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Inhibition of DNA Topoisomerase IIα Gene Expression by the p53 Tumor Suppressor

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DNA topoisomerase II (topo II) is an essential nuclear enzyme involved in major cellular functions such as DNA replication, transcription, recombination, and mitosis. While an elevated level of topo IIα is associated with cell proliferation, wild-type (wt) p53 inhibits the expression of various growth-stimulatory genes. To determine if p53 downregulates topo IIα gene expression, a murine cell line, (10)1val, that expresses a temperature-sensitive p53 was utilized. The (10)1val cells had significantly lower levels of topo IIα mRNA and protein following incubation for 24 h at 32°C (p53 with wt conformation) than at 39°C (p53 with mutant conformation). The effect of p53 on the human topo IIα gene promoter was determined by using luciferase reporter plasmids containing varying lengths of the topo IIα promoter transiently cotransfected into p53-deficient (10)1 cells together with wt or mutant p53 expression plasmids. Transcription from the full-length (bp −557 to +90) topo IIα promoter was decreased 15-fold by wt p53 in a concentration-dependent manner, whereas mutant p53 exerted much weaker inhibition. Consecutive deletion of the five inverted CCAAT elements (ICEs) from the topo IIα promoter reduced both the basal promoter activity and wt p53-induced suppression. Transcription of the minimal promoter (−32 to +90), which contains no ICE, was slightly stimulated by wt or mutant p53 expression. When point mutations were introduced into the most proximal ICE (−68), the inhibitory effect of wt p53 was alleviated and stimulation of topo IIα expression resulted. Our study suggests that wt p53 functions as a transcriptional repressor of topo IIα gene expression, possibly through a functional interaction with specific ICEs. Inactivation of wt p53 may reduce normal regulatory suppression of topo IIα and contribute to abortive cell cycle checkpoints, accelerated cell proliferation, and alterations in genomic stability associated with neoplasia.

DNA topoisomerase II (topo II) is a ubiquitous enzyme that can alter the topological state of DNA and untangle intertwined DNA helices (for reviews, see references 79 and 80). As such, topo II plays an essential role in several cellular events, such as replication (20), chromatin condensation (76), and sister chromatid segregation (20, 76). Functioning as a homodimer, topo II binds double-stranded DNA and attaches covalently to both strands of the helix, resulting in DNA breaks in each strand. Upon binding of ATP to the topo II-DNA complex, a second DNA helix can pass through the cleavable complex, and this is followed by hydrolysis of ATP and resealing of the cleaved double-stranded DNA (7, 10). The ability of topo II to pass duplex DNA molecules allows the enzyme to separate fully replicated DNA molecules prior to chromosome segregation (61). There are two topo II isoforms, topo IIα (170 kDa) and topo IIβ (180 kDa), present in mammalian cells. Although both enzymes are closely related in structure (72% identical amino acid residues [42]), they differ in important biochemical and pharmacological properties, including sensitivity to topo II-targeting drugs, thermal stability, cellular localization, and cell cycle regulation (22). While topo IIβ levels are relatively constant throughout the cell cycle, topo IIα expression is low in G_1, increases in S phase, and is maximal in G_2/M phase, which is consistent for the association of this isoform with DNA replication, mitosis, and cell proliferation (29, 84).

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The tumor suppressor protein p53 is a nuclear phosphoprotein whose inactivation either through mutation, selective interaction with cellular MDM2 protein, or viral oncogene products or via alteration in subcellular localization is strongly correlated with human cancer (5, 32, 77). The p53 protein has tetramerization, DNA binding, and transactivation functions residing in separate domains of the protein (14, 41). Depending on the specific cell type, the expression of wild-type (wt) p53 has been shown to result in G_1 checkpoint control of cell growth or apoptotic cell death (54, 56, 91). Convincing evidence suggests that p53-mediated cell cycle arrest at the G_1 checkpoint is induced by various types of DNA damage (43, 44, 48, 73). wt p53 can positively regulate expression of a number of downstream effector genes, including the GADD45 (44), $p21^{Waf1/Cip1}$ (24), mouse muscle creatine kinase (88), mdm-2 (59, 85), and cyclin G (62) genes. In addition, wt p53 negatively regulates a variety of genes that lack a p53 consensus binding site, including c-*fos* (28, 46), MDR1 (12), heat shock protein 70 (2), interleukin 6 (67), proliferating cell nuclear antigen (49, 71), O^6-methylguanine-DNA methyltransferase (33), as well as other viral and cellular promoters (71). It has been suggested that transcriptional repression by p53 results from its direct interaction with transcription factors such as TATA-binding protein (51, 58, 68), Sp1 (8), and CCAAT-binding factor (CBF) (2). Taken together, these observations strongly imply that p53 acts directly with the transcription machinery to modulate transcription. The loss or inactivation of wt p53 increases genomic instability (52, 87) and susceptibility to malignant transformation (34, 90). Defects in a cell cycle checkpoint may thus contribute to the genomic instability of cancer cells in which chromosomal rearrange-

ments, translocations, and gene amplification occur with increased frequency.

Treatment of cells with numerous agents that induce arrest of DNA replication and DNA strand breaks results in increased rates of gene amplification (reference 87 and references therein). Through breakage-fusion-bridge cycles, a series of chromosome breaks and rearrangements could result as the cells replicate damaged DNA and undergo inappropriate mitosis (11, 70, 83). Drug-induced topo IIα-mediated double-strand breaks could be an initiating step in the gene amplification process (19, 81). Therefore, the genomic instability associated with cells expressing mutant p53 may result from a loss of some other p53-controlled function(s) in addition to the known loss of p53 G_1/S checkpoint control. Since topo IIα activity is associated with important DNA functions, such as replication and recombination, through a DNA breakage-religation mechanism, one of the fundamental properties of p53 tumor suppressor may reside in its regulation of expression of the topo IIα gene.

