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Overlap of the p53-responsive element and cAMP-responsive element in the enhancer of human T-cell leukemia virus type I

(tumor-suppressor gene/transcriptional activation/DNA binding/functional domains/point mutants)

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ABSTRACT **The wild-type p53 protein suppresses transformation, but certain missense mutants of p53 can transform cells. Although the wild-type p53 protein contains a transcriptional activation domain, no p53-responsive element has been identified. Here, we identified the p53-responsive element within the Tax-responsive element [21-base-pair (bp) enhancer] of human T-cell leukemia virus type I. Mutation analysis of the 21-bp enhancer indicated that the 16-bp sequence containing the cAMP-responsive element and its surrounding sequence was responsible for p53-induced transactivation. This 16-bp sequence was demonstrated to bind specifically to wild-type human p53 protein *in vitro*. Using a series of deletion mutants of p53, we showed that almost the entire region of p53 is needed for the transactivating capacity. Furthermore, the transforming mutants of p53 were unable to act as transcriptional activators. The p53-responsive element identified here should be useful to analyze the mechanism by which p53 regulates expression of a set of genes with a negative effect on cellular growth.**

The wild-type p53 gene product suppresses transformation (1, 2) and negatively regulates cell growth (3–6). In contrast, certain p53 mutants can immortalize primary cells (7) and cooperate with the *ras* oncogene to transform primary rat embryo fibroblasts (8, 9). Loss of heterozygosity of the short arm of human chromosome 17p in the region coding for p53 in a number of human cancers suggested the implication of the p53 gene in many inherited and sporadic forms of malignancies in humans (10–12). Point mutations were also found in the human p53-encoding gene from various tumors (10–13). Most of these mutations are in four regions of the p53 protein that are highly conserved among different species (14). The mechanism by which p53 negatively controls cell proliferation remains unclear. The p53 protein binds to simian virus 40 large tumor antigen (15, 16), adenovirus E1B protein (17), and human papillomavirus 16 E6 proteins (18). The p53 protein inhibits the ability of large tumor antigen to mediate replication of a simian virus 40 origin-containing plasmid *in vitro* (19–21). The p53 protein also has a transactivating capacity (22–24), and the enhancer of the muscle-specific creatine kinase gene contains a p53-responsive element (25). However, the DNA sequence of the p53-responsive element is unknown. To clarify the relationship between the p53-induced transcriptional activation and cellular proliferation, identification of the p53-responsive element is now required.

In this report, we have identified the p53-responsive element in the 21-base-pair (bp) enhancer of human T-cell leukemia virus type I (HTLV-I). The p53-responsive element identified here has been shown to bind to p53 from a lysate of cells transfected with the p53 expression plasmid. Furthermore, we have analyzed the functional domains of p53 required for transactivating capacity depending on the p53-responsive element identified here.

MATERIALS AND METHODS

Plasmid Construction. The p53 expression plasmid (pact53) was constructed by joining the 5′-regulatory region of the chicken β-cytoplasmic actin gene and the human wild-type p53 cDNA as described (26). All plasmids designed to express mutant p53 proteins were generated from the plasmid pact53. Most mutants were constructed by site-specific mutagenesis (27); some mutants were made by using the appropriate restriction enzyme sites.

Chloramphenicol Acetyltransferase (CAT) Cotransfection Analysis. CAT cotransfection experiments were done as described (26). Mixtures of 5 μg of CAT reporter plasmid, 6 μg of the effector plasmid pact53 or pact1 lacking the p53-coding region, and 5 μg of the internal control plasmid pras-β-gal, in which the human c-*HRAS1* promoter was linked to the β-galactosidase gene (28), were transfected into CV-1 cells. The amounts of cell extract used for the CAT assays were normalized with respect to β-galactosidase activity. The degree of conversion was measured by using a Bioimage analyzer (Fuji). All CAT cotransfection experiments were repeated three or four times, and typical results are shown here. The differences between each set of experiments were within 60%. The CAT reporter plasmids in which the human c-*ERBB2*, epidermal growth factor receptor, c-*HRAS1*, c-*FOS*, chicken β-actin, Rous sarcoma virus, or simian virus 40 early promoter was linked to the CAT gene were described (29–34). The CAT reporter plasmids containing various portions of HTLV-I long terminal repeat or concatemers of synthetic oligonucleotides shown in Figs. 1 and 2 were described (35, 36).

