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p53

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13. ABSTRACT (Maximum 200 words) This research application is centered on testing the hypothesis that there are mechanisms of carcinogenesis involving functional inactivation of wild-type p53 besides direct genetic alteration in human breast cancer. The goal of this proposal is to identify mechanisms and clone genes which encode proteins which act on wild-type p53 and functionally inactivate it. Technical objectives include: (1) Determine the mechanisms by which rat embryo fibroblasts acquire resistance to the growth suppressing activity of p53. (2) Elucidate the underlying mechanism for cytoplasmic localization of wild-type p53 in particular breast tumor cell lines. (3) Utilize an expression cloning strategy to screen for novel regulators of p53 function. (4) Determine the relevance of these various mechanisms in human breast cancer. The overexpression or mutational activation of such genes will then be examined in human breast tumors to determine whether the genes encoding such proteins are indeed involved in novel mechanisms of carcinogenesis. Such approaches as outlined here can address two important issues. The first is to determine whether overexpression of certain regulators of p53 may be associated with a particular prognosis or a particular success rate for a type of therapy. Second, identification of relevant regulators of p53 can allow us to begin to use such protein-protein complexes as targets for therapeutic intervention.				
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FOREWORD

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Publication resulting from this research:

Tang, H., Zhao, K., Pizzolato, J.F., Fonarev, M., Langer, J.C., and Manfredi, J.J. (1998) Constitutive expression of the cyclin-dependent kinase inhibitor p21 is transcriptionally regulated by the tumor suppressor protein p53. *J. Biol. Chem.* **273**, 29156-29163.

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.

Meeting abstracts resulting from this research:

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).

ANNUAL REPORT - YEAR 2
Mechanisms of breast carcinogenesis involving wild-type p53
Career Development Award
(08/01/98-07/31/99)

INTRODUCTION

The tumor suppressor protein p53 is a sequence-specific DNA-binding protein which is capable of regulating transcription both by activating and repressing mechanisms (see Gottlieb and Oren, 1996; Ko and Prives, 1996; Levine, 1997). These activities appear to be central to its role in a cellular checkpoint at the G1 to S phase transition in response to DNA damage caused either by ionizing or ultraviolet radiation or by various chemical means (Kastan *et al.*, 1992; Kuerbitz *et al.*, 1992; Maltzman and Czyzyk, 1984). Induction of DNA damage causes an increase in p53 levels resulting in activation of transcription of target genes including p21, an inhibitor of cyclin-dependent kinases, which results in either growth arrest or apoptosis (Dulic *et al.*, 1994; El-Deiry *et al.*, 1994). Consistent with its role as a tumor suppressor, a high frequency of mutation of the p53 gene has been reported in a variety of human cancers, particularly those of the colon, lung, and bladder. In such cases there is also loss of expression of the remaining allele of p53 such that only a mutant p53 is expressed (Nigro *et al.*, 1989). Similarly, breast cancers show a high frequency of loss of expression of one allele of p53 but the remaining allele is mutated in only 20-30% of the cases (Deng *et al.*, 1994; Thompson *et al.*, 1992). These data suggested that either p53 is not directly relevant to the mechanism of oncogenesis in breast cancer or breast cancer cells may have alternate mechanisms for inactivation of p53 besides genetic alteration. The research funded by this award is centered on testing the hypothesis that there are novel mechanisms of carcinogenesis involving functional inactivation of this wild-type p53 that do not involve direct genetic alteration in this kind of tumor. Two approaches have been used. The first utilizes a breast carcinoma cell line, MCF7, in which p53 is aberrantly localized to the cytoplasm. The second has focussed on the study of a cell line which appears to be resistant to p53-dependent growth arrest. Preliminary studies have suggested that the overexpression of a novel 140 kd p53-binding protein may be responsible for the failure of this cell line to respond to p53. In the past year, our studies have focussed on examining aberrant subcellular localization of p53 which has been proposed to play an important role in the inactivation of p53 function in a subset of human breast tumors (Moll *et al.*, 1992). To test this notion, we examined the p53-dependent DNA damage pathway in two human breast tumor cell lines, MCF7 and ZR-75-1, which express cytoplasmically-localized p53 which is wild-type in sequence (Casey *et al.*, 1991; Guillot *et al.*, 1997; Sheikh *et al.*, 1994).

BODY

Results

Technical Objective #1. Determine the mechanisms by which rat embryo fibroblasts acquire resistance to the growth suppressing activity of p53.

During the last year, no progress has been made in the purification of the 140 kd protein. Rather, the focus has been on studying cytoplasmically localized p53 in MCF7 cells.

Technical Objective #2. Elucidate the underlying mechanism for cytoplasmic localization of wild-type p53 in particular breast tumor cell lines.

The human breast tumor cell line MCF7 has been shown to express a wild-type p53 protein (Casey *et al.*, 1991). Proliferating MCF7 cells were subjected to immunofluorescence analysis using an anti-p53 monoclonal antibody 1801. In contrast to a control incubation with Phosphate-buffered saline, staining with the anti-p53 antibody produced a strong fluorescent signal which was localized to the cytoplasm of MCF7 cells. To confirm this cytoplasmic localization, MCF7 cells were subjected to a biochemical fractionation procedure (Fig. 1). Cytoplasmic and nuclear extracts were prepared and cross-contamination was shown to be minimal. Lactate dehydrogenase was used as a cytoplasmic marker and was assayed enzymatically (Data not shown). Histone H1 was used as a nuclear marker and was assayed by immunoblotting. Quantitative comparisons demonstrated less than 5% cross-contamination between nuclear and cytoplasmic extracts (Fig. 1). These extracts were then subjected to SDS polyacrylamide gel electrophoresis and subsequent immunoblotting with a p53-specific monoclonal antibody. The majority of p53 protein was detected in the cytoplasmic extract of untreated MCF7 cells with very little p53 detected in the corresponding nuclear extract (Fig. 1A). In contrast, p53 was predominantly found in the nuclear fractions from four other human cell lines which are known to express wild-type p53: a breast tumor line (ZR-75-30, Fig. 1C), a glioblastoma line (U87-MG, Fig. 1F), an immortalized but not transformed breast epithelial line (MCF10F, Fig. 2E) and primary diploid fibroblasts (WI38, Fig. 1D). An additional breast tumor cell line which expresses wild-type p53, ZR-75-1, was also examined (Fig. 1B). Similar to MCF7 cells, the majority of the p53 in this cell line was also found in the cytoplasmic extract although a detectable level of p53 was also found in the nuclear extract as well (Fig. 1B).

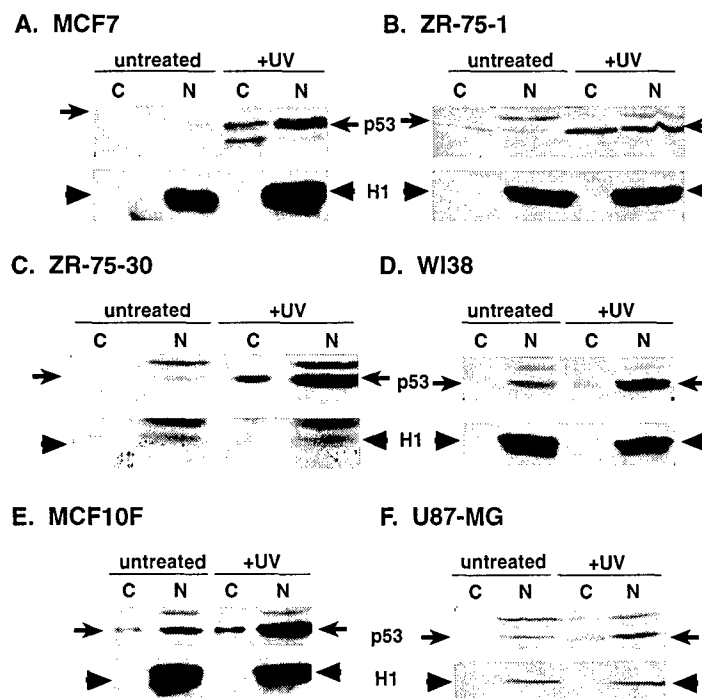


Fig. 1. Biochemical fractionation of six cell lines.

MCF7 (A), ZR-75-1 (B), ZR-75-30 (C), WI-38 (D), MCF10F (E), or U87-MG (F) cells were untreated or treated with 50 J/m² of ultraviolet light (+UV) and then incubated at 37°C for 20 to 24 hr prior to fractionation into cytosolic (C) or nuclear extracts (N) as described in Materials and Methods. 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 to represent extracts obtained from an equivalent number of cells.

Thus, two breast tumor cell lines have been identified which express a wild-type p53 that is predominantly localized to the cytoplasm. This is in contrast to other tumor cell lines as well as non-transformed and primary cells where the p53 is predominantly localized to the nucleus.