Many aspects of the biological function and activity of topo IIα in DNA replication and mitosis are fairly well characterized; however, the mechanisms of transcriptional regulation of the topo IIα gene at the promoter level are just beginning to be studied (26, 37, 40, 47, 53, 60). Effects of p53 on topo IIα transcription have until now been unexplored. In the present study, we show that expression of wt p53 severely inhibits topo IIα gene promoter activity. The loss of wt p53 as a transcriptional suppressor may lead to unregulated or inappropriate expression of topo IIα, resulting in the increased cell proliferation, chromosomal rearrangements, and/or gene amplifications seen in tumor cells.

MATERIALS AND METHODS

Materials. All cell culture media and supplements were purchased from Gibco-BRL (Gaithersburg, Md.) or BioWhittaker (Walkersville, Md.). Restriction enzymes and other nucleic acid-modifying enzymes and reagents were purchased from Promega (Madison, Wis.) or Gibco-BRL. ^{32}P-labeled nucleoside triphosphates were purchased from DuPont NEN (Boston, Mass.). Nitrocellulose filters (BA85) were bought from Schleicher & Schuell (Keene, N.H.), and Duralon-UV membranes were purchased from Stratagene (La Jolla, Calif.). Antibodies to wt and mutant p53 proteins (anti-p53 [Ab-7]) were purchased from Calbiochem (Cambridge, Mass.). Luminol/Enhancer solution and Stable Peroxide Solution were obtained from Pierce Chemical Company (Rockford, Ill.). Unless otherwise indicated, all other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Plasmids and cell culture. The human p53 expression plasmids and the empty vector used in this study have been described previously (5). All of the p53 constitutive expression constructs were produced with a cytomegalovirus promoter-enhancer expression vector. Briefly, a wt p53 cDNA was inserted into the pCMV-Neo-Bam vector to express p53 (originally named pC53-SN3) (5). Other vectors expressing mutant human p53 were similarly constructed. p53-175 is a mutant human p53 cDNA expression plasmid containing a substitution of histidine for arginine at amino acid (aa) 175, p53-281 has an aspartic acid-to-glycine mutation at aa 281, and p53-22/23 has mutations at aa 22 and 23. The (10)1 cell line, a spontaneously arising immortalized murine BALB/c embryo fibroblast line (34), contains large deletions in both p53 alleles and, consequently, is deficient in the p53 protein. The (10)1val cell line was developed from (10)1 cells by transfection with an expression vector, producing a temperature-sensitive p53 protein (85, 90). At 32°C, the p53 protein is predominantly in the wt conformation, whereas at 39°C, most of the p53 protein takes on a mutant form. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 25 μg of fungizone per ml in 5% CO_2 at 37°C.

Preparation of nuclear protein extracts. The (10)1 and (10)1val cells were plated 24 h before the duplicate plates of cells were shifted to either 39 or 32°C. After 24 h at the appropriate temperature, cells were trypsinized from the plate, washed once with ice-cold phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 1,000 × *g* for 4 min. The cell pellet was resuspended in hypotonic buffer, and the nuclear proteins were extracted as previously described (16). In all buffers, protease inhibitors were added just before use: phenylmethylsulfonyl fluoride and benzamidine at 1 mM each, aprotinin and leupeptin at 10 μg/ml, and pepstatin A at 1 μg/ml. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Richmond, Calif.). The proteins were stored in aliquots at −70°C.

SDS-polyacrylamide gel electrophoresis and Western blotting. For detection of topo II protein, a 100-μg sample of nuclear protein extract was mixed with loading buffer and, without boiling, loaded onto a 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS). The gel was run at 15 mA for 18 h. The separated proteins were transferred onto a nitrocellulose membrane by electroblotting. After being blocked with 5% nonfat dry milk, the nitrocellulose membrane was incubated with topo II antiserum (provided by Mary Danks, St. Jude Children's Research Hospital). Following incubation with goat anti-rabbit alkaline phosphatase (Sigma; diluted 1:1,000), the bound antibody was visualized by reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

For detection of p53 protein, 100 μg of cell lysate protein was mixed with loading buffer, boiled for 5 min, loaded onto a 12% polyacrylamide gel, electrophoresed, and transferred to a nitrocellulose membrane as described above. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline and incubated overnight with the p53 antibody (1:1,500 dilution). Following incubation with rabbit anti-sheep peroxidase second antibody (1:5,000 dilution), the p53 protein was visualized by using the Luminol/Enhancer and Stable Peroxide solutions as described by the manufacturer.

Isolation and analysis of mRNAs. The (10)1 and (10)1val cells were plated in 10-cm-diameter plates for 24 h at 37°C before being shifted to either 39 or 32°C for an additional 24 h. The cells were then harvested, and RNA was extracted for Northern blot analysis as described previously (82). Briefly, 10 μg of total mRNA was electrophoresed on a 1% agarose–6% formaldehyde gel and transferred to a Duralon-UV membrane, which was then UV cross-linked and subjected to prehybridization and hybridization with a topo IIα-specific cDNA probe. After hybridization, the membrane was washed and exposed to X-ray film for autoradiography. The same membrane was then stripped and rehybridized with a β-actin probe as an internal standard. The DNA probe used to analyze topo IIα mRNA was a 3-kb *Eco*RI fragment (+1.2 to +4.2 kb of the coding region) of the human topo IIα gene (75). A full-length cDNA clone of chicken cytoplasmic β-actin (15) was used as the β-actin probe.

