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# Relief of p53-mediated transcriptional repression by the adenovirus E1B 19-kDa protein or the cellular Bcl-2 protein

**(apoptosis/transcriptional regulation)**

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**ABSTRACT** **The p53 tumor suppressor gene product is a transcriptional regulatory protein. It activates transcription from promoters that contain a p53 DNA binding site but represses many promoters that lack its binding site. High-level expression of wild-type p53 can induce apoptosis in certain cell types, and this activity can be blocked by the adenovirus E1B 19-kDa oncoprotein or by the cellular Bcl-2 oncoprotein. Here we report that p53-mediated repression of promoters that lack a p53 binding site is abrogated by the E1B 19-kDa protein or Bcl-2 oncoprotein. In contrast, transcriptional activation by p53 still occurs in the presence of either protein. The fact that two oncoproteins capable of preventing p53-mediated apoptosis also block transcriptional repression by p53 raises the possibility that p53 might induce apoptosis, at least in part, by repressing transcription.**

Apoptosis is an active and well-defined process of cell death (1–3). It is essential for proper embryonic development and for maintaining homeostasis. Apoptosis also acts to remove abnormal cells, such as oncogenically transformed and virus-infected cells (2, 3). This is clearly exemplified by cells expressing the adenovirus E1A oncoprotein. Expression of the E1A protein in primary cells can efficiently induce cellular DNA synthesis and transient cell proliferation but is insufficient for oncogenic transformation (4, 5). This is because, in addition to inducing proliferation, E1A triggers apoptosis, which results in cell death (6–8). E1A-induced apoptosis is mediated by the p53 tumor suppressor protein (9, 10), and cell death can be blocked by the adenovirus E1B 19-kDa oncoprotein or the cellular *bcl-2* protooncogene product (8, 10), either of which can cooperate with E1A to fully transform cells.

Wild-type p53 has been shown to inhibit cell cycle progression and to suppress oncogenic transformation (11–13). It appears that p53 normally functions as a component of a $G_1$-phase checkpoint that blocks cell cycle progression in response to DNA damage (14–16). p53 is a transcriptional regulatory protein. It activates transcription from promoters that contain p53-specific DNA binding sites and represses many promoters that lack its binding site (for review, see ref. 17), and transcriptional activation by p53 is strongly correlated with its ability to block cell cycle progression (18). Recent work indicates that DNA damage can transiently induce p53; and this, in turn, activates expression of p53-responsive genes such as *WAF1* (19) whose product is able to arrest cell cycle progression (20).

p53 has also been implicated in apoptosis pathways. The intracellular level of p53 increases upon the expression of adenovirus E1A protein (9), and this in turn mediates the apoptosis effect of E1A (10). In addition, enhanced levels of p53 can directly induce apoptotic cell death in many cell types (21–25). The biochemical property of p53 that is responsible for this activity is still unknown. To investigate the mechanistic basis for its ability to mediate apoptosis, we examined whether the transcriptional regulatory properties of p53 might be altered by the adenovirus E1B 19-kDa protein or the cellular Bcl-2 protein, two oncoproteins that prevent p53-mediated apoptosis. Here we report that the transcriptional repression mediated by p53 is blocked by the E1B 19-kDa protein or Bcl-2 protein whereas transcriptional activation by p53 still occurs in the presence of either protein. The fact that two oncoproteins capable of preventing p53-mediated apoptosis also block transcriptional repression by p53 raises the possibility that p53 might induce apoptosis, at least in part, by repressing transcription.

## MATERIALS AND METHODS

**Plasmids.** pC53-SN3 (26) encodes wild-type human p53 and pCMV-19kDa (27) encodes the adenovirus E1B 19-kDa protein, both under control of the cytomegalovirus immediate-early promoter. pCMV-bcl2 was constructed by cloning a 1.8-kb *Eco*RI fragment containing the *bcl-2* cDNA from plasmid pSFFV-bcl2 (28) between *Eco*RI–*Hin*dIII ends generated by excision of the E1B 19-kDa protein coding region from pCMV-19kDa. pCMV-1 was derived from pCMV-19kDa by excision of the E1B 19-kDa protein coding region and religation of the vector. pTICAT (29) contains a chloramphenicol acetyltransferase (CAT) gene whose expression is controlled by a minimal promoter containing the TATA box from the adenovirus major late promoter and the initiator element from the terminal deoxynucleotidyltransferase gene. pSTICAT (N. Horikoshi and T.S., unpublished data) and p50-2CAT (30) are derivatives of pTICAT, containing SP1 binding sites and p53-responsive elements, respectively, located about 60 bp upstream of the transcriptional initiation site.

