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Wild-type and mutant p53 differentially regulate transcription of the insulin-like growth factor I receptor gene

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ABSTRACT **The insulin-like growth factor I receptor (IGF-I-R) plays a critical role in transformation events. It is highly overexpressed in most malignant tissues where it functions as an anti-apoptotic agent by enhancing cell survival. Tumor suppressor p53 is a nuclear transcription factor that blocks cell cycle progression and induces apoptosis. p53 is the most frequently mutated gene in human cancer. Cotransfection of Saos-2 (osteosarcoma-derived cells) and RD (rhabdomyosarcoma-derived cells) cells with IGF-I-R promoter constructs driving luciferase reporter genes and with wild-type p53 expression vectors suppressed promoter activity in a dose-dependent manner. This effect of p53 is mediated at the level of transcription and it involves interaction with TBP, the TATA box-binding component of TFIID. On the other hand, three tumor-derived mutant forms of p53 (mut 143, mut 248, and mut 273) stimulated the activity of the IGF-I-R promoter and increased the levels of IGF-I-R/luciferase fusion mRNA. These results suggest that wild-type p53 has the potential to suppress the IGF-I-R promoter in the postmitotic, fully differentiated cell, thus resulting in low levels of receptor gene expression in adult tissues. Mutant versions of p53 protein, usually associated with malignant states, can derepress the IGF-I-R promoter, with ensuing mitogenic activation by locally produced or circulating IGFs.**

The insulin-like growth factor I receptor (IGF-I-R) is a membrane-bound heterotetramer with ligand-induced tyrosine kinase activity (1, 2). IGF-I-R is constitutively expressed by most tissues, where it mediates the trophic and differentiative actions of the IGFs, IGF-I and IGF-II (3–5). The central role of the IGF-I-R during the cell cycle is demonstrated by the fact that overexpression of this receptor in BALB/c3T3 fibroblasts abrogates all requirements for exogenous growth factors (6). Furthermore, deletion of the IGF-I-R in mice by homologous recombination results in nonviable offspring (7, 8). In addition, there is compelling evidence that the IGF-I-R has a pivotal role in malignancy (9, 10). Thus, it is highly overexpressed in most tumors and cancer cell lines and, furthermore, fibroblast cell lines established from mouse embryos lacking the IGF-I-R cannot be transformed by any of a number of oncogenes, including the simian virus 40 large T antigen, activated *ras*, and others (11, 12).

The regulatory region of the IGF-I-R gene comprises a unique "initiator" motif, from which transcription is initiated *in vivo*, and that acts in concert with upstream Sp1 sites (13, 14). The region flanking the transcription start site is extremely G+C rich, with no obvious TATA or CAAT elements (14–17). When measured in transient transfection assays, the IGF-I-R promoter displays very high basal activity (14). Paradoxically, the expression of the receptor gene in normal adult tissues is extremely low (18), suggesting that in the postmitotic, fully differentiated cell the IGF-I-R promoter is under constitutive inhibitory control.

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Wild-type (wt) p53 is a tumor suppressor gene product that, in its hyperphosphorylated state, blocks progression of cells through the cell cycle (19). p53 is localized to the nucleus, where it functions as a DNA sequence-specific transcription factor (20, 21). It has been shown that p53 can either activate or suppress the activity of a number of target genes. Gene activation usually involves interaction of p53 with a specific consensus sequence, whereas it is thought that gene suppression involves interaction of p53 with the basal transcription machinery (22–24).

Mutations in the p53 gene are the most frequent mutations in human cancer (25, 26). The vast majority of these mutations occur in the central domain of the p53 molecule, which is the region involved in DNA binding. Because the IGF-I-R gene is highly overexpressed in most malignancies in which p53 is mutated, we investigated the potential regulation of the IGF-I-R gene by wt and mutant p53. The results obtained indicate that mutant p53 proteins have a stimulatory effect on promoter activity, whereas wt p53 suppresses the activity of the IGF-I-R promoter. These effects of p53 seem to involve its interaction with components of the basal transcription machinery. Due to the central role of the IGF-I-R in cell cycle progression and transformation, de-repression of IGF-I-R promoter by mutant p53 may constitute an important paradigm in tumorigenesis.

MATERIALS AND METHODS

Cell Cultures, Plasmids, and DNA Transfections. Saos-2 cells were obtained from the American Type Culture Collection. Saos-2 is a human osteogenic sarcoma-derived cell line in which both p53 alleles are deleted (27). RD is a human rhabdomyosarcoma cell line that exhibits a mutant p53 gene (Arg → Trp transition at codon 248) (28). RD cells were kindly provided by Lee Helman (National Cancer Institute, Bethesda, MD). Saos-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 5% calf serum. RD cells were grown in DMEM plus 10% FBS.

For transient cotransfection experiments, the following fragments of the IGF-I-R gene were subcloned upstream of a promoterless firefly luciferase reporter gene (pOLUC): −2350/+640, −476/+640, −455/+30, and −40/+640 (nucleotide 1 corresponds to the transcription initiation site). The basal promoter activity of these plasmids has been previously reported (14, 29).

