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p53 Stimulates Transcription from the Human Transforming Growth Factor α Promoter: a Potential Growth-Stimulatory Role for p53

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Physical and chemical agents can damage the genome. Part of the protective response to this damage is the increased expression of p53. p53, a transcription factor, controls the expression of genes, leading to cell cycle arrest and apoptosis. Another protective mechanism is the proliferative response required to replace the damaged cells. This proliferation is likely to be signaled by growth factors. In this communication, we show that the transforming growth factor α (TGF-α) gene is a direct target for p53-mediated transcriptional activation. In a stable cell line containing an inducible p53 construct, p53 induction leads to a threefold accumulation of the native TGF-α mRNA. In cotransfection assays using a TGF-α promoter reporter construct, we show that expression of wild-type but not mutant p53 increases transcriptional activity of the TGF-α promoter by approximately 2.5-fold. In vitro, wild-type p53 binds to a consensus binding site found in the proximal portion of the promoter, and this sequence is necessary for the p53 transcriptional response. Furthermore, this element confers p53 induction to the otherwise nonresponsive adenovirus major late promoter. In addition to these results, we found that the TGF-α promoter contains a nonconsensus but functional TATA box-binding protein-binding site approximately 30 bp upstream of the transcription start site. Although p53 can repress transcription from promoters containing a TATA box, the nonconsensus TGF-α TATA motif is resistant to this effect. On the basis of these results, we propose that p53 may play a dual role, which includes both the elimination of irreparably genetically damaged cells and the proliferative response necessary for their replacement, in the response to physical-chemical damage.

Multicellular organisms have developed protective mechanisms to cope with physical and chemical agents capable of damaging DNA. One mechanism is to eliminate, by cell growth arrest and apoptosis, those cells receiving the heaviest damage. The second mechanism involves the stimulation of proliferation of the less affected or undamaged cells to replace the defect resulting from cell death. Indeed, it is probably from this pool of proliferating cells that cancer arises. Recently, the transcription factor p53 has been implicated in coordinating part of these defensive mechanisms. p53 protein accumulates in the cell in response to DNA damage (31), and one of the consequences of this p53 induction in vivo is cell cycle arrest in G1 (21) and/or apoptosis (6, 26, 46). The cell cycle arrest by p53 requires the transcriptional modulation of target genes (13, 34, 41). p53 can stimulate transcription from several promoters (13) through sequence-specific recognition of consensus DNA elements in the responsive genes. These divalent p53-responsive elements confer binding of a homotetrameric complex of p53 (4, 12, 15). The N terminus of the p53 molecule contains a transcription activation domain. Activation of transcription by wild-type but not mutant p53 has been demonstrated both in vitro and in vivo. The cell cycle arrest induced by p53 has been attributed, in part, to the transcriptional induction by p53 of the gene encoding a 21-kDa inhibitor protein of cyclin-dependent kinases (11, 13). Certain cells such as murine thymocytes respond to DNA damage by undergoing apoptosis, a process also dependent on functional p53 (6, 26, 46). p53-induced apoptosis may not depend on the ability of p53 to activate transcription. A second role of p53 in transcriptional regulation is repression of transcription from promoters containing a TATA box (27). A molecular mechanism of the repression is not well understood; however, the sequence-specific DNA-binding activity of p53 is not required. p53 can directly interact with TATA box-binding protein (TBP) and may interfere with the binding of TBP to the TATA box (3, 25, 28, 40, 44). The role of this sequence-independent transcriptional regulation in cell cycle arrest or apoptosis is also not well defined.

In boundary epithelia, such as skin and colon, rapid replacement of damaged cells is required. The observed proliferative response to physical or chemical damage in such tissues is thought to be mediated through the activation of growth signals. For example, UV radiation has recently been shown to activate the tyrosine protein kinase activity intrinsic to the epidermal growth factor receptor (38) and to stimulate the secretion of one of the ligands for the epidermal growth factor receptor, transforming growth factor α (TGF-α) (14, 20, 30). Several signal transduction molecules downstream of the growth factor receptors, including those in the Ras-Raf-MAP kinase-AP1 pathway, have also been shown to be activated as part of the UV radiation response (8–10, 36, 38). The positive growth signals induced by this set of responses could potentially be countered by the growth-inhibitory response to p53, which also accumulates in the epithelial cell nuclei in response to UV radiation (18). Nevertheless, these potentially conflicting growth signals must ultimately result in the coordinated elimination of severely damaged cells and their replacement by the proliferating normal cells. The TGF-α released in response to UV radiation (14, 20, 30) might be one of the signals required for this proliferative response. For example, those cells sufficiently damaged to induce p53 expression and cell cycle arrest could still produce the growth factors sufficient to signal neighboring, less damaged cells to proliferate. We therefore studied the human TGF-α promoter (19, 37) to determine whether it contained elements that might account for its response to physical-chemical damage. This examination of the

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TGF-α promoter did not reveal potential binding sites for AP1, NF-κB, or the serum response factor. However, we did find two potential p53-binding sites in the TGF-α promoter. In this paper, we demonstrate that the TGF-α gene is indeed a direct target for p53 transcriptional activation. This observation supports the hypothesis that p53 not only is involved in the elimination of terminally damaged cells but may also contribute to the proliferative response that allows maintenance of the integrity of the epithelium following physical-chemical damage.