Construction of plasmids for transient expression. We used PCR techniques to generate derivatives of a human topo IIα promoter-chloramphenicol acetyltransferase construct kindly provided by I. D. Hickson (37). The following forward (fTIIα−557) and reverse (rTIIα+90) oligomers were used as primers to create TIIα−557 contain unique *Kpn*I and *Bgl*II sites (underlined), respectively: fTIIα−557, 5′GATCGGTACCGGGGTTGAGGCAGATGCCAG3′ (bp −557 to −538); and rTIIα+90, 5′GATCAGATCTGGTGACGGTCGTGAAGGGGC3′ (bp +90 to +71). Promoter deletions were created in the upstream sequences of TIIα−557 by using the following series of oligomers as forward primers, each containing a *Kpn*I site (underlined), in conjunction with the common reverse primer, rTIIα+90: fTIIα382, 5′GATCGGTACCGTTCCTGGAGAATAAACATC3′ (bp −382 to −363); fTIIα−252, 5′GATCGGTACCAGATTCCCTGTCAATCTCTC3′ (bp −252 to −233); fTIIα−182, 5′GATCGGTACCTAACCTGATTGGTTTATTCA3′ (bp −182 to −163); fTIIα−162, 5′GATCGGTACCAACAAACCCCGGCCAACTCA3′ (bp −162 to −143); fTIIα−142, 5′GATCGGTACCGCCGTTCATAGGTGGATATA3′ (bp −142 to −123); fTIIα−122, 5′GATCGGTACCAAAGGCAAGCTACGATTGGT3′ (bp −122 to −103); fTIIα−90, 5′GATCGGTACCAGACGGTGAGAGCGAGTCAG3′ (bp −90 to −71); and fTIIα−32, 5′GATCGGTACCTCAAGTGGAGCTCTCCTAAC3′ (bp −32 to −13). Bases are numbered with respect to the major transcription start site (designated +1) (37). PCR was carried out with Vent polymerase (New England Biolabs) as described previously (82). The PCR-amplified promoter fragments were cloned into the multiple cloning site of the pGL2-basic vector (Promega), upstream of the luciferase gene. All the construct DNAs were purified through columns of anion-exchange resin (Qiagen) and confirmed by restriction analysis and/or sequencing.

The topo IIα promoter construct with mutations in specific inverted CCAAT element (ICE) sites was produced with a forward primer, fTIIα−90mt, that extended through the nascent ICE sequence and altered the ICE sequence from ATTGG to CTGGA (double underlined) (5′GATCGGTACCAGACGGTGAGAGCGAGTCAGGGCTGGACTGGTCTGC3′ [−90 to −55]).

Transient transfections and luciferase, β-galactosidase, and protein assays. Cells were plated at 40% confluency in 12-well plates. After 24 h, the plasmid DNA (1 μg) was transfected into cells by calcium phosphate precipitation as described previously (82). Luciferase plasmids were cotransfected with 0.5 μg of pSV-β-galactosidase control vector (Promega) to monitor transfection efficiency. Five hours after transfection, the cells were treated with 15% glycerol for 2 min, washed, given fresh medium with or without drug, and grown for another 20 h before being harvested for various assays. The cell extracts were prepared with the reporter lysis buffer (Promega). The cell lysate was analyzed for luciferase activity, β-galactosidase activity, and protein concentration. Luciferase activity was measured with a luciferase assay system (Promega) by a luminometer with an autoinjector (Turner Designs). The β-galactosidase enzyme activity was determined by an assay system developed by Promega, and the protein concentrations were determined by the Bio-Rad protein assay. The experiments were performed in duplicate or triplicate, and the number of relative light units per microgram of protein in the cell extract was calculated since wt p53 severely inhibited the expression of the pSV-β-galactosidase vector (data not shown).

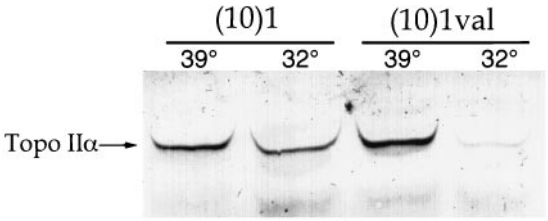

FIG. 1. Expression of wt p53 reduces topo IIα protein. (10)1 and (10)1val cells were plated and incubated at 37°C for 24 h before being shifted to either 39 or 32°C for another 24 h. Nuclear protein was extracted for Western blotting as described in Materials and Methods. The 170-kDa protein band reactive with topo II antiserum is indicated. (10)1val cells have predominantly wt p53 at 32°C but have mutant p53 at 39°C. (10)1 cells contain no p53 protein.

RESULTS

Expression of wt p53 reduces topo IIα protein. To study the effect of p53 expression on endogenous topo IIα protein, we used the murine fibroblast (10)1 cell line, which is negative for p53 protein, and the derivative (10)1val cell line, which expresses a temperature-sensitive p53 protein. When the (10)1val cells are grown at 32°C, the p53 protein is predominantly in the wt conformation, whereas at 39°C, most of the p53 protein is in the mutant conformation. Western blot analysis showed that (10)1val cells incubated at 32°C for 24 h had a dramatically decreased level of topo IIα protein compared with cells incubated at 39°C (Fig. 1). The decrease in topo IIα protein is a consequence of wt p53 and is not due to temperature effects, since shifting (10)1 cells to 32°C for the same time period had a minimal effect on the expression of topo IIα protein.

Expression of wt p53 decreases the steady-state level of topo IIα mRNA. The decreased amount of topo IIα protein in cells expressing wt p53 could result from either pretranslational or posttranslational events. With the same cell lines and temperatures as described for Fig. 1, the effect of p53 expression on endogenous topo IIα mRNAs was determined by Northern blot analysis. As shown in Fig. 2, the steady-state level of topo IIα mRNA was markedly decreased by wt p53 expression, as reflected by the sharp reduction of the topo IIα mRNA in (10)1val cells when they are incubated at 32°C for 24 h rather than at 39°C. Under the same conditions, the temperature had no effect on the level of topo IIα mRNA in (10)1 cells. When reprobed with radiolabelled β-actin cDNA as an internal loading control, the same membrane showed similar levels of β-actin mRNA at either temperature in both cell lines, indicating that wt p53 selectively inhibits topo IIα gene expression. Thus, wt p53 expression in (10)1val cells at the permissive temperature effectively decreases topo IIα mRNA levels, thus reducing topo IIα protein at a pretranslational level.

