The Tumor Suppressor Protein p53 Requires a Cofactor to Activate Transcriptionally the Human BAX Promoter*

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An important regulator of the proapoptotic BAX is the tumor suppressor protein p53. Unlike the p21 gene, in which p53-dependent transcriptional activation is mediated by a response element containing two consensus p53 half-sites, it previously was reported that activation of the BAX element by p53 requires additional sequences. Here, it is demonstrated that the minimal BAX response element capable of mediating p53-dependent transcriptional activation consists of two p53 half-sites plus an adjacent 6 base pairs (5'-GGGCCT-3'). This GC-rich region constitutes a "GC box" capable both of binding members of the Sp family of transcription factors, including Sp1 in vitro, and of conferring Sp1-dependent transcriptional activation on a minimal promoter in cells. Mutations within this GC box abrogated the ability of p53 to activate transcription without affecting the affinity of p53 for its binding site, demonstrating that these 6 bases are required for p53-dependent activation. In addition, a positive correlation was observed between the ability of p53 to activate transcription in cells and the ability of Sp1 to bind this response element in vitro. Mutations that inhibited Sp1 binding also blocked the ability of p53 to activate transcription through this element. Together, these results suggest a model in which p53 requires the cooperation of Sp1 or a Sp1-like factor to mediate transcriptional activation of the human BAX promoter.

The BCL-2 family of proteins are key mediators of the apoptotic response. One member of this family is the proapoptotic BAX. Preceding apoptosis, cytosolic BAX translocates to the mitochondria and homodimerizes. Homodimeric BAX then is thought to cause the release of cytochrome c (1–3) which subsequently functions as a coactivator of Apaf-1 in the cleavage of pro-caspase-9, initiating programmed cell death (4). BAX exists in equilibrium with two of its homologs, BCL-2 and BCL-XL. Consequently functions as a coactivator of Apaf-1 in the cleavage of pro-caspase-9, initiating programmed cell death (4). BAX exists in equilibrium with two of its homologs, BCL-2 and BCL-XL. Unlike BAX, these two homologs exert antiapoptotic effects by heterodimerizing with BAX in the mitochondria, blocking its ability to release cytochrome c (5, 6). Thus, an important determinant of the apoptotic response of a cell is the balance between the levels of BAX and BCL-2/BCL-XL. In this regard, regulation of the level of expression of BAX protein is key.

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p53-dependent Transactivation of bax

Demonstrate sequence-specific binding to the transcription factor Sp1. Mutational analysis of this “GC box” shows it to be required for p53-dependent activation, and a positive correlation between the ability of p53 to activate transcription in cells and the ability of Sp1 to bind this response element in vitro is observed. These results are consistent with a model in which p53 requires the cooperation of Sp1 or a Sp1-like factor to mediate transcriptional activation of the human BAX gene. This presents the intriguing possibility that regulation of this cofactor may represent a novel basis for the cell type-specific activity of the p53.

Experimental Procedures

Cells—The osteosarcoma Saos-2 cell line was maintained in a humidified tissue culture incubator at 37 °C with 5% CO2. Cells were grown in Dulbecco’s modified Eagle’s medium, containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Drosophila SL2 cells were cultured at 25 °C in Schneider’s Drosophila medium, containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Oligonucleotides—For use in electrophoretic mobility shift assays and for subsequent cloning into luciferase reporter plasmids, complementary single-stranded oligonucleotides were generated with an automated DNA synthesizer and pGEM-3ZF+ plasmids using the appropriate restriction enzymes (SacI and NheI). A 315 to 92 fragment was engineered to contain the NolI restriction site on the upstream side and the SacI restriction site on the downstream side. The –76 to +51 fragment was engineered to contain the SacI site upstream and the HindIII site downstream. Following PCR amplification each fragment was double-digested with the appropriate restriction enzymes (Nhel and NolI). A three-way ligation with the two PCR-generated fragments and pGL3-E1bTATA, double-digested with Nhel and HindIII, then was performed, replacing the BAX sequence from –113 to –104 with the NolI restriction site. The generation of pBAX –103/–93, pBAX –92/–83, and pBAX –113/–104 was accomplished as above with pBAX –113/–104 but using PCR-generated fragments corresponding to –315 to –104 and –92 to +51, respectively. The expression plasmid pCMV-p53-1, originally referred to as pCS5-SN3 (35), encodes the wild-type human p53 protein under the control of the cytomegalovirus promoter. The expression plasmid pRSV1 contains the 2.1-kilobase pair XhoI restriction fragment of p21 cloned downstream of the Actin 5C promoter (36). pPacU was generated by removing the 2.1-kilobase pair XhoI fragment from pRSV1.

