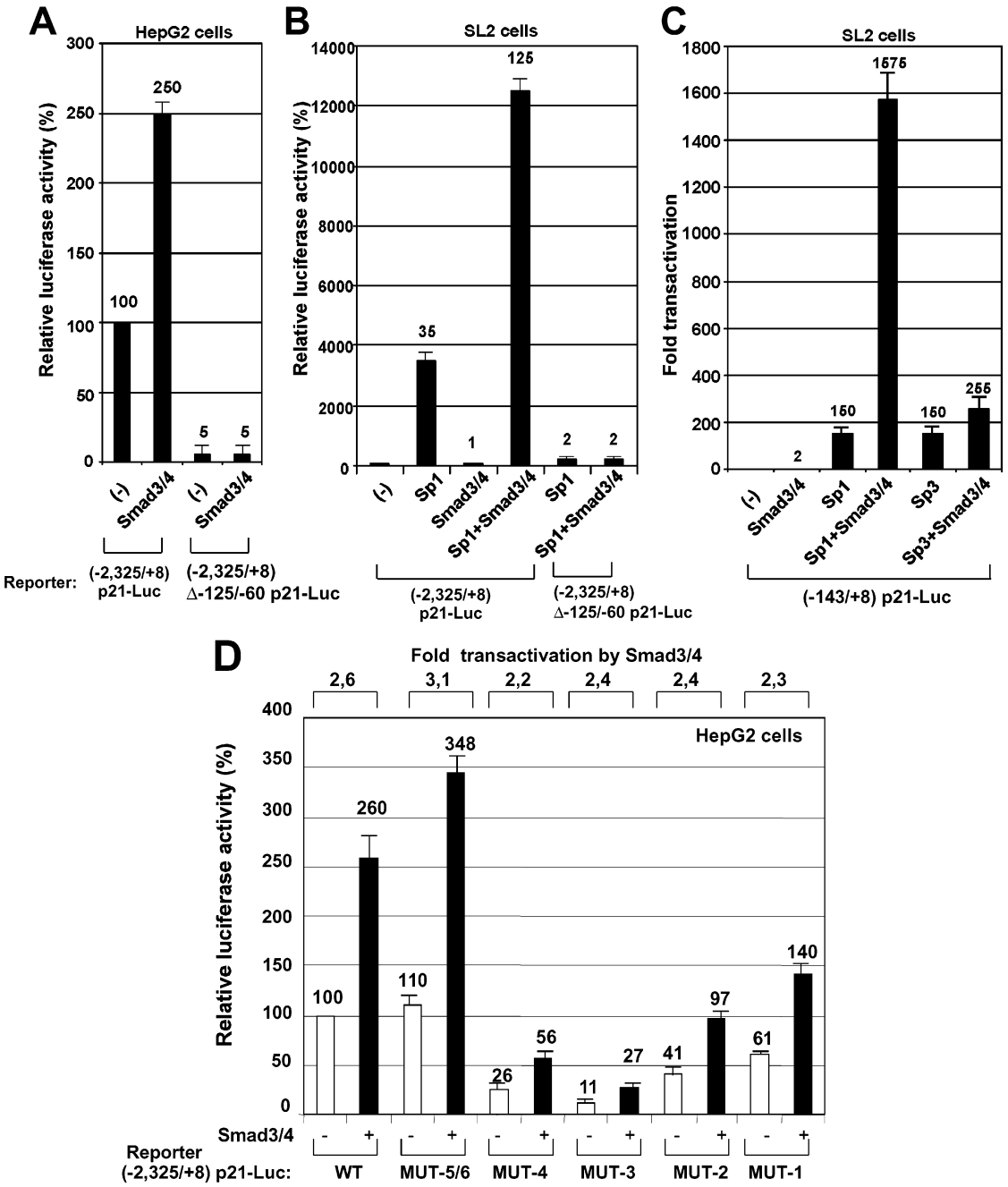

FIGURE 7: (A−D) Transactivation of the p21 promoter by Smad proteins is mediated by Sp1 but not by Sp3 and is not affected by mutations at individual elements 1−6. Panel A: HepG2 cells were cotransfected with the (−2325/+8) p21 Luc or the (−2325/+8) Δ −125/−60 p21 Luc constructs (1 μg) either alone (−) or in combination with 1 μg each of the expression vectors for human Smad3 and Smad4 proteins as indicated at the bottom of the graph. Actual values are shown on top of each bar. Panel B: SL2 cells were cotransfected with the (−2325/+8) p21 Luc or the (−2325/+8) Δ −125/−60 p21 Luc constructs (1 μg) either alone (−) or in combination with the vector pPac-Sp1 (50 ng) and the expression vectors for human Smad3 and Smad4 proteins (1 μg of each) as indicated at the bottom of the graph. The fold-transactivation of the p21 promoter by Sp1, Smad3/Smad4 independently or in combination is shown on top of each bar. Panel C: SL2 cells were cotransfected with the (−143/+8) p21 Luc construct (1 μg) either alone (−) or in combination with vectors pPac-Sp1 or pPac-Sp3 (50 ng) and expression vectors for human Smad3 and Smad4 proteins (1 μg of each) as indicated at the bottom of the graph. The fold-transactivation of the p21 promoter by Sp1, Sp3, and Smad3/Smad4 independently or in combination is shown on top of each bar. Panel D: HepG2 cells were cotransfected with the wild-type or mutant −2325/+8 p21 promoter−luciferase reporter vectors indicated at the bottom of the graph (1 μg) either alone (−, white bars) or in combination with expression vectors for human Smad3 and Smad4 proteins (1 μg of each) (+, black bars). Actual values are shown on top of each bar. The fold-transactivation of each p21 reporter construct by Smad3 and Smad4 proteins is shown on top of the graph.