MATERIALS AND METHODS

Plasmids. Four p53 expression vectors (effector plasmids) were used in this study. pC53-SN_3 (1) encodes wild-type human p53 under the control of the cytomegalovirus early promoter, while pC53-248_3 encodes a protein with an Arg-to-Trp mutation at codon 248 in the otherwise identical vector. pCEP53SN3 and pCEP53Δ80-393 (34) were used for the experiment described in Fig. 2C. These two plasmids are identical, except that the protein expressed from the latter lacks the first 80 amino acids of p53. All reporter plasmids contain the coding sequence for the firefly luciferase gene in conjunction with a sequence from the TGF-α promoter or the adenovirus major late promoter (AdMLP). The 1.1-kb TGF-α promoter construct is identical to pXP1/1.1 described earlier (37), except for the inclusion of a downstream sequence between positions +5 and +38. pG-181/+38 was described previously (42) and contains a 220-bp sequence of the TGF-α promoter. pG-181/+38AAN is a derivative of pG-181/+38 and includes restriction sites for *Apa*I, *Avr*II, and *Nco*I engineered at positions −65, −40, and −19, respectively, within the promoter sequence. Reporter activities of these two constructs are nearly identical in transfection assays. For construction of TATA-replacement vectors, the following double-stranded oligonucleotides were inserted between the *Avr*II and *Nco*I sites of pG-181/+38AAN: 5′-CTAGGCTGGGGCTATAAAAAGG-3′ and 5′-CATGCCTTTTATAGC CCCACG-3′ for the "good" TATA box and 5′-CTAGGTCTGCGTCG GCCGGTC-3′ and 5′-CATGGACCGGCCGACGCAGAC-3′ for the "poor" TATA box. For deletion of site I, the following double-stranded oligonucleotide was inserted between the *Apa*I and *Avr*II sites in pG-181/+38AAN: 5′-CTCCCGCGCGGATCC-3′ and 5′-CTAGGGATCCGCGCGGGAGC CGG-3′. pAdMLP1 contains a sequence between positions −38 and +11 of the AdMLP in the *Xho*I and *Bgl*II sites of a luciferase vector, pXP2. pAdEamI derives from pAdMLP1 and contains four copies of the site I sequence in a head-to-tail array upstream of the AdMLP. Since each site I sequence consists of two consensus binding motifs, there is a total of eight binding sites for p53. pSVK3 (Pharmacia, Piscataway, N.J.) was used to balance the amounts of plasmids in each transfection assay.

Northern (RNA) blot analysis. Dexamethasone was added to cells (29) at a concentration of 1 μM at various times prior to harvesting, and the RNA was isolated by the acid guanidine thiocyanate-phenol method of extraction (5). Twenty micrograms of the total was analyzed by electrophoresis on 1% agarose–6% formaldehyde, transferred, and UV cross-linked onto GeneScreen nylon membranes (Du Pont-New England Nuclear, Boston, Mass.). RNA was probed by hybridizing overnight with cDNA labeled with ^{32}P to a specific activity of approximately 2×10^9 cpm/μg by using the Deca Prime II kit from Ambion (Austin, Tex.). The TGF-α probe was a 900-bp fragment of the human TGF-α cDNA (24). The hexosaminidase A probe, consisting of a 1.6-kb sequence of the alpha-subunit of the lysosomal enzyme (23), was used for the purposes of RNA normalization.

Transfection assays. Approximately 10^7 MDA468 cells were electroporated with a total of 35 μg of plasmid DNA containing 20 μg of a reporter plasmid, 0 to 5 μg of an effector (p53) plasmid, 10 μg of pCMVβ-gal, and appropriate amounts of the balancer plasmid. Conditions and procedures for electroporation and enzymatic (luciferase and β-galactosidase) assays were described previously (43). Cells were harvested at 36 to 43 h after transfection. All experiments were done in triplicate, and transfection was repeated at least three times with each combination of plasmids. The luciferase activity was normalized to the activity of the β-galactosidase in the same lysate (ratio of luciferase to β-galactosidase activities). Cell numbers and the protein content in the lysate were determined and used also to control for variations in cellular yields. Neither the β-galactosidase nor cellular protein levels varied by more than 10% in the transfected cells.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared according to the method of Schreiber et al. (39) from approximately 10^7 transfected or mock-transfected cells. The probe used for the study of p53 binding contains the sequence from positions −65 to −15 of the TGF-α promoter. EMSA was carried out under standard conditions (43), using a 5% polyacrylamide gel and 0.5× TBE (Tris-borate-EDTA) as an electrophoresis buffer. Monoclonal antibodies were obtained from Oncogene Science (Uniondale, N.Y.) (pAb421 for p53 and C36 for Rb). For a supershift assay, nuclear extracts was preincubated with 100 ng of each antibody at 4°C for 30 min before being subjected to an EMSA. Recombinant TBP was obtained from Upstate Biotechnology Incorporated (Lake Placid, N.Y.). EMSA using TBP was performed according to Peterson et al. (33) with a probe containing the TGF-α promoter sequence between positions −40 and −20. For a competition assay, 25-fold excesses of TATA box oligonucleotides were added to selected reaction mixtures.

