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13. ABSTRACT *(Maximum 200 Words)*

Consistent with its function as a transcription factor, the ability to bind to DNA is central to the tumor suppressor activity of p53. Genetic alteration of p53 resulting in loss-of-function is a common event in human cancer. To identify novel mechanisms in human breast cancer involving functional inactivation of wild-type p53 besides such direct genetic alteration, cellular factors that regulate the ability of p53 to bind to DNA were identified and characterized. The high mobility group proteins HMG-1, HMG-2, and HMG-I all enhanced the sequence-specific binding of p53 to DNA *in vitro*, but only HMG-1 stimulated the transcriptional activity of p53 in cells. The transcriptional activator Sp1 bound in a sequence-specific manner to a distinct subset of p53 response elements, but, in contrast to p53, the binding of Sp1 to DNA was inhibited by HMG-1. An intact Sp1 binding site was shown to be required for efficient p53-dependent activation of the *bax* promoter. It is proposed that alterations in the interaction of HMG-1 and Sp1 with p53 may contribute to functional inactivation of p53 in human breast tumors.

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TABLE OF CONTENTS

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1. Resnick-Silverman, L., S. St Clair, M. Maurer, K. Zhao, and J. J. Manfredi. 1998. Identification of a novel class of genomic DNA-binding sites suggests a mechanism for selectivity in target gene activation by the tumor suppressor protein p53. Genes Dev **12:**2102-7.

2. Tang, H. Y., K. Zhao, J. F. Pizzolato, M. Fonarev, J. C. Langer, and J. J. Manfredi. 1998. Constitutive expression of the cyclin-dependent kinase inhibitor p21 is transcriptionally regulated by the tumor suppressor protein p53. J Biol Chem **273:**29156-63.

3. Thornborrow, E.C. and Manfredi, J.J. (1999) One mechanism for cell-type specific regulation of the *bax* promoter by the tumor suppressor p53 is dictated by the p53 response element. J. Biol. Chem. **274**: 33747-33755.

4. Thornborrow, E.C. And Manfredi, J.J. (2001) The tumor suppressor protein p53 requires a cofactor to transcriptionally activate the human *bax* promoter. Submitted.

FINAL REPORT
Regulation of the tumor suppressor activity of p53 in human breast cancer
IDEA
(08/01/97-07/31/00)

INTRODUCTION

Genetic alteration of p53 resulting in loss-of-function is a common event in human cancer. This research is centered on testing the hypothesis that there are novel mechanisms in human breast cancer involving functional inactivation of wild-type p53 besides such direct genetic alteration. Consistent with its function as a transcription factor, the ability to bind to DNA has been shown to be central to the tumor suppressor activity of p53. Thus, the immediate goal of this study is to identify and characterize activities in human cells which affect the ability of p53 to bind to DNA in a sequence-specific manner. During Year 1, the high mobility group protein HMG-1 was shown to stimulate the ability of p53 to bind to its sequence-specific binding site and two novel factors were identified which bind to a subset of p53 response elements in a sequence-specific manner. Studies in Year 2 focussed on these latter factors. Electrophoretic mobility shift assays suggest that the binding of p53 and these latter nuclear factors may be mutually exclusive. This suggests that the interaction of these factors with a subset of p53 response elements may regulate p53 target gene selectivity. In Year 3, one of these factors was shown to be the transcriptional activator Sp1 and studies demonstrated a critical role for Sp1 binding in p53-dependent activation of the *bax* promoter.

BODY

Results and Discussion

Identification of a novel class of genomic DNA-binding sites suggests a mechanism for selectivity in target gene activation by the tumor suppressor protein p53.

Two response elements for the tumor suppressor p53 have been identified in the promoter of the gene encoding the cyclin-dependent kinase inhibitor p21. Binding of a monoclonal antibody directed against the carboxyl terminus of p53 selectively enhanced binding by p53 to the upstream (5′ site) without affecting the affinity of p53 for the downstream (3′ site). Mutational analysis showed that a single base change can cause one site to behave similarly to the other site. These sites in the *p21* promoter represent two distinct classes of p53 response elements found in a variety of genes. The mAb 421-enhanced class was shown to include elements from the *gadd45*, *mdm2*, and *cyclin G* genes as well as the box A element from the *IGF-BP3* gene whereas the mAb 421-inhibited class included *bax*, the 3′ element from the *p21* promoter, a novel element from the *cdc25C* gene, and the box B element from the *IGF-BP3* gene. These results demonstrate the existence of two classes of p53 binding sites in the human genome and that the binding of p53 to these two classes of sites can be differentially regulated by binding of mAb 421. This is a novel example of the regulation of binding site selection by a transcription factor and suggests a possible mechanism for selectivity in target gene activation by the p53 protein.

The high mobility group proteins HMG-1, HMG-2, and HMG-I all enhance the sequence-specific binding of p53 to DNA in vitro, but only HMG-1 stimulates the transcriptional activity of p53 in cells.

The high mobility group protein HMG-1 enhances the sequence-specific binding of p53 to both of its response elements in the promoter of the gene that encodes the cyclin-dependent kinase inhibitor p21. The binding of the monoclonal antibody mAb 421 dramatically enhances sequence-specific binding to the upstream or 5′ response element whereas in the presence of mAb 421, the binding of p53 to the downstream or 3′ element is largely non-specific. HMG-1 enhanced the binding of p53 to the *p21* 3′ site in the presence of mAb 421, but this enhanced binding is not sequence-specific. Although the closely related HMG-2 and the unrelated HMG-I had similar effects on the DNA binding ability of p53 *in vitro*, only HMG-1 was capable of stimulating the transcriptional activity of p53 in cells. These results demonstrate that, in spite of their close sequence similarity, HMG-1 and HMG-2 are functionally distinct in their regulation of p53.

The transcriptional activator Sp1 binds in a sequence-specific manner to a distinct subset of p53 response elements, but, in contrast to p53, the binding of Sp1 to DNA is inhibited by HMG-1.

A cellular factor is identified which binds in a sequence-specific manner to the downstream or 3′ site but not to the upstream or 5′ site in the promoter of the human gene encoding the cyclin-dependent kinase inhibitor p21. This factor is shown to be the transcriptional activator Sp1 since the DNA-protein complex is supershifted by an anti-Sp1 antibody, the binding of the factor to the p21 3′ site is competed by an unlabeled oligonucleotide containing the consensus sequence for Sp1 binding, and Sp1 expressed as a recombinant baculovirus in insect cells binds to the p21 3′ site in a specific manner. Mutational analysis localizes the Sp1 binding site within the p21 3′ response element to the second half-site. A survey of genomic p53 response elements shows that Sp1 binds to a distinct subset of p53 response elements. The high mobility group proteins HMG-1, HMG-2, and HMG-I inhibit the binding of Sp1 to the p21 3′ site. In contrast, HMG-1 enhances the binding of p53 to both sites from the p21 promoter both in the absence and presence of Sp1. Mixing experiments demonstrate that the binding of p53 and Sp1 to the p21 3′ site is mutually exclusive. Thus, p53 and Sp1 can compete for binding to some p53 response elements but not others and this binding can be differentially regulated by high mobility group proteins such as HMG-1. This suggests a mechanism for selective regulation of the interaction of p53 with a subset of its response elements and may represent a basis for target gene selection by p53 under particular cellular conditions.

The p53 response element in the human bax promoter consists of overlapping p53 binding sites.

Previous studies had identified a 36 bp element derived from the *bax* promoter that conferred p53-dependent activation on a reporter construct and that p53 bound in a sequence-specific manner to this element. This element contains three overlapping sequences each of which could potentially bind to p53. Oligonucleotides containing each of these were synthesized and shown to bind to p53 in a sequence-specific manner although the binding of p53 to these sites was relatively weak. Only one of the sequences conferred p53-dependent activation on a reporter, and this activation was less than that seen with the full-length 36 bp element. Further mutational analysis demonstrated that the minimal element which binds to p53 *in vitro* and confers p53-dependent activation on a minimal promoter as well as the full-length 36 bp element is

contained within -113 to -83 which contains only two of the three binding sites. These results indicate that activation of the *bax* promoter by p53 is mediated by cooperative interaction of two overlapping, low affinity, p53 binding sites. This is in marked contrast to other p53 target genes, particularly *p21*, in which single, high affinity p53 binding sites mediate the activation. Electrophoretic mobility shift assays showed that the full-length 36 bp element from the *bax* promoter as well as each of the three binding sites fall into the class of p53 binding sites that is inhibited by mAb 421. Thus, p53-dependent activation of the *bax* promoter is distinguished in two ways: by the synergy of overlapping low affinity binding sites and by its differential response to mAb 421.

A high-affinity Sp1 binding site is located within the p53-response element of the human bax promoter and contributes to p53-dependent activation.

Deletion of the -113/-83 sequence in a luciferase reporter containing the human *bax* promoter completely abolishes p53-dependent transcriptional activation. As noted, this 31 bp consists of three half-sites (RRRCWWGYYY) for p53 binding in a row. Deletion of the first or last half-site reduces p53-dependent activation demonstrating that all three half-sites are necessary for this response in the context of the *bax* promoter itself. Although the second and third half-sites can mediate some p53-dependent activity alone when placed upstream of the minimal E1b promoter, maximal activation is only seen when all three half-sites are present. The first and second half-sites alone only confer robust p53-dependent activation when multimerized. Nevertheless, this result confirms that p53 bound to just the first two half-sites is capable of transcriptionally activating such a reporter. This latter suggests the possibility that the third half-site, although capable of interacting with p53, may serve as a binding site for another cellular factor that cooperates with p53 bound to the first two half-sites to mediate efficient transcriptional activation. Indeed, several such cellular factors have been identified. One of these is clearly the transcription factor Sp1. In EMSA, a DNA/protein complex is efficiently supershifted by an anti-Sp1 antibody and effectively competed by an unlabeled oligonucleotide containing the Sp1 consensus sequence for binding. The binding site for Sp1 was localized by further EMSA to the third-half site. To determine the relevance of this Sp1 binding site in p53-dependent transcriptional activation, 2 bp substitution mutants were constructed. One such mutant was shown to abolish an interaction of p53 with the third-half-site while retaining the ability to bind to Sp1 *in vitro*. A luciferase reporter containing these two substitution mutations was shown to confer 5-fold higher p53-dependent transcriptional activation on a minimal promoter than the unmutated element. Since this element can only bind p53 through the first and second half-sites, this demonstrates that the third-half site contributes to transcriptional activation by a mechanism that does not involve the direct interaction of p53. The most likely mechanism involves an interaction of some other cellular factor, possibly Sp1. The higher activation seen with the mutant suggests the possibility that p53 binding to the third-half site may interfere with this factor and hence an inability of p53 to interact with the third half site allows for more robust activation through the mutated element. Examination of the sequence of the third half-site shows that it resembles what is referred to as a "GC box". A large variety of known transcription factors interact with GC boxes, the most prominent of which is Sp1.

Adherence to Statement of Work

Technical Objective #1

Task 1:	Months 1-2	Completed
Task 2:	Months 3-6	Completed
Task 3:	Months 7-12	Completed

Technical Objective # 2

Task 4:	Months 6-12	Completed
Task 5:	Months 13-18	Completed
Task 6:	Months 11-14	Completed
Task 7:	Months 15-20	Completed

Technical Objective #3

Tasks 8:	Months 19-20	Completed
Tasks 9:	Months 21-26	Completed
Tasks 10	Months 27-32	Completed
Tasks 11:	Months 33-36	Completed

KEY RESEARCH ACCOMPLISHMENTS

- high mobility group proteins HMG-1, HMG-2, and HMG-I all enhance the DNA binding ability of p53
- HMG-1 but not the closely related HMG-2 stimulates the transcriptional activity of p53 in cells
- the transcriptional activator Sp1 binds in a sequence-specific manner to a subset of p53 response elements
- high mobiloity group proteins HMG-1, HMG-2, and HMG-I inhibit the binding of Sp1 to p53 response elements
- p53 requires a cofactor to transcriptionally activate the human *bax* promoter
- an intact Sp1 binding site is necessary for p53-dependent activation of the *bax* promoter

REPORTABLE OUTCOMES

Manuscripts

1. Resnick-Silverman, L., S. St Clair, M. Maurer, K. Zhao, and J. J. Manfredi. 1998. Identification of a novel class of genomic DNA-binding sites suggests a mechanism for selectivity in target gene activation by the tumor suppressor protein p53. Genes Dev **12:**2102-7.

2. Tang, H. Y., K. Zhao, J. F. Pizzolato, M. Fonarev, J. C. Langer, and J. J. Manfredi. 1998. Constitutive expression of the cyclin-dependent kinase inhibitor p21 is transcriptionally regulated by the tumor suppressor protein p53. J Biol Chem **273:**29156-63.

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4. Thornborrow, E.C. And Manfredi, J.J. (2001) The tumor suppressor protein p53 requires a cofactor to transcriptionally activate the human *bax* promoter. Submitted.

Abstracts

1. Resnick-Silverman, L., St. Clair, S., Zhao, K., Meng, J., and Manfredi, J.J. (1998) Identification of a novel class of genomic DNA binding sites suggests a mechanism for selectivity in target gene activation by the tumor suppressor protein p53. Ninth p53 Workshop (Crete, Greece).

2. Resnick-Silverman, L., St. Clair, S., Maurer, M., Zhao, K., Denburg, M., Meng, J., and Manfredi, J.J. (1998) Identification of a novel class of genomic DNA binding sites suggests a mechanism for selectivity in target gene activation by the tumor suppressor protein p53. 63rd Symposium: Mechanisms of Transcription (Cold Spring Harbor, NY).

3. Resnick-Silverman, L., Maurer M., Zhao, K., Meng, J., and Manfredi, J.J. (1998) Identification of a novel class of genomic DNA binding sites suggest mechanisms for selectivity in target gene activation by the tumor suppressor protein p53. Meeting on Cancer Genetics and Tumor Suppressor Genes (Cold Spring Harbor, NY).

4. Resnick-Silverman, L., Zhao, K., and Manfredi, J.J. (1998) The two p53 response elements in the p21 promoter mediate activation by distinct mechanisms and do not synergize in transcription. Meeting on Cancer Genetics and Tumor Suppressor Genes (Cold Spring Harbor, NY).

5. Thornborrow, E.C., Zhao, K., and Manfredi, J.J. (1998) Transcriptional activation of the bax promoter by the tumor suppressor p53 occurs in a distinct manner from that of other p53 target genes. Meeting on Cancer Genetics and Tumor Suppressor Genes (Cold Spring Harbor, NY).

6. Resnick-Silverman, L., St. Clair, S., Thornborrow, E., Maurer, M., Meng, J., Ream, A., and Manfredi, J.J. (1999) Target gene selection by p53 is regulated by multiple mechanisms. ICGEB Workshop: "p53: Twenty Years On" (Trieste, Italy).

7. Thornborrow, E.C. and Manfredi, J.J. (2000) A high-affinity Sp1 binding site is located within the p53-response element of the human bax promoter. Tenth p53 Workshop (Monterey, CA).

8. Manfredi, J.J., Maurer, M., Meng, J., Aidinis, V., and Resnick-Silverman, L. (2000) The high mobility group protein, HMG-1, but not the closely related HMG-2 enhances the transcriptional activity of the tumor suppressor protein p53. Era of Hope: Department of Defense Breast Cancer Program Meeting (Atlanta, GA).

Presentations

1. Identification of a novel class of genomic DNA binding sites suggest mechanisms for selectivity in target gene activation by the tumor suppressor protein p53
Meeting on Cancer Genetics and Tumor Suppressor Genes
Cold Spring Harbor, NY
August 21, 1998

2. Target gene selection by p53 is regulated by multiple mechanisms
ICGEB Workshop: p53: Twenty Years On
Trieste, Italy
May 21, 1999

3. Determinants of the cellular response to the tumor suppressor p53
San Raffaele Scientific Institute
Milan , Italy
May 24, 1999

4. Regulation of target gene selectivity by the tumor suppressor p53
Department of Pathology
State University of New York at Stony Brook
Stony Brook, NY
January 13, 2000

5. Determinants of the cellular response to the tumor suppressor p53
Department of Molecular Genetics, Biochemistry, and Microbiology
University of Cincinnati Medical Center
Cincinnati, OH
October 24, 2000

Funding applied for based on work supported by this award

Title of Project:	Determinants of cellular responses to p53
Sponsoring agency:	National Institutes of Health/National Cancer Institute
Number:	1 R01 CA86001-01A1
Grant type:	Research
Status:	Principal Investigator
Percent Effort:	25%
Total Project Period:	04/01/01-03/31/06
Total direct costs:	$875,000

This application was reviewed by the Pathology B Scientific Review Group and received a priority score of 183 and a percentile of 23.9.

CONCLUSIONS

Two cellular factors have been identified which influence the ability of p53 to bind to DNA and activate transcription. The high mobility group protein HMG-1 enhances the sequence-specific binding of p53 to DNA *in vitro* and stimulates the transcriptional activity of p53 in cells. The activator Sp1 binds in a sequence-specific manner to a subset of p53 response elements and this binding is regulated in a distinct manner from p53. In the case of p53-dependent activation of the *bax* promoter, an intact Sp1 binding site is shown to be required.

The long term goal is to determine the relevance of the interaction of p53 with the transcriptional activator Sp1 and the high mobility group protein HMG-1 in human breast cancer. Mechanistic studies will be pursued to determine whether and how the activities of Sp1 and HMG-1 are regulated. Human breast tumor samples would then be screened for alterations in the expression of these factors or their regulators which leads to effects on the DNA binding and transcriptional activity of p53. The identification of proteins which regulate wild-type p53 is an important focus for breast cancer research since the regulation, mechanism of action, and metabolism of such proteins would be central to our understanding of breast cancer and the aberrant expression of such proteins would represent novel important mechanisms of carcinogenesis.

LIST OF PERSONNNEL

Name	Role on project	Dates
James J. Manfredi	PI	8/1/97-7/31/00
Kathy Zhou	Technician	9/1/97-8/31/98
Amy Ream	Technician	9/1/98-7/31/99
Andrea DaCosta	Technician	8/1/99-7/31/00

APPENDICES

RESEARCH COMMUNICATION

Identification of a novel class of genomic DNA-binding sites suggests a mechanism for selectivity in target gene activation by the tumor suppressor protein p53

Lois Resnick-Silverman,[1] Selvon St. Clair,[1] Matthew Maurer,[1] Kathy Zhao,[1] and James J. Manfredi[1–3]

[1]Derald H. Ruttenberg Cancer Center and the [2]Brookdale Center for Molecular and Developmental Biology, Mount Sinai School of Medicine, New York, New York 10029 USA

There are two response elements for p53 in the promoter of the gene for the cyclin-dependent kinase inhibitor p21. The binding of p53 to the 5′ site was enhanced by incubation with monoclonal antibody 421, whereas the binding of p53 to the 3′ site was inhibited. Mutational analysis showed that a single-base change caused one element to behave like the other. A response element in the human *cdc25C* promoter is bound by p53 with properties similar to the 3′ site. These results identify two classes of p53-binding sites and suggest a mechanism for target gene selectivity by p53.

Received March 25, 1998; revised version accepted May 27, 1998.

The tumor suppressor protein p53 has been implicated in the cellular response to DNA damage and mediates either growth arrest or apoptosis, depending on particular cellular conditions (see Gottlieb and Oren 1996; Ko and Prives 1996; Levine 1997). It has also been implicated in a spindle checkpoint (Cross et al. 1995) and in the induction of either differentiation (Shaulsky et al. 1991; Aloni-Grinstein et al. 1993; Soddu et al. 1994) or senescence (Sugrue et al. 1997). The p53 protein is a transcription factor that binds in a sequence-specific manner to particular sites in the genome and activates transcription of target genes (for review, see Gottlieb and Oren 1996; Ko and Prives 1996; Levine 1997). Utilizing immunobinding assays involving the monoclonal antibody 421, a consensus binding site for p53 has been defined and consists of four pentameric repeats of RRRCW in which R is a purine and W represents either an A or T residue (El-Deiry et al. 1992; Funk et al. 1992; Halazonetis et al. 1993). Two palindromic pentamers (half-site) are juxtaposed to a second set of two palindromic pentamers, the two half-sites being separated by no insert or insertions from 1–13 bp (El-Deiry et al. 1992). Such a consensus site is consistent with the fact that p53 exists in solution and binds to DNA as a tetramer (Friedman et al. 1993). It has been proposed that to accommodate a symmetrical tetrameric p53 on such a site, the DNA must bend (Balagurumoorthy et al. 1995; Nagaich et al. 1997a,b). Studies to date have implicated the C residue at position 4 of each pentamer as essential for the binding of p53 to DNA (Halazonetis et al. 1993; Nagaich et al. 1997b).

The structure of p53 is consistent with its role as a transcription factor with identified domains that are responsible for transcriptional activation, sequence-specific DNA binding, and oligomerization as a tetramer. Previous studies have implicated the carboxy-terminal 30 amino acids of p53 as exerting a negative regulatory effect on the DNA-binding activity of the protein. Deletion of these carboxy-terminal 30 amino acids, phosphorylation of sites within this region by casein kinase II and protein kinase C, and the binding of bacterial DnaK in this region all will activate the DNA-binding activity of p53 (Hupp et al. 1992; Takenaka et al. 1995). Consistent with this, the mAb 421, which has an epitope in this carboxy-terminal region, activates the ability of p53 to bind to DNA (Funk et al. 1992; Hupp et al. 1992; Halazonetis et al. 1993; Mundt et al. 1997). Finally, a peptide derived from the carboxyl end of p53 has also been shown to stimulate the ability of p53 to interact with DNA, although not to the same extent as the activators identified previously (Hupp et al. 1995).

DNA damage induces expression of p53 protein which, in turn, transcriptionally activates expression of particular genes, most notably those that encode the cyclin-dependent kinase inhibitor p21. Consistent with this, cells that lack p21 expression have an impaired p53-dependent response to DNA damage (Brugarolas et al. 1995; Deng et al. 1995). The human *p21* promoter has been shown to contain two p53-responsive elements. Deletion analysis of reporter constructs containing the sequence of the human *p21* promoter identified a distal element located 2.3–2.5 kb and a proximal element located 1.1–1.5 kb from the start site of transcription (El-Deiry et al. 1993,1995; Macleod et al. 1995).

In this report we have confirmed the existence of two p53-responsive elements in the human *p21* promoter. One of these, the 3′ site, matches the consensus sequence for p53 DNA binding at 18 of 20 positions. Notably, there is a G residue in place of the C residue in the fourth position of the first pentamer (Fig. 1). In contrast to other known p53-binding sites, the binding of p53 to this 3′ site in the *p21* promoter is inhibited by mAb 421. This suggests the existence of a new class of genomic sites in which the binding of p53 may be regulated differentially. Because p53 has been implicated in a variety of cellular responses, an understanding of the mechanism for selection of target genes by p53 is central to understanding its biological functions. The results pre-

[*Key Words:* Tumor suppressor; DNA binding; sequence specificity; p53 protein; transcriptional activation; binding sites]
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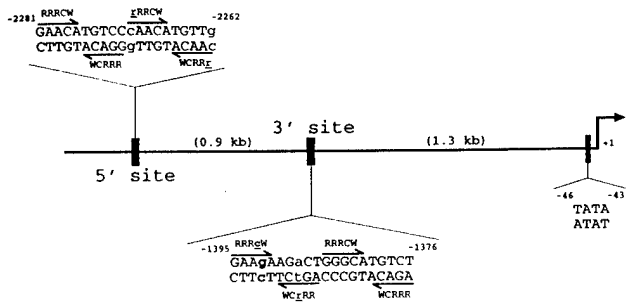

Figure 1. Schematic of *p21* promoter. Shown is 2.5 kb of the upstream sequence of the human *p21* promoter; 2.2 kb from the start site of transcription is a well-documented p53-binding site at positions -2281 to -2262. It matches the published consensus sequence for a p53 DNA binding site in 18 of 20 positions; the variations from the consensus are shown by lowercase letters. A second site with similarity to the consensus sequence is 1.3 kb upstream from the start site of transcription. Contained within positions -1395 to -1376, this sequence also matches the consensus at 18 of 20 positions. Note that in this 3′ site, the fourth position of the first pentamer contains a G rather than a C residue (shown in lowercase and boldface type). The position of the TATA box of the promoter is also indicated.

sented here suggest one potential mechanism for such site selection by p53.

Results

Two p53-responsive elements are present in the human p21 *promoter*

Using a series of deletion constructs, two p53-response elements had been identified previously in the human *p21* promoter (Macleod et al. 1995). A well-characterized element was located 2.4 kb upstream from the start site of transcription, and a second element had been suggested to be present 1.1–1.5 kb from the start of transcription (Fig. 1). We demonstrate that both a 20-bp 5′ element, located between -2262 and -2281, and a 20-bp 3′ element, located between -1376 and -1395, are each sufficient to transactivate a reporter gene in a p53-dependent manner (Fig. 2). Double-stranded synthetic oligonucleotides containing either one copy of the 5′ site or two copies of the 3′ site were inserted into a reporter vector, pGL3–E1bTATA, containing the *E1b* promoter upstream of a luciferase reporter gene. Although a single copy of the 3′ site conferred p53-dependent transcriptional activation on the minimal promoter (see Fig. 5B, below), two copies of the 3′ site showed a more pronounced effect and were used in the experiments described here (Fig. 2). Each reporter construct was cotransfected into p53-negative Saos-2 cells with empty vector or a plasmid expressing either human wild-type p53 or the human temperature-sensitive mutant $p53^{Ala143}$. The temperature-sensitive mutant $p53^{Ala143}$ is in a mutant conformation at 37°C. At this temperature, it is unable to activate p53-response elements. However, when shifted to 32°C, this mutant can assume a wild-type conformation and has been shown to activate some p53-responsive promoters (such as *p21*) but not others (such as Bax) (Friedlander et al. 1996). Cells were maintained at 37°C or shifted to 32°C, 17 hr prior to lysis. At 37°C, a luciferase reporter containing a single copy of the 5′ site was activated 1730-fold by wild-type p53, but only 2-fold by $p53^{Ala143}$. The reporter plasmid containing two copies of the 3′ site was activated 380-fold by wild-type p53, but again only 2-fold by $p53^{Ala143}$. The reporter vector lacking either response element was minimally activated by expression of either the wild-type or mutant p53 (Fig. 2A). At 32°C, the luciferase reporter containing a single copy of the 5′ site was activated 1154-fold by wild-type p53 and 792-fold in the presence of $p53^{Ala143}$. The reporter plasmid containing two copies of the 3′ site was activated 362-fold by wild-type p53 and 96-fold by the mutant $p53^{Ala143}$. A luciferase reporter plasmid containing the full-length *p21* promoter p21P was activated 16-fold by wild-type p53 and ninefold by the mutant $p53^{Ala143}$. At 32°C, the reporter vector lacking either response element was not activated by either wild-type or mutant p53 (Fig. 2B). These data confirm that there are two p53-response elements in the human *p21* promoter, each of which is sufficent to confer p53-dependent transcriptional activation on a luciferase reporter gene containing the minimal adenovirus *E1b* promoter. Additionally, neither the 5′ nor the 3′ site is activated by the temperature-sensitive mutant $p53^{Ala143}$ at 37°C, whereas at 32°C both sites are activated, although less so than by wild-type p53.

