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Wild-Type Human p53 Transactivates the Human Proliferating Cell Nuclear Antigen Promoter

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The wild-type p53 protein is a transcriptional activator implicated in the control of cellular growth-related gene expression. Here, using a number of different cell lines and transient-transfection–transcription assays, we demonstrate that at low levels, wild-type p53 transactivates the human proliferating cell nuclear antigen (PCNA) promoter. When expressed at a similar level, the tumor-derived p53 mutants did not transactivate the PCNA promoter. We identified a p53-binding site on the human PCNA promoter with which p53 interacts sequence specifically. When placed on a heterologous synthetic promoter, the binding site functions as a wild-type p53 response element in either orientation. Deletion of the p53-binding site renders the PCNA promoter p53 nonresponsive, showing that wild-type p53 transactivates the PCNA promoter by binding to the site. At a higher concentration, wild-type p53 inhibits the PCNA promoter but p53 mutants activate. Transactivation by p53 mutants does not require the p53-binding site. These observations suggest that moderate elevation of the cellular wild-type p53 level induces PCNA production to help in DNA repair.

The p53 tumor suppressor protein negatively regulates cell growth (reviewed in references 17, 28, 36, 39, 59, and 77). Mutations in the p53 gene are one of the most common somatic genetic changes found in human cancer (25, 28). Inactivation of the wild-type p53 gene through mutation leads to uncontrolled cell growth and oncogenesis (reviewed in reference 39). The mechanism of p53-mediated cell growth regulation has been a focus of interest in recent years.

Wild-type p53 shows properties of sequence-specific transcriptional activator. It is a sequence-specific DNA-binding protein (reviewed in references 17, 36, and 39). For efficient DNA binding, p53 requires two copies of the 10-bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, separated by 0 to 13 bp (18, 22). The contact between the DNA-binding domain of p53 and DNA has been identified by crystallographic analysis (10). The protein activates transcription from promoters with p53-binding sites (reviewed in references 17, 36, and 39). The DNA-binding domain of wild-type p53 resides between amino acids 100 and 300 (3, 58, 81), whereas transactivation function requires amino acids 1 to 42 (21, 57, 61, 76) of the protein. Tumor-derived p53 mutants are defective in this sequence-specific transactivation. Several prospective target genes whose expression may be positively influenced by wild-type p53 have been identified. These include the mouse muscle creatine kinase (*MCK*) (83), *MDM2* (2, 52), *CIP1*/*WAF1*/*p21* (19), *EGFR* (14), thrombospondin-1 (11), *GADD45* (31), *Bax* (50, 51) and cyclin G (54) genes.

We and others have found that overexpression of wild-type (but not mutant) human p53 inhibits the in vivo (13, 23, 37, 63, 70) or in vitro (60, 66, 69a) transcription of many cellular and viral promoters which lack p53-binding sites. Work from our laboratory as well as other laboratories demonstrates that wild-type and mutant p53 molecules bind to the TATA-binding protein (TBP) (29, 43, 46, 60, 66, 74). Free and DNA-bound p53 can bind to TBP, and free and DNA-bound TBP can interact with p53 (8, 14, 46). This interaction between p53 and TBP may activate or inhibit transcription, depending on whether TBP interacts with DNA-bound p53 or p53 interacts with DNA bound-TBP, respectively (14). However, p53-mediated transcriptional repression is released by adenovirus E1B or cellular Bcl-2 proteins, suggesting that the repression is a part of the apoptotic sequence of events (62, 67).

Besides TBP, both wild-type and mutant p53s interact with various members of transcription machinery such as Sp1 (4), CCAAT-binding factor (1), TFIIH (82), holo-TFIID (8, 43), and two subunits of the TFIID complex, $TAF_{II}40$ and $TAF_{II}60$ (72). Taken together, these observations strongly imply that p53 acts directly with the transcription machinery to modulate transcription. It has been suggested that p53 induces expression of growth suppressors to negatively regulate cell growth (19), although the exact mechanism(s) is not yet known.

Mutant p53 can transactivate cellular growth-related promoters in vivo (9, 13). p53 mutants activate promoters of genes expressing human proliferating cell nuclear antigen (PCNA), human epidermal growth factor receptor (EGFR), multiple drug resistance (MDR1), and human HSP70 in vivo (9, 13, 14, 16, 75, 84). Presumably, p53 mutants activate these promoters without directly binding to DNA, since mutants are known to be defective in DNA binding (3, 32, 34). The mutant p53-mediated transactivation may represent the gain of function of tumor-derived p53 by which it can activate a promoter even without a p53-binding site.

The role of wild-type p53 in arresting cell growth has been studied in several cell culture systems. p53 can induce cell cycle arrest in G_1 (15, 31, 42). Wild-type p53 levels increase in response to DNA-damaging agents such as ionizing radiation or chemotherapeutic agents. The increase in the p53 level may lead to arrest of cell growth at G_1. Increased p53 expression transcriptionally induces the expression of genes related to growth arrest, e.g., *GADD45* or *CIP1*/*WAF1*/*p21* (19, 31). Following DNA damage, cells respond by activating DNA repair processes that are partly controlled by p53 (31, 35, 44). The G_1 cell cycle arrest by wild-type p53 is thus probably needed to repair the damaged DNA. It is, however, not known whether p53 is directly involved in DNA repair.