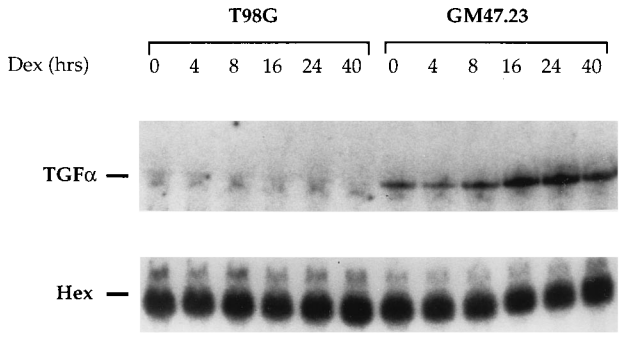

FIG. 1. Accumulation of TGF-α mRNA in response to the induction of p53 expression. A human glioma cell line (GM47.23) which had been stably transfected with a mouse mammary tumor virus-p53 construct (29) was used in these studies and compared with the parental cell line (T98G). The GM47.23 cells express wild-type p53 conditionally in response to dexamethasone. The cells were treated with 1 μM dexamethasone (Dex) for the indicated times prior to extraction of the RNA. Twenty micrograms of total RNA was subjected to Northern blot analysis and probed first for the TGF-α mRNA and then, after stripping, for the hexosaminidase A mRNA. Shown are the autoradiographic signals corresponding to the 4.8-kb TGF-α mRNA and the 1.8-kb hexosamindase A (Hex) mRNA.

Western blot (immunoblot), immunoprecipitation, and immunofluorescence microscopy analyses. Ten microliters of each nuclear extract was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A nitrocellulose blot was prepared, blocked with bovine serum albumin (BSA), and probed with the monoclonal antibody pAb421 followed by a goat anti-mouse immunoglobulin G antibody conjugated with alkaline phosphatase (Pierce, Rockford, Ill.). For immunoprecipitation, cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.9), 150 mM NaCl, 5 mM MgCl, 50 mM NaF, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Monoclonal antibody (pAb421 or pAb240) was added, and the lysate was incubated for 1 h at 4°C. The lysate was further incubated for 30 min after the addition of protein G-agarose (Oncogene Science). p53 in the immunocomplex was analyzed by Western blotting with the pAb421 antibody. For an immunofluorescence microscopy analysis, cells were fixed and permeabilized in cold methanol for 30 min. The cell samples were treated first with BSA and then with pAb421 or pAb240 antibody; this was followed by treatment with donkey anti-mouse immunoglobulin G antibody conjugated with a fluorophore, Texas Red (Jackson ImmunoResearch, West Grove, Pa.).

RESULTS

TGF-α mRNA accumulates in human glioma cells following the induction of p53 expression. Because the human TGF-α gene contains two potential divalent p53 consensus binding sites, the effect of p53 on the expression of this gene was examined. A human glioma-derived cell line was utilized for these studies. This cell line had been stably transfected with a mouse mammary tumor virus-p53 construct which allowed induction of p53 expression by exposure of the cells to dexamethasone (29). The parental cell line, which did not contain the inducible p53 construct, was also studied. The cells were exposed to dexamethasone, and at the indicated times, equal quantities of total RNA were analyzed by Northern blotting for TGF-α mRNA and the mRNA encoding hexosaminidase A. The addition of dexamethasone to the medium was staggered such that the time course ended at the same time for all cells. In the parental cell line, T98G, the usual 4.8-kb TGF-α mRNA was barely detectable at the exposure shown and dexamethasone had no effect on the abundance of this mRNA (Fig. 1). In contrast, in the GM47.23 cell line, which exhibits inducible p53, the TGF-α mRNA was detectable, even in the absence of dexamethasone induction. However, a clear, time-dependent