Transcriptional activity of the human topo IIα promoter in (10)1 cells. The decreased levels of topo IIα protein and mRNA resulting from expression of wt p53 prompted us to investigate the transcriptional regulation of topo IIα expression. Toward this end, human topo IIα promoter-luciferase reporter plasmids were constructed with varying lengths of 5′ promoter sequences (Fig. 3). (10)1 cells were used in transient-transfection assays because of their negative p53 background, which is ideal for studying the role of p53 in the transcriptional regulation of exogenously transfected gene promoters (68, 88). The reporter construct containing topo IIα promoter sequences from bp −557 to +90 (here regarded as the full promoter) gave maximal transcriptional activity. Stepwise deletion of the 5′ promoter sequences from the full promoter resulted in a concomitant decrease in luciferase activity (Fig. 3). The pattern of basal promoter activity of the topo IIα-luciferase constructs is in general agreement with previously reported results (37). Interestingly, the data obtained from various promoter deletion mutants indicated that multiple ICEs (indicated by asterisks in Fig. 3) in the topo IIα promoter may be important sites for the transcriptional activity of the promoter. In most instances, deletion of an ICE results in a significant drop in promoter activity (Fig. 3). For example, an ICE is the only known *cis*-acting element between bp −182 and −162 of the topo IIα promoter. The effect of deletion of the ICE is seen in the fourfold reduction in luciferase activity between pTIIα−182- and pTIIα−162-transfected cells. The reduced activity seen with deletions that did not include an ICE indicate that other regulatory *cis*-acting elements in the promoter may also play a role in controlling topo IIα gene expression.

Topo IIα promoter activity in (10)1val cells. Expression of wt p53 decreased expression of topo IIα mRNA and protein in (10)1val cells grown at 32°C. To test the effects of wt p53 on topo IIα promoter activity, the promoter-luciferase reporter constructs were transiently transfected, in duplicate, into (10)1val cells at 37°C and then shifted to 32 or 39°C for 20 h. Transfection of (10)1val cells with the full-length topo IIα promoter followed by incubation at 32°C to induce wt p53 expression resulted in a 60% reduction of luciferase activity compared with the activity in cells grown at 39°C. In contrast, pTIIα−32-transfected cells had an 80% higher level of luciferase activity at 32°C (Fig. 4). Parallel experiments conducted in (10)1 cells indicated that the lower temperature alone had no inhibitory effect on either promoter construct (data not shown). These results demonstrate that expression of p53 in the wt conformation decreased transcriptional activity of the topo IIα promoter and, more significantly, that this inhibition is related to the *cis* elements upstream of the minimal promoter sequence (bp −32 to +90).

wt p53 inhibits transcriptional activity of the human topo IIα promoter in a dose-related fashion. To study the effects of

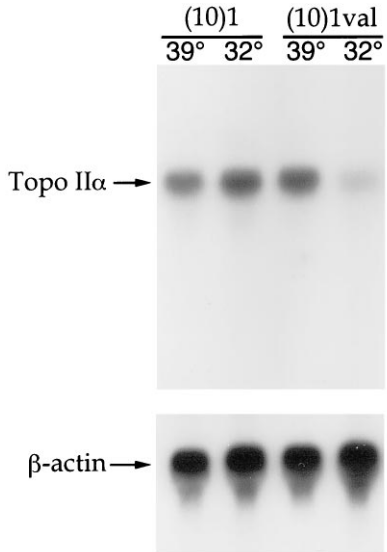

FIG. 2. Expression of wt p53 decreases the steady-state level of topo IIα mRNA. (10)1 and (10)1val cells were plated and incubated at 37°C for 24 h before being shifted to either 39 or 32°C for another 24 h. Total RNA was extracted for Northern blotting as described in Materials and Methods. The blot was first probed with a ^{32}P-radiolabeled insert of the human topo IIα gene. After being stripped, the membrane was reprobed with β-actin.