Transfections—Saos-2 cells were transfected using LipofectAMINE Plus Reagent (Life Technologies, Inc.). 2 × 106 cells were seeded into 35-mm plates. Cells were transfected 24 h later according to the manufacturer’s instructions. Cellular lysates were prepared 24 h post-transfection, and total protein concentration was determined by protein assay (Bio-Rad), and luciferase assays were quantitated using a luminometer (Microlight, Labvision). Drosophila SL2 cells were transfected using Cellfectin (Life Technologies, Inc.). 60-mm dishes were seeded with 2 × 106 cells in Schneider’s Drosophila medium containing 10% heat-inactivated fetal bovine serum but no penicillin or streptomycin. The DNA to be transduced was added to 500 μl of serum-free media containing 8 μl of Cellfectin reagent, mixed gently, and incubated at room temperature for 20 min. This mixture was added directly to the cells. 48 h post-transfection cells were lysed by sonication (6 × 20 s pulse). Total protein and luciferase activity was determined as above.

HeLa Cell Nuclear Extraction—Unless otherwise stated, all procedures were conducted at 4 °C. HeLa S3 cells were obtained as a packed cell pellet from the National Cell Culture Center (Minneapolis, MN). Cell pellets were resuspended in 5 volumes of Buffer A (10 mM HEPES, pH 7.6, 1.5 mM MgCl2, 0.5 mM DTT) and incubated on ice for 10 min. Cell pellets were then centrifuged at 500 × g for 12 min. The supernatant was removed, and the pellet was resuspended in two packed cell volumes of Buffer A. Cells were homogenized 10 times in a Dounce homogenizer with pestle A (tight). The resulting solution was centrifuged at 430 × g for 10 min to pellet the nuclei. The supernatant was decanted, and the pellet was recentrifuged at 24,000 × g for 20 min. The supernatant again was removed. The pellet was resuspended in 3 ml of Buffer C (20 mM HEPES, pH 7.6, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT) per 106 cells. The solution was homogenized 10 times with pestle B (loose). The resulting solution was transferred to a beaker and stirred for 30 min on ice. The solution then was centrifuged at 430 × g for 10 min. The supernatant was removed, and the extract was dialyzed against Buffer D (20 mM HEPES, pH 7.6, 20% glycerol, 0.1 mM KCl, 0.2 mM EDTA, 1.5 mM MgCl2, 0.5 mM DTT) for 5 h. The extract was clarified by centrifugation at 24,000 × g for 20 min. Nuclear extracts were aliquoted, frozen in a dry ice/ethanol bath, and stored at –70 °C.

Electrophoretic Mobility Shift Assay—Production of baculovirus-infected Sf9 cell extracts and purification of recombinant human p53 protein were done as described previously (34). Purified p53 protein, 1

The abbreviations used are: PCR, polymerase chain reaction; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assays.

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under “Experimental Procedures” with 1 µg of the indicated pBAX reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53+ (gray bars). 24 h post-transfection cells were lysed and assayed for total protein and luciferase activity as described under “Experimental Procedures.” The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to one S.D. The numbers above each bar indicate the fold activation for each reporter construct observed with pCMV-p53wt+ as compared with pCMV. A, the previously identified p53 response element is indicated by the dark gray box at −113 to −83. B, the three potential p53 half-sites are represented by the light gray (−113 to −104), white (−102 to −93), and dark gray (−92 to −83) boxes.

FIG. 1. All three potential p53 half-sites are required for the p53-dependent transcriptional activation of the human BAX promoter. A and B, Saos-2 cells were transfected as described under “Experimental Procedures” with 1 µg of the indicated pBAX reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53+ (gray bars). 24 h post-transfection cells were lysed and assayed for total protein and luciferase activity as described under “Experimental Procedures.” The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to one S.D. The numbers above each bar indicate the fold activation for each reporter construct observed with pCMV-p53wt+ as compared with pCMV. A, the previously identified p53 response element is indicated by the dark gray box at −113 to −83. B, the three potential p53 half-sites are represented by the light gray (−113 to −104), white (−102 to −93), and dark gray (−92 to −83) boxes.

RESULTS

All Three Potential p53 Half-sites Are Required for the p53-dependent Transcriptional Activation of the Human BAX Promoter—Previously it was demonstrated that in isolation the p53 response element from the human BAX promoter required sequences from three adjacent half-sites to confer p53-dependent transcriptional activation on a minimal promoter (37). To confirm the requirement of all three half-sites in the context of the BAX promoter, luciferase reporter plasmids were constructed with various deletions in the BAX promoter, both in and around the p53 response element, were cotransfected with either pCMV or a wild-type p53 expression vector into the p53-negative osteosarcoma Saos-2 cell line (Fig. 1). The previously characterized p53 response element of the BAX promoter is contained within the sequence from −113 to −83 from the start site of transcription.

