The Gene Encoding p202, an Interferon-inducible Negative Regulator of the p53 Tumor Suppressor, Is a Target of p53-mediated Transcriptional Repression*

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The p53 tumor suppressor protein regulates the transcription of regulatory genes involved in cell cycle arrest and apoptosis. We reported previously that overexpression of p202, an interferon-inducible negative regulator of cell growth, negatively regulates the transcriptional activity of p53. Now we identify the gene encoding p202 as one whose mRNA and protein expression decrease in cells following the expression of wild-type, but not mutant, p53. Furthermore, the levels of p202 also decrease after exposure of cells to ultra violet light, which correlate with increase in the levels of p53. We report that the sequence-specific DNA binding of p53 to the 5'-regulatory region of the 202 gene contributes to the transcriptional repression of the 202 gene. Interestingly, overexpression of p202 in cells induced to undergo p53-dependent apoptosis significantly delays this process, indicating that the negative regulation of the 202 gene by wild-type p53 is important to potentiate apoptosis.

The protein, p202, is an interferon (IFN) inducible phosphoprotein (52 kDa) whose ectopic expression in a variety of cell types retards proliferation (1–4). Interestingly, the reduced basal levels of p202 (in consequence of the expression of antisense RNA to 202) in fibroblasts, under reduced serum condition, increase the susceptibility to apoptosis (5) and overexpression of p202 in Rat-1 cells inhibits c-myc-induced apoptosis (6). Together, these observations support the notion that p202 participates in the regulation of apoptosis. However, the molecular mechanism(s) remains to be identified.

The p53 family genes include genes encoding p73, p63, and p53 (7). The p53 tumor suppressor gene from the family continues to hold distinction as the most frequently mutated gene in human cancer (7–11). This has stimulated efforts to understand the function of this gene in normal and neoplastic state. A large number of functions have been attributed to p53, including cell-cycle checkpoints and apoptosis (11). In human and rodent cells containing wild-type p53 genes, the p53 protein is induced by a variety of stimuli, including chemotherapeutic agents, oxidative stress, hypoxia, nucleotide depletion, and oncogenic expression. Accumulated data suggest that the high frequency of p53 mutations in human cancer reflect the ability of this protein to induce programmed cell death or apoptosis (7–11).

The underlying mechanism of tumor suppressor activity of p53 resides in part in its ability to bind DNA in a sequence-specific manner (8, 10). It has been reported that a substantial number of genes containing p53 binding site(s) are activated by p53 (8, 11). These include mdm2, p21, and gadd45. p21 and gadd45 have been implicated in p53-mediated cell cycle regulation. In addition to playing a role as a DNA binding-dependant activator, p53 has also been reported to negatively regulate the transcription of a number of genes (11), including those for presenilin (12), topoisomerase IIa (13, 14), map4 (15), hsp70 (16), and other viral and cellular promoters (17). In contrast to the transcriptional activation by p53, no consensus sequence has been found in the promoters that are repressed by p53. It was initially reported that only the promoters containing a TATA box are repressed by p53 (18). However, in at least two cases, it has been reported that the specific DNA binding of p53 in the regulatory region of these genes results in transcriptional repression (19, 20).

Several studies have implicated a role for transcriptional repression in p53-dependent apoptosis (9, 11). For example, proteins such as BCL2 have been shown to inhibit p53-mediated transcriptional repression, whereas transactivation and G1 arrest functions remain unaffected (21). Additionally, deletion of the proline-rich region (amino acids 60–90) in human p53, which contains five repeats of the PXXP motif (22), renders it defective at apoptosis induction and transrepression, but not transactivation (23–25).

The observation that p202 negatively regulates the transcriptional activity of p53 (26), coupled with the fact that a number of proteins that regulate p53 functions are in turn regulated by p53, led us to test if p53 regulates the expression of p202. Here we report that the expression of wild-type, but not mutant, p53 results in a decrease in the 202 RNA and protein. We show that p53 binds to a p53 DNA-binding consens-sus sequence present in the 5'-regulatory region of the 202 gene in gel mobility shift assays. Moreover, p53 represses the activity of a reporter gene, whose transcription is driven by the 5'-regulatory region of the 202 gene. Additionally, we demonstrate that overexpression of p202 significantly delays p53-induced apoptosis, suggesting that the decrease in p202 levels by wild-type p53 may be important for p53-induced apoptosis.

