Cell, Vol. 80, 293–299, January 27, 1995, Copyright © 1995 by Cell Press

Tumor Suppressor p53 Is a Direct Transcriptional Activator of the Human *bax* Gene

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Summary

The *bax* gene promoter region contains four motifs with homology to consensus p53-binding sites. In cotransfection assays using p53-deficient tumor cell lines, wild-type but not mutant p53 expression plasmids transactivated a reporter gene plasmid that utilized the *bax* gene promoter to drive transcription of chloramphenicol acetyltransferase. In addition, wild-type p53 transactivated reporter gene constructs containing a heterologous minimal promoter and a 39-bp region from the *bax* gene promoter in which the p53-binding site consensus sequences reside. Introduction of mutations into the consensus p53-binding site sequences abolished p53 responsiveness of reporter gene plasmids. Wild-type but not mutant p53 protein bound to oligonucleotides corresponding to this region of the *bax* promoter, based on gel retardation assays. Taken together, the results suggest that *bax* is a p53 primary-response gene, presumably involved in a p53-regulated pathway for induction of apoptosis.

Introduction

Inactivation of the p53 tumor suppressor gene occurs in over half of all human tumors, implying that loss of this gene represents a fundamentally important step in the pathogenesis of cancer (Hollstein et al., 1991; Vogelstein and Kinzler, 1992). The p53 protein functions at least in part as a transcriptional regulator and can transactivate cellular genes through sequence-specific interactions with DNA containing the sequence 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, typically in the context of two such sequence motifs separated by 0–13 bp (El-Deiry et al., 1992; Kern et al., 1992; Schärer and Iggo, 1992). At least two important events are regulated by p53 in connection with its function as a tumor suppressor. First, p53 has been shown to induce cell cycle arrest at the G1/S border (Mercer et al., 1990). This action of p53 has been ascribed to its ability to induce expression of a cellular gene *WAF1/CIP1* that encodes a 21 kDa inhibitor of G1 cyclin–dependent kinases (El-Deiry et al., 1993; Harper et al., 1993). Second, p53 can induce apoptosis. Expression of wild-type p53 in some p53-deficient tumor cell lines results in spontaneous cell death (Yonish-Rouach et al., 1991; Shaw et al., 1992). In other p53-deficient tumor cell lines, however, the mere restoration of p53 activity is insufficient to trigger apoptosis, but does render cells relatively more sensitive to induction of apoptosis by radiation and DNA-damaging chemotherapeutic drugs (Lowe et al., 1993a; Bristow et al., 1994; Fisher 1994). In this regard, it has been shown that the relative amounts of p53 transcriptional activity increase and levels of p53 protein become elevated through post-transcriptional mechanisms in cells exposed to radiation and DNA-damaging drugs, suggesting that p53 participates in a cellular system involved in genome surveillance and DNA repair (Lu and Lane, 1993; Zhan et al., 1993). The specific roles played by p53 in this process presumably include the arrest of cycling cells before S-phase to allow for repair of damaged DNA prior to DNA replication and the induction of apoptosis for cases in which the DNA damage is too severe to be properly repaired. Loss of p53 may thus contribute to the genomic instability common in tumor cells, both by allowing tumor cells to replicate damaged DNA, which would fix errors in the genome, and by promoting the survival of cells such that genomic alterations accumulate with time.

The effects of p53 on cell cycle arrest and apoptosis appear to be separable functions. For example, in tumor cell lines lacking p53 in which inducible restoration of p53 function results in cell cycle arrest and apoptosis, gene transfer–mediated elevations in the apoptosis-blocking Bcl-2 protein have been shown to interfere with p53-induced apoptosis without impairing p53-induced G1/S arrest (Wang et al., 1993; Selvakumaran et al., 1994; Ryan et al., 1994). Studies of p53-deficient transgenic mice (p53 "knockouts") have also demonstrated that p53 is required for the induction of apoptosis by γ radiation and by some DNA-damaging drugs in thymocytes, which are mostly noncycling cells in G0/G1 phase in vivo (Lowe et al., 1993b; Clarke et al., 1993). Loss of p53 function has also been shown to delay apoptosis resulting from growth factor deprivation in hemopoietic cells deprived of lymphokines, but it does not prevent G0/G1 arrest (Gottlieb et al., 1994). Finally, the importance of apoptosis regulation for in vivo suppression of tumor formation by p53 has been underscored recently by studies of transgenic mice in which tumor development was associated with loss of p53 and decreased rates of cell death rather than with increased rates of cell proliferation (Symonds et al., 1994).

