Modulation of Cellular and Viral Promoters by Mutant Human p53 Proteins Found in Tumor Cells

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Wild-type p53 has recently been shown to repress transcription from several cellular and viral promoters. Since p53 mutations are the most frequently reported genetic defects in human cancers, it becomes important to study the effects of mutations of p53 on promoter functions. We, therefore, have studied the effects of wild-type and mutant human p53 on the human proliferating-cell nuclear antigen (PCNA) promoter and on several viral promoters, including the herpes simplex virus type 1 UL9 promoter, the human cytomegalovirus major immediate-early promoter-enhancer, and the long terminal repeat promoters of Rous sarcoma virus and human T-cell lymphotropic virus type I. HeLa cells were cotransfected with a wild-type or mutant p53 expression vector and a plasmid containing a chloramphenicol acetyltransferase reporter gene under viral (or cellular) promoter control. As expected, expression of the wild-type p53 inhibited promoter function. Expression of a p53 with a mutation at any one of the four amino acid positions 175, 248, 273, or 281, however, correlated with a significant increase of the PCNA promoter activity (2- to 11-fold). The viral promoters were also activated, although to a somewhat lesser extent. We also showed that activation by a mutant p53 requires a minimal promoter containing a lone TATA box. A more significant increase (25-fold) in activation occurs when the promoter contains a binding site for the activating transcription factor or cyclic AMP response element-binding protein. Using Saos-2 cells that do not express p53, we showed that activation by a mutant p53 was a direct enhancement. The mutant forms of p53 used in this study are found in various cancer cells. The activation of PCNA by mutant p53s may indicate a way to increase cell proliferation by the mutant p53s. Thus, our data indicate a possible functional role for the mutants of p53 found in cancer cells in activating several important loci, including PCNA.

p53, a nuclear phosphoprotein, is expressed in normal cells and appears to be involved in the regulation of cellular proliferation (39; also see reference 33 for a review). Mutant p53 genes can cooperate with the *ras* oncogene to transform primary rodent fibroblasts (24, 29). Wild-type p53 has been shown to inhibit proliferation of transformed cells, suppress oncogene-mediated cell transformation, and eliminate the tumorigenic potential of tumor-derived cell lines (2, 3, 12-14, 16, 40, 44). Accordingly, p53 is considered to be an anti-oncogene or tumor suppressor gene. Somatic and germ line (in Li-Fraumeni syndrome) mutations of the p53 gene have been detected in a variety of human tumors; the mutations are concentrated in phylogenetically conserved sequence domains (26, 33, 36, 53). p53 mutations are the most frequently reported genetic defects in human cancer (26, 27, 47, 56, 58).

Mutant p53 proteins that are transforming or are found in tumor cells have properties that are different from those of the wild-type protein. Transforming mutants do not bind to simian virus 40 T antigen (57) but form a stable complex with a heat shock protein (hsc70) (17). Sequence-specific DNA binding by p53 has been reported (4, 30). Wild-type (but not mutant) p53 binds to the 21-bp repeats of the simian virus 40 early and late promoters (4) and to TGCCT repeats present in the human ribosomal gene cluster (30). Wild-type p53 inhibits simian virus 40 DNA replication in vivo and in vitro by forming complexes with T antigen and inhibiting the unwinding capability of T antigen, whereas the transforming

mutants do not (5, 18, 19, 59). Wild-type (but not mutant) p53 has recently been shown to inhibit *c-fos* transcription (20) and to repress transcription from several cellular and viral promoters (9, 20, 50). Interestingly, the wild-type p53 inhibited the multiple drug resistance gene promoter activity, whereas one mutant (p53 175 R→H) activated the promoter significantly (9).