There was no significant difference between the p53-dependent transactivation of either a reporter construct lacking sequences 5’ to the p53 response element (pBAX −127/+51) or the full-length promoter construct (pBAX −315/+51)(Fig. 1A). Deletion of a larger fragment, including the p53 response element (pBAX −76/+51), produced a reporter construct that was unresponsive to wild-type p53 (Fig. 1A). Furthermore, targeted deletion of the promoter region containing the p53 response element (pBAXΔ−126/−77) also produced a reporter plasmid that was unresponsive to wild-type p53 (Fig. 1A). These results show that −113 to −83 is the only region, within the 366-base pair promoter fragment investigated, that affects the ability of p53 to activate transcription.

The region from −113 to −83 contains three potential p53 half-sites (represented in Fig. 1B as the light gray, white, and dark gray boxes). The role of each of these half-sites in the p53-dependent activation of the BAX promoter was examined. Removal of the first half-site from −113 to −104 (pBAXΔ−113/−104) significantly reduced the ability of p53 to activate transcription through this promoter (Fig. 1B, compare 63-fold with pBAX −315/+51 to 7-fold with pBAXΔ−113/−104), whereas removal of the second (pBAXΔ−103/−93) or the third half-site (pBAXΔ−92/−83) completely abolished the ability of p53 to activate transcriptionally the promoter (Fig. 1B). Consistent with the above results, removal of the first and second half-sites in combination (pBAXΔ−113/−93) also abolished the ability of p53 to activate transcriptionally the promoter (Fig. 1B).
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83. Analysis of this region using a MatInspector search of the

These results demonstrate that, as was observed with the
isolated response element (37), p53 requires sequences from all
three potential half-sites to mediate transcriptional activation of
the BAX promoter.

The First Two Potential p53 Half-sites Constitute a Bona
Fide p53 Response Element—Each of the three potential p53
half-sites located in the BAX promoter from −113 to −83 closely
resembles the consensus sequence of 5’-RRRCWW-
GYY-3’ (represented in Fig. 2 by the light gray, white, and
dark gray boxes). The first, located at −113 to −104, deviates
from the consensus at 2 bases (−113 and −104). The second
half-site matches the consensus sequence at all 20 base pairs
and is located at −102 to −93. The third half-site is located at
−92 to −83 and deviates from the consensus at three bases
(−84, −85, and −88)(see Fig. 2). These three half-sites can
combine in different ways to produce a total of three possible
p53 complete binding sites (half-sites 1 and 2, 2 and 3, and 1
and 3). Previous studies demonstrated that in electrophoretic
mobility shift assays (EMSA), double-stranded oligonucleotides
representing both −113 to −93 (half-sites 1 and 2) and −102 to
−83 (half-sites 2 and 3) are capable of binding p53 in a
sequence-specific manner with similar affinities (37). When
cloned upstream of the adenovirus E1b 16 minimal promoter in
the pTATA luciferase reporter plasmid, however, the combination
of the first and second half-sites (−113 to −93) is unable to
mediate p53-dependent transcriptional activation (37). To ex-
amine further the ability of p53 to interact with this sequence in
cells, the −113 to −93 sequence was multimerized (as three
copies) and cloned into the pTATA luciferase reporter plasmid.
This reporter plasmid was cotransfected with either pCMV or a
wild-type p53 expression vector in the Saos-2 cell line (Fig. 2).
These three copies of this p53-binding site were capable of
mediating a significant degree of activation in response to p53
(Fig. 2, compare 4-fold with pTATA-113/−93 to 142-fold with
pTATA−113/−93)), demonstrating that the sequence from
−113 to −93 is indeed a bona fide p53 response element capa-
bile of both binding p53 in a sequence-specific manner in vitro
and mediating p53-dependent transcriptional activation in
cells. Confirming previous results, p53 was able to activate
transcription through the second and third half-sites (−102 to
−83), but this activation was significantly reduced as compared
with that mediated by all three half-sites combined (Fig. 2,
compare 44-fold with pTATA-102/−83 and 153-fold with
pTATA-113/−83). To test the ability of half-sites one and three
to mediate p53-dependent transcriptional activation, a syn-
thetic oligonucleotide corresponding to −113 to −83 of the BAX
promoter, with −102 to −93 scrambled to remove any contri-
bution of the second half-site, was cloned into the pTATA
reporter plasmid. This construct failed to be activated by p53
(Fig. 2, pTATA-113/−83(sc-102/-93)). The third half-site in
isolation (−92 to −83) also failed to mediate p53-dependent
transcriptional activation (Fig. 2, pTATA-92/−83).