Immunoprecipitation of the p53–DNA Complex and Methylation-Interference Analysis. CV-1 cells (10^6 cells per 10-cm dish) were transfected with 15 μg of the p53 expression plasmid pact53 or pact1 lacking the p53-coding region. Forty-eight hours after transfection, whole-cell extracts (WCE) were prepared from four dishes as follows. Cells were washed with phosphate-buffered saline (PBS), harvested, and suspended in 0.6 ml of the lysis buffer containing 20 mM Hepes (pH 7.9), 25% (vol/vol) glycerol, 0.42 M NaCl, 15 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. After freezing and thawing, the cell lysates were centrifuged at 100,000 × *g* for 5 min, and

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Abbreviations: CAT, chloramphenicol acetyltransferase; CRE, cAMP-responsive element; HTLV-I, human T-cell leukemia virus type I; WCE, whole-cell extracts.

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the supernatants (WCE) were rescued, frozen, and stored at −80°C. Immunoprecipitation was done as described by Kern *et al.* (37). Briefly, 30 ng of the ^{32}P-labeled DNA was incubated with 5 μl of WCE, 95 μl of binding buffer [20 mM Tris·HCl, pH 7.2/100 mM NaCl/10% (vol/vol) glycerol/1% Nonidet P-40/5 mM EDTA], 4 μl (0.4 μg) of pAb421, and 4 μl (0.4 μg) of pAb1801 (purified monoclonal antibodies to p53, Oncogene Science, Manhasset, NY) at 4°C for 30 min. Protein A-Sepharose (1.5 mg) and 12.5 μg of poly(dI·dC), in 25 μl of binding buffer, were added and mixed at 4°C for 30 min. The pellet was washed twice with binding buffer, and the DNA was extracted with phenol/chloroform, ethanol-precipitated, and dissolved in electrophoresis sample buffer. The fragments were separated on a Tris borate nondenaturing polyacrylamide gel and visualized by a Bioimage analyzer.

For the methylation-interference assay, the DNA labeled at one end was generated with T4 polynucleotide kinase. DNA (2×10^6 dpm) was methylated at guanine residues by using dimethyl sulfate, ethanol-precipitated, and dissolved in 10 μl of 3 mM Tris·HCl, pH 7.5/0.2 mM EDTA. A 0.5-μl vol was removed as the DNA control. A total of 4.5 μl was added to a binding reaction containing WCE prepared from CV-1 cells transfected with the p53 expression vector. The immunoprecipitated DNA was purified and ethanol-precipitated. The control DNA and precipitates of bound DNA were cleaved with piperidine at the methylated sites. Equivalent amounts of labeled DNA were separated on a denaturing polyacrylamide gel.

Immunoblot Analysis of p53 Mutants. A mixture of 15 μg of the plasmid DNA encoding various p53 proteins and 5 μg of the internal control plasmid pras-β-gal DNA was transfected into CV-1 cells. Forty-eight hours after transfection, the cells were harvested and lysed in SDS/sample buffer as described (38). At the same time, β-galactosidase activity was measured by using a cell sample to confirm that transfection efficiencies were similar. The solubilized proteins prepared from ≈1.5×10^6 cells were separated by SDS/10% PAGE and then transferred to a nitrocellulose filter. The filter was blocked with bovine serum albumin, and p53 protein was detected by sequential binding of the anti-p53 monoclonal antibody (pAb1801), secondary alkaline phosphatase-conjugated goat anti-mouse IgG antibody, and a color development reagent (Promega).