In order to determine the role mutant p53s may play in activating cell proliferation, we have studied the effect of mutant human p53 expression on the activity of the human proliferating-cell nuclear antigen (PCNA) promoter fused to a chloramphenicol acetyltransferase (CAT) reporter gene. We have also studied the effect on several viral promoter constructs to determine the specificity of p53 functions. Interestingly, mutants of p53 did activate the PCNA promoter significantly. Several viral promoters were also enhanced to various degrees. This suggests that wild-type p53 inhibits various promoters, in keeping with its tumor suppressor activity, but that the transforming mutants have the capacity to activate a cellular DNA replication gene. For the activating function of a mutant p53, a minimal promoter containing a TATA box is sufficient. However, the presence of an activating transcription factor (ATF)-binding site or cyclic AMP response element significantly improves the magnitude of activation. We demonstrate that activation by a mutant p53 is a direct effect and not an indirect release of inhibition from wild-type p53. Our data suggest that an activating mutation of p53 may contribute to the development of cancer by activating expression of crucial genes.

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FIG. 1. (a) Schematic representation of the p53 gene product. Conserved domains II to V are indicated by stippled areas. Positions of amino acid substitutions in the mutants that are used in this study are indicated below. (b) Effect of expression of wild-type and different mutant human p53s on the expression of PCNA promoter activity. HeLa cells were cotransfected with PCNA.CAT and pHCMV.Bam (vector alone) or pHCMV.Bam expressing either the wild type or one of the mutant p53s: c143A (V to A at amino acid 143), 175H (R to H at amino acid 175), c248W (R to W at amino acid 248), 273H (R to H at amino acid 273), or 281G (D to G at amino acid 281), as described in Materials and Methods.

MATERIALS AND METHODS

DNA plasmids. Wild-type and mutant human p53 expression plasmids (generously provided by Arnold J. Levine, Princeton University) utilize the human cytomegalovirus (CMV) major immediate-early promoter-enhancer (position -671 to +73) from the vector pHCMV-Neo-Bam (25). p53.cWT contains a wild-type p53 cDNA, whereas p53.c143A (Val to Ala at amino acid 143) and p53.c248W (Arg to Trp at amino acid 248) contain mutant p53 cDNAs (25). p53.175H (Arg to His at amino acid 175), p53.273H (Arg to His at amino acid 273), and p53.281G (Asp to Gly at amino acid 281) are mutant p53 cDNA genomic chimeras; all contain introns 2 through 4 (25). The neomycin resistance gene was removed from all plasmids by treatment with *HindIII* and *XbaI*. In the names of the mutants, "c" indicates cDNA clones and capital letters indicate amino acids.

The CAT plasmids described here all contain the *Escherichia coli* CAT gene under the transcriptional control of the following promoters: PCNA (human PCNA promoter) (45); CMV (human CMV major immediate-early promoter-enhancer) (10); UL9 (herpes simplex virus type 1 [HSV-1] UL9 gene promoter (11a); RSV (Rous sarcoma virus 3' long terminal repeat [LTR]) (11); and HTLV-I (human T-cell lymphotropic virus type I LTR) (52). The plasmids are designated as promoter name.CAT. PCNA.CAT was a generous gift by Gilbert Morris, Cold Spring Harbor Laboratory. PCNA(BstXI del).CAT was constructed by deleting sequences in the promoter region of PCNA.CAT (45) from position -1269 to -269. TATA.CAT, SP1.CAT, and ATF.CAT were generously provided by J. D. Gralla (60). TATA.CAT contains the TATA sequence (60) as the sole promoter element, SP1.CAT contains one SP1 site and a TATA element, while ATF.CAT contains an ATF-binding site and a TATA element.

Cell culture and transfection. Human cervical carcinoma (HeLa) cells were obtained from the American Type Culture Collection and propagated in minimum essential medium containing 10% fetal calf serum. Subconfluent cells were transfected by the calcium phosphate-DNA coprecipitation method with dimethyl sulfoxide shock at 4 h posttransfection (8, 22). In a typical experiment, 5×10^6 cells were cotransfected with 2.5 μ g of a CAT construct and 5 μ g of a p53 expression plasmid (or 5 μ g of the expression vector without p53 sequences, as a negative control). All transfection experiments were repeated several times. A 20 to 40% varia-

tion in activities was observed from one experiment to another. Thus, an increase or decrease in activity less than twofold may not be considered significant.

