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p53 Mutants Have Selective Dominant-Negative Effects on Apoptosis but Not Growth Arrest in Human Cancer Cell Lines

OSCAR N. AURELIO,† XIAO-TANG KONG, SWATI GUPTA, AND ERIC J. STANBRIDGE*

Department of Microbiology and Molecular Genetics, University of California—Irvine, College of Medicine, Irvine, California 92697-4025

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A bidirectional expression vector that allowed equal transcription of cloned wild-type and mutant p53 cDNAs from the same vector was developed. The vector was transfected into CaLu 6 lung carcinoma cells or Saos-2 osteosarcoma cells. All p53 mutants examined were recessive to wild-type p53 transactivation of *p21*$^{WAF1/CIP1}$ but dominant-negative for transactivation of *Bax*. An examination of effects on growth arrest and apoptotic pathways indicated that all mutants were recessive to wild type for growth arrest but only three of seven mutants were dominant negative for induction of apoptosis.

The tumor suppressor gene *p53* has been the subject of intense scientific inquiry due to the high frequency of mutations in human cancers (23, 27). A number of functions have been attributed to the wild-type p53 protein. Introduction of exogenous wild-type p53 into cancer cells and its subsequent overexpression result in growth arrest (3, 17, 19, 26). This growth arrest was initially shown to occur at the G_1/S cell cycle checkpoint (31). This phenotype is most likely due to the fact that p53 acts as a sequence-specific transactivator of genes containing a p53 consensus binding site in their promoters (16, 40). The chief mediator of p53-mediated G_1 arrest was shown to be the *p21*$^{WAF1/CIP1}$ gene, a cyclin–cyclin-dependent kinase complex inhibitor (14, 24, 46). In some cell types, introduction and overexpression of wild-type p53 resulted in apoptosis (43, 49). Thus, wild-type p53 acts as a tumor suppressor by causing a cell to either arrest growth or undergo apoptosis following activation by DNA-damaging events (28, 31, 43, 49).

Although this differential response to wild-type p53 overexpression would seem to be a cell-type-specific effect, recent studies indicate that specific domains and even specific residues of p53 may allow it to work in combination with the cellular milieu to bring about either a growth arrest or an apoptotic response (1, 21, 25, 32, 41). Interestingly, it has become clearer that some p53 mutations affect apoptosis but not growth arrest (25) or vice versa (1, 21, 32, 41). This has led to a model in which p53-mediated growth arrest and apoptotic pathways are regulated differently. These previous studies have also given further insight into the mechanism by which a cell that has only mutant p53 protein at its disposal is unable to elicit its tumor-suppressive effects.

The question arises as to what happens to the growth arrest and apoptosis pathways when mutant and wild-type p53 proteins are expressed in the same cell. Given that p53 binds to its DNA target sequences as a tetramer (11) and that a majority of p53 mutations found in human cancer are missense mutations (27), most mutant p53 monomers are capable of oligomerizing with wild-type p53 monomers. Moreover, the chance that heterotetramer formation occurs at steady-state conditions is probably increased due to the increased half-life of mutant p53 compared to wild-type p53 within a cell (18). Initial in vitro experiments in which mutant p53 was coexpressed with wild-type p53 indicated that the resulting heteromer adopted a mutant conformation (35). Investigations of this dominant-negative effect in vivo have yielded conflicting results. For nearly all mutants studied, dominant-negative effects are seen only when expression of the mutant p53 protein is in excess over wild type. Measurements of this phenomenon were primarily with transactivation assays using a consensus p53 binding site-luciferase-chloramphenicol acetyltransferase reporter construct (29, 39). One study which examined both transactivation and growth arrest parameters found that dominant-negative effects were seen in transactivation assays but not in growth arrest assays (20), indicating the importance of using several different assays to determine dominant-negative effects.

In the study reported here, we sought to determine whether there were differential dominant-negative effects on transcription regulation, growth arrest, and apoptosis with the use of a novel bidirectional expression vector. This system provides for the equal expression of wild-type and mutant p53 transcripts in the same cell. We found that dominant-negative effects are seen with only a subset of the p53 missense mutants examined and that this dominant-negative effect was manifested in the apoptotic process but not on growth arrest. Furthermore, although dominant-negative efforts were observed in *Bax* transactivation assays, these did not all correspond to dominant-negative apoptotic effects.

MATERIALS AND METHODS

Cell lines and conditions. Non-small-cell lung carcinoma CaLu 6 cells were obtained from C. C. Harris, and the osteosarcoma Saos-2 cell line was a gift from C. Prives (Columbia University, New York, N.Y.). Both lines are null for endogenous p53. All transfected cell lines were grown in Dulbecco modified Eagle medium with 10% fetal bovine serum (Bio-Whittaker).

Plasmids and construction of the bidirectional expression vector. Wild-type and mutant cDNAs for the experiment in Fig. 3a were cloned into pREP4 plasmids (Invitrogen). The bidirectional expression construct, pBIRP, was made through several intermediate constructs. In parallel, primers containing *Aat*II and *Nde*I sites and *Xho*I-compatible overhangs were annealed and then ligated into pBlueScript II (Stratagene) to make pBS-NaatX. The 2.1-kb *Aat*II/*Nde*I fragment from pBI (Clontech) was then subcloned into pBS-NaatX to make pBIBS. This pBI fragment contains the bidirectional promoter and a human β-globin poly(A) signal at one cloning site (site I) and a simian virus 40 poly(A) signal on the second cloning site (site II) that differ in approximately 400 bp in length of final processed transcripts when both sites contain identical length cDNAs. The 9.5-kb *Sal*I fragment from pREP9 (Invitrogen), containing a G418

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, University of California—Irvine, College of Medicine, Irvine, CA 92697-4025. Phone: (949) 824-7042. Fax: (949) 824-8598. E-mail: ejstanbr@uci.edu.