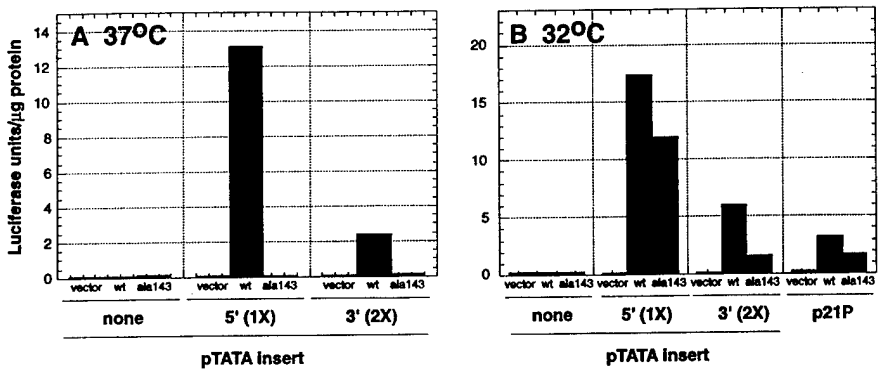

Figure 2. Two p53-response elements are present in the *p21* promoter. Saos-2 cells were transfected as described in Materials and Methods with 2 µg of the indicated reporter constructs in the absence or presence of either 50 ng of pCMV–p53wt, an expression vector encoding human wild-type p53 under the control of the CMV promoter (light and dark bars, respectively), or 50 ng of $pCMV–p53^{ala143}$, an expression vector encoding the temperature-sensitive mutant $p53^{Ala143}$ (solid bars). Cells were either maintained at 37°C (*A*) or shifted to 32°C 17 hr prior to lysis (*B*) and then were assayed for luciferase activity and total protein levels as described in Materials and Methods. The indicated values are the average of three (37°C) or four (32°C) independent experiments that had been performed in duplicate.

Two p53-binding sites are present in the human p21 *promoter*

Double-stranded oligonucleotides that contain the sequence of the 5′ and 3′ sites were synthesized and used in electrophoretic mobility shift assays (EMSAs). The 5′ site was bound by purified p53, and this binding was competed by an excess of unlabeled 5′ site (Fig. 3A, lanes 2–4), as well as an excess of the 3′ site (Fig. 3A, lanes 8–10), but not by an unlabeled oligonucleotide containing a mutated sequence of the 5′ site in which the C residue in the fourth position of each pentamer has been mutated to a T residue (Fig. 3A, lanes 5–7). The 3′ site competes approximately threefold less well than the 5′ site for the binding of p53 protein to a labeled 5′ site (Fig. 3A, lanes 2–4, and 8–10). The mAb 1801 supershifts the p53–5′ site complex efficiently, demonstrating the presence of p53 in that complex (Fig. 3A, lane 11). As has been reported previously for a p53 consensus site (Funk et al. 1992), mAb 421 also efficiently supershifts the p53–5′ site complex and in so doing enhances the binding that is seen (Fig. 3A, lane 12).

The 3′ site also was bound by purified p53, and the binding of p53 to a labeled 3′ site was effectively competed by the unlabeled 3′ site (Fig. 3B, lanes 2–4), as well as the unlabeled 5′ site (Fig. 3B, lanes 8–10) but not the mutated 5′ site with all four C residues altered (Fig. 3B, lanes 5–7). Consistent with the results of the competition analysis using the labeled 5′ site, the unlabeled 5′ site competed threefold better for binding to the labeled 3′ site as the unlabeled 3′ site (Fig. 3B, lanes 2–4, and 8–10). mAb 1801 again supershifted the p53–3′ site complex (Fig. 3B, lane 11) efficiently. Surprisingly, in contrast to the result with the labeled 5′ site, mAb 421 appeared to inhibit the binding of p53 to the 3′ site and supershifted what little DNA-binding complex that was detected only poorly (Fig. 3B, lane 12). This latter result suggests the intriguing possibility that the optimal binding of p53 to each of these sites may require different conformations of the p53 tetramer.

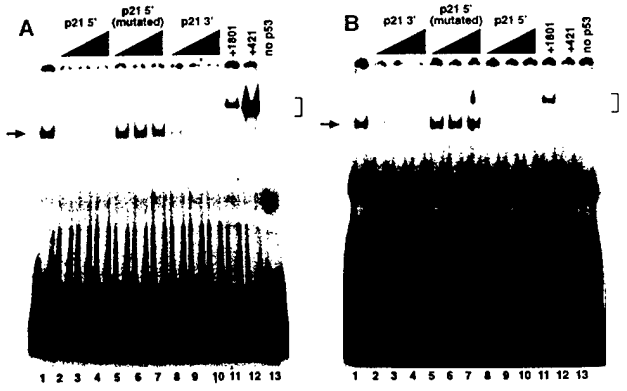

Figure 3. Monoclonal antibody enhances the binding of p53 to the 5′ site but inhibits the binding of p53 to the 3′ site. (*A*) An EMSA using as labeled probe the 5′ p53-binding site; (*B*) an EMSA using as labeled probe the 3′ p53-binding site. Five nanograms of purified p53 protein was incubated alone (lane *1*), in the presence of a 17-, 33-, or 50-fold excess of each unlabeled competitor, as indicated (lanes *2–10*), or in the presence of a 4 μl of mAb 1801 (lane *11*) or 4 μl of mAb 421 (lane *12*). p21 5′ (mutated) refers to a 5′ site in which the fourth postion of each pentamer has been mutated to a T residue. A sample that does not contain any p53 protein is shown in lane *13*. (→) The position of the p53–DNA complex; (]) the position of the supershifted p53–DNA antibody complex.

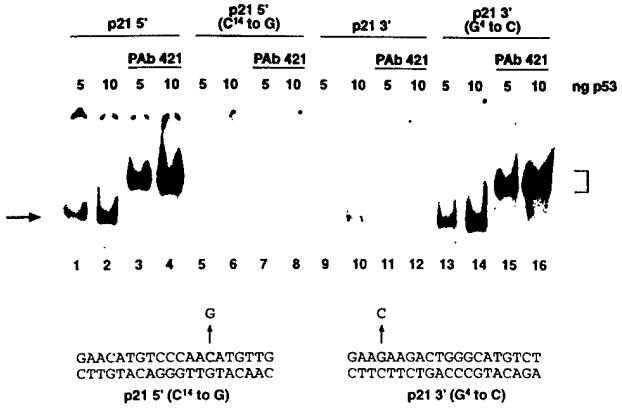

Figure 4. Mutational analysis demonstrates the importance of the C residue in the fourth position of a pentamer in responsiveness to mAb 421. Purified p53 (5 or 10 ng as indicated) was incubated with labeled probes containing the 5′ site (lanes *1–4*), the 5′ site with the residue at position 14 mutated to a G (C^{14} to G) (lanes *5–8*), the 3′ site (lanes *9–12*), or the 3′ site with the residue at position 4 mutated to a C (G^4 to C) (lanes *13–16*). The labeled probes had equivalent specific activities. Reactions were performed either in the absence (lanes *1,2,5,6,9,10,13,14*) or presence (lanes *3,4,7,8,11,12,15,16*) of mAb 421. The arrow (→) position of the p53–DNA complex; (]) position of the supershifted p53–DNA–antibody complex.

Mutation of a C residue affects responsiveness to mAb 421

The 3′ site diverges from the published consensus for p53-binding sites in two positions. The residue in position 4 of the first pentamer is a G (instead of a C residue), and the residue in position 3 of the second pentamer is an A residue (instead of a pyrimidine). The binding of p53 to the labeled 3′ site is inhibited in the presence of mAb 421 (Fig. 4, lanes 9–12). However, when a 1-bp change, G^4 to C residue, was engineered in this sequence and used as the labeled probe, the binding to p53 was enhanced in the presence of mAb 421 (Fig. 4, lanes 13–16). Thus, mutation of G^4 to a C, thereby creating a site with four consensus pentamers, allowed the binding of p53 to this site to be enhanced by mAb 421.

The reverse effect could be achieved through mutagenesis of the 5′ site. This site conforms to the consensus sequence in that a C residue is present in the fourth position of each pentamer. A labeled 5′ probe bound to increasing amounts of p53 (Fig. 4, lanes 1–2) and this binding could be enhanced in the presence of mAb 421 (Fig. 4, lanes 3,4). When mutated 5′ (C^{14} to G) was labeled and used in this EMSA, its binding to p53 was inhibited in the presence of 421 (Fig. 4, lanes 5–8) just like the 3′ site (Fig. 4, lanes 9–12). Therefore, the primary sequence of the response element can determine

whether the binding by p53 is enhanced or inhibited by antibody 421.

There is a p53-response element in the promoter of the human cdc25C *gene with properties similar to the 3′ site*

A search in the human genome database for other variant p53-binding sites that consist of four pentamers, only three of which contain C residues in the fourth position, was performed. A site in the promoter of the *cdc25C* gene, which encodes a cell cycle-regulated protein phosphatase that is necessary for progression into mitosis, was subjected to further analysis. A radiolabeled synthetic oligonucleotide containing a sequence from the human *cdc25C* promoter is bound by purified human p53 in an EMSA (Fig. 5A, lane 7). mAb 1801 supershifts this complex efficiently, whereas mAb 421 inhibits the binding of p53 to this site (Fig. 5A, lanes 8,9). Similar results were obtained with a radiolabeled oligonucleotide containing the sequence of the 3′ site of the *p21* promoter (Fig. 5A, lanes 4–6). These results are in contrast to the ability of mAb 421 to enhance and supershift the complex of p53 with a radiolabled oligonucleotide containing the 5′ site from the *p21* promoter (Fig. 5A, lanes 1–3). These results demonstrate that the site from the *cdc25C* promoter binds to p53 in the presence of mAb 421 with similar properties as the 3′ site from the *p21* promoter.

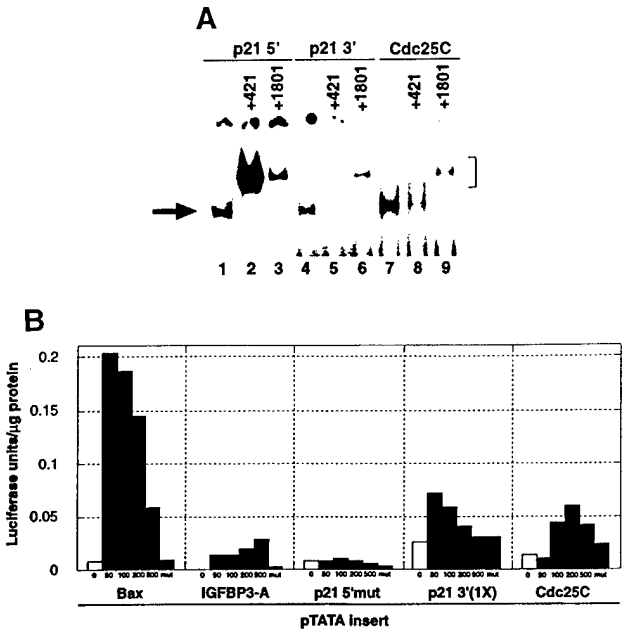

Figure 5. The p53-response element in the promoter of the human *cdc25C* gene has properties similar to the 3′ site. (*A*) An EMSA using as radiolabeled probe the 5′ site (lanes *1–3*), the 3′ site (lanes *4–6*), or the site from the *cdc25C* promoter (lanes *7–9*). The probes had an equivalent specific activity of labeling. Five nanograms of purified p53 was incubated in the absence (lanes *1,4,7*) or presence of either 4 µl of mAb 421 (lanes *2,5,8*) or the presence of 4 µl of mAb 1801 (lanes *3,6,9*). (→) The position of the p53–DNA complex; (]) position of the supershifted p53–DNA–antibody complex. (*B*) Saos-2 cells were transfected as described in Materials and Methods with 2 µg of the indicated reporter constructs in the absence (open bars) or presence of increasing amounts of pCMV–p53wt (50, 100, 200, or 500 ng, shaded bars), or 500 ng of pCMV–p53ala143 (solid bars). Appropriate amounts of the vector pCMV were added to each transfection mixture to maintain a total level of plasmid DNA of 2.5 µg. Cells were maintained at 37°C and assayed for luciferase activity and total protein levels as described in Materials and Methods. The indicated values are from a representative experiment that had been performed in duplicate.

To determine whether this p53-binding site from the *cdc25C* promoter can act as a p53-response element in cells, an oligonucleotide containing a single copy of the sequence of this site was inserted adjacent to the adenovirus *E1b* minimal promoter in a luciferase reporter plasmid. This construct was compared to constructs containing two previously characterized p53-response elements, namely one from the human bax promoter, and one of the two intronic sites found in the *IGFBP3* gene, the so-called box A site (Buckbinder et al. 1995; Miyashita and Reed 1995; Friedlander et al. 1996; Ludwig et al. 1996). These reporter constructs were compared to a plasmid containing a single copy of the 3′ site from the *p21* promoter and a plasmid containing a single copy of the 5′ site that contains all four C residues altered. Saos-2 cells were transfected with increasing amounts of the wild-type p53 expression vector in the presence of these various reporter constructs. Wild-type p53 activated reporters containing the Bax, IGFBP3-A, 3′ site, and Cdc25C sites, but not a reporter containing the mutated 5′ site (Fig. 5B). This demonstrates that the site from the *cdc25C* promoter is sufficient to confer p53-dependent transcriptional activation on a heterologous luciferase reporter construct. Thus, mAb 421 differentially affects the binding of p53 to two different genomic binding sites that can mediate p53-dependent transcriptional activation.

Discussion

The binding of the mAb 421 to p53 stimulates the ability of p53 to bind to one set of genomic sites that conform to a previously identified consensus sequence and inhibits its ability to bind to another set of genomic sites that deviate from that consensus. This ability to regulate the sequence selectivity of DNA binding by a transcription factor, even in an in vitro setting, is a novel finding. A provocative unanswered question is whether the inhibition seen in the presence of mAb 421 has a physiological counterpart in the cell such that the sequence-specific binding of p53 to elements such as the 3′ site is regulated. The relevance of the mAb 421 effect will remain an open question until cellular conditions are identified that produce selective inhibition of these variant p53-response elements. Previous studies suggest some possible mechanisms, including regulation by the coactivator p300 and phosphorylation by particular kinases. The coactivator p300 recently has been shown to stimulate the sequence-specific DNA-binding activity of p53 (Gu and Roeder 1997), and the DNA-binding activity of p53 can also be stimulated by phosphorylation by casein ki-

nase II, protein kinase C, and cyclin-dependent kinase (Meek et al. 1990; Takenaka et al. 1995; Wang and Prives 1995). Studies to examine the role of these different kinases, as well as p300, in the regulation of the ability of p53 to interact with elements such as the 3′ site in the *p21* promoter are essential to address these possibilities.

Depending on particular cellular conditions, the tumor suppressor protein p53 has been reported to induce growth arrest in both the G_1 and G_2 phases of the cell cycle, mediate an apoptotic response, or trigger alternatively a differentiation or a senescence pathway (see Gottlieb and Oren 1996; Ko and Prives 1996; Levine 1997; Sugrue et al. 1997). Because the DNA-binding activity of p53 appears to play a role in each of these physiological responses, the ability of p53 to select among various target genes to elicit a particular cellular response is central to the regulation of its biological function. To date, the identification of a mechanism for the regulation of target gene selectivity by p53 has been elusive. The results presented here, albeit under nonphysiological conditions, suggest one potential mechanism by which such selectivity may be achieved. It will be important to determine whether such a mechanism occurs during any of the various cellular responses to p53 and to identify the target genes that are relevant in each situation.

Materials and methods

Plasmids

The expression plasmids pCMV–$p53^{wt}$ and pCMV–$p53^{Ala143}$, encode the indicated human p53 protein under the control of the CMV promoter. These plasmids were referred originally to as pCMV–SN3 and pCMV–CX3, respectively. The reporter plasmid, pGL3–E1bTATA, was constructed by digesting a synthetic double-stranded oligonucleotide, GCGCGGTACCCTCGAGATGCATGAATTCGCTAGCGAGCTCAGGGTATATAATGAAGCTTGGCC, with *KpnI* and *HindIII* and cloning it into the pGL3–Basic vector (Promega), which had been double-digested with *KpnI* and *HindIII*. The resulting plasmid contains a multiple cloning region with the unique restriction sites, *KpnI*, *XhoI*, *NsiI*, *EcoRI*, *NheI*, and *SacI* upstream of the minimal adenovirus *E1b* promoter sequence and the coding region for firefly luciferase.

The following synthetic double-stranded oligonucleotides were digested with *KpnI* and either *NheI* [5′, 3′(1×), 3′(2×), Cdc25C, and Bax] or *SacI* (5′ mut and IGFBP3-A) and cloned into pGL3–E1bTATA, which had been double-digested with *KpnI* and either *NheI* or *SacI* to produce the appropriate reporter plasmids: 5′-AATTGGTACCGAACATGTCCCAACATGTTGGCTAGCGAATT; 3′(1×)–AATTCGGTACCGAAGAAGACTGGGCATGTCTGCTAGCGAATT; 3′(2×)–AATTCGGTACCGAAGAAGACTGGGCATGTCTGAAGAAGACTGGGCATGTCTGCTAGCGAATT; 5′ mut–AATTCGGTACCGAATATATCCCAATATATTGGAGCTCGAATT; Cdc25C–AATTCGGTACCGGGCAAGTCTTACCATTTCCAGAGCAAGCACGCTAGCGAATT; Bax–AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGGGCTATATTGCTAGCGAATT; and IGFBP3-A–AATTCGGTACCAAACAAGCCACCAACATGCTTTGGAGCTCGAATT.

Transfection of reporter constructs

Saos-2 cells were transfected using the DOTAP liposomal transfection reagent (Boehringer Mannheim) according to the manufacturer's instructions. Lysates were prepared, total protein concentration was determined, and luciferase assays were quantitated using a TD-20e Luminometer (Turner).

Purification and quantitation of human p53 protein

Sf9 cells that were infected with recombinant baculovirus were lysed in 20 mM HEPES (pH 7.4) containing 20% glycerol, 10 mM NaCl, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, 50 μM leupeptin, and 50 μg/ml aprotinin (lysis buffer). Nuclei were pelleted by centrifugation at 2300 rpm and then resuspended in lysis buffer containing 500 mM NaCl. Extracts were diluted to 100 mM NaCl, applied to a 0.5-ml Ni-NTA-agarose column (Qiagen) that was equilibrated with 20 mM HEPES containing 100 mM NaCl, and eluted with 200 mM imidazole containing 10 mM HEPES, (pH 7.4) and 5 mM NaCl. Fractions of 0.5-ml were collected, dialyzed against 10 mM HEPES (pH 7.4), 5 mM NaCl, 0.1 mM EDTA, 20% glycerol, and 1 mM DTT, aliquoted, and stored at −70°C.

EMSAs

Complementary single-stranded oligonucleotides were annealed, and ends were filled using the Klenow fragment of DNA polymerase to produce the following double-stranded oligonucleotides: p21 5′-AATTCTCGAGGAACATGTCCCAACATGTTGCTCGAGAATT; p21 3′-AATTCTCGAGGAAGAAGACTGGGCATGTCTTCTACCTCGAGAATT; p21 5′ (mutated)–AATTCTCGAGGAATATATCTTGAATTCTTCCTCGAGAATT; p21 5′ (C^{14} to G)–AATTCTCGAGGAACATGTCCCAAGATGTTGCTCGAGAATT; p21 3′ (G^4 to C)–AATTCTCGAGGAACAAGACTGGGCATGTCTTCTACCTCGAGAATT; and Cdc25C–AATTCTCGAGGGGCAAGTCTTACCATTTCCAGAGCAAGCACCTCGAGAATT.

Purified p53 protein, 3 ng of labeled double-stranded oligonucleotide, and hybridoma supernatant where appropriate, were incubated in a total volume of 30 μl of DNA binding buffer containing 20 mM HEPES (pH 7.5), 83 mM NaCl, 0.1 mM EDTA, 12% glycerol, 2 mM $MgCl_2$, 2 mM spermidine, 0.7 mM DTT, 133 μg/ml BSA, and 25 μg/ml poly[d(I–C)] for 30 min at room temperature. Samples were loaded on a native 4% acrylamide gel in 0.5× TBE and electrophoresed at 4°C at 200 V for 2 hr. The gel was dried and exposed to Kodak XAR-5 film using an intensifying screen at −70°C. Bands were quantitated using the Molecular Analyst Phosphorimaging system (Bio-Rad).

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Constitutive Expression of the Cyclin-dependent Kinase Inhibitor p21 Is Transcriptionally Regulated by the Tumor Suppressor Protein p53*

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The tumor suppressor protein p53 has been implicated in the response of cells to DNA damage. Studies to date have demonstrated a role for p53 in the transcriptional activation of target genes in the cellular response to DNA damage that results in either growth arrest or apoptosis. In contrast, here is demonstrated a role for p53 in regulating the basal level of expression of the cyclin-dependent kinase inhibitor p21 in the absence of treatment with DNA-damaging agents. Wild-type p53-expressing MCF10F cells had detectable levels of p21 mRNA and protein, whereas the p53-negative Saos-2 cells did not. Saos-2 cells were infected with recombinant retrovirus to establish a proliferating pool of cells with a comparable constitutive level of expression of wild-type p53 protein to that seen in untreated MCF10F cells. Restoration of wild-type but not mutant p53 expression recovered a basal level of expression of p21 in these cells. Constitutive expression of luciferase reporter constructs containing the p21 promoter was inhibited by co-transfection with the human MDM2 protein or a dominant-negative p53 protein and was dependent on the presence of p53 response elements in the reporter constructs. Furthermore, p53 in nuclear extracts of untreated cells was capable of binding to DNA in a sequence-specific manner. These results implicate a role for p53 in regulating constitutive levels of expression of p21 and demonstrate that the p53 protein is capable of sequence-specific DNA binding and transcriptional activation in untreated, proliferating cells.

The tumor suppressor protein p53 is a transcription factor that binds to DNA in a sequence-specific manner, has been implicated in the cellular response to DNA damage, and appears to play a role in a variety of cellular responses including growth arrest, apoptosis, differentiation, and senescence (1–4). Studies to date have documented a role for p53 in transcriptional activation of target genes in response to extracellular stimuli including DNA damage leading to a cellular response involving either growth arrest or apoptosis. DNA-damaging agents trigger an increase in p53 expression leading to activation of particular target genes most notably that of the cyclin-dependent kinase inhibitor, p21 (5). Consistent with this, cells that lack p21 expression have an impaired p53-dependent response to DNA damage (6, 7). This transcriptional activation of p21 expression is mediated by the interaction of p53 with two response elements located in the p21 promoter (8).

The DNA binding activity of p53 appears to be regulated by the terminal 30 amino acids of the protein. Phosphorylation by either casein kinase II or protein kinase C, acetylation by p300, and binding by a monoclonal antibody 421, or the bacterial dnaK protein all occur within this region of p53 and will activate the ability of p53 to bind to DNA in a sequence-specific manner *in vitro* (9–15). There have been several reports that the ability of p53 in nuclear extracts to bind to DNA requires the presence of antibody 421, leading to the notion that p53 exists in a latent form prior to DNA damage (10, 12). Consistent with this idea, microinjection of the antibody 421 into cells activates p53-dependent expression from reporter constructs (13, 16). Thus, it has been proposed that in untreated cells, the p53 protein exists in a latent state that is unable to bind to DNA and that the ability of p53 to activate target gene expression is not merely dependent on the increase in protein level but also requires post-translational modification of p53 to convert this latent form into a form that is active for DNA binding (12, 17). This notion is supported by studies demonstrating that p53 becomes phosphorylated at particular sites after treatment of cells with DNA-damaging agents (18, 19).

Prior to the cloning of the gene, it was noted that p21 was absent from cyclin/cyclin-dependent kinase complexes in cells lacking functional p53 (20). Other studies have noted that the level of p21 mRNA was much lower in fibroblasts and keratinocytes derived from mice containing a homozygous deletion of p53 as compared with the corresponding cells from mice expressing wild-type p53 (21–24). This suggests that p53 may play a role in the level of p21 expression in untreated, proliferating cells. The experiments presented here tested this idea directly and demonstrate that constitutive expression of the p21 protein in untreated cells is, indeed, dependent on p53 and thus implicate a role for p53 not only in the increased expression of p21 in response to DNA damage leading to either growth arrest or apoptosis but also in the basal level of expression of p21 in normally proliferating cells.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmid p21P contains 2.5-kb[1] of the human *p21* promoter inserted upstream of a firefly luciferase reporter gene in the

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[1] The abbreviations used are: kb, kilobase pair(s); CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; HMBA, *N,N'*-hexamethylene-bisacetamide.

vector pGL2 (Promega). The plasmid p21D2.1 has 2.1 kb at the 5′ end of the promoter sequence removed and lacks the two p53 response elements of the p21 promoter (25). The plasmid pRL-SV40 contains the SV40 enhancer and early promoter upstream of a Renilla luciferase reporter gene (Promega). The plasmid pCMV-hdm2 encodes the human MDM2 protein under control of the cytomegalovirus (CMV) promoter and the plasmid pCMV-p53Ala-143 encodes the tumor-derived mutant human p53 protein containing a missense mutation of valine to alanine at residue 143 (26).

Antibodies and Cells—Saos-2 and WI38 cells were obtained from the American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). MCF7 cells were maintained in RPMI medium containing 10% heat-inactivated FBS and 5 μg/ml insulin. MCF10F cells were grown in 50% DMEM and 50% Ham's F12 medium containing 5% horse serum, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 10 μg/ml insulin, and 500 ng/ml hydrocortisone. PA12-p53BN and PA12-p53EN are cell lines that produce recombinant retrovirus encoding human wild-type p53 or the mutant $p53^{His\text{-}273}$, respectively (27). These cell lines were grown in 10% FBS in DMEM containing high glucose and 400 μg/ml G418 sulfate. The hybridoma cell line producing the mouse monoclonal antibody 1801 was grown in DMEM containing 10% FBS. Hybridoma cell lines expressing the mouse monoclonal antibodies 421 and 419 were grown in 50% DMEM and 50% Fischer's medium containing 10% FBS. Monoclonal antibody 1801 specifically reacts with human p53 (28), 421 recognizes p53 from a variety of species, and 419 recognizes an epitope on the SV40 large T antigen (29). All cell lines were grown at 37 °C in a humid incubator containing 5% CO_2. Antibody against $p21^{WAF1, CIP1}$ was obtained commercially (Ab-1/clone EA10, Calbiochem). For treatment with ultraviolet light, the medium was removed, and the cells were exposed to ultraviolet light using a UV Stratalinker (Stratagene).