**Cell Culture and CAT Assays.** HeLa cells and SAOS-2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum. Cells ($1 \times 10^6$ cells per 10-cm plate) were transfected by the calcium phosphate precipitation method with 5 $\mu$g of the reporter plasmids, the indicated amount of pC53-SN3, and 0.5 $\mu$g of pCMV-19K or 1 $\mu$g of pCMV-bcl2. The total amount of DNA for each transfection was brought to 30 $\mu$g with salmon sperm DNA. Cells were harvested 48 hr after transfection, and CAT assays were performed as described (31). Results were quantified using a PhosphorImager. All assays were normalized for total protein concentration.

**RNase Protection Assay.** Cells were harvested 48 hr after transfection and total cellular RNA was prepared using TriZol (BRL) by following the manufacturer's instructions. A 10-$\mu$g portion of total cellular RNA from each sample was hybridized with probe ($4 \times 10^5$ cpm; $\geq 10^8$ cpm/$\mu$g) at 45°C

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Abbreviation: CAT, chloramphenicol acetyltransferase.

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for 12 hr. The $^{32}$P-labeled RNA probe corresponded to the first 243 nt of the CAT mRNA plus 158 nt of 5′ flanking sequence. Hybridized mixtures were digested with 5 units of RNase ONE (Promega) at 30°C for 1 hr, and the digestion products were resolved by electrophoresis on a denaturing 6% polyacrylamide gel and visualized by autoradiography.

**Southern Blot Hybridization.** HeLa cells were harvested 48 hr after transfection and nuclei were isolated by Dounce homogenization with 0.5% Nonidet P-40 in 10 mM Tris·HCl, pH 7.4/10 mM NaCl/3 mM $MgCl_2$. Isolated nuclei were then lysed in 100 mM NaCl/10 mM Tris·HCl, pH 8.0/25 mM EDTA/0.5% SDS/proteinase K (0.1 mg/ml) at 37°C for 10 hr. Total nuclear DNAs were purified and digested with *Bam*HI. Aliquots of DNA (10 μg) from each sample were subjected to electrophoresis on a 0.8% agarose gel and transferred to a GeneScreen*Plus* nylon membrane (New England Nuclear). The blot was hybridized with a $^{32}$P-labeled probe obtained by random-primed DNA synthesis using the ampicillin-resistance gene as the template.

**Immunoprecipitation.** At 48 hr after transfection, cells were labeled with $^{35}$S-Express protein labeling mixture (New England Nuclear) at 100 μCi/ml (1 Ci = 37 GBq) for 1 hr in methionine-free medium. They were then harvested and divided into two fractions, one for direct lysis of the entire cell and the other for preparation of nuclei by treatment with Nonidet P-40 in physiological saline followed by nuclear lysis. Lysis was carried out in 50 mM Tris·HCl, pH 8.0/5 mM EDTA/150 mM NaCl/0.5% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride at 4°C with vigorous vortex mixing. Cell or nuclear lysates were normalized to equivalent cpm ($5 \times 10^6$ cpm for whole cell lysates or $1 \times 10^6$ cpm for nuclear lysates), and immunoprecipitated with the p53-specific monoclonal antibody pAb1801 (Oncogene Science). The immunoprecipitated proteins were subjected to electrophoresis on an SDS/8% polyacrylamide gel and visualized by fluorography.