A wt p53 expression vector (pCB6.p53) was constructed by inserting an ≈1.6 kb *Xba*I fragment of the human p53 into the cytomegalovirus (CMV)-containing plasmid pCB6+ (30). An additional wt p53 (pC53-SN3) and three mutant expression vectors were kindly provided by Edward Mercer (Thomas

Abbreviations: IGF, insulin-like growth factor; IGF-I-R, insulin-like growth factor I receptor; wt, wild type; CMV, cytomegalovirus; β-gal, β-galactosidase; GST, glutathione *S*-transferase; TBP, TATA box-binding protein; f.p.u., footprint unit.

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Jefferson University, Philadelphia). pC53-SN3 encodes wt p53 in the pCMV-Neo-Bam vector (31). pC53-SCX3 encodes a mutant p53 harboring a Val → Ala mutation at position 143. pC53-248W contains an Arg → Trp mutation at position 248 and pC53-273H is a mutant p53 in which an Arg residue at position 273 was mutated to His. All three mutant p53 are in the pCMV-Neo-Bam expression vector.

Both cell lines were transiently transfected by the calcium phosphate method. For Saos-2 cells, we used a kit from 5 Prime–3 Prime Inc.; each 100-mm dish received 10 μg of reporter plasmid and 2.5 μg of expression vector. RD cells were transfected following the protocol described by Chen and Okayama (32), using 5 μg each of reporter and expression plasmids and 15 μg of salmon sperm DNA. Cells were harvested 48 h (Saos-2) or 72 h (RD) after transfection, and luciferase activities were measured as described (14).

In preliminary experiments, cells were cotransfected with a CMV-β-gal vector (β-gal, β-galactosidase), but since expression from the CMV promoter was found to be affected by p53, subsequent experiments were normalized to total protein, which was measured using a Bio-Rad kit. In a number of pilot experiments, normalization for transfection efficiency was done using a RAS-β-gal plasmid (33), kindly provided by Ronald Evans (The Salk Institute, San Diego). The levels of β-gal generated by this plasmid were not affected by p53 and the results obtained were essentially the same as those obtained using protein normalization.

Luciferase mRNA Measurements. After (48 h) transient transfection, Saos-2 cells were lysed in 4 M guanidinium isothiocyanate containing 0.01% 2-mercaptoethanol, and total RNA was prepared according to Chirgwin *et al.* (34). The integrity of the RNA was assessed by ethidium bromide staining of the 28S and 18S ribosomal RNA bands after gel electrophoresis. The levels of luciferase mRNA were determined by solution hybridization/RNase protection assay using 25 μg of total RNA as previously described (18). An antisense luciferase RNA probe was generated by linearization of the pGEM-luc DNA vector (Promega) with *Eco*RV and transcription with T7 RNA polymerase in the presence of [α-^{32}P]UTP. Hybridization of this 413-b probe with luciferase RNA results in a protected band of ≈365 bp. As an internal control, an 18S ribosomal antisense RNA probe that was labeled using the MEGAshortscript T7 kit (Ambion) was included in the hybridization reaction.

GST.p53 Preparation. Purified p53 protein was prepared as a glutathione *S*-transferase (GST) fusion protein. GST.p53 and GST plasmids (in pGEX-2 vector) were transformed into *Escherichia coli* XA-90 strain, and recombinant proteins were induced by addition of 1 mM isopropyl β-D-thiogalactoside (IPTG). Cells were harvested after 3 h, washed with phosphate-buffered saline, and resuspended in 10 ml of extraction buffer containing 50 mM Hepes (pH 7.4), 250 mM NaCl, 1 mM EDTA, 5 mM NaF, 1 mM dithiothreitol (DTT), 5 μg/ml each of aprotinin, leupeptin, and pepstatin A, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), and 1% Nonidet P-40. After sonication and centrifugation, the supernatant was mixed with GST-agarose beads for 2 h at 4°C. Beads were spun down, washed with extraction buffer, and proteins eluted with 25 mM reduced glutathione in extraction buffer. Peak fractions were dialyzed against 20 mM Hepes (pH 7.4), 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.1% Nonidet P-40.

Gel Retardation Assays. A fragment of the IGF-I-R promoter (−40 to + 115) encompassing the *in vivo* transcription initiation site was isolated by digestion of a genomic DNA clone with *Pml*I and *Xho*I. After purification from agarose gels, the fragment was dephosphorylated using calf intestinal phosphatase, and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The labeled probe was separated from unincorporated nucleotides using Elu-Tip columns (Schleicher and Schuell). Recombinant TATA box-binding protein (TBP) was purchased from Promega and used at a concentration of 1 footprint unit (f.p.u.) per reaction.