Northern Analysis—Total RNA was extracted from 5×10^6 cells using RNAzol (Tel-test), and Northern blot analysis was performed following conventional procedures, using a 2.1-kb full-length human p21 cDNA or human glyceraldehyde-3-phosphate dehydrogenase cDNA (Ambion) as probes.

Immunoblotting—Cells were lysed in a buffer containing 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate (SDS), 50 mM NaCl, 25 mM Tris-HCl, pH 7.5, and the protease inhibitors, phenylmethylsulfonyl fluoride (1 mM), aprotinin (50 μg/ml), and leupeptin (50 μg/ml) for 10 min on ice. Lysates were spun at 15,000 rpm for 10 min, and the supernatant was saved. Protein levels were determined by the bicinchoninic acid protein assay (Pierce). Appropriate amounts of total cellular protein were loaded on 10% SDS-polyacrylamide gels and electrophoresed at 150 V constant voltage for 3 h. Samples were transferred to nitrocellulose paper and probed with the appropriate antibody. Second antibody was a horseradish peroxidase-conjugated goat anti-mouse IgG, and the signal was detected by the enhanced chemiluminescence method (Amersham Pharmacia Biotech).

Establishment of Retrovirally Infected Cells Expressing Ectopic p53—The retrovirus-producing cell lines PA12-BN and PA12-EN were grown to 75% confluence and fed with fresh DMEM containing 10% FBS. After incubation at 37 °C for 16 h, the supernatant was harvested and filtered through a 0.2-μm filter. Old medium was removed from a subconfluent 60-mm dish of Saos-2 cells and replaced with 1 ml of filtered supernatant containing 8 μg/ml Polybrene. Dishes were rocked for 2 h at 37 °C in a humid incubator containing 5% CO_2 and then 3 ml of DMEM containing 10% FBS was added to the dish, and it was further incubated for 48 h. The cells were then trypsinized and replated in a 100-mm dish using DMEM containing 10% FBS and 400 μg/ml G418 sulfate. Cells were fed every 3 days with this same medium. After 2 weeks, the resulting drug-resistant colonies were pooled and passaged.

Incorporation of Bromodeoxyuridine—For detecting replicative DNA synthesis, cells were incubated with 10 μM bromodeoxyuridine for 30 min prior to fixation. The proportion of cells actively synthesizing DNA was quantitated by anti-bromodeoxyuridine immunoanalysis, and the total DNA content was analyzed by staining with propidium iodide as described previously (30). Cells were fixed with 70% ethanol for at least 2 h, resuspended in the following solutions in order: 0.25% paraformaldehyde in phosphate-buffered saline (PBS), 0.5 mg/ml ribonuclease A in PBS, 0.5% Triton X-100 in 0.1 N HCl, and finally distilled water. Samples were then heated at 97 °C for 10 min, immediately placed on ice for additional 10 min, and washed with 0.5% Tween 20 in PBS. The incorporation of bromodeoxyuridine was detected by monoclonal anti-bromodeoxyuridine antibody conjugated to fluorescein isothiocyanate (Becton Dickinson). Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson).

Transfection of Reporter Constructs—MCF7, MCF10F, or Saos-2 cells were transfected using the *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium salts liposomal transfection reagent (DOTAP, Boehringer Mannheim). One confluent 100-mm dish of cells was split into three 6-well dishes and incubated for 24 h. Cells were fed with complete medium containing serum and incubated for an additional 3 h. *N*-[1-(2,3-Dioleoyloxy)propyl]-*N,N,N*-trimethylammonium salts/DNA mixtures containing 2 μg of the relevant reporter plasmid plus 50 ng of the p53 expression plasmid or an equal amount of an empty vector plasmid were prepared according to the manufacturer's instructions and incubated at room temperature for 15 min. Serum-free medium was then added to the mixtures and used to replace the media in the wells. The dishes were incubated at 37 °C for 3 h, after which the transfection mix was removed and replaced with complete medium containing serum. After 48 h, the 6-well plates were placed on ice and washed once with PBS. The cells were then lysed by scraping into 120 μl of Reporter Buffer (Promega Luciferase Assay System), and samples were spun for 1 min at 14,000 rpm at 4 °C. Total protein concentration was determined using a commercially available assay (Bio-Rad). 40 μl of each sample was warmed to room temperature and mixed with luciferase assay substrate that was reconstituted with Luciferase Assay Buffer (Promega). Light emission was determined in a TD-20e luminometer (Turner).

Preparation of Nuclear and Cytosolic Extracts—Nuclear and cytosolic extracts were performed as described by Graeber *et al.* (31). Cells were homogenized in 10 mM Tris-HCl, pH 7.4, containing 10 mM NaCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, and 100 μM Na_3VO_4 and spun at 10,000 rpm for 30 s. The supernatant was saved as the cytosolic extract. The pellet was repacked by spinning at 14,000 rpm for 1 min and then nuclei were suspended in a nuclear extraction buffer (20 mM Hepes, pH 7.5, containing 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$,, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 50 μg/ml aprotinin), incubated at 4 °C for 1 h, and spun at 14,000 rpm for 10 min. This supernatant was saved as the nuclear extract. Lactate dehydrogenase activity was assayed according to Ramirez *et al.* (32), and histone levels were determined by immunoblotting using an anti-histone antibody that reacts with an epitope that is present on all five histone proteins (H11-4, Boehringer Mannheim). Such assays showed less than 10% cross-contamination between cytosolic and nuclear extracts.

Electrophoretic Mobility Shift Assays—The specific probe that was used for binding, TCGAGCCGGGCATGTCCGGGCATGTCCGGGCATGTC, contains the high affinity binding sequence identified by Halazonetis *et al.* (11) named by them BC or BB.9. In the competition experiments, the nonspecific oligonucleotide (referred to as Sens-1), TCGAAGAAGACGTGCAGGGACCC, was used. Complementary single-stranded oligonucleotides were annealed by incubation at 95 °C for 4 min, 65 °C for 10 min, and then gradually brought to room temperature. Ends were filled using the Klenow fragment of DNA polymerase to produce a labeled double-stranded oligonucleotide. Appropriate amounts of extracts (1–7 μl) were mixed with 1 ng of labeled double-stranded oligonucleotide in a total reaction mixture of 30 μl containing 6 μl of 5× electrophoretic mobility shift assay buffer (100 mM Hepes, pH 7.9, 0.5 mM EDTA, 50% glycerol, 10 mM $MgCl_2$), 1.5 μl of 40 mM spermidine, 1.5 μl of 10 mM dithiothreitol, 1 μl of 500 μg/ml double-stranded poly(dI/dC), and 5–13 μl of water with a final salt concentration of 85 mM. The amount of total protein per reaction was normalized, and the reactions were carried out at room temperature for 30 min. For antibody supershift analysis, 2 μl of the appropriate undiluted hybridoma supernatant was added. His-tagged human p53 was produced by infection of insect cells with a recombinant baculovirus and purified by nickel-agarose chromatography and used as a positive control (52). Samples were electrophoretically separated on a native 4% polyacrylamide gel at 4 °C at 200 V for 2 h. After drying, gels were exposed to Kodak XAR film at −70 °C with an intensifying screen.

RESULTS

MCF10F Cells Express Detectable Levels of p21 mRNA and Protein, Whereas p53-Negative Saos-2 Cells Do Not—Previous studies have noted that either fibroblasts or keratinocytes from mice that were homozygously deleted for p53 expressed lower basal levels of p21 mRNA as compared with fibroblasts or keratinocytes from mice expressing both alleles of the wild-type p53 gene (21–24). To characterize further a role for p53 in the basal level of expression of p21, the p53-negative cell line

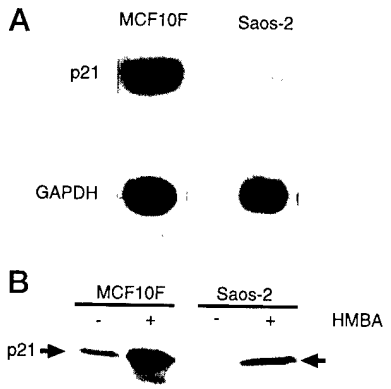

FIG. 1. **MCF10F cells express detectable levels of p21 mRNA and protein, whereas p53-negative Saos-2 cells do not.** *A,* RNA was extracted from wild-type p53-expressing MCF10F and p53-negative Saos-2 cells, and Northern analysis was performed as described under "Experimental Procedures." Blots were probed with either a cDNA for human p21 or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as indicated. *B,* MCF10F or Saos-2 cells were left untreated or treated with 10 mM HMBA for 16 h. Whole cell extracts were prepared and subjected to electrophoresis and subsequent immunoblotting using an anti-p21 antibody as described under "Experimental Procedures."

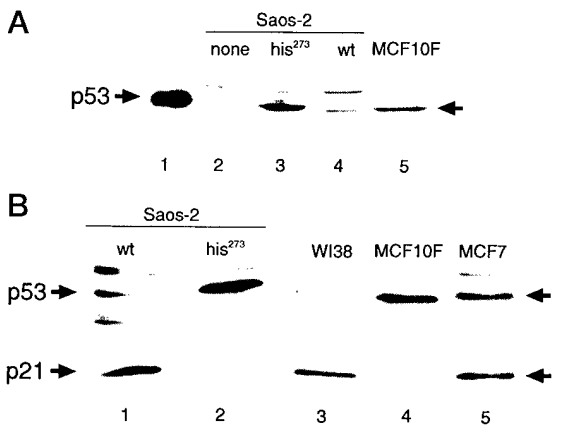

FIG. 2. **Retroviral infection of Saos-2 cells restores expression of p53 and p21.** *A,* Saos-2 cells were infected with a recombinant retrovirus expressing either wild-type p53 or the mutant $p53^{His\text{-}273}$. Selection was performed in G418 sulfate, and drug-resistant pools of cells were obtained. Equivalent amounts of total cellular extract of parent Saos-2 cells (*lane 2*), the His^{273}-expressing Saos-2 cells (*lane 3*), the wild-type (*wt*) p53-expressing Saos-2 cells (*lane 4*), or MCF10F cells (*lane 5*) were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with an anti-p53 antibody. Purified human p53 is shown in *lane 1. B,* equivalent amounts of total cellular extract of Saos-2 cells expressing either wild-type p53 (*lane 1*) or the His^{273} mutant p53 (*lane 2*) were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with either an anti-p53 or anti-p21 antibody.

Saos-2 was compared with the wild-type p53-expressing cell line MCF10F. Total RNA was extracted from each cell line, and Northern analysis was performed. The p53-negative Saos-2 cell line expressed low levels of p21 mRNA as compared with the wild-type p53-expressing MCF10F cells (Fig. 1*A*). Total cellular extracts of each cell line were subjected to SDS-polyacrylamide gel electrophoresis and subsequent immunoblotting with an anti-p21-specific antibody (Fig. 1*B*). MCF10F cells expressed a detectable level of p21, whereas the level of p21 expression in Saos-2 cells was undetectable. To confirm that Saos-2 cells retained the ability to synthesize p21, both MCF10F and Saos-2 cells were treated with 10 mM *N,N'*-hexamethylenebisacetamide (HMBA). HMBA is a non-retinoid, differentiating agent that has previously been shown to induce p21 expression in a p53-independent manner (24). Treatment of Saos-2 cells with HMBA induced expression of p21 demonstrating that Saos-2 cells retained the ability to synthesize p21. Thus, both the level of protein and messenger RNA for p21 were much higher in the p53-expressing MCF10F cells than in the p53-negative Saos-2 cells.

Retroviral Infection of Saos-2 Cells Restores Expression of p53 and p21—Previous studies have shown that restoration of wild-type p53 expression through transfection of a suitable expression plasmid did not allow for establishment of stable cell lines expressing wild-type p53 (33–37). This was presumably due to the fact that plasmid transfection results in a high level of expression of p53 which is incompatible with cell proliferation. Chen *et al.* (27) utilized recombinant retroviral infection to restore a level of wild-type p53 expression in Saos-2 cells that was comparable to that seen in normal cells and that was compatible with continued proliferation of these cells. To that end, Saos-2 cells were infected with recombinant retroviruses expressing either wild-type human p53 or the mutant human $p53^{His\text{-}273}$, and pools of G418 sulfate-resistant cells were established. Immunoblotting of whole cell extracts from these drug-resistant pools demonstrated that both wild-type (Fig. 2*A, lane 4*) and mutant (Fig. 2*A, lane 3*) p53 expression could be detected in comparison to the parent cells which are p53-negative (Fig. 2*A, lane 2*). Furthermore, the pool of Saos-2 cells expressing wild-type p53 expressed a level that is comparable to the endogenous p53 level in MCF10F cells (Fig. 2*A, lane 5*). Consistent with previous observations, this level of expression of wild-type p53 that was obtained using recombinant retroviral infection was sufficiently low to allow the cells to continue to grow (Table I). These drug-resistant pools were labeled with bromodeoxyuridine and subjected to flow cytometric analysis to demonstrate that they were actively incorporating DNA. Indeed, the pools expressing wild-type p53 had a similar percentage of bromodeoxyuridine-positive cells as the parent cell line, the pool expressing mutant p53, or the wild-type p53 expressing cell lines WI38, MCF10F, or MCF7 (Table I). These pools were then examined for the level of p21 expression. Immunoblotting of whole cell extracts demonstrated that Saos-2 cells expressing wild-type but not mutant p53 expressed a level of p21 that was comparable to that of WI38 or MCF7 cells and, in fact, was greater than that seen with MCF10F cells (Fig. 2*B*). Thus, restoration of expression of wild-type p53 in a p53-negative cell line also restored a basal level of expression of p21.

Constitutive Expression of Luciferase Reporter Constructs Containing the p21 Promoter Is p53-dependent—The observation that reintroduction of p53 expression in Saos-2 cells restored a basal level of p21 expression (Fig. 2) suggests that in the absence of DNA damage, p53 regulates expression of p21. To test directly this notion and to confirm that such regulation is at the level of transcription, wild-type p53-expressing MCF7 cells were transfected with a luciferase reporter construct containing 2.4 kb of the human p21 promoter. To determine whether the basal level of expression that is observed was p53-dependent, an expression plasmid for the human MDM2 protein was co-transfected with the reporter. Mdm2 binds to p53 and inhibits its transcriptional activity, apparently by targeting the p53 protein for degradation (19, 38–41). Transfection of MCF7 cells with a luciferase reporter construct under control of the p21 promoter, p21P, confirmed a basal level of activation of the p21 promoter (Figs. 3 and 4*A*). Co-transfection of an expression plasmid encoding human MDM2 protein caused repression of that basal level of expression (Fig. 3 and 4*A*). Deletion of the p53-binding sites from this reporter (p21P 2.1) resulted in a complete loss of basal luciferase activity (Fig. 3). In contrast, co-transfection of the plasmid encoding Mdm2

TABLE I
Incorporation of bromodeoxyuridine into the DNA of various cell lines

Cell line	Bromodeoxyuridine positive cells[a]
	%
WI38	12
MCF10F	22
MCF7	27
Saos-2	24
Saos-2 (wt)[b]	23
Saos-2 (His^{273})[b]	24

[a] Cells were labeled with 1 μM bromodeoxyuridine for 30 min, fixed, and processed for flow cytometric analysis as described under "Experimental Procedures."

[b] Pools of G418 sulfate-resistant Saos-2 cells that had been infected with recombinant retrovirus expressing either wild-type (wt) or mutant (His^{273}) p53 proteins.

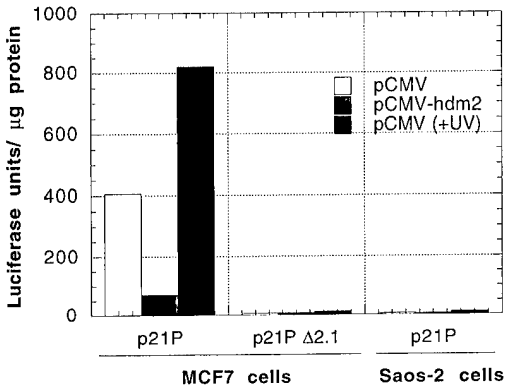

FIG. 3. **Deletion of the p53 response elements results in loss of basal expression of a luciferase reporter containing the p21 promoter.** MCF7 cells or Saos-2 cells were transfected as described under "Experimental Procedures" with 2 μg of p21P or p21P 2.1 and either 50 ng of pCMV or pCMV-hdm2 as indicated. 18 h prior to lysis, a set of wells containing only pCMV were treated with 50 J/m^2 of ultraviolet light, indicated by *+UV*. 48 h after transfection, cells were washed, lysed, and assayed for luciferase activity and total protein levels as described under "Experimental Procedures." The indicated values are from a representative experiment that had been performed in duplicate.

into the p53-negative Saos-2 cells had no effect on the low level of luciferase activity seen in these cells from the same reporter construct (Figs. 3 and 4*C*). Treatment of MCF7 cells with ultraviolet light induced expression of the full-length p21 promoter construct but not the construct that lacks the p53-binding sites (Fig. 3). Furthermore, treatment of Saos-2 cells with ultraviolet light had no effect on the expression of luciferase from the full-length p21 promoter reporter construct (Fig. 3). These latter results are consistent with the fact that MCF7 cells express a functional wild-type p53 protein (42–44).[2]

The ability of the Mdm2 protein to repress basal expression from a luciferase reporter containing the full-length p21 promoter was confirmed in the wild-type p53-expressing cell line MCF10F (Fig. 4*B*). Similar to MCF7 cells (Fig. 4*A*) and in contrast to the p53-negative Saos-2 cells (Fig. 4*C*), co-transfection of the expression plasmid for Mdm2 protein inhibited the constitutive level of expression that is seen with the reporter p21P. The apparently low level of basal expression seen in Saos-2 cells further strengthens the notion that the basal level of expression is p53-dependent. To confirm that this is indeed the case and not a reflection of different transfection efficiencies by the various cell lines, MCF7, MCF10F, and Saos-2 cells were co-transfected with p21P and an additional reporter that contains Renilla luciferase under the control of the SV40 enhancer and early promoter. This latter reporter construct was used to normalize for transfection efficiency. The results of this analysis demonstrated that, indeed, both MCF7 and MCF10F have a much higher basal level of expression of p21P than the p53-negative Saos-2 cells (Fig. 4*D*).

To provide further evidence that the basal expression that is seen upon transfection of MCF7 cells with p21P is p53-dependent, MCF7 and Saos-2 cells were co-transfected with p21P and increasing amounts of an expression plasmid encoding a dominant-negative mutant $p53^{Ala\text{-}143}$ (45). Increasing amounts of the $p53^{Ala\text{-}143}$ expression plasmid repressed the basal level of expression of p21P in MCF7 cells but not in Saos-2 cells (Fig. 5). Thus, co-transfection of either a dominant-negative p53 or the human MDM2 protein, both of which are capable of inhibiting the endogenous wild-type p53, caused repression of the basal level of expression from the reporter construct containing the p21 promoter. In contrast, co-transfection of the dominant-negative p53 or the human MDM2 protein into the p53-negative Saos-2 cells had no effect on the low level of luciferase activity seen in these cells from the same reporter construct.

p53 in Nuclear Extracts of Untreated Cells Is Capable of Binding to DNA in a Sequence-specific Manner—The data presented thus far implicate a role for endogenous p53 in transcriptional activation of the p21 promoter in untreated proliferating cells. If this is the case, then this endogenous p53 should be capable of binding to DNA. To examine this, electrophoretic mobility shift assays utilized nuclear extracts from three different wild-type p53-expressing cell lines, WI38, MCF10F, and MCF7 to demonstrate that, indeed, the endogenous p53 was capable of binding to DNA prior to DNA damage. All three cell lines were either untreated or treated with 50 J/m^2 of ultraviolet light and then were fractionated into nuclear and cytosolic extracts. Immunoblotting for p53 demonstrated that prior to DNA damage, WI38 and MCF10F cells express a p53 that was primarily localized to the nucleus (Fig. 6, *lanes 1* and *2*, and *5* and *6*), whereas the p53 in untreated MCF7 cells was present primarily in the cytoplasm with a low level detectable in the nuclear fraction (Fig. 6, *lanes 9* and *10*). After treatment with ultraviolet light, the p53 levels increased substantially in all three cell lines (Fig. 6, *lanes 3–4, 7* and *8*, and *11–12*). Extracts were assayed for a cytoplasmic marker, lactose dehydrogenase,[3] as described under "Experimental Procedures" and were immunoblotted for a nuclear marker, histone H1 (Fig. 6, *lower panel*). It is estimated that there was less than 10% cross-contamination between the cytoplasmic and nuclear extracts using these markers.

Nuclear and cytoplasmic extracts from untreated and UV-treated MCF cells were normalized for level of p53 protein and used in an electrophoretic mobility shift assay using a consensus p53-binding site as radiolabeled probe (Fig. 7). Both the nuclear and cytoplasmic extracts from UV-treated cells demonstrate a shifted complex with a similar mobility as that of purified p53 (Fig. 7, *lanes 11* and *14*). The p53-specific antibody 1801 efficiently supershifted this complex, whereas the nonspecific antibody 419 did not (Fig. 7, *lanes 12–13* and *15–16*). The extracts from untreated cells contained a shifted complex with a similar mobility as purified p53 (Fig. 7, *lanes 5* and *8*). Incubation with the p53-specific antibody 1801 produced a slower migrating complex but did not substantially affect the original protein-DNA complex (Fig. 7, *lanes 6* and 9). The nonspecific antibody 419 had no such effect (Fig. 7, *lanes 7* and *10*). This result suggests that there is p53 in these extracts which is capable of binding to DNA, but there is also an addi-

[2] H.-Y. Tang, K. Zhao, J. Langer, S. Waxman, and J. J. Manfredi, submitted for publication.

[3] H.-Y. Tang and J. J. Manfredi, unpublished observations.

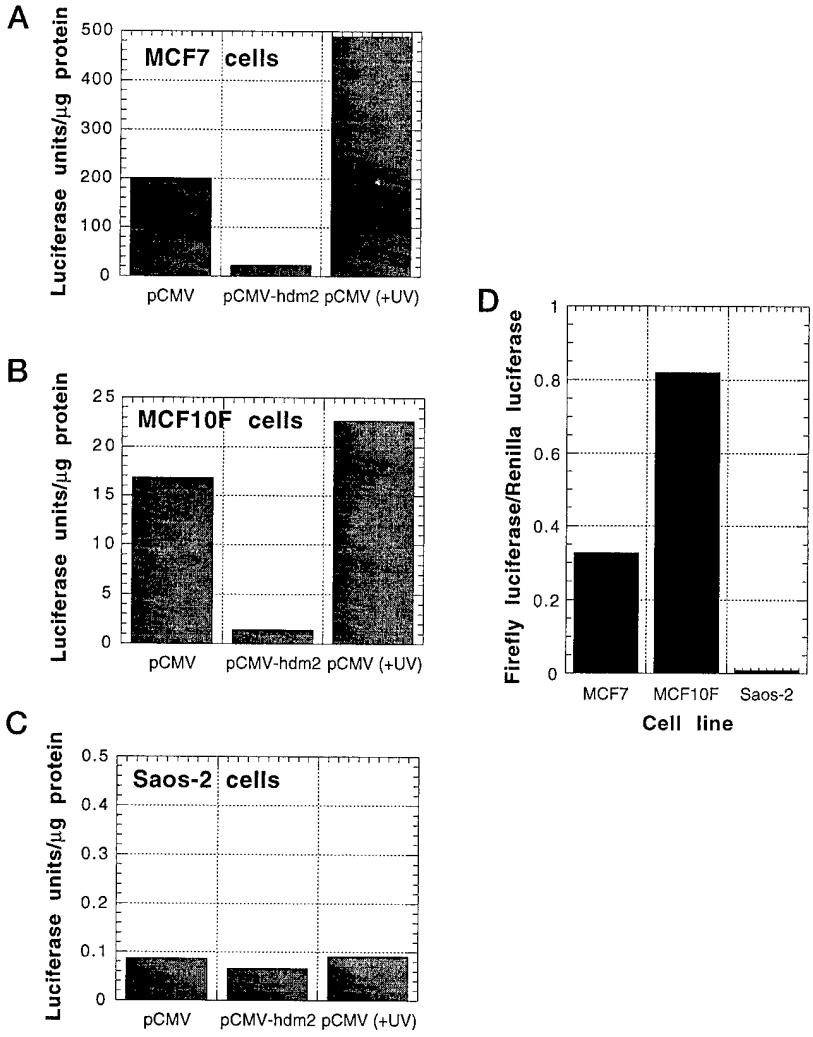

FIG. 4. **Ectopic expression of the human MDM2 protein represses basal expression of a luciferase reporter containing the p21 promoter.** MCF7 cells (*A*), MCF10F cells (*B*), or Saos-2 cells (*C*) were transfected as described under "Experimental Procedures" with 2 μg of p21P and either 50 ng of pCMV or pCMV-hdm2 as indicated. 48 h later cells were washed, lysed, and assayed for luciferase activity and total protein levels as described under "Experimental Procedures." 18 h prior to lysis, a set of wells containing only pCMV were treated with 50 J/m^2 of ultraviolet light, indicated by *+UV*. The *bars* represent the average of three independent experiments that had been performed in duplicate. *D*, MCF7 cells, MCF10F cells, or Saos-2 cells were transfected as described under "Experimental Procedures" with 2 μg of p21P and 50 ng of pRL-SV40. 48 h later cells were washed, lysed, and assayed for luciferase activity and total protein levels as described under "Experimental Procedures." The *bars* represent the average of two independent experiments that had been performed in duplicate.

tional DNA-binding protein that is distinct from p53 which produces a shifted complex of similar mobility as purified p53. To test this, nuclear extracts of untreated MCF7 cells were immunoprecipitated with an anti-p53 antibody to clear all detectable p53 protein from the extract as determined by immunoblotting.[4] This extract was compared in an electrophoretic mobility shift assay with a comparable extract that had been immunoprecipitated with the nonspecific antibody 419 as a control as well as a nuclear extract for UV-treated cells that had similarly been immunoprecipitated with 419 (Fig. 8*A*). Incubation of the p53-specific antibodies 1801 or 421 with untreated nuclear extracts resulted in the detection of slow migrating DNA-protein complexes that were not present in the absence of antibody (Fig. 8*A*, *lanes 2* and *3*). These slower migrating complexes were not seen in an extract that had been cleared of p53 by immunoprecipitation but were present in extract that had been immunoprecipitated with a nonspecific antibody (Fig. 8*A*, *lanes 5* and *6* and *8* and *9*). Clearing of p53 from the extract had no effect on the protein-DNA complex that migrated to a similar mobility as the p53-DNA complex, confirming that there is a DNA-binding protein in the extract which is distinct from p53. Extracts from UV-treated cells were used to identify the p53-DNA complex that was confirmed by its ability to be efficiently supershifted by both 1801 and 421 (Fig. 8*A*, *lanes 11* and *12*). To determine that the binding that was seen was sequence-specific, competition experiments were performed (Fig. 8*B*). Nuclear extract from untreated MCF7 cells was used in an electrophoretic mobility shift assay in the presence of increasing amounts of either specific probe, BB.9, or a nonspecific probe, Sens-1. Since it was difficult to detect the p53-DNA complex in the absence of antibody, the competition was also performed in the presence of the p53-specific antibody 1801. Increasing amounts of unlabeled BB.9 (Fig. 8*B*, *lanes 9–11*) competed well for the binding to 1801-supershifted complexes, whereas increasing amounts of Sens-1 (Fig. 8*B*, *lanes 12–14*) did not. The faster migrating complex that did not appear to contain p53 was similarly competed suggested that the binding of this protein is also sequence-specific (Fig. 8*B*).