## RESULTS

**Transcriptional Repression by Wild-Type p53 Is Blocked by E1B 19-kDa Protein or Bcl-2 Protein.** We first examined the effect of E1B 19-kDa or Bcl-2 protein expression on p53-mediated repression of the minimal promoter in pTICAT (Fig. 1*a*). We employed HeLa cells for the analysis since they contain very low endogenous levels of p53 (32). When pTICAT was cotransfected with increasing amounts of pC53-SN3, a wild-type human p53 expression vector, CAT expression was repressed by p53 in a dose-dependent fashion. Neither E1B 19-kDa or Bcl-2 protein significantly influenced expression of the reporter gene in the absence of exogenously added p53 (Fig. 2, and data not shown). However, when p53 was present, E1B 19-kDa and Bcl-2 proteins not only blocked repression but also caused expression to be activated in a p53 dose-dependent fashion. The highest level of p53 tested repressed CAT expression by a factor of ≈6 in the absence of E1B 19-kDa or Bcl-2 protein, but activated expression in their presence by a factor of ≈4 (Fig. 1*a*). As a result, either E1B 19-kDa or Bcl-2 protein increased CAT expression by a factor of ≈25 in the presence of p53, causing p53 to activate a promoter that it normally represses. The same result was observed when pSTICAT was used as the reporter plasmid, which is identical to pTICAT except that it contains upstream Sp1 binding sites to increase the basal level of transcription (Fig. 1*a*), and when experiments were performed in SAOS-2 cells that lack endogenous p53 (data not shown). The analyses displayed in Fig. 1*a* were performed at a constant concentration of the E1B 19-kDa (0.5 μg) or Bcl-2 (1.0 μg) effector plasmid. Higher input levels of these plasmids did not enhance their effect on p53 function (data not shown). In these experiments, the expression of E1B 19-kDa or Bcl-2

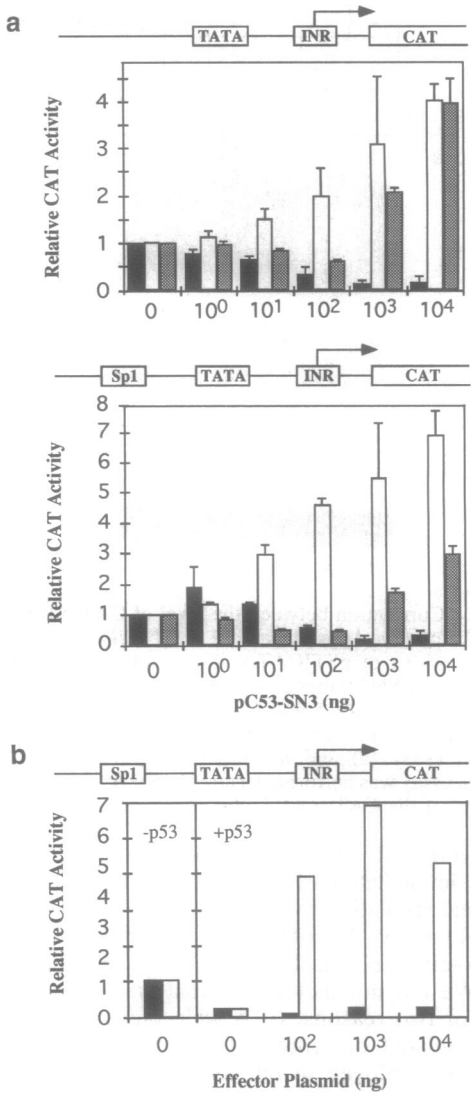


FIG. 1. (*a*) Effects of the adenovirus E1B 19-kDa protein and the cellular Bcl-2 protein on transcriptional repression by p53. Promoter elements present in each reporter plasmid (inr, initiator element; TATA, TATA motif; Sp1, Sp1 binding site; p53RE, p53 response element) are diagrammed above each experiment. Five micrograms of pTICAT (*Upper*) or pSTICAT (*Lower*) reporter plasmid were cotransfected into $1 \times 10^6$ HeLa cells with increasing amounts of pC53-SN3, a wild-type p53 expression vector. The regulation of CAT gene expression by p53 alone (solid bars), p53 + 0.5 μg of plasmid for E1B 19-kDa protein (open bars), and p53 + 1 μg of plasmid for Bcl-2 protein (shaded bars) was monitored. Inclusion of plasmids expressing E1B 19-kDa or Bcl-2 protein did not significantly change the expression of the reporter plasmid in the absence of p53. The bar graphs display the average with standard deviation of two experiments for each reporter construct. (*b*) Block of p53-mediated repression is not a direct effect of the presence of the cytomegalovirus immediate-early promoter. Five micrograms of the reporter plasmid (pSTICAT) was used in each experiment. − p53 and + p53 indicate the absence or the presence, respectively, of 1 μg of pC53-SN3 plasmid in the transfection reaction mixture. Increasing amounts of the expression vector alone (pCMV-1, solid bars) or the recombinant plasmid carrying the E1B 19-kDa region (pCMV-19kDa, open bars) were included in the transfection as indicated.