Sp1 Binds with Sequence Specificity to and Activates Tran-
scription through the p53 Response Element from the Human
BAX Promoter—We previously reported the identification of a
nuclear factor, termed Binder of BAX 1 (BoB1), that interacts
with sequence specificity with the same region of the human
BAX promoter that is required for p53-dependent transcrip-
tional activation (37). These previous studies demonstrated
that this factor binds to sequences within the region of −102
to −83. Analysis of this region using a MatInspector search of the
TRANSFAC data base (38, 39) showed that it contains a se-
quence that potentially could bind the transcription factor Sp1.
To test this, a synthetic oligonucleotide corresponding to −102
to −83 of the BAX promoter was used as a radiolabeled probe
in an EMSA with HeLa cell nuclear extract (Fig. 3). As reported
previously for Saos-2 (37), HeLa cell nuclear extract contains a
factor that demonstrated marked sequence specificity for the
labeled BAX probe. This factor was successfully competed by
increasing amounts of unlabeled probe (Fig. 3, lanes 7–9) as
well as by increasing amounts of oligonucleotide corresponding
to the DNA-binding consensus sequence of Sp1 (Fig. 3, lanes
13–15). This binding was specific, as an oligonucleotide corre-
sponding to the 5’ p53 response element from the human p21
promoter failed to compete for binding (Fig. 3, lanes 10–12). In
addition, this factor was successfully bound by an anti-Sp1
antibody, as demonstrated by a “supershifted” complex (Fig. 3,
lanes 2 and 3), whereas a control anti-p300 antibody failed to
bind the factor (Fig. 3, lanes 4 and 5). Together, these data demon-
Fig. 3. Sp1 binds with sequence specificity to the p53 response element from the human BAX promoter. An electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the −102/−83 sequence from the human BAX promoter as radiolabeled probe. 8 μg of HeLa cell nuclear extract was incubated with 3 ng of the probe alone (lanes 1 and 6), in the presence of 2 or 4 μl of anti-Sp1 antibody (Ab) (lanes 2 and 3, respectively), 2 or 4 μl of anti-p300 antibody (lanes 4 and 5, respectively), a 100-, 200-, or 300-fold molar excess of either the unlabeled BAX −102/−83 oligonucleotide (lanes 7–9) or p21 5′-oligonucleotide (lanes 10–12), or a 10-, 20-, or 30-fold molar excess of the unlabeled Sp1 consensus oligonucleotide (lanes 13–15). The arrows indicate the positions of the Sp1-DNA complex and the supershifted complex containing antibody, Sp1, and DNA.

To delineate further the sequences important for Sp1 binding, oligonucleotides were synthesized that replaced portions of the BAX sequence with corresponding sequence from the p21 5′ p53 response element. The sequence from −102 to −83 in the BAX promoter contains two p53 half-sites (−102 to −93 and −92 to −83), and the p21 5′ element also consists of two p53 half-sites. Hybrid oligonucleotides were synthesized in which the first of the two half-sites in the BAX element was combined with the second half-site of the p21 5′ element and vice versa. The oligonucleotide corresponding to −102 to −83 of the BAX promoter again was used as a radiolabeled probe with HeLa nuclear extract in an EMSA (Fig. 4). Competitions, using unlabeled probe as well as the oligonucleotides corresponding to the p21 5′ element and the two hybrid elements, were conducted. Sp1 bound the radiolabeled probe (Fig. 4, lane 1) and was recognized by an anti-Sp1 antibody (Fig. 4, lane 2) but not by a control anti-CBP antibody (Fig. 4, lane 3). Both unlabeled probe and the Sp1 DNA-binding consensus site oligonucleotide effectively competed for Sp1 binding (Fig. 4, lanes 4–5 and 12–13, respectively), whereas the p21 5′ element did not (Fig. 4, lanes 10–11). Consistent with the notion that Sp1 binds DNA through GC box regions, the hybrid oligonucleotide in which the first half-site is derived from the p21 sequence and the second half-site from the BAX sequence (−92 to −83, 5′-GGGCCGTGGGG-3′) effectively competed for Sp1 binding (Fig. 4, lanes 8–9), whereas the other hybrid oligonucleotide that replaces this GC-rich region with sequence from the p21 5′ element demonstrated a significantly reduced affinity for Sp1 binding (Fig. 4, lanes 6–7). These data indicate that Sp1 binds to sequence within −92 to −83 of the BAX promoter.

To determine whether or not Sp1 can interact with this element in cells, a pTATA luciferase reporter plasmid containing −113 to −77 of the human BAX promoter was cotransfected with increasing amounts of an Sp1 expression vector into the Sp1-deficient Drosophila SL2 cell line (Fig. 5). Expression of Sp1 successfully activated transcription of this reporter and yet failed to activate transcription of a control plasmid containing the 5′ p53 response element of the p21 promoter (Fig. 5). Consistent with the in vitro EMSA results, this confirms that Sp1 is capable of activating transcription through the p53 response element of the human BAX promoter.