At present it remains unclear how p53 induces apoptosis. Recently, however, we obtained evidence that restoration of p53 in a murine leukemia cell, M1, was associated with increases in *bax* mRNA and protein. Furthermore, these increases in *bax* gene expression were accompanied by simultaneous decreases in the steady-state levels of *bcl-2* mRNA and protein (Miyashita et al., 1994a; Selvakumaran et al., 1994). Bcl-2 and Bax are homologous proteins that have opposing effects on cell life and death, with Bcl-2 serving to prolong cell survival and Bax acting as an accelerator of apoptosis (reviewed by Reed, 1994). The Bcl-2 and Bax proteins can form heterodimers in cells (Oltvai et al., 1993). Furthermore, the interaction of Bcl-2 with Bax appears to be important for the ability of Bcl-2 to block cell death, based on analysis of Bcl-2 mutants (Yin et al., 1994; Sato et al., 1994). The effects of p53 on *bcl-2* gene expression may be mediated at least in part by a *cis*-acting p53 negative-response element located in the 5′ untrans-

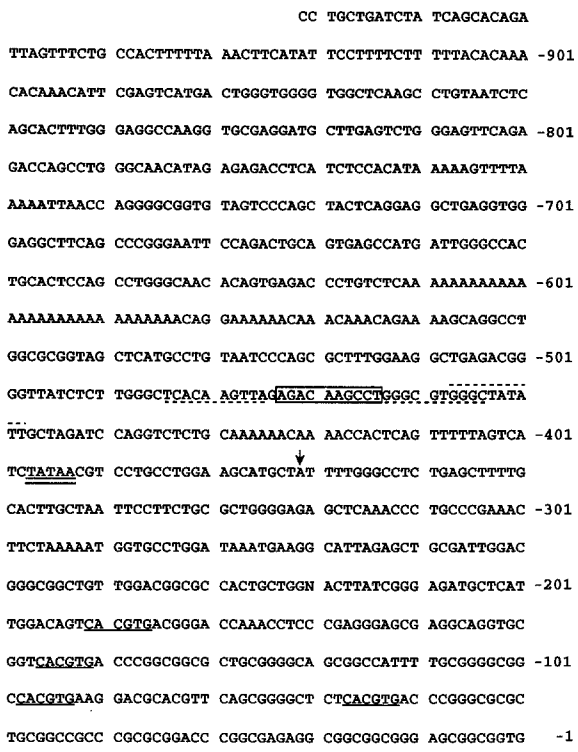

Figure 1. Nucleotide Sequence of the Human *bax* Gene Promoter and 5′UTR

The nucleotide sequence of the *bax* gene corresponding to the first 972 bp upstream of the translation initiation site is shown. The approximate transcription start site is indicated by an arrow. The perfect (10 of 10 matches) p53-binding site consensus sequence is boxed, and the three imperfect p53-binding sites are either underlined or overlined with dashes. The TATAA box is double underlined. The CACGTG motifs are single underlined.

lated region (5′UTR) of the *bcl-2* gene (Miyashita et al., 1994b).

To investigate the mechanisms by which p53 regulates *bax* gene expression, we molecularly cloned and determined the partial DNA sequence of the human *bax* gene. Functional analysis of the *bax* gene promoter indicates that this apoptosis-inducing gene is a direct target of p53. These findings thus provide the first example of a pro-apoptotic gene that is directly regulated by p53.

