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Nuclear Factor-Y Binding to the Topoisomerase IIα Promoter Is Inhibited by Both the p53 Tumor Suppressor and Anticancer Drugs

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ABSTRACT

Expression of the human DNA topoisomerase IIα (topo IIα) gene is positively regulated by the binding of the nuclear factor Y (NF-Y) transcription factor to four of five inverted CCAAT boxes (ICBs) located in its promoter. We have demonstrated previously that expression of the p53 tumor suppressor inhibits human topo IIα promoter activity in murine (10)1 cells. In this report, we demonstrate that the inhibition of topo IIα gene expression by wild-type p53 correlates with the decreased binding of the transcription factor NF-Y to the first four ICBs of the topo IIα promoter. The expression of mutant p53 does not affect the binding of NF-Y. In NIH3T3 cells, we show that topo II-targeted drugs inhibit the binding of NF-Y to ICB sites in the topo IIα promoter. This effect is seen not only with drugs that result in DNA strand breaks but also with drugs that inhibit the catalytic activity of topo II, and even with the mitotic spindle inhibitor, vinblastine. Further experiments with p53-null (10)1 cells treated with these same drugs also demonstrate decreased NF-Y binding to the topo IIα ICBs. The data presented points to the existence of both p53-dependent and -independent mechanisms for regulating NF-Y binding to ICBs in the topo IIα promoter and thus the modulation of topo IIα gene expression.

The efficient transcription and replication of DNA requires changes in its double-strand topology at specific times during the cell cycle. These topological alterations are carried out by the ubiquitously expressed, homodimeric nuclear protein, topoisomerase II (topo II). Topo II can relieve supercoiling that results during DNA replication and is an essential enzyme for decatenation of sister chromatids at mitosis. The action of topo II involves the cleavage of one DNA double strand, the passage of a transfer double strand through the break, and religation of the cleaved DNA. During the decatenation cycle, topo II is covalently bound to the cleaved DNA strand, forming an intermediate topo II-DNA cleavable complex. The expression of topo IIα is lowest in the G_1 phase, increases as the cells traverse S phase, and reaches a maximum at the G_2/M phase interface (Woessner et al., 1991). This observation is consistent with the association of increased topo IIα with DNA replication, mitosis, and proliferation.

Various transcriptional mechanisms regulate the expression of topo IIα. The promoter region of the topo IIα gene contains five inverted CCAAT boxes (ICBs), two GC boxes, and an ATF site (Hochhauser et al., 1992). Topo IIα expression is increased in rapidly proliferating cells after exposure to heat shock (Matsuo et al., 1993) and by the ras oncogene (Chen et al., 1999). On the other hand, topo IIα expression is down-regulated by wild-type p53 (Wang et al., 1997) or confluence-induced growth arrest (Isaacs et al., 1996). Specific ICBs have been implicated in the regulation of topo IIα expression by confluence arrest (Isaacs et al., 1996) heat-shock response (Furukawa et al., 1998), p53 (Wang et al., 1997), and cell cycle dependence (Falck et al., 1999).

Topo IIα is an important target for a variety of clinically useful anticancer agents. The topo II poisons (i.e., etoposide, VP-16) act by stabilizing the topo II-DNA cleavable complex (Corbett and Osheroff, 1993), which results in DNA double strand breaks. The topo II catalytic inhibitors (i.e., aclarubicin) inhibit topo IIα activity at a step other than the formation of the cleavable complex (Drake et al., 1989).

Whereas increased levels of topo IIα correlate with increased cellular proliferation, induction of the tumor suppressor p53 leads to negative regulation of proliferation. After DNA damage, the activity and level of p53 increases

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ABBREVIATIONS: topo IIα, α isoform of DNA topoisomerase type II; ICB, inverted CCAAT box; NF-Y, nuclear factor Y; VP-16, etoposide; VM-26, teniposide; *m*-AMSA, amsacrine; wt, wild type; DMEM, Dulbecco's modified Eagle's medium.

significantly, which may lead to either cycle arrest at the G_1 checkpoint or apoptotic cell death (Kastan et al., 1991). A structural change in p53 facilitates its sequence-specific binding to DNA (Cho et al., 1994) and increases expression of genes involved in cycle arrest or apoptosis. Gene expression is up-regulated by wild-type (wt) p53 for genes such as *GADD45* (Kastan et al., 1992), $p21^{Waf1/Cip1}$ (El-Deiry et al., 1993), *mdm-2* (Momand et al., 1992), and *cyclin G* (Okamoto and Beach, 1994) that contain a p53 binding site. In contrast, genes lacking a p53 consensus binding site, like *c-fos* (Kley et al., 1992), *mdr1* (Chin et al., 1992), *hsp70* (Agoff et al., 1993), and *O^6-methylguanine-DNA methyltransferase* (Harris et al., 1996) are down-regulated by wt p53. Evidence suggests the repression by wt p53 results from its direct interaction with factors such as TATA-binding protein (Liu et al., 1993), Sp1 (Borellini and Glazer, 1993), CCAAT binding factor (Agoff et al., 1993), and transcriptional coactivators such as p300/cAMP-response element-binding protein (Ravi et al., 1998) and p300/cAMP-response element-binding protein-associated factor (Scolnick et al., 1997). On the other hand, mutant p53 exhibits an attenuated transcriptional repression activity that may reflect a lack of association/interaction with these or other transcription factors (Zambetti and Levine, 1993).