The untreated MCF7 cells used in these experiments expressed an endogenous p53 that is localized primarily in the cytoplasm (Fig. 6). The DNA binding results were subsequently confirmed in WI38 and MCF10F cells in which the p53 is primarily nuclear prior to DNA damage (Fig. 6). Nuclear and cytoplasmic extracts from untreated and UV-treated WI38 and MCF10F cells were normalized for level of p53 protein and used in similar electrophoretic mobility shift assays (Fig. 9). Both the nuclear and cytoplasmic extracts from UV-treated cells from both cell lines demonstrated a shifted complex with a similar mobility as that of purified p53 (Fig. 9, *A* and *B*, *lanes 11* and *14*). The p53-specific antibody 1801 efficiently supershifted this complex, whereas the nonspecific antibody 419 did not (Fig. 9, *A* and *B*, *lanes 12* and *13*, and *15* and *16*). As seen with extracts from MCF7 cells, the extracts from untreated WI38 or MCF10F cells contained a shifted complex with a similar mobility as purified p53 (Fig. 9*A*, *lane 5*, and 9*B*, *lanes 5* and *8*). Incubation with the p53-specific antibody 1801 pro-

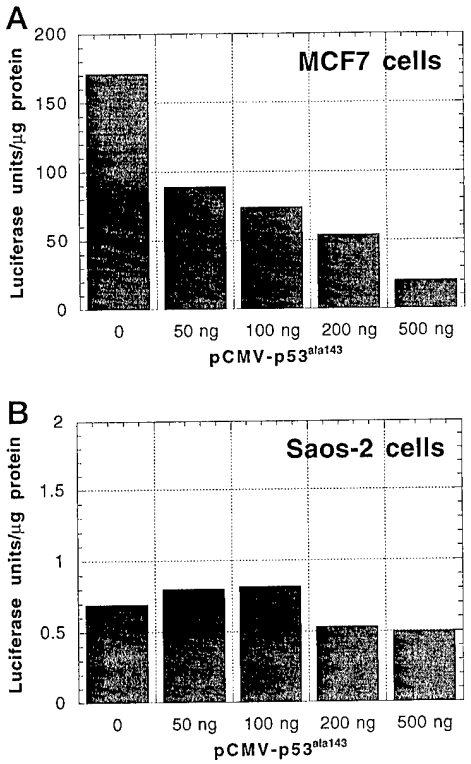

FIG. 5. **Ectopic expression of the mutant human p53$^{Ala-143}$ protein represses basal expression of a luciferase reporter containing the p21 promoter in a dose-dependent manner.** MCF7 cells (*A*) or Saos-2 cells (*B*) were transfected as described under "Experimental Procedures" with 2 μg of p21P alone or in the presence of 50, 100, 200, or 500 ng of pCMV-p53$^{Ala-143}$ as indicated. 48 h later cells were washed, lysed, and assayed for luciferase activity and total protein levels as described under "Experimental Procedures." The indicated values are from a representative experiment that had been performed in duplicate.

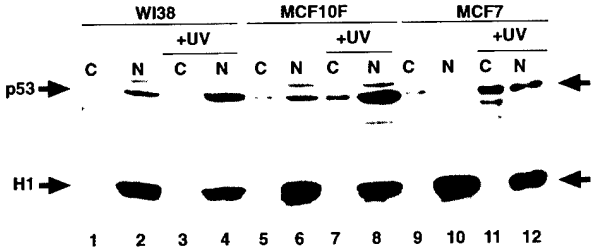

FIG. 6. **Biochemical fractionation demonstrates that nuclear p53 levels increase upon UV treatment in WI38, MCF10F, and MCF7 cells.** WI38, MCF10F, or MCF7 cells were untreated or treated with 50 J/m^2 of ultraviolet light (+*UV*) and then incubated at 37 °C for 20 h prior to fractionation into cytosolic (*C*) or nuclear extracts (*N*) as described under "Experimental Procedures." Samples were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with the anti-p53 monoclonal antibody 1801 or a polyclonal antibody directed against histone H1 as indicated. The protein level for the cytosolic samples ranged from 60 to 120 μg. For each cell line, the cytosolic and nuclear samples were normalized so that the loaded samples were obtained from an equivalent number of cells.

duced a slower migrating complex but did not substantially affect the original protein-DNA complex (Fig. 9, *A* and *B*, *lanes 6* and *9*). The nonspecific antibody 419 had no such effect (Fig. 9, *A* and *B*, *lanes 7* and *10*). These results are consistent with those seen with extracts of MCF7 cells demonstrating that there is p53 in these extracts that is capable of binding to DNA but there is also an additional DNA-binding protein that is distinct from p53 that produces a shifted protein-DNA complex of a similar mobility as p53. The presence of a slower migrating shifted complex that was induced by the p53-specific antibody

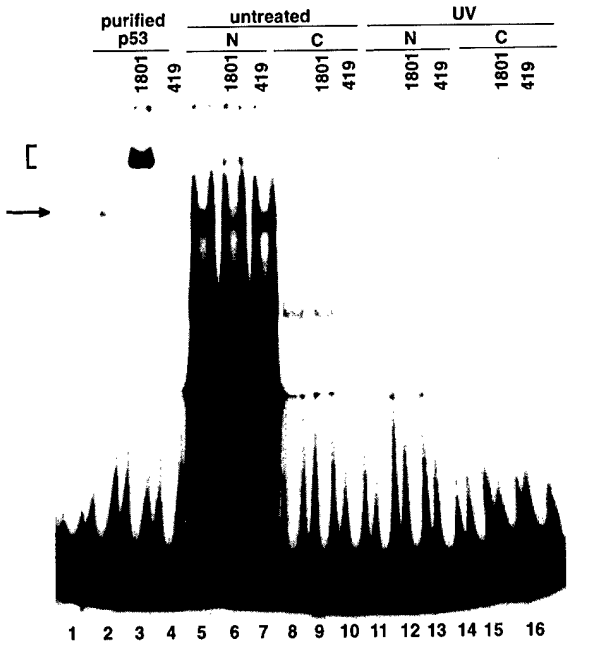

FIG. 7. **p53 in nuclear extracts of untreated MCF7 cells binds to DNA in an electrophoretic mobility shift assay.** Nuclear and cytosolic extracts were prepared from MCF7 cells that were untreated (*lanes 5–10*) or 24 h after treatment with 50 J/m^2 of ultraviolet light (*lanes 11–16*). Electrophoretic mobility shift assay was performed as described under "Experimental Procedures." 1 ng of radiolabeled probe (BB.9) was incubated in the absence of protein (*lane 1*), or in the presence of 0.5 μg of human purified human p53 (*lanes 2–4*) or the appropriate amount of nuclear (*lanes 5–7* and *11–13*) or cytosolic (*lanes 8–10* and *14–16*) extracts as indicated. The level of p53 loaded in cellular extracts was normalized by adjusting the total protein. Incubations were performed either with no addition (*lanes 1, 2, 5, 8, 10,* and *14*) or in the presence of either 1801 (anti-p53 antibody, *lanes 3, 6, 9, 12,* and *15*) or 419 (anti-SV40 large T antigen, *lanes 4, 7, 10, 13,* and *16*). The *arrow* indicates the position of the p53-DNA complex, and the *bracket* indicates the position of the supershifted p53-DNA-antibody complex.

1801 but not the nonspecific antibody 419 in untreated extracts from all three cell lines is consistent with the notion that the endogenous p53 in these cells is capable of binding to DNA in untreated, proliferating cells.

DISCUSSION

Previous studies have suggested that p53 exists in a latent or inactive form in untreated cells and that upon DNA damage not only does the p53 level increase but the p53 itself is modified in some way to activate it for DNA binding and transcriptional activation. Reintroduction of p53 into the p53-negative cell line Saos-2 restored a constitutive level of expression of the cyclin-dependent kinase inhibitor p21 (Fig. 2). A similar result has been reported upon similar retroviral infection of a p53-negative peripheral neuroepithelioma cell line (46). These results suggest that the endogenous p53 in untreated, proliferating cells may be capable of transcriptionally regulating p21 expression. Indeed, experiments involving transfection of wild-type p53-expressing MCF7 cells with a luciferase reporter construct containing the p21 promoter have confirmed that this is the case (Figs. 3–5). A reporter containing the full-length p21 promoter but not a promoter construct in which the p53 response elements have been deleted demonstrated a basal level of expression in MCF7 or MCF10F cells but not in p53-negative Saos-2 cells (Fig. 4). This basal level of expression was inhibited by coexpression of either a dominant-negative p53 or the human MDM2 protein (Figs. 3–5). These results imply that the endogenous p53 in MCF7 or MCF10F cells is capable of binding to DNA. This was directly tested through the use of electro-

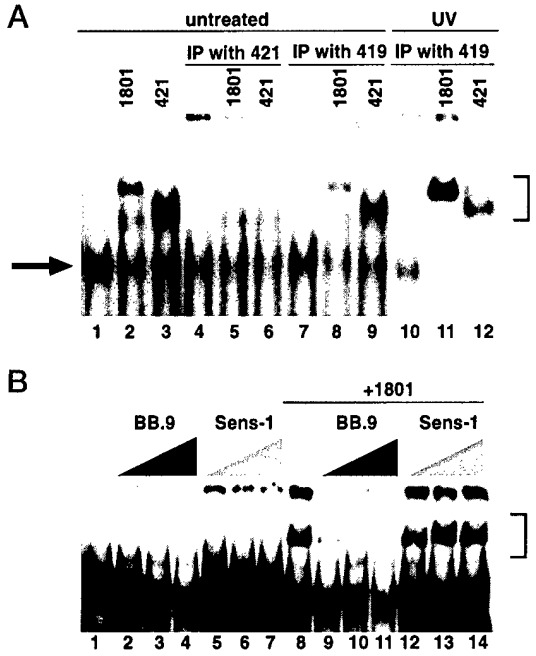

FIG. 8. **p53 in nuclear extracts of untreated MCF7 cells binds to DNA in a sequence-specific manner.** *A*, nuclear extracts of MCF7 cells that were untreated (*lanes 1–9*) or treated with 50 J/m^2 ultraviolet light (UV, *lanes 10–12*) were used directly (*lanes 1–3*) or immunoprecipitated with either an anti-p53 antibody 421 (*lanes 4–6*) or an anti-SV40 large T antigen antibody 419 (*lanes 7–12*). The resulting supernatants were used in an electrophoretic mobility shift assay using 1 ng of radiolabeled probe (BB.9). Incubations were performed either in the absence (*lanes 1, 4, 7,* and *10*) or presence of 1801 (anti-p53 antibody, *lanes 2, 5, 8,* and *11*) or presence of 421 (anti-p534 antibody, *lanes 3, 6, 9,* and *12*). The *arrow* to the *left* indicates the position of the p53-DNA complex, and the *bracket* to the *right* indicates the position of the supershifted p53-DNA-antibody complex. *B*, 1 ng of radiolabeled probe (BB.9) was incubated with an appropriate amount of nuclear extract alone (*lanes 1* and *8*), or in the presence of increasing amounts of unlabeled BB.9 oligonucleotide (*lanes 2–4* and *9–11*) or increasing amounts of a nonspecific oligonucleotide, Sens-1 (*lanes 5–7* and *12–14*). Incubations were performed either in the absence (*lanes 1–7*) or presence of 1801 (anti-p53 antibody, *lanes 8–14*). The *bracket* to the *right* indicates the position of the supershifted p53-DNA-antibody complex.

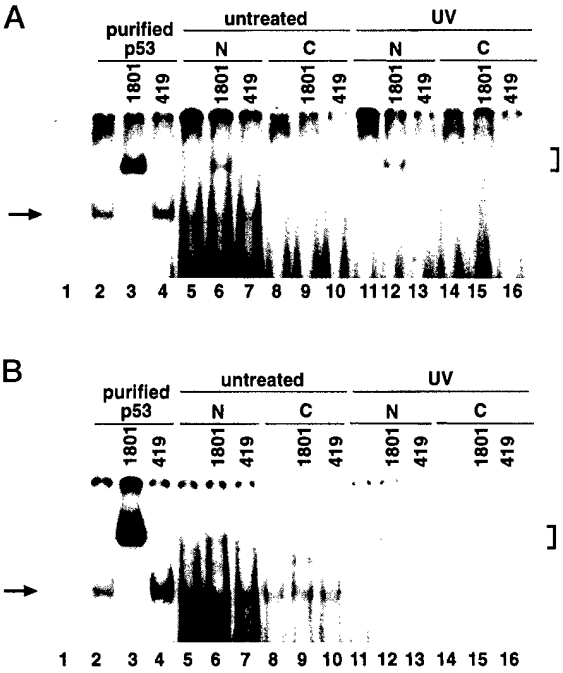

FIG. 9. **p53 in nuclear extracts of untreated WI38 or MCF10F cells binds to DNA in an electrophoretic mobility shift assay.** Nuclear and cytosolic extracts were prepared from WI38 (*A*) or MCF10F (*B*) cells that were untreated (*lanes 5–10*) or 24 h after treatment with 50 J/m^2 of ultraviolet light (*lanes 11–16*). Electrophoretic mobility shift assay was performed as described under "Experimental Procedures." 1 ng of radiolabeled probe (BB.9) was incubated in the absence of protein (*lane 1*), or in the presence of 0.5 μg of human purified human p53 (*lanes 2–4*) or the appropriate amount of nuclear (*lanes 5–7* and *11–13*) or cytosolic (*lanes 8–10* and *14–16*) extracts as indicated. The level of p53 loaded in cellular extracts was normalized by adjusting the total protein. Incubations were performed either with no addition (*lanes 1, 2, 5, 8, 10,* and *14*), or in the presence of either 1801 (anti-p53 antibody, *lanes 3, 6, 9, 12,* and *15*) or 419 (anti-SV40 large T antigen, *lanes 4, 7, 10, 13,* and *16*). The *arrow* to the *left* indicates the position of the p53-DNA complex, and the *bracket* to the *right* indicates the position of the supershifted p53-DNA-antibody complex.

phoretic mobility shift assays (Figs. 7–9). Although untreated extracts from either MCF7, MCF10F, or WI38 cells contain a DNA-binding protein other than p53 which is capable of shifting a specific radiolabeled probe representing a p53 consensus binding site, experiments in the presence of a p53-specific monoclonal antibody 1801 clearly demonstrated that p53 in these extracts could bind to DNA in a sequence-specific manner (Figs. 7–9). Taken together, these results demonstrate that endogenous p53 in untreated, proliferating cells is capable of binding DNA and activating transcription. Thus, p53 is implicated as playing a role in constitutive expression of a particular target gene, that of the cyclin-dependent kinase inhibitor p21, in proliferating cells in the absence of treatment with DNA-damaging agents.

Attempts to demonstrate the ability of p53 from nuclear extracts to bind DNA have often relied on the use of monoclonal antibody 421. The epitope for 421 is located in the carboxyl end of p53, a region that has been suggested to have a negative effect on the sequence-specific DNA binding of p53 (10–15). Studies *in vitro* have demonstrated that 421 can stimulate the binding of wild-type p53 and in some cases can activate select tumor-derived mutant p53 proteins that are incapable of binding to DNA in the absence of antibody (10–12, 14, 47). In the electrophoretic mobility shift analyses performed here, care was taken to avoid the use of monoclonal antibody 421 for these reasons. The presence of a BB.9-binding protein that is not p53 in the untreated extracts made it necessary to supershift gel shift complexes containing p53 in order to detect the complex of p53 with the probe (Figs. 7–9). Use was made of the antibody 1801 that has an epitope on p53 near the amino-terminal end of the protein (28). Studies have demonstrated that in contrast to 421, 1801 does not restore DNA binding activity to mutant p53 proteins (48). It does, however, exert an enhancing effect on the ability of p53 to bind to DNA, but this is due to the ability of 1801 to stabilize p53 against thermal denaturation that occurs during the incubations that are performed to detect specific DNA binding (48, 49). Hence, it is unlikely that 1801 is conferring on the p53 in untreated cell extracts an ability to bind to DNA that this p53 would not otherwise have. Thus, the supershifted complexes produced by incubation with 1801 do indeed reflect the ability of endogenous p53 in the cell to interact in a specific manner with DNA.

Studies utilizing mice that have been homozygously deleted for p53 have shown that the majority of tissues express p21 in a p53-independent manner. Only in the spleen was there substantial differences in p21 expression between p53-null and p53-expressing animals (24). These results indicate that in addition to the p53-dependent mechanism demonstrated here, there must also be p53-independent mechanisms for the regulation of basal levels of p21 expression.

Nevertheless, treatment of cells with DNA-damaging agents clearly inhibits cellular proliferation and involves an increase

in p21 expression that is p53-dependent (1–3). It is reasonable to expect that cells growing *in vitro* experience a low level of oxidative DNA damage, and there may be damage resulting from errors during DNA synthesis. This low level of DNA damage may be responsible for activation of a subset of the p53 protein in the cell leading to transcriptional activation of particular target genes at a low level. Indeed it is likely that cells *in vivo* are subjected to similar low levels of DNA damage. Thus, the results presented here do not necessarily contradict the notion that upon DNA damage, p53 may, in fact, be modified in some way to increase its ability to bind DNA and transcriptionally activate target genes. Post-translational modification of p53 upon DNA damage of cells has been documented, and some studies suggest that this modification may be necessary to achieve the full induction of p53 target gene expression that is seen after treatment with DNA-damaging agents (18, 19).

The human gene for thrombospondin-1 has previously been identified as a target for transcriptional activation by p53 (50). Studies leading to this observation demonstrated that fibroblasts from early passage cells obtained from Li-Fraumeni patients constitutively expressed thrombospondin-1, but later passage cells that had lost expression of p53 no longer secreted thrombospondin-1. Transfection studies demonstrated that the thrombospondin-1 promoter was a target for transcriptional activation by p53, although a specific binding site for p53 in this promoter has yet to be identified (50). As thrombospondin-1 has anti-angiogenic activity, the observation that its constitutive expression is p53-dependent is consistent with the role of p53 as a tumor suppressor. The experiments reported here with the p21 promoter confirm the ability of p53 to transcriptionally regulate constitutive expression of particular target genes in proliferating cells, thereby suggesting a mechanism that is consistent with the report of p53-dependent expression of thrombospondin-1 in proliferating human fibroblasts (50).

Chen *et al.* (27) demonstrated that expression of wild-type but not mutant p53 in Saos-2 cells by retroviral infection will inhibit the ability of these cells to grow in soft agar and grow as tumors in nude mice. Similar results were obtained by retroviral infection of a p53 null peripheral neuroepithelioma cell line (51). As with the studies reported here (Fig. 2 and Table I), the level of p53 that was expressed in the cells in both these studies was sufficiently low to allow the cells to continue to proliferate albeit at a slower rate than the parent cell lines (27, 51). The implication of these observations is that this low level of p53 is capable of suppressing the oncogenic phenotype in these cells suggesting that the ability of p53 to transcriptionally regulate constitutive expression of select target genes may, therefore, play a role in its ability to function as a tumor suppressor. The increased tumorigenicity that results from the loss of basal expression of these p53-dependent targets would then contribute to the selective pressure for the loss of wild-type p53 function in human tumors.

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One Mechanism for Cell Type-specific Regulation of the *bax* Promoter by the Tumor Suppressor p53 Is Dictated by the p53 Response Element*

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Key to the function of the tumor suppressor p53 is its ability to activate the transcription of its target genes, including those that encode the cyclin-dependent kinase inhibitor p21 and the proapoptotic Bax protein. In contrast to Saos-2 cells in which p53 activated both the *p21* and *bax* promoters, in MDA-MB-453 cells p53 activated the *p21* promoter, but failed to activate the *bax* promoter. Neither phosphorylation of p53 on serines 315 or 392 nor an intact C terminus was required for p53-dependent activation of the *bax* promoter, demonstrating that this differential regulation of *bax* could not be explained solely by modifications of these residues. Further, this effect was not due to either p73 or other identified cellular factors competing with p53 for binding to its response element in the *bax* promoter. p53 expressed in MDA-MB-453 cells also failed to activate transcription through the p53 response element of the *bax* promoter in isolation, demonstrating that the defect is at the level of the interaction between p53 and its response element. In contrast to other p53 target genes, like *p21*, in which p53-dependent transcriptional activation is mediated by a response element containing two consensus p53 half-sites, activation by p53 of the *bax* element was mediated by a cooperative interaction of three adjacent half-sites. In addition, the interaction of p53 with its response element from the *bax* promoter, as compared with its interaction with its element from the *p21* promoter, involves a conformationally distinct form of the protein. Together, these data suggest a potential mechanism for the differential regulation of p53-dependent transactivation of the *bax* and *p21* genes.

The tumor suppressor protein p53 is an important regulator of cellular growth. The *p53* gene is mutated in the majority of human cancers (1, 2), suggesting that loss of p53 may play an important causative role in oncogenesis. The p53 protein has been implicated in several diverse growth-related pathways, including apoptosis, cell cycle arrest, and senescence (3–5). The ability of p53 to function as a sequence-specific DNA-binding protein appears to be central to the function of p53 as a tumor suppressor (6, 7). At its N terminus, the p53 protein contains a potent transcriptional activation domain (8) that is linked to a central core domain that mediates sequence-specific DNA binding (9–11). Both of these domains have been shown to be important for p53-mediated growth suppression (12). The importance of the DNA binding domain is further highlighted by the fact that the major mutational hot spots from human cancers are found in this domain (13), and several of these mutations have been shown to abolish the ability of p53 to function as a transcriptional activator (14–16).

A DNA consensus sequence through which p53 binds and activates transcription has been identified. This sequence consists of two palindromic decamers of 5′-RRRCWWGYYY-3′ (where R is a purine, Y is a pyrimidine, and W is an adenine or thymine) separated by 0–13 bp,[1] forming four repeats of the pentamer 5′-RRRCW-3′ alternating between the top and bottom strands of the DNA duplex (17–19). This arrangement is consistent with the notion that p53 binds DNA as a homotetramer (20–23). Through sequences similar to this consensus, p53 has been shown to activate the transcription of many genes, including *bax*, *p21*, *mdm2*, *gadd45*, *IGF-BP3*, and *cyclin G* (24–31). Data are consistent with a model in which DNA damage leads to the phosphorylation of p53 as well as the subsequent stabilization of p53 and activation of its DNA binding capability (32–35). Consequently, p53-mediated transcription of its target genes increases. When compared with alternate p53 targets, such as the cyclin-dependent kinase inhibitor *p21*, evidence suggests that the *bax* gene is differentially regulated by p53. Several tumor-derived p53 mutants have been identified that are capable of activating transcription through the promoter of the *p21* gene but not through the *bax* promoter (36–39). This has been correlated with an inability of the mutants both to bind the p53 response element of the *bax* promoter and to trigger apoptosis (36, 38, 39). Such studies with these tumor-derived p53 mutants suggest that a failure in the ability of p53 to activate the *bax* gene may play an important role in tumor formation and progression. As such, a complete understanding of the transcriptional regulation of the *bax* promoter by p53 may yield important information relevant to our understanding of tumorigenesis.

Previous studies have demonstrated that the *bax* promoter is differentially regulated by wild-type p53 in a cell type-specific manner (40). Here the osteosarcoma Saos-2 and the breast carcinoma MDA-MB-453 cell lines were used as a model system to explore the potential mechanisms for this differential regulation. In the Saos-2 cell line, transfected wild-type p53 effectively activated transcription through both the *p21* and *bax* promoters. In contrast, p53 expressed in the MDA-MB-453 cell line was capable of activating transcription through the *p21* promoter as well as the p53 response elements of the *p21*, *cyclin G* and *cdc25C* promoters but failed to do so through

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[1] The abbreviations used are: bp, base pair(s); mAb, monoclonal antibody.

either the *bax* promoter or the isolated p53 response element derived from the *bax* promoter. Neither p53 phosphorylation at serine 315 or serine 392 nor an intact C terminus was required for activation of the *bax* promoter, demonstrating that the observed defect in MDA-MB-453 cells could not be explained solely by modifications of these residues. In addition, neither the p53 homolog p73 nor other cellular factors that are capable of binding the p53 response element of the *bax* promoter explained the differential regulation of the *bax* promoter. Detailed analysis of the interaction of p53 with the *bax* promoter, however, demonstrated that unlike other well characterized p53 response elements, like that of the *p21* gene, in which p53-dependent transcriptional activation is mediated by a response element containing two consensus p53 half-sites, the response element of the *bax* promoter consists of three adjacent half-sites that cooperate to bring about complete activation by p53. In addition, it appears that p53 exists in a distinct conformation when bound to its response element from the *bax* promoter as compared with when it is bound to the 5′-response element of the *p21* promoter. Together, these data suggest a potential mechanism for the cell type-specific differential regulation of *bax* by p53.

MATERIALS AND METHODS

Oligonucleotides—For use in electrophoretic mobility shift assays and for subsequent cloning into luciferase reporter plasmids, complementary single-stranded oligonucleotides were annealed to produce double-stranded oligonucleotides with the indicated sequences: Bax, AATTCGGCTACCTCACAAGTTAGAGACAAGCCTGGGCGTGGGCTATATTGTAGCGAATT; OligoA, AATTCGGTACCTCACAAGTTAGAGACAAGCCTGCTAGCGAATT; OligoB, AATTCGGTACCAGACAAGCCTGGGCGTGGGCGCTAGCGAATT; OligoC, AATTCGGTACCAGACAAGCCTTTTACGGGGCTATATTGCTAGCGAATT; OligoAB, AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGGGCGCTAGCGAATT; OligoAC, AATTCGGTACCTCACAAGTTAGAGACAAGCCTTTTACGGGGCTATATTGCTAGCGAATT; OligoBC, AATTCGGTACCAGACAAGCCTGGGCGTGGGCTATATTGCTAGCGAATT; p21–5′-AATTCGGTACCGAACATGTCCCAACATGTTGGCTAGCGAATT; p21–3′(2x), AATTCGGTACCGAAGAAGACTGGGCATGTCTGAAGAAGACTGGGCATGTCTGCTAGCGAATT; Cyclin G-AATTCGAGCTCCAAGGCTTGCCCGGGCAGGTCTGGGTACCGAATT; Cdc25C(2x), AATTCGGTACCGGGCAAGTCTTACCATTTCCAGAGCAAGCACGCTAGCAGGCCTGTGCTTGCTCTGGAAATGGTAAGACTTGCCCAGATCTAATATTG; and Sens-1, TCGAAGAAGACGTGCAGGGACCCTCGA.