CAT assay. Cells were harvested at 48 h posttransfection and lysed by three successive cycles of freezing and thawing. Extracts were normalized for protein concentration and assayed for CAT enzyme activity (21). CAT activity was detected by thin-layer chromatographic separation of [14 C] chloramphenicol from its acetylated derivatives, and it was quantitated by cutting out radioactive spots from the thin-layer chromatograph plate after autoradiography.

RESULTS

Activation of PCNA promoter activity by mutants of human p53. The PCNA gene encodes a nuclear protein that acts as an auxiliary factor of DNA polymerase δ and is presumably a part of the cellular replication machinery (1, 54). Growth suppression induced by the wild-type p53 protein is accompanied by a down-regulation of PCNA expression (43). More recently we determined that wild-type p53 inhibited the function of the PCNA promoter in HeLa cells (55). Effects of different mutants of p53 on PCNA promoter function were examined in the present study. The mutants are: c143A, 175H, c248W, 273H, and 281G. These mutants were chosen because they contain the frequently mutated amino acid residues found in tumors (26, 33) (Fig. 1a). These residues fall in or near domains II to IV, which are highly conserved in vertebrate species (53). PCNA.CAT (45) was cotransfected into HeLa cells by the calcium phosphate precipitation technique as described in Materials and Methods with the pHCMV.Bam expression vector alone or with the plasmid expressing either the wild-type or one of the mutant forms of p53 (c143A, 175H, c248W, 273H, or 281G). After 48 h, CAT activity (PCNA promoter activity) was assayed in these cells. While wild-type p53 inhibited PCNA.CAT activity in transient assays by fourfold, the mutants did not (Fig. 1b). In fact, most of the mutants enhanced PCNA promoter activity significantly (4- to 11-fold) (Table 1).

Effect of mutants of p53 on various viral promoters. We have used various viral promoters to determine whether all of them are activated by mutant p53s. These included the CMV early promoter-enhancer (CMV.CAT) (10), HSV-1 UL9 promoter (UL9.CAT) (11a), RSV LTR (RSV.CAT) (11), and HTLV-I LTR (HTLV.CAT) (52). The promoter activities were determined by CAT assays after cotransfecting the respective promoter constructs with the pHCMV.Bam expression vector alone or with the plasmid expressing

FIG. 2. Effect of expression of wild-type and mutant human p53s in HeLa cells on the expression of CMV immediate-early promoter activity (a), HSV-1 UL9 promoter activity (b), RSV LTR promoter activity (c), and HTLV-I LTR promoter activity (d). Assays were carried out as described in the legend to Fig. 1 and in Materials and Methods.

either the wild-type or one of the five mutants of p53 into HeLa cells (Fig. 2; Table 1). The experiments were repeated several times, with qualitatively similar results. Representative examples are shown in Fig. 2. Although all the promoters were inhibited significantly by the expression of wild-type p53, mutants of p53 had a relatively moderate enhancing or no effect on the expression of the various promoter.CAT constructs. There were, however, significant differences between the extent of activation by different mutants of p53 and different promoters. In fact, in the case of UL9.CAT, some of the mutants had a slight inhibitory effect on the CAT activity.

Effect of p53 expression on different promoter activities in the Saos-2 cell line. The effect of wild-type and mutant p53s on promoter functions in a cell line devoid of endogenous p53 was determined by using Saos-2 cells. This was necessary for two reasons. (i) HeLa cells express human papillomavirus type 18 E6 protein (51a) which may influence the