Nuclear factor-Y (NF-Y) is a heterotrimeric protein composed of NF-YA, NF-YB, and NF-YC subunits (Maity and de Crombrugghe, 1998). It functions as a transcription factor whose DNA binding domain is created by the interaction of highly conserved regions located in the three subunits (Maity et al., 1992). NF-Y specifically recognizes a CCAAT box motif found in the promoter and enhancer regions of many genes (Mantovani, 1998). These sites are typically located in the proximal promoter region from −80 to −60 bp upstream of the transcription start site. Biochemical studies demonstrate that the DNA binding domain of the NF-YB and NF-YC subunits associate through a protein-protein histone-fold "handshake" motif (Sinha et al., 1996). The NF-YA subunit interacts only with the NF-YB:NF-YC heterodimer, suggesting that the NF-YB:NF-YC histone-fold is critical in creating a functional NF-Y CCAAT box DNA binding complex. In this report, we describe experiments in which wt p53 and certain anticancer drugs induce the down-regulation of topo IIα gene expression through inhibition of the binding of NF-Y to specific ICBs in the topo IIα promoter.

Materials and Methods

Materials. All cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA), BioWhittaker (Walkersville, MD), or Atlanta Biologicals (Norcross, GA). ^{32}P-Labeled deoxycytidine triphosphate was purchased from PerkinElmer Life Sciences (Boston, MA). Mouse anti–NF-YA (clone YA-1a) was purchased from BD Pharmingen (San Diego, CA). The ICBp90 antibody was a gift from Dr. Christian Bronner (Strasbourg, France) (Hopfner et al., 2000). Unless otherwise specified, all other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). VM-26 (teniposide) was a gift from Bristol-Myers Squibb Co. (Princeton, NJ). Unless otherwise indicated, all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Plasmids and Cell Culture. The human p53 expression plasmids (a gift from Gerard Zambetti, St. Jude Children's Research Hospital, Memphis, TN) and the pCMV-Neo-Bam control vector used in this study have been described previously (Wang et al., 1997). The mutant p53-22/23 vector contains mutations at amino acids 22 and 23 of the expressed p53 protein. The dominant negative NF-YA vector (Δ4-YA13m29) (a gift from Dr. Roberto Mantovani, University of Milan, Italy) contains mutations in three amino acids in the DNA binding domain (Mantovani et al., 1994). The (10)1 cell line (a gift from Gerard Zambetti) is a spontaneously immortalized murine BALB/c embryo fibroblast line, containing large deletions in both *p53* alleles; consequently, it is completely deficient in p53 protein. The (10)1val cell line was developed by transfection of (10)1 cell with a temperature-sensitive p53 expression vector (Wang et al., 1997, and references within). The p53 protein is predominately in the wt conformation at 32°C, and in the mutant conformation at 39°C. The mouse NIH3T3 cell line was obtained from American Type Culture Collection (Manassas, VA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin, in humidified 5% CO_2/95% air atmosphere at 37°C.

Transient Transfection of (10)1 Cells. (10)1 cells were cultured in 150-mm plates at a cell density of 2.5×10^6 cells/plate for 24 h. A mixture of 2 μg of the control vector, wt p53 vector, or mutant p53-22/23 vector DNA and 50 μl of LipofectAMINE (Invitrogen) (1 μg/50 μg) were incubated at room temperature in 10 ml of DMEM without serum for 30 min. Culture media on the cells was replaced with the DNA-LipofectAMINE mixture, the cells were incubated for 5 h at 37°C, and then 20 ml of DMEM with serum was added without aspirating off the DNA-LipofectAMINE mixture. Twenty-four hours after transfection, the media was aspirated off, the cells washed once with 1× phosphate-buffered saline, and fresh DMEM with serum was added. The cells were allowed to grow for an additional 24 h before being harvested with trypsin-EDTA for preparation of nuclear extract.

Preparation of Nuclear Extracts. After transient transfection, cells were harvested and centrifuged at 1000 × g for 4 min. The cell pellet was resuspended in hypotonic buffer and the nuclear proteins were extracted as described previously (Danks et al., 1988). In all buffers, protease inhibitors were added just before use: phenylmethylsulfonyl fluoride and benzamidine at 1 mM each, aprotinin, soybean trypsin inhibitor 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, Hercules, CA). The nuclear extracts were stored in aliquots at −80°C.

Preparation of Cell Lysates. After the designated drug treatment, cells were harvested and collected by centrifugation at 1000 × g for 4 min. The cell pellet was resuspended in 120 μl RIPA buffer (50

Fig. 1. Oligomers containing ICBs for topo IIα. Numbers indicate base positions relative to the topo IIα transcription start site (see Hochhauser et al., 1992). ICBs are underlined and mutations are indicated by boxed small letters.