MCF7 cells were treated with either ionizing radiation or ultraviolet light and subjected to dual-labeling with propidium iodide and bromodeoxyuridine followed by flow cytometric analysis to determine the cell cycle distribution and extent of active DNA synthesis. Treatment with ionizing radiation dramatically decreased the number of cells that were positive for bromodeoxyuridine incorporation and caused an accumulation of cells in the G1 and G2 phases of the cell cycle. Although treatment with ultraviolet light similarly reduced the number of bromodeoxyuridine-positive cells, the overall distribution of cells in the different phases of the cell cycle was not substantially changed from untreated cells. Similar results were obtained after treatment of p53-negative cells with ultraviolet light confirming that this UV-induced cell cycle response is not necessarily indicative of a functional p53. A variety of cell lines including both MCF7 and ZR-75-1 were then treated with ionizing radiation and then analyzed in a similar manner (Table 1). All five cell lines which express a wild-type p53 (MCF7, ZR-75-1, ZR-75-30, MCF10F, and WI-38) demonstrated an increase in cells in the G1 phase of the cell cycle and a reduction of cells that incorporated bromodeoxyuridine indicative of an inhibition of DNA synthesis (Table 1). In contrast, two p53-negative cell lines (Saos-2 and HL-60) showed a decrease in cells in the G1 phase and an increase in cells in the S phase of the cell cycle upon treatment with ionizing radiation (Table 1). Thus, the two cell lines which express cytoplasmically localized wild-type p53 responded to ionizing radiation treatment in a similar manner as cell lines which express a wild-type p53 which is localized to the nucleus.

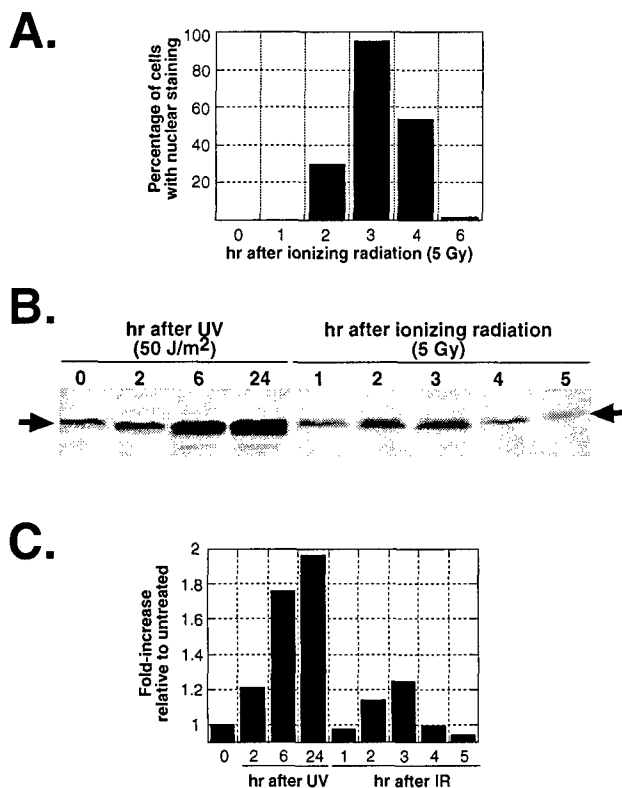


Fig. 2. (A) Level of nuclear staining for p53 correlates with an increase in p53 protein levels. MCF7 cells were grown on coverslips and either left untreated (0 hr), or treated with 5 Gy of ionizing radiation and then incubated at 37°C for the indicated time periods. Cells were then fixed and processed for immunofluorescence as described in Materials and methods. Fields of cells were counted and the resulting percent of cells with nuclear staining for p53 is shown in the bar graph. (B) MCF7 were treated with either 50 J/m² of ultraviolet light or 5 Gy of ionizing radiation as described in Materials and methods and then incubated at 37°C for the indicated times. Whole cell extracts were prepared and samples were electrophoresed on a 10% SDS polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with the anti-p53 monoclonal antibody 1801. 100 µg of total protein was loaded in each lane. (C) Densitometric analysis of the autoradiogram shown in b.

Table 1 Status of p53 and the cell cycle distribution in response to ionizing radiation in various cell lines

Cell line	Cell type	p53 status	Treatment	% of cells ^a		
				G0/G1	S	G2/M
MCF7	breast carcinoma	wild-type	control	50	28	21
			5 Gy	67	8	25
ZR-75-1	breast carcinoma	wild-type	control	75	16	8
			5 Gy	81	4	15
ZR-75-30	breast carcinoma	wild-type	control	75	15	10
			5 Gy	82	4	14
MCF10F	breast epithelium	wild-type	control	51	27	21
			5 Gy	60	12	27
WI-38	lung fibroblast	wild-type	control	57	12	29
			5 Gy	59	1	36
Saos-2	osteosarcoma	null	control	30	24	43
			5 Gy	13	32	52
HL-60	promyelocytic leukemia	null	control	37	24	5
			5 Gy	11	45	37

^a Data were determined utilizing a flow cytometric assay with dual-parameter staining (propidium iodide and bromodeoxyuridine) and are presented from a single representative experiment for each line.

Induction of p53 protein levels has been shown to result when cells carrying wild-type p53 are treated with DNA damaging agents. MCF7 cells were treated with non-lethal doses of ionizing radiation (5 Gy) or ultraviolet light (50 J/m²), and the level of p53 protein was determined by immunoblotting analysis (Fig. 2B) and quantitated by densitometry (Fig. 2C). The level of p53 increased significantly within 2 hr after either 5 Gy of ionizing radiation or 50 J/m² of ultraviolet light treatment (Fig. 2B-C). However, each of these treatments produced different kinetics of the p53 induction. In cells treated with ionizing radiation, the p53 levels increased and peaked at 2 to 3 hr after treatment

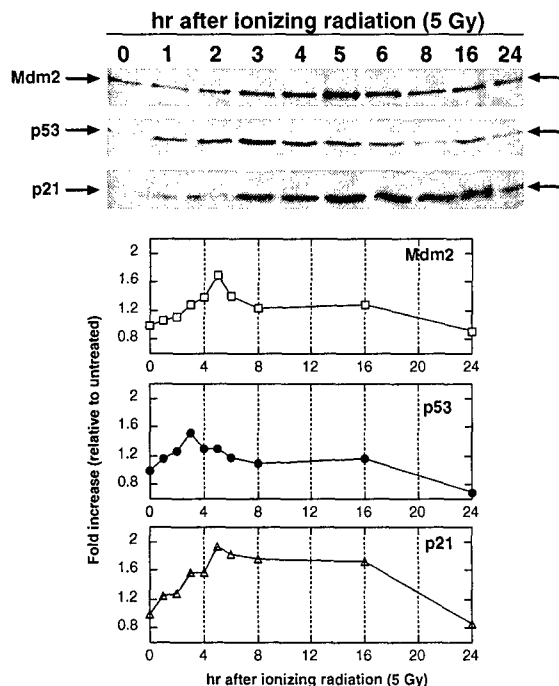


Fig. 3. Levels of Mdm2, p53 and p21 proteins in MCF7 cells after treatment with ionizing radiation. Cells were treated with 5 Gy of ionizing radiation and then incubated at 37°C for the indicated times. Whole cell extracts were prepared and samples were electrophoresed on a 10% SDS polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with either the anti-Mdm2 antibody, the anti-p53 monoclonal antibody 1801, or the anti-p21 antibody as indicated. 100 µg of total protein was loaded in each lane. Densitometric analysis was performed on the autoradiograms and the results were plotted in the graphs as shown.

and then fell back quickly to basal level, whereas in cells treated with ultraviolet light, a more profound and sustained (up to 24 hr) increase in p53 levels was observed (Fig. 2B-C).

The subcellular localization of the p53 was then examined by immunofluorescent staining of MCF7 cells after treatment with a variety of DNA damaging agents including ionizing radiation, ultraviolet light, and treatment with adriamycin. MCF7 cells were treated with either ionizing or ultraviolet radiation, then incubated for 24 hr, or treated with adriamycin continuously for 20 hr, and the localization of p53 was examined. In contrast to untreated cells where the detectable p53 protein is present in the cytoplasm, the intensity of the p53 protein staining increased and was shifted to the nucleus after each treatment. Strong nuclear staining of p53 was observed in cells 3 hr after ionizing radiation or 24 hr after treatment with ultraviolet light. Similar results were seen after treatment of MCF7 cells with adriamycin (1 μ g/ml) for 20 hr. This increase in nuclear staining correlated with the levels of protein (Fig. 2). With 5 Gy of ionizing radiation, maximal nuclear staining of p53 was observed 3 hr after treatment while the protein level peaked and this staining shifted back to the cytoplasm at 6 hr as the p53 protein level dropped to its basal level (Fig. 2).