Several laboratories have reported that mild UV irradiation induces expression of PCNA and this induction is parallel to

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the induction of p53 (6, 26, 73). PCNA is involved in simian virus 40 DNA replication functioning with DNA polymerase δ (79) as well as in nucleotide excision repair of DNA (68). After DNA damage, the elevated wild-type p53 level induces CIP1/WAF1/p21 expression, which may inhibit cellular DNA replication by interacting with PCNA and interfering with PCNA's replication function (78). However, p21/CIP1/WAF1 does not inhibit PCNA's DNA repair functions (41). Moreover, PCNA is also found complexed with GADD45, which might be a link between the repair machinery and p53-dependent cell cycle checkpoint (69). Under these conditions an elevated PCNA concentration may control the rate of DNA repair. Therefore, it is important to determine the effect of wild-type p53 on PCNA expression.

The effect of elevated expression of wild-type p53 on PCNA expression has been previously studied. Deb et al. (13) have shown that transient expression of wild-type p53 using a cytomegalovirus immediate-early promoter in HeLa cells inhibits the PCNA promoter. Mercer et al. (47) have reported that expression of wild-type p53 in a human glioblastoma cell line using a dexamethasone-inducible mouse mammary tumor virus promoter downregulates PCNA expression. Jackson et al. (30) reported that wild-type p53 activates the PCNA promoter in HeLa cells but represses it in CV1, CHO, L929, and Saos-2 cells. Previously, Mack et al. (45), using C33A cells which harbor a mutant p53 (R-273 to C) (64), showed that wild-type p53 does not significantly affect the human PCNA promoter. Since mild UV irradiation is accompanied by elevated expression of p53 and upregulation of PCNA expression, we sought to determine whether p53 regulates PCNA expression in a dose-dependent manner.

Using a number of different cell lines and transient-transfection–transcription assays, we demonstrate that low levels of wild-type p53 transactivate the human PCNA promoter. When expressed at a similar level, tumor-derived p53 mutants cannot transactivate the PCNA promoter. We report the identification of a wild-type human p53-binding site on the human PCNA promoter with which p53 interacts sequence specifically. The identified p53-binding site can function as a wild-type p53 response element in either orientation when placed on a heterologous synthetic promoter. Deletion of the p53-binding site renders the PCNA promoter p53 nonresponsive, showing that wild-type p53 transactivates the PCNA promoter by binding to the site. At a higher concentration, wild-type p53 inhibits the PCNA promoter but tumor-derived p53 mutants activate. Transactivation by p53 mutants does not require the p53-binding site. These observations suggest that moderate elevation of the cellular wild-type p53 level induces PCNA production to help in DNA repair.

MATERIALS AND METHODS

DNA plasmids. Wild-type and mutant human p53 expression plasmids (generously provided by Arnold Levine and Bert Vogelstein) utilize the human cytomegalovirus major immediate-early promoter. These expression plasmids were described earlier (70). Five point mutants of p53 were used: p53-143A (V-143 to A), p53-175H (R-175 to H), p53-248W (R-248 to W), p53-273H (R-273 to H), and p53-281G (D-281 to G) (27). In this paper, the expression plasmids have been designated pCMV.143A and pCMV.175H, etc. Plasmid TATA.CAT is a gift from J. D. Gralla (80).

The original PCNA.CAT plasmid was generously provided by Gilbert Morris (53). It contains the human PCNA promoter sequence from −1269 to +60 upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene. The intact PCNA promoter sequences (−1269 to +60) and promoter deletion mutants generated either by restriction enzyme treatments or by PCR procedures were cloned upstream of the CAT gene in the pCATbasic vector (Promega). These promoter deletion mutants are shown in Table 2. To identify the p53 response element, the PCNA promoter was sequentially deleted from the 5′ or 3′ end and was cloned upstream of the TATA box in plasmid TATA.CAT. This plasmid contains a TATA box in a polylinker upstream of the CAT gene. These recombinant plasmids are called X-TATA.CAT, where X indicates the name of the promoter mutant (see Table 2).

PG13.CAT (13 p53-binding sites upstream of the polyomavirus minimal early promoter) was a kind gift from Bert Vogelstein (33). ,

Cell culture and transfection. Established cell lines Saos-2 (human osteosarcoma), HeLa (human cervical carcinoma), H1299 (human non-small-cell lung carcinoma), Vero (monkey kidney), and NIH 3T3 (mouse embryo) were obtained from the American Type Culture Collection and propagated as suggested. Subconfluent cells were transfected by the calcium phosphate-DNA coprecipitation method with or without dimethyl sulfoxide shock 4 h posttransfection (5, 14, 38). In a typical experiment, 5×10^6 cells were cotransfected with 2.5 μg of a CAT construct and 100 ng of a p53 expression plasmid (pCMV.p53). The total DNA in each experiment was balanced to 10 μg by using plasmid pCMV (the expression vector without p53 sequences). All transfection experiments were repeated multiple times, and qualitatively similar results were obtained.

CAT assay. Cells were harvested 36 to 40 h after transfection and suspended in a buffer containing 250 mM Tris-HCl (pH 7.8). Cells were lysed by three successive cycles of freezing and thawing. Extracts were normalized for protein concentration and assayed for CAT enzyme activity (24). Since wild-type p53 inhibits and mutant p53 activates different promoters to various extents, it was not possible for us to normalize extracts for the activity of an internal control such as pSVβ-gal (β-galactosidase under the control of the simian virus 40 early promoter). This plasmid (pSVβ-gal), however, was used as an internal control to confirm the effect of p53. Thus, transfection of increasing amounts of pCMV.p53 activated PCNA.CAT and inhibited pSVβ-gal. CAT activity was detected by thin-layer chromatographic separation of [^{14}C]chloramphenicol from its acetylated derivatives followed by autoradiography. Acetylated and nonacetylated chloramphenicols were quantitated by using a PhosphorImager (Molecular Dynamics).