TABLE 1

Effects of wt and mutant p53 on topo IIα promoter constructs in (10)1 cells

(10)1 cells were cotransfected with topo IIα promoter constructs and either wt or mutant p53 expression vector. p53 values are expressed as a percentage of the luciferase activity in lysates from cells cotransfected with the control CMV-neo-Bam vector only. The values are the mean ± 1 S.D. of activities from triplicate samples in a representative experiment.

Topo IIα Construct	No. of ICBs	Relative Luciferase Activity	wt p53	Mutant p53
			% of control	
pT IIα-32	0	1	147 ± 15	151 ± 14
pT IIα-90	1	4.9	55 ± 8	82 ± 8
pT IIα-142	2	12.8	25 ± 3	98 ± 10
pT IIα-252	3	132	12 ± 1	62 ± 1
pT IIα-382	4	202	11 ± 2	87 ± 23

mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid and 1% NP-40). The lysed cells were homogenized by multiple passes through a pipette tip, incubated on ice for 30 min, and then centrifuged at 12,500 rpm for 10 min. The supernatant was collected as the cell lysate and the protein concentration determined by the Bio-Rad protein assay.

Electrophoretic Mobility Shift and Supershift Assays. The human topo IIα CCAAT box oligomers used in these experiments were as shown in Fig. 1. The oligomers were annealed to their respective complementary strands designed to create 2 to 4 base overhangs at the 5′ end. The resulting overhangs were filled in with ^{32}P-dCTP using Klenow fragment of DNA polymerase and the incorporated dCTP determined by scintillation counting. Nuclear proteins (1.5 μg) from the (10)1 cell line transfected with either the wt p53, mutant p53 or control vector, along with poly dIdC (1 μg) (Roche Molecular Biochemicals, Indianapolis, IN) and 70,000 cpm/lane of labeled ICB oligonucleotide were incubated at room temperature for 30 min in a binding buffer consisting of 20 mM HEPES pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% glycerol and 50 mM NaCl. For the supershift assays 1 μl of the specific antibody was also included in the binding reaction with the labeled oligomer and the nuclear extract. To check for background protein binding, 10 μg of BSA was added to specific control samples. For competition studies, 1 μl (10 fold) of the unlabeled oligo was included in the binding reaction. The DNA-protein complexes were resolved on a 6.5% nondenaturing polyacrylamide gel run in Tris-glycine buffer (25 mM Tris, pH 8.5, 200 mM glycine and 1 mM EDTA) at 4°C. The gels were dried and exposed to Kodak BIOMAX MR film (Eastman Kodak Co.) with intensifying screens at −80°C. In the studies with drug-treated cells, the following drugs were used: vinblastine, etoposide (VP-16), amsacrine (mAMSA), teniposide (VM-26), aclarubicin, cisplatin (CDDP), camptothecin, mitoxantrone, or ellipticine. The cells were exposed to the drugs for 20 h.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting. For detection of p53 protein, 30 μg of nuclear extract protein was denatured by boiling in loading buffer for 5 min and loaded onto a 12% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS). The gel was run at 200 V for 30 min. The separated proteins were transferred onto a nitrocellulose membrane by electroblotting. The gel was subsequently stained to confirm the equivalent loading and transfer of proteins. After being blocked in 5% nonfat dry milk, the nitrocellulose membrane was incubated overnight in a 1:500 dilution of rabbit anti-human p53 antibody. After incubation with goat anti-rabbit alkaline phosphatase antibody (1:500 dilution) the bound antibody was visualized by reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. For NF-Y protein, 40 mg of cell lysate protein was loaded onto a 7.5% polyacrylamide minigel and run at 150 V for 1.0 h. The nitrocellulose membrane was incubated overnight in a 1:1000 dilution of mouse anti-NF-YA antibody. After incubation with an anti-mouse alkaline phosphatase antibody (1:1000 dilution), the bound antibody was visualized as above.

Luciferase Assays. (10)1 cells were cultured in 12 well plates (7×10^4 cells/well) for 24 h before transfection as described above with slight modifications. Briefly, 1 μg of the topo IIα-luciferase reporter construct DNA and 0.5 μg of the dominant negative NF-YA vector (Δ4YA13m29) and/or 0.1 μg of the wt p53 vector was mixed with 4 μl LipofectAMINE (1 μg/2.7 μl) in 0.5 ml of DMEM without serum for 30 min at room temperature. The DNA-LipofectAMINE mixture was added to the cells for 2.5 h and the transfections completed as described above. After 24 h in fresh media, cells were washed in phosphate-buffered saline, lysed with 200 μl of passive lysis buffer (Promega), and centrifuged for 10 s to remove cell debris. The cell lysate was analyzed for luciferase activity using the Promega luciferase substrate as described by the supplier. Luciferase activity was measured on a luminometer (Turner Designs). The lysate protein concentration was determined using the Bio-Rad protein dye reagent.