† Present address: Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032.

resistance marker and EBNA-1 episomal origin of replication, was the recipient of the 2.1-kb *Xho*I fragment from pBIBS, resulting in the final cloning vector, pBIRP. The 1.8-kb *Kpn*I/*Xho*I fragment containing wild-type p53 cDNA was taken from pA/E/53/C/Rb/B- (a gift from K. Wills, CANJI, Inc.), and the base overhangs were filled in with Klenow fragment and deoxynucleoside triphosphates. This cDNA has a proline codon at codon 72. The resulting fragment was cloned into the *Eco*RV site on site I of pBIRP. All mutant cDNAs were derived from an original 1.8-kb wild-type p53 cDNA *Bam*HI fragment excised from pCMV Neo Bam (a gift from B. Vogelstein, The Johns Hopkins University, Baltimore, Md.) and subcloned into pBlueScript II. These mutant p53 cDNAs were then excised again with *Not*I and *Sal*I and subcloned into site II of pBIRP. The alternate versions of the codon 72 polymorphism in wild-type p53 were tested functionally and found to have no bearing on function (data not shown). Additionally, cloning of wild-type p53 into either site I or site II was found to have no effect on function (data not shown).

Transcriptional regulation assays and Western blot analysis. The *WAF1* promoter (WWP luc) and *Bax* promoter (Bax-luc) constructs were gifts from B. Vogelstein and J. C. Reed, respectively. CaLu 6 or Saos-2 cells (6×10^5) were plated into each well of a six-well plate. The next day, 3 μg of either WWP-luc or Bax-luc was cotransfected with 1 μg of a p53 expression plasmid by the Lipofectin procedure (GIBCO BRL). The p53 expression plasmid consists of wild-type or mutant p53 cDNA cloned into the *Bam*HI site of pREP4 (Invitrogen). Also cotransfected with the p53 expression plasmid and the firefly luciferase reporter (e.g., WWP-luc or Bax-luc) was an internal control plasmid, pCMV-RL, expressing renilla luciferase (Promega). Each transfection of the different p53 expression plasmids was done in duplicate for each experiment, and the experiment was repeated at least four times. The ratio of firefly to renilla luciferase was calculated for each transfection, and the results were expressed as the fold transactivation relative to wild-type p53 averaged over the set of experiments.

Western blots were performed with cell pellets from one of the transient transfections used for studying transactivation of the WWP-luc construct. The protein extraction and polyacrylamide gel electrophoresis for Western blots for analysis of the bidirectional vector expression were performed as described elsewhere (9). The protein was then immunoblotted onto an Immobilon-P nylon membrane (Millipore), incubated with anti-p53 antibody DO-1 (1:500 dilution; Santa Cruz Biotechnology) followed by peroxidase-conjugated anti-mouse immunoglobulin G (Santa Cruz), and developed with the Amersham enhanced chemiluminescence detection system according to the manufacturer's protocol.

Colony inhibition assays. CaLu 6 cells (2×10^5) were plated in six-well tissue culture plates and allowed to adhere overnight. The next day cells were transfected with 6 μg of a pREP 4-derived expression vector (encoding a hygromycin resistance gene) by the Lipofectin procedure and allowed to incubate for 16 h. Selection for hygromycin-resistant colonies was started 48 h posttransfection. After 12 to 14 days of selection, hygromycin-resistant colonies were fixed and stained with crystal violet–50% methanol. Colonies of ca. 10 or more cells were counted. When the bidirectional vector was used, G418 was substituted for hygromycin. Reported numbers reflect the average of at least three independent experiments.

Northern blot analysis. Total RNA from transfected CaLu 6 cells was isolated with Trizol Reagent (GIBCO BRL) according to the manufacturer's directions. Poly(A) RNA enrichment was performed by passing total RNA over oligo(dT)-cellulose (Pharmacia). Running of RNA on a denaturing formaldehyde agarose gel and blotting onto a nylon membrane (Amersham Life Science) were performed by standard techniques. The probe was generated by single-strand labeling on a thermocycler (MJ Research) using an antisense primer and a p53 template cDNA.

Transfection of Saos-2 cells for fluorescence-activated cell sorting (FACS) analysis. The day prior to transfection, 10^6 Saos-2 cells were seeded into 25-cm^2 tissue culture flasks. The following day, the cells were transfected with the various p53 expression vectors identified above via calcium phosphate precipitation, using a Cal-Phos kit (Clontech). Floating and adherent cells were then harvested at the indicated times and fixed in 70% ethanol for at least 2 h at −20°C. The cells were then rehydrated in phosphate-buffered saline (PBS)–150 μg of RNase A per ml for 30 min at room temperature, pelleted, gently resuspended in a 1:100 dilution of mouse anti-p53 primary antibody DO-1 (Santa Cruz), diluted in PBS–2.5% bovine serum albumin (BSA), and incubated at room temperature for 30 min. After two PBS washes, the cells were resuspended in a 1/40 dilution of goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody (DAKO) in PBS–2.5% BSA and incubated for 30 min at room temperature in the dark. After two PBS washes, the cells were resuspended in PBS containing propidium iodide (1 μg/ml). The samples were then filtered through Spectra Mesh filters (Spectrum). Samples were analyzed on a FACScan cell scanner (Becton-Dickinson), and the data were analyzed by the ModFIT/LT software (Verity Software). Cells were gated to remove doublets and very small debris and gated for high FITC fluorescence; 10,000 events were acquired from every sample. Reported percentages were the averages of three separate experiments, and statistical computation was performed with the SAGE (Statistical Analysis for Genetic Epidemiology) computer program, version 3.0 (Case Western Reserve University).