Expression of proteins by transient transfection of corresponding expression plasmids. The expression of different proteins by their corresponding expression plasmids after transfections was verified by Western blot (immunoblot) analysis using appropriate monoclonal antibodies and anti-mouse immunoglobulin G conjugated with alkaline phosphatase (Promega) by following the supplier's protocol. Monoclonal antibody PAb1801 (Ab2; Oncogene Science) against p53 was used as the primary antibody to detect p53.

Purification of wild-type and mutant human p53. Human wild-type p53 and p53-281G proteins were purified by immunoaffinity chromatography using recombinant baculoviruses expressing the corresponding proteins as described before (5, 46).

Interaction of p53 with the PCNA promoter. (i) Electrophoretic mobility shift analysis (EMSA). For EMSA of p53 interactions with the PCNA promoter, fragment P5 (−272 to −46 [see Table 2]) was isolated, purified, and dephosphorylated with calf intestinal alkaline phosphatase. The DNA fragment was then labeled at the ends with T4 polynucleotide kinase and [γ-^{32}P]ATP. DNA-binding reactions were carried out in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–1 mM dithiothreitol–50 mM NaCl–5% glycerol. Immunopurified human p53 (100 ng) was incubated with the labeled probe (0.5 to 1 ng, 20,000 cpm) for 15 min after a 15-min preincubation with 1 μg of poly(dI-dC) (Pharmacia) at room temperature. For competition experiments, unlabeled P5 fragment or double-stranded oligonucleotides containing p53-binding sites with or without mutation were added to the binding reaction before the addition of the labeled probe. DNA-protein complexes were separated by 5% polyacrylamide gel electrophoresis in 0.25× Tris-borate-EDTA buffer at 200 V for 2 h.

The sequences of the wild-type and mutant oligonucleotides used in this study were as follows: wild-type, 5′-GATCCTTGCCTTGCCTGGACTTGCCTGG CCTTGCCTTTTCTTTC-3′; mutant, 5′-GATCCGAAAGAAAATTAAATTA CATTAAAGTCCATTAAATTAAAG-3′.

(ii) DNase I footprinting. For DNase I footprinting the same probe as in EMSA was used except that the probe was asymmetrically labeled at the 3′ end. Footprint analyses were carried out with the fragment containing the −272 to −46 sequence of PCNA by following a protocol described by Bargonetti et al. (3). For each assay 0.4 to 0.6 ng of labeled probe (20 to 30,000 cpm) was incubated with immunopurified wild-type p53 or the mutant p53-281G for 30 min at room temperature. The reaction mixture was then treated with DNase I (Boehringer Mannheim; 0.06 U in 4 μl of 100 mM $MgCl_2$–50 mM $CaCl_2$) for 10 s at room temperature. Following digestion, 70 μl of a stop solution (2 M ammonium acetate, 100 mM EDTA, 0.2% sodium dodecyl sulfate, 100 μg of salmon sperm DNA) was added, and the mixture was phenol extracted and precipitated in ethanol. The protected fragments were analyzed by 8% polyacrylamide–urea gel electrophoresis.

RESULTS

Effect of wild-type p53 expression on the human PCNA promoter activity. To determine whether wild-type p53 regulates the human PCNA promoter in a dose-dependent manner, we performed in vivo transcription analyses. PCNA.CAT was

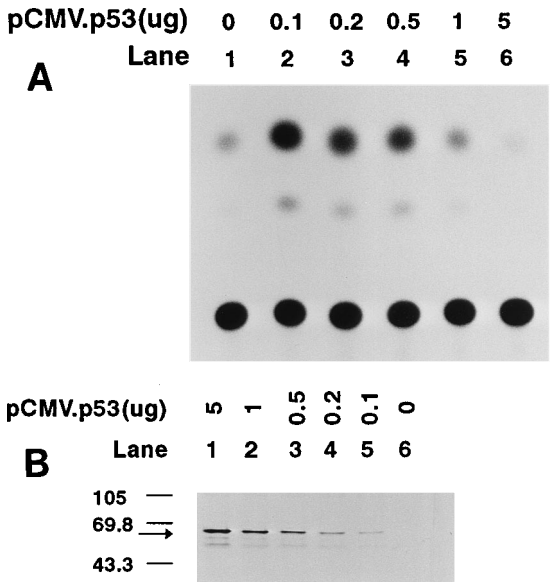

FIG. 1. Dose-dependent activation of the human PCNA promoter by wild-type p53 in Saos-2 cells. Saos-2 cells were transfected with 2.5 μg of PCNA.CAT (53), containing the upstream sequence of the PCNA gene from −1265 to +60, and increasing amounts of pCMV.p53 (wild-type p53 expression plasmid). After incubation at 37°C for 36 to 40 h, cells were harvested and the cell extracts containing equal amounts of protein were analyzed for CAT activity (A) and p53 expression by Western blot analysis (B). One hundred nanograms of pCMV.p53 enhanced the CAT activity 5.5-fold (compare lanes 1 and 2). Further increases in the amount of pCMV.p53 lowered the activation, showing a 3.6-fold inhibition of CAT activity with 5 μg of pCMV.p53 (compare lanes 1 and 6). The result of the Western blot analysis (B) indicates a gradual increase in p53 expression as the amount of pCMV.p53 was increased in the transfection.