Results

Binding of Transcription Factors to the ICBs of the Topo IIα Promoter Is Inhibited by the Expression of wt p53. Several laboratories have documented the vital role of multiple ICBs in the topo IIα promoter for expression of the topo IIα gene in both human and rodent cell lines (Ng et al., 1995; Herzog and Zwelling, 1997; Wang et al., 1997; Takano et al., 1999). Earlier studies from our lab have demonstrated that successive deletions of each of the five ICBs from the topo IIα promoter results in progressively reduced promoter activity in mouse embryo fibroblast (10)1 cells and that the activity of the topo IIα promoter is specifically inhibited by wt p53 but not by mutant p53 (Wang et al., 1997 and Table 1). Specific mutations disrupting the CCAAT sequence of certain ICBs eliminated the p53-induced inhibition of topo IIα promoter activity. The effect of p53 expression on the binding of transcription factors to the topo IIα promoter was studied using nuclear extracts from the p53-null (10)1 cells transiently transfected with control, wt, or mutant p53-expressing vector. Electrophoretic mobility shift assays were conducted with labeled oligomers representing the five ICBs of the topo IIα promoter to ascertain distinctions in factor binding.

The oligomers comprising ICB-1, -3, and -4 exhibited strong binding to a transcription factor found in nuclear extracts from cells transfected with the control vector (Fig. 2). Adding antibody for the NF-YA subunit to the mixture of labeled oligomer and nuclear extract caused a distinct shift in the electrophoretic mobility of the band corresponding to the bound transcription factor. Because all three subunits of the NF-Y complex are required for DNA binding, the supershift with NF-YA confirms that the factor binding to the oligomer is NF-Y. Nuclear extracts from (10)1 cells transfected with the wt p53 vector exhibited a dramatic decrease in the amount of the NF-Y transcription factor binding to these ICB oligomers relative to the extracts from cells trans-

fected with the control vector. Again, supershift analysis with the NF-YA antibody substantiates that the NF-Y complex binding to ICB-1, -3, and -4 is decreased in wt p53-transfected cells. This decrease in NF-Y binding was not seen in nuclear extracts from cells transfected with a vector expressing a mutant p53. There was no significant difference in the binding of NF-Y to these oligomers with nuclear extracts from either control or mutant p53 transfected cells.

In similar mobility shift assays conducted with the ICB-2 oligomer, there was a different and significantly weaker banding pattern with control nuclear extracts than the binding pattern seen with ICB-1, -3, and -4 (Fig. 3). However, there is still a distinct supershift of a specific band with the NF-YA antibody, indicating that NF-Y does bind to this ICB-2 oligomer. Using nuclear extracts from cells transfected with wt p53, the bands binding to the ICB-2 oligomer that correspond to NF-Y are no longer detectable. As expected, nuclear extracts from mutant p53-transfected cells show no reduction in NF-Y binding to the ICB-2 oligomer relative to control nuclear extract.

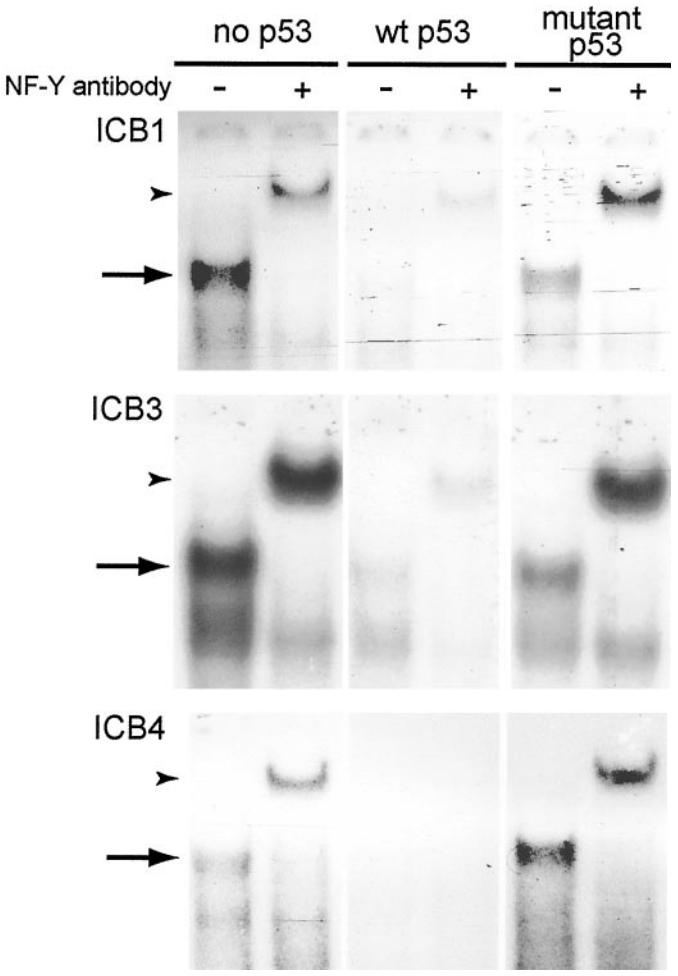

Fig. 2. wt p53, but not mutant p53, inhibits binding of NF-Y to topo IIα ICBs. Nuclear extracts were prepared from p53-null (10)1 cells that had been transfected with the control vector (no p53), the wt p53 expression vector, or the mutant p53-22/23 expression vector. In three separate experiments, nuclear extract proteins (1.5 μg) were incubated with labeled duplex oligomers that contained the sequence for the indicated ICB, and gel mobility shift assays were performed as described under *Materials and Methods*. The arrow indicates the position of the NF-Y band, and the arrowhead indicates the position of the NF-Y band supershifted with antibody to NF-YA.