next tested the relative contribution of elements 1−6 to the Smad-mediated transactivation of the p21 promoter in HepG2 cells. For that purpose, the wt p21 promoter and the p21 promoters mutated at elements 1−6 were cotransfected into HepG2 cells in the absence or in the presence of expression vectors for human Smad3 and Smad4 proteins, and their activity was determined by luciferase assays. As shown in Figure 7D, all mutated p21 promoters were transactivated by Smad3 and Smad4 proteins to a similar extent as the wt promoter (2−3-fold). Thus, the transactivation of the p21

promoter by Smad proteins was not affected by mutations at individual elements 1−6.

DISCUSSION

High Levels of Human p21 Promoter Activity in Hepatocytes Are Determined by the Functional Synergism between Proximal and Distal Regulatory Regions. In our previous studies, we established that high levels of p21 promoter activity in HepG2 cells are determined by the combination of distal enhancer as well as proximal promoter elements (*22*). We showed that the distal −2325/−2260 p21 promoter region binds with high affinity the p53 tumor suppressor protein (*8*). A single copy of this region was sufficient to induce high levels of activity when placed next to the proximal p21 promoter region −215/+8 (*8*). Additional factors bound to the −2260/−215 p21 promoter region also contribute to hepatic p21 gene transcription since deletion of this region reduced drastically p21 promoter activity to 20% relative to the control in HepG2 cells (*22*). Responsive elements for vitamin D (−779/−765), retinoic acid (−1212/−1194), dexamethasone (−1270/−1256), vitamin E (−1928/−1920), and interleukin 6 (−696/−688) have been identified and characterized (*23* and references cited therein). On the other hand, an internal deletion of the proximal −125/−60 region of the p21 promoter that contains multiple binding sites for members of the Sp1 family abolished p21 promoter activity in HepG2 cells despite the presence of the distal enhancer regions (*22* and this study). These studies indicated that the functional synergism between p53 and other factors bound to distal p21 promoter regions and Sp1 and related factors that occupy proximal elements is very important for optimal p21 promoter activity in liver cells and possibly in other cell types as well.