To confirm the results that were observed with immunocytochemistry, the level of p53 protein in both cytoplasmic and nuclear compartments was assayed after biochemical fractionation 20 hr after ultraviolet light treatment in MCF7 cells (Fig. 1A). The level of p53 protein was increased in both the cytoplasmic and the nuclear fractions after DNA damage. The five other human lines which express a wild-type p53 were also examined. Upon ultraviolet light treatment, like MCF7 cells, the level of p53 protein in ZR-75-1 cells

A. ZR-75-1

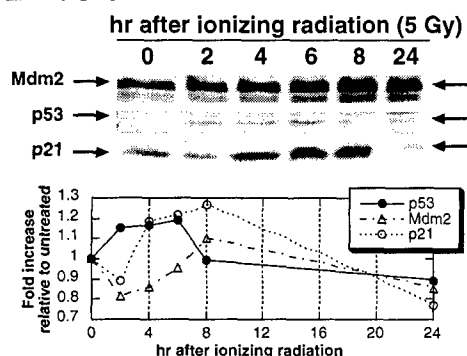
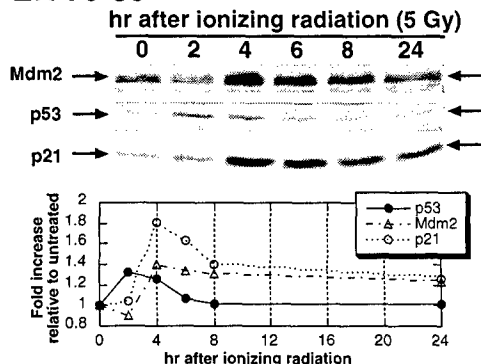


Fig. 4. Levels of Mdm2, p53 and p21 proteins in ZR-75-1 and ZR-75-30 cells after treatment with ionizing radiation. Cells were treated with 5 Gy of ionizing radiation and then incubated at 37°C for the indicated times. Whole cell extracts were prepared and samples were electrophoresed on a 10% SDS polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with either the anti-Mdm2 antibody, the anti-p53 monoclonal antibody 1801, or the anti-p21 antibody as indicated. 100 μ g of total protein was loaded in each lane. Densitometric analysis was performed on the autoradiograms and the results were plotted in the graphs as shown.

B. ZR-75-30



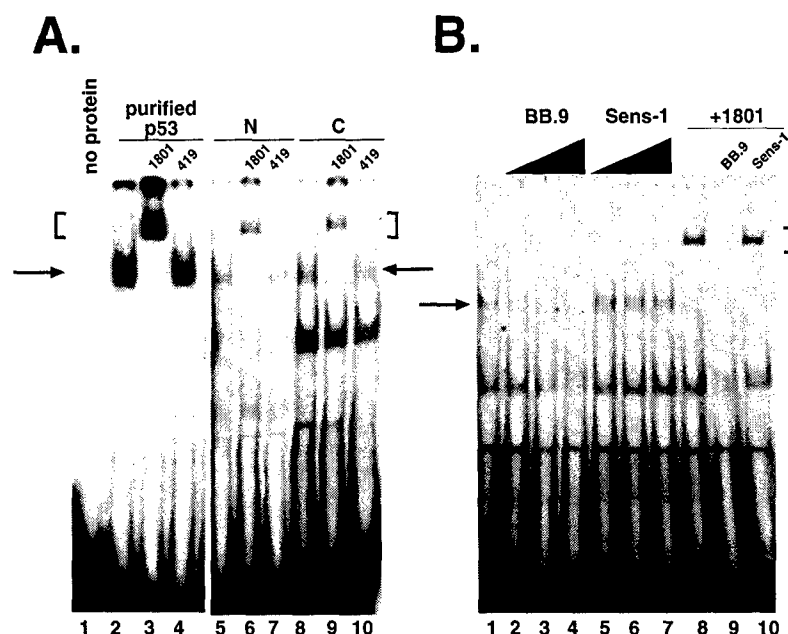


Fig.5. Sequence-specific DNA binding of p53 in nuclear extracts from ultraviolet light-treated MCF7 cells. Nuclear and cytosolic extracts were prepared from MCF7 cells 24 hr after treatment with 50 J/m² of ultraviolet light. Electrophoretic mobility shift assays were performed as described in Materials and methods. (A) 1 ng of radiolabeled probe (BB.9) was incubated in the absence of protein (Lane 1), or in the presence of purified human p53 (Lanes 2-4) or the appropriate amount of nuclear (Lanes 5-7) or cytosolic (Lanes 8-10) 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, and 8), or in the presence of either 1801 (anti-p53 antibody, Lanes 3, 6, and 9) or 419 (anti-SV40 large T antigen, Lanes 4, 7, and 10). (B) 1 ng of radiolabeled probe (BB.9) was incubated with an appropriate amount of nuclear extract alone (Lane 1), or in the presence of increasing amount of unlabeled BB.9 oligonucleotide (Lanes 2-4) or increasing amounts of a non-specific oligonucleotide, Sens-1 (Lanes 5-7). In addition, incubations were performed in the presence of 1801 (anti-p53 antibody) in the absence (Lane 8) or presence of unlabeled BB.9 (Lane 9) or Sens-1 (Lane 10).

increased in both compartments (Fig. 1B-C). In contrast to these two breast tumor lines, three of the other lines (ZR-75-30, WI38, and U87-MG) showed nuclear localization both before and after DNA damage (Fig. 1C, D, F). Interestingly, MCF10F cells had a low percentage of cytoplasmic p53 retention before and after treatment with ultraviolet light (Fig. 1E). As previously noted, histone H1 protein was only observed in the nuclear extracts and the lactate dehydrogenase activity in the nuclear fraction was less than 5.2 ± 0.7% of that seen in cytosolic extracts (averaged for all samples).

The level of p53, p21, and Mdm2 proteins were determined by immunoblotting using extracts of MCF7 cells after treatment with ionizing radiation (Fig. 3). Following the p53 induction, the level of both p21 and Mdm2 protein increased by 3 hr and peaked at 5 hr after irradiation. However, the level of p21 remained elevated up to 24 hr while the Mdm2 protein returned to basal level within 8 hr. The level of p53, Mdm2, and p21 was also examined following ionizing radiation in ZR-75-1 which have a cytoplasmically localized p53 and ZR-75-30 cells which express a nuclear p53 by immunoblotting (Figure 4). The level of Mdm2 and p21 proteins were increased following the induction of p53 in both cell lines, however, with different kinetics. In the ZR-75-30 line, like MCF7, the p53 level peaked within 2 to 4 hr and dropped back to basal value within 6 hr whereas in ZR-75-1 cells, p53 induction started as early as 2 hr and peaked at 6 hr after treatment. The

kinetics of Mdm2 and p21 induction corresponded to the changes of p53 level in both lines. Level of Mdm2 and p21 peaked at 8 hr in ZR-75-1 cells and at 4 hr in ZR-75-30.

To examine the ability of the nuclear p53 in MCF7 cells to bind to a specific DNA sequence was examined using an electrophoretic mobility shift assay (EMSA) before and after treatment with ultraviolet light (Fig. 5). Similar levels of p53 protein were used in each lane by loading different amounts of total protein. The retarded bands resulting from DNA binding were observed with either purified human p53 (used as a positive control) or both cytosolic and nuclear extract of MCF7 cells after treatment with ultraviolet light (Fig. 5A). The retarded bands were supershifted with a specific p53 antibody, 1801, but not with 419, an anti-SV40 large T antigen antibody, suggesting that p53 protein was present in the retarded complex (Fig. 5A).

Our recent studies have 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 (Tang *et al.*, 1998). 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. Further, 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.

During the course of these experiments, an analysis of the molecular basis for p53-dependent upregulation of p21 gene expression was begun (Resnick-Silverman *et al.*, 1998). It was shown that 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 upstream or 5' site was enhanced by incubation with a monoclonal antibody 421, whereas the binding of p53 to the downstream or 3' site was inhibited. Mutational analysis showed that a single base change caused one element to behave like the other. The sites in the p21 promoter are representative of two distinct classes of p53 response elements found in a variety of genes and mutational analysis showed that the sequence determinants that distinguish the two classes of elements are complex. This ability of the antibody to either enhance or inhibit DNA binding by p53 was dependent on non-specific high molecular weight DNA and could be regulated by particular high mobility group proteins. These results are consistent with an allosteric model for regulation of the sequence-specific DNA binding by p53 and argue that the conformation of p53 bound to a particular genomic site is determined by multiple factors including the binding to non-specific DNA, an interaction with HMG-1, as well as the sequence of the response element itself.

Technical Objective #3. Utilize an expression cloning strategy to screen for novel regulators of p53 function.

As the research proposed for this aim is dependent on further studies under Aims #1 and 2, no progress was made on this specific aim.

Technical Objective # 4. Determine the relevance of these various mechanisms in human breast cancer.

As the research proposed for this aim is dependent on Aim #3, no progress was made on this specific aim.

Discussion

Previous studies have demonstrated that ionizing radiation induces a p53-dependent G1 arrest which is only detectable in the presence of a functional wild-type p53. Cell lines which express either no p53 or a mutant p53 do not arrest in response to ionizing radiation (Kastan *et al.*, 1991; Kastan *et al.*, 1992; Kuerbitz *et al.*, 1992). Expression of wild-type p53 after transfection of p53-null cell lines results in restoration of a G1 growth arrest in response to ionizing radiation (Kuerbitz *et al.*, 1992). Normal cells derived from mice which are homozygously deleted for p53 are resistant to ionizing radiation-induced growth arrest but remain responsive to other types of treatment (Kastan *et al.*, 1992). Finally, selection for cell lines resistant to ionizing radiation results in the appearance of clones containing a genetically altered p53 (Wazer *et al.*, 1994). This is in contrast to other DNA damaging agents in which this is not the case. For example, resistance to chemotherapeutic drugs can result from altered uptake of the drugs across the cell membrane and ultraviolet light also induces a p53-independent G1 arrest making these two methods of DNA damage unsuitable for such use. Thus, responsiveness to ionizing radiation represents a strong criteria for the presence of a functional wild-type p53 in a particular cell line.

