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

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

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

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

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

EXPERIMENTAL PROCEDURES

Plasmids—The plasmid p21P contains 2.5-kb1 of the human p21 promoter inserted upstream of a firefly luciferase reporter gene in the

1 The abbreviations used are: kb, kilobase pair(s); CMV, cytomegalo-

virus; DMM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine

serum; PBS, phosphate-buffered saline; HMBA, N,N’-hexamethyl-

ene-bisacetamide.
vector pGL2 (Promega). The plasmid p21Δ2.1 has 2.1 kb at the 5’ end of the promoter sequence removed and lacks the two p35 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 containing serine 167 (CMVC) and the plasmid pCMV-p53Δ3Ala-143 encodes the tumor-derived mutant human p53 protein containing a missense mutation of valine to alanine at residue 143 (26).

**Antibodies and Cells—**Saos-2 and WI38 cells were obtained from the American Type Culture Collection and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). MCF7 cells were maintained in RPMI medium containing 10% heat-inactivated FBS and 5 μg/ml insulin. MCF10F cells were grown in 50% DMEM and 50% Ham’s F12 medium containing 5% horse serum, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 10 μg/ml insulin, and 500 ng/ml hydrocortisone. PA12-p53BN and PA12-p53EN are cell lines that produce recombinant retrovirus encoding human wild-type p53 or the mutant p53^53R27S, respectively (27). These cell lines were grown in 10% FBS in DMEM containing high glucose and 400 μg/ml G418 sulfate. The hybridoma cell line producing the mouse monoclonal antibody 1801 was grown in DMEM containing 10% FBS. Hybridoma cell lines expressing the mouse monoclonal antibodies 421 and 419 were grown in 50% DMEM and 50% Fischer’s medium containing 10% FBS. Monoclonal antibody 1801 specifically reacts with human p53 protein containing a missense mutation of valine to alanine.

**Preparation of Nuclear and Cytosolic Extracts—**Nuclear and cytosolic extracts were prepared as described by Greber et al. (31). Cells were washed twice with PBS, and then nuclei were suspended to replace the media in the reaction. The was incubated at 37 °C for 5 h, after which the transfection mixture was removed and replaced with complete medium containing serum. After 48 h, the 6-well plates were placed on ice and washed once with PBS. The cells were then lysed by scraping into 120 μl of PBS and 0.5% Triton X-100, and sonicated and nuclear extracts. 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 (H1-4, Boehringer Mannheim). Such assays showed less than 10% cross-contamination between cytosolic and nuclear proteins.

**Electrophoretic Mobility Shift Assays—**The specific probe that was used for binding, TCGAGCCGGGATCTGCGGGATCTGCGGGGATGTC, contains the high affinity binding sequence identified by Halazonetis et al. (11) named by them BC or BB,9. In the competition experiment, the non-specific oligonucleotide (refers to 30 min of 1A, TCGAAGAGGCTGCAGGGGACC, was used. Complementary single-stranded oligonucleotides were annealed by incubation at 95 °C for 5 min, 65 °C for 10 min, and then gradually brought to room temperature. Ends were filled using the Klenow fragment of DNA polymerase to produce a labeled double-stranded oligonucleotide. Appropriate amounts of extracts (1–7 μl) were mixed with 1 ng of labeled double-stranded oligonucleotide in a total reaction mixture of 30 μl containing 6 μl of 5× electrophoretic mobility shift assay buffer (100 mM Hepes, pH 7.9, 0.5 mM EDTA, 50% glycerol, 10 mM MgCl₂, 1.5 μl of 40 μM spermidine, 1.5 μl of 10 mM dithiothreitol, 1 μl of 500 μg/ml doublestranded poly(dC)₃, and 5–13 μl of water with a final salt concentration of 55 mM. The amount of total protein per reaction was normalized, and the reactions were carried out at room temperature for 30 min. For antibody supershift analysis, 2 μl of the appropriate undiluted hybridoma supernatant was added. His-tagged human p53 was produced by infection of insect cells with a recombinant baculovirus and purified by nickel-agarose chromatography and used as a positive control (52). Samples were electrophoretically separated on a native 4% polyacrylamide gel at 4 °C at 200 V for 2 h. After drying, gels were exposed to Kodak XAR film at −70 °C with an intensifying screen.