protein was controlled by the cytomegalovirus immediate-early promoter. To rule out a direct effect of the cotransfected promoter on p53-mediated repression, an experiment was performed in which the activity of the expression vector (pCMV-1) alone was compared to the recombinant vector carrying the E1B 19-kDa protein coding sequence (pCMV-

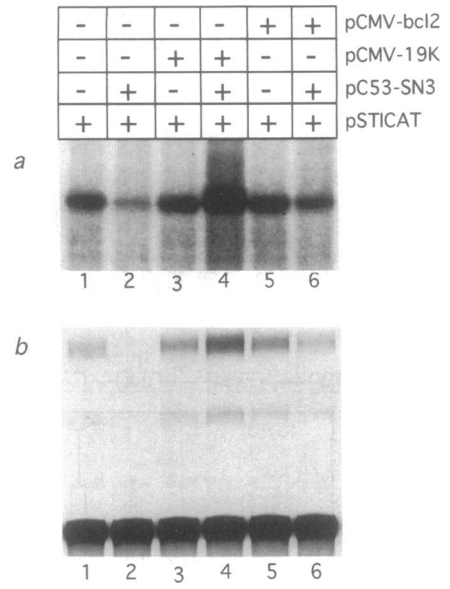


FIG. 2. Correlation between the level of CAT mRNA and CAT enzymatic activity. Approximately $1 \times 10^6$ HeLa cells were cotransfected with 5 μg of pSTICAT and 1 μg of the following effector plasmids: pC53-SN3 (lanes 2, 4, and 6), pCMV-19K (lanes 3 and 4), and pCMV-bcl2 (lanes 5 and 6). Cells were harvested 48 hr after transfection and then divided into two fractions: one for RNase protection assay (*a*) and the other for CAT assay (*b*). RNase protection assay was performed using a probe that corresponds to the first 243 nt of the CAT mRNA plus 158 nt of 5′ flanking sequence.

19kDa) (Fig. 1*b*). The expression vector with the cytomegalovirus immediate-early promoter had no effect on transcriptional regulation by p53.

All CAT assays described in this paper were normalized for total protein concentration of the extracts that were analyzed. We did not include internal standards in the CAT assays for two reasons. (*i*) A wide range of promoters are either activated or repressed by p53 under the range of conditions employed in our assays, making it difficult to select a promoter that would not be affected. (*ii*) We wanted to avoid inclusion of additional promoters that could influence results by competing for binding to cellular factors. Therefore, instead of including internal standards, we performed each experiment using multiple concentrations of the p53 effector plasmid and we repeated each experiment at least three times with consistent results.

A good correlation was observed between the levels of CAT mRNA determined by RNase protection (Fig. 2*a*) and CAT enzymatic activity (Fig. 2*b*). This leads us to conclude that the increase in CAT expression mediated by p53 in the presence of E1B 19-kDa or Bcl-2 protein occurred at the level of mRNA accumulation.

In some assays, Bcl-2 failed to activate expression of the reporter in the presence of p53 as efficiently as E1B 19-kDa protein (Fig. 1*a Lower*), or Bcl-2 substantially relieved repression but failed to mediate activation in the presence of p53 (Fig. 2). We don't know the reason for this variability, but it is clear that in several dozen assays, Bcl-2 always substantially relieved p53-mediated repression and generally caused transcriptional activation in the presence of p53.