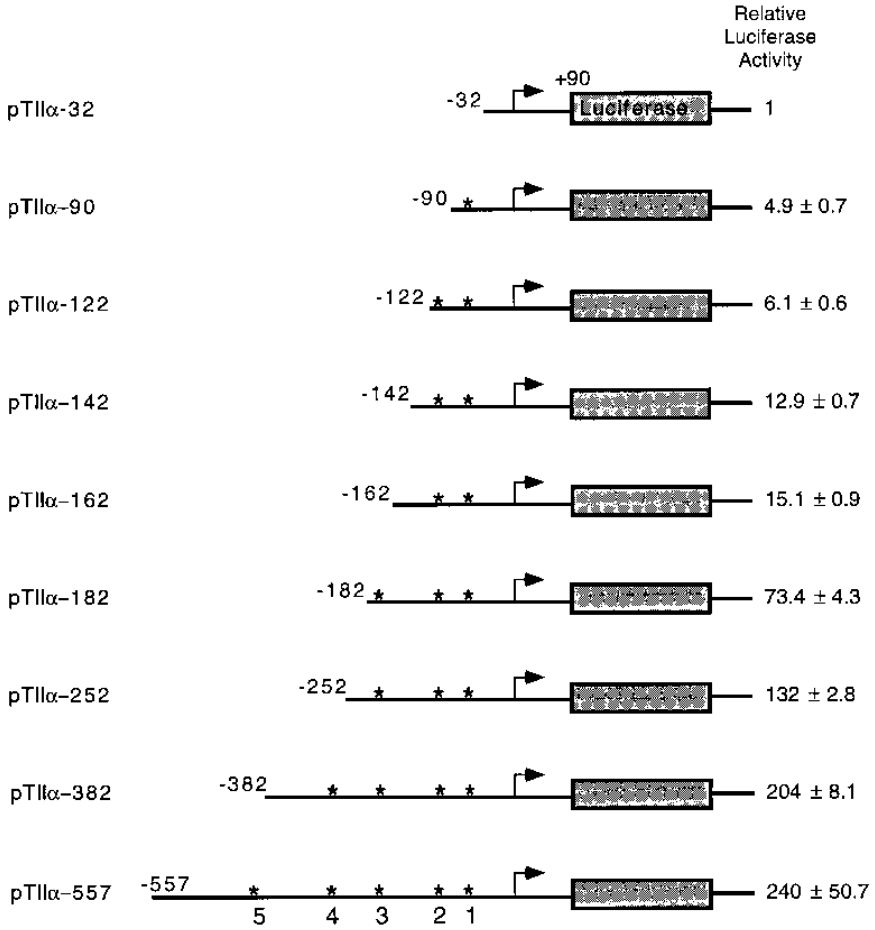

FIG. 3. Schematic linear map of human topo IIα promoter-luciferase reporter plasmids and their transcription activity in (10)1 cells. Plasmids containing various lengths of human topo IIα promoter 5′ of the luciferase gene were constructed as described in Materials and Methods. The sequence numbers in the map correspond with those published by Hochhauser et al. (37), with the major transcription start site set to +1 (indicated by the arrow). The positions of ICEs are indicated by the asterisks and are numbered starting with the ICE closest to the major transcription start site. The base numbers of the 5′ A's of the ICEs are −68, −108, −175, −259, and −389 for ICEs 1 to 5, respectively. Plasmid DNA (1 μg) was transfected into (10)1 cells by calcium phosphate precipitation. Five hours after transfection, the cells were glycerol shocked, washed, given fresh medium with 10% FBS, and grown at 37°C for another 20 h before being harvested for determination of luciferase activity. Each experiment was repeated at least twice with triplicate samples. The values shown on the right are the averages of the relative promoter activities of triplicate samples from a representative experiment.

p53 expression on the topo IIα gene promoter, (10)1 cells were cotransfected with pTIIα−557 and various amounts of expression plasmids encoding wt or mutant p53 (Fig. 5). Transfection with the wt p53 expression plasmid at levels as low as 10 ng caused a significant inhibition (60%) of the topo IIα promoter activity. Increasing the amount of wt p53 plasmid used in the transfection resulted in enhanced inhibition of the topo IIα promoter activity; 0.1 μg of p53 decreased the promoter activity 8-fold, while 1.0 μg of p53 caused a maximal 15-fold inhibition of the full promoter activity (Fig. 5). The greater amounts of p53 plasmid did not increase the inhibition proportionally, indicating a saturable mechanism for this p53 function. The possibility that transcription in general is suppressed due to wt p53 expression (i.e., as a result of apoptosis) was discounted by use of a wt p53 response reporter which was dramatically induced by wt p53 in (10)1val cells (85, 88) (data not shown).

Transfection with low levels of mutant p53 expression plasmid had no significant effects on topo IIα promoter activity, and at levels as high as 1.0 μg, inhibition was only approximately 50% (Fig. 5). The relatively low-level (2-fold) inhibition of the topo IIα promoter activity exhibited with high levels of mutant p53 reflects the drastically reduced inhibitory ability of the mutant p53s compared to the 15-fold inhibition obtained with the wt p53.

Western blots of protein from transfected-cell lysates were performed to confirm that wt and mutant p53 proteins were being produced in the cells transfected with the p53 expression vectors. With transfection of 1.0 μg of the p53 construct DNA, similar levels of wt and mutant p53 proteins were detected (Fig. 6). These results eliminate the possibility that the lack of significant inhibition of topo IIα promoter activity by mutant p53 is because the mutant protein is not being produced in the transfected cells.

Inhibition of the human topo IIα promoter by wt p53 may be mediated by ICEs. To delineate possible *cis* elements involved in p53-mediated transcriptional inhibition of the topo IIα gene, we tested the effects of p53 on topo IIα promoter constructs of varying lengths. Cotransfection of either wt or mutant p53 slightly stimulated the luciferase activity driven by the minimal topo IIα promoter (pTIIα−32) (Fig. 7). However, when the promoters were extended to include more 5′ upstream sequence, inhibition of the promoter activity by wt p53 was observed with the pTIIα−90 promoter and markedly in-

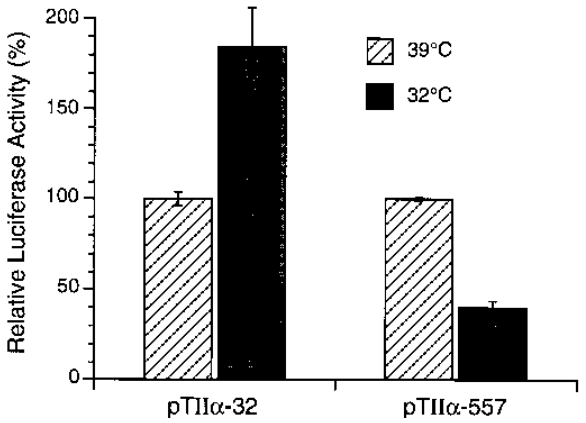

FIG. 4. Topo IIα promoter activity in (10)1val cells. (10)1val cells were transfected with the promoter constructs by calcium phosphate precipitation. Five hours after transfection of the indicated plasmid (1 μg), the cells were glycerol shocked, washed, given fresh medium with 10% FBS, and grown at 39 or 32°C for another 20 h before being harvested for determination of luciferase activity. Each experiment was repeated at least twice with triplicate samples. Values are expressed as percentages of the control activity (100%) in lysates from cells incubated at 39°C and are the means ± 1 standard deviation (error bars) of luciferase activities from triplicate samples in a representative experiment. Refer to Fig. 3 for plasmid identification.

creased to a maximum level of 15-fold with the pTII−382 promoter (Fig. 7). In contrast, mutant p53s containing either a single amino acid mutation that disrupts DNA-binding activity (p53-175) (45) or a double mutation in the acidic domain that blocks transactivation (p53-22/23) (50) showed no significant transrepression of the topo IIα promoter regardless of the length of the 5′ promoter region. Similar to the positive role of ICEs in topo IIα transcription (Fig. 3), the wt p53-induced inhibition of topo IIα promoter activity may also be ICE related. The minimal promoter pTIIα−32 contained no ICE and was actually stimulated by wt p53 as opposed to being inhibited. Addition of the promoter sequence including a single ICE (pTIIα−90) resulted in significant promoter inhibition by wt p53, and the inclusion of additional ICE-containing promoter sequences correlates with increased inhibition of topo IIα promoter activity (e.g., compare pTIIα−162 and pTIIα−182 in Fig. 7).