EXPERIMENTAL PROCEDURES

Cell Cultures, Antibodies, and Reagents—Murine AKR-2B cells (originally a gift from Dr. H. L. Moses, Vanderbilt University, Nashville, TN), murine fibroblasts (between passages 7 and 15) derived from a p53-null mouse (generously provided by Dr. Gigi Lozano, University of...
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Texas M. D. Anderson Cancer Center, Houston, TX (10.1 Val5 (27), and Vm10 cells (28) (generously provided by Dr. Arnold Levine, Rockefeller University, New York, NY), human C-33A (from American Type Culture Collection), a cervical carcinoma cell line with a mutant p53, were grown in Dulbecco's modified Eagle's medium (with high glucose) supplemented with 10% fetal bovine serum in an incubator with 5% CO2. Val5 and Vm10 cells were maintained at 39 °C. If so indicated, recombinant IFN (Universal Type-1, Research Diagnostic, Inc., Flanders, NJ) (1000 units/ml) was added to subconfluent cultures as described previously (29).

Plasmas and Generation of Stable Cell Lines—The 202 reporter plasmid (28) containing the 5′-flanking sequence (0.8 kb) of the 202 gene (30, 31), was constructed by ligating HindIII-PstI fragment (nucleotides from 1 to 804 in Fig. 7 in Ref. 32) from plasmid pBA into the pGL3 basic vector (without any enhancer and promoter sequences) vector (from Promega, Madison, WI). Polymerase chain reaction (PCR)-based mutagenesis was performed with a primer containing the desired point mutations in p53CS1 sequence thus changing the sequence to 5′-CTAGAATACTTTACTACATCCT-3′ (altered bases underlined). The resulting PCR fragment containing the 5′-regulatory region was subcloned into the pGL3 basic vector, resulting in p53CS1mut-luc reporter plasmid. Similarly, a PCR-based approach was used to generate deletions in the 5′-regulatory region of the 202 gene, resulting in deletions corresponding to the nucleotides from −485 to −282 (delp53CS1) and delp53CS1 to −122 (from the 5′ end of the transcription initiation site in Fig. 7 of Ref. 32). The deletions included the p53CS1 site alone (delp53CS1) or the p53CS1 and p53CS2 (delp53CS1 and p53CS2) sites in the 202 gene. The PCR fragments were ligated into the pGL3 basic vector, resulting in the reporter plasmid delp53CS1-luc and delp53CS1 and p53CS2-luc. The reporter plasmid hdm2-luc was generously provided by Dr. Carol Prives (Columbia University, New York, NY). pRL-TK reporter vector allowing expression of Renilla luciferase was purchased from Promega (Madison, WI). The plasmid pCMV53Val135, encoding the temperature-sensitive mutant of murine p53 (33), was generously provided by Dr. Moshe Oren (Weizmann Institute, Rehovot, Israel). pCMH65K53 encoding wild-type murine p53 was provided by Dr. Stanley Fields (University of Washington, Seattle, WA). pCMV53C2 plasmid encoding wild-type human p53 was purchased from CLONTECH Inc. (Palo Alto, CA). Bidirectional expression plasmids pBIRP175H and pBIRP248W, allowing coexpression of both wild-type and the dominant negative mutants of p53 (34), were generously provided by Dr. E. J. Stanbridge (University of California, Irvine, CA). Plasmid p53A62–91 (25) was provided by Dr. X. Chen (Medical College of Georgia, Augusta, GA).

To express the temperature-sensitive mutant of p53, murine AKR-2B fibroblasts were cotransfected with plasmid pCMV53Val135 and pCMV (in 1:10 ratio) and the transfected cells were selected in G418 (500 μg/ml). After 2 weeks, the drug-resistant colonies (>70) were pooled and maintained at 37 °C (favoring the mutant conformation of p53). If so indicated, cells were shifted to 32 °C (favoring the wild-type conformation of p53) for indicated times. If so indicated, AKR-2B fibroblasts were exposed to p53-null medium and were exposed to UV-C light (using UV-Stratalinker) without any growth medium.

Vm10 cells (these cells express a temperature-sensitive mutant of p53 and c-myc) (28) were transfected with plasmid pcDNA3.1–202 or empty vector (pcDNA3.1) and the transfected cells were selected at 39 °C in medium containing Zeocin (100 μg/ml) for about 2 weeks. More than 100 Zeocin-resistant colonies transfected with p202-encoding plasmid or empty vector were pooled for further studies. To initiate apoptosis in the pooled transfected cells, cells were cultured without Zeocin and were shifted to the permissive temperature (32.5 °C) for the indicated times.