Unlabeled ICB-1, -2, or -3 oligomers were able to abrogate the NF-Y binding to ICB-4, signifying that ICB-1, -2, -3, and -4 bind the same transcription factor. Mutation of the ATTGG sequence in the oligomers to CTGGA abolished the binding of NF-Y, indicating that this consensus sequence is essential. However, substitution of the base pair immediately 5′ of the ICB-1 did not affect the NF-Y binding to the oligomer (data not shown).

Experiments were conducted to determine whether the inhibition of NF-Y binding to the ICBs was caused by physical sequestration of NF-Y by the p53 protein. Various concentrations of purified exogenous p53 protein were incubated with (10)1 nuclear extracts for 15 min before determination of the binding of NF-Y to the ICBs of the topo IIα promoter. The preincubation of nuclear extracts with exogenous p53 protein did not significantly affect the binding of NF-Y, indicating that in vitro wt p53 protein does not inhibit NF-Y binding by physical interaction or sequestration (data not shown).

The ICB-5–containing oligomer exhibited strong binding to a protein factor with a banding pattern distinct from the other four ICBs (Fig. 4). In addition, there was no evidence of a shift in mobility of the protein binding to ICB-5 when NF-YA antibody was incubated with the nuclear extract. This result indicates that the factor binding to the ICB-5 oligomer is not NF-Y. Interestingly, with nuclear extract from cells transfected with wt p53, the binding of this factor to ICB-5 was almost completely blocked. Nuclear extract from mutant p53-transfected cells shows no reduction in factor binding relative to the control nuclear extract. Unlabeled ICB-4 oligomer was unable to compete with the binding of the transcription factor to the labeled ICB-5 oligomer, demonstrating that this factor is distinct from the NF-Y factor. As with the NF-Y binding to the other ICBs, mutation of the ICB-5 ATTGG site to CTGGA eliminated the binding of this factor. However, mutation of two A bases at positions 2 and 4 base pairs 5′ of the ICB-5 did not decrease factor binding.

In an attempt to identify the factor(s) binding to ICB-5, nuclear extracts were incubated with antibodies to known

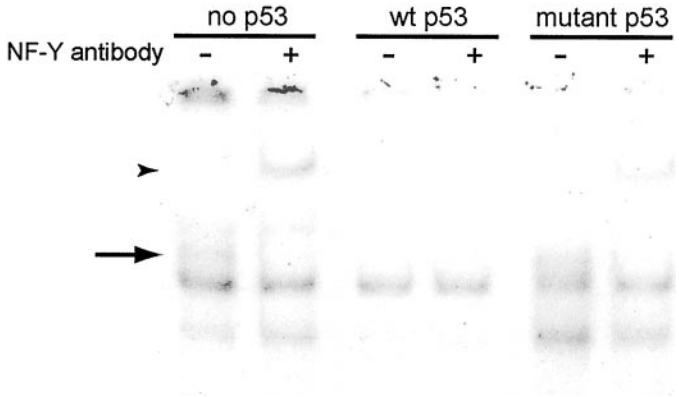

Fig. 3. wt p53, but not mutant p53, inhibits binding of NF-Y to topo IIα ICB-2. Nuclear extracts were prepared from p53-null (10)1 cells that had been transfected with the control vector (no p53), the wt p53 expression vector, or the mutant p53-22/23 expression vector. Nuclear extract proteins (1.5 μg) were incubated with labeled duplex oligomers that contain the sequence for ICB-2, and gel mobility shift assays were performed as described under *Materials and Methods*. The arrow indicates the position of the NF-Y band, and the arrowhead indicates the position of the NF-Y band supershifted with antibody to NF-YA.

nuclear factors that might have been predicted to bind to this ICB or its close flanking regions. Gel shift assays indicated no change in the mobility of this factor with antibodies to ICBp90, CCAAT displacement protein, CCAAT enhancer binding protein-α, -β, or -γ, or TATA binding protein. A weak supershifted band was observed with an antibody to nuclear factor 1, but the major bands binding to the ICB-5 oligomer were not affected by the nuclear factor 1 antibody.

Inhibition of Topo IIα Promoter Activity by Dominant-Negative NF-Y. The present study shows that transient transfection with wt p53 inhibits the binding of NF-Y to the ICBs of the topo IIα promoter. Our previous results demonstrate that transient transfection with wt p53 inhibits topo IIα promoter activity (Wang et al., 1997). We wanted to determine whether the inhibition of topo IIα promoter activity by wt p53 was related to the ability of p53 to inhibit the binding of NF-Y to the ICBs of the topo IIα promoter. To further confirm the relationship of inhibition of NF-Y binding to decreased topo IIα promoter activity, a dominant negative NF-Y vector (Δ4YA13m29) was transfected into (10)1 cells with or without cotransfection of wt p53. Luciferase assays were performed to examine the effect of the mutant NF-Y vector on topo IIα promoter-luciferase reporter expression. The dominant-negative NF-YA can associate with the endogenous NF-YB and NF-YC, but the resultant heterotrimer does not bind to ICBs to activate transcription. Thus expression of the dominant-negative NF-YA should inhibit the binding of a functional NF-Y complex to the ICBs of the topo IIα promoter in a manner independent of the effect of wt p53.