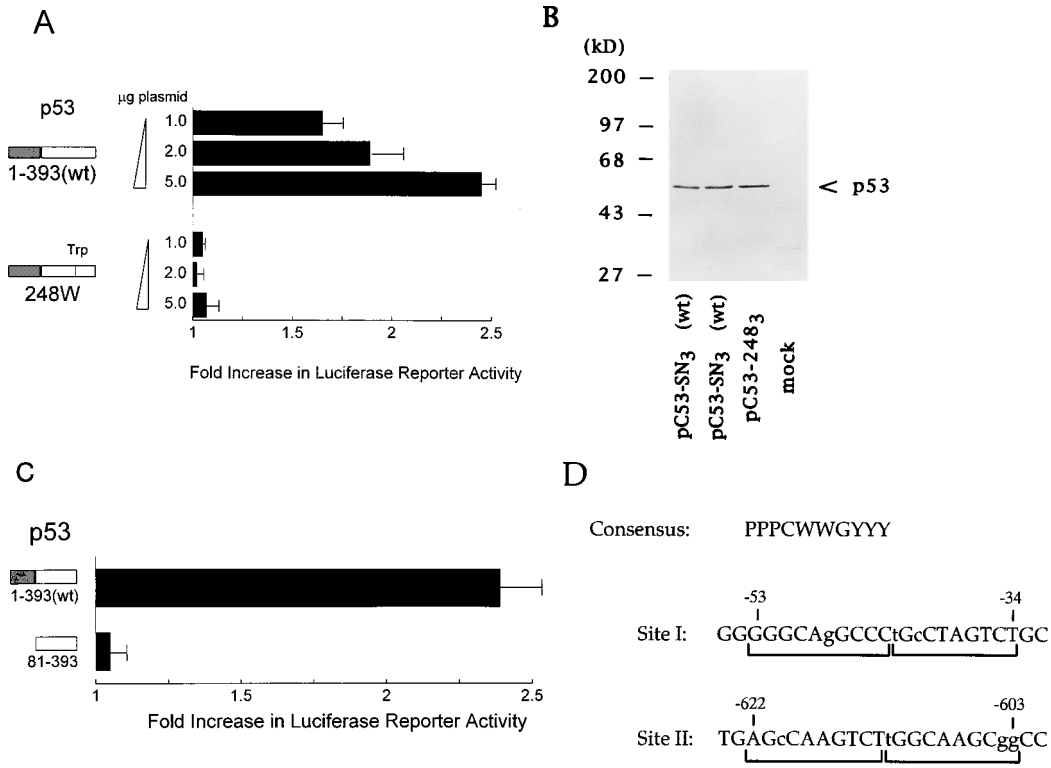

FIG. 2. TGF-α promoter activity is induced by expression of wild-type but not mutant p53. (A) A 1.1-kb TGF-α promoter-luciferase reporter plasmid was transfected into MDA468 cells along with 0, 1, 2, or 5 μg of either a wild-type [1-393(wt)] or a mutant (248W) p53 expression vector. The mutant p53 plasmid encodes a protein with Trp at codon 248 instead of Arg. The reporter activity in the presence of an indicated amount of p53 plasmids is expressed as a fold increase over the activity of the reporter plasmid alone. All values were normalized against the activity of β-galactosidase expressed from a cotransfected pCMVβ-gal. Results of three independent triplicate experiments were averaged. The standard deviations are indicated. (B) A Western blot showing expression of p53 proteins in nuclear extracts. Nuclear extracts were prepared from MDA468 cells that had been transfected with either no effector plasmid (mock), pC53-SN_3 (wild-type), or pC53-248_3 (mutant). The blot was probed with the p53 monoclonal antibody pAb421. (C) The N-terminal activation domain of p53 is required for induction of TGF-α promoter activity. Cells were transfected with the 1.1-kb TGF-α promoter construct and one of the p53 expression plasmids encoding either wild-type p53 [1-393(wt)] or p53 with the first 80 amino acids deleted (81-393). These plasmids are otherwise identical to each other in sequence but different from the constructs used in the experiment shown in panel A. The expression of each protein was confirmed by immunoprecipitation by pAb421, which recognizes p53 in both the wild-type and mutant conformations, and by pAb240, which is specific for mutant proteins (data not shown). The truncated p53 was expressed at a level that was comparable to or higher than that of the wild-type protein. pAb240 precipitated only a small fraction of the truncated p53 protein present in the lysate, indicating that the majority of the protein exists in the wild-type conformation. The normalized luciferase activity and standard deviations are expressed as a fold increase as described for panel A. (D) Potential p53-binding sites in the TGF-α promoter. The sequences of the two potential p53-binding sites, with their nucleotide positions relative to the transcription start site (+1), are shown. Lowercase letters indicate those nucleotides that do not match the consensus sequence, which is also shown.

accumulation of the TGF-α mRNA occurred in response to dexamethasone (Fig. 1). This increase in the TGF-α signal was evident as early as 8 h into the time course, with a maximal accumulation seen at 16 h. Densimetric analysis of the hybridization signals indicated that the TGF-α mRNA signal at 16 and 24 h was 2.5- to 3-fold higher than in the untreated cells. In contrast, the hexosaminidase A mRNA showed equal abundance in T98G and GM47.23 cells and was not affected by dexamethasone. These results indicate that TGF-α gene expression is responsive to p53 but do not indicate that the TGF-α gene is a direct target for p53 transactivation.

TGF-α promoter activity is induced by the expression of wild-type but not mutant p53 in MDA468 cells. To determine whether TGF-α gene transcription is directly regulated by p53, the effects of p53 on TGF-α transcription were examined by a transient transfection assay. A reporter plasmid containing the 1.1-kb promoter region of the human TGF-α gene was cotransfected into MDA468 cells with human p53 expression vectors. MDA468 cells, a human mammary carcinoma cell line, contain a single p53 allele which encodes a protein with a missense mutation at codon 273 (Arg to His) (32). This mutant p53 protein neither binds a consensus p53-binding sequence nor does it behave as a dominant-negative mutant in transcription assays (45). Cotransfection of the wild-type p53 expression plasmid (pC53-SN_3) resulted in the induction of TGF-α promoter activity in a dose-dependent manner (Fig. 2A). The maximal induction observed was, reproducibly, 2.5-fold in multiple triplicate experiments, agreeing reasonably well with the Northern blotting results. An expression vector encoding a missense mutant p53-248W (Arg-248 to Trp) failed to induce TGF-α promoter activity. As shown in Fig. 2B, the 248W protein was expressed at a level similar to that of wild-type p53 in nuclear extracts prepared from the transfected cells. The 248W mutant is defective in sequence-specific DNA binding. Thus, these results indicate that a functional p53 protein is required for the activation of the TGF-α promoter. This conclusion was confirmed by the failure of the TGF-α promoter plasmid to respond to a p53 molecule lacking the N-terminal transactivation domain (34) (Fig. 2C). Thus, both DNA-binding and transactivation functions are necessary for p53 to stimulate the transcriptional activity of the TGF-α promoter.