Gel retardation assays were performed by preincubating TBP protein, GST.p53 protein (100 ng), or a combination of both proteins, in 9 μl of 20 mM Hepes (pH 7.5), 70 mM KCl, 12% glycerol, 0.05% Nonidet P-40, 100 μM $ZnSO_4$, 0.05 M DTT, 1 mg/ml bovine serum albumin, and 0.1 mg/ml poly(dI·dC), in the presence or absence of the indicated unlabeled DNA competitor, on ice. After 15 min, 75,000 dpm (20–120 pg) of the labeled fragment was added, and the reaction was incubated for an additional 10 min. Changes in mobility were assessed by electrophoresis through a 5% polyacrylamide gel that was run at 250 V for 2 h at 4°C. After fixation in 10% acetic acid, the gels were autoradiographed at −70°C.

***In Vitro* Transcription Assays.** The DNA template used in *in vitro* transcription reactions includes 476 bp of 5′-flanking region and 640 bp of 5′-untranslated region. The fragment was isolated from vector DNA by *Hin*dIII digestion and purified by agarose gel electrophoresis.

In vitro transcriptions were performed as described (35). Briefly, 500 ng of the DNA template were incubated with HeLa whole-cell extract (12.5 to 44 μg protein) (36) in the presence of 420 μm each of ATP, GTP, and CTP, and 15 μCi of [α-^{32}P]UTP (400 Ci/mmol; 1 Ci = 37 GBq), in a final volume of 18 μl. The composition of the reaction buffer was as follows: 8.33 mM Hepes (pH 7.9), 42 mM KCl, 5.2 mM $MgCl_2$, 42 μM EDTA, 7% glycerol, and 1 mM DTT. Where indicated, α-amanitin was added to a final concentration of 10 μg/ml. Purified GST.p53 fusion protein (or GST control) was added at a concentration of 25 to 150 ng per reaction. Transcription reactions were incubated at 30°C for 1 h, and were terminated by the addition of 20 mM Tris·HCl (pH 7.8), 150 mM NaCl, and 0.2% SDS. Following extraction with phenol-chloroform and precipitation with ethanol, the transcription products were resolved on a 4% polyacrylamide gel containing 7 M urea, which was run at 350 V for 2 h. Wet gels were autoradiographed at −70°C.

RESULTS

Suppression of IGF-I-R Promoter by wt p53. Activation of the IGF-I-R constitutes a basic requirement for progression through the cell cycle. Because wt p53 specifically blocks this process, whereas mutant p53 proteins are unable to halt cell proliferation, we examined whether wt p53 can suppress the activity of the IGF-I-R promoter. For this purpose, coexpression studies were performed by using an IGF-I-R promoter fused to a luciferase reporter gene [p(−2350/+640)LUC], together with wt p53 expression vectors. Transfections were performed in the human osteosarcoma cell line Saos-2 that, due to the lack of endogenous p53, provides a "clean" background for this type of study. As shown in Fig. 1*A*, increasing amounts of pCB6.p53 suppressed promoter activity in a dose-dependent manner. Maximal suppression was seen with 2.5 μg of expression vector, at which concentration promoter activity was 11.7 ± 1.6% (mean ± SEM of six experiments, each in duplicate) of control levels. Likewise, expression vector pC53-SN3 suppressed promoter activity, although to a lesser extent. Thus, at 2.5 μg of input DNA, the activity of the IGF-I-R promoter was 46.9 ± 5.7% (mean ± SEM; $n = 3$ experiments, each in duplicate) of control values (Fig. 1*A*).

Experiments were also performed in the rhabdomyosarcoma cell line, RD (Fig. 1*B*). In this cell line, pCB6.p53 reduced promoter activity to 25.4 ± 3.1% of control levels (mean ± SEM, $n = 6$), whereas pC53-SN3 reduced it to 58.8 ± 5.2% ($n = 2$).

Transcriptional Repression of IGF-I-R/Luciferase Fusion mRNA by wt p53 *in Vivo*. To establish whether the specific repressive effect of wt p53 *in vivo* was indeed mediated at the level of transcription, RNA was prepared from Saos-2 cells that were transiently transfected with pCB6.p53, and the levels of luciferase mRNA were measured by means of a sensitive