The Ability of Sp1 to Bind the p53 Response Element of the BAX Promoter in Vitro Correlates with the Ability of p53 to Activate Transcription through This Element in Cells—To explore the significance of the Sp1-binding site to the ability of p53 to activate transcription through the BAX promoter, nucleotide substitutions were identified that differentially affected the ability of p53 to activate transcription through its response element in the BAX promoter (−113 to −83). Two mutated forms of the p53 response element from the BAX promoter, in which the indicated guanine bases were replaced with adenines (Fig. 6A, GG−92/−91AA and GG−85/−84AA), were cloned into the pTATA luciferase reporter plasmid. In cotransfection assays with a wild-type p53 expression vector in
the Saos-2 cell line, substitution of bases −92 and −91 completely abolished the ability of p53 to activate transcription through this element (Fig. 6A, compare −113/−83 to GG−92/−91AA), whereas substitution of bases −85 and −84 did not inhibit the ability of p53 to mediate transcriptional activation through this element (Fig. 6A, compare −113/−83 and −113/−93), demonstrating the requirement for this Sp1-binding sequence in the p53-dependent transcriptional activation of this element.

Both of these mutant sequences were assayed for their ability to bind purified p53 in an EMSA. An oligonucleotide corresponding to −113 to −77 of the BAX promoter was used as a radiolabeled probe with purified p53 in an EMSA (Fig. 6B). Competitions were performed with increasing amounts of an oligonucleotide corresponding to −113 to −83 of the BAX promoter and the two mutant oligonucleotides. When compared with the wild-type oligonucleotide, both mutant oligonucleotides displayed a slightly decreased affinity for p53 (Fig. 6B, compare lanes 2–4 with lanes 5–7 and 8–10; Fig. 6C). Compared with one another, however, both mutant oligonucleotides demonstrated a comparable affinity for p53 (Fig. 6, A and B), suggesting that the differences in p53-dependent transcriptional activation observed in Fig. 6A are not due to differences in the affinity of p53 for the two sequences. In contrast, the abilities of the two mutant sequences to bind Sp1 differed (Fig. 6, D and E). An oligonucleotide corresponding to −113 to −77 of the BAX promoter was used as radiolabeled probe with extract from SF9 cells expressing recombinant human Sp1 protein in an EMSA (Fig. 6D). Sp1 bound the probe and was recognized by an anti-Sp1 antibody (Fig. 6D, lanes 1–2). Sp1 binding was successfully competed by unlabeled BAX −113/−83 oligonucleotide as well as by the GG−85/−84AA mutated oligonucleotide (Fig. 6D, lanes 3–4 and 9–11, respectively; Fig. 6E). The GG−92/−91AA mutant, however, demonstrated a significant decrease in affinity for Sp1 (Fig. 6D, compare lanes 3–5 and 9–11 to lanes 6–8; Fig. 6E).

The results with the GG−85/−84AA mutant presented in Fig. 6 suggest that not all of the bases contained within the third potential half-site of the p53 response element are required for p53-dependent transcriptional activation. To identity the minimal sequence elements required to mediate p53-dependent transcriptional activation, a series of oligonucleotides was synthesized in which each of the 10 bases of the third potential half-site (−92 to −83) was individually replaced. These mutant oligonucleotides then were cloned into the pTATA luciferase reporter plasmid and tested for their responsiveness to p53 in a cotransfection assay in the Saos-2 cell line (Fig. 7). Consistent with the results in Fig. 6A, substitution of the bases at either −85 or −84 did not inhibit the ability of p53 to activate transcription through this element (Fig. 7, G−85T and G−84T). Furthermore, substitution of −86 and −83 also failed to affect significantly the ability of p53 to activate transcription (Fig. 7, compare −113/−83 to G−86T and C−83A). Substitution of the base at −87, however, significantly reduced the ability of p53 to activate transcription through this element (Fig. 7, compare −113/−83 to T−87G). Together, these results suggest that the minimal response element consists of sequence from −113 to −87, with −86 to −83 being dispensable for p53-dependent transactivation.