It is now demonstrated that two human breast carcinoma cell lines, MCF7 and ZR-75-1, which express a cytoplasmic wild-type p53, can respond to DNA damaging agents in a p53-dependent manner. This finding is supported by three observations. First, p53 protein levels increase and the cells undergo growth arrest after treatment with DNA damaging agents. It has been reported that upon DNA damage, cells that express a functional, wild-type p53 show an increase in the level of p53 protein and a drop in replicative DNA synthesis due to an arrest in the G1 phase of the cell cycle. It should be noted that in MCF7 cells, the pattern of cell cycle distribution upon growth arrest depended on the type of DNA damage that was used. Ionizing radiation caused cells to arrest at both the G1 and G2 phases of the cell cycle, however after treatment with ultraviolet light, cells arrested in all phases of the cell cycle including the S phase. This is consistent with the findings of others demonstrating an acute cessation of replicative DNA synthesis upon treatment with ultraviolet light (Lu and Lane, 1993). Second, by using biochemical fractionation, it is shown that MCF7 and ZR-75-1 cells express a cytoplasmically localized p53 and upon either ionizing radiation- or ultraviolet light-induced damage, the protein level of p53 increases in both the nucleus and the cytoplasm (Fig. 1A-B). In this assay, we have included two separate markers (lactate dehydrogenase activity and level of histone H1) to determine cross-contamination between the nuclear and cytoplasmic fractions. Two other cells lines (WI-38 and U-87 MG) express a p53 which is localized to the nucleus before and after DNA damage, suggesting that the cytoplasmic p53 that is detected in MCF7 cells is not due to a technical artifact of extract preparation (Fig. 1D, F). Moreover,

the biochemical fractionation confirmed the results seen with immunocytochemical staining in MCF7 cells. Before treatment, the p53 is cytoplasmically localized and 20 hr after ultraviolet light treatment, the level as well as staining of p53 were increased in both the cytoplasm and the nucleus. It has previously been shown that treatment with DNA-damaging agents causes an accumulation of p53 in the nucleus. Few studies, however, have reported the cellular localization of p53 in untreated cells, most likely due to the inability to detect the relative low level of p53 protein that is present prior to DNA damage. Here cytoplasmic staining for p53 in untreated MCF7 cells and nuclear accumulation of p53 in response to DNA damage is detected. Third, the expression of both p21 and Mdm2 proteins was elevated following p53 induction in MCF7 and ZR-75-1 cells (Fig. 34). The genes encoding p21 and Mdm2 are downstream targets of p53. Induction of p21 protein has been linked to G1 arrest by modulating the activity of cyclin dependent kinases. Mdm2, on the other hand, binds to p53 and targets it for degradation suggesting that it plays a role in a feedback loop. Overall, these observations suggest that cytoplasmic localization of p53 does not prevent the cellular response to DNA damage via a p53-dependent pathway and demonstrate that two breast cell lines that express cytoplasmic p53 are not resistant to DNA damage-induced growth arrest.

Since the p53-dependent constitutive expression of p21 is also intact in MCF7 cells, this argues that cytoplasmic localization of p53 does not affect the ability of p53 to control basal levels of p21. Taken together, these studies argue that cytoplasmic localization of wild-type p53 does not functionally inactivate p53 and that MCF7 cells are not a suitable model system for identifying a novel mechanism of inactivation of p53.

Adherence to Statement of Work

Technical Objective #1

Task 1:	Months 1-5	In progress
Task 2:	Months 6-17	In progress
Task 3:	Months 18-27	In progress

Technical Objective #2

Task 4:	Months 1-5	Completed
Task 5:	Months 6-17	Pending
Task 6:	Months 18-23	Pending
Task 7:	Months 24-35	Pending
Task 8:	Months 36-39	Pending

Technical Objective #3

Tasks 9-11:	Months 13-39	Pending
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Technical Objective #4

Tasks 12:	Months 40-48	Pending
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It was hoped that by the start of Year 3, sufficient material would be available for microsequencing. Although this goal was not achieved, it is expected that purification will be completed within 2 months at the latest.

KEY RESEARCH ACCOMPLISHMENTS

- cytoplasmic wild-type p53 responds to DNA damage
- cell lines expressing cytoplasmic wild-type p53 are not resistant to p53-induced growth arrest
- constitutive expression of the cyclin-dependent kinase inhibitor p21 is regulated by p53
- p53 response elements can be classified

REPORTABLE OUTCOMES

Manuscripts

Tang, H., Zhao, K., Pizzolato, J.F., Fonarev, M., Langer, J.C., and **Manfredi, J.J.** (1998) Constitutive expression of the cyclin-dependent kinase inhibitor p21 is transcriptionally regulated by the tumor suppressor protein p53. *J. Biol. Chem.* **273**, 29156-29163.

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.

Abstracts

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).

Presentations

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

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

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 CA86001-01
Grant type:	Research
Status:	Principal Investigator

Percent Effort: 25%
Total Project Period: 04/01/00-03/31/05
Total direct costs: \$875,000

This application was reviewed by the Pathology B Scientific Review Group and received a priority score of 263 and a percentile of 55.1.

CONCLUSIONS

The goal of the research contained within this proposal is to identify and clone genes which encode proteins which act on wild-type p53 and functionally inactivate it. Two potential mechanisms have been identified: the overexpression of a 140 kd p53 binding protein in the VS4-6 cell line and the cytoplasmic localization of p53 in the breast tumor cell line MCF7. Recent studies have suggested that MCF7 cells are not a suitable model for studying a mechanism of inactivation of p53. Hence in the coming year, emphasis will be placed on the purification and cloning of the 140kd protein. In addition, in the coming funding period, an expression cloning strategy will be employed in an attempt to identify novel p53 regulators and the overexpression or mutational activation of the genes that have been identified by this approach will then be examined in human breast tumors to determine whether the genes encoding such proteins are indeed involved in novel mechanisms of breast carcinogenesis.

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APPENDICES

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 activa-

tion 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-kb¹ 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 μ g/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 μ g/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-273}, respectively (27). These cell lines were grown in 10% FBS in DMEM containing high glucose and 400 μ g/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% CO₂. 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 $\times$ 10⁶ 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 μ g/ml), and leupeptin (50 μ g/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- μ m 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 μ g/ml Polybrene. Dishes were rocked for 2 h at 37 °C in a humid incubator containing 5% CO₂ 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 μ g/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 μ M bromodeoxyuridine for 30 min prior to fixation. The proportion of cells actively synthesizing DNA was quantitated by anti-bromodeoxyuridine immunofluorescence, 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 μ g 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 μ l 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 μ l 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 MgCl₂, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, and 100 μ M Na₃VO₄ 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 MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 μ M leupeptin, and 50 μ g/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 μ l) were mixed with 1 ng of labeled double-stranded oligonucleotide in a total reaction mixture of 30 μ l containing 6 μ l of 5 $\times$ electrophoretic mobility shift assay buffer (100 mM Hepes, pH 7.9, 0.5 mM EDTA, 50% glycerol, 10 mM MgCl₂), 1.5 μ l of 40 mM spermidine, 1.5 μ l of 10 mM dithiothreitol, 1 μ l of 500 μ g/ml double-stranded poly(dI/dC), and 5–13 μ l 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 μ l 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

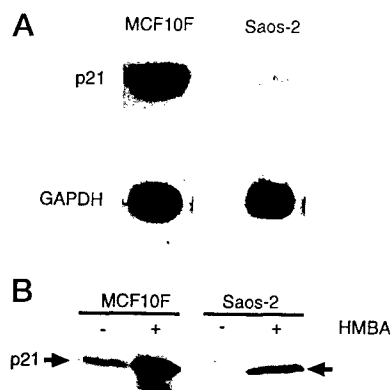


FIG. 1. MCF10F cells express detectable levels of p21 mRNA and protein, whereas p53-negative Saos-2 cells do not. A, RNA was extracted from wild-type p53-expressing MCF10F and p53-negative Saos-2 cells, and Northern analysis was performed as described under "Experimental Procedures." Blots were probed 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."

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. 1A). Total cellular extracts of each cell line were subjected to SDS-polyacrylamide gel electrophoresis and subsequent immunoblotting with an anti-p21-specific antibody (Fig. 1B). 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'*-hexamethylene-bisacetamide (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-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. 2A, lane 4) and mutant (Fig. 2A, lane 3) p53 expression could be detected in comparison to the parent cells which are p53-negative (Fig. 2A, 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. 2A, lane 5). Consistent with previous observations, this level of

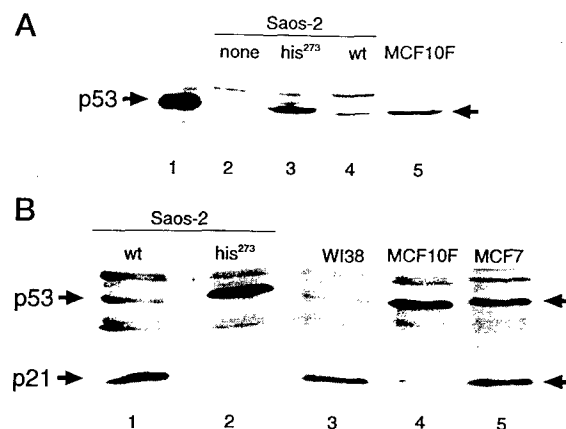


FIG. 2. Retroviral infection of Saos-2 cells restores expression of p53 and p21. A, Saos-2 cells were infected with a recombinant retrovirus expressing either wild-type p53 or the mutant p53^{His-273}. Selection was performed in G418 sulfate, and drug-resistant pools of cells were obtained. Equivalent amounts of total cellular extract of parent Saos-2 cells (lane 2), the His²⁷³-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 His²⁷³ 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.