Plasmids—The expression plasmids pCMV-p53^{wt}, pCMV-p53^{V143A}, and pCMV-p53^{S392A}, originally referred to as pC53-SN3 (41), pC53-SCX3 (14), and pCMVhup53ala392 (42), respectively, encode the indicated human p53 protein under the control of the cytomegalovirus promoter. The expression plasmid pCMV-p53$^{\Delta 370-393}$, originally referred to as pCB6+p53Δ370 (43), encodes p53, under the control of the cytomegalovirus promoter, with a point mutation introducing a stop codon at amino acid 370. The expression plasmids pB-p53^{S315A} and pB-p53^{S315D}, originally referred to as Bhup53ala315 (42) and Bhup53asp315 (42), respectively, encode the indicated human p53 protein under the control of the human B-actin promoter. The expression vector pCMV-p73α encodes wild-type p73 under the control of the cytomegalovirus promoter (44). The luciferase reporter plasmid p21P contains the 2.4-kilobase pair *Hin*dIII fragment from the *p21* promoter cloned into the pGL2-Basic vector (45). The luciferase reporter plasmid pBax contains the 370-bp *Sma*I/*Sac*I fragment from the *bax* promoter cloned into the pGL3-Basic vector (29). The following synthetic double-stranded oligonucleotides were digested with *Kpn*I and *Nhe*I and cloned into pGL3-E1bTATA (46), which also had been double-digested with *Kpn*I and *Nhe*I to produce pTATA vectors with corresponding names: Bax, OligoA, OligoB, OligoC, OligoAB, OligoBC, OligoAC, p21–5′, p21–3′ (x2), Cyclin G, and Cdc25C (x2) (46).

Cell Lines—The osteosarcoma Saos-2 cell line and the breast carcinoma MDA-MB-453 cell line were maintained in a humidified tissue culture incubator at 37 °C with 5% CO_2. Saos-2 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. MDA-MB-453 cells were grown in RPMI medium, containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml insulin.

Transfections—Unless otherwise indicated, 1×10^5 cells were seeded into 35-mm plates. Cells were transfected 24 h later using the DOTAP liposomal transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Cellular lysates were prepared 48 h post-transfection, total protein concentration was determined by protein assay (Bio-Rad), and luciferase assays were quantitated using a commercially available kit (Promega) and a TD-20e Luminometer (Turner).

Nuclear Extracts—All procedures were conducted at 4 °C. For each 100-mm dish, cells were washed three times with 5 ml of phosphate-buffered saline. Cells then were scraped into 500 μl of lysis buffer (20 mM HEPES, pH 7.5, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 50 μg/ml aprotinin) and centrifuged at 500 × *g* for 5 min. Pellets were resuspended in 200 μl of nuclear extraction buffer (lysis buffer containing 500 mM NaCl) and incubated end-over-end for 60 min. Samples were centrifuged at 18,000 × *g* for 10 min. Nuclear extracts were aliquoted, quick-frozen in liquid nitrogen, and stored at −70 °C.

Electrophoretic Mobility Shift Assays—Purification of human p53 protein and electrophoretic mobility shift assays using this purified p53 were conducted as described previously (46). In brief, Sf9 cells that were infected with recombinant baculovirus expressing His-tagged p53 were lysed in 20 mM HEPES, pH 7.4, containing 20% glycerol, 10 mM NaCl, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 50 μg/ml aprotinin (Buffer L). Nuclei were pelleted by centrifugation at 2300 rpm and then resuspended in Buffer L containing 500 mM NaCl. Extracts were diluted to 100 mM NaCl with Buffer L, applied to a 0.5-ml nickel nitriloacetic acid agarose column (Qiagen) that was equilibrated with 20 mM HEPES containing 100 mM NaCl and eluted with 200 mM imidazole containing 10 mM HEPES, pH 7.4, and 5 mM NaCl. Fractions of 0.5 ml were collected, dialyzed against 10 mM HEPES, pH 7.4, 5 mM NaCl, 0.1 mM EDTA, 20% glycerol, and 1 mM dithiothreitol, aliquoted, and stored at −70 °C.

Purified p53 protein or nuclear extract was incubated with 3 ng of radiolabeled double-stranded oligonucleotide and hybridoma supernatant where appropriate in a total volume of 30 μl of DNA binding buffer, containing 20 mM $MgCl_2$, 2 mM spermidine, 0.7 mM dithiothreitol, 1 mg/ml bovine serum albumin, and 25 μg/ml poly[d(I-C)] for 30 min at room temperature. Samples were loaded on a native 4% acrylamide gel and electrophoresed in 0.5× TBE at 225 V for 2 h at 4 °C. Gels were dried and exposed to Kodak XAR-5 film using an intensifying screen at −70 °C. Bands were scanned and quantitated using the Molecular Analyst Imaging Densitometer (Bio-Rad).

SDS-Polyacrylamide Gel Electrophoresis and Western Blot—Cells were lysed in 150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 10 μg/ml aprotinin. The protein concentration of each sample was determined using the Bio-Rad Protein Assay. Samples containing equal amounts of protein were electrophoresed in a 10% polyacrylamide gel. Following electrophoresis, protein was transferred to nitrocellulose and probed with a 1:1 mixture of the anti-p53 mouse monoclonal antibodies 1801 and 421. The secondary antibody was a horseradish peroxidase-conjugated goat anti-mouse IgG, and the signal was detected by the enhanced chemiluminescence method (Amersham Pharmacia Biotech).

RESULTS

Wild-type p53 Fails to Activate Transcription through the p53 Response Element of the bax Promoter in the Breast Carcinoma MDA-MB-453 Cell Line—Wild-type p53 expressed in the breast carcinoma MDA-MB-453 cell line is unable to activate transcription through the *bax* promoter or through the isolated p53 response element of the *bax* promoter (Figs. 1*A* and 2*A*). Luciferase reporter plasmids containing either the *p21* promoter or the *bax* promoter were transfected into the p53-negative Saos-2 or MDA-MB-453 cell line with pCMV vector, increasing amounts of a plasmid expressing wild-type p53, or a plasmid expressing the mutant p53^{V143A}. In the Saos-2 cell line, wild-type p53 effectively activated transcription of reporter constructs containing either the *p21* or *bax* promoters. In contrast, wild-type p53 expressed in the MDA-MB-453 cell line, although still capable of activating transcription of a reporter containing the *p21* promoter, failed to activate transcription through a construct containing the *bax* promoter (Fig. 1*A*). Western blots

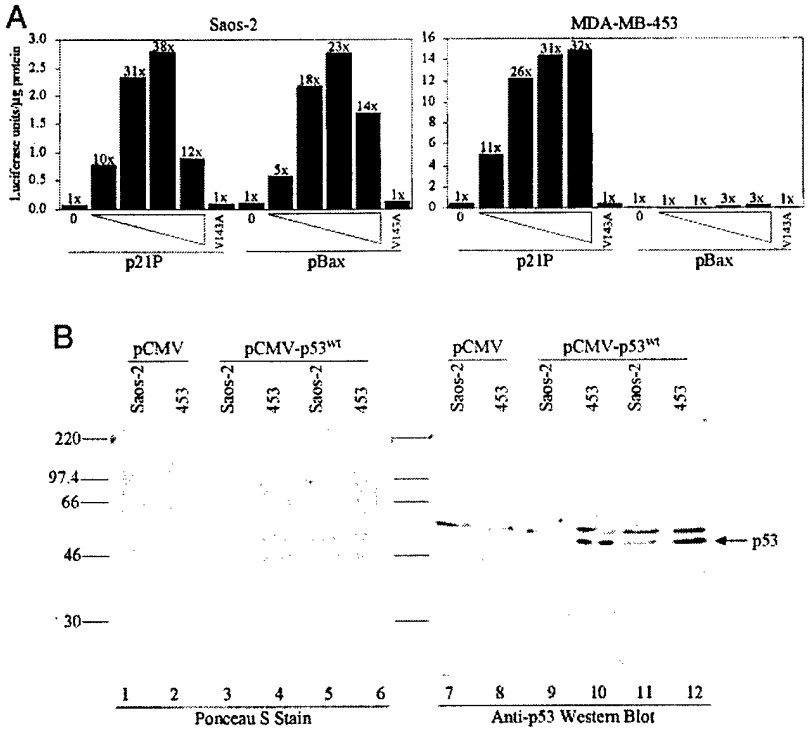

FIG. 1. **Expression of wild-type p53 in MDA-MB-453 cells fails to activate transcription through the *bax* promoter.** *A*, Saos-2 and MDA-MB-453 cells were transfected as described under "Materials and Methods" with 2 μg of the indicated reporter constructs in the presence of 0, 5, 50, 100, or 200 ng of pCMV-p53^{wt} or 50 ng of pCMV-p53^{V143A}. 48 h post transfection cells were lysed and assayed for total protein and luciferase activity as described under "Materials and Methods." Appropriate amounts of the vector pCMV were added to each transfection mixture to maintain a constant level of total plasmid DNA of 2.2 μg/sample. The indicated values are the average of three independent experiments each performed in duplicate. The *numbers* above each *bar* indicate the fold activation for each reporter construct observed with pCMV-p53^{wt} or pCMV-p53^{V143A} as compared with pCMV. *B*, 1×10^6 cells of either Saos-2 (*lanes 7*, *9*, and *11*) or MDA-MB-453 (*lanes 8*, *10*, and *12*) were seeded in 100-mm plates and subsequently transfected with 10 μg of either empty pCMV (*lanes 7* and *8*) or pCMV-p53^{wt} (*lanes 9–12*). 48 h post transfection cells were lysed and assayed for p53 expression levels by Western blot (*lanes 7–12*) as described under "Materials and Methods." Following immunodetection the blot was stained with Ponceau S to confirm that equal amounts of protein were loaded in each lane (*lanes 1–6*). Each lane contains 60 μg of total protein, and each lane represents an independent transfection.

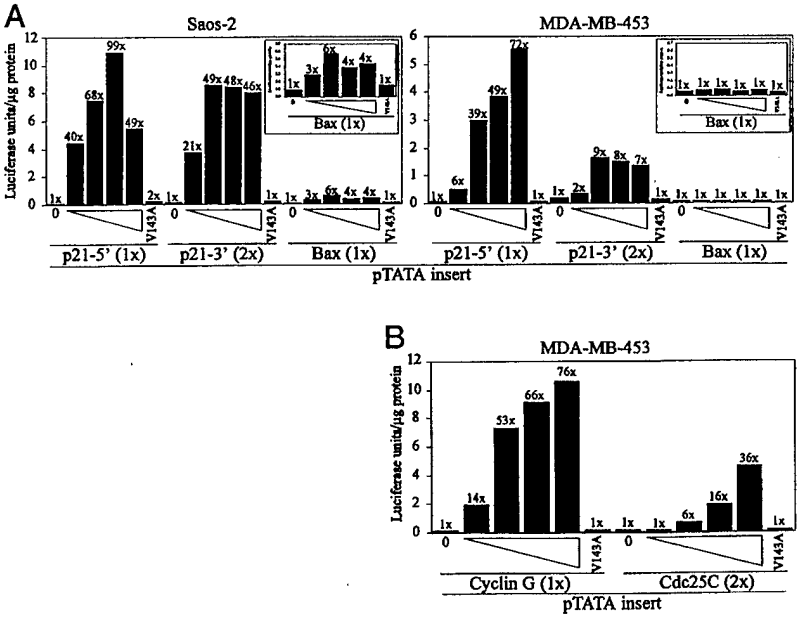

FIG. 2. **Expression of wild-type p53 in MDA-MB-453 cells fails to activate transcription through a 37-bp element of the *bax* promoter.** Saos-2 (*A*) and MDA-MB-453 (*A* and *B*) cells were transfected as described under "Materials and Methods" with 2 μg of the indicated reporter constructs in the presence of 0, 5, 50, 100, or 200 ng of pCMV-p53^{wt} or 50 ng of pCMV-p53^{V143A}. 48 h post transfection cells were lysed and assayed for total protein and luciferase activity as described under "Materials and Methods." Appropriate amounts of the vector pCMV were added to each transfection mixture to maintain a constant level of plasmid DNA of 2.2 μg/sample. The Bax (1x) data are enlarged for clarity (*inset A*). The plots shown in the *insets* have the same scale. The indicated values are the average of three independent experiments each performed in duplicate. The *numbers* above each *bar* indicate the fold activation for each pTATA construct observed with pCMV-p53^{wt} or pCMV-p53^{V143A} as compared with pCMV.

demonstrated that p53 was expressed to equivalent levels in the two cell lines (Fig. 1*B*, compare *lane 9* with *lane 10* and *lane 11* with *lane 12*), if not slightly higher in MDA-MB-453 (Fig. 1*B*, *lanes 10* and *12*), suggesting that the failure of p53 to activate transcription through the *bax* promoter is not due to decreased levels of p53 protein expression.

To determine whether the isolated p53 response element of the *bax* promoter was sufficient for this differential effect, synthetic oligonucleotides corresponding to the p53 response elements of the *p21* and *bax* promoters were cloned into the pGL3-E1bTATA luciferase reporter vector, upstream from the minimal adenovirus *E1b* promoter. Each reporter construct again was transfected into either the Saos-2 or MDA-MB-453 cell line with pCMV vector, increasing amounts of the wild-type

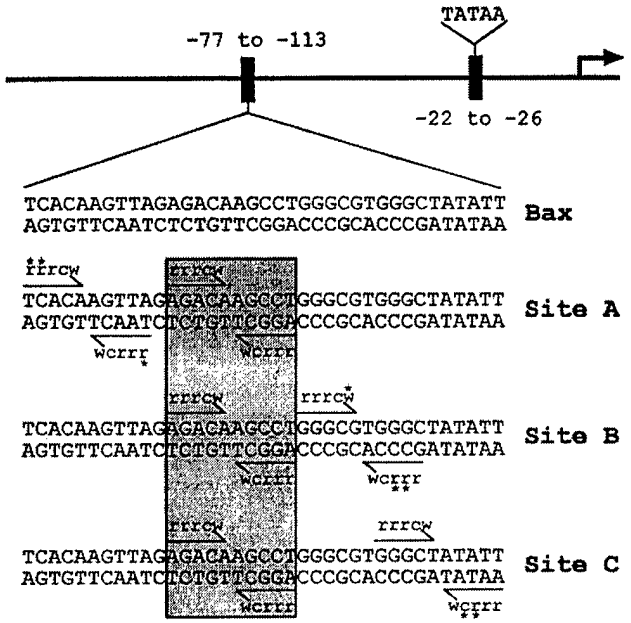

FIG. 3. **Schematic of the p53 response element of the human *bax* promoter.** The previously identified p53 response element of the *bax* promoter is located at −113 to −77 from the transcriptional start site. Based on the p53 consensus binding site, there exists, within this 37-bp sequence, three potential, overlapping p53 binding sites. These putative binding sites are labeled Site A (−113 to −93), Site B (−102 to −83), and Site C (−102 to −77). The *arrows* indicate the four quarter sites that constitute each proposed p53 binding site. The p53 consensus sequence is indicated above each *arrow* with *r* representing purine and *w* representing either an adenine or thymine base. Bases in the *bax* sequence that vary from this consensus are indicated by *asterisks*. The perfect half-site shared by each potential binding site is highlighted by the *gray box*. The position of the TATA box for the *bax* promoter (−22 to −26) also is indicated.

p53 expression plasmid, or the mutant $p53^{V143A}$ expression plasmid. In the Saos-2 cell line, wild-type p53 effectively activated transcription of constructs containing either the 5′ or the 3′ p53 response elements from the *p21* promoter (Fig. 2*A*), as well as a construct containing the p53 response element of the *bax* promoter (Fig. 2*A*, *inset*). As observed with the promoter constructs, wild-type p53 expressed in the MDA-MB-453 cell line failed to activate transcription via an *E1b* reporter plasmid containing the p53 response element of the *bax* promoter (Fig. 2*A*, *inset*), whereas activating reporters containing either the 5′ or the 3′ element of the *p21* promoter (Fig. 2*A*). Expression of wild-type p53 in MDA-MB-453 cells also activated transcription of reporters containing the p53 response elements of the *cyclin G* and *cdc25C* genes (Fig. 2*B*). Thus, the defect in p53-dependent transcriptional activation of the *bax* promoter appears to be at the level of the interaction of p53 with its response element.

The p53 Response Element of the bax Promoter Consists of Overlapping Binding Sites for p53—The data presented in Figs. 1 and 2 demonstrate that in MDA-MB-453 cells there is a defect in wild-type p53-dependent activation via the 37-bp p53 response element of the *bax* promoter, as compared with the 5′ p53 response element of the *p21* promoter. To understand the molecular mechanism mediating this differential regulation of the p53 response elements, the interaction between p53 and its response element from the *bax* promoter was examined in detail by electrophoretic mobility shift assays. Previous studies localized the p53 response element of the *bax* promoter to a 37-bp region at −113 to −77 from the start site of transcription (29). An examination of the nucleotide sequence of this 37-bp element revealed three potential p53 binding sites, termed Site A, Site B, and Site C (Fig. 3), that correspond to the consensus site for p53 binding (17–19). Site A consists of the first 21 bp of the 37-bp response element, with two potential p53 half-sites separated by a 1-bp insert. The first half-site contains three bases that vary from the consensus (two purine-to-pyrimidine changes in the first quarter-site and one in the second quarter-site). The second half-site of Site A matches the consensus sequence in all 10 bases. Site B consists of 20 bp including this same "consensus" half-site and a second half-site downstream, separated by no intervening sequences. Site B diverges from the consensus at three bases (the A/T is a G in the last position of the third quarter-site, and there are two purine-to-pyrimidine changes in the fourth quarter-site). Site C consists of 26 bp and includes the same half-site noted in Sites A and B, separated from a second half-site by a 6-bp insert. Site C contains two variations from the consensus sequence (a C to A change and a purine-to-pyrimidine change both in the fourth quarter site). Of note is the spatial relationship of these three potential p53 binding sites. The three sites overlap one another with the consensus half-site (−102 to −93) common to each. Because of this shared half-site, the binding of p53 to one site excludes its simultaneous binding to either of the other sites. Therefore, if one assumes that p53 binds as a tetramer (20–22), then only one site can be occupied at any given time.

To identify which of these putative binding sites are responsible for the interaction between p53 and the *bax* promoter, synthetic double-stranded oligonucleotides were constructed to model each site (Table I). The Bax oligonucleotide contained the complete 37-bp p53 response element from the *bax* promoter. Oligo A contained the 21 bp corresponding to Site A, whereas Oligo B contained the 20 bp corresponding to Site B. Oligo C consisted of the 26 bp corresponding to Site C; however, because of the sequence overlap between Sites B and C the 6 bp separating the two half-sites in Site C were scrambled to abolish any potential contribution from Site B. Each oligonucleotide contained identical flanking sequences that allowed for its subsequent cloning into a luciferase reporter plasmid. The relative affinities of these oligonucleotides for p53 were assessed by electrophoretic mobility shift assay. Purified p53 bound the labeled Bax oligonucleotide containing the entire 37-bp p53 response element (Fig. 4*A*, *lane 1*), and this binding was effectively competed by an excess of the same, unlabeled oligonucleotide (Fig. 4*A*, *lanes 2–4*). Unlabeled Oligo A, Oligo B, and Oligo C also successfully competed for p53 binding (Fig. 4*A*, *lanes 5–7*, *8–10*, and *11–13*). For comparison, an unrelated control oligonucleotide, Sens-1, was unable to compete for p53 binding (Fig. 4*A*, *lanes 14–16*, and *B*), demonstrating that the binding of p53 to Oligos A, B, and C is specific. In each case, however, the binding of p53 to the isolated sites was weaker than that observed with the entire 37-bp response element (Fig. 4*B*). These data suggest the possibility that in the context of the entire p53 response element of the *bax* promoter there is a cooperative interaction between the overlapping p53 binding sites that allows for enhanced p53 binding.

The ability of purified p53 to directly bind to these oligonucleotides in electrophoretic mobility shift assays was then examined. A labeled oligonucleotide corresponding to the 5′ p53 response element of the *p21* promoter was used as a positive control for p53 binding (Fig. 5, *lanes 1–3*). The *p21*-5′ oligonucleotide was bound by p53 and was effectively supershifted by mAb 1801, a p53 N-terminal-specific monoclonal antibody (Fig. 5, *lane 2*). In addition, the labeled Bax oligonucleotide, corresponding to the entire p53 response element of *bax*, as well as those corresponding to Site A, Site B, and Site C were also bound by purified p53 (Fig. 5, *lanes 4*, *7*, *10*, and *13*) and were supershifted by mAb 1801 (Fig. 5, *lanes 5*, *8*, *11*, and *14*). This binding, however, was weaker than that observed with the

TABLE I
Synthetic oligonucleotides used in electrophoretic mobility shift assays and transfection assays

Name of oligonucleotide	Nucleotide sequence[a]
Bax	5′-aattcggtacc**TCACAAGTTAGAGACAAGCCTGGGCGTGGGCTATATT**gtagcgaatt-3′
Oligo A	5′-aattcggtacc**TCACAAGTTAGAGACAAGCCT**gctagcgaatt-3′
Oligo B	5′-aattcggtacc**AGACAAGCCTGGGCGTGGGC**gctagcgaatt-3′
Oligo C	5′-aattcggtacc**AGACAAGCCT**tttacg**GGGCTATATT**gctagcgaatt-3′
Oligo AB	5′-aattcggtacc**TCACAAGTTAGAGACAAGCCTGGGCGTGGGC**gctagcgaatt-3′
Oligo AC	5′-aattcggtacc**TCACAAGTTAGAGACAAGCCT**tttacg**GGGCTATATT**gctagcgaatt-3′
Oligo BC	5′-aattcggtacc**AGACAAGCCTGGGCGTGGGCTATATT**gctagcgaatt-3′
p21–5′	5′-aattcggtacc**GAACATGTCCCAACATGTTG**gctagcgaatt-3′

[a] The bold capital letters represent the sequences taken from the *bax* and *p21* promoters. Bases that participate in the formation of potential p53 binding sites are indicated by underlining. The lowercase letters indicate sequences not derived from either the *bax* or *p21* promoters.

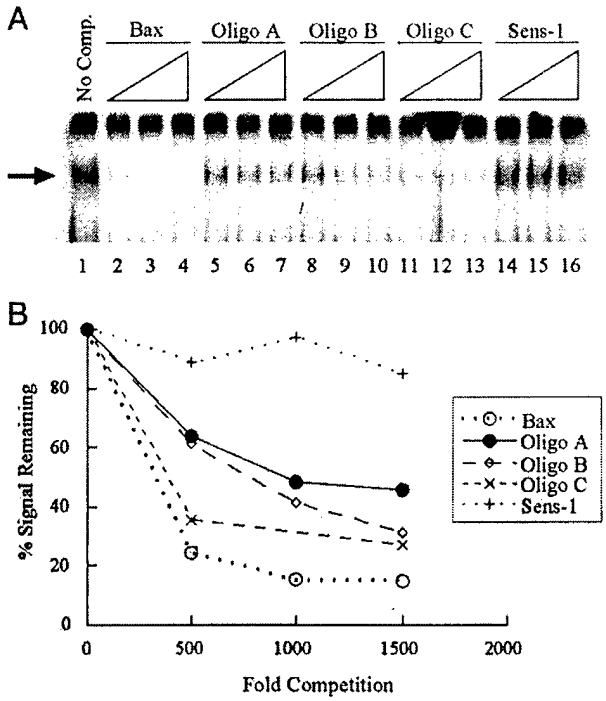

FIG. 4. **The p53 response element of the *bax* promoter contains three overlapping p53 binding sites.** *A*, an electrophoretic mobility shift assay was performed using the Bax oligonucleotide as radiolabeled probe. 50 ng of purified p53 was incubated with 3 ng of the probe alone (*lane 1*) or in the presence of a 500- (*lanes 2, 5, 8, 11,* and *14*), 1000- (*lanes 3, 6, 9, 12,* and *15*), or 1500-fold (*lanes 4, 7, 10, 13,* and *16*) molar excess of the indicated unlabeled competitors. The Sens-1 oligonucleotide (*lanes 14–16*) was used as a nonspecific control. The *arrow* indicates the position of the p53-DNA complexes. Bands were quantitated by densitometry and expressed as a percentage of the no competition signal (*lane 1*) (*B*). The 1000x point of the Oligo C competition (*lane 12*) was not included because of an artifactual streak in the lane that interfered with quantitation.

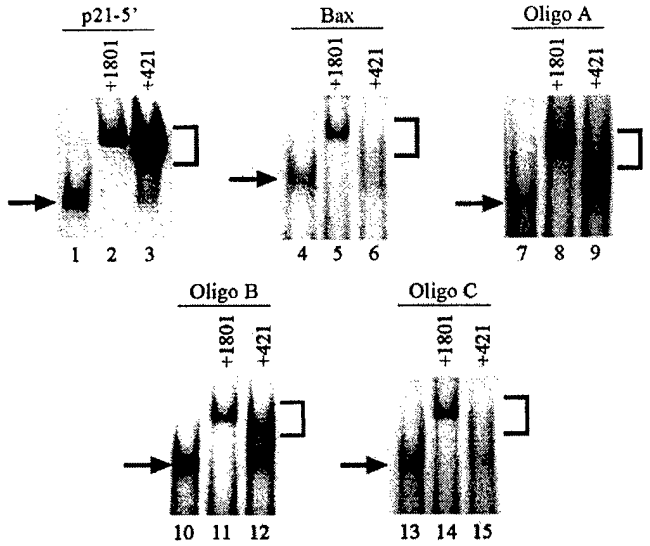

FIG. 5. **Monoclonal antibody 421 enhances the binding of p53 to the *p21* site but inhibits the binding of p53 to the *bax* sites.** An electrophoretic mobility shift assay was performed, incubating either 5 ng (*lanes 1–3*) or 50 ng (*lanes 4–15*) of purified p53 with 3 ng of the indicated radiolabeled probes in the absence (*lanes 1, 4, 7, 10,* and *13*) or presence of monoclonal antibodies 1801 (*lanes 2, 5, 8, 11,* and *14*) or 421 (*lanes 3, 6, 9, 12,* and *15*). The *arrows* indicate the positions of the p53-DNA complexes, and the *brackets* indicate the positions of the supershifted antibody-p53-DNA complexes.

p21-5′ site, requiring approximately 10-fold more p53 to generate a detectable band shift.