As shown in Fig. 5, cotransfection of the dominant-negative NF-YA vector significantly decreased topo IIα promoter activity relative to the topo IIα promoter constructs alone. This effect was seen with all the topo IIα promoter vectors, except for pTIIα-32, which does not contain an ICB site. As seen with the expression of wt p53, the relative decrease in promoter activity was greater for the topo IIα promoter vectors containing multiple ICBs. Cotransfection of both the dominant-negative NF-Y and wt p53 vectors resulted in no significant increase in inhibition of the promoter activity of the various topo IIα constructs containing multiple ICBs. These data substantiate the earlier findings with wt p53 that inhibition of the binding of a functional NF-Y complex to the ICBs in the topo IIα promoter results in a coordinate decrease in promoter activity.

Effect of Anticancer Drugs on the Binding of NF-Y to the ICBs of Topo IIα in Cells Containing Endogenous p53. Many of the topo II-targeted anticancer drugs result in DNA double-strand breaks. Other anticancer drugs cause DNA damage by alternative means, such as the blocking of mitosis or inhibition of topoisomerase I activity. These types of DNA damage are known to induce p53, leading to cell-cycle arrest and apoptosis. Exogenous expression of wt p53 inhibits the binding of NF-Y to the ICBs of the topo IIα promoter and decreases topo IIα expression. Therefore, one might predict that treatment of cells containing a functional wt p53

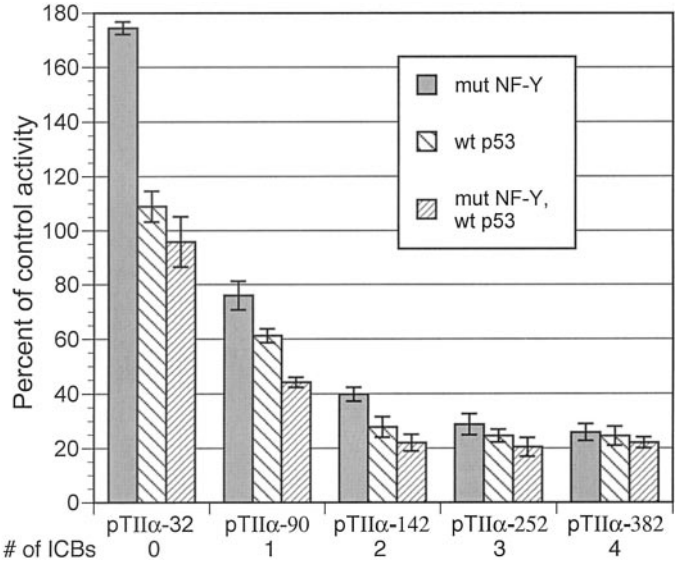

Fig. 5. Both wt p53 and dominant-negative NF-Y decrease topo IIα promoter activity. Cell lysates were prepared from p53-null (10)1 cells that had been transfected with a topo IIα promoter- luciferase vector and the wt p53 expression vector, the dominant negative NF-Y expression vector, or both. The luciferase activity was determined as described under *Materials and Methods*, and is expressed as a percentage of the activity of the specific topo IIα promoter construct alone. The positions of the ATTGG comprising ICB-1 to -5 are −68, −108, −175, −259, and −389, respectively (see Fig. 1). The number of the topo IIα construct indicates the 5′-most base included in that construct. The number of ICBs in each of the constructs is indicated at the bottom of the graph.

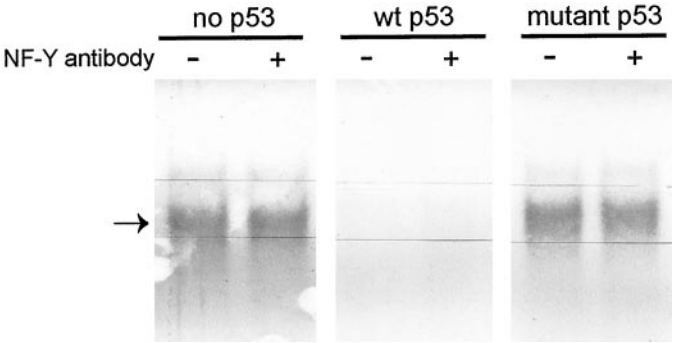

Fig. 4. wt p53, but not mutant p53, inhibits binding of a transcription factor to ICB-5 of the topo IIα promoter. Nuclear extracts were prepared from p53-null (10)1 cells that had been transfected with the control vector (no p53), the wt p53 expression vector, or the mutant p53-22/23 expression vector. Nuclear extract proteins (1.5 μg) were incubated with labeled duplex oligomers that contained the sequence for ICB-5, and gel mobility shift assays performed were as described under *Materials and Methods*. The arrow indicates the position of the major unknown band binding to ICB-5 that is inhibited by p53.