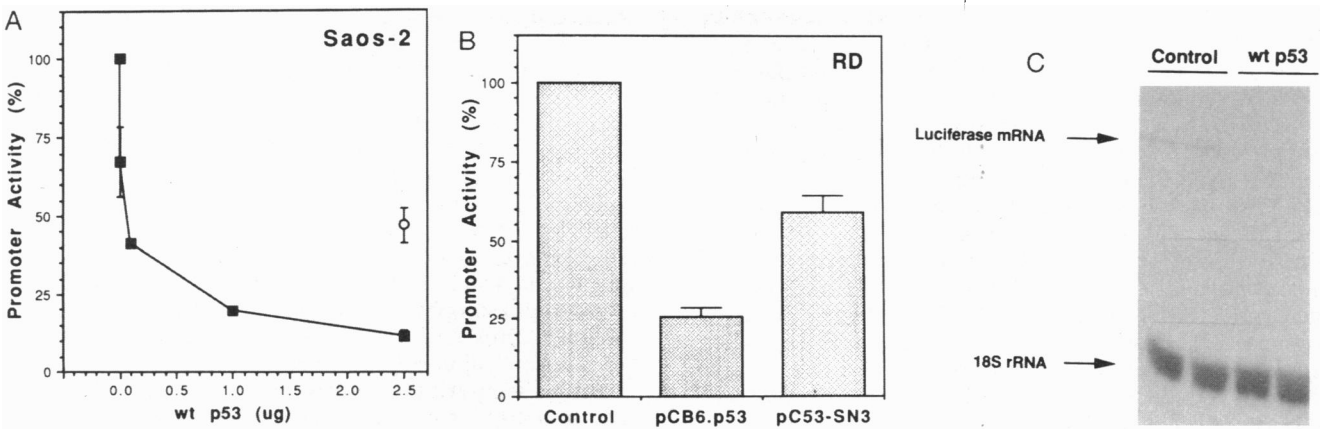

FIG. 1. Regulation of IGF-I-R promoter activity by wt p53. (*A*) The reporter plasmid p(−2350/+640)LUC (10 μg) was cotransfected into Saos-2 cells with increasing amounts of the wt p53 expression vector pCB6.p53 (■) or with 2.5 μg of pC53-SN3 (○) using the calcium phosphate method. The values of luciferase activity shown are expressed as a percentage of the levels seen in the absence of p53. Experiments were performed between three and six times, each time in duplicate. Where not shown, the SEM bars are smaller than the symbol size. (*B*) p(−2350/+640)LUC (5 μg) and pCB6.p53 or pC53-SN3 (5 μg) were cotransfected into RD cells, and luciferase activity was measured after 72 h. The results are expressed as percentage of the luciferase levels generated by cotransfecting the empty expression vectors (pCB6+ or pCMV-Neo-Bam, respectively). Experiments were done between two and six times, each in duplicate. (*C*) Saos-2 cells were transiently cotransfected with p(−2350/+640)LUC and pCB6.p53 as indicated above, lysed in 4 M guanidinium thiocyanate after 48 h, and the levels of the IGF-I-R/luciferase fusion mRNA were measured by solution hybridization/RNase protection assay. The autoradiogram was exposed for 17 days.

solution hybridization/RNase protection assay. As shown in Fig. 1*C*, wt p53 reduced the levels of luciferase mRNA by ≈70%. Because wt p53 did not affect the levels of the constitutively expressed 18S ribosomal RNA (Fig. 1*C*), we can infer that the effect of p53 on luciferase mRNA is not the result of a generalized transcriptional "switch-off."

Localization of the Promoter Region Responsible for Transcriptional Repression. A consensus DNA binding half site for wt p53 has been identified (Pu-Pu-Pu-C-A/T-T/A-G-Py-Py-Py) and shown to mediate most of the gene stimulatory effects of wt p53 (37). Sequencing analysis of the IGF-I-R promoter region (including 2.3 kb of 5′-flanking region and the complete 943-bp 5′-untranslated region) revealed the presence of multiple sites highly related to the consensus sequence. Fig. 2 *Upper* shows the location of sites that conform to the consensus sequence at least at 8 of 10 nucleotides in each half site.

To examine whether the presence of those potential p53 binding sites correlated with the effect of p53, coexpression studies were performed using IGF-I-R promoter/reporter plasmids containing different portions of 5′-flanking and 5′-untranslated sequences, together with the pCB6.p53 expression vector (Fig. 2). Wt p53 suppressed promoter activity of all four constructs assayed (constructs 1–4), regardless of the various combinations of 5′-flanking and 5′-untranslated regions involved. The basal promoter activity of p(−40/+640)LUC, which lacks most of the 5′-flanking region, was extremely low, though p53 was still able to reduce those levels. Wt p53 did not affect the luciferase levels generated by pOLUC (construct 5).

The results obtained indicate that the suppressive effect of p53 on IGF-I-R promoter activity was independent of the potential binding sites present in this region. Because the only region in common to all of the constructs is the fragment surrounding the "initiator" element, we sought to characterize the interactions of p53 with this region.

Interaction of p53 with Components of the Basal Transcription Machinery. TBP, in addition to being essential for transcription from TATA-containing promoters, is required for transcription initiation of promoters that, like the IGF-I-R gene, contain Sp1 binding sites and an "initiator" element but lack a TATA box (38). To study the interaction between TBP and p53 in regulation of IGF-I-R promoter, gel retardation assays were performed using a labeled DNA fragment (−40/+115) comprising the initiator region, together with purified TBP (1 f.p.u.) or GST.p53 (100 ng), or a combination of both proteins. Incubation of TBP with the labeled probe generated two retarded bands (Fig. 3*A*) whose appearance was prevented by addition of an ≈600-fold molar excess of the unlabeled probe (Fig. 3*C*). Furthermore, the formation of the DNA-TBP complexes was abolished by addition of GST.p53 to the binding reaction. In addition, incubation of this fragment with GST.p53 generated one retarded band, both in the absence and presence of TBP (Fig. 3*A*).