Previously our laboratory reported two distinct classes of p53 binding sites based on their responses to the C-terminal-specific mAb 421 (46). p53 binding to one class of sites, which includes the *p21*-5′ site, is enhanced in the presence of mAb 421, whereas binding to the second class of sites is inhibited by mAb 421. Confirming our original observation, p53 binding to the *p21*-5′ site was enhanced in the presence of mAb 421 (Fig. 5, *lane 3*). Binding of p53 to the Bax oligonucleotide as well as to Oligo C, however, was inhibited in the presence of mAb 421 (Fig. 5, *lanes 6* and *15*). The binding of p53 to Oligos A and B displayed an intermediate phenotype, in which mAb 421 failed to effectively supershift the p53-oligonucleotide complexes and failed to enhance p53 binding to the oligonucleotides (Fig. 5, *lanes 9* and *12*). In either case, the data are consistent with the notion that binding to each of the *bax* sites as compared with the *p21*-5′ site may require a conformationally distinct form of p53.

Overlapping, Low Affinity p53 Binding Sites Synergize for Complete p53-dependent Transactivation through the p53 Response Element of the bax Promoter—The Bax oligonucleotide as well as Oligo A, Oligo B, and Oligo C were cloned into the pGL3-E1bTATA luciferase reporter vector upstream from the adenovirus minimal *E1b* promoter. Each reporter construct was transfected with the pCMV empty vector, a plasmid expressing wild-type p53, or a plasmid expressing the temperature-sensitive $p53^{V143A}$ mutant into the p53-negative Saos-2 cell line (Fig. 6). At 37 °C the $p53^{V143A}$ mutant fails to activate transcription through p53-responsive promoters. At 32 °C, however, this mutant adopts a wild-type conformation and has been shown to activate some p53-responsive promoters (such as *p21*) but not others (such as *bax*) (36, 38). At 37 °C, wild-type p53 activated transcription through the complete 37-bp response element of the *bax* promoter (Fig. 6*A*). In addition, wild-type p53 activated transcription through Oligo B; however, this activation was significantly lower than that observed with the complete response element (21-fold compared with 67-fold). Although Oligos A and C both showed sequence-specific binding to p53 in an electrophoretic mobility shift assay (Fig. 4), p53 failed to activate transcription, to any significant

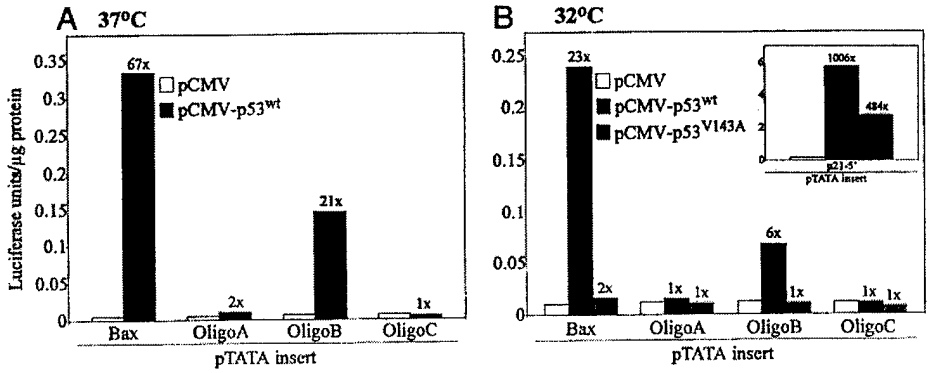

FIG. 6. **Site B is sufficient to confer p53-dependent transactivation, but the level of transactivation is lower than that observed with the complete 37-bp response element.** Saos-2 cells were transfected as described under "Materials and Methods" with 2 μg of the indicated reporter constructs and 50 ng of either empty pCMV (*white bars*), the wild-type p53 expression vector pCMV-p53^{wt} (*black bars*), or the temperature-sensitive p53 expression vector pCMV-p53^{V143A} (*gray bars*). Cells were maintained either at 37 °C (*A*) or shifted to 32 °C 24 h prior to lysis (*B*). Luciferase activity and total protein levels were assayed as described under "Materials and Methods." The pTATA-p21–5′ reporter construct (*B inset*) was used as a positive control for the pCMV-p53^{V143A} expression vector. The indicated values are the averages of three independent experiments each performed in duplicate. The *numbers* above each *bar* indicate the fold activation for each pTATA construct observed with pCMV-p53^{wt} or pCMV-p53^{V143A} as compared with pCMV.

degree, through either sequence (Fig. 6*A*, 2- and 1-fold, respectively). The same pattern of activation was observed with wild-type p53 at 32 °C (Fig. 6*B*). Similar to observations made with the *bax* promoter (36, 38), the temperature-sensitive p53^{V143A} mutant at 32 °C failed to activate transcription through any of the isolated p53 binding sites of the *bax* promoter (Fig. 6*B*, *gray bars*). The p53^{V143A} mutant, however, did successfully activate transcription through the *p21*-5′ response element inserted into the same pGL3-E1bTATA reporter vector (Fig. 6*B*, *inset*).

The transfection data demonstrate that Site B can mediate p53-dependent activation but that the level of activation conferred by this sequences is one-third of that observed with the complete 37-bp response element. To analyze which additional sequences in the 37-bp element are necessary for full activation, another set of synthetic double-stranded oligonucleotides was constructed (Table I). Oligo AB contained the 31 bp that correspond to the overlapping Sites A and B. Oligo AC consisted of the 37-bp response element; however, the 6 bp separating the two half-sites in Site C were scrambled to abolish any potential contribution from Site B. Oligo BC contained the 30 bp corresponding to the overlapping Sites B and C. Again, each oligonucleotide contained identical flanking sequences that allowed for its subsequent cloning into a luciferase reporter plasmid. These oligonucleotides were analyzed by electrophoretic mobility shift assay. Purified p53 bound the labeled Bax oligonucleotide containing the entire 37-bp p53 response element of the *bax* promoter (Fig. 7*A*, *lane 1*), and this binding was effectively competed by an excess of the same, unlabeled oligonucleotide (Fig. 7*A*, *lanes 2–4*). Oligo BC, as well as Oligo AC failed to compete for p53 binding to any greater degree than Oligo B (Fig. 7*A*, compare *lanes 11–13* and *14–16* with *lanes 5–7*). Oligo AB, however, effectively competed for p53 binding (Fig. 7*A*, *lanes 8–10*). This competition was in the same range as that observed with the complete Bax oligonucleotide (Fig. 7*B*), suggesting that the two oligonucleotides share a similar affinity for the purified p53.

Each double-stranded oligonucleotide was inserted into the pGL3-E1bTATA reporter vector upstream of the adenovirus minimal *E1b* promoter and transfected into Saos-2 cells with either empty vector or the wild-type p53 expression vector (Fig. 8). Wild-type p53 effectively activated transcription through the 37-bp p53 response element of the *bax* promoter (60-fold) and to a lesser extent through Oligo B (13-fold). In contrast, p53 failed to significantly activate transcription through either Oligo A (2-fold) or Oligo C (1-fold). Consistent with the results of the electrophoretic mobility shift assays, p53 activated transcription through Oligo AB to a greater extent than through Oligo B (61-fold compared with 13-fold). This activation was in the same range as that observed with the complete p53 response element (61-fold compared with 60-fold). Both Oligos BC and AC failed to mediate any significant p53-dependent transactivation (4-fold and 1-fold respectively). These data confirm that in contrast to other p53 response elements, like the *p21*-5′ site, in which two adjacent p53 half-sites mediate transcriptional activation, the p53 response element of the *bax* promoter consists of three half-sites that cooperate to bring about full activation.

Two Nuclear Factors Selectively Interact with the p53 Response Element of the bax Promoter but Are Not Responsible for Its Differential Regulation in MDA-MB-453 Cells—Given that the defect in the ability of p53 to activate transcription of *bax* is at the level of the interaction between p53 and its response element in the *bax* promoter, one potential mechanism to explain the failure of p53 to activate transcription of *bax* in MDA-MB-453 cells might be that cellular factors exist in this cell line that can selectively compete p53 for binding to the *bax* promoter. To investigate this possibility, the labeled Bax oligonucleotide was used as a probe with MDA-MB-453 cell nuclear extract in an electrophoretic mobility shift assay. Four distinct nuclear factors bound this oligonucleotide (Fig. 8*A*, *lane 1*). Three of these factors, labeled *BoB1* and *BoB2* (binder of *bax* 1 and 2), and *n.s.*, were effectively competed by an excess of this same unlabeled oligonucleotide (Fig. 8*A*, *lanes 2–4*). The band labeled *n.s.* also was competed effectively by Oligos A, B, and C, as well as by the *p21*-5′ oligonucleotide (Fig. 8*A*, *lanes 2–16*), suggesting that this factor is a nonspecific (*n.s.*) DNA-binding protein. In contrast, the bands labeled *BoB1* and *BoB2* were effectively competed by an excess of unlabeled Oligo B but were not competed by Oligo A, Oligo C, or the *p21*-5′ oligonucleotide, demonstrating sequence specificity for Oligo B (Fig. 8*A*, compare *lanes 8–10* with *lanes 5–7* and *11–16*). The band shifts produced with nuclear extract of MDA-MB-453 cells were unaffected by the presence of anti-p53 antibodies (data not shown). In addition, BoB1 and BoB2 failed to bind the *p21*-5′ oligonucleotide, as well as oligonucleotides corresponding to the p53 response element of the *gadd45* gene and the 3′ element of the *mdm-2* gene (Fig. 8*A*, *lanes 14–16*, and data not shown). These results demonstrate the identification of two novel nuclear factors that display sequence specificity for the same region of the *bax* promoter that we have shown to be

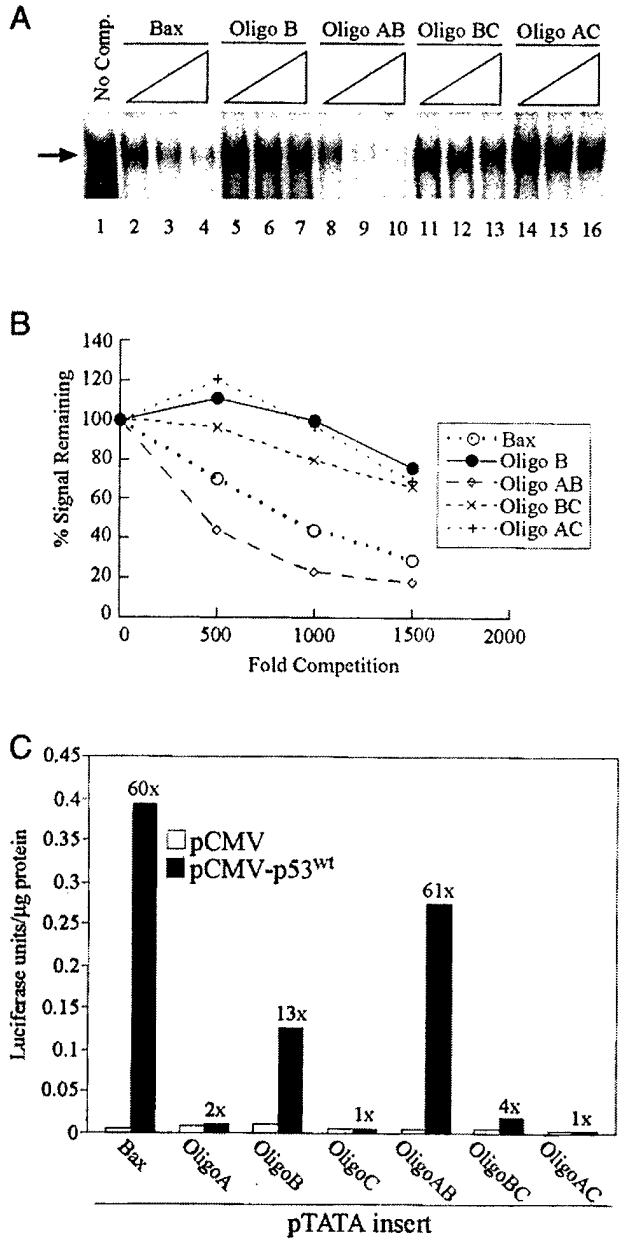

FIG. 7. **Overlapping binding sites synergize in p53 binding and in p53-dependent transactivation.** *A*, an electrophoretic mobility shift assay was performed using the Bax oligonucleotide as radiolabeled probe. 50 ng of purified p53 was incubated with 3 ng of the probe alone (*lane 1*) or in the presence of a 500- (lanes 2, 5, 8, 11, 14), 1000- (*lanes 3, 6, 9, 12,* and *15*), or 1500-fold (*lanes 4, 7, 10, 13,* and *16*) molar excess of the indicated unlabeled competitors. The *arrow* indicates the position of the p53-DNA complexes. *B*, bands were quantitated by densitometry and expressed as a percentage of the no competition signal (*lane 1*). *C*, Saos-2 cells were transfected as described under "Materials and Methods" with 2 μg of the indicated reporter constructs and 50 ng of either pCMV (*white bars*) or the wild-type p53 expression vector pCMV-p53^{wt} (*black bars*). 48 h post transfection luciferase activity and total protein levels were assayed as described under "Materials and Methods." The indicated values are the averages of three independent experiments each performed in duplicate. The *numbers* above each *black bar* indicate the fold activation for each pTATA construct observed with pCMV-p53^{wt} as compared with pCMV.

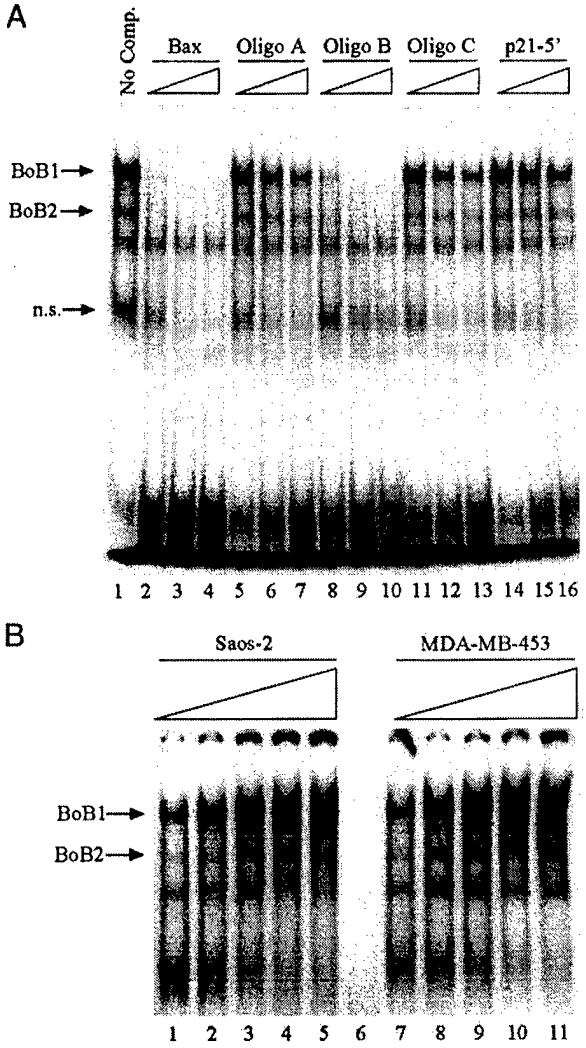

FIG. 8. **Nuclear extracts from MDA-MB-453 cells contain two factors that bind in a sequence-specific manner to the 37-bp p53 response element of the *bax* promoter.** *A*, an electrophoretic mobility shift assay was performed using the Bax oligonucleotide as radiolabeled probe. 2 μl (9 μg of total protein) of MDA-MB-453 nuclear extract was incubated with 3 ng of the probe alone (*lane 1*) or in the presence of a 10- (*lanes 2, 5, 8, 11,* and *14*), 50- (*lanes 3, 6, 9, 12,* and *15*), or 100-fold (*lanes 4, 7, 10, 13,* and *16*) molar excess of the indicated unlabeled competitors. *BoB1* and *BoB2* indicate the positions of the two sequence-specific DNA-binding factors, and *n.s.* indicates the position of a nonspecific band. BoB1 and BoB2 levels are equivalent in MDA-MB-453 and Saos-2 nuclear extracts. *B*, an electrophoretic mobility shift assay was performed using the Bax oligonucleotide as radiolabeled probe. 0 (*lane 6*), 4 (*lanes 1* and *7*), 8 (*lanes 2* and *8*), 12 (*lanes 3* and *9*), 16 (*lanes 4* and *10*), and 20 μg (*lanes 5* and *11*) of either Saos-2 (*lanes 1–5*) or MDA-MB-453 (*lanes 7–11*) nuclear extract was incubated with 3 ng of the probe. *BoB1* and *BoB2* indicate the positions of the two sequence-specific binding factors.

essential for p53-dependent transcriptional activation.

The identification of nuclear factors that showed sequence specificity for the p53 response element of the *bax* promoter suggests a potential mechanism for the differential activation of a reporter construct containing the *bax* promoter in MDA-MB-453 cells. To explore this possibility, the levels of BoB1 and BoB2 in Saos-2 (Fig. 8*B*, *lanes 1–5*) and MDA-MB-453 (Fig. 8*B*, *lanes 7–11*) nuclear extracts were compared by electrophoretic mobility shift assay, using the Bax oligonucleotide as radiolabeled probe. No significant difference in BoB1 or BoB2 levels was observed between nuclear extracts from these two cell lines (Fig. 8*B*, compare *lanes 1–5* with *lanes 7–11*) that had been normalized by total protein. These results suggest that BoB1 and BoB2 levels, as assessed by electrophoretic mobility shift assay, cannot explain the differential effects observed with wild-type p53 on its response element from the *bax* promoter in MDA-MB-453 cells as compared with Saos-2 cells.

The p53 Homolog p73 Does Not Selectively Inhibit the Ability of p53 to Activate Transcription through the bax Promoter—In

addition to BoB1 and BoB2, the p53 homolog p73 was examined as a potential explanation for the inability of wild-type p53 to activate transcription through the *bax* promoter in MDA-MB-453 cells. Saos-2 cells were transfected with a wild-type p53 expression vector, increasing amounts of an expression vector for p73α and either the p21P or pBax luciferase reporter constructs (Fig. 9). In the absence of p73, p53 activated transcription through both the *p21* (12-fold) and *bax* (48-fold) promoters. The addition of increasing amounts of p73 failed to inhibit the ability of p53 to activate transcription through either the *p21* or *bax* promoters, suggesting that p73 is not responsible for the differential activation observed with these two promoters in the MDA-MB-453 cell line.

An Intact C Terminus Is Not Required for p53-dependent Transcriptional Activation of the bax Promoter—Previous studies have demonstrated that C-terminal phosphorylation on serines 315 (47–49) and 392 (50) as well as acetylation of the C terminus (51) functionally alter the DNA binding characteristics of p53. Further, the ability of the C-terminal-specific mAb 421 to enhance the DNA binding activity of p53 has been proposed to be functionally similar to deletion of the last 30 amino acids of p53. In both cases, the binding of p53 to certain response elements is enhanced (50). As mAb 421 inhibits binding of p53 to the *bax* element, the effect of deletion of the terminal 30 amino acids was also examined. Saos-2 cells were transfected with either the p21P or pBax luciferase reporter plasmid and increasing amounts of pCMV-p53^{wt}, pB- p53^{S315A}, pB- p53^{S315D}, pCMV-p53^{S392A}, or pCMV-p53$^{\Delta 370-393}$ expression vector (Fig. 10). In each case p53 effectively activated transcription through both the *p21* and the *bax* promoters, suggesting that neither phosphorylation of serine 315 or serine 392 nor an intact C terminus is required for the p53-dependent transactivation of the *bax* promoter. As compared with wild-type p53, each phosphorylation mutant activated transcription through the *p21* promoter to an equal or greater extent. Although these mutants, S315A, S315D, and S392A, also clearly activated transcription through the *bax* promoter (up to 18-, 16-, and 24-fold, respectively), this level of activation was consistently lower than that observed with the wild-type p53 (up to 72-fold), suggesting that although loss of phosphorylation on either of these residues alone does not completely inhibit the ability of p53 to activate transcription through the *bax* promoter they may contribute in a partial manner.

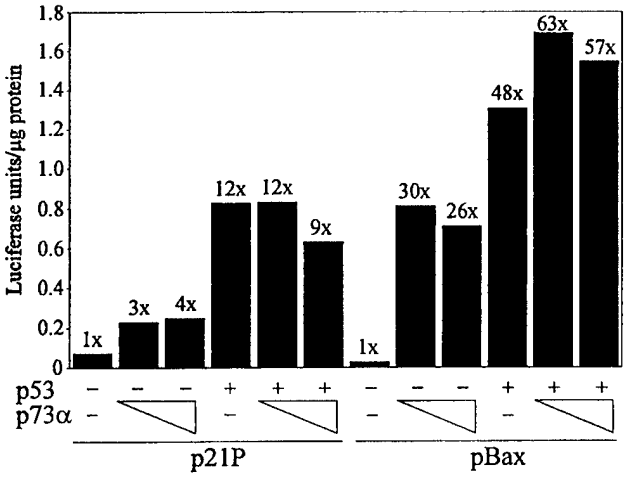

FIG. 9. **The p53 homolog p73 does not selectively inhibit the ability of p53 to activate transcription through the *bax* promoter.** Saos-2 cells were transfected as described under "Materials and Methods" with 2 μg of either the p21P or pBax luciferase reporter plasmids, 0 ng (−) or 50 ng (+) of pCMV-p53^{wt}, and 0 (−), 50, or 100 ng of pCMV-p73α. 48 h post transfection cells were lysed and assayed for total protein and luciferase activity as described under "Materials and Methods." Appropriate amounts of the vector pCMV were added to each transfection mixture to maintain a constant level of plasmid DNA of 2.1 μg/sample. The indicated values are the average of three independent experiments each performed in duplicate. The *numbers* above each *bar* indicate the fold activation for each reporter construct observed with pCMV-p53^{wt} and/or pCMV-p73α as compared with pCMV.

DISCUSSION

The data presented in this report demonstrate that wild-type p53 expressed in the osteosarcoma Saos-2 cell line successfully activated transcription through the promoters of both the cyclin-dependent kinase inhibitor *p21* and the proapoptotic *bax*. In contrast, p53 expressed in the breast carcinoma MDA-MB-453 cell line was capable of activating transcription through the *p21* promoter but failed to do so through the *bax* promoter (Fig. 1*A*). A luciferase reporter construct containing the 37-bp p53 response element from the *bax* promoter displayed the same differential response to p53 as the reporter containing the complete promoter (Fig. 2). This suggests that the 37-bp p53 response element alone is sufficient to mediate this differential regulation and argues in favor of the notion that the differential effect depends on an inherent difference in the interaction of p53 with its response elements in the *bax* and *p21* promoters. In this regard, the data demonstrate three distinct differences

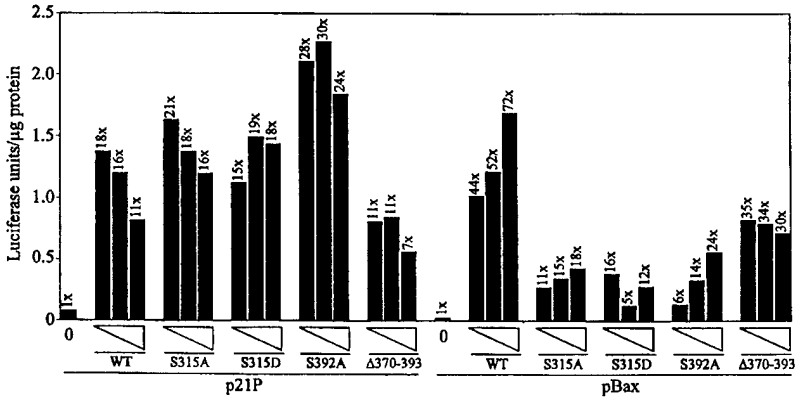

FIG. 10. **An intact C terminus is not required for p53-dependent transcriptional activation of the *bax* promoter.** Saos-2 cells were transfected as described under "Materials and Methods" with 2 μg of either the p21P or pBax luciferase reporter plasmids and 0, 50, 100, or 200 ng of pCMV-p53^{wt} (*WT*), pB-p53^{S315A} (*S315A*), pB-p53^{S315D} (*S315D*), pCMV-p53^{S392A} (*S392A*), or pCMV-p53$^{\Delta 370-393}$ (*Δ370–393*). 48 h post transfection cells were lysed and assayed for total protein and luciferase activity as described under "Materials and Methods." Appropriate amounts of the vector pCMV were added to each transfection mixture to maintain a constant level of total plasmid DNA of 2.2 μg/sample. The indicated values are the average of three independent experiments each performed in duplicate. The *numbers* above each *bar* indicate the fold activation for each reporter construct observed with each p53 expression vector as compared with pCMV.

between the p53 response elements from these two promoters. First, unlike the *p21*-5′ element, which consists of two consensus p53 half-sites that form a high-affinity p53 response element, the response element of the *bax* promoter consists of three half-sites that cooperate in mediating p53-dependent transactivation (Fig. 7). Second, the studies with the C-terminal-specific mAb 421 suggest that the binding of p53 to its response element in the *bax* promoter, as compared with its binding to other response elements, involves a conformationally distinct form of p53 (Fig. 5). Finally, two novel nuclear factors, termed BoB1 and BoB2, were identified that demonstrated sequence-specific binding to the same region of the *bax* promoter that was essential for p53-dependent transactivation and failed to bind to the 5′ element of the *p21* promoter (Fig. 8).

The fact that the binding of p53 to the *bax* element, unlike that to the *p21*-5′ element, failed to be enhanced by the addition of mAb 421 (Fig. 5) indicates that the binding of p53 to these two sequences may require conformationally distinct forms of p53. Thus, the inability of p53 to activate transcription through the *bax* promoter in certain cell lines, like MDA-MB-453, may be due to an altered post-translational modification that prevents p53 from acquiring the correct conformation for binding. Alternatively, binding to the *bax* element may induce a distinct conformational change in p53, as compared with when it is bound to the *p21*-5′ element, that subsequently allows it to interact with a distinct set of additional regulatory factors, and the cell type-specific regulation is at the level of these additional regulators. This latter scenario has been observed with the transcription coactivator OCA-B. OCA-B is a B-cell-specific coactivator that markedly enhances transcription mediated by Oct-1 or Oct-2 through the octamer sequence of immunoglobulin promoters but fails to activate transcription mediated by the same Oct-1 or Oct-2 activators through octamer sequences in the histone H2B gene (52). Consistent with the notion that mAb 421 is revealing a conformational distinction significant to the observed differential regulation of *bax*, the ability of wild-type p53 to activate transcription through the *p21*-3′ response element, to which the binding of p53 also is inhibited by mAb 421 (46), was significantly decreased in MDA-MB-453 cells as compared with Saos-2 (Fig. 2).