The human hsp70 promoter, which like the topo IIα promoter does not contain a TATA box, is predominately regulated by a CCAAT element, located at position −70, and the transactivation factor CBF (57). Further studies have shown that transcription of the hsp70 gene is repressed by p53, indicating a direct protein-protein interaction between p53 and CBF (2). Since CCAAT elements are functional in either orientation (30), it seemed appropriate to determine if the ICEs in the topo IIα promoter were associated with the p53-induced repression. As a direct test of the role of ICEs in the p53 inhibition of topo IIα promoter activity, a topo IIα-luciferase construct that contained mutations in the first ICE (pTIIα−90mt) was produced. This mutant construct was cotransfected with the wt p53 expression vector into (10)1 cells. The results, shown in Fig. 8, demonstrate a shift in the effect of p53 on the topo IIα promoter activity back to that seen with the minimal promoter (pTIIα−32). Instead of a relative 50 to 60% inhibition of activity by wt p53, the mutant ICE construct, pTIIα−90mt, stimulated promoter activity twofold. This result is similar to the 1.4- to 1.6-fold stimulation consistently seen for the minimal promoter, which does not contain an ICE, with either wt or mutant p53 expression (Fig. 4, 7, and 8).

DISCUSSION

Functional inactivation of p53 is the single most common event in human malignancies and occurs in at least 50% of all

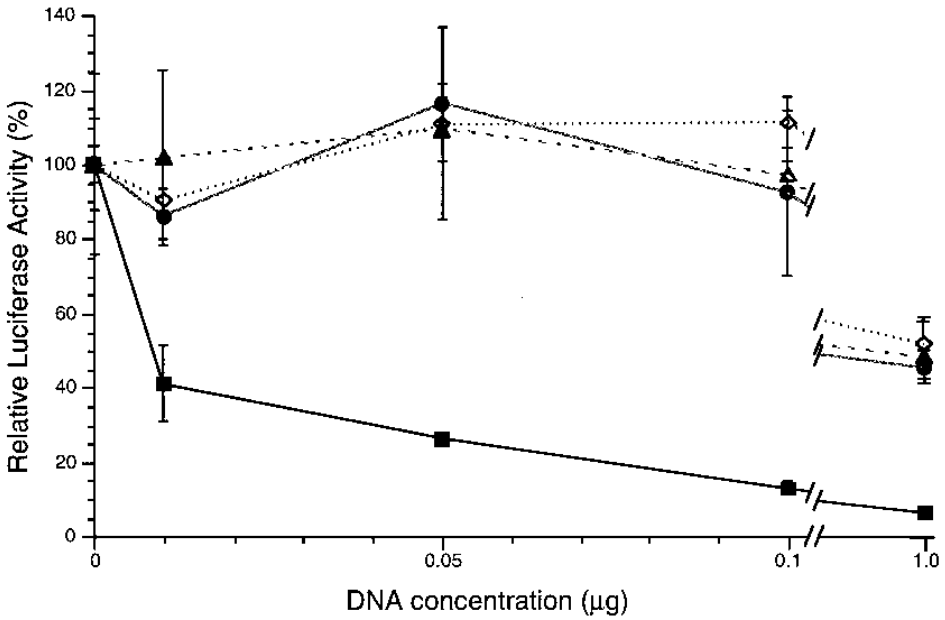

FIG. 5. Effects of different amounts of p53 on topo IIα promoter pTIIα−557 activity in (10)1 cells. (10)1 cells were cotransfected with pTIIα−557 (1.0 μg) and various amounts of wt or mutant p53 expression plasmid. The total DNA for each transfection was brought up to 2.0 μg with the CMV-Neo-Bam control vector. Five hours after transfection, the cells were glycerol shocked, washed, given fresh medium with 10% FBS, and grown at 37°C for an additional 20 h before being harvested for luciferase assay. Each experiment was repeated four times with duplicate samples. Values are expressed as percentages of the control activity (100%) in lysates from cells cotransfected with pTIIα−557 and the CMV-Neo-Bam control vector and are the means ±1 standard deviation (error bars) of luciferase activities from duplicate samples in a representative experiment. ■, wt p53; ●, p53-175; ▲, p53-281; ◇, p53-22/23. Refer to Fig. 3 for plasmid identification.

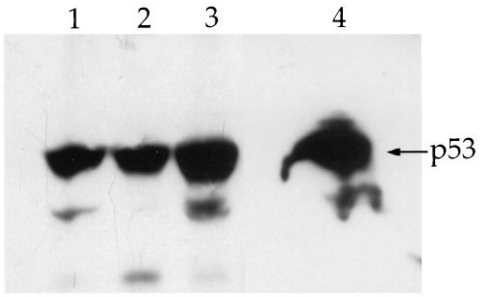

FIG. 6. Expression of wt and mutant p53 in transfected (10)1 cells. (10)1 cells were transfected with wt or mutant p53 expression plasmid DNA (1.0 μg) without any topo IIα promoter vector. The cells were glycerol shocked, washed, and incubated as described in the legend to Fig. 4 before cell lysates were prepared. Western blotting was conducted as described in Materials and Methods. Lane 1, wt p53; lane 2, p53-175; lane 3, p53-22/23; lane 4, baculovirus-expressed control p53 protein.

cancers (32, 77). By interfering, directly or indirectly, with the activity of genes whose products are needed for ongoing cell proliferation, p53 may restrict growth and prevent tumor progression (89). Functional loss of wt p53 results in the loss of both cell cycle checkpoint control and regulation of growth-related gene products in the tumor cells. In this study, we have demonstrated that expression of wt p53 in (10)1 cells results in a significant reduction of both topo IIα mRNA and protein, most likely as a consequence of p53-induced inhibition of topo IIα gene transcription. Our results place topo IIα among the several growth-related genes under the regulatory control of wt p53.