The GC-Rich Motifs of the Proximal p21 Promoter Bind Selectively Sp1 Family Members. The proximal p21 promoter region between nucleotides −125 and −45 contains several GC-rich motifs which are homologous or identical with the classical consensus binding site for the ubiquitous transcription factor Sp1 and related proteins. Several binding sites for the transcription factor E2F as well as three E boxes have also been identified in the same region (*23*).

Previous studies have established that GC boxes, which are present in many ubiquitous, tissue-specific, and viral genes, are occupied by members of the Sp1/XKLF family of transcription factors (*26, 40*). This family consists of at least 16 different mammalian members, all of which contain a highly conserved DNA binding domain with 3 C2H2-type zinc fingers (*26, 40, 41*). A similar motif is found in the *Drosophila melanogaster* regulatory protein Krüppel. Certain members of this family (Sp1, Sp3, Sp4, BTEB1, TIEG2) recognize classical Sp1 binding sites with similar affinities (*28, 42, 43*). Sp2 does not bind to the classical GC box but rather shows a preference for GT elements due to structural differences with the other members of this family (*44*). Similar to Sp2, EKLF, UKLF, and BKLF have a binding preference for GT boxes over classical GC boxes (*45*−*48*).

Using gel mobility shift, supershift, and competition experiments, we established that the six GC-rich motifs of the proximal p21 promoter bind exclusively Sp1 and Sp3 (and a truncated form of Sp3) with different affinities (Figures 1−3). The affinity of Sp1 and Sp3 for these sites follows the order: element 3 > element 1 = element 4 = element 5/6 ≫ element 2 (Figure 2B). These results indicated that sequences flanking the classical GC-rich motifs also determine the binding characteristics, i.e., the affinity and specificity, of Sp1 and related proteins for these motifs. The truncated form of Sp3 was not observed in gel mobility shift experiments using extracts from SL2 cells transfected with an expression vector for human Sp3 (Figures 1C and 3B), suggesting that this protein possibly represents an isoform of Sp3 existing in hepatic nuclear extracts. This is in agreement with previous studies showing that in addition to the full-length, 115 kDa, Sp3 protein, two smaller 70−80 kDa Sp3 species exist in mammalian cells which arise from internal translation initiation sites (*26*). However, the role of all these Sp3 species in eukaryotic gene regulation remains uncertain.

The close proximity of the six GC-rich elements of the proximal p21 promoter prompted us to investigate potential mutual exclusivity or cooperativity in binding of Sp1 and Sp3 to these sites. Mutually exclusive binding of Sp1 factors to the p21 promoter could result from steric hindrance, especially in cases where the sites are extremely close to each other such as elements 5 (−60/−55) and 6 (−55/−50). In fact, these two sites are overlapping, each site sharing at least one nucleotide with the other. In gel mobility shift assays, a p21 probe containing both elements 5 and 6 (p21 −63/−45) formed complexes with identical electrophoretic mobilities as the complexes formed by p21 probes containing single elements such as p21 (−87/−70) (element 3) or p21 (−124/−109) (element 1) (Figure 1E). A similar observation was made using an oligonucleotide containing both elements 1 and 2 (−123/−97). In gel mobility shift assays, this probe formed complexes which had identical electrophoretic mobility with the complexes formed by p21 probes containing only element 1 (−124/−109) (Figure 1E). The competition analysis of Figures 2 and 3 and the transactivation experiments of Figure 4 clearly established that element 1 is a strong binding site for Sp1 and Sp3 and plays the most important role in the transactivation of the p21 promoter by these two factors. In contrast, element 2, which contains a sequence with perfect homology with the consensus 5′ GGGCGG 3′ binding site for Sp proteins, is an extremely weak binding site for Sp1 and Sp3 (Figures 1−3). Despite the fact that binding of Sp1 and Sp3 to element 2 is almost undetectable in gel electrophoretic mobility shift assays, mutagenesis of element 2 abolished binding of Sp1 and Sp3 from the −123/−97 p21 probe (Figure 1E) and reduced significantly the activity of the −2325/+8 p21 promoter in HepG2 and COS-7 cells [by 57% and 76%, respectively (Figure 5B)]. Unexpectedly, this mutation (MUT2) had a more severe effect on the activity of the p21 promoter than mutations in the adjacent element 1 (MUT1) which is a strong binding site [23% and 5% reduction in HepG2 and COS-7 cells, respectively (Figure 5B)]. Based on these observations, we are tempted to speculate that the specific nucleotide substitutions introduced into element 2 not only affect the binding of Sp proteins to this specific site but somehow interfere with binding of Sp factors to adjacent elements as well. This interference could be the result of changes in the conformation of the DNA in the vicinity of element 2 caused by the nucleotide substitutions incorporated into this element.