Flow Cytometric Analysis—Flow cytometry was performed on single cell suspensions on adherent (after trypsin and EDTA treatment) as well as the floating cells after pooling them. Briefly, for cell cycle analysis, cells were stained with propidium iodide (50 μg/ml, Sigma) and subjected to flow cytometry using a Coulter Epics XL-MCL flow cytometer as described previously (35). Apoptosis was measured by the accumulation of cells with a sub-G1 DNA content.

Immunoblotting—To detect p202 levels, cells were collected from plates in phosphate-buffered saline, resuspended an immunoassay lysis buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (50 μg/ml leupeptin, 50 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride), and incubated at 4 °C for 30 min. The cell lysates were sonicated briefly before centrifugation at 14,000 rpm in a microcentrifuge for 10 min. The supernatants were collected, and equal amounts of proteins were processed for immunoblotting as described previously (29). The p202 polyclonal antibody has been described previously (29). Antibodies to p21 (sc-6246) and p53 (sc-6243) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For gel-shift assays, anti-p53 monoclonal antibodies (clone 421) and anti-E2F-1 antibodies (clone KH95) were purchased from Oncogene Science (Boston, MA).

Northern Blotting—Total cytoplasmic RNA was isolated from cells and subjected to Northern blotting, followed by hybridization with the 202-specific cDNA probe (HindII fragment).

DNA Fragmentation Assays—Assays were performed on single cell suspensions on adherent (after trypsin/EDTA treatment) as well as the floating cells after pooling them. Cells (about 2−5 × 10⁶) were lysed in 20 μl of lysis buffer (20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 0.8% sodium dodecyl sulfate). To lyses 10 μl of RNase A/T1 mixture mix (500 and 200,000 units/ml, respectively) was added, and the mixture was incubated at 37 °C for 2 h. Proteinase K solution (10 μl of 20 mg/ml) was added, and the samples were incubated overnight at 50 °C. Samples were mixed with 6× DNA loading buffer (30% glycerol and 0.25% bromophenol blue) before their analysis on 1.5% agarose gel. The agarose gel was run at 2 W/cm until the dye reached to the end of the gel. The gel was stained with ethidium bromide and photographed.

Gel Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared from murine AKR-2B cells treated with UV-C (5 mJ/cm²) for 24 h or untreated cells as described previously (36). Equal amounts of nuclear proteins or the recombinant purified p53 protein (250−500 ng; purchased from Santa Cruz Biotechnology) were used for binding to labeled oligonucleotides (labeled using T4 polynucleotide kinase and annealed into double-stranded oligonucleotides) containing either the p53 DNA binding consensus sequence (purchased from Santa Cruz Biotechnology) or the 202 gene p53 DNA binding sequence (purchased from Oncogene Science). The set of oligonucleotides used to probe the DNA is shown as the complementary sequence (see Table I for sequence). For supershift assays, nuclear extract proteins or the recombinant p53 were incubated with

FIG. 1. Expression of wild-type p53 results in a decrease in p202 levels. A, upper panel, murine AKR-2B fibroblasts, stably transfected with plasmid p53Val135, were incubated at 37 °C (lanes 1 and 3) or 32 °C (lanes 2 and 4) for 1 day (lanes 1 and 2) or 2 days (lanes 3 and 4). Total cytoplasmic RNA was isolated and analyzed by northern hybridization using the 202-specific cDNA probe. The arrow indicates the location of the 202-specific mRNA. Middle panel, the blot in upper panel was stripped and reprobed with the β-actin-specific probe. The arrow indicates the location of actin-specific RNA. Lower panel, the cytoplasmic RNA applied on the agarose gel was visualized with ethidium bromide staining to control for equal amounts of RNA loading.

B, AKR-2B cells, stably transfected with plasmid p53Val135, were incubated at 37 °C (lanes 1 and 3) or 32 °C (lanes 2 and 4) for 2 days. Total cell lysates prepared from untreated cells (lanes 1 and 2) or cells that had been treated with IFN (lanes 3 and 4) were analyzed by immunoblotting using an anti-p202 antisera, anti-p53, and anti-p21. P68 protein (29) served as a control for loading of equal amounts of proteins. The location of a protein band is indicated by an arrow. C, Val5 cells were incubated at 39 °C (lanes 1 and 2) or 32 °C (lanes 3 and 4) after they had been treated with IFN (1000 units/ml for 24 h) (lanes 2 and 4) or left untreated (lanes 1 and 3). The total cell lysates prepared from untreated cells (lanes 1 and 2) or cells were analyzed by immunoblotting using anti-p53, anti-p21, anti-p202, or anti-β-actin antibodies. The location of a protein band is indicated by an arrow.
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**RESULTS**