RESULTS

Presence of the p53-Responsive Element in the 21-bp Enhancer Element of HTLV-I. To identify the p53-responsive element, we did cotransfection experiments with the p53 expression plasmid and a reporter plasmid in which the bacterial CAT gene was linked to various test promoters/enhancers. In our CAT cotransfection experiments without an internal control plasmid, such as pras-β-gal, which is usually used to normalize the transfection efficiencies, the activity of the human c-*HRAS1* promoter was not affected by p53, but the Rous sarcoma virus long terminal repeat promoter activity was greatly reduced by p53 (data not shown). Therefore, we used pras-β-gal in which the human c-*HRAS1* promoter was linked to the β-galactosidase gene as an internal control plasmid in our CAT cotransfection analysis. Cotransfection of most of the CAT reporter plasmids, 18 of 20 plasmids, with the p53 expression plasmid into CV-1 cells resulted in an 83–30% decrease in CAT activity. Results with typical reporter plasmids are shown in Fig. 1*A*. It is unlikely that all of these plasmids contain a common *cis*-element that is responsible for the p53-induced transrepression. When the reporter plasmid prasCAT1, which contains the human c-*HRAS1* promoter, was used, the level of CAT activity was not affected by p53. Among the 20 reporter plasmids used, the level of CAT activity of only one reporter plasmid WT

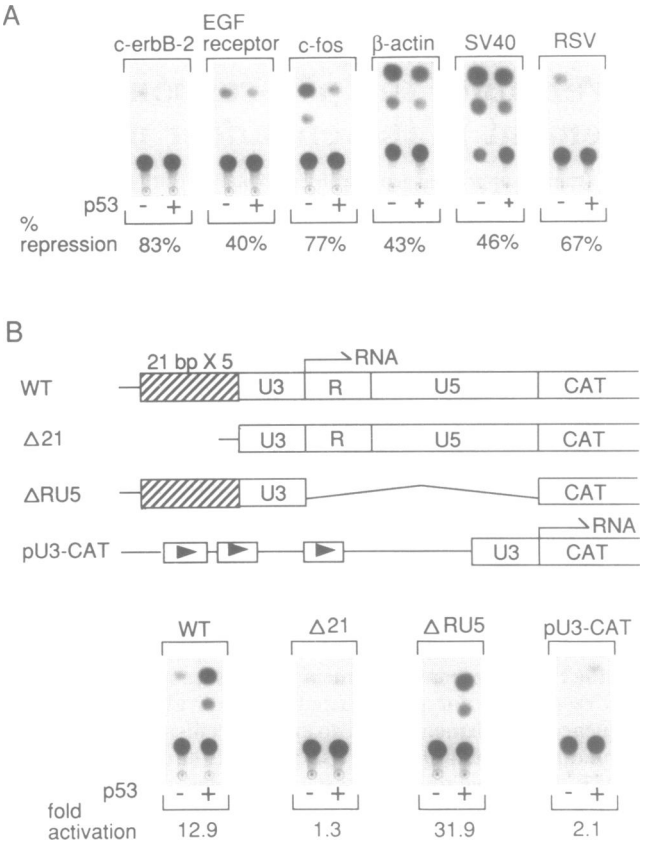

FIG. 1. Transactivation by the human wild-type p53. (*A*) Transrepression of various enhancers/promoters by p53. Mixtures of CAT reporter plasmid DNA in which the 5′-regulatory region of the gene shown above was linked to the CAT gene, the wild-type human p53 expression plasmid DNA(+) or the plasmid pact1 lacking the p53 cDNA(−), and pras-β-gal plasmid DNA were transfected into African green monkey kidney cells, CV-1. Forty-eight hours after transfection, CAT activities were assayed. (*B*) Transactivation of tandem repeats of the HTLV-I 21-bp enhancer by p53. The CAT constructs shown above were used for cotransfection.

was stimulated (13-fold) by p53 (Fig. 1*B*). In plasmid WT, the 21-bp enhancer element of HTLV-I, through which the product of the *tax* gene of HTLV-I (Tax) can transactivate expression of HTLV-I, and the core promoter of the HTLV-I long terminal repeat were linked to the CAT gene (36). We used deletion mutants of the promoter/enhancer region of WT5 in cotransfection experiments (Fig. 1*B*). Deletion of the tandem repeats of the 21-bp enhancer (Δ21) completely abolished the p53-induced transactivation, but removal of the downstream region from the RNA start site (ΔRU5) did not affect the p53-induced transactivation. Thus, five tandem repeats of 21-bp enhancer of HTLV-I can mediate the p53-induced transactivation.