cotransfected into Saos-2 cells (devoid of endogenous p53) along with increasing amounts (100 ng to 5 μg) of the wild-type p53 expression plasmid (pCMV.p53). In each transfection the total amount of plasmid DNA was kept constant (10 μg) by the addition of expression vector (pCMV). After 40 h of incubation, cells were harvested and CAT assays were performed using cell extracts containing equal amounts of protein. These experiments have been repeated several times with qualitatively similar results. One representative CAT assay result is shown in Fig. 1A. The data indicate that cotransfection with 100 ng of pCMV.p53 (compare lanes 1 and 2) raised the PCNA promoter activity significantly (5.5-fold). Cotransfection with increasing amounts of pCMV.p53 led to a decrease in transcriptional activation. Transfection with 5 μg of pCMV.p53 showed 3.6-fold repression of the PCNA promoter (compare lanes 1 and 6), similar to what was reported earlier (13). The Saos-2 cell extract containing equal amounts of protein was also subjected to Western analysis using a monoclonal antibody against p53 (PAb1801; Oncogene Science). The result shows that p53 expression increased when Saos-2 cells were transfected with increasing amounts of pCMV.p53 and PCNA.CAT (Fig. 1B). These results suggest that at low levels, wild-type p53 activates the PCNA promoter, whereas at higher levels it inhibits the PCNA promoter. It is possible that the different effects of wild-type p53 on the PCNA promoter observed earlier (13, 30, 45, 47, 64) were due to variation in the amount of functional wild-type p53 present in a cell. For example, HeLa cells express human papillomavirus 18 E6, which perhaps reduces the effective concentration of wild-type p53 (65), while in the case of C33A cells the mutant p53 may poison the wild-type protein (37, 49).

TABLE 1. Comparative activation of PCNA.CAT,[a] EGFR.CAT (14), and PG13.CAT (33) with increasing amounts of pCMV.p53

Amt of pCMV.p53 (μg)	Fold activation[b] of:		
	PCNA.CAT	EGFR.CAT	PG13.CAT
0.1	4.86 ± 0.88	4.00 ± 1.58	78.5 ± 14.3
1.0	1.48 ± 0.58	8.19 ± 2.52	210 ± 65
5.0	0.69 ± 0.37	7.88 ± 1.09	237 ± 66

[a] In each case, 2.5 μg of reporter plasmid was used.
[b] Represents the p53-mediated increase in CAT activity (percent chloramphenicol acetylated) in comparison to that of the expression vector (pCMV) alone.

To determine whether the p53-mediated stimulation of PCNA promoter activity followed by a decline is common for other p53 response elements, we chose two reporter plasmids, PG13.CAT (contains 13 p53-binding sites [33]) and EGFR.CAT (contains p53 response element of human EGFR promoter [14]). Each of these reporter plasmids was transfected into Saos-2 cells with increasing amounts of pCMV.p53 as described for the earlier experiment. After 40 h of incubation, cells were harvested and CAT assays were performed using cell extracts containing equal amounts of protein. Averages (with standard deviations) of CAT assay results obtained from three identical sets of experiments are shown in Table 1. The results show that the p53-mediated activation of PG13.CAT or EGFR.CAT did not decline with increases in pCMV.p53. These results are consistent with earlier reports (14, 71). Therefore, p53-mediated activation of the PCNA promoter is more sensitive to higher levels of p53.

The wild-type p53 response element on the PCNA promoter is located between −238 and −199 of the PCNA promoter sequence. To identify the p53 response element on the PCNA promoter, we undertook a promoter deletion analysis. Initially, we cloned the PCNA promoter (P1) and two deletion mutants (P2 and P3) upstream of the CAT gene in the pCATbasic vector as shown in Table 2. To identify the p53 response element, each of these CAT plasmids was cotransfected with 100 ng of pCMV.p53 (or expression vector alone) into Saos-2 cells. Data presented in Table 2 indicate that the P3 fragment did not show any transactivation whereas both P2 and P1 did. These data suggest that the wild-type p53 response element is localized between −272 and −45 on the PCNA promoter.

In order to further localize the p53 response element and to determine whether the response element functions in the context of a minimal promoter, we cloned deletion mutants of the PCNA promoter (Table 2) upstream of the TATA box of plasmid TATA.CAT. This plasmid has a TATA element driving the CAT gene (80). Thus, the P4 fragment harbors the PCNA promoter sequence from −1265 to −46 and the P5 fragment contains the sequence from −272 to −46. To determine whether the p53 response element can function in both orientations, we also cloned the P5 fragment in reverse orientation (P6, −46 to −272 [Table 2]) in TATA.CAT. Each of these reporter plasmids was transfected into Saos-2 cells in the presence of pCMV.p53 or pCMV (100 ng each). Results of in vivo transcription analysis with these constructs have been summarized in Table 2. P4-TATA.CAT (−1265 to −46), P5-TATA.CAT (−272 to −46), and P6-TATA.CAT (−46 to −272) were transactivated by wild-type p53. Further deletion of the promoter from the 5′ end (P7, −219 to −46) resulted in a loss of p53 response. Also, the basal promoter (TATA.CAT) was not activated by cotransfection with pCMV.p53 (Table 2). These results suggest that the wild-type p53 response element

TABLE 2. Wild-type p53 response element on the human PCNA promoter between −238 and −199 of the promoter sequence

Sequences used[a]	Construct name	Fold activation[b]
-1265_____+60	P1.pCATbasic	6.73 ± 0.89
-272_____+60	P2.pCATbasic	3.46 ± 1.3
-45___+60	P3.pCATbasic	0.42 ± 0.17
	TATA.CAT	1.02 ± 0.29
-1265_____-46	P4-TATA.CAT	41.5 ± 16.2
-272_____-46	P5-TATA.CAT	20.0 ± 6.9
-46_____-272	P6-TATA.CAT	31.0 ± 8.0
-219___-46	P7-TATA.CAT	1.2 ± 0.45
-238__-199	P8-TATA.CAT	42.8 ± 15.16

[a] P1, P2, and P3 sequences were cloned in pCATbasic vector (Promega) upstream of the CAT gene in the proper orientation. The remaining sequences (P4 to P8) were cloned in a TATA.CAT plasmid (80) that contains a minimal TATA element upstream of the CAT gene. Reporter plasmid (2.5 μg), 100 ng of pCMV.p53, and equal amounts of vector (pCMV) were used in each experiment.