**Expression of Wild-type p53 Results in a Decrease in p202 Levels**—To test if the expression of wild-type p53 regulates the expression of p202, we generated a stable cell line of murine AKR-2B fibroblasts (we chose this cell line because the basal levels of p202 RNA and protein can be detected in this cell line) constitutively expressing the temperature-sensitive mutant of murine p53 (p53Val135) (33). While incubation of these cells at 37°C (the nonpermissive temperature) favors mutant conformation of p53, upon shift of cells to 32°C (the permissive temperature) favors a wild-type conformation of p53 (33). Using this cell system, we found that the expression of wild-type p53 significantly decreased the steady-state levels of the 202 RNA (Fig. 1A) and the decrease was apparent 24 h after shift of cells to the permissive temperature (compare lane 1 with lane 2). Additionally, the decrease in the 202 RNA was accompanied by a decrease in the basal levels of p202 (Fig. 1B, compare lane 1 with lane 2) and also the interferon-induced levels of p202 (Fig. 1B, compare lane 3 with lane 4). Furthermore, the decrease in p202 levels was accompanied by an increase in p21^WAF1/CIP1^ levels, a known transcriptional target of the p53 tumor suppressor (8, 10).

Because AKR-2B cells express wild-type p53, which could affect the regulation by a transgene (p53Val135) by dimeriza-
tion (9), we utilized the Val5 cell line (in these cells both alleles of p53 are deleted) (27), which also expresses the temperature-sensitive mutant of p53. As shown in Fig. 1C, these cells had higher levels of p202 at the nonpermissive temperature, which were comparable with the p202 levels in IFN-treated cells (compare lane 2 with lane 1), and shift of these cells to the permissive temperature resulted in a decrease in p202 levels (compare lane 3 with lane 1). Moreover, the decrease in p202 levels was accompanied by an increase in p21WAF1/CIP1 levels. Interestingly, as noted above (Fig. 1B), the IFN treatment of these cells at the permissive temperature resulted in only a moderate increase in p202 levels. Together, these observations provide support to the idea that the expression of wild-type p53 negatively regulates the steady-state levels of the 202 RNA and protein in these cell lines.

Wild-type p53 Binds Strongly to One of the Two p53 DNA Binding Consensus Sequence Present in the 5′-Regulatory Region of the 202 Gene—The 5′-regulatory region of the 202 gene, which is immediately upstream to the transcription initiation sites, has been characterized (30–32). It does not contain a TATA box (31, 32). Therefore, to identify a potential mechanism(s) by which wild-type p53 regulates the expression of the 202 gene, we searched the 5′-regulatory region of the 202 gene for the presence of other cis-regulatory elements that are shown to be involved in p53-mediated transcriptional regulation of genes. Our search revealed that the regulatory region of the 202 gene contains several cis-acting elements (Fig. 2A), including two potential p53 DNA binding sites (indicated as p53CS1 and p53CS2), shown to be involved in the transcriptional regulation of genes by p53 (8, 11). Interestingly, as shown in Table I, the two potential p53 DNA binding sites p53CS1 and p53CS2 in the 5′-regulatory region of the 202 gene, like other known p53 DNA binding sites in other p53-responsive genes (37, 38), contain C/A/T/Y/T/A/G at the core of the ten base palindrome, which is important for the DNA binding activity of p53 (37, 38). However, as noted in Table I, these two potential p53 DNA binding sites in the 202 gene contain variations (in the case of p53CS1, 5 variations out of 20 nucleotides, and, in the case of p53CS2, 4 out of 20 nucleotides) from the p53 DNA binding consensus sequence (38).