Mutation Analysis of the 21-bp Enhancer. The 21-bp enhancer contains a sequence similar to the cAMP-responsive element (CRE) and half of the NF-κB site. To identify the p53-responsive element more precisely, we have used a series of point and deletion mutants of the 21-bp enhancer (ref. 36, Fig. 2). The tandem repeats of synthetic oligonucleotides shown in Fig. 2 were inserted into an enhancerless HTLV-I promoter linked to the CAT gene, and the generated constructs were used for cotransfection experiments. The B2 mutant, which has a mutation in the core sequence of CRE, was not responsive to p53. Two mutations (B1 and A2) in the CRE core region and the 5′ region of the CRE also dramatically abolished the transactivating capacity. Introduction of mutations into the 3′ region of the CRE (C1 and C2) also

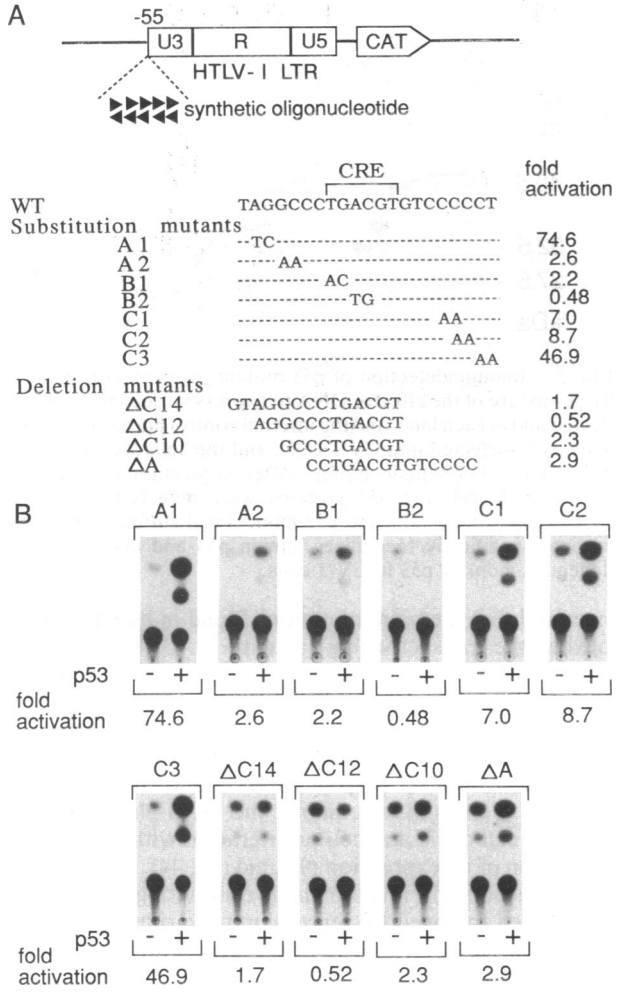

FIG. 2. Mutation analysis of the HTLV-I 21-bp enhancer. (*A*) Structure of CAT reporter DNAs containing a concatemer of the mutated enhancer. Constructs were made by inserting the concatemers of synthetic oligonucleotides shown above into the 5′ end of the HTLV-I promoter as described (36). For the substitution mutants, only substituted bases are shown (−, same as in WT). CAT cotransfection assays were done as described in Fig. 1, and the degree of transactivation by p53 is indicated on the right. LTR, long terminal repeat. (*B*) Transient expression of CAT activity. The CAT constructs shown in *A* were used for cotransfection.