Results

The Human *bax* Gene Promoter Contains Potential p53-Binding Sites

The human *bax* gene was cloned and the DNA sequence was determined for the first ~1000 bp upstream of the translation intitiation site, corresponding to the presumptive promoter and 5′UTR of the *bax* gene (Figure 1). A TATAA box is located 398 bp upstream from the open reading frame in the human gene. A transcription start site was mapped by primer extension to a position ~22 bp downstream of the TATAA homology element. Within the 5′UTR of the *bax* gene are four CACGTG motifs that represent potential binding sites for several transcription factors, including Myc and its homologs Max, Mad, and Mxi-1, as well as upstream stimulatory factor and related transcription factors (Blackwood and Eisenman, 1991; Zervos et al., 1993; Ayer et al., 1993; Pognonec et al., 1992).

Located ~70 bp 5′ of the TATAA box, at positions −486 bp to −448 bp in the *bax* gene, are one perfect and three imperfect consensus sites for p53 binding (Figure 1). In the *bax* gene promoter, the perfect 10-bp consensus site (−474 bp to −465 bp) is flanked on the 5′ side by a sequence that shares 7 of 10 matches with the consensus sequence, including a stretch of 7 of 7 matches in the core of this motif. This second potential p53-binding site is separated from the perfect site by a single nucleotide. On the 3′ side, separated from the perfect p53-binding site by 0 bp or 6 bp, respectively, are two additional imperfect 10-bp motifs that have either 7 of 10 or 8 of 10 matches with the consensus sequence.

The *bax* Gene Promoter Is p53 Responsive

To explore the functional significance of the potential p53-binding sites identified in the *bax* gene promoter, reporter gene plasmids were constructed that contained the region of *bax* from −687 bp to −318 bp, which includes the TATAA box and transcription start site as well as the upstream p53 consensus sites. This DNA segment was subcloned upstream of chloramphenicol acetyltransferase (CAT) in a promoterless plasmid. Cotransfection assays were then performed in which the *bax*–CAT plasmid was introduced into p53-deficient human tumor cell lines with expression plasmids encoding wild-type p53, a mutant inactive form of p53 (His-179→Glu), or no p53 protein.

In H358 human lung cancer cells, cotransfection with plasmid DNA encoding wild-type p53 protein resulted in strong transactivation of the *bax* gene promoter, with a ~60-fold increase in CAT activity compared with cells cotransfected with plasmid encoding the mutant p53(179) or no p53 protein (Figure 2A). Similarly, wild-type but not mutant p53 strongly transactivated the *bax*–CAT plasmid by ~30-fold in the p53-deficient osteosarcoma line Saos-2 (Figure 2B). Transaction by wild-type p53 of the *bax*–CAT plasmid was also seen in the human cervical cancer line HeLa, which has reduced p53 activity in part owing to the presence of E6 protein from human papilloma virus, as well as in a p53-deficient human prostate cancer line, Tsu-pr1 (Figure 2B). Taken together, these data indicate that p53 can be a strong transactivator of the *bax* promoter in a variety of types of human tumor lines.

The Potential p53-Binding Sites in *bax* (−486 bp to −448 bp) Are Sufficient for Transactivation

To begin to delineate the minimal region within the *bax* gene responsible for transactivation by p53, a 94-bp DdeI–DdeI fragment from the *bax* gene promoter (−508 bp to −415 bp) that contains the four potential p53-binding sites was subcloned upstream of a minimal promoter in the plasmid pA10–CAT. In addition, a mutant version of this region from the *bax* promoter, with point mutations in 3 of the 4 potential p53-binding sites, was created by site-directed mutagenesis. When cotransfected into H358 cells with wild-type p53, CAT constructs containing this 94-bp frag-