There are two potential p53-binding sites in this 1.1-kb region of the TGF-α promoter (Fig. 2D). One element is located approximately 600 bp upstream of the transcription start site

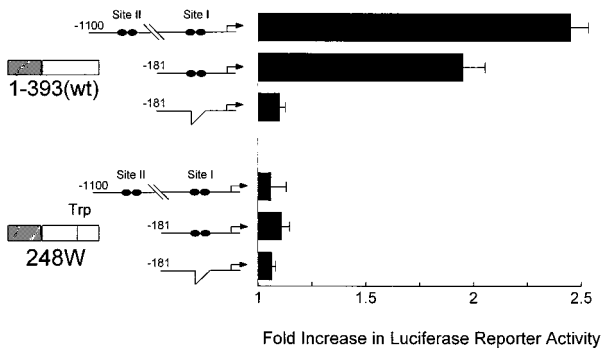

FIG. 3. Site I is required for the induction of TGF-α promoter activity by p53. Cotransfection and reporter assays were done as described for Fig. 2. Three reporter plasmids were tested (shown schematically on the left) in this experiment. The first plasmid is identical to the 1.1-kb promoter plasmid described in the legend to Fig. 2 and contains the two potential p53-binding sites described in the legend to Fig. 2D. The second reporter lacks the sequence distal to position −181 in the TGF-α promoter but retains the site I binding motif. The third plasmid contains an internal deletion that has removed site I from the proximal TGF-α promoter. The average of three independent results is shown, as is the standard deviation. 1-393(wt) and 248W, wild-type and mutant p53 expression vectors, respectively.

(site II), while the other (site I) is found between positions −53 and −34, relative to the start site. p53-binding sites have been identified in several genes (13). Most of these sequences are located one or more kilobases distal to the transcription start site either in a 5′ or 3′ direction. As in other bona fide p53-binding sites, both sites in the TGF-α promoter consist of two tandemly repeated 10-bp sequences, each closely resembling the consensus binding motif (17 and 16 matches of 20, respectively).

Deletion of sites I and II renders the TGF-α promoter insensitive to p53 expression. To determine if p53 directly activates the TGF-α promoter, we first used the proximal 220-bp region of the promoter which lacks site II but retains the more proximal site I. This segment of the promoter is still responsive to wild-type p53 (approximately twofold) but not to the 248W mutant (Fig. 3). To determine if site I is required for this activation, we deleted this site from the 220-bp promoter. As shown in Fig. 3, the deletion of both site I and site II virtually eliminated the p53-dependent activation. In the absence of coexpressed p53, the activity of the proximal promoter was not affected by the deletion, suggesting that it did not cause a general alteration in the structure and function of the TGF-α promoter. Instead, only the p53-dependent transcriptional activation was abolished. These results strongly suggest that site I is an essential p53-responsive element in the proximal portion of the TGF-α promoter. Site II appears to contribute to the transcriptional activation by p53, but its independent role has not been firmly established.

Site I supports transcriptional induction by p53 in conjunction with the AdMLP. If site I is truly a p53-responsive element, it should be able to transfer the p53 inducibility to a heterologous promoter. Therefore, we placed the sequence of site I upstream of the AdMLP and examined its effects on the promoter activity. Four copies of site I (a total of 8 binding sites; Fig. 2D) were cloned into the vector. Such a tandem array of transcription factor binding sites often allows synergistic activation of transcription. As shown in Table 1, cotransfection of the wild-type p53 plasmid resulted in very strong (200- to 500-fold) induction of promoter activity only in the presence of the site I sequence. Indeed, the activity of the AdMLP alone is repressed by the p53 expression. This induction also depended on a functional p53 protein. The larger p53 induction seen with this chimeric promoter, compared with that seen with the wild-type TGF-α promoter, may also suggest that other elements in the native promoter modulate the p53 effect.

TABLE 1. TGF-α site I confers p53 responsiveness to the AdMLP[a]

Plasmid	Luciferase reporter activity in expt:		
	1	2	3
AdMLP alone	1	1	1
AdMLP + p53 (wt)	0.44	0.29	0.36
AdMLP + p53-258W	0.98	1.0	
AdMLP-(site I)$_4$ alone	2.0	3.4	2.4
AdMLP-(site I)$_4$ + p53	510	790	920
AdMLP-(site I)$_4$ + p53-258W	1.8	3.3	

[a] MDA468 cells were cotransfected with one of the p53 expression plasmids and a luciferase reporter plasmid containing either the AdMLP alone or the AdMLP plus four tandem repeats of the site I sequence. All luciferase activities were normalized against the activity of β-galactosidase expressed from cotransfected pCMVβ-gal. The normalized luciferase reporter activities are expressed relative to the activity observed from the AdMLP alone. Results of three independent experiments are indicated.

To confirm that this effect of p53 on transcription from this chimeric reporter was not a result of the cotransfection assay, this same reporter plasmid containing the TGF-α site I upstream of the AdMLP was transiently transfected into T98G and GM47.23 cells. These cells were treated as described before with dexamethasone for the indicated times (Fig. 4). As shown in Fig. 4, dexamethasone had a minimal effect on transcription from this promoter in the parental cell line (T98G), whereas a 600-fold induction of reporter activity was seen with the p53-expressing cell line (GM47.23). The time course was virtually identical to the time course observed for the accumu-

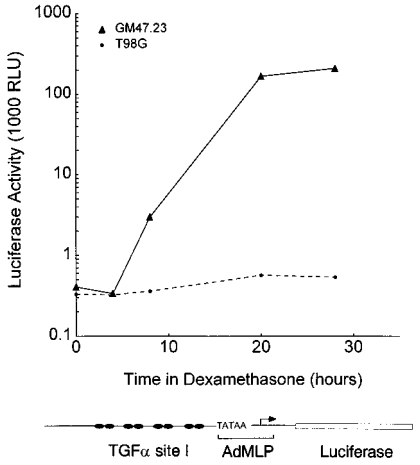

FIG. 4. TGF-α site I confers p53 responsiveness in cells that conditionally express p53. The human glioma cell line (GM47.23) which had been stably transfected with a mouse mammary tumor virus-p53 construct (29) was used in these studies and compared with the parental cell line (T98G). The GM47.23 cells express wild-type p53 conditionally in response to dexamethasone. The cells were transiently transfected with the chimeric promoter containing the multimerized TGF-α site I placed upstream of the AdMLP (Table 1). The transfected pool of cells was distributed into replicate tissue culture plates. Eighteen hours later, treatments with 1 μM dexamethasone for the indicated times were started in a staggered manner such that the luciferase reporter assays were performed at the same time at the end of the experiment. Shown are the luciferase activities performed in triplicate at each time point in the GM47.23 and T98G cells. Shown also is a schematic representation of the reporter plasmid containing TGF-α site I upstream of the AdMLP. RLU, relative light units.