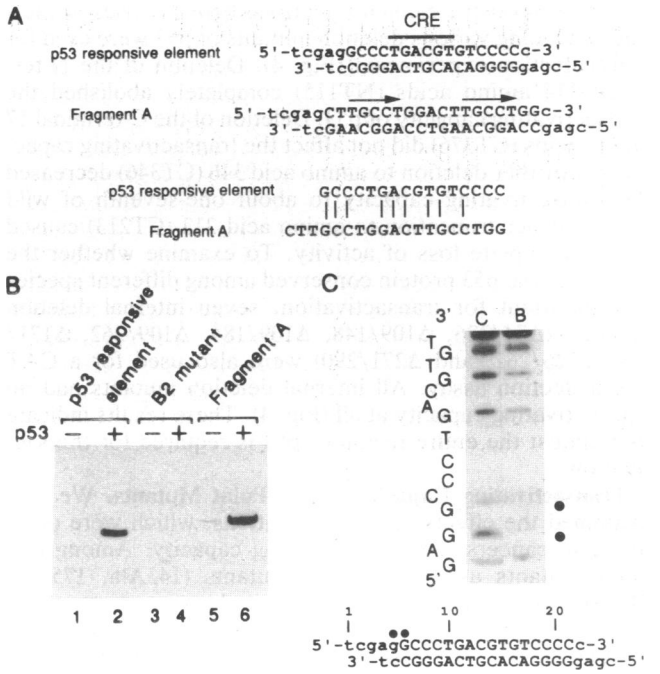

FIG. 3. Binding of the p53-responsive element to p53 from a lysate of cells transfected with the p53 expression plasmid. (*A*) DNA fragments used for an immunoprecipitation assay. The nucleotide sequences of the p53-responsive element and fragment A-containing repeats of TGCCT (37) are shown by an uppercase letter, and their flanking sequences are indicated by a lowercase letter. The homology between the p53-responsive element and the fragment A is also shown. The identical nucleotides are connected. (*B*) Detection of DNA fragments bound by p53 by an immunoprecipitation assay. The DNA fragment containing six tandem repeats of the p53-responsive element, the B2 mutant, or the fragment A was end-labeled and incubated with lysates from CV-1 cells transfected with the p53 expression vector (p53, +) or the control vector lacking the p53-coding region (p53, −). After immunoprecipitation with anti-p53, bound DNA fragments were recovered and separated on a nondenaturing 4% polyacrylamide gel. (*C*) Methylation-interference assay. DNA containing the p53-responsive element was end-labeled on the upper strand and partially methylated at guanine residues. Fragments retaining p53-binding activity were selected by immunoprecipitation with cell lysates of CV-1 cells transfected with p53 expression vector. Bound (lane B) and control (lane C) DNA samples were cleaved at methylated guanines and separated on 20% denaturing gel. Dots represent methylation-sensitive sites.

decreased the degree of p53-induced transactivation partially. Four deletion mutants of the 21-bp enhancer (ΔC14, ΔC12, ΔC10, and ΔA) also failed to mediate the p53-induced transactivation. In contrast, introduction of mutations into the regions far from the CRE (A1 and C3) stimulated the p53-induced transactivation, and A1 and C3 were stimulated by p53 74.6- and 46.9-fold, respectively. The bases changed in these mutants may affect but appear not to be essential for the p53-induced transactivation. Based on these results, we concluded that the sequence of the p53-responsive element is 5′-GCCCTGACGTGTCCCC-3′. Thus, the p53-responsive element overlaps the CRE.

Binding of p53 to the p53-Responsive Element. To examine whether the p53-responsive element identified here directly binds to p53, we have used an immunoprecipitation assay. The DNA fragment containing six tandem repeats of the p53-responsive element (Fig. 3*A*) was end-labeled with ^{32}P and incubated with p53 from a lysate of CV-1 cells transfected with the p53 expression plasmid. Labeled DNA fragments that bound to p53 were then recovered by immunoprecipitation with monoclonal antibodies against p53. As a control, we also used the fragment containing six tandem repeats of the fragment A sequence. The fragment A contains repeats of the sequence TGCCT and was recently shown to bind to p53 by Kern *et al.* (37). Furthermore, a homology was found between the nucleotide sequences of p53-responsive element and fragment A (Fig. 3*C*). Both DNA fragments containing the p53-responsive element or the fragment A sequence bound reproducibly to p53 (Fig. 3*B*). In contrast, the DNA fragment containing the sequence of the B2 mutant (see Fig. 2) did not bind to p53 (Fig. 3*B*). We then studied the requirements for binding at the single nucleotide level by using a methylation-interference assay. The DNA fragment containing the p53-responsive element was methylated *in vitro* and immunoprecipitated after binding to p53. The bound DNA was then cleaved with piperidine at methylated residues and separated by electrophoresis on a sequencing gel. The results (Fig. 3*C*) demonstrated that methylation at guanine residues at nucleotide positions 5 and 6 significantly interfered with binding. These results indicate that wild-type p53 proteins can bind to the p53-responsive element identified here.