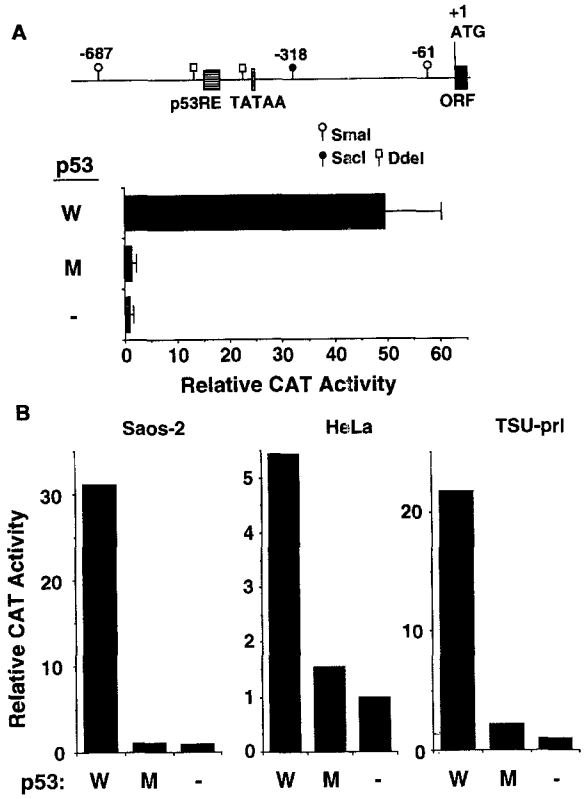

Figure 2. The *bax* Gene Promoter Is p53 Responsive

A SmaI–SacI restriction fragment containing −687 bp to −318 bp of the human *bax* gene was subcloned upstream of CAT in the promoterless plasmid pUCSV0CAT and was cotransfected into various p53-deficient tumor cells with pCMV–β-gal and either pCMV–$p53_{wt}$ (W), pCMV–$p53_{179}$ (M), or pRc/CMV (minus sign) plasmid DNAs. CAT activity was measured 2 days later and was normalized relative to β-gal. Previous studies showed that p53 does not influence the activity of the pCMV–β-gal plasmid (Miyashita et al., 1994b). In (A), H358 lung carcinoma cells were employed for reporter gene assays (mean standard deviation (mean ± SD, n = 3). The *bax* gene promoter region tested is shown schematically at the top. In (B), Saos-2 osteosarcoma, HeLa cervical cancer, and TSU-prl prostate cancer cells were employed for reporter gene assays. The data are representative of 2 of 2 experiments.

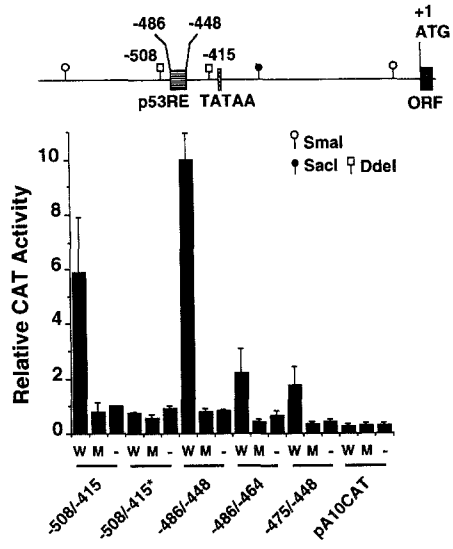

Figure 3. The −486 bp to −448 bp Region of the *bax* Gene Promoter Is Sufficient for Conferring p53-Positive Responsiveness on a Heterologous Promoter

Various fragments of the *bax* gene promoter were subcloned upstream of a minimal promoter in the plasmid pA10CATBS and were cotransfected into H358 cells with plasmids that encode wild-type p53 (W), mutant p53 (M), or no p53 (minus sign) protein. CAT activity was measured 2 days later and was normalized relative to β-gal (mean ± SD, n = 3). The region of the *bax* promoter analyzed is shown schematically at the top. The asterisk indicates a mutant version of the protein with alterations in potential p53-binding sites (see Experimental Procedures).

ment were transactivated by ~6- to 8-fold compared with cells that received plasmids encoding mutant p53(179) or no p53 protein. In contrast, CAT reporter gene plasmids containing mutations in the potential p53-binding sites were unresponsive to p53 (Figure 3). Thus, the −508 bp to −415 bp segment of the *bax* gene promoter is sufficient to confer p53-dependent regulation on a heterologous promoter.