[b] Represents the p53-mediated increase in CAT activity (percent chloramphenicol acetylated) in comparison to that of the expression vector (pCMV) alone.

resides between −272 and −46, the response element can function in both the orientations, and the PCNA promoter sequence from −219 to −46 does not contain a functional p53 response element.

Transcriptional activation of P5-TATA.CAT by wild-type p53 was inhibited by the human oncoprotein MDM2 and simian virus 40 T antigen (data not shown). Since MDM2 blocks the transactivation domain of p53 (5, 7, 52, 55, 56) and T antigen blocks the DNA-binding ability of the tumor suppressor (12a, 20, 48), these data suggest that the transactivation and the p53-binding domains of wild-type p53 are needed for this transactivation. Thus, P5-TATA.CAT may harbor a p53-binding site.

Next, we generated another promoter construct, P8-TATA.CAT, containing the PCNA promoter sequence from −238 to −199, which harbors a p53-binding region (241 to 219) as determined by an in vitro footprint experiment described below (see Fig. 5). When transfected into Saos-2 cells in the presence and absence of 100 ng of pCMV.p53 (or pCMV), P8-TATA.CAT was activated significantly by wild-type p53 (Table 2), showing that the wild-type p53 response element is within the sequence between −238 and −199. P5-TATA.CAT showed higher basal activity than P8-TATA.CAT, suggesting that the PCNA promoter sequence present in P5-TATA.CAT helps transcription. For this reason, we used P5-TATA.CAT for subsequent analysis. Quantitation of acetylated and nonacetylated chloramphenicol revealed that p53-mediated activation of P4-, P5-, P6-, and P8-TATA.CAT did not vary significantly in repeated experiments (Table 2).

Wild-type p53-mediated activation of the human PCNA promoter can be observed in different cell lines. To determine whether the activation of the PCNA promoter by low levels of wild-type p53 is cell type specific, we cotransfected P5-TATA.CAT (2.5 μg) and pCMV.p53 (100 ng) or the expression vector alone into HeLa (human cervical carcinoma), NIH 3T3 (murine embryo), H1299 (human lung carcinoma), and Vero (monkey kidney) cells. Since HeLa cells express the human papillomavirus E6 protein that binds and degrades p53 (65), we transfected 200 ng of pCMV.p53 in HeLa cells. The results of these transient-transfection–transcription assays (the p53-mediated increase in CAT activity [percentage of chloramphenicol acetylated] in comparison to that of the expression vector [pCMV] alone) were as follows: H1299, 7.62-fold ± 2.91-fold; NIH 3T3, 4.30-fold ± 1.54-fold; Vero, 3.57-fold ± 2.21-fold; and HeLa, 3.13-fold ± 0.40-fold. The data show that in all four cell lines wild-type p53 activates the human PCNA promoter, suggesting that this activation is not cell type specific.

At low levels, tumor-derived p53 mutants do not activate the human PCNA promoter. Tumor-derived p53 mutants can transactivate the PCNA promoter (13). Therefore, we wished to determine whether this mutant p53-mediated transactivation is also dose dependent. We cotransfected P5-TATA.CAT with 100 ng of a plasmid expressing wild-type p53 (pCMV.p53) or one of the tumor-derived mutants of p53 (pCMV.143A, pCMV.175H, pCMV.248W, pCMV.273H, and pCMV.281G) into Saos-2 cells. The CAT assay results are summarized in Table 3. Clearly, at low levels, wild-type p53 transactivated the P5-TATA.CAT while the mutants failed to do so. Western blot analysis shown in Fig. 2 also indicates that all p53 proteins (wild type or mutant) were expressed sufficiently, suggesting that lack of expression cannot be a reason for mutant p53's inability to transactivate the PCNA promoter (see below for activation by mutant p53 at higher expression plasmid concentrations). These data suggest that P5-TATA.CAT harbors a sequence-specific wild-type p53-binding site, since tumor-derived mutants are known to be defective in DNA binding (32, 34).

TABLE 3. Failure of low levels of mutant p53 to activate the human PCNA promoter[a]

p53 expression plasmid	Fold activation[b]
pCMV.p53	14.88 ± 3.28
pCMV.143A	0.59 ± 0.39
pCMV.175H	0.66 ± 0.41
pCMV.248W	0.68 ± 0.33
pCMV.273H	0.88 ± 0.45
pCMV.281G	1.05 ± 0.55

[a] In each case, 2.5 μg of P5-TATA.CAT and 100 ng of pCMV.p53 (wild type or mutant) were used.

[b] Represents the p53-mediated increase in CAT activity (percent chloramphenicol acetylated) in comparison to that of the expression vector (pCMV) alone.

A tumor-derived p53 mutant does not require the wild-type p53 response element for transactivation of the human PCNA promoter. Since mutant p53 (p53-281G) transactivates the PCNA promoter when expressed at a higher level (13), we tested whether the mutant p53 protein has the same promoter sequence requirements for transactivation. For this we have used P7-TATA.CAT, which contains PCNA promoter sequences from −219 to −46. P7-TATA.CAT is not transactivated by wild-type p53 (Table 2). Figure 3A shows the CAT assay results obtained after transfection of Saos-2 cells with PCNA.CAT, P5-TATA.CAT, and P7-TATA.CAT in the presence and absence of the p53-281G expression plasmid. p53-281G activated all three promoters, including P7 (lane 6), which cannot be activated by wild-type p53 (Table 2). This indicates that mutant p53 does not require the wild-type p53 response element for transactivating the PCNA promoter.