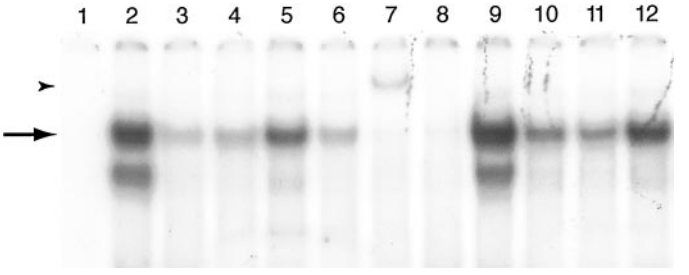

Fig. 6. Treatment of NIH3T3 cells with anticancer drugs can block the binding of NF-Y to ICBs of the topo IIα promoter. Nuclear extracts were prepared from NIH3T3 cells that had been treated for 20 h with the indicated drug. The nuclear proteins (1.5 μg) were incubated with labeled duplex oligomer that contain the sequence for ICB-1, and gel mobility shift assays were performed as described under *Materials and Methods*. The arrow indicates the position of the NF-Y band, and the arrowhead indicates the position of the NF-Y band supershifted with antibody to NF-YA. The lanes are: 1, no nuclear extract; 2, no drug; 3, vinblastine (10 μM); 4, VP-16 (10 μM); 5, *m*-AMSA (5 μM); 6, VM-26 (5 μM); 7, VM-26 (5 μM) plus NF-YA antibody; 8, aclarubicin (5 μM); 9, cisplatin (10 μM); 10, camptothecin (5 μM); 11, mitoxantrone (1 μM); 12, ellipticine (5 μM).

gene with anticancer drugs would have similar inhibitory effects on NF-Y binding. Nuclear extracts were prepared from NIH3T3 cells treated with various anticancer drugs for 20 h. Mobility shift assays with the ICB-1 oligomer of the topo IIα promoter and nuclear extracts from NIH3T3 cells treated with vinblastine, aclarubicin, VP-16, or VM-26 exhibited a significant decrease in NF-Y binding (Fig. 6). Treatment of NIH3T3 cells with *m*-AMSA, mitoxantrone, ellipticine, and camptothecin induced a moderate decrease in NF-Y binding. Nuclear extracts from cisplatin-treated NIH3T3 cells did not exhibit any decrease in NF-Y binding to ICB-1. In lane 7, antibody to NF-Y was added to the nuclear extract to confirm that the decreased band represents NF-Y. This decrease in NF-Y binding with treatment of NIH3T3 cells with anticancer agents correlates directly with the effect of VM-26 treatment on topo IIα promoter activity. We have found that treatment of NIH3T3 cells with 5 μM VM-26 can inhibit promoter activity up to 75%, as measured by relative luciferase activity with the topo IIα promoter constructs containing three or four ICBs (see Table 1).

Effect of Anticancer Drugs on the Binding of NF-Y to the ICBs of Topo IIα in Nuclear Extracts from p53-Null Cells. From our initial experiments, it seemed that the inhibition of NF-Y binding to the ICBs of the topo IIα promoter occurs because of the expression of wt p53. However, with the exception of vinblastine- and aclarubicin-treated cells, western blots with nuclear extracts from drug-treated NIH3T3 cells did not show a detectable induction of p53 protein expression (Fig. 7). This unanticipated result led us to question whether the induction of p53 in drug-treated cells was essential for the inhibition of NF-Y binding to ICBs. Gel-shift assays were conduced with the ICB-1 oligomer and nuclear extracts from the p53-null (10)1 cells treated with anticancer drugs for 20 h. The drug concentrations used were the same as those in the NIH3T3 experiments, except that 0.1 μM aclarubicin was used instead of 5 μM. Interestingly enough, nuclear extracts from the drug-treated p53-null (10)1 cells also exhibited an inhibition of NF-Y binding to ICB-1 as seen in the studies with drug-treated NIH3T3 cells (Fig. 8). In lane 7, antibody to NF-Y was added to the nuclear extract to confirm that the decreased band represents NF-Y.

Similar experiments were conducted with the ICB3 and ICB4 oligomers and nuclear extracts from both NIH3T3 and (10)1 cells treated with VM-26 or cisplatin. The results were the same as for ICB1: VM-26 inhibited NF-Y binding to either oligomer, whereas cisplatin had no effect on binding. These results demonstrate that expression of wt p53 is not essential for anticancer drugs to induce inhibition of NF-Y binding to ICBs of the topo IIα promoter. The shifting of two major bands with the ICB oligomers is a consistent result in all our experiments and is consistently seen in publications looking at NF-Y binding to ICBs (Yun et al., 1999; Hu et al., 2000; Jung et al., 2001). The exact identity of the lower band is not known (Hu et al., 2000); however, both bands can be competed with unlabeled oligonucleotides containing ICBs. In our studies, expression of p53 or treatment with certain drugs can decrease the binding to varying degrees of NF-Y and the protein comprising the lower band.