**E1B 19-kDa or Bcl-2 Protein Does Not Change the Nuclear Level of the Transfected DNAs or Block the Expression of p53 Protein.** Since it was previously reported that E1B 19-kDa protein can increase gene expression by stabilizing transfected plasmid DNA (33), the nuclear concentration of reporter DNAs was determined by DNA blot analysis. E1B 19-kDa or Bcl-2 protein did not influence reporter plasmid levels at the time when CAT assays were performed (Fig. 3),

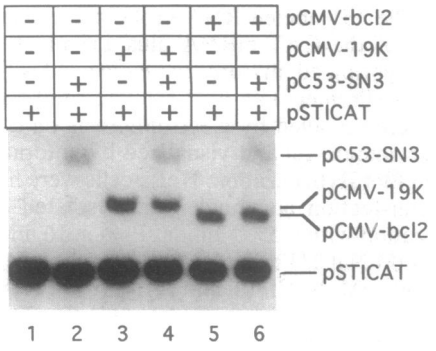


FIG. 3. Determination of plasmid DNA levels in the nuclei of transfected cells by Southern blot analysis. Approximately $1 \times 10^6$ HeLa cells were plated and then cotransfected with 5 μg of reporter plasmid, pSTICAT, and 1 μg of various effector plasmids. Lanes: 1, pSTICAT; 2, pSTICAT + pC53-SN3; 3, pSTICAT + pCMV-19K; 4, pSTICAT + pC53-SN3 + pCMV-19K; 5, pSTICAT + pCMV-bcl2; 6, pSTICAT + pC53-SN3 + pCMV-bcl2.

ruling out the possibility that an increased plasmid copy number was responsible for elevated reporter expression.

We also asked whether E1B 19-kDa or Bcl-2 protein abrogated p53-mediated repression by interfering with the expression of p53. Nuclear localization of p53 was not altered; and, rather than blocking expression, either E1B 19-kDa or Bcl-2 protein increased the level of p53 protein by a factor of ≈6 (Fig. 4). The increase was not surprising because the expression of p53 was directed by the cytomegalovirus immediate-early promoter, which can be repressed by p53 itself (34). E1B 19-kDa and Bcl-2 proteins presumably release this repression, resulting in a higher level of p53.

Thus, E1B 19-kDa and Bcl-2 proteins did not alter the levels of plasmid DNA, reduce p53 expression, or alter its nuclear localization.

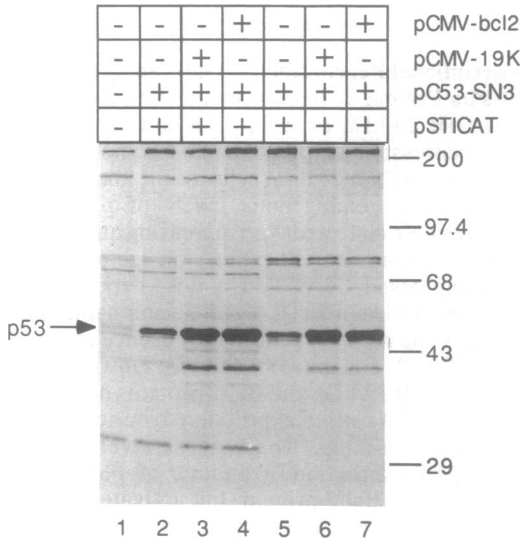


FIG. 4. Immunoprecipitation analysis of p53. Approximately $1 \times 10^6$ HeLa cells were transfected with no DNA (lane 1), 1 μg of pC53-SN3 (lanes 2 and 5), 1 μg of pC53-SN3 + 1 μg of pCMV-19K (lanes 3 and 6), or 1 μg of pC53-SN3 + 1 μg of pCMV-bcl2 (lanes 4 and 7). Cells were labeled and harvested. Labeled cells were divided into two fractions, one for direct lysis and the other for nuclei preparation followed by nuclear lysis. Whole cell lysates were used in lanes 1–4; nuclear lysates were used in lanes 5–7. Cell or nuclear lysates were normalized to equivalent cpm ($5 \times 10^6$ cpm for whole cell lysates or $1 \times 10^6$ cpm for nuclear lysates) and were immunoprecipitated with anti-p53 monoclonal antibody pAb1801. The arrow indicates the position of p53. Molecular masses (in kDa) are on the right.