The four potential p53-binding sites in the *bax* gene promoter reside within a 39-bp region (−486 bp to −448 bp). Therefore, we subcloned oligonucleotides comprising this sequence upstream of a minimal promoter in pA10–CAT. In cotransfection experiments, wild-type p53 strongly transactivated (~10- to 15-fold) CAT constructs containing this 39-bp sequence. In contrast, cotransfection of this CAT reporter gene construct with plasmids expressing the inactive p53(179) protein or no p53 protein resulted in little CAT production. Thus, this 39-bp element from the *bax* gene is sufficient for p53-mediated transactivation. Reporter gene constructs that contained only portions of this 39-bp element were far less effective with regards to p53-mediated transactivation.

p53 Binds to the *bax* Gene p53 Response Element In Vitro

Gel retardation assays were performed to determine whether the p53 protein can potentially bind to oligonucleotides containing the 39-bp (-486 bp to -448 bp) sequence corresponding to the p53-response element in *bax*. For these experiments, wild-type p53 and mutant p53(179) proteins were produced in Sf9 cells using recombinant baculoviruses, and extracts from these cells were incubated with ^{32}P-labeled DNA probes containing either the 39-bp *bax* gene sequence or a mutant version containing nucleotide substitutions in 3 of the 4 p53-binding site motifs. Antibodies directed against p53 were also included in some samples to help stablize the in vitro interaction of p53 with target DNAs (Hupp et al., 1992). As shown in Figure 4, proteins present in the Sf9 cells used to produce p53 proteins bound nonspecifically to both the wild-type and mutant DNA probes, irrespective of the presence or absence of p53 proteins (lanes 2–9). However, when the wild-type p53 protein was incubated with wild-type DNA probe, a complex with shifted gel mobility was detected (Figure 4, lane 4). Moreover, when the combination of wild-type p53 protein, wild-type DNA probe, and anti-p53 anti-

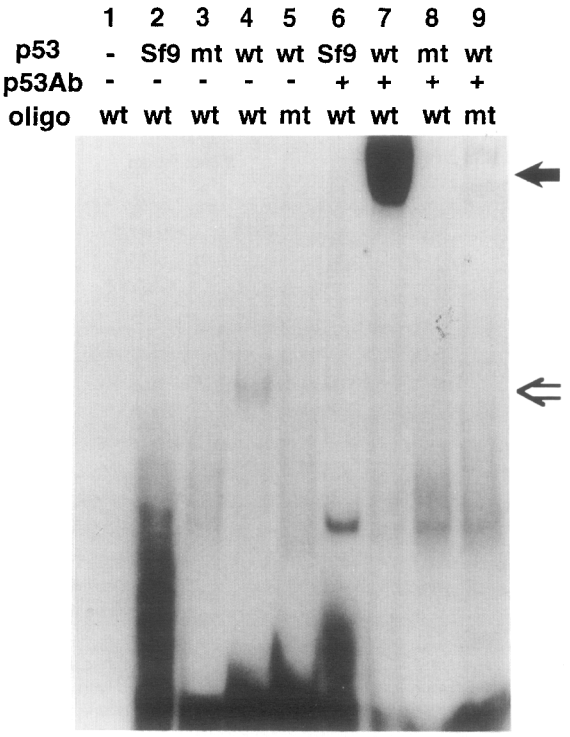

Figure 4. p53 Can Bind In Vitro to an Oligonucleotide Probe Representing the *bax* Gene Region

Lysates prepared from uninfected Sf9 cells or cells infected with recombinant baculoviruses encoding wild-type p53 (wt, top line) or mutant 179 p53 (mt, top line) were incubated with ^{32}P-labeled oligonucleotide (oligo, bottom line) DNA probes containing the −486 bp to −448 bp region of *bax* (wt, bottom line) or a mutant version with four nucleotide substitutions (mt, bottom line), together with (plus sign) or without (minus sign) monoclonal antibodies (Abs) against p53 as indicated. DNA–protein complexes were size fractionated in nondenaturing polyacrylamide gels and were detected by autoradiography. The open and closed arrows indicate the presence of the shifted and supershifted complexes, respectively. In lane 1, the DNA probe was subjected to electrophoresis directly without prior incubation with cell lysates or antibodies.

body was employed, the band detected in lane 4 of Figure 4 was replaced by a supershifted band (lane 7). In contrast, substitution of mutant p53 for wild-type p53 protein, mutant DNA probe for the wild-type sequence, or different monoclonal antibodies for the anti-p53 antibodies resulted in a failure to detect mobility-shifted complexes in these gel retardation assays (Figure 4, lanes 8–9; data not shown).