Fluorescent TdT-mediated dUTP-biotin nick end labeling (TUNEL) analysis. Saos-2 cells were plated onto sterile acid-washed glass coverslips. The following day, cells were transfected with 6 μg of the indicated expression vector by calcium phosphate coprecipitation and incubated for 48 h. Cells were then fixed in 1.5% paraformaldehyde in PBS for 20 min and washed with PBS. Fixed cells were permeabilized with 0.1% Triton X-100–PBS for 5 min then washed with PBS. Anti-p53 antibody DO-1 (Santa Cruz) was applied to the cells at a 1:200 dilution (in 2.5% BSA–PBS) for 30 min at room temperature then washed with PBS. Rabbit anti-mouse tetramethyl rhodamine isocyanate-conjugated antibody (DAKO) at a 1:1,000 dilution was added to the cells for 30 min at room temperature. Following a brief wash in PBS, the cells were FITC labeled for DNA fragmentation with terminal deoxynucleotidyltransferase (TdT) supplied in the ApoTAG Direct kit (ONCOR) and used according to the manufacturer's protocol for immunocytochemistry. Nuclei were then stained with 4′,6-diamidino-2-phenylindole (DAPI) after termination of the TdT reaction and prior to slide mounting with an antifade solution. The percentages reported are the averages of three separate experiments, and statistical computation was performed with the SAGE computer program.

RESULTS

p53 mutants are recessive for transactivation of $p21^{WAF1/CIP1}$ but dominant negative for transactivation of *Bax*. p53 mutants previously found in human cancers were analyzed for the ability to perform wild-type p53-associated functions. We included in this study five mutations that are localized to the core domain and represent some of the most common missense mutations found in human cancer (27). This panel includes mutants 143a (Val-to-Ala substitution), 175h (Arg to His), 245c (Gly to Cys), 248w (Arg to Trp), and 273h (Arg to His). Included are two additional germ line-derived mutants that lie within the hinge domain that connects the sequence-specific DNA binding domain to the tetramerization domain. These two mutants, 305m (Lys-to-Met substitution) and 325v (Gly to Val), were previously shown (2) to have retained cell cycle arrest but not apoptotic functions in the absence of wild-type p53. Previous studies determined that the p53 mutants (other than 305m and 325v) were impaired in the ability to transactivate the $p21^{WAF1/CIP1}$ promoter and that all of the mutants used in this study were impaired in the ability to transactivate the *Bax* gene promoter (2, 14, 29, 36, 39, 41). We reconfirmed these phenotypes in the present study in both CaLu 6 and Saos-2 cells (data not shown).

We sought to determine possible dominant-negative effects that the mutants would have when coexpressed with wild-type p53. Earlier studies of the dominant-negative effects of mutant p53 protein on wild-type p53 protein functions used cotransfection of two expression vectors, one for mutant p53 and one for wild-type p53 (12, 29, 39). While the overall phenotype of the transfected cell population was assessed in each of these studies, there is no guarantee that each transfected cell received and expressed both the mutant and wild-type expression vectors. An attempt was made to remedy this technical problem by using bicistronic expression vectors with an internal ribosome entry site (IRES) mediating translation of one of the cistrons (20). In this case, however, it is possible that differences in the efficiency of IRES-mediated translation initiation, relative to cap-dependent initiation, would lead to different levels of expression of the mutant and wild-type p53 protein. This possibility was not ascertained in the above-referenced study. In preliminary studies we indeed noted such disparate levels of expression (data not shown).

In an attempt to overcome this important problem, we constructed an episomally replicating bidirectional expression vector that allowed transcription initiation from the same regulatory element (Fig. 1A). The vector is a variation of the one developed by Baron et al. (4) for use with the tetracycline-regulated expression system (22). Important additions to the vector were a G418 resistance marker and an episomal origin of replication, both of which made the vector useful for colony inhibition assays. The tetracycline-responsive element, which

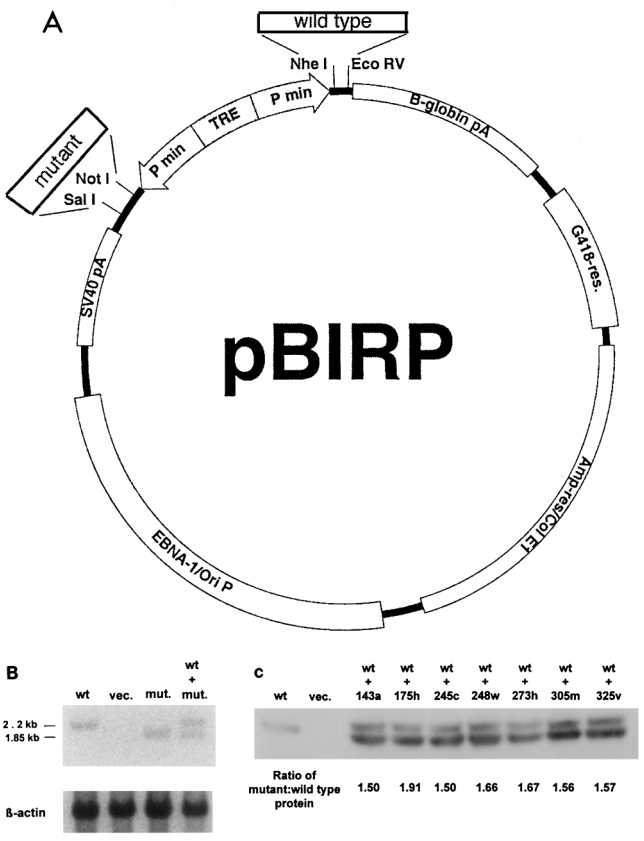

FIG. 1. Coexpression of wild-type and mutant p53 from the bidirectional expression vector, pBIRP. (A) Diagram of the bidirectional expression vector, pBIRP. Wild-type and/or mutant p53 cDNAs are cloned into the indicated restriction sites on the vector. P min, minimal promoter; TRE, tetracycline-responsive element; res, resistance; pA, poly(A); SV40, simian virus 40. (B) Representative Northern blot of CaLu 6 cells transiently transfected with the pBIRP vector (vec.) containing wild-type (wt) and/or mutant (mut.) p53 cDNA. (C) Western blot of lysates from cells transfected with bidirectional vectors bearing the indicated wild-type and mutant p53 cDNAs. Also indicated are the ratios of mutant to wild-type protein.

serves as the *cis*-acting regulatory element of the vector, contains multiple copies of the tetracycline operator sequence. An expression vector producing a chimeric protein consisting of the DNA binding domain of the tetracycline repressor and the transactivation domain of the herpes simplex virus 1 VP16 was previously stably transfected into both CaLu 6 and Saos-2 cell lines. In the absence of tetracycline, the chimeric tetracycline transactivator protein is able to bind to the regulatory element and drive overexpression from the two minimal promoters. All experiments in this study were done in the absence of tetracycline.