Next, we tested if p53 could bind to the p53CS1 or p53CS2 sequence in gel-mobility shift assays. For this purpose, we used nuclear extracts from AKR-2B cells (these cells contain wild-type p53) untreated or after exposure to low levels (5 mJ/m²) of UV-C treatment. As shown in Fig. 2B (left panel), p53 in these nuclear extracts specifically bound to a p53 consensus sequence in gel mobility shift assays and the complex was competed well with 20-fold excess of cold consensus sequence oligonucleotide (compare lane 3 with lane 4). Furthermore, the complex was supershifted after incubation with antibodies to p53 (antibody pAb 421) that are known to improve the binding of p53 to its cognate site (39). However, no such supershift was seen after incubation with an isogenic control antibody to E2F-1 (compare lane 5 with lane 6). Interestingly, incubation of nuclear extracts with a labeled oligonucleotide containing the p53CS1 sequence resulted in binding of p53 (see Fig. 2B, right panel) and the binding was also competed out well by 20-fold excess of cold oligonucleotide containing the p53 DNA binding consensus sequence (compare lane 8 with lane 7). Additionally, the p53 complex was supershifted after incubation with antibodies to p53 (antibody pAb 421). However, no such supershift was seen after incubation with an isogenic control antibody to E2F-1 (compare lane 9 with lane 10). Thus, these experiments indicated that, in these assays, p53 specifically bound to the 202 p53CS1 sequence and the p53 consensus sequence with a comparable affinity.

| Gene of origin | p53 consensus site* |
|----------------|---------------------|
| Consensus      | PuPuPuC (A/T) (T/A) |  
| gadd45         | GAGACATGT CTAGATTGCTGG |  
| p21            | GAGACATGT CCAACATGTCG |  
| mdr2a          | GCTCAAGTCT TGAGACAGTCG |  
| mdr2b          | AGCTAGCT GTGACAGTCG |  
| RGC-w          | GGACTTGCCT GGAGACAGTCG |  
| If202(p53CS1)  | CCCAGATCTGCCTGACAGTCG |  
| If202(p53CS2)  | CACGGTATCT TTGACAGTCG |  

* A bold letter indicates deviation from the p53 DNA binding consensus sequence. Arrows indicate orientation of pentamers. The center bases of consensus sequence are underlined.

Because the p53CS2 sequence in the 5′-regulatory region of the 202 gene differs from the p53CS1 and the p53 consensus sequence (see Table I), we tested its ability to bind p53 in gel-shift assays. As shown in Fig. 2C, oligonucleotide containing a p53 DNA binding consensus sequence bound to p53 (lane 2) and incubation with anti-p53 antibodies resulted in supershift of p53-oligonucleotide complex (lane 3). As expected from our above experiment (Fig. 2B), the 202 p53CS1 sequence also bound to p53 to a comparable extent (compare lane 5 with lane 2) and incubation with anti-p53 antibodies resulted in a supershift of p53-oligonucleotide complex (compare lane 6 with lane 3). However, the p53CS2 sequence bound to p53 weakly (compare lane 8 with lanes 2 and 5) and incubation with anti-p53 antibodies did not result in a significant supershift of p53-oligonucleotide complex (compare lane 9 with lanes 3 and 6). Thus, together, these experiments indicated that in these assays p53 can specifically bind to one of the two (p53CS1) p53 consensus sequence present in the 5′-regulatory region of the 202 gene with an affinity comparable to that of p53 DNA binding consensus sequence.

Because in nuclear extracts p53 is bound to other nuclear proteins, which could facilitate binding of p53 to an oligonucleotide, we tested if the recombinant purified p53 can bind to an oligonucleotide containing the p53CS1 sequence from the 202 gene. As shown in Fig. 2D, purified recombinant p53 bound to an oligonucleotide containing the p53 DNA binding consensus sequence (lanes 2 and 3) and the p53-oligonucleotide complex was supershifted upon incubation with anti-p53 antibodies (lanes 4 and 5). Incubation of recombinant p53 with an oligonucleotide containing the p53CS1 sequence did not result in a significant binding to the oligonucleotide (lanes 7 and 8). However, incubation of p53 with anti-p53 antibody resulted in a significant increase in DNA binding and supershift (lanes 9 and 10). These experiments, thus, clearly demonstrated that in gel mobility shift assays one of the two p53 DNA binding sites (p53CS1) present in the 5′-regulatory region of the 202 gene can specifically bind to the recombinant purified p53 and p53 in nuclear extracts. Therefore, these experiments provide further support to the idea that the specific DNA binding of p53 in the 5′-regulatory region of the 202 gene may contribute to the transcriptional regulation of the 202 gene.