Several TATA box-containing promoters exhibit specific gene suppression by wt p53, whereas TATA-less promoters may be refractory to this inhibition (58). However, the topo IIα promoter differs from other known wt p53-suppressed genes in that it does not contain a TATA box consensus site. The topo IIα promoter elements that do appear to play a significant role in transcription are the ICEs, of which there are five (37, 40, 60, 63). Studies of the hamster topo IIα promoter have shown that the ICEs are functionally important for topo IIα promoter activity and that these sites form complexes with ICE-binding proteins (60). Recent work on the regulation of the human topo IIα promoter in confluence-arrested cells demonstrated that the second ICE is involved in the down-regulation of topo IIα expression (40). The present study confirms that the ICEs are positively involved in human topo IIα transcription (Fig. 3).

Topo IIα promoter activity is efficiently repressed by wt p53 but not by the mutants p53-175 and p53-281, which do not bind DNA (6, 23, 45). Disruption of the DNA-binding domain of p53 may induce a conformational change altering its interaction with factors that mediate promoter repression. To help define the factor(s) that may control p53 transrepression, we have mapped a wt p53 repression element in the topo IIα promoter to the most proximal ICE at position −68 (Fig. 7 and 8). Interestingly, Wu and coworkers have demonstrated that wt p53 transrepresses the human heat shock promoter through a CCAAT element and physically interacts with a CBF (2, 57). The attenuated transcriptional-repression activity of the mutant p53s may reflect a lack of association with a similar CBF. Additional studies are required to define the specificity of this association by identifying the sites of interaction on both proteins and by testing the ability of various mutant p53 proteins to associate with CBF, related CBF family members, or other CCAAT-binding proteins.

Previous studies have demonstrated that wt p53 requires its N-terminal acidic domain to interact with the TATA-binding protein (38, 51, 68, 74) and other transcription-associated factors (TAFs) (55, 72, 86), resulting in repression of promoter activity. For example, double point mutations in the acidic domain of p53 (amino acids 22 and 23) block the association of p53 with *Drosophila* $TAF_{II}40$ and $TAF_{II}60$ and their human homologs, TAF_{II} 31/32 and TAF_{II} 70 (55, 72). Our data demonstrate that p53-22/23 is markedly impaired for inhibition of the topo IIα promoter (Fig. 5 and 7), and these data are consistent with a functional link between p53, TAFs, and topo IIα gene transcription.

The dose-dependent effect of wt and mutant p53 proteins on

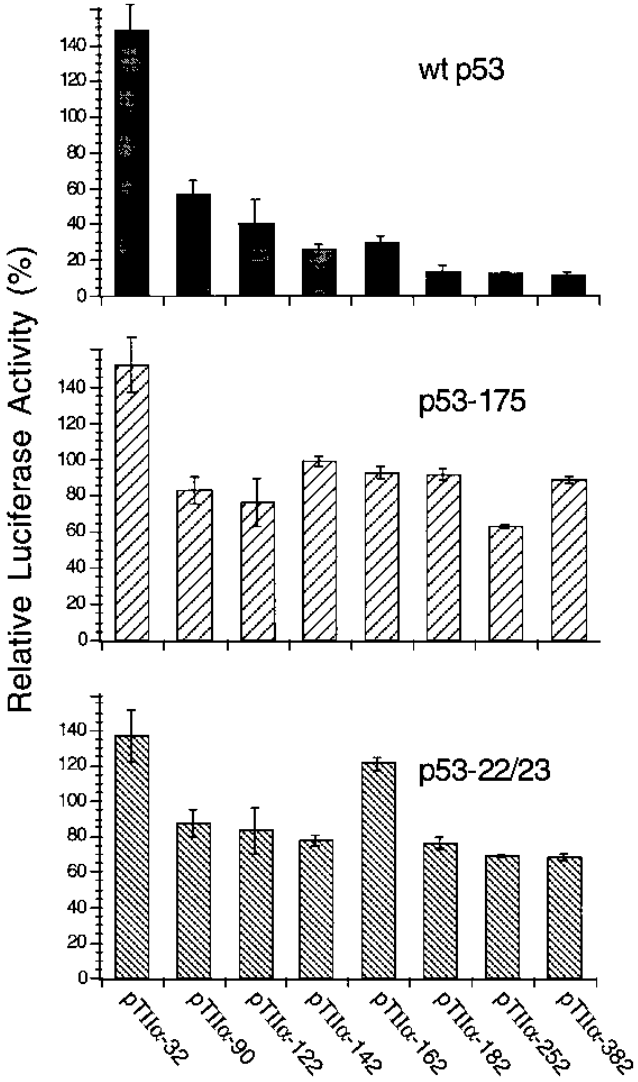

FIG. 7. Effects of wt or mutant p53 on various topo IIα promoters in (10)1 cells. (10)1 cells were cotransfected with topo IIα promoter constructs (1.0 μg) and wt or mutant p53 expression plasmids (0.1 μg). The amount of DNA in each transfection was normalized with empty vector. Five hours after transfection, the cells were glycerol shocked, washed, given fresh medium with 10% FBS, and grown at 37°C for an additional 20 h before being harvested for determination of luciferase activity. Each experiment was repeated at least twice with duplicate samples. Values are expressed as percentages of the luciferase activity in lysates from cells cotransfected with the control CMV-Neo-Bam vector only and are the means ± 1 standard deviation (error bars) of activities from triplicate samples in a representative experiment. Refer to Fig. 3 for plasmid identification.