Within the C terminus, phosphorylation of serines 315 (47–49) and 392 (50, 53–55) as well as acetylation of lysines 370, 372, and 373 (51) have been shown to enhance the DNA binding (47–51), transcriptional activation (53, 54), and growth suppressor (55) functions of p53. In fact, Scheidtmann and coworkers (49, 54) have suggested that phosphorylation of serines 315 and 392 alters the ability of p53 to both bind to and activate transcription through the p53 response element of the *bax* promoter, in particular. Given these results and the observation that the C-terminal-specific mAb 421 inhibits the binding of p53 to the *bax* element (Fig. 5), we investigated whether or not these particular post-translational modifications could explain the observed defect in the ability of wild-type p53 to activate transcription through the *bax* promoter in the MDA-MB-453 cell line. The results in Fig. 10 demonstrate that although mutation of either serine 315 or serine 392 to alanine slightly decreases the ability of p53 to activate transcription through the *bax* promoter, as compared with the *p21* promoter neither phosphorylation of 315 or 392 nor an intact C terminus is required for p53 to effectively activate transcription through either the *bax* or *p21* promoters. Because the data presented here address each modification independently of the others, the possibility still exists that some combination of these modifications, or other C-terminal modifications not addressed here, may have a more significant impact on the ability of p53 to activate transcription through the *bax* promoter.

The identification of two novel nuclear factors, BoB1 and BoB2, that showed sequence specificity for the same region of the *bax* promoter that was essential for p53-dependent transactivation (Figs. 6 and 8) suggested an alternate explanation for the observed defect in MDA-MB-453 cells. Preliminary results indicated that the binding of p53 and BoB1 or BoB2 to the p53 response element of the *bax* promoter were mutually exclusive, suggesting that these factors may compete with p53 for binding (data not shown). These factors demonstrated a strong affinity for the *bax* element and poor affinity for the *p21*-5′ element. In addition, BoB1 and BoB2 were found to display a moderate affinity for the *p21*-3′ element (data not shown). Correspondingly, the level of p53-dependent activation of the reporter construct containing this 3′ element was reduced in MDA-MB-453 cells when compared with its level of activation in Saos-2 cells (Fig. 2). These results suggested an inverse relationship between the affinity of these binding factors for a particular sequence and the ability of that sequence to mediate p53-dependent transcriptional activation in MDA-MB-453. When the levels of these factors in MDA-MB-453 and Saos-2 cells were compared, however, there was no discernable difference observed (Fig. 8*B*), suggesting that although these factors still may have some significance to the p53-dependent transactivation of *bax*, they do not explain the observed defect in the MDA-MB-453 cell line. One could hypothesize that the p53 homolog p73 might function in a manner analogous to that originally proposed for the BoB1 and BoB2 binding factors. Given the sequence homology between the DNA-binding domains of p53 and p73, it is reasonable to speculate that p73 can bind DNA at p53 response elements and, therefore, may compete with p53 for binding. The results presented here, however, do not support such a hypothesis. Expression of p73α was unable to inhibit the ability of p53 to activate transcription through either the *bax* or *p21* promoters (Fig. 9). In fact, p73 was found to be a potent activator of transcription through the *bax* promoter (Fig. 9, up to 30-fold).

The identification of tumor-derived p53 mutants that selectively fail to activate transcription through the *bax* promoter and subsequently fail to undergo apoptosis (36–39) suggests that the ability of p53 to activate transcription through the *bax* promoter is important to the tumor suppressor function of p53. The Bax protein, in fact, has been shown to play an important role both in inhibiting tumor progression and in promoting the apoptosis of tumor cells in response to DNA-damaging agents like those used in the treatment of cancer (56–62). Studies have shown that decreased Bax levels are significantly associated with tumor cell resistance to chemotherapy (56, 58) and that increased expression of Bax is sufficient to sensitize at least certain tumor cell types to apoptotic stimuli (57, 60, 61, 63). In addition, the p53-dependent transcriptional activation of the *bax* gene has been shown to be important both in inhibiting tumor formation and progression (59, 62, 64) and in promoting apoptosis in response to radio and chemotherapy (59, 63). As such, understanding the mechanism of p53-dependent regulation of the *bax* gene will provide new insights into the processes of tumor formation and progression, as well as the development of tumor resistance to treatment. The data presented here identify several characteristics that differentiate the p53 response element of the *bax* promoter from other p53 response elements, such as the *p21*-5′ element. These characteristics suggest a potential mechanism for the cell type-specific regulation of the *bax* promoter by p53, as seen with the MDA-MB-453 and Saos-2 cell lines. The data demonstrate that in this model system the defect in the ability of wild-type p53 to activate transcription through the *bax* promoter is at the level of the interaction between p53 and its response element and

that this interaction appears to involve a conformationally distinct form of p53 interacting with a unique arrangement of three half-sites. It is reasonable to speculate that the mechanism responsible for the failure of wild-type p53 to activate transcription through the *bax* promoter in MDA-MB-453 cells may also be relevant to the inhibition of *bax* induction observed both in tumor formation and progression and in tumors that are resistant to apoptosis-inducing treatments.

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The tumor suppressor protein p53 requires a cofactor to transcriptionally activate the human *bax* promoter

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Running title: p53-dependent transactivation of *bax*

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Summary

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 six base pairs (5'-GGGCGT-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 six 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.

Introduction

The bcl-2 family of proteins are key mediators of the apoptotic response. One member of this family is the proapoptotic Bax. Proceeding 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. 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.

An important regulator of *bax* gene expression is the tumor suppressor protein p53 (7,8). The p53 protein has been implicated in several growth-related pathways, including apoptosis and cell-cycle arrest (9,10). The ability of p53 to function as a sequence-specific DNA binding protein appears to be central to its role as a tumor suppressor (11,12). At its amino-terminus, the protein contains a potent transcriptional activation domain (13) which is linked to a central core domain that mediates sequence-

specific DNA binding (14-16). Both of these domains have been shown to be important for p53-mediated growth suppression (17).

A DNA consensus sequence through which p53 binds and activates transcription has been identified. This sequence consists of two palindromic decamers of 5'-RRRCWWGYYY-3' (where R is a purine, Y is a pyrimidine, and W is an adenine or thymine) separated by 0-13 bp, forming four repeats of the pentamer 5'-RRRCW-3' alternating between the top and bottom strands of the DNA duplex (18,19). Through sequences similar to this consensus, p53 has been shown to activate the transcription of many genes, including *bax*, *p21*, *mdm2*, *gadd45*, *IGF-BP3*, and *cyclin G* (8,20-26). When compared to alternate p53 targets, studies demonstrate that the *bax* gene is differentially regulated by wild-type p53 in a cell type-specific manner (7,27,28). In the mouse, p53-dependent regulation of *bax* expression following ionizing radiation is seen in the prostate, thymus, spleen, small intestine, and lung, as well as sympathetic, Purkinjie, and olfactory cortical neurons. In the kidney, heart, liver, and brain, however, no p53-dependent regulation of *bax* is observed (7,27). Further, the myeloid leukemia ML-1, Burkitt's lymphoma WMN and AG876, and lymphoblastoid NL2 and FWL cell lines induce *bax* following ionizing radiation, while the fibroblast AG1522 and WI38, colorectal carcinoma RKO, and osteosarcoma U2-OS cell lines fail to do so (28). In addition, several tumor-derived p53 mutants have been identified that are capable of

activating transcription through the promoter of the *p21* gene but not through the *bax* promoter (29-32). This correlates with an inability of these mutants to trigger apoptosis (29,31,32), suggesting that a failure in the ability of p53 to transactivate the *bax* gene may play an important role in tumor formation anc progression. Supporting this, Yin *et al.* demonstrated that Bax is an obligatory downstream effector for the p53-mediated apoptosis that attenuates choroid plexus tumor growth in the TgT121 mouse model (33). Thus, a complete understanding of the transcriptional regulation of the *bax* promoter by p53 may yield important information relevant to our understanding of tumorigenesis.

Here is presented a detailed analysis of the p53 response element located in the promoter of the human *bax* gene. The minimal *bax* response element capable of mediating p53-dependent transcriptional activation is found to consist of two p53 half-sites plus an adjacent six base pairs (5'-GGGCGT-3') that 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 co-factor may

represent a novel basis for the cell-type specific control of the proapoptotic *bax* by wild-type p53.

Experimental Procedures

Cells

The osteosarcoma Saos-2 cell line was maintained in a humidified tissue culture incubator at 37°C with 5% CO_2. 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 annealed to produce double-stranded oligonucleotides with the indicated sequences:

Bax-113/-77,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGGGCTATATTGCTA

GCGAATT; Bax-113/-83,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGGGCGCTAGCGAAT

T; Bax-113/-92,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGCTAGCGAATT; Bax-102/-83,

AATTCGGTACCAGACAAGCCTGGGCGTGGGCGCTAGCGAATT; Bax-113/-83(sc-102/-93), AATTCGGTACCTCACAAGTTAGCTCACCTAAGGGGCGTGGGCGCTAGCGAATT; Bax(-113/-93)3, AATTCGGTACCTCACAAGTTAGAGACAAGCCTCACTGGTCACAAGTTAGAGACAAGCCTCACTGGTCACAAGTTAGAGACAAGCCTGCTAGCGAATT; Bax-92/-83, AATTCGGTACCGGGCGTGGGCGCTAGCGAATT; p21-5', AATTCGGTACCGAACATGTCCCAACATGTTGGCTAGCGAATT; Bax/p21-5' hybrid, AATTCGGTACCAGACAAGCCTCAACATGTTGGCTAGCGAATT; p21-5'/Bax hybrid, AATTCGGTACCGAACATGTCCGGGCGTGGGCGCTAGCGAATT; Sp1 Consensus, ATTCGATCGGGGCGGGGCGAGC; BaxGG-92/-91AA, AATTCGGTACCTCACAAGTTAGAGACAAGCCTAAGCGTGGGCGCTAGCGAATT; BaxGG-85/-84AA, AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGAACGCTAGCGAATT; BaxG-92A, AATTCGGTACCTCACAAGTTAGAGACAAGCCTAGGCGTGGGCGCTAGCGAATT; BaxG-91A, AATTCGGTACCTCACAAGTTAGAGACAAGCCTGAGCGTGGGCGCTAGCGAATT; BaxG-90A, AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGACGTGGGCGCTAGCGAAT

T ; Bax C-89A ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGAGTGGGCGCTAGCGAAT

T ; Bax G-88A ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCATGGGCGCTAGCGAAT

T ; Bax T-87G ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGGGGGCGCTAGCGAAT

T ; Bax G-86T ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTTGGCGCTAGCGAAT

T ; Bax G-85T ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGTGCGCTAGCGAAT

T ; Bax G-84T ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGGTCGCTAGCGAAT

T ; Bax C-83A ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGGGAGCTAGCGAAT

T ; Bax sc-92/-83 ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTTTTATGTTTAGCTAGCGAATT

; Bax sc-86/-83 ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTTTTAGCTAGCGAAT

T.

Plasmids

The following synthetic double-stranded oligonucleotides were digested with KpnI and NheI and cloned into pGL3-E1bTATA (34), which also had been double-digested with KpnI and NheI to produce pTATA vectors with corresponding names: Bax-113/-83, Bax-113/-93, Bax-102/-83, Bax-113/-83(sc-102/-93), Bax(-113/-93)3, Bax-92/-83, Bax-133/-77, p21-5', BaxGG-92/-91AA, BaxGG-85/-84AA, BaxG-92A, BaxG-91A, BaxG-90A, BaxC-89A, BaxG-88A, BaxT-87G, BaxG-86T, BaxG-85T, BaxG-84T, BaxC-83A, Bax sc-92/-83, Bax sc-86/-83. pBax-315/+51, pBax-127/+51, and pBax-76/+51 were generated by PCR amplification of the appropriate fragments from the original pBax luciferase reporter plasmid (8). Upstream primers were engineered with the NheI restriction site. Downstream primers contained the HindIII restriction site. Following PCR, products were digested with both NheI and HindIII and cloned into pGL3-E1bTATA which was also double digested with NheI and HindIII, removing the adenovirus *E1b* minimal promoter. To construct pBaxΔ-126/-77, PCR amplification of the original pBax was used to generate two fragments corresponding to -315 to -127 and to -76 to +51 from the start site of transcription. The -315 to -127 fragment was engineered to contain the NheI 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 (NheI and SacI or SacI and HindIII). A three way ligation with the two PCR-generated fragments and pGL3-E1bTATA, double-digested with NheI and HindIII, then was performed, replacing the *bax* sequence from -126 to -77 with the SacI restriction site. To construct pBaxΔ-113/-104, PCR amplification of the original pBax was used to generate two fragments corresponding to -315 to -114 and to -103 to +51 from the start site of transcription. The -315 to -114 fragment was engineered to contain the NheI restriction site on the upstream side and the NcoI restriction site on the downstream side. The -103 to +51 fragment was engineered to contain the NcoI site upstream and the HindIII site downstream. Following PCR amplification each fragment was double-digested with the appropriate restriction enzymes (NheI and NcoI or NcoI and HindIII). A three way ligation with the two PCR-generated fragments and pGL3-E1bTATA, double-digested with NheI and HindIII, then was performed, replacing the *bax* sequence from -113 to -104 with the NcoI restriction site. The generation of pBaxΔ-103/-93, pBaxΔ-92/-83, and pBaxΔ-113/-93 was accomplished as above with pBaxΔ-113/-104 but using PCR-generated fragments corresponding to -315 to -104 and -92 to +51, -315 to -93 and -82 to +51, and -315 to -114 and -92 to +51 respectively. The expression plasmid pCMV-p53^{wt}, originally referred to as pC53-SN3 (35), encodes the wild-type human p53 protein under the control of the cytomegalovirus promoter. The expression plasmid pPacSp1 contains the 2.1 kb XhoI restriction fragment of Sp1 cloned downstream of the

Actin 5C promoter (36). pPacU was generated by removing the 2.1 kb XhoI fragment from pPacSp1.

Transfections

Saos-2 cells were transfected using Lipofectamine Plus Reagent (GibcoBRL, Life Technologies). 2 x 10^5 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, total protein concentration was determined by protein assay (Bio-Rad), and luciferase assays were quantitated using a commercially available kit (Promega) and a TD-20e Luminometer (Turner). *Drosophila* SL2 cells were transfected using Cellfectin (GibcoBRL, Life Technologies). 60-mm dishes were seeded with 2 x 10^6 cells in Schneider's *Drosophil*a media containing 10% heat-inactivated fetal bovine serum, but no penicillin or streptomycin. The DNA to be transfected 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 then was added directly to the cells. 48 h post transfection cells were lysed by sonication (6 x 20 sec 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 (10mM HEPES pH7.6, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT) and incubated on ice for 10 min. Cells then were centrifuged at 500xg 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 centrifuges at 430xg for 10 min to pellet the nuclei. The supernatant was decanted and the pellet was recentrifuged at 24,000xg for 20 min. The supernatant again was removed. The pellet was resuspended in 3 ml of Buffer C (20mM HEPES pH7.6, 25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT) per 10^9 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 24,000xg for 30 min. The resulting nuclear extract was dialyzed against Buffer D (20mM HEPES pH7.6, 20% glycerol, 0.1M KCl, 0.2mM EDTA, 1.5mM $MgCl_2$, 0.5mM DTT) for 5 h. The extract was clarified by centrifugation at 24,000xg 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 was done as previously described (34). Purified p53 protein, extract from Sf9 cells expressing recombinant human Sp1 protein, or HeLa cell nuclear extract was incubated with 3 ng of radiolabeled double-stranded oligonucleotide and antibody (Sp1 PEP-2X, p300 N-15X, and CBP 451X, Santa Cruz Biotechnology), where appropriate, in a total volume of 30 μl of DNA-binding buffer (20mM HEPES pH7.5, 83mM NaCl, 0.1mM EDTA, 12% glycerol, 2mM $MgCl_2$, 2mM spermidine, 0.7mM DTT, and 25 μg/ml poly d(I-C)) for 20 min at room temperature. Samples were loaded on a native 4% acrylamide gel in 0.5X TBE and electrophoresed at 4°C at 225V for 2 h. The gel was dried and exposed to Kodak XAR film using an intensifying screen at -70°C. Phosphorimaging and densitometry data were collected with a Personal Molecular Imager FX and a GS-710 Calibrated Imaging Densitometer (Bio-Rad), and analyzed with Quantity One software (Bio-Rad).

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 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 (Figure 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)(Figure 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 (Figure 1A). Further, 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 (Figure 1A). These results show that -113 to -83 is the

only region, within the 366 bp 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 Figure 1B as the light grey, white, and dark grey boxes). The role of each of these half-sites in the p53-dependent activaiton 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 (Figure 1B, compare 63-fold with pBax-315/+51 to 7-fold with pBaxΔ-113/-104), while removal of the second (pBaxΔ-103/-93) or the third half-site (pBaxΔ-92/-83) completely abolished the ability of p53 to transcriptionally activate the promoter (Figure 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 transcriptionally activate the promoter (Figure 1B). 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 resemble the consensus sequence of 5'-RRRCWWGYYY-3' (represented in

Figure 2 by the light grey, white, and dark grey boxes). The first, located at -113 to -104, deviates from the consensus at two 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 Figure 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. When cloned upstream of the adenovirus *E1b* 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 further examine 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 (Figure 2). These three copies of this p53 binding site were capable of mediating a significant degree of activation in response to p53 (Figure 2, compare 4-fold with pTATA-113/-93 to 142-fold with pTATA$(-113/-93)_3$), demonstrating that the sequence from -113 to -93 is indeed a *bona fide* p53 response element capable 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 to that mediated by all three half-sites combined (Figure 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 synthetic oligonucleotide corresponding to -113 to -83 of the *bax* promoter, with -102 to -93 scrambled to remove any contribution of the second half-site, was cloned into the pTATA reporter plasmid. This construct failed to be activated by p53 (Figure 2, pTATA-113/-83(sc-102/-93)). The third half-site in isolation (-92 to -83) also failed to mediate p53-dependent transcriptional activation (Figure 2, pTATA-92/-83).

Sp1 binds with sequence-specificity to and activates transcription 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 transcriptional 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 database (38,39) showed that it contains sequence 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 (Figure 3). As previously reported for Saos-2, 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 (Figure 3, lanes 7-9) as well as by increasing amounts of oligonucleotide corresponding to the DNA-binding consensus sequence of Sp1 (Figure 3, lanes 13-15). This binding was specific, as an oligonucleotide corresponding to the 5' p53 response element from the human *p21* promoter failed to compete for binding (Figure 3, lanes 10-12). In addition, this factor was successfully bound by an anti-Sp1 antibody, as demonstrated by a "super-shifted" complex (Figure 3, lanes 2 and 3), while a control anti-p300 antibody failed to bind the factor (Figure 3, lanes 4 and 5). Together, these data demonstrate that Sp1 can bind a portion of the p53 response element from the human *bax* promoter in a sequence-specific manner.

To further delineate 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 (Figure 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 (Figure 4, lane 1) and was recognized by an anti-Sp1 antibody (Figure 4, lane 2) but not by a control anti-CBP antibody (Figure 4, lane 3). Both unlabeled probe and the Sp1 DNA-binding consensus site oligonucleotide effectively competed for Sp1 binding (Figure 4, lanes 4-5 and 12-13 respectively), while the *p21*-5' element did not (Figure 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'-GGGCGTGGGC-3') effectively competed for Sp1 binding (Figure 4, lanes 8-9), while the other hybrid oligonucleotide which replaces this GC-rich region with sequence from the *p21*-5' element demonstrated a significantly reduced affinity for Sp1 binding (Figure 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 a Sp1 expression vector into the Sp1-deficient *Drosophila* SL2 cell line (Figure 5). Expression of Sp1 successfully activated transcription of this reporter, yet failed to activate transcription of a control plasmid containing the 5' p53 response element of the *p21* promoter (Figure 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 (Figure 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 (Figure 6A, compare -113/-83 to GG-92/-91AA), while substitution of bases -85 and -84 did not (Figure 6A). As

observed in Figures 1 and 2, removal of the third potential half-site (-92 to -83) inhibited the ability of p53 to mediate transcriptional activation through this element (Figure 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 radiolabeled probe with purified p53 in an EMSA (Figure 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 to the wild-type oligonucleotide both mutant oligonucleotides displayed a slightly decreased affinity for p53 (Figure 6B, compare lanes 2-4 with lanes 5-7 and 8-10; Figure 6C). Compared to one another, however, both mutant oligonucleotides demonstrated a comparable affinity for p53 (Figure 6A and B), suggesting that the differences in p53-dependent transcriptional activation observed in Figure 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 (Figure 6D 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 (Figure 6D). Sp1 bound the probe and was recognized by an anti-Sp1 antibody (Figure 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 (Figure 6D, lanes 3-4 and 9-11 respectively; Figure 6E). The GG-92/-91AA mutant, however, demonstrated a significant decrease in affinity for Sp1 (Figure 6D, compare lanes 3-5 and 9-11 to lanes 6-8; Figure 6E).

The results with the GG-85/-84AA mutant presented in Figure 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 identify the minimal sequence elements required to mediate p53-dependent transactivation, a series of oligonucleotides was synthesized in which each of the ten 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 (Figure 7). Consistent with the results in Figure 6A, substitution of the bases at either -85 or -84 did not inhibit the ability of p53 to activate transcription through this element (Figure 7, G-85T and G-84T). Further, substitution of -86 and -83 also failed to significantly affect the ability of p53 to activate transcription (Figure 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 (Figure 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 ten nucleotides from -92 to -83 (Figure 8A, sc-92/-83). The four bases from -86 to -83 were substituted as indicated to generate the second mutant oligonucleotide (Figure 8A, sc-86/-83). Each oligonucleotide was cloned into the pTATA vector, and tested for its responsiveness to p53 in a cotransfection assay (Figure 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 ten bases of the third potential half-site (-92 to -83) are replaced, showed little to no response to p53 (Figure 8A, compare pTATA-113/-93 to pTATA-113/-83 and pTATAsc-92/-83). In contrast, the second mutant, in which only the last four bases of the element (-86 to -83) are replaced, was efficiently activated by p53 (Figure 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 to the wild-type sequence (Figure 8B, compare lanes 2-4 to lanes 8-10 and 11-13; Figure 8C). When compared to each other, there was no significant difference in the affinity of p53 for the

two mutant sequences (Figure 8B, compare lanes 8-10 to 11-13; Figure 8C). This suggests that the differences in transcriptional activation observed in Figure 8A cannot be explained by differences in p53 affinities. Further, the oligonucleotide corresponding to -113 to -93 displayed a similar affinity for p53 as the two mutant oligonucleotides (Figure 8B, compare lanes 5-7 to lanes 8-10 and 11-13; Figure 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 (Figure 8D, compare lanes 2-4 to lanes 8-10; Figure 8E), while the ability of the sc-92/-83 mutant to bind Sp1 was significantly reduced compared to the wild-type sequence (Figure 8D, compare lanes 2-4 to lanes 5-7; Figure 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-dependent transcriptional activation on a minimal promoter (Figure 2). Immediately adjacent to this p53 binding site are six base pairs that are GC-rich in nature (-92 to -87: 5'-GGGCGT-3'). These six 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 (Figures 1B, 2, 6A, and 8A). The addition of these bases to the -113/-93 sequence appears to have little effect on the affinity of p53 for this sequence (Figure 8B and C), consistent with a model in which these six bases function to recruit a co-activator as opposed to simply enhancing p53 binding. Further, these six base pairs mediate sequence-specific binding to the Sp1 transcription factor (Figures 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 (Figures 6 and 8). In addition, the results with electophoretic mobility shift assays with the GG-92/-91AA mutant oligonucleotide (Figure 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 positions of each half-site. The GG-92/-91AA 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* (Figure 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 a 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 super-shift EMSA identified other Sp-family members as minor components of the Sp1-DNA complex (E. C. Thornborrow and J. J. Manfredi, unpublished data). Further, 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 (E. C. Thornborrow and J. J. Manfredi, unpublished data). 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) which 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 (Figure 9). In this model, cells that are

permissive to p53-dependent upregulation of the *bax* gene express both p53 and the cofactor and these proteins function together to transcriptionally activate the gene. In those cells that fail to show p53-dependent *bax* expression one can propose three possible mechanisms to explain the apparent failure of wild-type p53 to activate the *bax* gene (Figure 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. While 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 a 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, BTEB1/AP-2rep, and BKLF/EKLF, this competition has ramifications on gene expression (60-62). In each case, transcriptional activation by one

family member is repressed by the other member by competing for the same DNA-binding site. The data in this report, in combination with the previous studies of the Sp-family of transcription factors, support a model in which the regulation of a required cofactor controls cell-type specific p53-dependent expression of the *bax* gene.

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 corresponding resistance to apoptotic stimuli, as well as, a shorter survival period also has been observed in a number of human tumor types, including breast, ovarian, pancreatic, colorectal, and non-Hodgkin lymphoma (65-69). In addition, in colon and gastric cancers of the microsatellite mutator phenotype mutational inactivation of the *bax* gene has been shown to confer a strong survival advantage during tumor clonal evolution (70). Complimenting these data are observations showing that overexpression of the Bax protein in certain tumor cell lines both sensitizes these cells to chemotherapy- and radiation-induced apoptosis and reduces their ability to form tumors in SCID mice (71-73). Together, these results strongly support a tumor suppressor role for the Bax protein.

An important regulator of the *bax* gene is the tumor suppressor protein p53. Several reports have demonstrated the significance of the p53-Bax pathway in tumor suppression. Both the identification of tumor derived p53 mutants that selectively fail 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 *bax* is an obligatory downstream effector of p53 in the suppression of choroid plexus tumor growth (33) suggest that the ability of p53 to activate transcription through the *bax* promoter is important to the tumor suppressor function of p53. Further, the resistance of certain tumor cell lines to radiation therapy is associated with a failure of wild-type p53 to induce *bax* expression (28,74), and certain human tumors have been identified that are genetically wild-type for both *p53* and *bax*, and, yet, fail to express significant levels of Bax protein (75). Thus, a complete understanding of the transcriptional regulation of the *bax* gene by the tumor suppressor p53 may provide important information concerning both the molecular origins of cancer as well as the development of tumor resistance to certain cancer treatments.

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Figure Legends

Figure 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^{wt} (grey 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 standard deviation. The numbers above each bar indicate the fold activation for each reporter construct observed with pCMV-p53^{wt} as compared with pCMV. (A) The previously identified p53 response element is indicated by the dark grey box at -113 to -83. (B) The three potential p53 half-sites are represented by the light grey (-113 to -104), white (-102 to -93), and dark grey (-92 to -83) boxes.

Figure 2. The first two potential p53 half-sites constitute a *bona fide* p53 response element.