Expression of the Wild-type p53 Results in a Decrease in the Activity of a Reporter Gene Whose Expression Is Driven by the 5′-Flanking Sequence of the 202 Gene—To test if p53 transcriptionally regulates the 202 gene, we transfected murine fibroblasts derived from a p53-null mouse or human C-33A cells (these cells are null for p53) with the 202-luc reporter plasmid, a luciferase reporter driven by a 800-base pair fragment from the 5′-flanking sequence of the 202 gene (Fig. 2A), together with varying amounts of pCMV-p53 plasmid encoding wild-type murine p53. As shown in Fig. 3A, the expression of the
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Expression of the wild-type p53 in transient transfection assays results in a decrease in the activity of the 202-luc reporter gene. A, murine fibroblasts (passages 7–15) derived from p53-null mouse were transfected with the 202-luc, murine fibroblasts derived from p53-null mouse were transfected with the 202-luc, or a plasmid encoding a deletion mutant of p53 (25). Consistent with an earlier report (25), the expression of this mutant of p53 weakly

Acetylation of p53 upon coexpression of cofactor p300, a histone acetyltransferase, has been shown to potentiate the repression of α-fetoprotein gene (20). Therefore, we tested if coexpression of p300 with p53 affects p53-mediated transcriptional repression of the 202 gene. We found that the expression of p300 alone or its coexpression with wild-type p53 did not affect the activity of the 202-luc reporter in p53-null fibroblasts (data not shown). Therefore, it is unlikely that the acetylation of p53 by p300 contributes to the transcriptional repression of the 202 gene.

We next tested if the expression of a mutant of human p53 (p53mt135), which is defective in the specific DNA binding activity (40), affects the 202-luc reporter activity. As shown in Fig. 3B, transfection of a plasmid encoding wild-type murine or human p53 resulted in decreased activity of the 202-luc reporter (compare second and third columns with first column). However, transfection of a plasmid encoding p53mt135 only moderately decreased the activity of the 202-luc reporter as compared with a plasmid encoding wild-type human p53 (compare fourth and third columns). Curiously, no such decrease by the expression of p53mt135 was seen under conditions in which transfection of nanogram amounts of a plasmid encoding wild-type p53 still decreased the activity of the 202-luc (see Fig. 3C) (in this experiment, we used lesser amounts of the expression plasmids to rule out repression because of excess amounts of plasmid). We also tested how coexpression of two dominant negative mutants of human p53 with wild-type p53 affects p53's ability to repress the 202-luc reporter activity. For this purpose, we utilized bi-directional plasmids (pBIRP) allowing coexpression of equal amounts of wild-type and the dominant negative mutant of p53 (34). As shown in Fig. 3D, coexpression of wild-type p53 together with the dominant negative mutant 175H or 248W did not result in a decrease in the activity of 202-luc (compare lane 1 with lanes 3 or 4). Instead, coexpression of these mutants of p53 with wild-type p53 moderately increased the activity of the 202-luc reporter. Thus, these observations indicated that the specific DNA binding activity of p53 is required for transcriptional repression of the 202 gene.

Because the transcriptional repression of the genes by p53 is shown to involve the PXXP domain in p53 (23–25), we tested if this domain in p53 is needed for the repression of the 202 gene. As shown in Fig. 3E, the expression of a deletion mutant of human p53 (p53(A62–91)), which lacks the five PXXP motifs, did not result in repression of the 202-luc reporter activity. To rule out the possibility that the lack of repression of the 202-luc reporter activity in the above experiments was due to the lack of expression of mutant p53, we used hdm2-luc reporter plasmid, which is shown to be weakly responsive to the expression of the above deletion mutant of p53 (25). Consistent with an earlier report (25), the expression of this mutant of p53 weakly
stimulated the activity of hdm2-luc reporter. Thus, these experiments indicated that the FXXP motif in p53 is important for the transcriptional repression of the 202-luc reporter activity.

Next, to examine the relative contribution of the two p53 DNA binding sites (p53CS1 and p53CS2) in p53-mediated transcriptional repression of the 202 gene, we deleted either both p53 DNA binding sites (delp53CS1 and CS2) or only one site (delp53CS1) from the 5′-regulatory region of the 202 gene and performed reporter assays. As shown in Fig. 3F, these deletions in the 5′-regulatory region of the 202 gene relieved p53-mediated repression of the 202-luc activity. Because an oligonucleotide containing the p53CS1 sequence bound to p53 in gel-mobility shift assays (Fig. 2), we tested if mutations in the p53CS1 site could relieve p53-mediated transcriptional repression of the 202-luc activity. As shown in Fig. 3F, site-directed mutagenesis of p53CS1 p53 DNA binding site in the 202 gene, that has been shown to result in abrogation of the specific DNA binding activity of p53 (37), relieved murine p53-mediated repression of the 202-luc activity. Additionally, the repression mediated by expression of human p53 was also diminished by the CS1 mutation (data not shown). These observations, thus, raise the possibility that p53 utilizes the p53CS1 site in the 202 gene to regulate the expression of the 202 gene.