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. 2B). 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 4A). Co-transfection of an expression plasmid encoding human MDM2 protein caused repression of that basal level of expression (Fig. 3 and 4A). 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 μ M 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 (His²⁷³) p53 proteins.

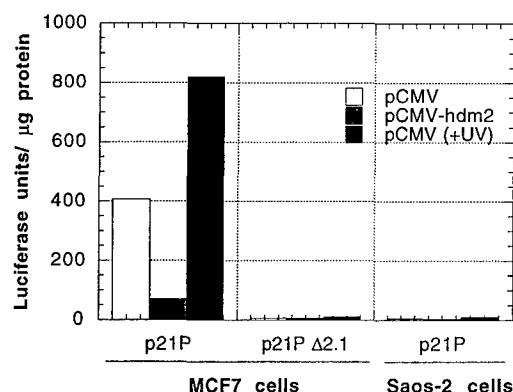


FIG. 3. Deletion of the p53 response elements results in loss of basal expression of a luciferase reporter containing the p21 promoter. MCF7 cells or Saos-2 cells were transfected as described under "Experimental Procedures" with 2 μ g of p21P or p21P 2.1 and either 50 ng of pCMV or pCMV-hdm2 as indicated. 18 h prior to lysis, a set of wells containing only pCMV were treated with 50 J/m² 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 4C). 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. 4B). Similar to MCF7 cells (Fig. 4A) and in contrast to the p53-negative Saos-2 cells (Fig. 4C), 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. 4D).

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-143} (45). Increasing amounts of the p53^{Ala-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.

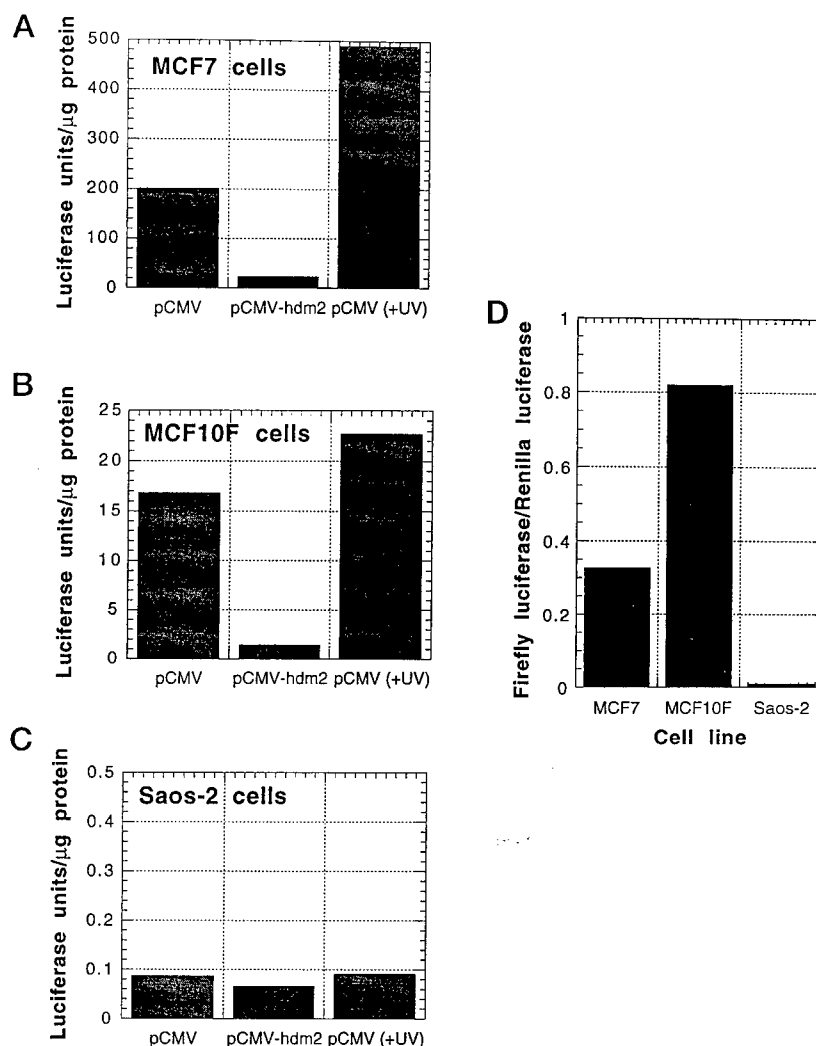
p53 in Nuclear Extracts of Untreated Cells Is Capable of Binding to DNA in a Sequence-specific Manner—The data presented thus far implicate a role for endogenous p53 in transcriptional activation of the p21 promoter in untreated proliferating cells. If this is the case, then this endogenous p53 should 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/m² 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 μ g 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/m² 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 μ g 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 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. 8A). 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. 8A, 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. 8A, 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. 8A, lanes 11 and 12). To determine that the binding that was seen was sequence-specific, competition experiments were performed (Fig. 8B). 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. 8B, lanes 9–11) competed well for the binding to 1801-supershifted complexes, whereas increasing amounts of Sens-1 (Fig. 8B, 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. 8B).

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. 9A, lane 5, and 9B, lanes 5 and 8). Incubation with the p53-specific antibody 1801 pro-

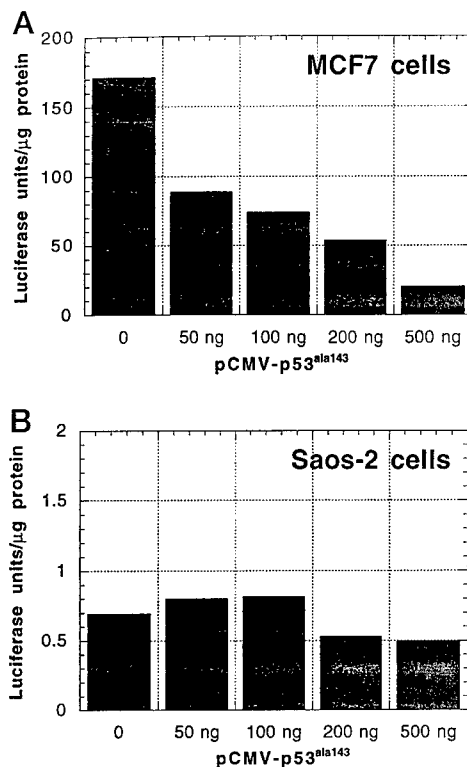


FIG. 5. Ectopic expression of the mutant human p53^{Ala-143} protein represses basal expression of a luciferase reporter containing the p21 promoter in a dose-dependent manner. MCF7 cells (A) or Saos-2 cells (B) were transfected as described under "Experimental Procedures" with 2 μg of p21P alone or 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.

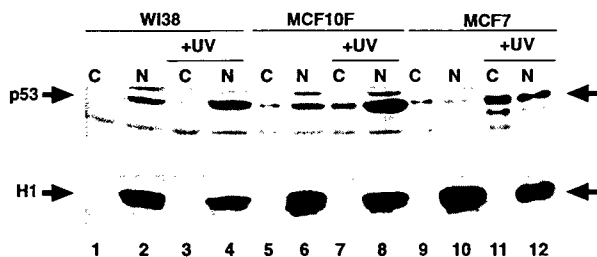


FIG. 6. Biochemical fractionation demonstrates that nuclear p53 levels increase upon UV treatment in WI38, MCF10F, and MCF7 cells. WI38, MCF10F, or MCF7 cells were untreated, or treated with 50 J/m² of ultraviolet light (+UV) and then incubated at 37 °C for 20 h prior to fractionation into cytosolic (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

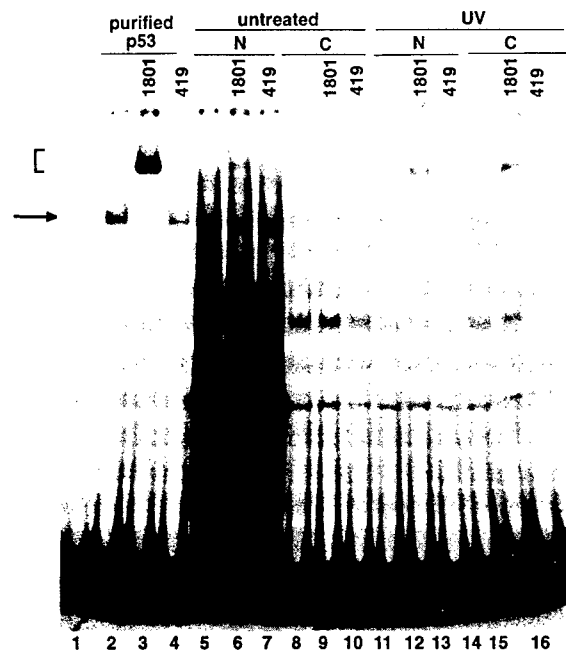


FIG. 7. p53 in nuclear extracts of untreated MCF7 cells binds to DNA in an electrophoretic mobility shift assay. Nuclear and cytosolic extracts were prepared from MCF7 cells that were untreated (lanes 5–10) or 24 h after treatment with 50 J/m² of ultraviolet light (lanes 11–16). Electrophoretic mobility 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-