Discussion

p53 protein suppresses tumor formation in vivo at least in part through its ability to induce cell death (Symonds et al., 1994). In addition, loss of p53 appears to play a significant role in the treatment of cancer, based on clinical correlative studies (Thor et al., 1992; Sun et al., 1992; Visakorpi et al., 1992). Presumably, the reason for the prognostic significance of p53 loss can be explained in part by the following observations: nearly all cytotoxic drugs commonly used in the treatment of cancer, as well as radiation, have in common the ability to induce tumor cell death through apoptosis (reviewed by Reed, 1994); and the relative sensitivity of tumor cells to induction of apoptosis by drugs and radiation is modulated by p53 in many cases (reviewed by Fisher, 1994). Until now, the mechanisms by which p53 loss renders tumor cells more resistant to induction of apoptosis by anticancer drugs and radiation have remained obscure. The data presented here, however, suggest that one contributor may be the loss of p53-induced elevations in *bax* gene expression. In this regard, it was reported recently that p53-mediated induction of new gene expression is not obligatory for triggering apoptosis in response to UV radiation in a pituitary tumor cell line inasmuch as actinomycin D and cycloheximide did not impair the process (Caellas et al., 1994). When examined within the context of our findings on p53-mediated regulation of *bcl-2* and *bax* gene expression (Miyashita et al., 1994a, 1994b; Selvakumaran et al., 1994), this recent report suggests that p53-stimulated elevations in *bax* gene expression are not required for apoptosis, but it does not exclude a potentially important role for p53-induced reductions in *bcl-2* expression. However, the extent to which p53-mediated transactivation of gene expression is not required for p53-dependent apoptotic responses may be cell-type specific. For example, we have shown that most types of neuronal cells contain what appear to be relatively high levels of Bax protein (Miyashita et al., 1994a; Krajewski et al., 1994). It could therefore be that sufficient levels of Bax protein are already present in some types of cells, such as the pituitary tumor cell line employed recently to explore the issue of requirements for new gene expression for p53-induced apoptosis (Caelles et al., 1994).

Regardless of whether there exists an obligatory requirement for stimulation of *bax* gene expression in all types of cells in which p53 has been shown to induce apoptosis, it seems reasonable to propose that p53-mediated elevations in Bax protein levels would at least render cells more susceptible to apoptotic cell death. The relative ratio of Bax and Bcl-2 proteins has been hypothesized to be a major determinant of cellular vulnerability to apoptosis (Oltvai et al., 1993). Based on studies with *bcl-2* knockout mice and experiments using antisense approaches to reduce *bcl-2* expression, it appears that lowering the ratio of Bcl-2 to Bax may often be insufficient, by itself, to trigger apoptosis, but it does render cells relatively more sensitive to induction of cell death by various apoptotic stimuli, including γ radiation and cytotoxic anticancer drugs (Nakayama et al., 1993; Veis et al., 1993; Kitada et al., 1993, 1994). Likewise, p53 probably does not "induce" apoptosis per se but rather adjusts the relative sensitivity of cells so that apoptosis can be triggered more easily in response to stimuli that activate the endogenous cell death pathway or when cells are confronted with clashes in their signals for cell cycle control (Wu and Levine, 1994; Fisher, 1994). Thus, as pertains to anticancer drugs and radiation, for example, elevations in p53 activity have been shown to shift the dose-response curve to the left, allowing for induction of tumor cell death by relatively lower concentrations of drugs or lower doses of radiation. In those cases for which the mere reintroduction of functional p53 into a

p53-deficient cell was shown to cause cell death and no additional stimulus (such as radiation or exposure to a DNA-damaging drug) was required, it should be borne in mind that some types of neoplastic cells may have high rates of spontaneous DNA damage owing to crippled DNA repair machinery, generation of DNA-damaging reactive oxygen species due to altered cellular metabolism, or other causes. Therefore, taken together, the data presented here and elsewhere are consistent with a model in which the induction of elevations in p53 activity by DNA-damaging agents or other means can lower the resistance of cells to apoptotic stimuli through p53-mediated effects on *bcl-2* and *bax* gene expression. These p53-induced changes in *bcl-2* and *bax* gene expression would alter the ratio of Bcl-2 and Bax proteins, placing the cells into a state of enhanced susceptibilty to apoptosis.