Wild-type p53 binds to the human PCNA promoter sequence specifically. Since we were successful in identifying a segment on the PCNA promoter that responds positively to wild-type p53 in transfection and transient-expression assays, we tested whether wild-type p53 can bind to the promoter sequence specifically, as is the case with other promoter enhancers that can be activated by wild-type p53 (2, 19, 33, 51, 54). For this purpose, we have used EMSA with the P5 promoter segment (−272 to −46) (Table 2) radiolabeled at its ends as a probe. The radioactive probe was incubated with immunopurified wild-type p53 that was generated by recombinant baculovirus and purified as described elsewhere (46). The general procedure for the assay has been described in Materials and Methods. Figure 4A shows an autoradiogram from one such analysis. Lanes 1 and 3 both show a retarded band in the presence of immunopurified wild-type p53. This band was supershifted (lane 2) to a higher position in the gel by the addition of the anti-p53 monoclonal antibody DO1 (Santa Cruz), showing the presence of p53 in the protein-DNA complex at the retarded band position. This supershifting was not observed when a nonspecific monoclonal antibody (monoclonal antibody against human EGFR) was used in the place of the anti-p53 monoclonal antibody (lane 1). Lane 4 shows that DO1 alone cannot bind to DNA. Thus, these results indicate that wild-type p53 binds to the PCNA promoter in vitro to the same sequence that gets activated by wild-type p53 in vivo.

We next checked the specificity of the PCNA promoter binding by wild-type p53. Competition experiments were performed using unlabeled P5 fragment and double-stranded oligonucleotides harboring wild-type or mutated sites for wild-type p53 binding. Sequences of both the oligonucleotides and binding conditions of the competition experiments have been described in Materials and Methods. Figure 4B shows the result of one such competition experiment. Data shown in the figure indicate that the unlabeled P5 fragment (lanes 3 and 4)

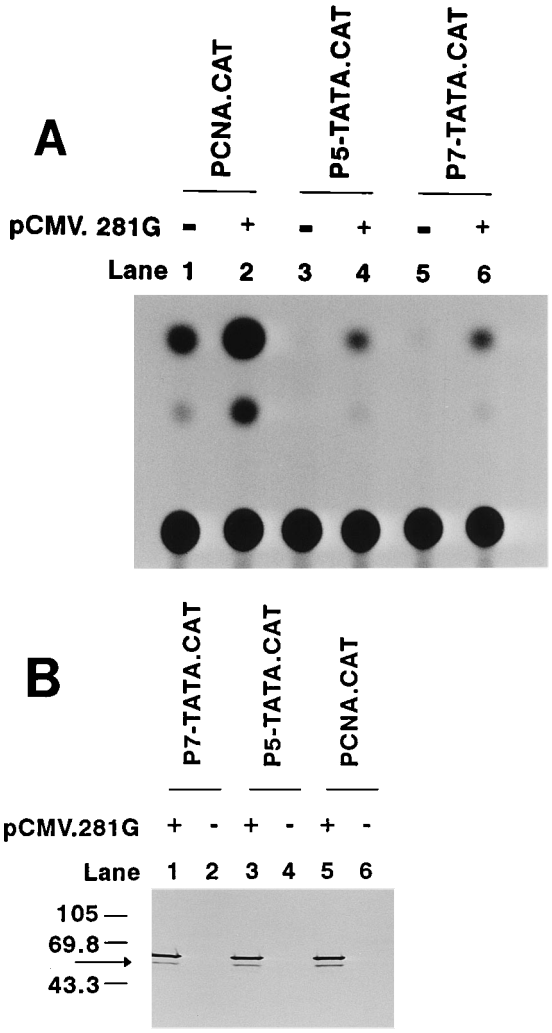

FIG. 3. p53-281G does not require the wild-type p53-binding site to activate the PCNA promoter. The full-length PCNA promoter or one of the deletion mutants (2.5 μg) was cotransfected into Saos-2 cells with 5 μg of the p53-281G expression plasmid (or expression vector pCMV). Cell extracts containing equal amounts of proteins were used for CAT assays (A) and Western blot analysis (B). All three promoters were transactivated by p53-281G. P7-TATA.CAT does not contain the predicted p53-binding site.

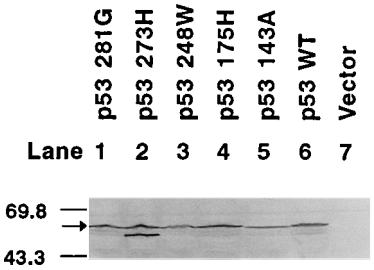

FIG. 2. Expression of low levels of mutant p53. Saos-2 cells were transfected with 100 ng of pCMV.p53, either the wild type or one of the tumor-derived mutants (or expression vector alone without insert), and 2.5 μg of P5-TATA.CAT. Cell extracts containing equal amounts of proteins were used for Western blot analysis depicting the level of expressed p53 proteins.