effect of p53 expressed by transfection, and (ii) HeLa cells are known to produce translatable p53 mRNA (41). If a mutant p53 were produced in HeLa cells, the observed inhibition by the wild-type p53 may then be a simple release of activation of promoters by a mutant p53. Saos-2 cells are human osteosarcoma cells that do not produce detectable p53 because of a genomic deletion (12, 40). Expression of several promoters, PCNA.CAT, HSV-1 UL9.CAT, RSV.CAT, and CMV.CAT, was studied in these cells. As shown in Fig. 3, all the promoters were affected to a lesser extent than that observed in HeLa and Vero cells (55; this communication). The reason is not clear at present. The extent of inhibition of PCNA.CAT (49% reduction in activity) and UL9.CAT (46.7% reduction in activity) does not seem to be significantly high, while those of RSV LTR.CAT (78.1% reduction) and CMV.CAT (74.4%) are slightly more striking. Mutant p53s were also tested for their effect on PCNA.CAT in Saos-2 cells. The data in Fig. 4 demonstrate that PCNA.CAT expression was activated significantly by the p53 mutants in a similar fashion as it was in HeLa cells. The mutants enhanced the activity relative to vector alone as follows: c143A, 5.2-fold; 175H, 7.5-fold; c248W, 8.7-fold; 273H, 10-fold; and 281G, 11.7-fold.

Minimal promoter elements required for transactivation by a mutant p53. To determine the minimal promoter element necessary for transactivation by a mutant p53 (p53.281G), we used the PCNA promoter as the starting point, since it showed the highest activation by mutants of p53 (Fig. 1 and 2). We first constructed a PCNA.CAT deletion mutant by deleting sequences from -1269 to -269. Figure 5 shows the CAT expression obtained by using this deletion mutant in

TABLE 1. Effect of wild-type and mutant p53 expression on different promoter activities in HeLa cells

Promoter	% Activity relative to vector alone					
	Wild-type p53	c143A	175H	c248W	273H	281G
PCNA	25.3	183.0	406.0	1,086.8	570.6	867.7
CMV	10.8	114.2	122.3	113.0	133.9	107.8
HSV-1 UL9	4.5	57.9	53.7	56.7	113.1	457.0
RSV LTR	6.0	95.7	149.2	198.8	264.1	360.6
HTLV-I LTR	26.3	241.5	207.3	231.7	270.9	263.5

FIG. 3. Effect of expression of wild-type human p53 on the activity of different promoters in Saos-2 cells. Saos-2 cells do not have endogenous p53. The assays were carried out as described in the legend to Fig. 1 and in Materials and Methods.

HeLa cells in the presence of vector alone or of a mutant p53-expressing plasmid. It is clear that the mutant p53 could activate the PCNA.CAT deletion mutant significantly. This promoter has potential binding sites for several transcription factors (45). Among these are three SP1 and three ATF/CREB (cyclic AMP recognition binding protein) binding sites (Fig. 6). Therefore, we tested whether p53.281G can activate a minimal promoter with a TATA box alone or with a TATA box and one SP1-binding site or a TATA box and one ATF/CREB-binding site. These promoters have been cloned upstream of a CAT gene as described recently (60) (Fig. 6). The synthetic ATF/CREB site used in ATF.CAT corresponds 100% with the first ATF/CREB site in PCNA (BstXI del).CAT. However, it is possible that a similar (or identical) factor(s) may bind into the other two sites also. Figure 7 (also Table 2) shows the results of a transfection assay with these constructs. Promoter activities were determined in the presence of vector alone or of vector expressing wild-type or a mutant (281G) p53. Clearly, maximum activation was observed in the case of the ATF.CAT construct (Table 2). The other two promoters were marginally affected. Therefore, it seems that the mutant p53 may functionally interact with the ATF-binding site to activate tran-

FIG. 4. Effect of expression of wild-type and mutant human p53s on the promoter activity of PCNA.CAT in Saos-2 cells. Experiments were performed as described in the legend to Fig. 1 and in Materials and Methods.

FIG. 5. Effect of expression of mutant p53s on the promoter activity of PCNA(BstXI del).CAT in HeLa cells. PCNA promoter sequences from -1269 to -269 were deleted in this construct. Assays were performed as detailed in the legend to Fig. 1 and in the text.

scription. Recently, Morris and Mathews (46) demonstrated that E1A activated the PCNA promoter through the ATF/CREB site located 50 nucleotides upstream from the transcription initiation site of the promoter. This suggests that E1A and the mutant p53 may function through identical sequence elements.