Though the *bax* gene promoter clearly has the potential to respond to p53, the findings presented here do not exclude the possibility that other *cis*-acting elements within the *bax* gene can mediate p53-independent transactivation of this gene or can modulate the influence of p53 on it. In this regard, our previous analysis of tissues from p53 knockout mice using immunoblotting and immunohistochemical techniques demonstrated that Bax protein levels are markedly reduced in some tissues such as prostate epithelium, central and peripheral neurons, and small intestine, but are not noticably perturbed in others (Miyashita et al., 1994a). Thus, tissue-specific factors appear to influence the extent to which p53 is required for basal expression of *bax* in vivo. It may be of relevance in this regard that four CACGTG motifs are located within the 5′UTR of the *bax* gene. This hexameric DNA sequence has been shown to represent a potential binding site for several transcription factors, including Myc, its homologs (Blackwood and Eisenman, 1991; Zervos et al., 1993; Ayer et al., 1993), and upstream stimulatory factor, a ubiquitously expressed transcription factor the activity of which is controlled by redox mechanisms (Pognonec et al., 1992). Given that Myc has been reported to induce apoptosis under some circumstances (Asken et al., 1991; Evan et al., 1992; Bissonnette et al., 1992), it will be important in the future to determine whether Myc is an additional transactivator of *bax* and whether this accounts for the ability of Myc to induce apoptosis.

Experimental Procedures

Library Screening

A human placental DNA library in the cosmid vector pWE15 (Stratagene) was screened by using a ^{32}P-labeled mouse *bax* cDNA (Miyashita et al., 1994a). One of the resulting cosmid clones, pTM597-2, was digested with BamHI, producing a ~4000-bp fragment (containing the *bax* gene promoter) that was subcloned into pBluescript SKII (Stratagene). This generated the plasmid pTM-604-4, which was sequenced.

CAT Reporter Gene Plasmids

A 371-bp SmaI–SacI fragment (*bax*, −318 bp to −687 bp) from pTM604-4 was subcloned into the HindIII site of the promoterless CAT plasmid, pUCSV0CAT, by blunt-end ligation, which produced the plasmid pTM667-3. A 94-bp DdeI–DdeI fragment (*bax*, −415 bp to −508 bp) from pTM604-4 was subcloned by blunt-end ligation into the BglII site of the minimal promoter CAT reporter plasmid pA10CATBS, which contains an SV-40 early-region promoter in which the enhancer was deleted to produce the plasmid pTM672-6 (Spalholz et al., 1987).

Oligonucleotides 5′-GATCTCACAAGTTAGAGACAAGCCTG-3′(oligomer A) and 5′-TCGACAGGCTTGTCTCTAACTTGTGA-3′(oligomer B), corresponding to *bax* sequences from −464 bp to −486 bp, were annealed to produce a double-stranded DNA fragment that was subcloned into the BglII and SalI sites of pA10CATBS, producing pTM672-14. The plasmid pTM672-18 was similarly produced by annealing the oligomers 5′-GATCGAGACCAAGCCTGGGCGTGGGCTATATTG-3′ (oligomer C) and 5′-TCGACAATATAGCCCACGCCCAGGCTTGTCTC-3′ (oligomer D), corresponding to *bax* from −448 bp to −475 bp. To create a reporter gene plasmid containing nucleotides −448 bp to −486 bp of *bax*, the oligomers A and D were annealed, filled in with Klenow fragment in the presence of dNTPs, and treated with T4 DNA kinase and ATP, and then the resulting fragment was subcloned into the BglII site of pA10CATBS by blunt-end ligation.