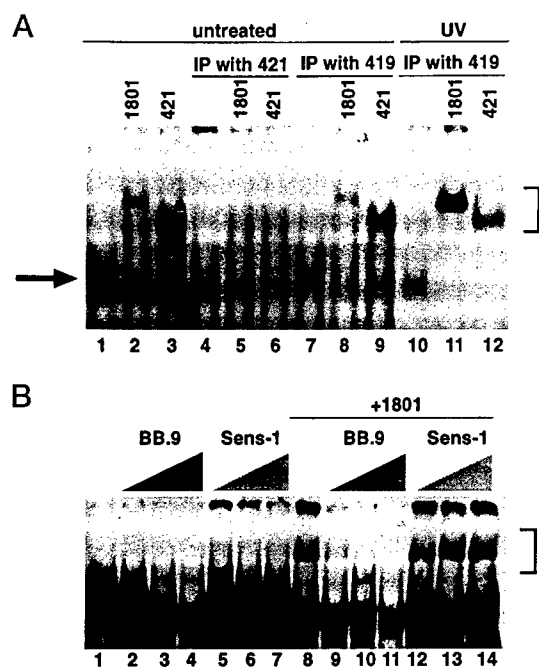


FIG. 8. p53 in nuclear extracts of untreated MCF7 cells binds to DNA in a sequence-specific manner. A, nuclear extracts of MCF7 cells that were untreated (lanes 1–3) or treated with 50 J/m² ultraviolet light (UV, lanes 4–12) were used directly (lanes 1–3) or immunoprecipitated with either an anti-p53 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-p53 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.

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

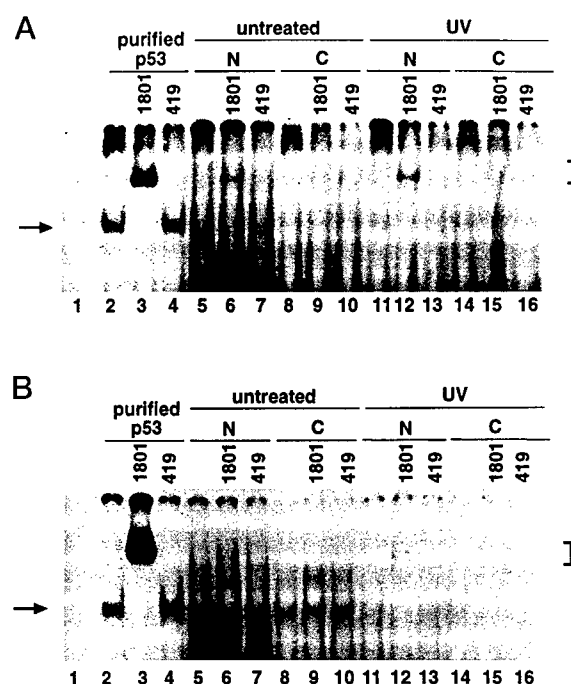


FIG. 9. p53 in nuclear extracts of untreated WI38 or MCF10F cells binds to DNA in an electrophoretic mobility shift assay. Nuclear and cytosolic extracts were prepared from WI38 (A) or MCF10F (B) cells that were untreated (lanes 5–10) or 24 h after treatment with 50 J/m² of ultraviolet light (lanes 11–16). 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 μ g 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.

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.

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

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

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

MATERIALS AND METHODS

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% CO₂. 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.

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 μ M leupeptin, and 10 μ g/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. 1A and 2A). 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. 1A). Western blots

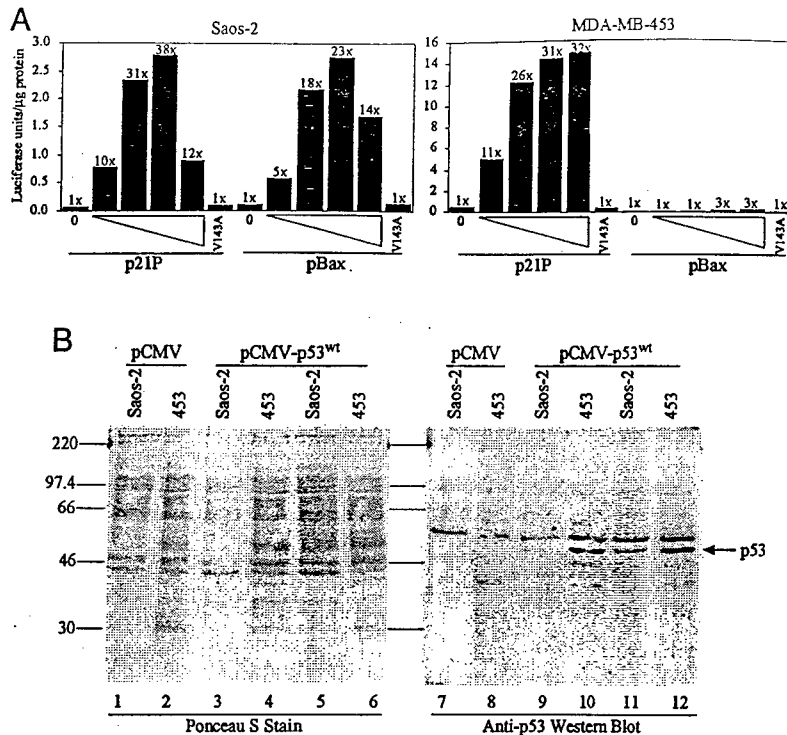
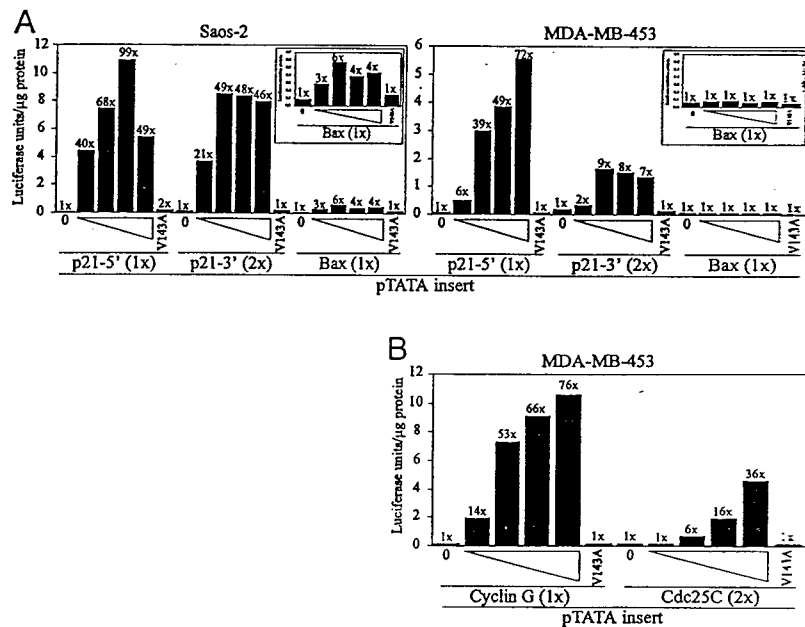


FIG. 1. Expression of wild-type p53 in MDA-MB-453 cells fails to activate transcription through the *bax* promoter. A, Saos-2 and MDA-MB-453 cells were transfected as described under "Materials and Methods" with 2 μ g of the indicated reporter constructs in the presence of 0, 5, 50, 100, or 200 ng of 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 μ g/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 μ g 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 μ g 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 μ g 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 μ g/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. 1B, compare lane 9 with lane 10 and lane 11 with lane 12), if not slightly higher in MDA-MB-453 (Fig. 1B, 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