DISCUSSION

Several groups recently reported an inhibitory activity of wild-type p53 on different cellular and viral promoters (9, 20, 50, 55). Chin et al. (9) demonstrated that wild-type p53 inhibited the MDR1 gene promoter but a mutant (p53.175H) significantly activated it. Santhanam et al. (50) and Ginsberg et al. (20) also examined a few mutants, including p53.143A, and reported that mutants were less inhibitory to various promoters. The results described in the present communication show that overexpression of wild-type human p53 can,

FIG. 6. Schematic representation of PCNA(BstXI del).CAT, TATA.CAT, SP1.CAT, and ATF.CAT. Prominent transcription factor binding sites are depicted. Individual clones are described in the text. The following are the sequences of sites located on the promoters: PCNA(BstXI del).CAT, TGACGA (ATF/CREB), GAG GCGGG (SP1), GCGGCGGG (SP1), GCGGCGGG (SP1), ACGTCG (ATF/CREB), and CGACGT (ATF/CREB); TATA.CAT, TATA AAA (TATA); SP1.CAT, CCGCGCC (SP1) and TATAAAA (TATA); and ATF.CAT, TCGTCA (ATF/CREB) and TATAAAA (TATA).

FIG. 7. Effect of wild-type or a mutant (p53.281G) p53 on promoter activities of TATA.CAT, SP1.CAT, and ATF.CAT in HeLa cells. TATA.CAT has a single TATA box as the sole transcription element, SP1.CAT has one SP1 site and the TATA box, and ATF.CAT has an ATF-binding site and a TATA box. Assays were carried out as described in the text.

as expected, exert an inhibitory effect on a variety of viral promoters as well as on the cellular PCNA promoter (4- to 25-fold; Table 1), whereas mutants of p53 have either no activity or, for some promoters, positively activating roles. The PCNA promoter was most significantly up-regulated (Table 1). Different mutants of p53 have different quantitative effects on promoter activities (Table 1). Previously we reported that PCNA.CAT activity in HeLa cells was reduced by 41.2% (less than a twofold reduction) by c143A, whereas the data presented in Fig. 1b of this report suggested an increase by 83% (less than a twofold increase). Because of the inherent variability of transfection experiments and because we could not normalize the transfection efficiencies (all the promoters that we tested so far were affected by p53), we have chosen not to consider an effect significant for which a promoter activity has been altered by less than twofold. Thus, in our hands, except for HTLV-I LTR, all the promoter constructs studied (Table 1, PCNA.CAT, CMV.CAT, UL9.CAT, and RSV LTR.CAT) were not affected significantly by the mutant p53.c143A.

It is significant that some of the mutants of p53 have an activating role in increasing the promoter activity of a key DNA replication gene, PCNA (6, 7, 28, 42, 48). It will be important to determine the effect of these proteins on the expression of other crucial genes like DNA polymerase α and replication protein A (7, 54). It is also possible that the mutated p53s will activate genes that are directly involved in inducing cell proliferation, like *c-myc*, *c-fos*, and *c-jun* (23). The expression of a p53 with a mutation at any of the four amino acid positions 175, 248, 273, or 281 correlates with their presence in tumor cells (26, 33). It remains to be determined what role these activating mutations have in establishing tumor formation. Recently, an elegant work by

Zambetti et al. (62) demonstrated that a mutant p53 (c143A) is required for maintenance of the transformed phenotype in cells transformed with p53 plus *ras* cDNAs. It is therefore possible that an activating function (transcriptional activation) is necessary and responsible for maintenance of the transformed phenotype.