To more precisely map the sites of interaction of TBP with the IGF-I-R promoter fragment, competition was performed using six 24-mer oligonucleotides covering the region between

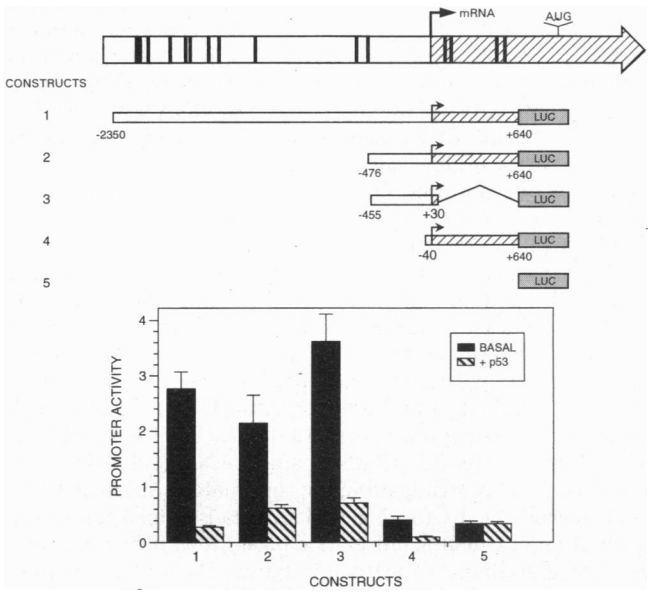

FIG. 2. Suppression of IGF-I-R promoter activity by wt p53. (*Upper*) The 5′-flanking (open bar) and 5′-untranslated (hatched bar) regions of the IGF-I-R gene contain multiple sites that are highly related to the putative p53 consensus site. Each bar denotes a potential site composed of two half motifs (Pu-Pu-Pu-C-A/T-T/A-G-Py-Py-Py), each containing at least eight of ten nucleotides, separated by 0–13 bp. The arrow indicates the transcription start site. Saos-2 cells were cotransfected with 10 μg of IGF-I-R reporter plasmids [or pOLUC (construct 5)] and 2.5 μg of pCB6.p53 (or empty pCB6+, results designated as basal). LUC, luciferase cDNA (not shown to scale). (*Lower*) Values are luciferase units normalized per total protein (×10³).