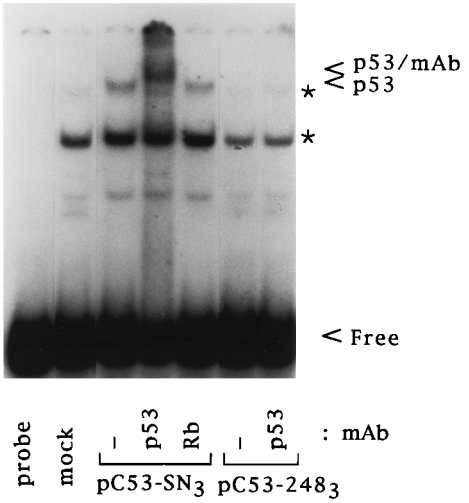

FIG. 5. p53 in nuclear extracts from transfected MDA468 cells binds site I. An EMSA was conducted with nuclear extracts prepared from cells transfected with no effector plasmid (mock), pC53-SN_3, or pC53-248_3. The probe was a double-stranded oligonucleotide containing the sequence of site I. In selected reactions, a monoclonal antibody against p53 (pAb421) was included to indicate the presence of p53 in the complex. The p53-specific complex and the supershift produced by the p53 antibody are indicated by arrowheads. The strong intensity of the supershift is probably due to a stabilizing effect of pAb421 (15, 17). An Rb antibody (C36) serves as a specificity control for the immunological reaction. Asterisks indicate nonspecific complexes that are present in all extracts regardless of p53 expression. Similar background bands have been reported by others (15, 45). The nonspecific bands are not recognized by the p53 antibody.

lation of the native TGF-α mRNA, and the magnitude of the response was comparable to that in the cotransfection assay (Table 1). Thus, the presence of site I is sufficient for conferring p53-dependent transcription. On the basis of these observations, together with the results presented in Fig. 3, we conclude that site I can mediate transcriptional activation by p53.

p53 binds site I. The functional data in conjunction with the sequence homology suggest that site I may directly interact with p53. To test this idea, we prepared nuclear extracts from cells transfected with p53 expression plasmids and used them in EMSAs. A double-stranded oligonucleotide containing the sequence of site I was radiolabeled and used as a probe. Consistent with other reports (15, 45), extracts from untransfected cells contained two or more nonspecific DNA-binding activities (mock; Fig. 5). However, an additional band that migrated more slowly than the background bands was observed with the pC53-SN_3-transfected cell extracts. This band could be inhibited by an oligonucleotide containing the p53-binding site from p21 (data not shown). A monoclonal immunoglobulin G to p53 (pAb421) replaced this complex with a new, even lower mobility band, indicating the presence of p53 in the complex. The antibody-induced supershift had greater intensity than the original band generated by the extract and probe only. This is in agreement with the reported property of pAb421 to stabilize the p53-DNA complex (15, 17). As a control for antibody specificity, a monoclonal immunoglobulin G to the Rb protein was included in a separate reaction, and this antibody did not produce a supershift (Fig. 5). The extract containing the 248W p53 failed to yield the specific complex. Arg-248 is directly involved in the minor groove interaction of p53 with DNA (4), perhaps explaining the inability of the 248W mutant protein to bind the consensus site (45). Finally, pAb421 had no effects when added to the extract containing the 248W mutant protein (Fig. 3) or by itself (not shown). These results indicate that site I is specifically recognized by p53. In addition, the failure of mutant p53 to activate the TGF-α promoter most likely results from its inability to bind site I.

A nonconsensus TATA box is located immediately downstream of the p53-binding site (site I). p53 can selectively repress transcription from TATA box-containing promoters but not transcription directed by an initiator (27). This repression does not require direct binding of p53 to the promoter and appears to be mediated by interaction of p53 with TBP. The TGF-α promoter lacks a consensus TATA motif and uses an initiator for the accurate transcription initiation (42). However, it has not been determined whether the TGF-α promoter truly lacks a functional TATA box, in that a nonconsensus TATA motif may also mediate the binding of TFIID (16). Interestingly, we noticed a region of extensive homology between the human and rat TGF-α promoters positioned approximately 30 bp upstream of the transcription start site (Fig. 6A) (2, 19). In this region, not only is a contiguous 19-bp sequence almost perfectly conserved, but the distance between the start site and the conserved region differs by only one base. Furthermore, within this 19-bp stretch is a motif, TTTTTCCCCC, that is somewhat reminiscent of the T-rich strand of the TATA box contained in the AdMLP. To assess whether this motif functions as a TATA box and to address a potential relationship to the neighboring p53-binding site, we replaced this native sequence with either a consensus TATA motif (from the AdMLP) or an unrelated sequence (Fig. 6B). Substitution with the good (AdMLP) TATA box increased the promoter activity by about 2.5-fold, while the promoter with the unrelated sequence was only half as active as the wild-type promoter (Fig. 6C). This result is consistent with the idea that this motif in the TGF-α promoter participates in the interaction with TFIID. Indeed, recombinant TBP is capable of directly binding to a labeled oligonucleotide containing the TGF-α motif (Fig. 6D) in an EMSA. This binding was inhibited by both the TTTT TCCCCC sequence itself and the AdMLP TATA box but not by the poor TATA box that also functioned poorly in the transfection assay. These results indicate that the TGF-α promoter contains a nonconsensus TATA motif that is, nevertheless, recognized by TBP.