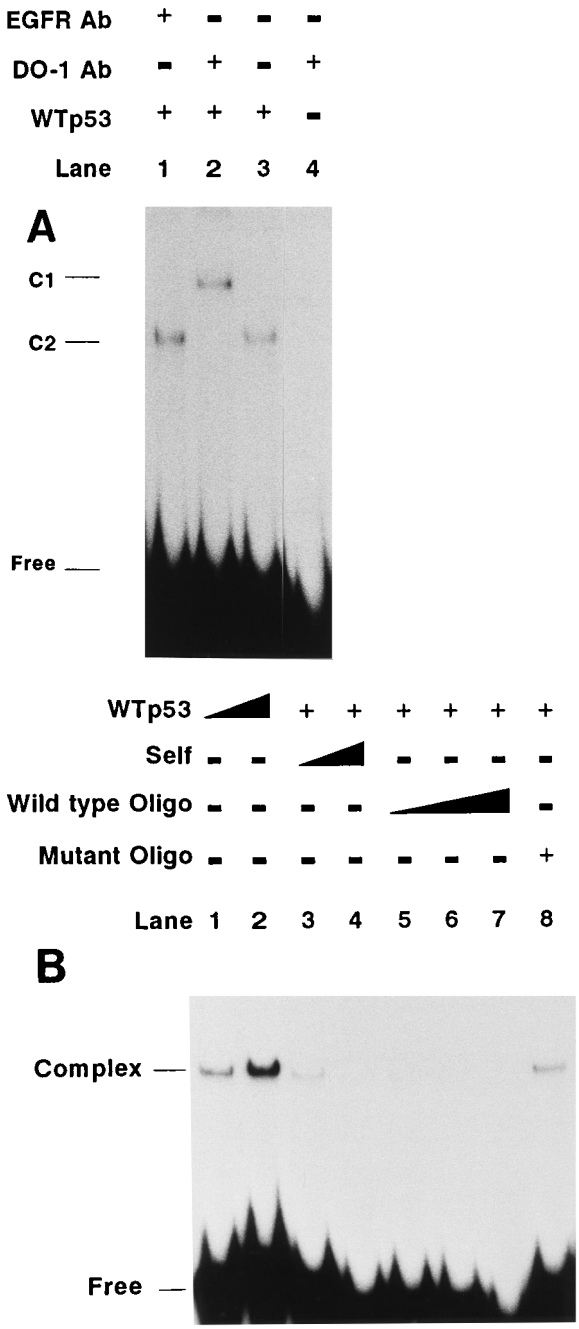

FIG. 4. Wild-type human p53 binds to the human PCNA promoter. (A) EMSA of wild-type p53 binding to the PCNA promoter. The PCNA promoter segment P5 (−272 to −46 [Fig. 3A]) was labeled at the ends with ^{32}P by kinasing and incubated with purified wild-type human p53 in the presence and absence of p53 antibody (DO-1 Ab) or a nonspecific antibody (EGFR Ab) as described in Materials and Methods and Results. The protein-DNA complexes were resolved in a native gel and autoradiographed. Two complexes (C1 and C2) are indicated. C2 was formed when wild-type p53 was incubated with the probe in the absence of any antibodies, and C1 is the supershifted complex in the presence of anti-p53 antibody (DO1). (B) Sequence-specific binding of wild-type p53 to the PCNA promoter. In this case, the DNA binding was carried out in the presence or absence of increasing amounts (100 and 200 ng) of unlabeled PCNA promoter fragment; P5 (Self, lanes 3 and 4); 50, 100, and 200 ng of double-stranded oligonucleotides (Oligo) with wild-type p53-binding sites (lanes 5, 6, and 7); or 200 ng of mutated p53-binding sites (lane 8) as outlined in Materials and Methods. All lanes received 100 ng (lane 2 received 200 ng) of immunopurified human wild-type p53 (wt p53). Data shown indicate that self DNA or double-stranded oligonucleotides with wild-type p53-binding sites competed the most strongly, showing sequence-specific interaction.

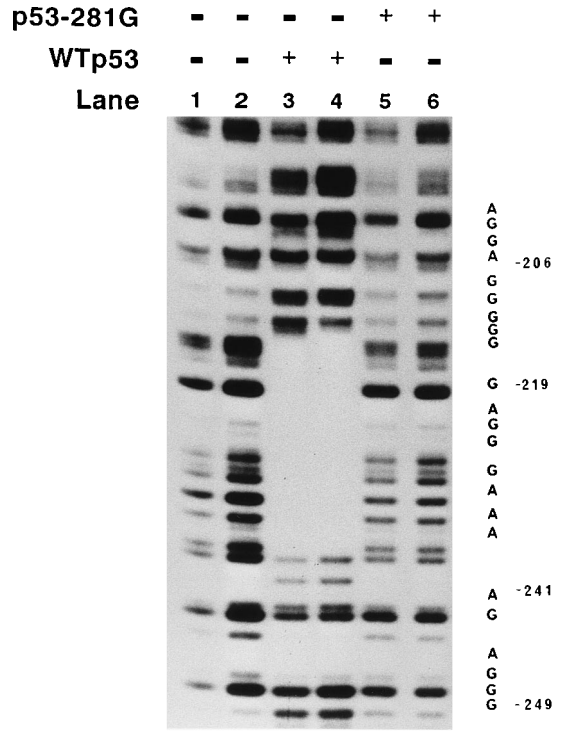

FIG. 5. DNase I footprint of human wild-type p53 on the human PCNA promoter. DNase I footprint analysis was carried out with wild-type p53 (wt p53) or mutant p53-281G as described in Materials and Methods. The DNA probe used was radiolabeled at one end only. Lanes 1 and 2 received 50 and 100 ng of bovine serum albumin, lanes 3 and 4 received 50 and 100 ng of immunopurified human wild-type p53, and lanes 5 and 6 received 50 and 100 ng of immunopurified human p53-281G protein, respectively. The protected sequences were identified by electrophoresing a G+A Maxam and Gilbert ladder of the same fragment. The sequences and the nucleotide positions are shown on the right.

and the wild-type oligonucleotide (lanes 5, 6, and 7) were far more successful in inhibiting PCNA promoter binding than the mutant oligonucleotides (lane 8). Therefore, wild-type p53 specifically interacts with the PCNA promoter sequence between −272 and −46.