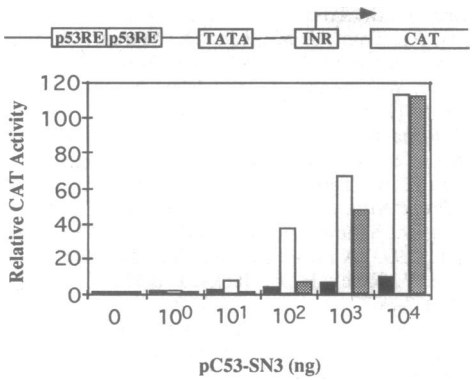


FIG. 5. Effects of E1B 19-kDa protein and Bcl-2 protein on transcriptional activation by p53. The reporter plasmid was p50-2CAT. The promoter controlling this reporter includes two copies of a p53 responsive element (p53RE) and its constituents are diagrammed as in Fig. 1. Five micrograms of p50-2CAT was cotransfected into $1 \times 10^6$ HeLa cells with increasing amounts of pC53-SN3. The regulation of CAT gene expression by p53 alone (solid bars), p53 + 0.5 $\mu$g of plasmid for E1B 19-kDa protein (open bars), and p53 + 1 $\mu$g of plasmid for Bcl-2 (shaded bars) was monitored. Results of a single experiment are shown. The experiment was repeated three times, producing consistent results, although at the highest concentration ($10^4$ ng) of p53 expression plasmid (pC53-SN3), the extent to which E1B 19-kDa and Bcl-2 proteins enhance p53-mediated transcriptional activation was variable, ranging from ≈3-fold to 12-fold.

**E1B 19-kDa or Bcl-2 Protein Does Not Block Transcriptional Activation by p53.** Finally, we tested whether p53-mediated activation of a promoter with p53-response elements can be altered by E1B 19-kDa or Bcl-2 protein (Fig. 5). p50-2 contains a CAT gene whose expression is directed by a control region composed of two p53-binding sites upstream of the basal promoter in pTICAT. Expression from p50-2 was stimulated by p53 in a dose-dependent manner. In the presence of E1B 19-kDa or Bcl-2 protein, p53-mediated activation of this promoter was enhanced by a factor of ≈8. The increased level of activation could result from a change in the function of p53, E1B 19-kDa protein, or Bcl-2 protein. Perhaps transcriptional activation observed for p53 is normally the net result of repression by one population of p53 and activation by a second functionally distinct p53 population. The increased activation in the presence of the E1B 19-kDa or Bcl-2 protein could result from conversion of the entire p53 population to the hypothetical activating form. Alternatively, the enhanced activation could simply result from the increased levels of p53 that accumulate in the presence of E1B 19-kDa or Bcl-2 protein (Fig. 4).

## DISCUSSION

A series of observations argue that transcriptional repression is probably a normal physiologically relevant activity of p53. (*i*) Only wild-type but not mutant p53 can repress transcription (31, 34–40), correlating repression with normal function of the tumor suppressor protein. (*ii*) Some promoters lacking a p53 response element are not repressed by p53. A class I major histocompatibility complex promoter (35), the Ha-ras1 promoter (41, 42), the human epidermal growth factor receptor promoter (41), the proliferating cell nuclear antigen promoter (43), and promoter constructs that lack "TATA" motifs (43) have been reported to be resistant to p53-mediated repression. Thus, repression by p53 exhibits promoter selectivity. (*iii*) The strongest argument for physiological relevance derives from the ability of two oncoproteins, E1B 19-kDa and Bcl-2 proteins, to regulate the ability of the p53 tumor suppressor to repress transcription (Figs. 1 and 2).

Transcriptional repression by p53 is not simply blocked by E1B 19-kDa and Bcl-2 proteins. Rather, these oncoproteins somehow alter the nuclear milieu so that promoters that are normally repressed by the tumor suppressor become activated (Figs. 1 and 2). Enhanced transcriptional activation of promoters with p53 response elements might be another consequence of these putative changes (Fig. 5). The altered response to p53 is not restricted to basal promoters. The human c-*fos* promoter is also repressed by p53 but it is activated by coexpressing p53 with E1B 19-kDa or Bcl-2 protein (data not shown). In addition, the increased expression of p53 in the presence of E1B 19-kDa or Bcl-2 protein (Fig. 4) suggests that repression by p53 of the cytomegalovirus immediate-early promoter (which controls p53 expression in these experiments) is abrogated.