Expression of p202 Decreases the Rate of p53-induced Apoptosis—To investigate how the decrease in the basal levels of p202, after the physiological increases in the levels of p53, affect p53-induced apoptosis, we overexpressed p202 in murine Vm10 cell line. These cells express a temperature-sensitive mutant of p53 (p53Val135) and c-myc (28). Upon shift of temperature to the permissive temperature (32.5 °C), these cells undergo p53-induced apoptosis in about 14 h (28). As shown in Fig. 5A, overexpression of p202 at the permissive temperature in these cells significantly reduced number of cells morphologically appearing apoptotic. Flow cytometry of these cultures for the presence of sub-G1 cells revealed that overexpression of p202 in these cells significantly (up to 10% after 19 h and 50% after 36 h) reduced the extent of sub-G1, cells (Fig. 5B). Additionally, the analysis of the genomic DNA content from both adherent and floating cells in these cultures (transfected with vector) revealed that these cells indeed exhibited a significant laddering of DNA, a hallmark of apoptosis, at the permissive temperature (Fig. 5C, compare lane 1 with lane 3), but not at the nonpermissive temperature, and overexpression of p202 significantly (about 50%) reduce it (compare lane 3 with lane 4). Thus, these experiments provide support to the idea that the transcriptional repression of the 202 gene by the wild-type p53 may be important for a rapid induction of p53-induced apoptosis.

**DISCUSSION**

In the present study, we demonstrate that wild-type p53 negatively regulates the levels of p202 by repressing the transcription of the 202 gene. We used two approaches to investigate if the expression of wild-type p53 regulates the basal levels of p202. In the first approach, we used two murine cell lines (AKR-2B and Val5) constitutively expressing a temperature-sensitive mutant of p53. Upon incubation of these cell lines at the permissive temperature, which favors the wild-type conformation of p53, both the 202 RNA and protein levels decreased significantly (Fig. 1). As expected, the decrease in
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p202 levels was accompanied by an increase in the levels of p21\(WAF1/CIP1\), a known transcriptional target of p53 (9, 10). In the second approach, we exposed murine AKR-2B cells to UV-C light (which results in increase in p53 levels in these cells) to test if the physiological increase in p53 levels correlate with decrease in p202 levels. We found that UV treatment of cells, which resulted in a decrease in the levels of p202 (Fig. 4), was correlated with increases in the levels of p53 and p21\(WAF1/CIP1\) (Fig. 4). The decrease was also evident at the 202 RNA levels (data not shown). Thus, these two approaches revealed that increases in p53 levels are associated with decreases in p202 protein and mRNA, thus suggesting that p53 may negatively regulate p202 expression.

Unlike the activation of gene expression, the mechanism of repression of gene expression by wild-type p53 is not well defined and p53 has been shown to repress transcription of genes by different mechanisms (11, 41, 42). These mechanisms include: (i) the binding of p53 to the TATA-binding proteins, which are bound to the TATA containing promoters (11, 18); (ii) the recruitment of mSin3a and histone deacetylases by p53 to the gene promoter (41); (iii) the sequence-specific DNA binding of p53 to the consensus sequence present in the regulatory region of genes (19, 20); and (iv) the interactions of p53 with other cis-acting elements, which include SP-1 (16), CCAAT (43), and AP-1 (44). Because the 5'-flanking sequence of the 202 gene does not contain a TATA box (30, 31), it is conceivable that the mechanism(s) by which p53 represses the transcription of the 202 gene is independent of TATA-binding proteins (45–47). Consistent with this prediction, we found that the expression of adenovirus-encoded E1A protein, which is shown to relieve p53-mediated transcriptional repression of genes by interacting with the TATA-binding protein (46), did not relieve p53-mediated transcriptional repression of the 202-luc reporter activity (data not shown).

The presence of two potential p53 DNA binding sites (37, 38) in the 5'-regulatory region of the 202 gene (Table 1) prompted us to test if p53 regulates the expression of the 202 gene through the specific DNA binding. These experiments revealed that: (i) one of the two potential p53 DNA binding site (p53CS1, Fig. 2A) present in the 5'-regulatory region of the 202 gene specifically bound to p53 in gel-mobility shift assays (Fig. 2, C and D); (ii) in transient transfection assays, the expression of wild-type p53, but not various mutants of p53 (defective in the specific DNA binding activity), resulted in a decrease in the 202-luc reporter activity (Fig. 3); and (iii) site-directed mutagenesis of the p53CS1 sequence in the 202 gene or its deletion relieved p53-mediated transcriptional repression of the 202-luc reporter activity (Fig. 3F). Thus, our observations are consistent with the possibility that the sequence-specific DNA binding activity of p53 contributes to the transcriptional repression of the 202 gene. Therefore, the 202 gene joins the class of genes whose transcriptional repression by p53 depends on the sequence-specific DNA binding activity of p53 (19, 20).