It was shown previously that a mutant Sp1 that lacks the C-terminal domain D (amino acids 703−778) (Figure 4I) binds to GC motifs as efficiently as wild-type Sp1 but cannot transactivate, in a synergistic manner, promoters that contain multiple Sp1 binding elements (*30*). In transient transfection experiments in SL2 cells (Figure 4G), we showed that the Sp1 (83−702) mutant transactivated the −2325/+8 p21 promoter 12-fold. This transactivation was 75% lower than the transactivation achieved by wild-type Sp1 (47-fold). Furthermore, the Sp1 mutant lacking domain D acted in a dominant negative manner and repressed the Sp1-mediated transactivation of the p21 promoter in SL2 cells possibly by antagonizing with Sp1 for the same elements present on the p21 promoter (Figure 4G). These findings suggested that the strong transactivation of the p21 promoter by Sp1 may result from cooperative interactions between Sp1 molecules bound simultaneously to multiple sites.

Another issue that remains to be clarified is the role played by element 3 in the regulation of the p21 promoter by Sp proteins. It was shown in Figures 4F and 6A that mutagenesis of element 3 in the context of the −215/+8 or the −2325/+8 p21 promoter increased the Sp1- and Sp3-mediated transactivation of the p21 promoter in SL2 cells. On the other hand, the same mutations in element 3 caused a dramatic 90% reduction in basal −2325/+8 p21 promoter activity in HepG2 cells (Figure 5B). The difference in results with p21 promoter mutated in element 3 in the two experimental systems (HepG2 and SL2 cells) could be due to the relative expression level of the Sp1 and Sp3 proteins in these systems. It is possible that the mutation in element 3 has a negative effect on Sp1- and Sp3-mediated transactivation of the p21 promoter when these two transactivators are expressed at lower, perhaps more physiological, concentrations, as is the case in HepG2 cells, but not when these proteins are overexpressed in SL2 cells.

Alternatively, the contradictory findings regarding the role of element 3 in p21 promoter regulation could be attributed to the functional status of Sp1 or Sp3 molecules occupying this specific site in different cell environments. Previous studies have shown that Sp3 contains a repression function in domain C located between the second glutamine-rich activation domain and the first zinc finger domain (*49*). This repression domain includes a lysine residue, which is acetylated in vivo and in vitro by CBP and p300 but not by P/CAF (*27*). Interestingly, acetylation of Sp3 correlates with its transactivation potential. Thus, acetylated Sp3 acts as an inhibitor whereas nonacetylated Sp3 is a potent transactivator (*27*). Interestingly, strong enhancement in Sp1-mediated transactivation of the p21 promoter in SL2 cells was observed by an Sp1 mutant lacking domain C (Koutsodontis et al., unpublished results). These findings suggested that acetylation of Sp1 or Sp3 proteins bound to element 3 of the proximal p21 promoter could play an important regulatory role under basal or stimulatory conditions.

Contribution of Individual Sp1/Sp3 Binding Elements to the Smad-Inducible Activity of the p21 Promoter. We have shown previously that certain members of the Smad family of TGFβ signaling effectors transactivate the p21 promoter in HepG2 cells via physical and functional interactions with Sp1 bound to the proximal −125/−60 p21 promoter region (*22*). In another study, it was found that TGFβ induces p21 gene transcription in HaCaT cells via element 3, which was termed TβRE (TGFβ responsive element) (*18*). It was subsequently reported that the response of p21 gene transcription to TGFβ in these cells is mediated by the MEK pathway (*50*).