Additional modifications were made to the *p53* cDNAs cloned into the vector. First, the wild-type cDNA was cloned into the cloning site of the vector that contains a human β-globin polyadenylation signal, while mutant cDNAs were cloned into the other cloning site that contains a shorter simian virus 40 polyadenylation signal. This makes an approximately 400-bp-longer 3′ noncoding region mRNA for the wild-type transcript than the mutant transcript and detectable migration differences of transcripts on a Northern blot (Fig. 1B). Second, the wild-type and mutant *p53* cDNAs code for alternate versions of a characterized polymorphism at codon 72 (6, 34). The proline version of the polymorphism (arginine being the alternative) occurs in approximately 10% of all *p53* alleles in the general population yet shows no functional differences from the alternate version of the polymorphism (reference 6 and data not shown). However, the polymorphism does allow for differential migration of protein products on a denaturing polyacrylamide gel (Fig. 1C) and has been used in a similar manner to differentiate p53 products from different cDNAs in previous studies (9, 34). It should be noted that the ratios of mutant to wild-type protein levels ranged from 1.50 to 1.91, most likely due to the varying increased half-lives of mutant p53s compared to wild-type protein (18).

Bidirectional expression vectors were transiently cotransfected with the $p21^{WAF\text{-}1/CIP\text{-}1}$ luciferase reporter in both the human non-small-cell lung cancer line CaLu 6 and the human osteosarcoma cell line Saos-2, and transactivation levels were measured (Fig. 2A). Both of these cell lines are null for endogenous p53 expression (8, 10). In contrast to the transfection of expression vectors containing only mutant *p53* (2, 14, 29, 36, 39, 41), the coexpression of wild-type p53 within the same cell results in significant transactivation. As expected (2), the hinge domain mutants, 305m and 325v, showed no dominant-negative effect. This was also true of the DNA binding domain mutants. Although some decrease in transactivation levels was observed, the inhibition was not more than a 30% decrease relative to wild-type alone.

Bidirectional expression vectors were also transiently cotransfected with the *Bax* luciferase reporter into both CaLu 6 and Saos-2 cells, and transactivation levels were measured (Fig. 2B). In contrast to the $p21^{WAF\text{-}1/CIP\text{-}1}$ results, all of the mutants displayed dominant-negative effects on wild-type p53-mediated transactivation of the *Bax* promoter. This dominant-negative effect is particularly evident in the DNA binding domain mutants that gave 75 to 80% inhibition in CaLu 6 cells and 65 to 70% inhibition in Saos-2 cells.

Because it is possible in some conditions to have overall biological effects, such as apoptosis, in the absence of transactivation-competent p53 (25 and 27), we sought to investigate dominant-negative effects on the biological effects of growth arrest and apoptosis.

No dominant-negative effects are seen on growth arrest. The ability of the mutant p53s to cause overall cell growth arrest in the absence of wild-type p53 was first assessed in a colony inhibition assay (Fig. 3A). CaLu 6 cells were transfected with the vectors expressing the indicated p53 protein. Compared to the vector control, containing no p53 cDNA, wild-type p53 inhibited colony growth 25-fold, on average. Most of the mutants examined, however, had colony counts similar to that of the vector control, demonstrating loss of growth inhibition. The hinge domain mutants, as seen previously (2), retained the ability to arrest colony growth.

Dominant-negative effects on overall growth arrest were then examined (Fig. 3B). Although the average colony count from each of the mutant and wild-type combinations is slightly higher ($\leq$15), each shows noticeably fewer colonies formed than an empty vector expressing neither mutant nor wild-type p53. The hinge domain mutants, which were previously shown to inhibit colony growth similarly to wild-type p53 (2), had no adverse effects on inhibition when coexpressed with wild-type p53. Wild-type p53 is thus able to elicit nearly all of its growth-inhibitory effects even in the presence of mutant p53 proteins within the cell. Recall that all mutants were recessive to wild type for transactivation of $p21^{WAF1/CIP1}$ (Fig. 2A). Given that $p21^{WAF1/CIP1}$ is the effector of p53-mediated G_1 arrest (46), there is a good correlation between these colony inhibition results and the $p21^{WAF1/CIP1}$ transactivation results. In keeping with such a correlation, we wished to determine if the domi-