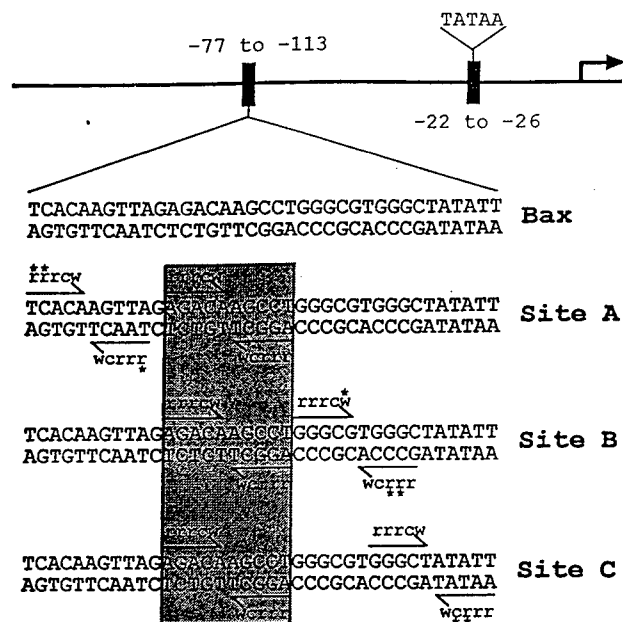


FIG. 3. Schematic of the p53 response element of the human *bax* promoter. The previously identified p53 response element of the *bax* promoter is located at -113 to -77 from the transcriptional start site. Based on the p53 consensus binding site, there exists, within this 37-bp sequence, three potential, 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, *Y* representing pyrimidine, 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) is also 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. 2A), as well as a construct containing the p53 response element of the *bax* promoter (Fig. 2A, 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. 2A, inset), whereas activating reporters containing either the 5' or the 3' element of the *p21* promoter (Fig. 2A). 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. 2B). 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. 4A, lane 1), and this binding was effectively competed by an excess of the same, unlabeled oligonucleotide (Fig. 4A, lanes 2–4). Unlabeled Oligo A, Oligo B, and Oligo C also successfully competed for p53 binding (Fig. 4A, lanes 5–7, 8–10, and 11–13). For comparison, an unrelated control oligonucleotide, Sens-1, was unable to compete for p53 binding (Fig. 4A, 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. 4B). 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

T1

F4

F5

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 gtagcgaatt-3'
Oligo B	5'-aattcggtacc AGACAAGCCTGGGCGTGGGCG gtagcgaatt-3'
Oligo C	5'-aattcggtacc AGACAAGCCT tttacg GGGCTATATT gtagcgaatt-3'
Oligo AB	5'-aattcggtacc TCACAAGTTAGAGACAAGCCTGGGCGTGGGCG gtagcgaatt-3'
Oligo AC	5'-aattcggtacc TCACAAGTTAGAGACAAGCCT tttacg GGGCTATATT gtagcgaatt-3'
Oligo BC	5'-aattcggtacc AGACAAGCCTGGGCGTGGGCGTATATT gtagcgaatt-3'
p21-5'	5'-aattcggtacc GAACATGTCCCAACATGTTG gtagcgaatt-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.

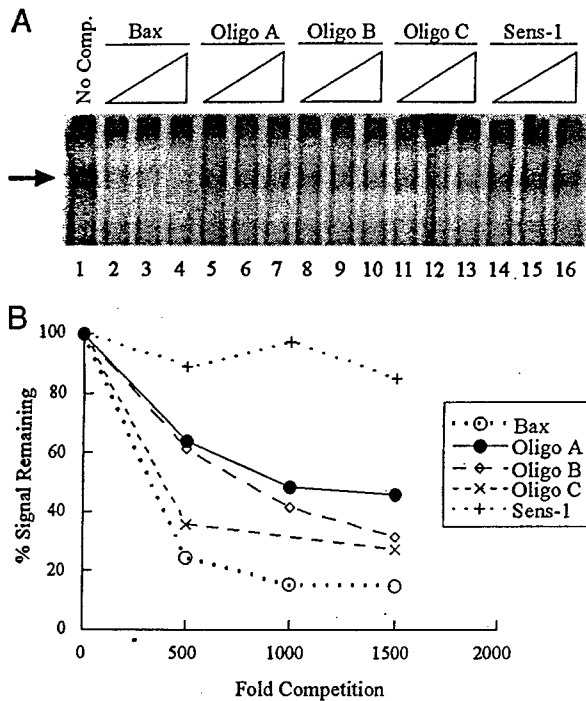


FIG. 4. The p53 response element of the *bax* promoter contains three overlapping p53 binding sites. A, an electrophoretic mobility shift assay was performed using the Bax oligonucleotide as radiolabeled probe. 50 ng of purified p53 was incubated with 3 ng of the probe alone (lane 1) or in the presence 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.

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

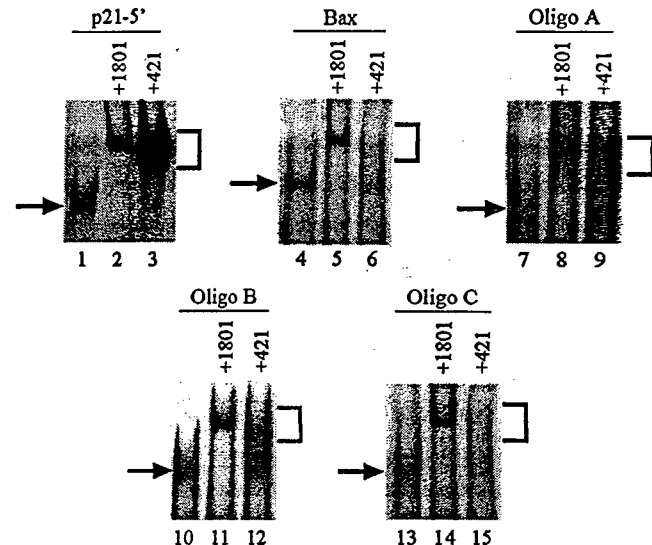


FIG. 5. Monoclonal antibody 421 enhances the binding of p53 to the *p21* site but inhibits the binding of p53 to the *bax* sites. An electrophoretic mobility shift assay was performed, incubating either 5 ng (lanes 1-3) or 50 ng (lanes 4-15) of purified p53 with 3 ng of the indicated radiolabeled probes 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.

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. 6A). 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

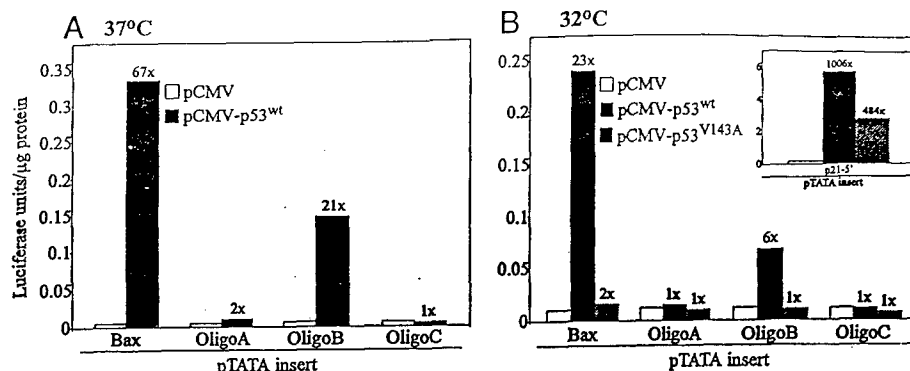


FIG. 6. Site B is sufficient to confer p53-dependent transactivation, but the level of transactivation is lower than that observed with the complete 37-bp response element. Saos-2 cells were transfected as described under "Materials and Methods" with 2 μ g of the indicated reporter constructs and 50 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. 6A, 2- and 1-fold, respectively). The same pattern of activation was observed with wild-type p53 at 32 °C (Fig. 6B). 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. 6B, 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. 6B, 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. 7A, lane 1), and this binding was effectively competed by an excess of the same, unlabeled oligonucleotide (Fig. 7A, lanes 2-4). Oligo BC, as well as Oligo AC failed to compete for p53 binding to any greater degree than Oligo B (Fig. 7A, compare lanes 11-13 and 14-16 with lanes 5-7). Oligo AB, however, effectively competed for p53 binding (Fig. 7A, lanes 8-10). This competition was in the same range as that observed with the complete Bax oligonucleotide (Fig. 7B), 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. 8A, 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. 8A, 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. 8A, lanes 5-16), 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. 8A, 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. 8A, 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

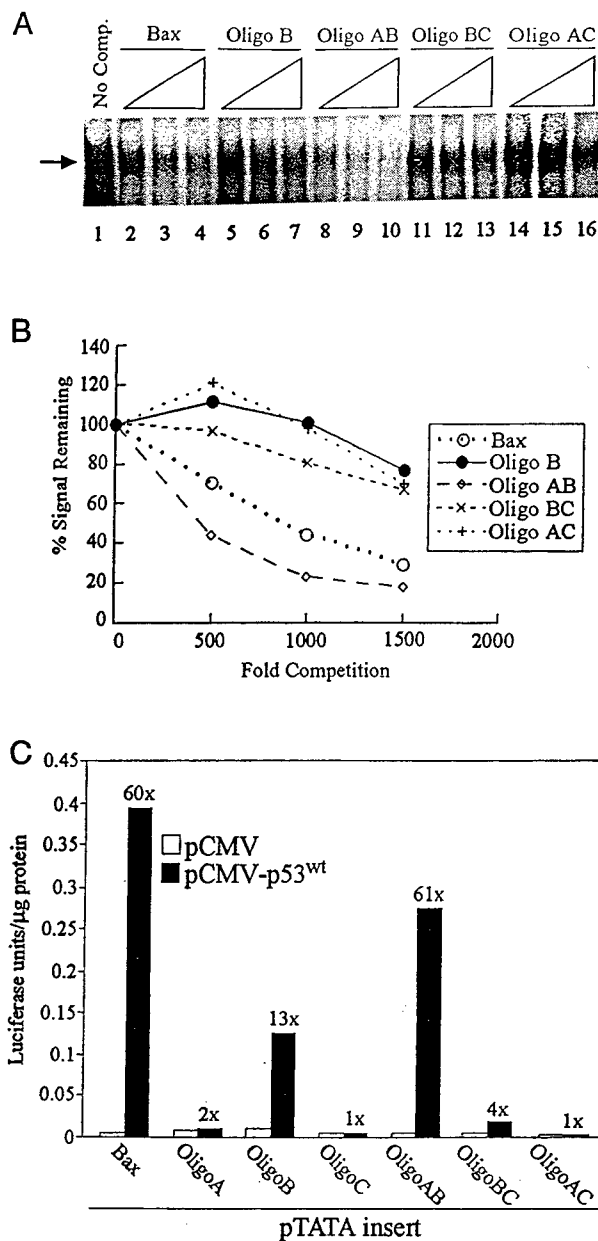


FIG. 7. Overlapping binding sites synergize in p53 binding and in p53-dependent transactivation. A, an electrophoretic mobility shift assay was performed using the Bax oligonucleotide as radiolabeled probe. 50 ng of purified p53 was incubated with 3 ng of the probe alone (lane 1) or in the presence of 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 μ g 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.