A mutant version of the *bax*–CAT plasmid containing the 94-bp DdeI–DdeI fragment, with four nucleotide substitutions within the consensus p53-binding sites, was created by site-directed mutagenesis using a PCR overlap extension technique (Ho et al., 1989). The following amplification primers were used with Pfu heat-stable DNA polymerase (Stratagene) and pTM604-4 as a template: 5′-GAAGATCTGAGACGGGGTTATCTCTT-3′ (BglII site underlined); 5′-CGCGTCGACTGAGTGGTTTTGTTTTTT-3′ (SalI site underlined); 5′-AAGTTAGAGA**T**A**T**G**C**TGGGCG**T**AGG-3′; and 5′-CC**T**ACGCCCAG**CATTA**TCTCTAACTT-3′ (mutations in bold). The final PCR product was gel purified, digested with BglII and SalI, and subcloned into the BglII and SalI sites of pA10CATBS to produce the plasmid pTM688-2. Introduction of the expected mutations was confirmed by DNA sequencing.

Transfections and Reporter Gene Assays

Cells at ~70% confluence in 6-well (~35 mm) plates were transfected using 30 μg of lipofectin (GIBCO BRL), 3 μg of CAT reporter gene plasmid, 1 μg of pCMV–β-gal plasmid, and 3 μg of either pCMV–$p53_{wt}$, pCMV–$p53_{179}$, or pRc/CMV plasmid DNA for 16 hr, essentially as described previously (Miyashita et al., 1994b). After 48 hr, transfected cells were resuspended in 50 μl of 0.25 M Tris (pH 7.8), subjected to three freeze-thaw cycles, and centrifuged at 16,000 × g for 5 min to obtain supernatants for measurements of CAT and β-gal activity as described (Miyashita et al., 1994b).

Production of p53 Proteins Using Recombinant Baculoviruses

The plasmids pCMV–$p53_{wt}$ and pCMV–$p53_{179}$ were cut with HindIII, the ends were filled in using Klenow fragment, and then they were further cut with XbaI, thus liberating the p53-coding sequences, which were subcloned into the baculovirus transfer vector pVL1393 Pharmingen that had been digested with SmaI and XbaI. Sf9 cells were then cotransfected with recombinant baculovirus transfer vector DNAs and BaculoGold viral DNA, as recommended by the manufacturer, thus producing recombinant baculoviruses. For p53 production, Sf9 cells were infected by a high-titer stock solution of recombinant baculoviruses at a multiplicity of infection of 10. Cells were harvested after ~3 days and lysed in 1% NP-40, 150 mM NaCl, 5 mM EDTA, 10% glycerol, and 50 mM Tris (pH 8.0), containing protease inhibitors. The clarified cell lysates were dialyzed against electrophoretic mobility shift assay (EMSA) buffer.

EMSA

Oligomers A and D were annealed and filled in using Klenow fragment in the presence of [α-^{32}P]dCTP. A similar DNA probe was prepared that contained four nucleotide substitutions using the oligomers 5′-GATCTCACAATTTAGAGATAATGCTG-3′ and 5′-TCGACAATATAGCCTACGCCCAGCATTATCTC-3′. Sf9 cell lysates (2 μg of total protein) containing wild-type p53, p53(179), or no p53 protein were preincubated in some cases with monoclonal anti-p53 antibodies (a combination of 0.5 μg of DO-1 [Santa Cruz Biotechnology] and 0.5 μg of Pab421 [Oncogene Science]), and then they were incubated for 10 min at ~25°C with 0.5 μg of sonicated salmon sperm DNA and 7 μl of EMSA buffer (20 mM HEPES [pH 7.5], 0.1 M NaCl, 1.5 mM $MgCl_2$, 10 mM dithiothreitol, 20% glycerol, 0.1% Triton X-100, 1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin). The ^{32}P-labeled double-strand oligomers (4 × 10^5 cpm) were then added. After 20 min at ~25°C, the samples were subjected to electrophoresis in native 4% polyacryl-

amide gels using 1 x TBE. The gels were then dried and exposed to X-ray film (XAR; Kodak) at −80°C with intensifying screens.

Acknowledgments

Correspondence should be adressed to J. C. R. This work was supported in part by grants from the American Cancer Society (DHP32C) and the National Institutes of Health (CA60181). J. C. R. is a Scholar of the Leukemia Society of America.

Received September 30, 1994; revised November 3, 1994.

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