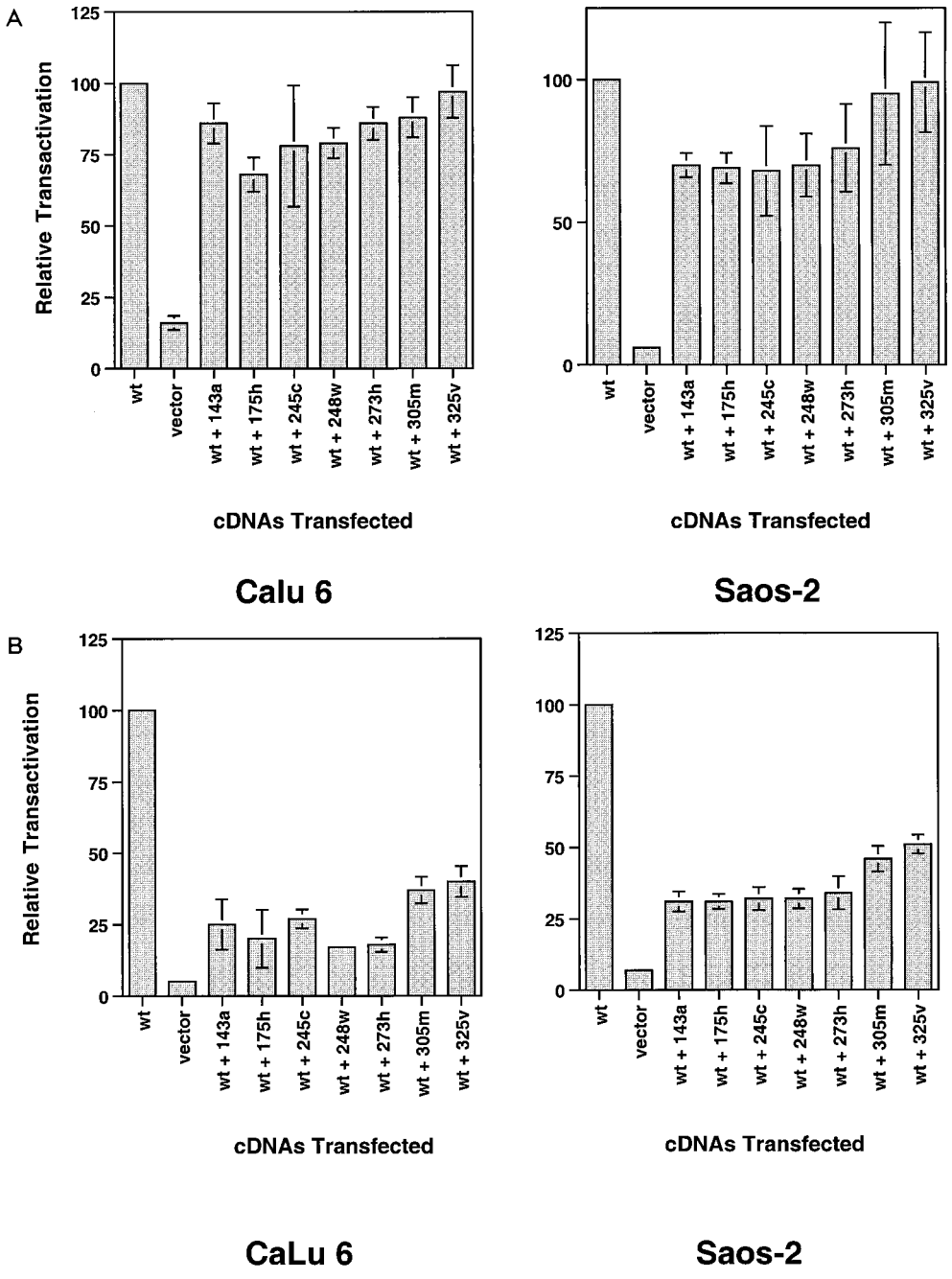

FIG. 2. Effects of equal coexpression of wild-type and mutant p53 on transactivation of p53-responsive promoters. (A) p53 mutants are recessive to wild-type (wt) p53 for transactivation of $p21^{WAF1/CIP1}$ promoter. (B) p53 mutants have a dominant-negative effect on *Bax* promoter transactivation.

nant-negative effects seen on *Bax* transactivation would show an expected dominant-negative effect on apoptosis.

Dominant-negative effects on apoptosis are seen with some but not all p53 mutants. An assessment of the ability of mutant p53 to induce overall apoptosis was assessed in Saos-2 cells, which are highly apoptotic in response to overexpression of wild-type p53 (10, 32, 41, 42). One assay involved transient transfection of Saos-2 cells with a p53 expression vector and subsequent double staining of cells, first for exogenous p53 expression and then for DNA degradation, an indicator of apoptosis, using a fluorescent TUNEL assay (Table 1). In the assay, cells that expressed exogenous mutant or wild-type p53 were noted and then assessed for the percentage of p53-positive cells that were also TUNEL positive. The p53 mutants lost nearly 60% of the ability to induce apoptosis compared to wild-type p53. Mock-transfected cells showed 2 to 3% apoptosis for the overall cell population (data not shown).

A second assay for induction of apoptosis was performed by FACS (Table 2). In this assay, transiently transfected Saos-2 cells were doubly stained with the anti-p53 antibody DO-1 (and FITC-labeled secondary antibody) and the DNA dye propidium iodide. This allowed for gating of the analyzed popu-

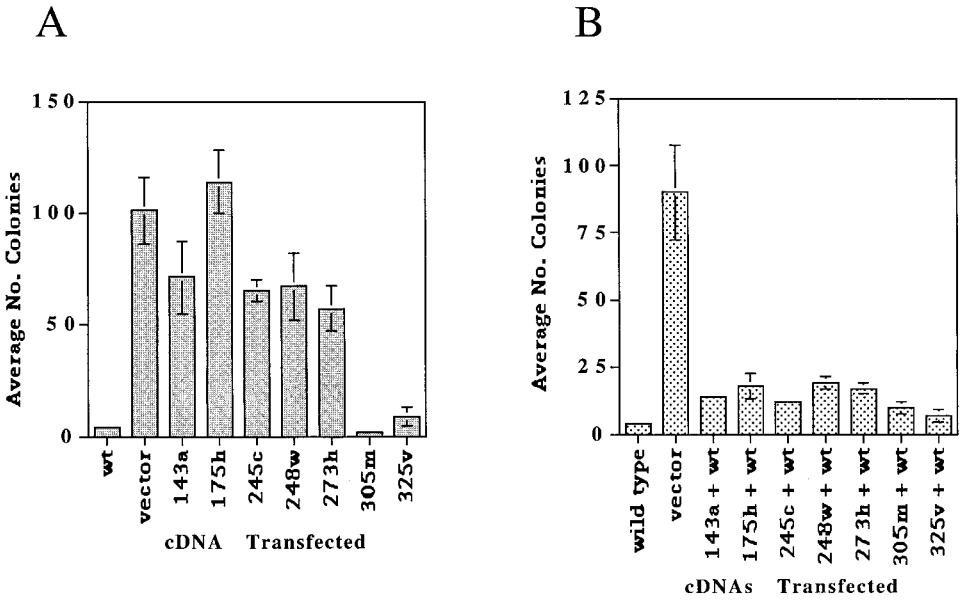

FIG. 3. Growth (colony) inhibition assays. Expression vectors expressing the indicated cDNAs were transfected into CaLu 6 cells and subjected to drug selection. Colonies were counted after 12 to 14 days selection. The results are the average of three separate experiments. (A) Most p53 mutants have lost the ability to inhibit cell growth. wt, wild type. (B) p53 mutants are recessive to wild-type p53 when coexpressed.