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

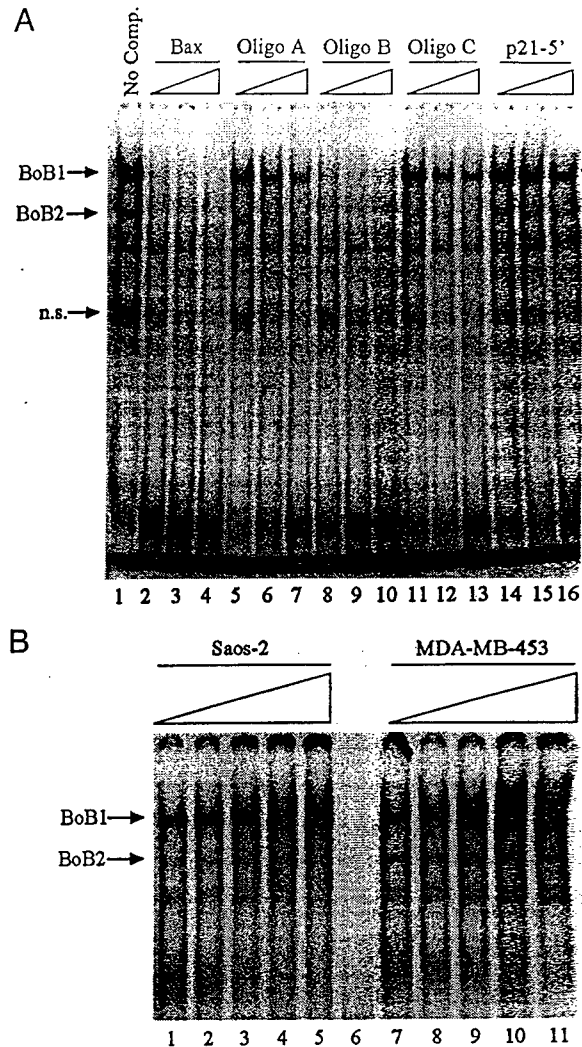


FIG. 8. Nuclear extracts from MDA-MB-453 cells contain two factors that bind in a sequence-specific manner to the 37-bp p53 response element of the *bax* promoter. A, an electrophoretic mobility shift assay was performed using the Bax oligonucleotide as radiolabeled probe. 2 μ l (9 μ g of total protein) 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. 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 μ g (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.

BoB2 in Saos-2 (Fig. 8B, lanes 1-5) and MDA-MB-453 (Fig. 8B, 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. 8B, 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, 73 Does Not Selectively Inhibit the Ability of p53 to Activate Transcription through the bax Promoter—In

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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 p73 α 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 Δ ^{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.

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. 1A). 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

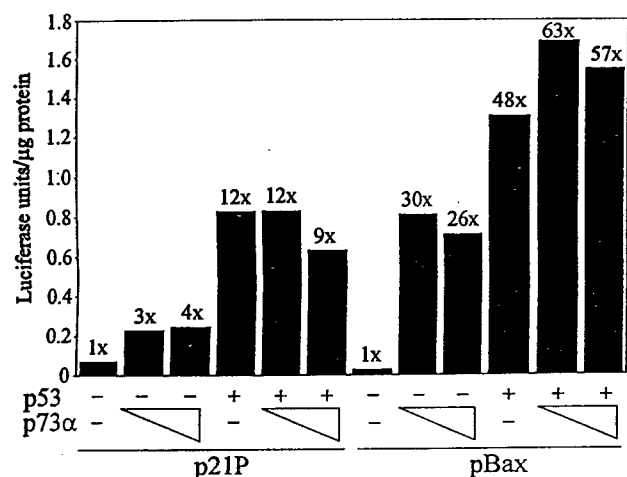


FIG. 9. The p53 homolog p73 does not selectively inhibit the ability of p53 to activate transcription through the *bax* promoter. Saos-2 cells were transfected as described under "Materials and Methods" with 2 μ g of either the p21P or pBax luciferase reporter plasmids, 0 ng (-) or 50 ng (+) of pCMV-p53^{wt}, and 0 (-), 50, or 100 ng of pCMV-p73 α . 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 μ g/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-p73 α as compared with pCMV.

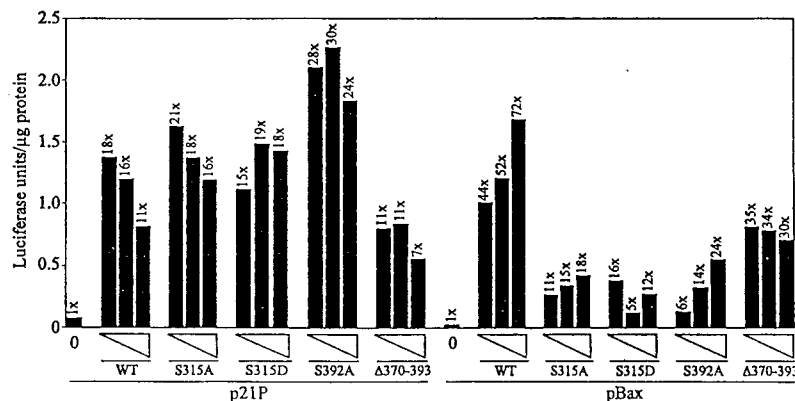


FIG. 10. An intact C terminus is not required for p53-dependent transcriptional activation of the *bax* promoter. Saos-2 cells were transfected as described under "Materials and Methods" with 2 μ g of either the p21P or pBax luciferase reporter plasmids and 0, 50, 100, or 200 ng of pCMV-p53^{wt} (WT), pB-p53^{S315A} (S315A), pB-p53^{S315D} (S315D), pCMV-p53^{S392A} (S392A), or pCMV-p53 Δ ^{370–393} (Δ 370–393). 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 μ g/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 co-workers (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. Thus, 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. 8B), 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 p73 α 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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TARGET GENE SELECTION BY p53 IS REGULATED BY MULTIPLE MECHANISMS

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Depending upon particular cellular conditions, the tumor suppressor protein p53 induces growth arrest or apoptosis. Since the DNA binding activity of p53 plays a role in each of these responses, the ability of p53 to select among various target genes to elicit a particular cellular outcome may be central to the regulation of its biological function. Three mechanisms have been identified which can contribute to the regulation of target gene selection by p53.

First, two classes of p53 response elements have been identified by examining the effect of monoclonal antibody 421 (mAb 421) on the sequence-specific DNA binding of p53 in electrophoretic mobility shift assays (EMSA). Incubation with mAb 421 enhanced the binding of p53 to one set of response elements but inhibited binding to another set. A comparison of these elements as well as mutational analysis of sites from the *p21* promoter has defined some of the sequence determinants that distinguish the two classes of elements. Further, this ability of mAb421 to either enhance or inhibit DNA binding by p53 was dependent on the presence of non-specific high molecular weight DNA and could be regulated by particular high mobility group proteins. The results are consistent with p53 adopting distinct conformations when bound to different subsets of response elements.

Second, two novel factors which bind to a subset of p53 response elements in a sequence-specific manner have been identified. These factors appear to be distinct from p53 and its homologs. EMSA studies have suggested that the binding of p53 and these nuclear factors may be mutually exclusive, suggesting that the interaction of these factors with a subset of p53 response elements is a mechanism for regulation of p53 target gene selectivity.

Third, additional sequence elements besides the p53 binding sites appear to be involved in p53-dependent regulation of the *p21* and *cdc25C* genes. The 3' site in the *p21* promoter requires an additional element to confer p53-dependent activation on a minimal promoter. The p53 site in the *cdc25C* promoter confers p53-dependent activation, however, the presence of an additional *cdc25C* promoter element causes p53 to repress transcription through the same p53 binding site. Thus, sequence context can determine the functional consequences of p53 binding.

Understanding the basis for target gene selection by p53 has implications for cancer treatment. The optimal therapeutic response to DNA damage caused by chemotherapeutic agents is apoptosis rather than cell cycle arrest. Elucidating the molecular mechanisms that regulate target gene selectivity by p53 may give insight into the ability of p53 to trigger apoptosis versus arrest and lead to more effective therapeutic intervention.