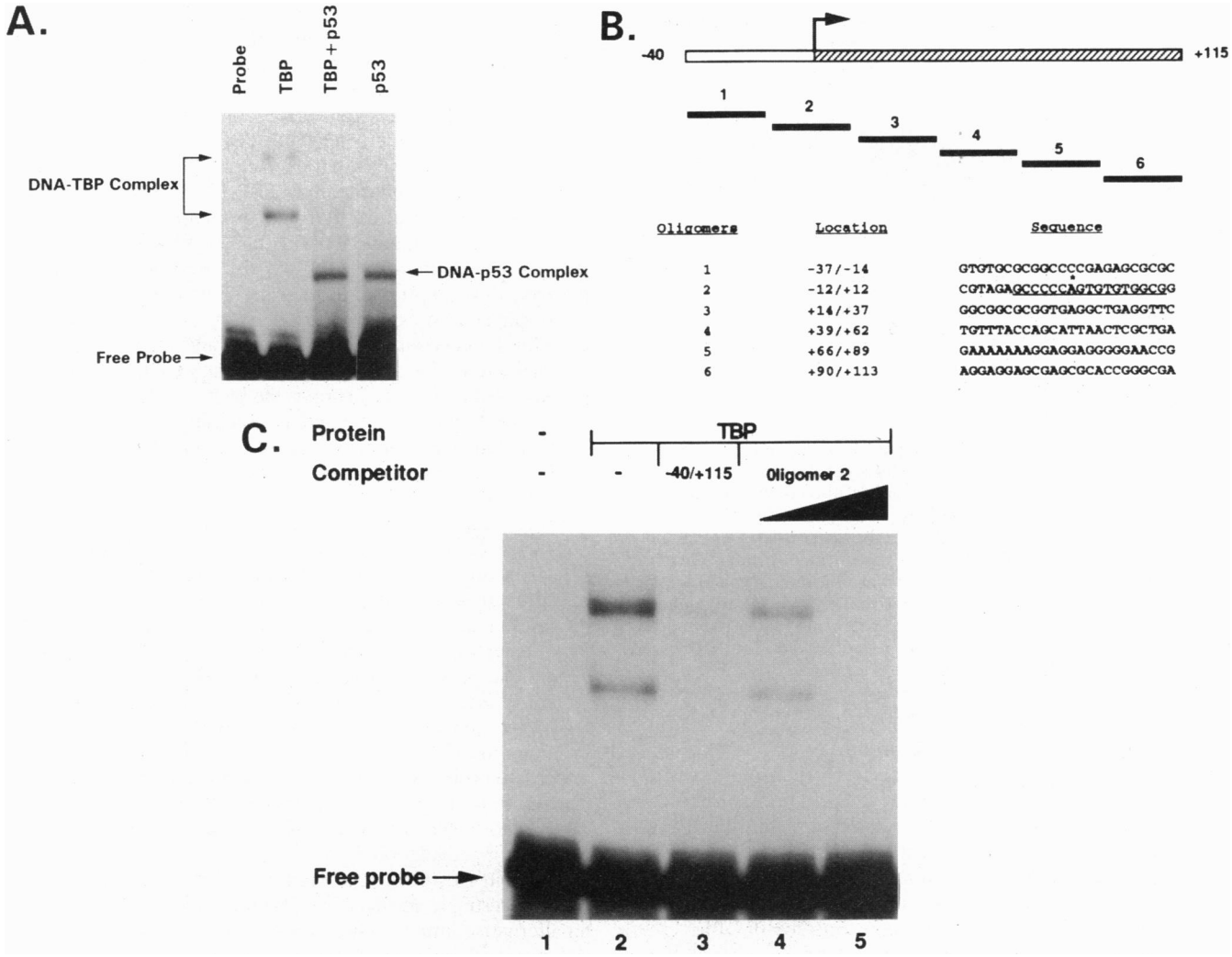

FIG. 3. Gel retardation analysis of the IGF-I-R transcription start site with TBP and GST.p53. (*A*) A DNA fragment extending from −40 to +115 was end-labeled with [γ-^{32}P]ATP and used in binding reactions with TBP (1 f.p.u.), GST.p53 (100 ng), or both proteins. (*B*) Location of oligomers used in competition assays. The underlined bases in oligomer 2 correspond to the "initiator" motif, and the asterisk denotes the first transcribed nucleotide, as determined by primer extension assay (15). (*C*) Competition experiments were performed by incubating the labeled −40/+115 fragment with TBP (1 f.p.u.) in the absence (lane 2) or presence of excess unlabeled probe (60 ng, lane 3) or oligomer 2 (20–200 ng, lanes 4 and 5, respectively).

nucleotides −40 and +115 (Fig. 3*B*). The only oligomer that was able to prevent the formation of DNA–TBP complexes was oligomer 2, which encompasses the "initiator" element (Fig. 3*C*). None of the other five oligomers had a significant effect on TBP binding (data not shown), suggesting that TBP binds specifically to the transcription start site. In addition, none of the six oligomers was able to compete out the retarded band generated by p53 (data not shown), indicating that p53 binds nonspecifically to this DNA fragment.

***In Vitro* Transcription Assays.** To demonstrate that the action of wt p53 on IGF-I-R promoter was a direct effect at the transcriptional level, *in vitro* transcription reactions were performed using a DNA template (−476 to +640) comprising the proximal promoter region. This fragment has been previously shown to exhibit high levels of promoter activity in functional assays (29). Furthermore, this fragment contains a number of Sp1 sites that are required for transcription initiation (14). Incubation of HeLa cell extracts, which contain low endogenous levels of p53 (39), with the IGF-I-R promoter template, resulted in transcription initiation that was inhibited by α-amanitin (10 μg/ml), a specific RNA polymerase II inhibitor. The levels of transcription observed decreased with increasing amounts of HeLa extract, consistent with transcriptional suppression of the IGF-I-R gene by endogenous p53 and/or other tumor suppressors (Fig. 4*A*). Addition of exogenous purified GST.p53 (25 to 150 ng) abolished transcription in a dose-dependent fashion (Fig. 4*B*). Intriguingly, under the *in vitro* conditions assayed, transcription started from a site located ≈100 bp downstream from the initiator element. The location of this alternative site was corroborated using a number of overlapping DNA templates in *in vitro* transcription assays (data not shown).

Stimulation of IGF-I-R Gene Transcription by Mutant p53. To address the question whether overexpression of the IGF-I-R gene in human malignancies can result from lack of inhibition by mutant p53, Saos-2 cells were cotransfected with an IGF-I-R promoter/luciferase reporter plasmid together with expression vectors encoding mutant versions of p53. Whereas wt p53 in the same vector (pC53-SN3) suppressed promoter activity to 47% of control levels (Fig. 1*A*), pC53-SCX3, pC53-248W, and pC53-273H mutants stimulated its activity to 227%, 319%, and 406% of control values, respectively (Fig. 5*A*).

Increased levels of luciferase activity were associated with increased concentrations of IGF-I-R/luciferase fusion mRNA, (200% to 570% of control), as detected by solution hybridization/RNase protection assays (Fig. 5 *B* and *C*). These results thus indicate that mutant p53 proteins can induce transcription from the IGF-I-R gene *in vivo*.