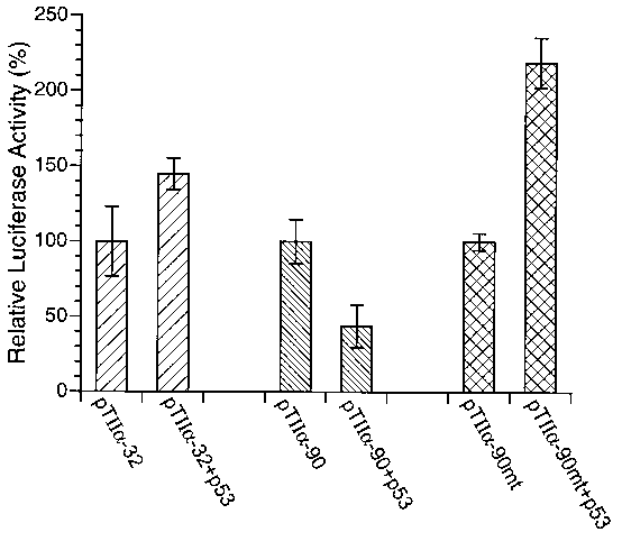

FIG. 8. Effects of ICE mutations on p53-induced inhibition of topo IIα promoter activity. (10)1 cells were cotransfected with normal or mutant ICE topo IIα promoter constructs (1.0 μg) and either wt p53 expression plasmids or the empty control vector (0.5 μg). Conditions for the transfection and luciferase assay were as described in the legend to Fig. 7. For each topo IIα promoter construct, the luciferase activity in the cells cotransfected with the empty control vector is set to 100%.

the activity of the full-length topo IIα promoter was determined by varying the amount of p53 expression plasmid used in the cotransfection assays (Fig. 5). The results showed that as little as 10 ng of the wt p53 vector significantly inhibited topo IIα promoter activity (60% inhibition), and when the amount of wt p53 vector was increased to 1.0 μg, the level of inhibition increased to greater than 90%. Three mutant p53 expression plasmids showed no significant repression of topo IIα promoter activity when low levels of vector were used in the transfection. However, when 1.0 μg of mutant p53 expression plasmid was used, up to 50% inhibition of activity resulted. The combined data suggest that wt p53 repression of topo IIα is DNA sequence specific and mediated by factors such as CBF, as opposed to a more nonspecific repression that can be induced by high levels of mutant p53s.

wt p53-mediated G_1 cell cycle arrest appears to be dependent on its transactivation function to induce the expression of negative growth regulators, such as WAF1 and GADD45 (24, 44). In contrast, p53-mediated programmed cell death in certain cell types does not appear to require this transactivation function (9, 78). Several lines of indirect evidence suggest that p53-induced cell death may be dependent, at least in part, on transrepression (65, 69). For example, mutant p53-22/23 is deficient in transactivation and transrepression functions and concomitantly fails to elicit G_1 cell cycle arrest or programmed cell death (18, 50, 66). In addition, while conditional expression of wt p53 in baby Fisher rat kidney cell lines that stably express the adenovirus E1A gene product induces apoptosis (17), coexpression of Bcl-2 or the adenovirus E1B 19K protein rescues these cells from death (13, 17). Subsequent studies demonstrated that the Bcl-2 and E1B 19K proteins inhibit wt p53 transrepression but not transactivation (65, 69). It remains a formal possibility that in certain cell types, wt p53 may induce an imbalance of growth signals that ultimately results in apoptosis by repressing genes that are required for cell growth, such as topo IIα, at the same time that expression of a dominant oncogene, such as E1A, promotes cell cycle progression.

In response to DNA-damaging agents, cells either arrest progression through the cell cycle in order to repair the damage or progress into apoptosis. Both cell cycle arrest and apoptosis require p53. The expression of p53 has been shown to arrest cells in G_1, before DNA synthesis occurs in S phase (43, 49). It has recently been reported that p53 can also control a G_2/M checkpoint (1). Arrest of cell growth at a G_2/M checkpoint in the cell cycle is seen when p53 is expressed without the complication of DNA damage and is associated with increased expression of p21/WAF1, a cyclin-dependent kinase inhibitor. Studies involving the use of topo II inhibitors that do not produce DNA damage (bisdioxopiperazines) have provided evidence for a topo II-dependent G_2 checkpoint that may be distinct from the G_2 damage checkpoint (3, 21). This alternate G_2 checkpoint may be sensitive to the catenation state of the DNA. Topo II is the essential enzyme for full chromosome condensation and decatenation of chromatids. The function of G_2 phase is to allow adequate decatenation of replicated DNA for mitosis to occur correctly (21). It is intriguing to speculate that the p53-induced G_2 arrest could be linked to the topo II-dependent G_2 arrest through p53 repression of topo IIα gene expression as demonstrated in the present study. In the normal cell cycle, topo IIα levels are highest at the G_2/M phase, just prior to mitosis (84). wt p53-induced inhibition of topo IIα gene expression could prevent the production of topo IIα at the levels required to progress through a G_2/M checkpoint.

Although it has yet to be directly demonstrated in vivo, the results presented in this study provide strong evidence for the regulation of topo IIα by wt p53 in a defined cell system. Additional, unpublished data from our laboratory indicate that treatment of NIH 3T3 cells with topo I- or topo II-targeted drugs, which create DNA strand breaks and should thereby induce p53, results in a decrease in topo IIα mRNA and promoter activity. Given that p53 is functionally inactive in a majority of neoplastic cells, the following reports provide support for this linkage: (i) neoplastic cells have been reported to have levels of topo II that are 5 to 10 times higher than those in normal cells (35, 36, 64); (ii) in non-small-cell lung cancer, a higher level of topo IIα expression is associated with a higher cell proliferation rate, and the expression of topo IIα was higher in tumor cells than in normal lung cells (27); and (iii) more specifically, studies of various lymphoma cell lines with mutant p53 have demonstrated higher levels of topo II than in similar cell lines with wt p53 (25). The linkage of p53 and topo II is further supported when the importance of p53 in the maintenance of genomic stability is considered (11, 52, 87). Topo II plays a significant role in recombination and genomic stability (81) but under certain conditions can induce genomic instability through illegitimate recombination (4, 31, 39). Thus, the proper regulation of topo II levels may be one of several factors involved in the ability of p53 to enhance genomic stability. Studies are under way to more precisely define the role that p53 regulation of topo IIα expression may have in genomic stability, cell proliferation, and neoplastic transformation.

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