Although our observation that point mutations in the p53CS1 site (or its deletion) in the 202 gene relieve p53-mediated transcriptional repression of the 202-luc activity provides support to the idea that p53-mediated transcriptional repression of the 202 gene depends on the specific DNA binding activity of p53, our observations do not completely rule out the possibility that other mechanism(s), such as interactions of DNA-bound p53 with other transcription factors, also contribute to the regulation of the 202 gene. Because in addition to the p53 DNA-binding sites (p53CS1 and p53CS2), the 5'-flanking sequence of the 202 gene also contains other cis-acting elements, such as CCAAT enhancer, SP-1, and an AP-1-like site, shown to be involved in p53-mediated transcriptional repression of the genes (16, 43, 44), further work will be needed to identify the relative contributions of these cis-elements in p53-mediated transcriptional repression of the 202 gene.

Curiously, the proline-rich region in human p53, which contains five PXXP motifs (where P represents proline and X any amino acid) (22), was found to be dispensable for transactivation and cell cycle arrest mediated by p53 (23–25). However, the proline-rich region was found to be necessary for transrepression and apoptosis mediated by p53 (24, 25). Our observation that the expression of a deletion mutant of p53, lacking the proline-rich region (amino acids 62–91), was not able to repress the transcription of the 202 gene (Fig. 3E) raises the possibility that the mechanism(s) by which wild-type p53 negatively regulates the transcription of the 202 gene depends on the presence of PXXP motifs in p53. Similar to the 202 gene, the requirement for the p53 proline-rich region was also demonstrated for the transcriptional regulation of the PIG3 gene (24).

Our previous studies revealed that the decreased levels of
p202 in fibroblasts increase the propensity of cells to undergo apoptosis (5). Interestingly, in a recent study overexpression of p202 was found to inhibit c-myc-induced apoptosis in Rat-1 cells, which harbor a wild-type p53 (6). Together, these observations support the notion that p202 participates in the regulation of apoptosis. Consistent with this notion, our observations provide additional evidence that the increased levels of p202 in Vm10 cells significantly delay p53-induced apoptosis (Fig. 5B). Therefore, it is likely that the decrease in the basal endogenous levels of p202, after the physiological increases in the levels of wild-type p53, contribute to the potentiation of p53-induced apoptosis. Additionally, because p202 negatively regulates the transcriptional activity of p53 (26) and other factors (for example, NF-kB and E2F-1) (49, 50), with which p53 cooperates to induce apoptosis (48, 51), the transactivation of the 202 gene by p53 may be part of an important cascade of events by which p53 induces apoptosis. However, it remains to be seen how the decrease in the levels of p202 following the expression of wild-type p53 affect the activity of these other transcription factors.

p53-mediated biological effects in cultured cells, which include the growth arrest and apoptosis, are shown to be dependent on the levels of p53 expressed, the type (for example UV or ionizing radiation exposure) and the extent of stress (different doses of UV), and the cell type (11, 23). Therefore, it is conceivable that p53-mediated transcriptional repression of the 202 gene depends on these factors, and further work will be needed to examine these possibilities.

The identification of the 202 gene as a p53-repressible gene in the present study provides a novel feedback mechanism by which p53 negatively regulates the expression of a negative regulator. Our findings also raise the interesting possibility which p53 cooperates to induce apoptosis (48, 51), the transcriptional repression of the 202 gene by p53 may be part of an important cascade of events by which p53 induces apoptosis. However, it remains to be seen how the decrease in the basal endogenous levels of p202, after the physiological increases in the levels of wild-type p53, contribute to the potentiation of p53-induced apoptosis. Additionally, because p202 negatively regulates the transcriptional activity of p53 (26) and other factors (for example, NF-kB and E2F-1) (49, 50), with which p53 cooperates to induce apoptosis (48, 51), the transactivation of the 202 gene by p53 may be part of an important cascade of events by which p53 induces apoptosis. However, it remains to be seen how the decrease in the levels of p202 following the expression of wild-type p53 affect the activity of these other transcription factors.

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