To confirm that the bases from −86 to −83 are not required for p53-dependent transcriptional activation, two additional mutant oligonucleotides were synthesized. The first mutant was generated by replacing all 10 nucleotides from −92 to −83 (Fig. 8A, sc−92/−83). The 4 bases from −86 to −83 were substituted as indicated to generate the second mutant oligonucleotide (Fig. 8A, sc−86/−83). Each oligonucleotide was cloned into the pTATA vector and tested for its responsiveness to p53 in a cotransfection assay (Fig. 8A). As observed with the reporter plasmid in which the sequence from −92 to −83 is removed entirely (pTATA−113/−93), the first mutant, in which all 10 bases of the third potential half-site (−92 to −83) are replaced, showed little to no response to p53 (Fig. 8A, compare pTATA−113/−93 to pTATA−113/−83 and pTATAsc−92/−83). In contrast, the second mutant, in which only the last 4 bases of the element (−86 to −83) are replaced, was efficiently activated by p53 (Fig. 8A, compare 312-fold with pTATA−113/−83 to 323-fold with pTATAsc−86/−83). This result demonstrates that the minimal p53 response element in the BAX promoter consists of sequence from −113 to −87. In an EMSA both mutants displayed a decreased affinity for p53 as compared with the wild-type sequence (Fig. 8B, compare lanes 2–4 to lanes 8–10 and 11–13; Fig. 8C). When compared with each other, there was no significant difference in the affinity of p53 for the two mutant sequences (Fig. 8B, compare lanes 8–10 to 11–13; Fig. 8C). This suggests that the differences in transcriptional activation observed in Fig. 8A cannot be explained by differences in p53 affinities. Furthermore, the oligonucleotide corresponding to −113 to −93 displayed a similar affinity for p53 as the two mutant oligonucleotides (Fig. 8B, compare lanes 5–7 to lanes 8–10 and 11–13; Fig. 8C) consistent with the idea that, in the case of the two mutants, p53 is interacting with the first and the second half-sites only. The sc−86/−83 mutant oligonucleotide efficiently competed for Sp1 binding in an EMSA (Fig. 8D, compare lanes 2–4 to lanes 8–10; Fig. 8E), whereas the ability of the sc−92/−83 mutant to bind Sp1 was significantly reduced compared with the wild-type sequence (Fig. 8D, compare lanes 2–4 to lanes 5–7; Fig. 8E), further strengthening the correlation between Sp1 binding in vitro and p53 activation in cells.

**DISCUSSION**

The data presented in this report demonstrate that the minimum p53 response element in the BAX promoter consists of the sequence from −113 to −87 from the start site of transcription. This sequence contains a p53-binding site (−113 to −93) that can function as a *bona fide* response element as demonstrated by its ability when multimerized to confer p53-depend-
ent transcriptional activation on a minimal promoter (Fig. 2).

Immediately adjacent to this p53-binding site are 6 base pairs that are GC-rich in nature (292 to 287: 5'-GGGCGT-3'). These 6 bases are required for p53-dependent transcriptional activation as deletion or mutation of this region in the context of either the promoter or the isolated response element completely abrogates the ability of p53 to activate transcription through this sequence (Figs. 1B, 2, 6A, and 8A). The addition of these bases to the 2113/277 sequence appears to have little effect on the affinity of p53 for this sequence (Fig. 8, B and C), consistent with a model in which these 6 bases function to recruit a co-activator as opposed to simply enhancing p53 binding. Furthermore, these 6 base pairs mediate sequence-specific binding to the Sp1 transcription factor (Figs. 3, 4, 6D, and 8D), and a positive correlation is seen between the ability of Sp1 to bind this element in vitro and the ability of p53 to mediate transcriptional activation through its response element in cells (Figs. 6 and 8). In addition, the results with electrophoretic mobility shift assays with the GG292/291AA mutant oligonucleotide (Fig. 6B) are not consistent with the published p53 DNA-binding consensus sequence of (RRRCWWGYYY)2 (18, 19). This consensus allows for a purine in the first three posi-

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**FIG. 6.** A mutant element that fails to bind Sp1 in vitro also fails to confer p53-dependent transcriptional activation in cells. A, Saos-2 cells were transfected as described under “Experimental Procedures” with 1 μg of the indicated pTATA reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53wt (gray bars). 24 h post-transfection cells were lysed and assayed for total protein and luciferase activity as described under “Experimental Procedures.” The indicated values are the average of five independent experiments each performed in duplicate. Error bars correspond to 1 S.D. The GC-rich region that binds Sp1 is shown by the boxed sequence. Bases in the wild-type sequence that were mutated are shown in gray with the corresponding mutations indicated above. B, an electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the –113/–77 sequence from the human BAX promoter as radiolabeled probe. 50 ng of purified p53 was incubated with 3 ng of the probe alone (lane 1) or in the presence of a 500-, 1000-, or 1500-fold molar excess of either the unlabeled BAX –113/–83 oligonucleotide (lanes 2–4), the GG –92/–91AA oligonucleotide (lanes 5–7), or the GG –85/–84AA oligonucleotide (lanes 8–10). The arrow indicates the position of the p53-DNA complex. D, bands were quantitated by densitometry. C, an electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the –113/–77 sequence from the human BAX promoter as radiolabeled probe. Extract from Sf9 cells expressing human recombinant Sp1 protein was incubated with 3 ng of the probe alone (lane 2), in the presence of anti-Sp1 antibody (lane 1), or in the presence of a 50-, 100-, or 200-fold molar excess of either the unlabeled BAX –113/–83 oligonucleotide (lanes 3–5), the GG –92/–91AA oligonucleotide (lanes 6–8), or the GG –85/–84AA oligonucleotide (lanes 9–11). The arrow indicates the position of the Sp1-DNA complex, and the asterisk indicates the position of the supershifted anti-Sp1-DNA complex. E, bands were quantitated by densitometry.
tions of each half-site. The GG\textsubscript{92}/GG\textsubscript{91}AA mutant contains a conservative substitution of purines (adenines) for purines (guanines) and, as such, does not represent a substantive change in terms of the p53 DNA-binding consensus sequence. This substitution, however, did produce a significant decrease in the ability of p53 to bind to this oligonucleotide in vitro (Fig. 6B), suggesting that, in these limited circumstances, the p53 DNA-binding sequence involves greater specificity than implied by the consensus.