Domain Analysis of p53. To identify the functional domains of p53 required for the transactivating capacity dependent on

the p53-responsive element identified here, a series of plasmids encoding various deletion mutants of p53 were used for cotransfection experiments (Fig. 4). Deletion of the N-terminal 114 amino acids (NT115) completely abolished the transactivating capacity of p53. Deletion of the C-terminal 17 amino acids (CT376) did not affect the transactivating capacity, but further deletion to amino acid 346 (CT346) decreased the transactivating capacity to about one-seventh of wild type. Further truncation to amino acid 213 (CT213) caused almost complete loss of activity. To examine whether the regions of the p53 protein conserved among different species are important for transactivation, seven internal deletion mutants (Δ74/326, Δ109/148, Δ109/184, Δ109/262, Δ171/184, Δ226/262, and Δ271/290) were also used for a CAT cotransfection assay. All internal deletion mutants had no transactivating capacity at all (Fig. 4). These results indicate that almost the entire region of p53 is required for transactivation.

Transactivating Capacity of p53 Point Mutants. We also examined the effects of point mutations, which were often found in cancers, on transactivating capacity. Among five point mutants used here, four mutants (143Ala, 175His, 248Trp, and 273His) were found in human cancers (for review, see refs. 39 and 40). The point mutation for Ala-135 → Val allows the mouse p53 protein to cooperate with activated Ras to transform primary rat embryo fibroblasts (41). Comparison of the amino acid sequence between human and mouse p53 indicates that amino acid 135 of the mouse p53 corresponds to amino acid 138 of human p53. Therefore, we also made the construct 138Val, in which alanine at amino acid 138 was replaced by valine. All five point mutants lacked transactivating capacity (Fig. 4). The levels of all deletion and point mutant proteins in transfected cells were not appreciably different from wild-type p53 (Fig. 5), indicating that the loss of transactivating capacity was not from any decrease in protein stability.

DISCUSSION

Our results indicate that the p53-responsive element overlaps CRE and the Tax-responsive element in the HTLV-I enhancer and that the point mutations found in human cancers abolished the transactivating capacity.

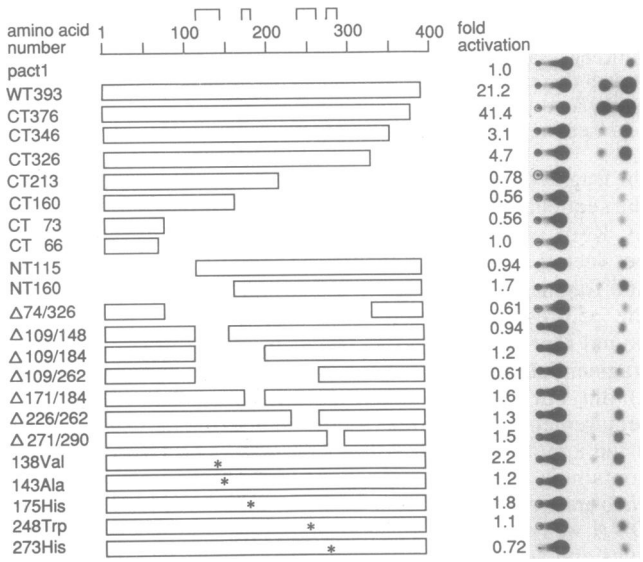

FIG. 4. Transactivation by p53 mutants. The four regions in the p53 protein, which are highly conserved among different species, are indicated on the top. p53 mutants are schematically represented and are named according to the position of the mutation and the substituted amino acid. CAT cotransfection assays were done by using the effector plasmid to express p53 mutant shown in each lane, and the results are summarized on the right.