lation for the DNA content of cells expressing exogenous p53. DNA damage, indicative of apoptosis, is measured as the cells having a sub-G_1 DNA content. As in the TUNEL assay, the p53 mutants were unable to induce apoptosis. Each mutant gave a lower percentage of p53-positive cells with a sub-G_1 DNA content, relative to wild-type p53, at each time point measured. Thus, in the absence of wild-type p53, there is a good correlation between the loss transactivation of *Bax* and the lack of induction of overall apoptosis.

We wished to determine whether the observed dominant-negative effects on *Bax* transactivation also resulted in dominant-negative effects on overall induction of apoptosis. The bidirectional expression vectors described above were transiently transfected into the Saos-2 cells, and the ratio of TUNEL positive to p53 positive cells was determined for each sample (Table 3). In contrast to the transactivation results shown in Fig. 2A, only three mutants showed dominant-negative effects on the induction of apoptosis: 245c, 248w, and 273h. The other mutant–wild-type combinations had apoptosis levels similar to that of the wild type alone and were thus recessive to wild type.

This trend was also seen when transiently transfected cells were monitored for the induction of apoptosis by FACS analysis (Fig. 4). The same three mutants were dominant negative over wild-type p53-mediated apoptosis, whereas the remaining mutants were recessive. Although there were measurable differences in the sub-G_1 DNA content (apoptosis) seen in the dominant-negative mutant–wild-type transfectants compared to the recessive mutant–wild-type combinations, detectable G_1 arrest was noted for every mutant–wild-type combination (Fig. 4A). Thus, a dominant wild-type p53-mediated cell cycle arrest was seen irrespective of whether a dominant-negative effect on apoptosis induction was detected.

DISCUSSION

It is becoming increasingly apparent, through the study of p53 mutants, that p53 regulates growth arrest and apoptotic pathways differentially (1, 2, 32, 41, 42). These studies described phenotypes that occurred when there was only a p53 mutant product expressed. Investigations of *p53* status in natural cancer progression models suggest that *p53* mutations may play a role early or late in progression, depending on the cancer in question. For example, in colorectal cancer, *p53* mutations appear relatively late in progression (15), whereas in skin cancers it appears to be a relatively early event (5). In spite of the fact that the majority of *p53* mutations are missense—suggesting a gain-of-function mutation where a single mutant allele may influence progression—virtually all cancers have both p53 alleles compromised by mutations or deletions (37). Indeed, in studies examining afflicted members of Li-Fraumeni syndrome families, where there is a heterozygous germ line mutation, loss of the wild-type allele is found in tumors but not in surrounding normal tissue (33, 44). However, it seems likely that at least some p53 missense mutants may influence progression, possibly by acting as dominant-negative mutants. This

TABLE 1. Quantitation of apoptosis by TUNEL staining[a]

Construct	p53-induced apoptosis	CI
Wild type	34.10	0.28–0.34
143a	13.08	0.03–0.07
175h	13.23	0.04–0.07
245c	13.39	0.04–0.07
248w	12.53	0.03–0.06
273h	13.36	0.04–0.07
305m	18.22	0.07–0.12
325v	18.28	0.07–0.12

[a] Expression vectors bearing the indicated cDNAs were transiently transfected into Saos-2 cells, and the cells were stained for p53 expression and TUNEL. p53-induced apoptosis was calculated as the percentage of TUNEL-positive cells of all cells expressing the indicated p53 cDNA. The results of three separate experiments were subjected to a two-variance statistical analysis compared to wild-type p53 to arrive at a confidence interval (CI) and *P* value. The *P* value for each sample was <0.0005 and thus considered statistically significantly different from the value for wild-type p53.

TABLE 2. FACS analysis for detection of DNA degradation

Time (h)	% Sub-G_1 DNA content[a]							
	Wild type	Vector	143a	175h	245c	248w	273h	305m
24	3.67 ± 1.2	1.98 ± 1.0	2.01 ± 0.81	2.05 ± 0.91	2.40 ± 0.73	1.89 ± 1.0	2.14 ± 0.40	3.68 ± 2.40
48	12.53 ± 1.30	4.69 ± 1.60	3.58 ± 0.93	5.04 ± 1.20	3.79 ± 1.90	4.07 ± 1.40	4.02 ± 1.60	5.65 ± 1.50
72	20.44 ± 2.60	5.24 ± 1.90	5.78 ± 0.83	4.85 ± 1.30	5.60 ± 1.70	5.78 ± 1.30	4.98 ± 1.80	6.18 ± 1.80

[a] Expression vectors bearing the indicated cDNAs were transiently transfected into Saos-2 cells. Values are the average percent sub-G_1 DNA content were recorded at each time point posttransfection. Values shown are the averages of three separate experiments.

postulate is somewhat controversial, with evidence provided both for (12, 29) and against (20) it. The uncertain outcome of these experiments may be explained, in part, by experimental designs that facilitate various levels of expression of mutant and wild-type p53 and by a reliance of transactivation assays as the major determinant of dominant-negative function.

We have therefore reexamined this issue and, in an attempt to circumvent the problems outlined above, used bidirectional vectors that provide for equal levels of expression of mutant and wild-type p53 within the same cell and multiple assays for biological function.