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-p53^{wt} (grey 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 standard deviation. The numbers above each bar indicate the fold activation for each reporter construct observed with pCMV-p53^{wt} as compared with pCMV. The sequence of the *bax* promoter from -113 to -83 is given at the top of the figure. Potential p53 quarter-sites are indicated by the solid bars above and below the sequence. Bases that deviate from the p53 DNA-binding consensus sequence are indicated by asterisks. The three potential half-sites are indicated by the brackets labeled 1, 2, and 3 respectively, and are represented graphically as the light grey, white, and dark grey boxes respectively. The vertical arrow above the *bax* sequence indicates the one base pair insert between the first and second half-sites.

Figure 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 (lane 1 and 6), in the presence of 2 or 4 μl of anti-Sp1 antibody (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 super-shifted complex containing antibody, Sp1, and DNA.

Figure 4. The Sp1 binding site is localized to a GC-rich region of the p53 response element.

(A) 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 (lane 1), in the presence of 4 μl of anti-Sp1 antibody (lane 2), 4 μl of anti-CBP antibody (lane 3), a 100- or 200-fold molar excess of either the unlabeled Bax-102/-83 oligonucleotide (lanes 4 and 5), p21-5' oligonucleotide (lanes 10 and 11), Bax/p21-5' hybrid oligonucleotide (lanes 6 and 7) or p21-5'/Bax hybrid oligonucleotide (lanes 8 and 9), or a 10- or 20-fold molar excess of the unlabeled Sp1 Consensus oligonucleotide (lanes 12 and 13). The arrows indicate the positions of the Sp1-DNA and the super-shifted

antibody-Sp1-DNA complexes. (B) The sequences of the human *bax* promoter from -102 to -83 from the start site of transcription (grey boxes) and the human *p21* promoter from -2281 to -2262 from the start site of transcription (white boxes; corresponding to the p21-5' oligonucleotide) are shown. Each sequence is divided into two with the first half indicated as A and the second half indicated as B. Oligonucleotides in (A) are represented graphically according to this color and letter scheme. For example, the Bax/p21-5' hybrid oligonucleotide which corresponds to the first half of the *bax* sequence followed by the second half of the *p21* sequence is indicated by a grey box labeled A followed by a white box labeled B.

Figure 5. Sp1 can activate transcription through the p53 response element of the human *bax* promoter.

Drosophila SL2 cells were transfected as described under Experimental Procedures with 2 μg of the indicated pTATA reporter constructs in the presence of 0, 300, 600, or 900 ng of pPacSp1. Appropriate amounts of the vector pPacU were added to each transfection mixture to maintain a constant level of total plasmid DNA of 2.9 μg/sample. 48 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 four

independent experiments expressed as the fold activation for each reporter plasmid with pPacSp1 as compared with pPacU. Error bars correspond to one standard deviation.

Figure 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-p53^{wt} (grey 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 one standard deviation. 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 grey 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 BaxGG-92/-91AA oligonucleotide (lanes 5-7), or the BaxGG-85/-84AA oligonucleotide (lanes 8-10). The arrow indicates the position of the

p53-DNA complex. The vertical bar between lanes 4 and 5 represents the removal of irrelevant lanes from the gel. (C) Bands were quantitated by densitometry. (D) 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 BaxGG-92/-91AA oligonucleotide (lanes 6-8), or the BaxGG-85/-84AA oligonucleotide (lanes 9-11). The arrow indicates the position of the Sp1-DNA complex and the asterisk indicates the position of the super-shifted Ab-Sp1-DNA complex. The vertical bar between lanes 5 and 6 represents the removal of irrelevant lanes from the gel. (E) Bands were quantitated by densitometry.

Figure 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 μg of the indicated pTATA reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53^{wt} (grey 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 standard deviation. 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 grey with the corresponding mutations indicated above.

Figure 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-p53^{wt} (grey 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 standard deviation. The numbers above each bar indicate the fold activation for each reporter construct observed with pCMV-p53^{wt} 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 grey 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.

Figure 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, such as phosphorylation "P", glycosylation "G", or acetylation "A". (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 grey circle, the dotted oval, and the cross-hatched triangle respectively.

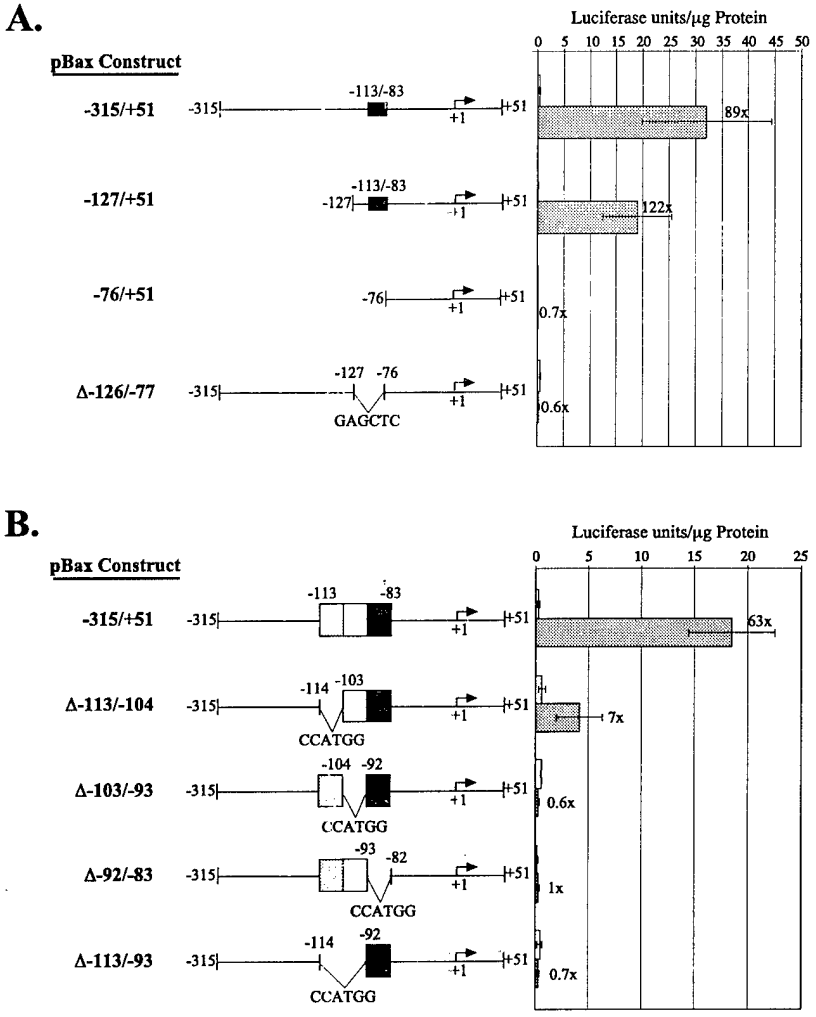

A.
pBax Construct
Luciferase units/μg Protein
0 5 10 15 20 25 30 35 40 45 50
-315/+51
-315
-113/-83
+1
+51
89x
-127/+51
-127
-113/-83
122x
-76/+51
-76
0.7x
Δ-126/-77
-315
-127 -76
GAGCTC
0.6x
B.
pBax Construct
Luciferase units/μg Protein
0 5 10 15 20 25
-315/+51
-113 -83
63x
Δ-113/-104
-114 -103
CCATGG
7x
Δ-103/-93
-104 -92
0.6x
Δ-92/-83
-93 -82
1x
Δ-113/-93
-114 -92
0.7x

Fig. 1

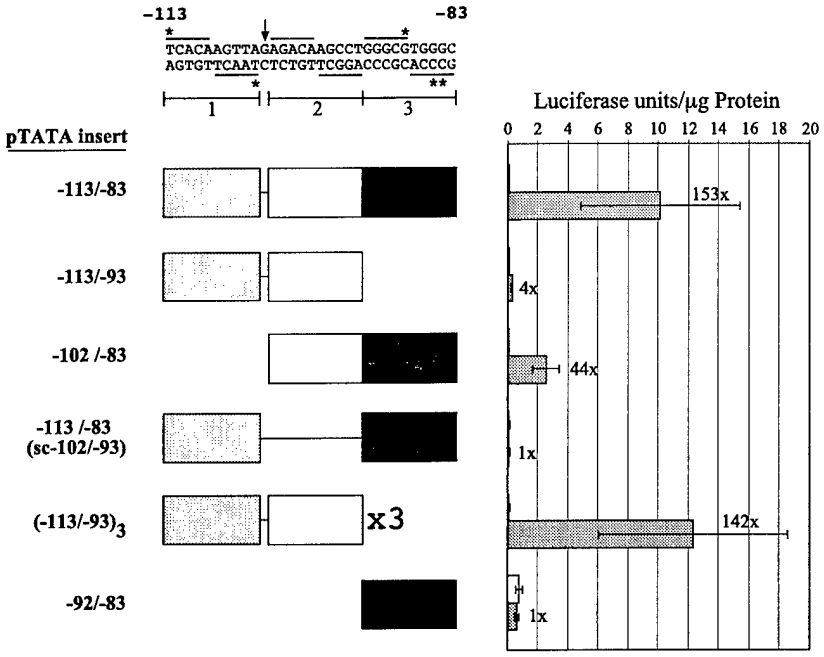

-113
-83
TCACAAGTTAGAGACAAGCCTGGGCGTGGGC
AGTGTTCAATCTCTGTTCGGACCCGCACCCG
1
2
3
pTATA insert
-113/-83
-113/-93
-102 /-83
-113 /-83
(sc-102/-93)
(-113/-93)3
x3
-92/-83
Luciferase units/µg Protein
0 2 4 6 8 10 12 14 16 18 20
153x
4x
44x
1x
142x
1x

Fig. 2

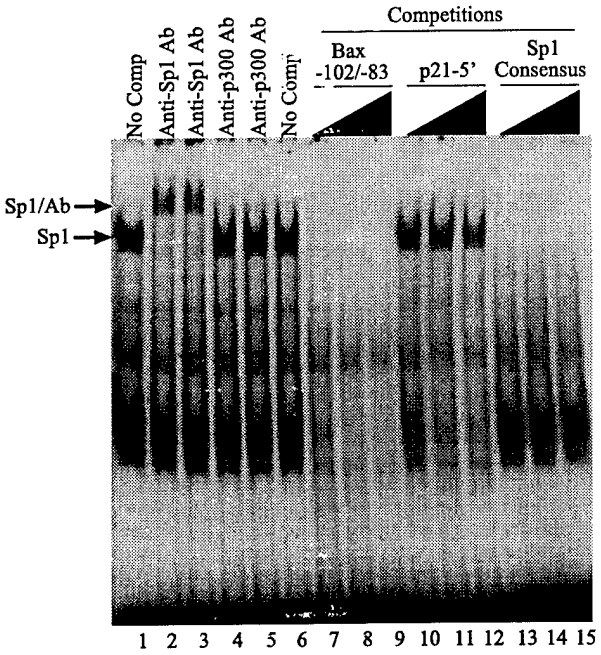
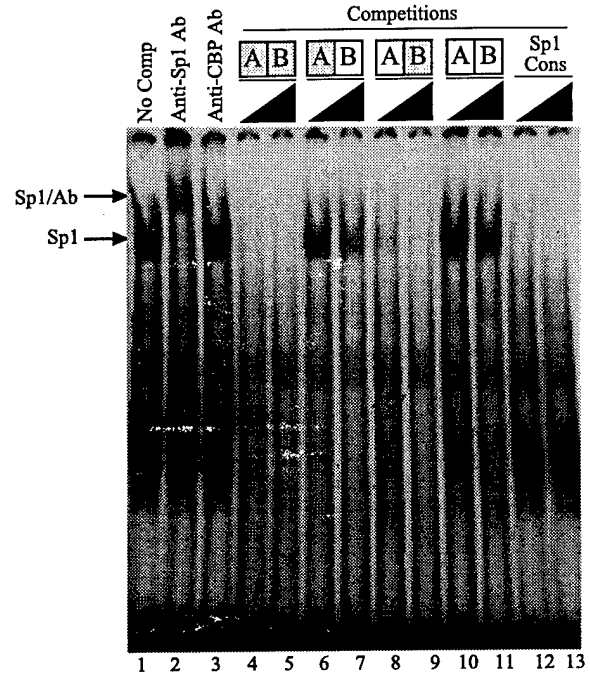

Competitions
Bax -102/-83
p21-5'
Sp1 Consensus
No Comp
Anti-Sp1 Ab
Anti-Sp1 Ab
Anti-p300 Ab
Anti-p300 Ab
No Comp
Sp1/Ab
Sp1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Fig. 3

A.


Competitions
No Comp
Anti-Sp1 Ab
Anti-CBP Ab
A B
A B
A B
A B
Sp1 Cons
Sp1/Ab
Sp1
1 2 3 4 5 6 7 8 9 10 11 12 13

B.

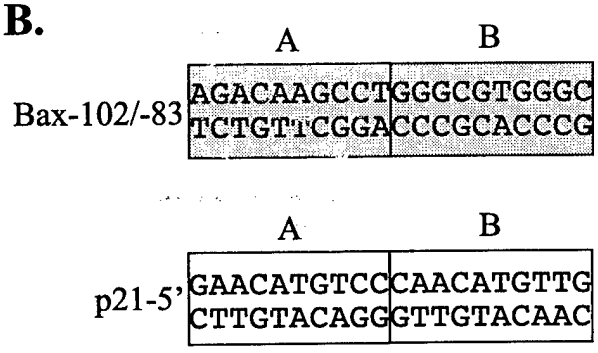

A
B
Bax-102/-83
AGACAAGCCTGGGCGTGGGC
TCTGTTCGGACCCGCACCCG
A
B
p21-5'
GAACATGTCCCAACATGTTG
CTTGTACAGGGTTGTACAAC

Fig. 4

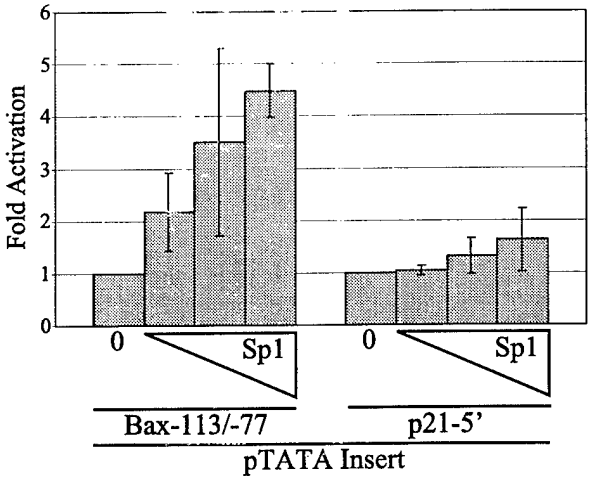

Fold Activation
6
5
4
3
2
1
0
0
Sp1
0
Sp1
Bax-113/-77
p21-5'
pTATA Insert

Fig. 5

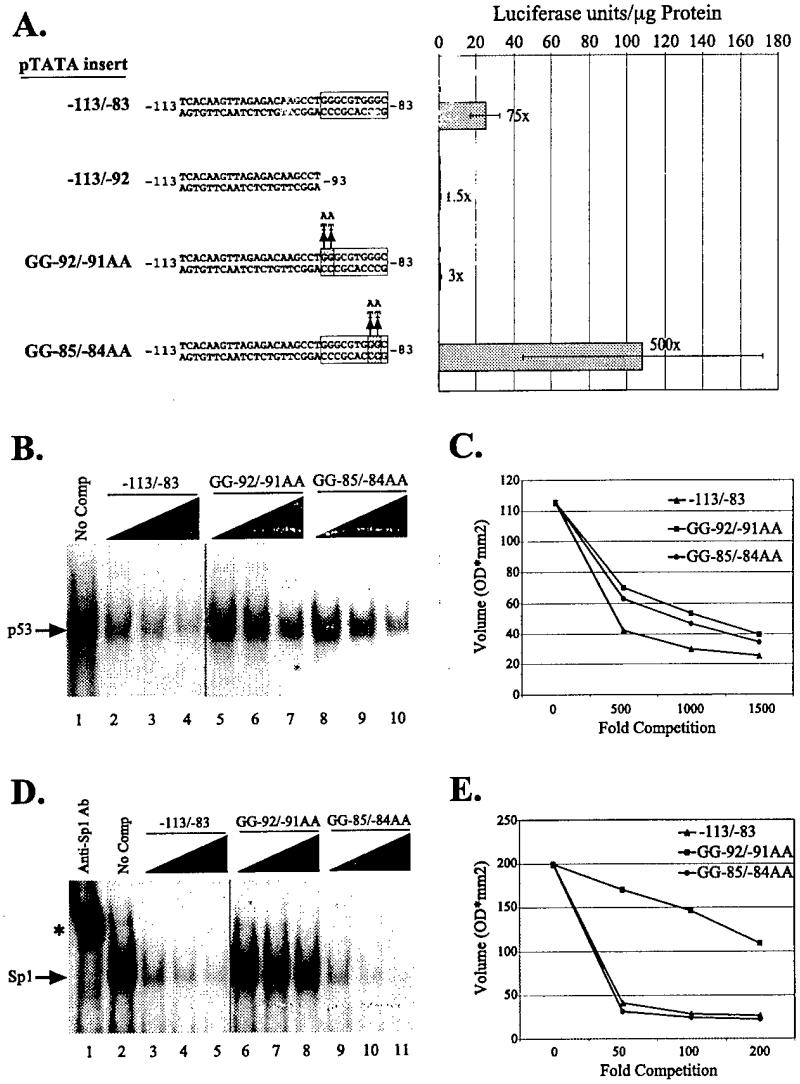

A.
pTATA insert
-113/-83
-113/-92
GG-92/-91AA
GG-85/-84AA
Luciferase units/μg Protein
0 20 40 60 80 100 120 140 160 180
75x
1.5x
3x
500x
B.
No Comp
-113/-83
GG-92/-91AA
GG-85/-84AA
p53
1 2 3 4 5 6 7 8 9 10
C.
Volume (OD*mm2)
-113/-83
GG-92/-91AA
GG-85/-84AA
Fold Competition
D.
Anti-Sp1 Ab
No Comp
-113/-83
GG-92/-91AA
GG-85/-84AA
Sp1
1 2 3 4 5 6 7 8 9 10 11
E.
Volume (OD*mm2)
-113/-83
GG-92/-91AA
GG-85/-84AA
Fold Competition

Fig. 6

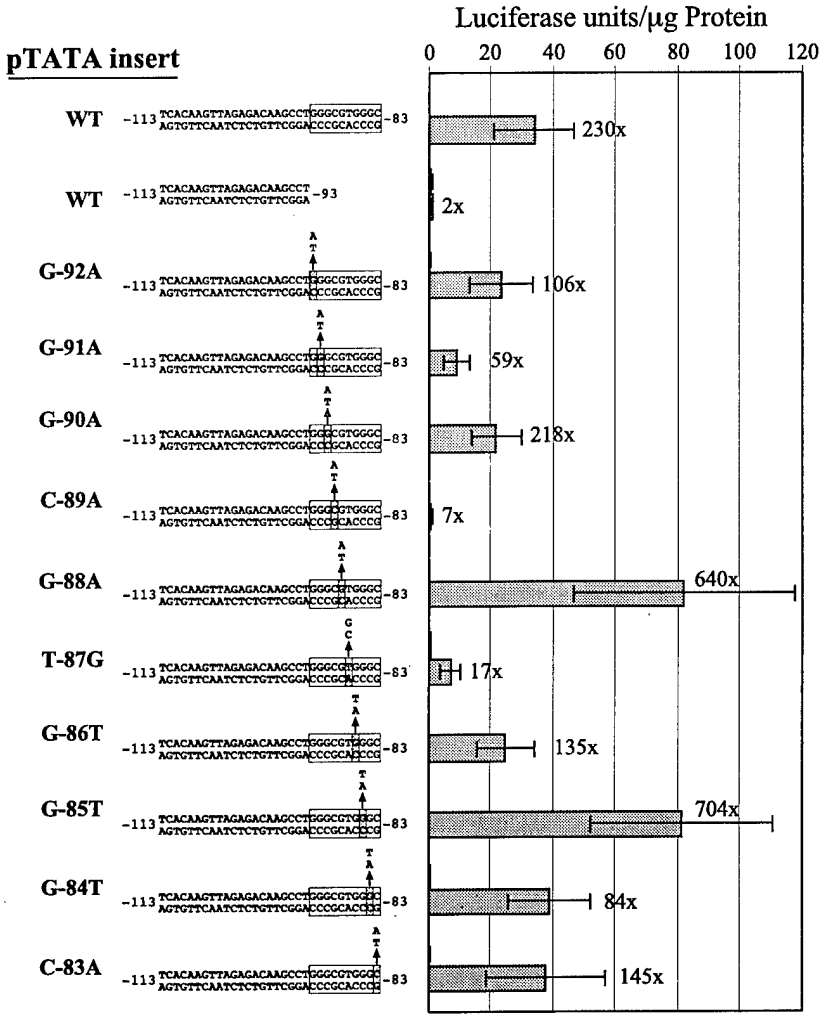

pTATA insert
Luciferase units/μg Protein
0
20
40
60
80
100
120
WT
WT
G-92A
G-91A
G-90A
C-89A
G-88A
T-87G
G-86T
G-85T
G-84T
C-83A
230x
2x
106x
59x
218x
7x
640x
17x
135x
704x
84x
145x

Fig. 7

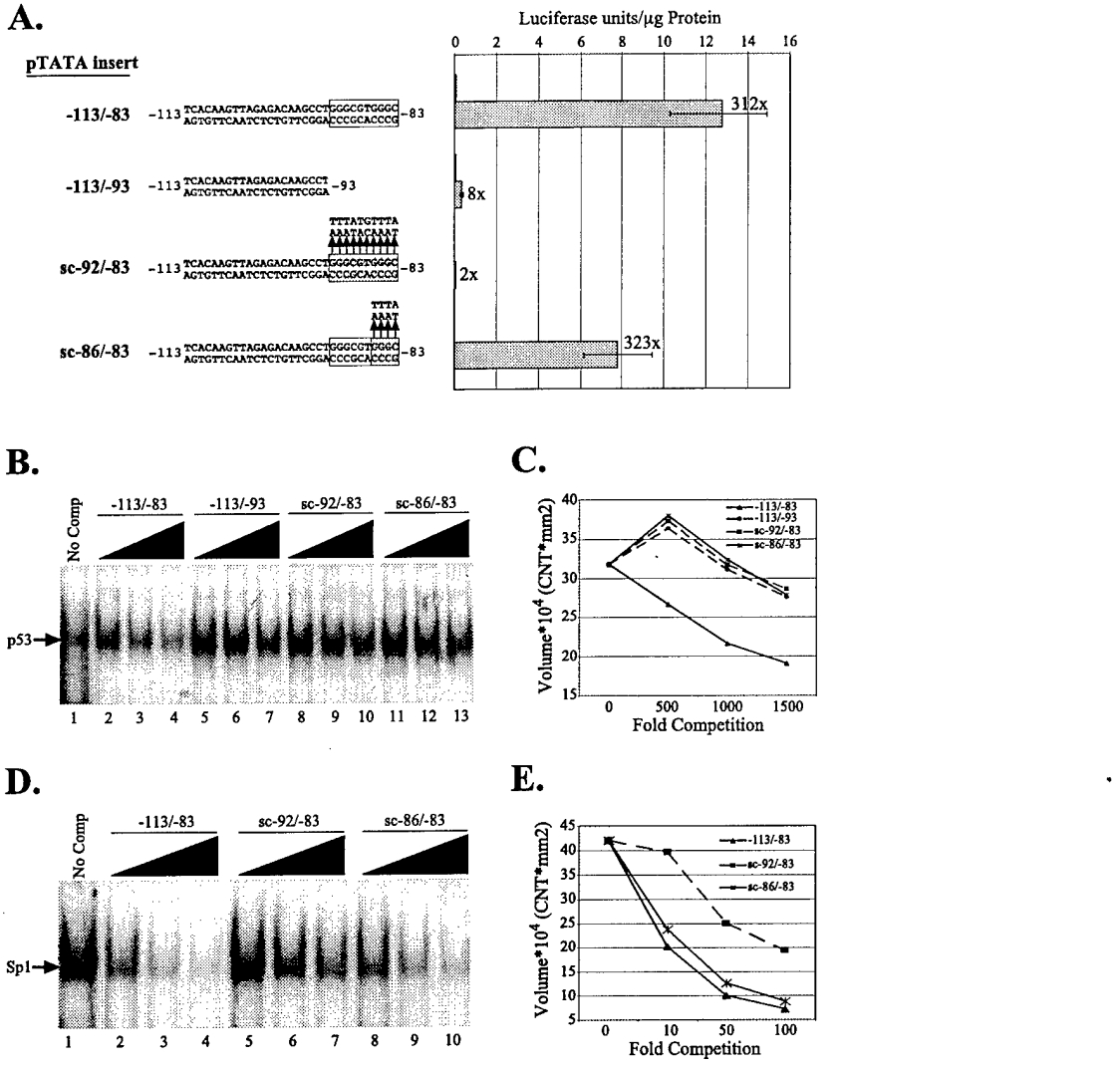

A.
pTATA insert
-113/-83
-113/-93
sc-92/-83
sc-86/-83
Luciferase units/µg Protein
312x
8x
2x
323x
B.
No Comp
-113/-83
-113/-93
sc-92/-83
sc-86/-83
p53
C.
Volume*10^4 (CNT*mm2)
Fold Competition
D.
Sp1
E.

Fig. 8

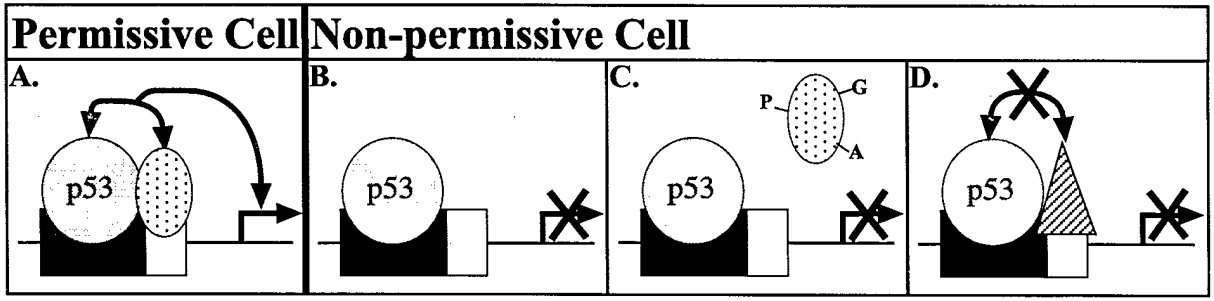

Permissive Cell
Non-permissive Cell
A.
B.
C.
D.
p53
p53
p53
p53
P
G
A

Fig. 9