Previous studies have suggested a connection between p53 and Sp1. The two proteins physically interact under certain circumstances (40–42), and, transcriptionally, p53 and Sp1 have been shown to function in a cooperative manner in some settings and an antagonistic manner in others (41, 43, 44). In addition to p53, Sp1 has been found to synergize with other transcription factors, including YY1 and SREBP (45–47). Studies with the Sp family of transcription factors, however, are complicated by the fact that there are at least 16 mammalian members of this family. Due to marked conservation in the DNA-binding domain, many of these family members have similar if not identical in vitro DNA binding characteristics (48, 49). Originally, this led to the misclassification of many GC boxes solely as Sp1-binding sites because of the ubiquitous nature of Sp1 and the fact that it was the first family member cloned. Given this, the possibility exists that the true in vivo cofactor required for the p53-dependent transactivation of the BAX promoter is an Sp1-related family member that is obscured in in vitro assays by the sheer abundance of Sp1 in nuclear extracts from tissue culture cells. Consistent with this, antibodies used in a supershift EMSA identified other Sp family members as minor components of the Sp1-DNA complex.2 Furthermore, cotransfection assays in the Sp1-deficient Drosophila SL2 cell line failed to demonstrate cooperation between Sp1 and p53 in transcriptionally activating the p53 response element of the BAX promoter.2 The Drosophila assays, however, are difficult to interpret as the ability of p53 alone to activate transcription through a control plasmid was significantly impaired in the SL2 cell line. Complicating interpretation of the results in the Drosophila system is the recent identification of a Drosophila p53 homolog (50, 51) that may affect the ability of transiently expressed human p53 to function properly in this system.

Regardless of whether the cofactor required for the p53-dependent transactivation of the BAX promoter is Sp1 or a related family member, the requirement of this cooperating protein suggests a model for the observed cell type- and tumor type-specific regulation of the BAX gene by wild-type p53 (Fig. 9). In this model, cells that are permissive to p53-dependent up-regulation of the BAX gene express both p53 and the cofactor, and these proteins function together to activate transcriptionally the gene. In those cells that fail to show p53-dependent

2 E. C. Thornborrow and J. J. Manfredi, unpublished data.

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**Fig. 7.** Mutational analysis shows that the BAX promoter sequence from -86 to -83 is not required for p53-dependent transcriptional activation. Saos-2 cells were transfected as described under “Experimental Procedures” with 1 \( \mu g \) of the indicated pTATA reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53\textsuperscript{wt} (gray bars). 24 h post-transfection cells were lysed and assayed for total protein and luciferase activity as described under “Experimental Procedures.” The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to 1 S.D. The GC-rich region that binds Sp1 is shown by the boxed sequence. Bases in the wild-type sequence that were mutated are shown in gray with the corresponding mutations indicated above.
BAX expression, one can propose three possible mechanisms to explain the apparent failure of wild-type p53 to activate the BAX gene (Fig. 9). First, the required cofactor may be absent, either due to mutation or due to cell type-specific limitations on its expression. Second, this factor may be inactivated by post-translational modification. Finally, another factor that cannot cooperate with p53 may compete with the cofactor for binding to its site in the BAX promoter. Data with the Sp family of transcription factors support each of these possibilities. Although several of the Sp family members, like Sp1, are ubiquitously expressed, other members of the family display high degrees of tissue specificity (48, 49). Even the ubiquitously expressed family members fluctuate in levels under particular cellular conditions (52–55). Sp1 mRNA, for example, varies up to 100-fold depending on the cell type and developmental stage of the mouse (56). Consistent with a model of post-translational modification, certain Sp family members, including Sp1 and EKLF, are phosphorylated, glycosylated, and acetylated (57–59). Finally, given the high level of conservation in the DNA-binding domain of the Sp family of transcription factors, it is not surprising that DNA binding competition can be observed between various members of this family. In certain cases, including Sp1/Sp3, BTB1/AP-2rep, and BKL/EKLF, this competition has ramifications on gene expression (60–62). In each