The clearest evidence for a lack of dominant-negative effects was provided by the growth inhibition assays. None of the mutants studied produced this effect. p53-mediated growth inhibition is generally accepted to be orchestrated by transactivation of the $p21^{WAF1/CIP1}$ gene. In accordance with this notion, all mutants are recessive to wild type in transactivation of the $p21^{WAF1/CIP1}$ promoter-luciferase reporter assay (Fig. 2A). el-Deiry and colleagues have also reported that mutant p53 proteins do not inhibit the transactivation of p21 by wild-type p53 (13). However, these studies were undertaken with cotransfections and were not controlled for equal levels of expression. It is, of course, possible that growth inhibition, as measured by the colony assay, is due to p53-mediated apoptotic events. A possible correlate of this was that the mutants were found to behave in a dominant-negative fashion in a *Bax* promoter transactivation assay (Fig. 2B). This interesting difference in the capacity of mutant p53 proteins to transactivate promoters, relative to wild type, has been noted previously (32, 42), although the mechanism is unknown.

The observation of dominant-negative effects on *Bax* promoter transactivation provided an attractive scenario since Bax protein is known to promote apoptosis (38, 48). However, with respect to dominant-negative effects, this attractive correlation broke down when actual effects on apoptosis were measured. Only three of the seven mutants studied showed clear dominant-negative effects in the apoptosis assays (Table 3 and Fig. 4). Thus, effects on *Bax* promoter transactivation were not the determining feature for apoptotic regulation and also did not support the notion that the growth arrest assays were measuring exclusively apoptosis-inducing functions.

TABLE 3. Quantitation of dominant-negative effects on apoptosis by TUNEL staining[a]

Construct	p53-induced apoptosis	CI	*P*
Wild type	33.80	0.28–0.35	
Wild type +:			
143a	28.28	0.20–0.25	0.0329
175h	28.81	0.21–0.26	0.0579
245c	12.68	0.04–0.06	0.0001*
248w	12.62	0.04–0.06	0.0001*
273h	13.95	0.05–0.06	0.0001*
305m	27.33	0.19–0.24	0.0116
325v	27.81	0.19–0.24	0.0197

[a] Bidirectional expression vectors bearing the indicated p53 cDNAs were transiently transfected into Saos-2 cells. p53-induced apoptosis, confidence interval (CI), and *P* are as described in the footnote to Table 1. Samples with *P* values of <0.0005 (*) are considered to have a statistically significant difference in value compared to wild-type p53.

Kern et al. studied the effects of three of the mutants used in the present study, 175h, 248w, and 273h (30). Using the synthesized PG13 promoter, they found that the mutants inhibited transactivation by cotransfected wild-type *p53* nearly 70% when cotransfected at a 1-to-1 ratio and 85 to 95% when cotransfected at a 3-to-1 mutant-to-wild-type ratio. However, a study by Unger et al. showed that while mutant 248w was dominant negative at the 3-to-1 ratio, 273h was recessive to wild-type p53 in transactivation of a similar promoter (46).

The effect of mutant-to-wild type expression vector ratio is clearly an issue in these findings. Crook and colleagues, using the PG13 promoter system, showed that dominant-negative effects increased with an increase in the ratio of mutant to wild-type p53 (12). It is noteworthy that mutant p53 protein has a longer half-life than wild-type protein (18), and thus mutant p53 protein monomers outnumber wild-type monomers at steady-state conditions, even when coexpressed at equal levels. Thus, when the mutant ratio increases, the level of mutant protein will be disproportionately larger. Under physiological conditions, for example, in affected Li-Fraumeni syndrome family members, there are equal numbers (1:1) of mutant and wild-type *p53* alleles. Thus, it is likely that the most appropriate measure of potential dominant-negative effects will be in those cells that express equivalent levels of wild-type and mutant p53 mRNAs.

A study of the crystal structure of the sequence-specific DNA binding domain of p53 showed that this domain was susceptible to drastic conformational changes due to single amino acid substitutions (11). All of the three dominant-negative substitutions (at codons 245, 248, and 273) are at residues critical for overall structure. Residues 248 and 273 directly contact the DNA helix, and any substitutions to the wild-type glycine at residue 245 affect the positioning of L2 and L3, two amino acid loops that interact with the minor groove. Residue 143, in contrast, interacts with fewer structures. Residue 175 also affects L2 and L3 positioning, but only some (not all) substitutions may affect function, as was shown in a study (42) on substitutions at residue 175. Thus, substitutions at residues 143 and 175 may not affect the overall structure and function of a heterotetramer as drastically as substitutions at residues 245, 248, and 273, although the substitutions at residues 143 and 175 may have still conferred subtle effects on heterotetramer structure. Virtually nothing is known about the effects that the hinge domain mutations (at residues 305 and 325) have on p53 function. It is possible that they do not affect the structure and function of the overall tetramer due to their position at a flexible domain of p53. This effect on the conformation of