The mechanism by which the transcriptional response to p53 is altered by the E1B 19-kDa and Bcl-2 proteins remains unclear. It seems unlikely that the altered transcriptional response results from the formation of a complex between p53 and E1B 19-kDa or Bcl-2 protein since we have been unable to detect interactions between these proteins either by coimmunoprecipitation from extracts of cotransfected cells or by capture of *in vitro*-translated oncoproteins with a glutathione *S*-transferase–p53 fusion protein (data not shown). We also have failed to detect a conformational change in the p53 protein in response to expression of the E1B 19-kDa protein by immunoprecipitation of p53 with conformation-sensitive antibodies (data not shown). p53 might indirectly alter the Bcl-2 and E1B 19-kDa proteins so that they can activate transcription. Although we cannot rule out this possibility, we do not favor it since neither of these proteins has been shown to have intrinsic transcriptional-activating potential. Alternatively, the E1B 19-kDa and Bcl-2 proteins might induce an alteration in p53 so that it activates promoters that it normally represses. This possibility seems somewhat more plausible since p53 has both activation and repression potential. Finally, it is possible that the functions of the tumor suppressor and the oncoproteins might remain unchanged, but their combined activities might lead to transcriptional activation of the reporter genes. In this scenario, the indirect but strong activating event would presumably override the continued repressive activity of p53, causing a promoter that is normally repressed by p53 to be activated.

p53 can suppress cellular proliferation by arresting cells in the $G_1$ phase of the cell cycle (11, 44) or by inducing apoptosis (9, 10, 21–25). $G_1$ arrest by p53 appears to result from its ability to activate transcription (18). One of the consequences of transcriptional activation by p53 is the induction of WAF1 (19), which inhibits the activity of $G_1$ cyclin-dependent kinases and blocks the $G_1$-to-S phase transition (20). The mechanism underlying the induction of apoptosis by p53, however, remains a mystery. E1B 19-kDa and Bcl-2 proteins can both block p53-mediated apoptosis (6, 8, 10), and we show here that both proteins can also block p53-mediated transcriptional repression but not activation (Figs. 1 and 5). The correlation between the ability of E1B 19-kDa and Bcl-2 proteins to block apoptosis and their ability to alleviate p53-mediated repression raises the possibility that p53 might induce apoptosis, at least in part, by repressing transcription.

It has been shown recently that p53 can simultaneously activate expression of a gene termed *bax* and inhibit expression of the *bcl-2* gene, both at the level of mRNA accumulation (45, 46). The Bax protein and the Bcl-2 protein apparently have opposite effects on cell death: Bax accelerates apoptosis, whereas Bcl-2 promotes cell survival (47). Bax can heterodimerize with Bcl-2, and the ratio between these two proteins seems to determine cell survival or death. Thus, p53 might induce apoptosis at least in part by altering the intracellular ratio of Bax and Bcl-2 proteins. It is not yet known whether p53 directly inhibits transcription of the *bcl-2* gene;

the downregulation could be an indirect effect. Nevertheless, the inhibition raises the possibility that p53 might induce apoptosis by directly inhibiting expression of the *bcl-2* gene. Adenovirus E1B 19-kDa protein might relieve p53-mediated apoptosis by blocking repression of *bcl-2* gene expression by p53. In a similar vein, the induction of Bax mRNA accumulation suggests that p53-mediated transcriptional activation might also contribute to apoptosis.

The adenovirus E1B gene encodes two proteins unrelated in their primary sequence, either of which can inhibit E1A-mediated apoptosis (8) and cooperate with E1A proteins to oncogenically transform cells (48, 49). p53 appears to be in the pathway through which E1A induces apoptosis (9, 10), and it is noteworthy that both E1B oncoproteins alter p53 function. The E1B 55-kDa protein blocks p53-mediated transcriptional activation (18, 50), and the E1B 19-kDa protein blocks p53-mediated repression (Figs. 1 and 2). As suggested above, both transcriptional regulatory activities of p53 might be involved in the induction of apoptosis, and inhibition of either activation or repression might block the process. Alternatively, the E1B 55-kDa protein, which binds directly to p53 (51) may prove to block p53-mediated repression and activation. If this is true, then it is conceivable that both proteins might cooperate with the E1A protein, at least in part, by blocking the transcriptional repression activity of p53.

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