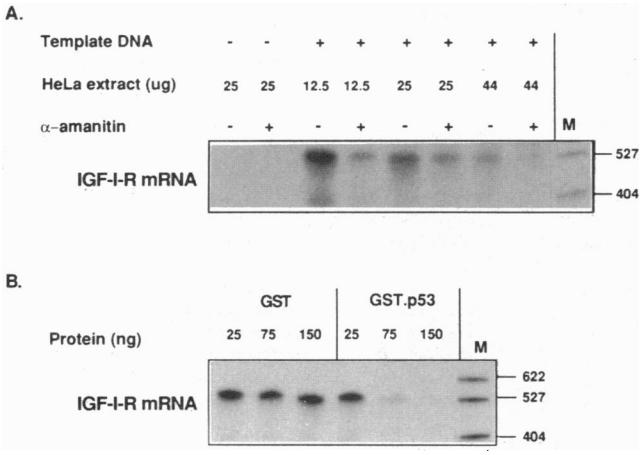

FIG. 4. *In vitro* transcription assays. (*A*) Dose-dependent suppression of IGF-I-R gene transcription by HeLa cell extracts. Increasing amounts of whole-cell HeLa extracts were incubated with a purified DNA template extending from −476 to +640, and with ATP, GTP, CTP, and [α-^{32}P]UTP for 1 h at 30°C, in the absence or presence of α-amanitin (10 μg/ml). The labeled transcription products were resolved on a 4% denaturing gel, which was autoradiographed for 8 days. (*B*) Suppression of IGF-I-R gene transcription by exogenous GST.p53. *In vitro* transcription assays were performed using 12.5 μg of HeLa extract and increasing amounts of purified GST or GST.p53 protein. M, pBR322/*Msp*I molecular mass marker.

DISCUSSION

We have identified the IGF-I-R promoter as a molecular target for tumor suppressor p53. The IGF-I-R promoter is a highly regulated, TATA-less, "initiator"-containing promoter that directs transcription of very high levels of receptor mRNA at embryonic and early postnatal stages (18). The abundance of this transcript significantly decreases at adult stages, whereas malignant states associated with augmented cellular proliferation are characterized by a rebound in gene expression (9, 10).

The results of this study demonstrate that, in spite of the presence of potential p53 binding sites both upstream and downstream of the IGF-I-R gene transcription start site (a finding which is usually associated with genes stimulated by p53), wt p53 suppresses transcription from the IGF-I-R promoter both *in vivo* and *in vitro*. The difference in the extent of suppression between Saos-2 and RD cells may be due to the different p53 backgrounds in both cell lines. Thus, overexpression of wt p53 in Saos-2, a cell line that lacks any endogenous p53, resulted in inhibition of promoter activity by 88%, whereas the effect of p53 transfection in RD cells, which express an endogenous p53 mutated at codon 248, was comparatively lower (75% inhibition).

The mechanism of action of p53 on the IGF-I-R promoter seems to involve its interaction with TBP, the TATA-box binding subunit of the general initiation factor, TFIID. Results of gel retardation assays indicate that TBP binds specifically to the "initiator" element of the IGF-I-R promoter, whereas p53 displays a nonspecific interaction with this DNA fragment. However, p53 precludes binding of TBP to the promoter region, most probably through protein–protein interaction. As a result, TBP is no longer able to assemble a functional transcription initiation complex. Direct contact between wt p53 and TBP was previously demonstrated using affinity chromatography (40). The region of p53 involved in this interaction is the highly acidic N-terminal 73 amino acid fragment that functions as a transcriptional activation domain. Oncogenic versions of p53 mutated in their DNA-binding core domain are impaired in their ability to bind TBP, possibly due to overall conformational changes.

Specific repression of transcription by wt p53 has been previously postulated to be limited to TATA-containing promoters (41). This proposal was based on the observation that a number of "initiator"-containing promoters, including the proliferating cell nuclear antigen (PCNA), c-Ha-*ras*, and the epidermal growth factor receptor, are immune to the effects of p53 (42). In addition, a synthetic promoter containing an "initiator" element downstream of a simian virus 40 21-bp activator element was unaffected by coexpression with wt p53, whereas an homologous promoter in which the "initiator" was replaced by the adenovirus major late promoter TATA box was significantly repressed (41). The results of the present study provide evidence for a novel class of "initiator" element-containing promoters that are susceptible to inhibitory regulation by p53, though it is still unknown what elements in the IGF-I-R promoter confer upon it sensitivity to p53. Interestingly, results of *in vitro* transcription assays point to an alternative initiation site, which differs by ≈100 bp from the site previously shown to function *in vivo* (14, 15). The reason for this discrepancy is presently unknown, though it may reflect the preference of members of the basal transcription complex to contact specific DNA sequences in the 5′-untranslated region under *in vitro* conditions. Thus, it is conceivable that the mechanism for p53 regulation of IGF-I-R gene transcription *in vitro* may differ from the *in vivo* mechanism, though with identical end results, i.e., suppression of IGF-I-R promoter. Alternatively, the