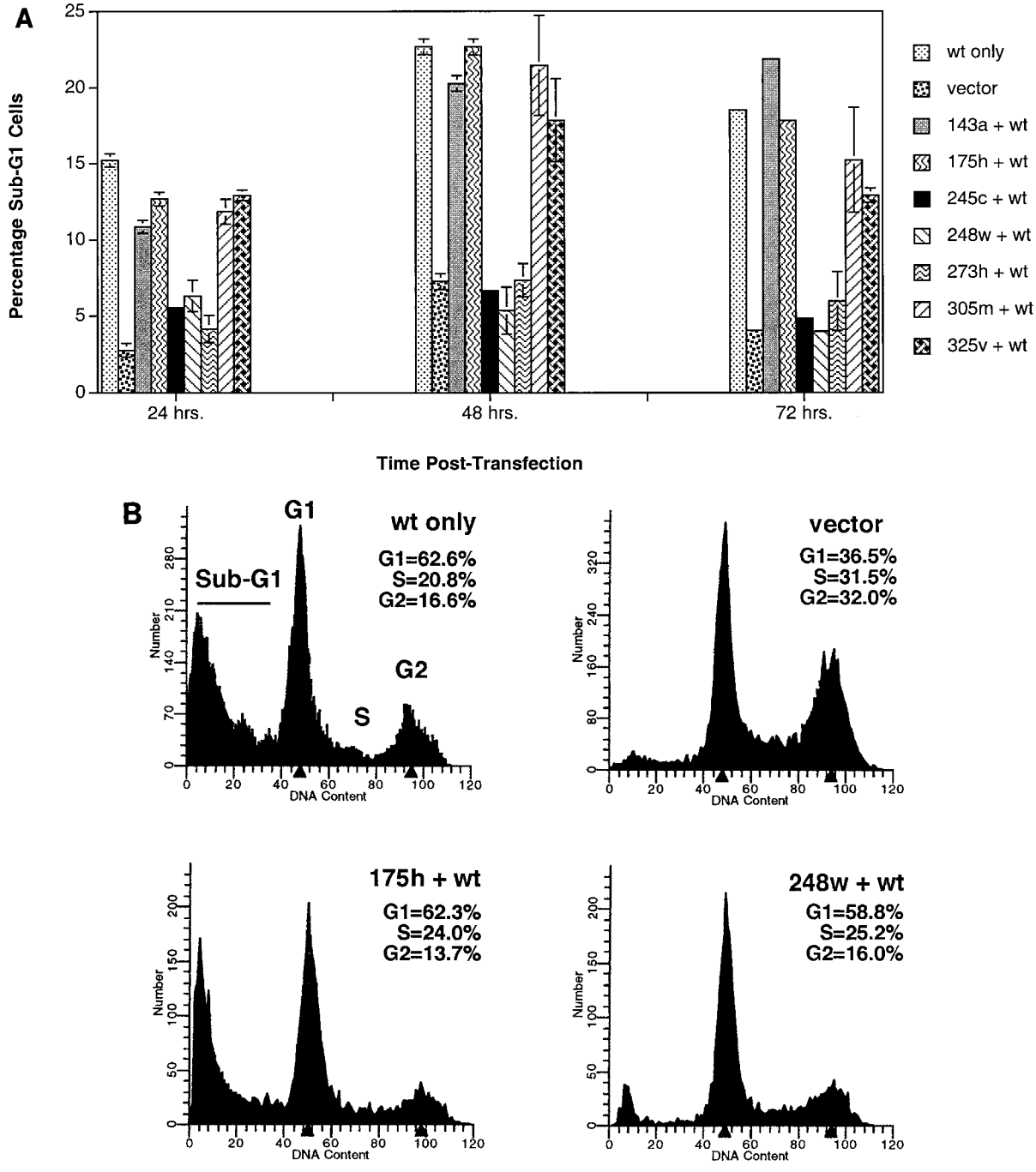

FIG. 4. FACS analysis of dominant-negative effects on apoptosis. (A) Bar graph illustrating dominant-negative effects of only a fraction of the p53 mutants. wt, wild type. (B) Representative FACS profiles illustrating a dominant-negative (248w) and a recessive (175h) p53 mutant.

heterotetramers could have various effects on interactions with DNA and/or other proteins under certain contexts. As a consequence, heterotetramers may transactivate differently at one p53-responsive promoter compared to another.

Thus, there are several possible explanations for the differential dominant-negative effects on transactivation of $p21^{WAF1/CIP1}$ versus the transactivation of *Bax*. The first is that the promoters for $p21^{WAF1/CIP1}$ and *Bax* have one and four p53 consensus binding sites, respectively (14, 36). Dominant-negative effects are not seen on the $p21^{WAF1/CIP1}$ promoter but are seen in the *Bax* promoter (Fig. 2), perhaps because fewer binding sites need to be occupied for transactivation of $p21^{WAF1/CIP1}$ than *Bax*. This means that less fully functional (i.e., all wild-type monomers) p53 is needed to transactivate $p21^{WAF1/CIP1}$ than *Bax*. This would also suggest that apoptotic genes, in general, require more functional p53 than growth arrest genes, $p21^{WAF1/CIP1}$ being the major effector of G_1 arrest (46). Another possibility is that pathway-specific accessory factors may be needed for induction of the growth arrest or apoptotic pathways. Thus, even though presence of one or

more mutant p53 monomers may allow binding to natural promoters such as $p21^{WAF1/CIP1}$ and *Bax*, the critical *Bax* cofactor may not be able to interact with the mutant–wild-type hetero-oligomer as a result of slight conformational changes due to the presence of mutant oligomers. Yet another possibility is that it may be a combination of these two models that accounts for the differential effects on transactivation of $p21^{WAF1/CIP1}$ and *Bax*.

The $p21^{WAF1/CIP1}$ gene appears to be the main effector of p53-mediated G_1 arrest (46). However, *Bax* is not the only p53-regulated gene that has been shown to be involved in the apoptotic pathway (7), and there may be several additional, as yet uncharacterized genes in the pathway that are regulated by p53. This may also explain why apoptosis is seen in some tissues in *Bax*-deficient mice (30). It may also explain why, whereas all of the mutants examined in this study displayed dominant-negative effects for *Bax* transactivation (Fig. 2B), only three had a dominant-negative effect on overall apoptosis (Table 3 and Fig. 4). Furthermore, it is possible that under certain conditions p53-mediated apoptosis does not require transactivating ability. Haupt and colleagues showed that apoptosis can occur in HeLa cells with transactivation-deficient p53 (25). Also, Wang and colleagues found that XPB and XPD helicase interaction with the carboxy terminus of p53, rather than the transactivation domain in the amino terminus, may be important for apoptosis (48). Obviously many factors may play a role in p53 regulation of apoptosis and growth arrest, including different pathways that regulate the transcription of different subsets of downstream effectors or through different protein-protein interactions, or indeed a combination of the two (25, 33, 41, 42, 47). We also wish to emphasize that we have observed these phenomena in two transformed cell lines. Whether the results that we have presented here are applicable generally to biological systems remains to be determined.

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