p53-dependent transcription repression is not supported by the TTTTTCCCCC motif of the TGF-α promoter. In TATA-containing promoters that lack a p53-binding site, p53 represses transcription (25, 27, 28, 40). By deleting the p53-binding sites in the TGF-α promoter, we could test whether the TTTTTCCCCC motif could support transcriptional repression by p53. As shown in Fig. 3, deletion of the p53-binding sites results in a promoter that is practically insensitive to p53. We have noted that this version of the promoter occasionally responds to wild-type p53 with a slight induction (not more than 17%) but never with transcriptional repression. This observation indicates that the nonconsensus TATA box in the TGF-α promoter does not support p53-dependent repression. This result was also observed with HeLa cells and in experiments with the same promoter in a different plasmid background (data not shown). Instead, the repression requires a consensus TATA box, in that p53 expression repressed transcription from the promoter containing the AdMLP TATA box (Table 1). It appears that when TBP is recruited to the TGF-α promoter, it is somehow inaccessible to p53 if p53 is not bound to DNA. In this respect, the TGF-α promoter behaves like a TATA-less promoter.

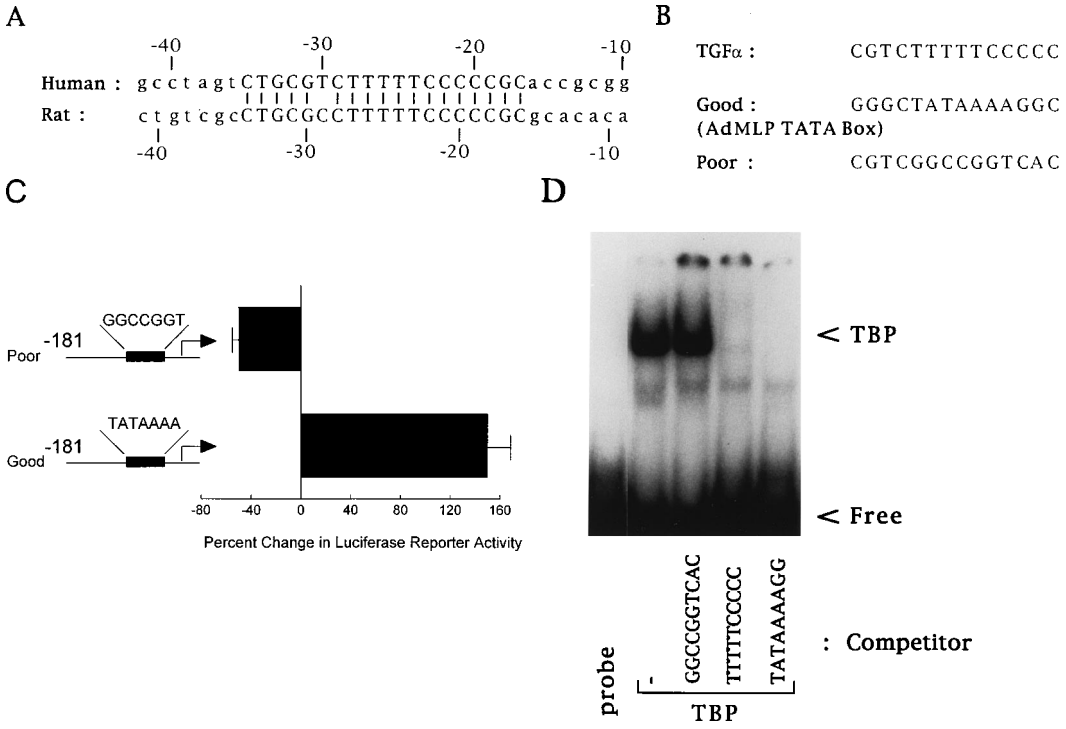

FIG. 6. The TGF-α promoter contains a nonconsensus TATA box approximately 30 bp upstream of the transcription start site. (A) Sequence comparison of the human and rat TGF-α promoters at the −30 region. A 19-bp conserved region is shown by uppercase letters, with vertical lines indicating identical nucleotides in the two sequences. Nucleotide positions relative to the respective transcription start sites are also shown. (B) Sequences of TATA motifs used in the following sets of experiments. The good motif is the TATA box from the AdMLP. (C) Replacing the TGF-α sequence with the good TATA motif increases the promoter activity, while the poor TATA box has an opposite effect. Reporter plasmids used in this experiment contain the proximal 220-bp TGF-α promoter sequence and one of the motifs shown in panel B at the −30-bp region. Except for this substitution, all reporter constructs are identical. Normalized luciferase activities (averages of three experiments) are expressed relative to that from the wild-type promoter. (D) Recombinant TBP binds the TGF-α TATA motif in an EMSA. The probe for the EMSA is a double-stranded oligonucleotide containing the TATA-like motif of the TGF-α promoter. Competitors were the TATA motifs shown in panel B present at a 25-fold molar excess relative to the probe.