The mutant p53.281G activated the PCNA promoter in the Saos-2 cell line which does not have a detectable p53 gene (12, 40). This experimental result suggests that the mutant p53 directly interacts with the process leading to gene expression. However, at this stage one cannot rule out a general activation of cellular growth as the cause of the PCNA promoter activation. A direct role becomes more possible, because the mutant p53.281G specifically activates a minimal promoter containing an ATF/CREB-binding site. It is, therefore, possible that the mutant p53 is functionally interacting with the transcriptional factor ATF/CREB, resulting in an increase of PCNA promoter activity because the PCNA promoter has multiple ATF/CREB-binding sites (45, 46). Sequence analysis indicates that other promoters (CMV immediate-early, RSV LTR, and HTLV-I LTR) that were also activated by mutants of p53 have discernible sequences resembling binding sites for ATF/CREB. In order to verify this model and to establish the mechanism, one first needs to determine whether purified p53.281G will activate PCNA.CAT or ATF.CAT in vitro. E1A has been found to interact with ATF (34, 37, 38) and TFIID (32). It has been postulated that E1A may bridge ATF and TFIID (32). It is intriguing that the PCNA promoter is also activated by E1A interacting with an ATF/CREB site (46). Whether a mutant p53 has a similar role mechanistically has yet to be determined.

It is interesting that the wild-type p53 is an inhibitor, whereas a mutant p53 is an activator, of gene expression. At present it is not clear whether the mode of action of these two proteins focuses on the same step of growth or transcription. The observations reported in the present communication suggest a functional involvement of a mutant p53 with the ATF/CREB-binding site or with ATF/CREB directly in activating transcription. The nature of the interaction is as yet unknown. ATF/CREB can be activated by the cyclic AMP-mediated signal transduction pathway as a result of external stimuli (35). This raises the possibility that the effect of an external stimulus can be magnified by the action of a mutant p53 in activating expression of crucial genes in a cell. It remains to be seen whether these factors, mutant p53 and transcription factor(s), interacting at ATF/CREB-binding sites can directly cooperate in increasing the transcription rate. Our observation that a mutant p53 can activate an important cellular promoter by interacting with the ATF/CREB-binding site allows one to speculate that an interaction between signal transduction pathways and a mutant tumor suppressor may activate the route to tumor development.

p53 represents a relatively small molecule with many puzzling functions. Wild-type p53 acts as a transcriptional activator in a human cell line and in yeast cells on artificial promoters with p53-binding sites (31, 51); the activating mutants cannot. Wild-type p53 can activate the muscle creatine kinase promoter which has a p53-binding site (61, 61a). Also, p53.GAL4 fusion proteins containing the amino-terminal 73 amino acids of p53 can enhance transcription in yeast and mammalian cells (15, 49). This activation can be blocked by mutations, found in tumors, that are activating for transformation (26, 33, 47). These observations clearly indicate that p53 has a strong transactivation domain. Yet, in

TABLE 2. Effect of wild-type and mutant p53 expression on different synthetic promoters in HeLa cells

Promoter	% Activity relative to vector alone	
	Wild type	281G
TATA	70.0	320.0
SP1	40.0	173.3
ATF	20.6	2,582.4

mammalian cell transfection systems, wild-type p53 acts as a strong inhibitor of gene expression (9, 20, 50, 55). The mechanism by which p53 exerts this inhibition is unclear. Another striking feature is the ability of a mutant p53 to activate transcription through an ATF/CREB-binding site (this study). One can try to build the following model to take these facts into account. Wild-type p53 has a strong transactivation domain that only becomes accessible to transcription factors on the promoter when the protein binds to its binding site on the promoter sequence. Under normal circumstances when the protein is not bound to a DNA, a cellular factor or a typical normal protein conformation prevents p53 from interacting with a transcription factor like ATF/CREB. A mutated p53 on the other hand, because of either altered conformation or inability to bind to a cellular factor, is free to interact with transcription factors on growth-related genes, activating their expression. Because of the mutation, the mutant cannot bind to promoters with p53-binding sites and thus fails to control cell growth. Thus, it can be speculated that the transactivation motif can be activated either by a mutation in the gene or by binding of the wild-type protein on its binding site. In the inactive normal stage, it may inhibit the general transcriptional process by interacting with promoter DNA or a generalized transcription factor like TFIID in a transcription-antagonistic fashion. Another possibility to explain the general inhibition of transcription is by activating the expression of a gene synthesizing a transcriptional inhibitor. This promoter may have a p53-binding site for p53-mediated activation.

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