**Fig. 8.** The minimal element from the BAX promoter that confers p53-dependent transcriptional activation consists of a single p53-binding site and an adjacent Sp1-binding site. A, Saos-2 cells were transfected as described under “Experimental Procedures” with 1 μg of the indicated pTATA reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53wt (gray bars). 24 h post-transfection cells were lysed and assayed for total protein and luciferase activity as described under “Experimental Procedures.” The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to 1 S.D. The numbers above each bar indicate the fold activation for each reporter construct observed with pCMV-p53wt as compared with pCMV. The GC-rich region that binds Sp1 is shown by the boxed sequence. Bases in the wild-type sequence that were mutated are shown in gray with the corresponding mutations indicated above. B, an electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the −113/−83 sequence from the human BAX promoter as radiolabeled probe. 50 ng of purified p53 was incubated with 3 ng of the probe alone (lane 1) or in the presence of a 500-, 1000-, or 1500-fold molar excess of either the unlabeled BAX −113/−83 oligonucleotide (lanes 2–4), the BAX −113/−93 oligonucleotide, the BAX sc −92/−83 oligonucleotide (lanes 5–7), or the BAX sc −86/−83 oligonucleotide (lanes 8–10). The arrow indicates the position of the p53-DNA complex. C, bands were quantitated by phosphorimaging. D, an electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the −113/−83 sequence from the human BAX promoter as radiolabeled probe. Extract from Sf9 cells expressing human recombinant Sp1 protein was incubated with 3 ng of the probe alone (lane 1) or in the presence of a 10-, 50-, or 100-fold molar excess of either the unlabeled BAX −113/−83 oligonucleotide (lanes 2–4), the BAX sc −92/−83 oligonucleotide (lanes 5–7), or the BAX sc −86/−83 oligonucleotide (lanes 8–10). The arrow indicates the position of the Sp1-DNA complex. E, bands were quantitated by phosphorimaging.
The ability of the proapoptotic BAX to function as a tumor suppressor protein has been substantiated by several studies. In certain mouse models, BAX has been shown to be an important mediator of p53-dependent apoptosis and a suppressor of oncogenic transformation, with loss of BAX leading to accelerated rates of tumor growth, increased tumor numbers, larger tumor mass, and decreased survival rates (63, 64). A significant correlation between decreased BAX expression and both a shorter survival period also have been observed in a number of human tumor types, including breast, ovarian, pancreatic, colorectal, and gastric cancers of the microsatellite mutator phenotype (71–73). Together, these results strongly support the tumor suppressor role for the BAX protein. In SCID mice (71–73), the ability of the tumor suppressor protein p53 to activate transcription through the BAX promoter and subsequently fail to induce apoptosis (29–32) as well as the TgT121 transgenic studies that demonstrate that the BAX gene, p53, and the required cofactor cooperate to mediate activation. In cells that do not support the p53-BAX pathway, three possible mechanisms may explain the apparent failure of wild-type p53 to activate the BAX gene: A. the cofactor may be absent due to mutation or to cell-type-specific limitations on its expression. C. the cofactor may be inactivated by post-translational modification as follows: P, phosphorylation; G, glycosylation; or A, acetylation. D. another factor that cannot cooperate with p53 may compete with the required cofactor for binding to its site in the BAX promoter. The p53-binding site (–113 to –93) is represented by the black box. The Sp1-binding site (–93 to –87) is represented by the white box. p53, the required cofactor, and the inhibitory factor are represented by the gray circle, the dotted oval, and the cross-hatched triangle, respectively.

**Fig. 9.** Model for the cell type-specific regulation of the BAX promoter by the tumor suppressor protein p53. A, in cells that are permissive to p53-dependent transcriptional activation of the BAX gene, p53 and the required cofactor cooperate to mediate activation. In cells that do not support the p53-BAX pathway, three possible mechanisms may explain the apparent failure of wild-type p53 to activate the BAX gene. B, the cofactor may be absent due to mutation or to cell-type-specific limitations on its expression. C, the cofactor may be inactivated by post-translational modification as follows: P, phosphorylation; G, glycosylation; or A, acetylation. D, another factor that cannot cooperate with p53 may compete with the required cofactor for binding to its site in the BAX promoter. The p53-binding site (–113 to –93) is represented by the black box. The Sp1-binding site (–93 to –87) is represented by the white box. p53, the required cofactor, and the inhibitory factor are represented by the gray circle, the dotted oval, and the cross-hatched triangle, respectively.

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