DISCUSSION

The TGF-α promoter is a target for p53. The experiments described here provide strong evidence that transcription from the TGF-α promoter is subject to direct regulation by p53. This observation may implicate p53 in the proliferative response to DNA damage from physical-chemical injury. Such DNA damage results in the accumulation of p53 in the nucleus (18, 31). To isolate this induction of p53 activity from other effects of DNA damage, we used a cell line in which p53 was inducible through a heterologous promoter (29). We showed that p53 induction resulted in the accumulation of the TGF-α mRNA in these cells. We conclude that this effect on the TGF-α mRNA results from direct activation of the TGF-α gene by p53. First, segments of the TGF-α promoter, extending to position −1100 or −181, are stimulated by wild-type p53. This stimulation is reproducible and in clear contrast to the lack of response seen with the TGF-α promoter with deletions of the p53-binding sites. Thus, the induction is dependent on the presence of these sites and, therefore, is not the consequence of nonspecific activation by overexpressed p53. Of note, the muscle creatine kinase promoter, in which the p53 element was also tested in the context of the native promoter, responds to p53 to a comparable degree in a cotransfection assay (47). Second, a naturally occurring mutant of p53 (248W) fails to stimulate transcription from the TGF-α promoter. This mutant contains a missense mutation within the DNA binding domain and, as a result, exhibits no significant sequence-specific DNA-binding activity (45). Similarly, deletion of the first 80 amino acids of p53, which are part of the major transcriptional activation domain, renders p53 inactive at inducing TGF-α promoter activity. These observations exclude the possibility that cotransfection of p53 expression vectors resulted in an increased TGF-α promoter activity by a nonspecific mechanism which is independent of the transcription activator function of p53. Third, the ability of the proximal p53-binding site (site I) to form a complex in EMSA is dependent on the presence of wild-type p53 in the nuclear extracts. The antibody-specific supershift indicates that this complex contains p53. Together with the sequence similarity between site I and the consensus p53-binding site, the most obvious explanation for this observation is that p53 directly binds site I. Fourth, four tandem repeats of site I act as a strong p53-dependent enhancer for the AdMLP, a minimal promoter containing merely a TATA box and an initiator. This transcriptional activation is again dependent upon a p53 molecule which is competent at both binding DNA and activating transcription. Thus, site I is both necessary and sufficient for p53-dependent promoter activation. Taken all together, these results indicate that the TGF-α promoter is transcriptional target p53.

Potential importance of p53-mediated TGF-α induction in the DNA damage response. p53 normally has a short half-life in the cell, but following DNA damage it is stabilized and accumulates in the nucleus (31). In barrier epithelia exposed to UV or chemical damage, p53 may play a dual role in the epithelial reconstruction following such damage. First, the activation of p53 could induce apoptosis (6, 26, 46), thus eliminating those

cells with irreparably damaged DNA. Simultaneously, p53 could stimulate production of TGF-α by the surrounding cells and/or the dying cells themselves. It remains to be seen whether this level of p53 response is sufficient to activate transcription of the TGF-α gene in vivo to produce physiologically relevant amounts of this growth factor. However, TGF-α is a potent mitogen and an inducer of cell motility, and this growth factor, deposited at the site of cell death, could be recognized as a homing receptor and proliferation signal for cells of the basal epithelial layer, which could then replace the lethally damaged cells. This dual role of p53 in the repair response may ironically contribute to carcinogenesis in the long run. That is, p53 contributes to immediate homeostasis by removing cells with severely damaged DNA but stimulating the production of growth factors that are required for the proliferative response that results in the replacement of the arrested cells with the less damaged neighbors. Nevertheless, these wounded cells may harbor DNA damage too subtle to result in sustained cell cycle arrest or apoptosis yet sufficient to result in carcinogenesis, as a result of mutations in growth regulatory genes.

Transcription repression by p53. Previously, the TGF-α promoter was classified as a TATA-less promoter (19). However, our experiments suggest that efficient transcription of this gene requires a nonconsensus TATA motif present approximately 30 bp upstream of the transcription start site. Our data demonstrate that TBP directly binds to this motif in the TGF-α gene. Furthermore, transfection studies demonstrated a correlation between the strength of the TATA motifs as a TBP-binding site and its promoter activity.

Transcription repression by p53 has been observed only with promoters containing a TATA box but no p53-binding sites (25, 27, 28, 40). Our observation that the TGF-α promoter is not repressed by p53 when the p53-binding sites are removed suggests that the mere presence of a TBP-binding site is not sufficient for the repression. The TGF-α promoter also contains an initiator element (42). Presumably, this initiator recruits a TBP-containing protein complex in collaboration with the weak upstream TATA box. We speculate that, although TBP can directly interact with the TGF-α promoter at the −30-bp region, it is in a configuration and/or association with a distinct set of proteins (e.g., TBP-associated factors), which distinguishes it from TBP bound to a consensus TATA box. In this model, the p53 interaction domain of TBP may not be exposed to free p53 molecules. Evidence exists in support of multiple forms of TBP-containing complexes (7). Alternatively, TBP may not be involved in transcription from the TGF-α promoter in vivo. This is unlikely, however, in light of our results as well as recent reports indicating that TFIID itself can interact with initiator elements (22, 35). That the TGF-α TATA box does not support the usual p53-mediated transcriptional repression is consistent with the overall stimulatory action of p53 on the TGF-α promoter.

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