After determining sequence-specific interaction between wild-type p53 and the PCNA promoter sequence from −272 to −46, we performed DNase I footprinting experiments to identify the sequences where wild-type p53 interacts on the promoter. For DNase I footprint analysis we have utilized the same promoter fragment used in EMSA except that the probe was labeled at one end only. Figure 5 depicts a typical footprint of wild-type p53 on the PCNA promoter (lanes 3 and 4). No DNA binding was found when a purified mutant protein, p53-281G, was used (lanes 5 and 6), showing that mutations in the conserved domain of p53 inhibit sequence-specific binding of p53 to the PCNA promoter. Comparison of the DNA sequence (−241 to −219) protected by wild-type p53 with the consensus p53-binding sites identified by El-Deiry et al. (18) indicated only two mismatches, suggesting sequence similarity with the consensus p53-binding site.

DISCUSSION

In this communication we demonstrate that low levels of wild-type p53 transcriptionally activate the human PCNA promoter. When expressed at a similar level, tumor-derived p53 mutants failed to activate the PCNA promoter. Transient ex-

pression of higher levels of wild-type p53 inhibits the promoter activation in vivo, whereas tumor-derived p53 mutants significantly activate the promoter under similar conditions. Perhaps this follows the physiologically achievable concentrations of these proteins. For wild-type p53, since its half-life is small, the usual concentration in normal cells will be very low. DNA damage may enhance the p53 concentration to a level appropriate for activation of the PCNA promoter. However, a further increase in wild-type p53 as achieved by transient transfection may even cause inhibition of the PCNA promoter. This is consistent with what we (13) and others (30, 45, 47) have observed. Transient expression of wild-type p53 from 5 μg of pCMV.p53, however, is high and may not reflect a physiological situation. Mutant p53 is usually found in relatively higher concentrations in tumor cells because of increased stability (40). Thus, it is possible that the high levels of mutant p53 often present in cancer cells activate PCNA gene transcription. However, in this report the levels of mutant p53 expressed transiently by our expression plasmids have not been compared with the levels of mutant p53 found in cancer cells.

EMSA and DNase I footprint analysis show that the wild-type p53 sequence specifically interacts with the PCNA promoter from −238 to −212. The identified sequence (5′-TC-C**AcAtATGCCCGGACTTGTTC**TGCG-3′) bears sequence similarity (boldface letters) to the consensus p53 binding site identified earlier (18), and only two mismatches (lowercase letters) were found. When a segment containing the wild-type p53-binding site was placed upstream of a TATA box in a heterologous system, the promoter became wild-type p53 responsive. This indicates that the p53-binding site can function as a p53 response element independent of the total context of the promoter (Table 2). We have also shown that the wild-type p53 response element can function bidirectionally, a property reported for other wild-type p53 response elements (14, 83). Significantly, mutant p53-mediated transactivation of the PCNA promoter does not require the wild-type p53 binding site. A PCNA promoter deletion mutant nonresponsive to wild-type p53 can be activated by p53-281G (Fig. 3A), suggesting that mutant p53 must use some other mechanism to transactivate the promoter. Since p53-281G fused with the GAL4 DNA-binding domain activates a promoter with GAL4-binding sites (12), it seems likely that the mutant protein binds to a transcription factor with a binding site on the promoter and thereby gets recruited to the promoter.

Although the PCNA promoter has a p53 response element that can be used for transactivation by wild-type p53, so far three reports indicate the opposite, that wild-type p53 overexpression represses the PCNA transcription (13, 30, 47). The results presented here demonstrate that the level of wild-type p53 is critically important to determine the effect of wild-type p53 on the PCNA promoter activation. At a low concentration wild-type p53 transactivates the promoter, while at a relatively higher concentration it represses the promoter (Fig. 1). It is possible that previous reports of the effect of wild-type p53 on the endogenous PCNA level were based on the use of relatively high levels of p53. Because of the way in which those cell lines were derived, multiple p53 expression plasmids might have been integrated into the genome, resulting in relatively high endogenous concentrations of p53. The stable transfectants generated by Mercer et al. used a hormone-inducible promoter to express p53, which might produce high levels of p53 when induced by the hormones (47). In the future it will be important to introduce a single copy of either a temperature-sensitive p53 mutant (e.g., p53-143A) or wild-type p53 under stringent control (so that it does not express constitutively) in a cell lacking endogenous p53 and then determine the effect of wild-type p53 expression on the endogenous PCNA level.

The presence of a positive wild-type p53 response element on the PCNA promoter suggests a role of wild-type p53 in modulating PCNA levels, at least under certain conditions. Interestingly, mild UV irradiation and serum stimulation increase wild-type p53 and PCNA levels simultaneously (26). This covariance may indicate a direct relationship between PCNA and p53 levels. Moreover, the recent findings about the interaction of GADD45 and PCNA suggest that p53 plays a role in transiently activating PCNA. PCNA might then interact with GADD45 to help the nucleotide excision repair (69). Lastly, as pointed out earlier, it is possible that wild-type p53 induces PCNA production for rapid repair of damaged DNA.

The observations reported in this communication thus raise the possibility that mild DNA damage moderately elevates the wild-type p53 level in cells and in turn increases PCNA expression, presumably to assist DNA repair. Excessive increases in the wild-type p53 level may lead to inhibition of PCNA expression as a result of apoptosis. p53 mutants found in cancer cells are usually expressed at a relatively high level which may constitutively induce PCNA expression, resulting in hyperproliferation of the cells.

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