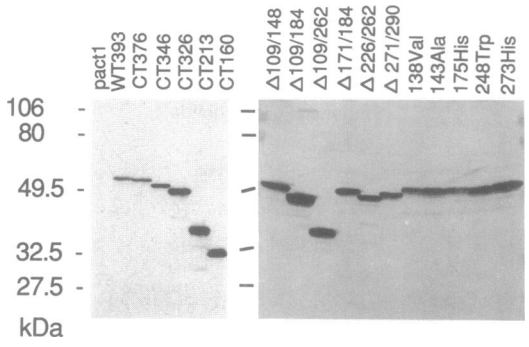

FIG. 5. Immunodetection of p53 mutant proteins in transfected cells. A mixture of the effector DNA to express various p53 proteins, indicated above each lane, and the internal control plasmid pras-β-gal DNA was transfected into CV-1 cells, and the cells were harvested and lysed in SDS/sample buffer. After separation on SDS/10% polyacrylamide gel, the p53 proteins were detected by anti-p53 monoclonal antibody. The anti-p53 monoclonal antibody (pAb1801) used here specifically recognizes human p53 and may not detect endogenous monkey p53 in CV-1 cells.

Cotransfection of many CAT reporter plasmids with p53 expression plasmid significantly decreased the level of CAT activity (Fig. 1*A*). During review of this manuscript, Ginsberg *et al.* (42) also reported that p53 can down-modulate the activity of a number of promoters. Some transcriptional activators, such as MyoD, inhibit the G_0–S phase of transition of the cell cycle and cell proliferation within 18 hr after introduction of its expression plasmid (43, 44). When growth inhibition occurs, the levels of labile transacting factors might be decreased followed by repression of various promoters/enhancers. The p53-induced transrepression of many promoters/enhancers observed here may occur by such a mechanism.

The p53-responsive element overlaps CRE in the HTLV-I enhancer, and mutation in the core sequence of CRE abolished the p53-induced transactivation. When we used CAT plasmids containing CRE derived from various genes, however, p53 did not stimulate CAT activity (data not shown). These results indicate that CRE is distinct from the p53-responsive element. p53 could stimulate the CAT expression only slightly (2.1-fold) from the native HTLV-I enhancer/promoter (Fig. 1*B*, pU3-CAT). Although the native HTLV-I enhancer contains three repeats of the 21-bp enhancer, sequences of the enhancers are not identical. Therefore, the native HTLV-I enhancer may contain only one copy of the p53-responsive element, and the presence of one copy may not be sufficient for significant transactivation by p53. Alternatively, the transcription factor(s) that bind to the sequence around the 21-bp enhancer in the U3 region may interfere with transactivation by p53.

Identification of the sequence of p53-responsive element allowed us to analyze the functional domains of p53 required for the transactivating capacity and the effects of point mutations in the p53 gene found in cancers on the transactivating capacity. Requirement of the N-terminal portion for transactivating capacity is consistent with the report (22) that the transcriptional activation domain is located in the N-terminal 73 residues. Our results, however, indicate that almost the entire region of p53 is necessary for transactivation. Consistent with these results, all five point mutations, which were found in human cancers or altered p53, become transformation competent, abolished transactivating capacity. These results strongly suggest that p53 regulates cellular growth negatively by regulating transcription of a set of genes.

Recently the wild-type p53-immunopurified proteins were shown to bind to the GC boxes in the simian virus 40 early promoter and the TGCCT repeated element (45, 46), although whether p53 itself contacts those sequences is unclear. Comparison of the sequence of p53-responsive element identified here with that of TGCCT repeated element indicates that 9 of 16 nucleotides are identical (Fig. 3*A*). Both the p53-responsive element and the TGCCT repeated element bound to p53 from a lysate of cells transfected with the p53 expression plasmid (Fig. 3*B*). However, neither the bacterially expressed p53 proteins nor the *in vitro*-translated p53 proteins bound to tandem repeats of the 21-bp enhancer of HTLV-I (data not shown). These results suggest that p53 proteins require a modification such as phosphorylation or a cofactor to function as an activator of transcription that interacts with the p53-responsive element identified here. Because p53 binds to the viral transcription factors (15–18), p53 quite possibly forms a complex with another cellular protein and binds to the p53-responsive element like the Myc/Max complex (46). Finally, the p53-responsive element identified here may provide a clue to clarify what kind of genes are regulated by p53 and the mechanism by which p53 regulates cellular proliferation.

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