**RESULTS**

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

**Transfection of Reporter Constructs—**MCF7, MCF10F, or Saos-2 cells were transfected using the N-[1-(2,3-dioleoyloxy)propyl]-N,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 left undisturbed for 24 h. At the end of this period, the supernatant was harvested and filtered.
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 \(N,N\)-hexamethylenebisacetamide (HMBA). HMBA is a non-retinoid, differentiating agent that has previously been shown to induce p21 expression in a p53-independent manner (24). Treatment of Saos-2 cells with HMBA induced expression of p21 demonstrating that Saos-2 cells retained the ability to synthesize p21. Thus, both the level of protein and messenger RNA for p21 were much higher in the p53-expressing MCF10F cells than in the p53-negative Saos-2 cells.

**Retroviral Infection of Saos-2 Cells Restores Expression of p53 and p21**—Previous studies have shown that restoration of wild-type p53 expression through transfection of a suitable expression plasmid did not allow for establishment of stable cell lines expressing wild-type p53 (33–37). This was presumably due to the fact that plasmid transfection results in a high level of expression of p53 which is incompatible with cell proliferation. Chen et al. (27) utilized recombinant retroviral infection to restore a level of wild-type p53 expression in Saos-2 cells that was comparable to that seen in normal cells and that was compatible with continued proliferation of these cells. To that end, Saos-2 cells were infected with recombinant retroviruses expressing either wild-type human p53 or the mutant human p53\(^{His-273}\), 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 cell 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 p53 expression was maintained when this cell line expressed low levels of p21 mRNA as compared with the wild-type p53-expressing MCF10F cells (Fig. 2A, lane 3). In contrast, co-transfection of the plasmid encoding human MDM2 protein caused repression of p21 expression (Fig. 3).
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Table I

| Cell line | Bromodeoxyuridine positive cells |
|-----------|---------------------------------|
| WI38      | 12                              |
| MCF10F    | 22                              |
| MCF7      | 27                              |
| Saos-2    | 24                              |
| Saos-2 (wt) | 23                         |
| Saos-2 (His273) | 24                  |

* Cells were labeled with 1 μM bromodeoxyuridine for 30 min, fixed, and processed for flow cytometric analysis as described under “Experimental Procedures.”

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. Immunoblots 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, 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, 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 untreated cells contained 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-

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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.
tional DNA-binding protein that is distinct from p53 which produces a shifted complex of similar mobility as purified p53. To test this, nuclear extracts of untreated MCF7 cells were immunoprecipitated with an anti-p53 antibody to clear all detectable p53 protein from the extract as determined by immunoblotting. 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 419 (Fig. 8A, lanes 5 and 6). 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-
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 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-p53Ala-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. 5. Ectopic expression of the mutant human p53Ala-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-p53Ala-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 W138, MCF10F, and MCF7 cells. W138, 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.

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-
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FIG. 8. p53 in nuclear extracts of untreated MCF7 cells binds to DNA in a sequence-specific manner. A, nuclear extracts of MCF7 cells that were untreated (lanes 1–9) or treated with 50 J/m² ultraviolet light (UV, lanes 10–12) were used directly (lanes 1–3) or immunoprecipitated with either an anti-p53 antibody 421 (lanes 4–6) or an anti-SV40 large T antigen antibody 419 (lanes 7–12). The resulting supernatants were used in an electrophoretic mobility shift assay using 1 ng of radiolabeled probe (BB.9). Incubations were performed either in the absence (lanes 1, 4, 7, and 10) or presence of 1801 (anti-p53 antibody, lanes 2, 5, 8, and 11) or presence of 421 (anti-p53 antibody, lanes 3, 6, 9, and 12). The arrow to the left indicates the position of the supershifted p53-DNA complex, and the bracket to the right indicates the position of the supershifted p53-DNA-antibody complex. However, although untreated extracts from either MCF7, MCF10F, or WI38 cells contain a DNA-binding protein other than p53 which is capable of shifting a specific radiolabeled probe representing a p53 consensus binding site, experiments in the presence of a p53-specific monoclonal antibody 1801 clearly demonstrated that p53 in these extracts could bind to DNA in a sequence-specific manner (Figs. 7–9). Taken together, these results demonstrate that endogenous p53 in untreated, proliferating cells is capable of binding DNA and activating transcription. Thus, p53 is implicated as playing a role in constitutive expression of a particular target gene, that of the cyclin-dependent kinase inhibitor p21, in proliferating cells in the absence of treatment with DNA-damaging agents.

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

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

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

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

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

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