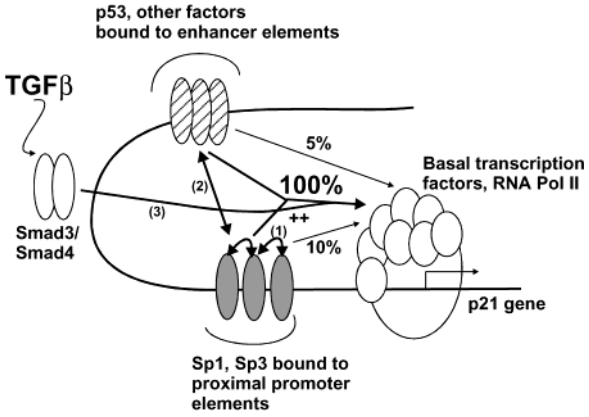

FIGURE 8: Schematic representation showing protein−protein interactions that could account for the hepatic activity of the human p21 promoter. These interactions include: (1) cooperative interactions between Sp1 and Sp3 proteins bound to the proximal promoter; (2) functional interactions between Sp1 and Sp3 with p53 and other factors bound to distal p21 enhancer elements; and (3) physical and functional interactions between Sp1 and soluble factors such as Smad3 and Smad4 proteins under cell stimulatory conditions. Maximal hepatic promoter activity (100%) is obtained by various combinations of factors acting on the proximal p21 promoter region that binds Sp1 and Sp3.

In the present study, we showed that Smad3/Smad4 proteins transactivated the p21 promoter in SL2 cells only in the presence of Sp1 (Figure 7C). This finding is in agreement with a previous study showing that a GAL4-Sp1 hybrid protein could be transcriptionally induced by TGFβ in HaCaT cells whereas GAL4-Sp3 could not (*20*). Thus, our previous and present findings suggest that the 2-fold transactivation of the −2325/+8 p21 promoter by Smad proteins in HepG2 cells is due to the functional cooperation between Smads and Sp1 bound to the proximal p21 promoter.

The data presented in Figure 7D indicated that none of the individual elements 1−6 were absolutely required for the transactivation of the −2325/+8 p21 promoter by the Smad3/Smad4 proteins, suggesting that mutagenesis of individual elements was possibly compensated by the interaction of Smads with Sp1 molecules bound to adjacent elements. However, none of the p21 promoters mutated at elements 1−4 had levels of activity comparable to the activity of the wild-type p21 promoter in HepG2 cells, even in the presence of Smad3 and Smad4 proteins (Figure 7D). These findings suggest that cellular responses to the growth inhibitory function of TGFβ could be determined by the basal activity of the p21 promoter in the responsive cells.

Figure 8 is a schematic representation of the regulatory interactions that occur on the p21 promoter and determine the basal and the TGFβ-inducible activity of this promoter in HepG2 and possibly other cell types. According to this model, Sp1 and Sp3 molecules bound to multiple elements present in the proximal p21 promoter contribute by 10% to basal p21 promoter activity in the absence of distal enhancer regions. Deletion of the proximal elements 1−4 caused a 95% reduction in hepatic p21 promoter activity (Figure 7A), suggesting that the factors that bind to the upstream enhancer elements contribute 5% to the promoter activity in the

absence of the proximal GC-rich motifs. Optimal p21 promoter activity (100%) is accomplished by functional interactions between factors bound to proximal and distal elements. Functional interactions between Sp1 and p53 have been established recently by our group (*8*). The p21 promoter activity could be further enhanced by soluble factors such as certain members of the Smad family that physically and functionally interact with Sp1 bound to the proximal region. Thus, the basal activity of the p21 promoter determines the magnitude and the effectiveness of cellular responses to different extracellular stimuli such as TGFβ. Understanding in detail the transcriptional regulatory interactions occurring on the p21 promoter could allow the development of therapeutic strategies for the management of uncontrolled cellular proliferation, which is caused by the escape of cells from differentiation programs and leads to cancer.

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