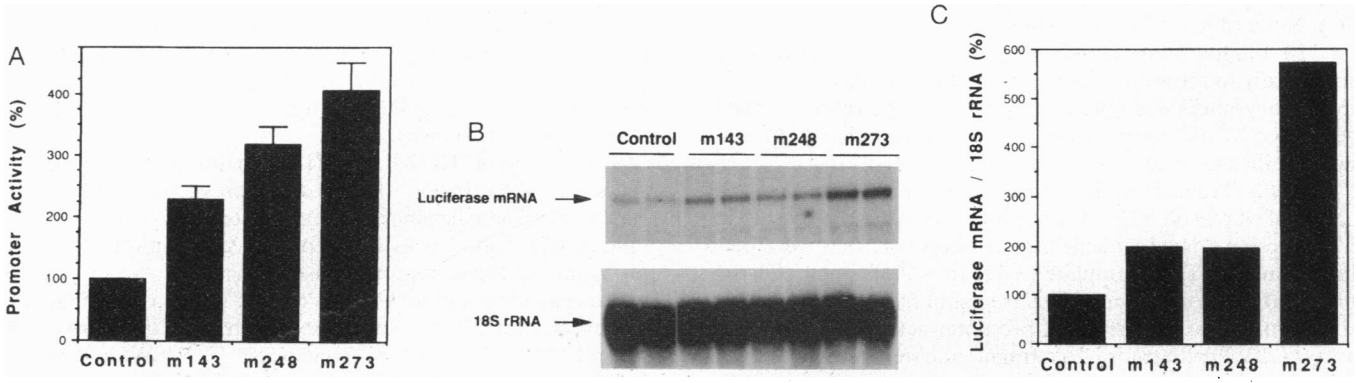

FIG. 5. Stimulation of IGF-I-R gene transcription by mutant p53 proteins *in vivo*. (*A*) Activation of IGF-I-R promoter activity. Saos-2 cells were cotransfected with 10 μg of the reporter plasmid p(−2350/+640)LUC and 2.5 μg of the expression vectors pC53-SCX3, pC53-248W, and pC53-273H (or empty pCMV-Neo-Bam plasmid, as a control). The levels of luciferase activity are expressed as percentage of control values. Experiments were performed between three and four times, each in duplicate. (*B*) Transcriptional stimulation by mutant p53. RNA was prepared from Saos-2 cells that were transiently cotransfected with reporter and expression vectors, as indicated above, and the levels of IGF-I-R/luciferase fusion mRNA were determined by solution hybridization/RNase protection assay. The autoradiogram was exposed for 28 days. (*C*) Quantitation of IGF-I-R/luciferase fusion mRNA. The autoradiogram shown in *B* was scanned using National Institutes of Health IMAGE software (version 1.55). The levels of luciferase mRNA, normalized to those of 18S rRNA, are expressed as percentage of controls.

discrepancy between *in vivo* and *in vitro* results may be due to technical reasons in *in vitro* transcription assays.

The IGF-I-R has an important role as an inhibitor of apoptosis, both *in vivo* and *in vitro* (43). Activation of this receptor by IGFs protects cells from apoptotic death in a number of models. For example, IGF-I was shown to inhibit the etoposide-induced apoptosis in BALB/c3T3 fibroblasts overexpressing the IGF-I-R. This protective effect of IGF-I was less marked in parental BALB/c3T3 cells and it was totally absent in fibroblasts lacking the IGF-I-R (44). Similar results were seen *in vivo* using a biodiffusion chamber that allows passage of nutrients and proteins but excludes the inward or outward flow of intact cells. In this model, a decrease in the number of IGF-I-Rs was associated with massive cell death. Furthermore, overexpression of the receptor protects cells from apoptosis (45). Because wt p53 is a potent inducer of apoptosis, we may speculate that the effect of p53 on apoptosis is mediated, at least partially, through suppression of the IGF-I-R promoter. Lack of inhibition of the IGF-I-R gene by mutant p53 in malignant states may help expand a cell population that is otherwise destined to die. Furthermore, additional components of the IGF-signaling system have been shown to be modulated by p53. Thus, the expression of IGF-II P3 transcripts is reduced by wt p53 (46). On the other hand, the IGF-binding protein 3 (IGF-BP3) has been shown to be induced by wt, but not mutant, p53 (47). Because IGF-BP3 is an inhibitor of mitogenic signaling by IGFs, it follows that p53 can regulate the IGF system both at the level of availability of IGF ligands and at the level of activity of the IGF receptor.

However, suppression of the IGF-I-R promoter is not limited to p53. We have recently demonstrated that WT1, a tumor suppressor involved in the etiology of Wilms tumor, binds both upstream and downstream of the IGF-I-R gene transcription start site by means of its zinc finger domain and suppresses promoter activity in functional assays (29, 48). In addition, overexpression of WT1 in G401 cells was associated with a decrease in the endogenous levels of IGF-I-R and with a reduction in IGF-I-mediated cellular proliferation and anchorage-independent growth (49).

In conclusion, we have presented evidence for the suppression of IGF-I-R gene transcription by wt p53, and for its stimulation by mutant p53. When combined with our previous results on tumor suppressor WT1, a novel paradigm for tumorigenesis emerges. According to this model, the expression of the IGF-I-R gene is constitutively inhibited in the terminally differentiated cell by local (i.e., WT1) as well as by more ubiquitous (i.e., p53) tumor suppressors. As a result of this negative control, cells remain at a postmitotic state and out of the cell cycle. Mutation of tumor suppressors or activation of oncogenes, two events usually associated with malignancy, can de-repress the IGF-I-R gene promoter with increased production of cell-surface receptors and augmented mitogenic activation by locally produced or circulating IGFs.

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