Effect of Anticancer Drugs and wt p53 Expression on the Endogenous Levels of NF-Y Protein. We have demonstrated thus far that both cellular expression of wt p53 and treatment of cells with specific anticancer drugs results in a substantial decrease in the binding of NF-Y to the ICBs of the topo IIα promoter. We next checked the protein levels of NF-Y in the treated cells to determine whether the decreased binding was the result of decrease levels of available NF-Y protein. Western blots were performed with cell lysates from the p53-null (10)1 cells treated with various drugs for 20 h or with lysates from the (10)1val cells incubated at either 32°C (wt p53) or 39°C (mutant p53) for 20 h. We did not observe a significant change in endogenous NF-Y protein levels in the drug-treated cells compared with the untreated cells (Fig. 9A). Similarly, there was no significant difference in the NF-Y protein levels in cells expressing wt p53 compared with those expressing mutant p53 (Fig. 9B), indicating that anticancer drugs or wt p53 expression do not affect the endogenous protein levels of NF-Y. For independent confirmation of the effect of cell-cycle arrest on NF-Y protein levels, (10)1 cells were serum-starved or treated with aphidicolin or nocodazole to block the cell cycle at distinct points. Serum-starved cells were arrested in G_1, before entry into S (71% in G_1), and aphidicolin-treated cells were blocked in both G_1 (52%) and S (42%) phases. Nocodazole treatment arrested 86% of cell in G_2. Western blots of cell lysates indicate that cell cycle arrest in any of these states does not significantly affect the protein levels of NF-Y (Fig. 9C).

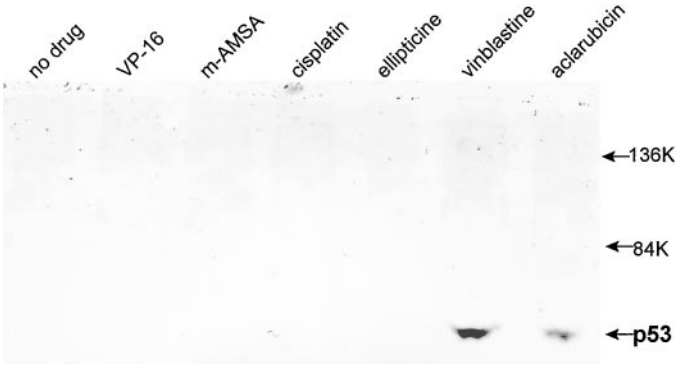

Fig. 7. Effect of drug treatment on p53 expression of NIH3T3 cells. Cell lysates were prepared from NIH3T3 cells that had been treated for 20 h with the indicated drug. A Western blot with antibody to p53 was performed as described under *Materials and Methods*. The lanes are: 1, no drug; 2, VP-16 (15 μM); 3, *m*-AMSA (10 μM); 4, cisplatin (30 μM); 5, ellipticine (1 μM); 6, vinblastine (10 μM); and 7, aclarubicin (0.1 μM).

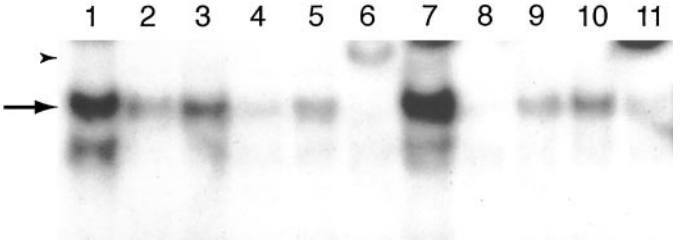

Fig. 8. Treatment of p53-null (10)1 cells with anticancer drugs can block the binding of NF-Y to ICBs of the topo IIα promoter. Nuclear extracts were prepared from (10)1 cells that had been treated for 20 h with the indicated drug. The nuclear proteins (1.5 μg) were incubated with labeled duplex oligomer that contain the sequence for ICB-1, and gel mobility shift assays were performed as described under *Materials and Methods*. The arrow indicates the position of the NF-Y band, and the arrowhead indicates the position of the NF-Y supershifted with antibody to NF-YA. The lanes are: 1, no drug; 2, vinblastine (10 μM); 3, VP-16 (10 μM); 4, *m*-AMSA (5 μM); 5, VM-26 (5 μM); 6, VM-26 (5 μM) plus NF-YA antibody; 7, cisplatin (10 μM); 8, aclarubicin (0.1 μM); 9, camptothecin (5 μM); 10, mitoxantrone (1 μM); 11, ellipticine (5 μM).

Discussion

Using promoter deletion constructs and mutated ICB consensus sites, it has been well documented that the ICBs in the topo IIα promoter are required for optimal transcriptional activity (Hochhauser et al., 1992; Ng et al., 1995; Park et al., 1995; Herzog and Zwelling, 1997). Isaacs et al. (1996) first indicated that the transcription factor complex NF-Y binds to the ICBs of the topo IIα promoter, and this NF-Y-specific binding has been confirmed by others (Herzog and Zwelling, 1997; Wang et al., 1997), including this present report. Work in our laboratory and the laboratory of Ian Hickson has demonstrated that expression of wt p53, but not mutant p53, represses the transcription of the topo IIα promoter (Sandri et al., 1996; Wang et al., 1997). The present report provides data showing that the p53-induced decrease in topo IIα promoter activity results from the wt p53-dependent inhibition of transcription factor NF-Y binding to the four proximal ICBs of the topo IIα promoter. With each of the ICB oligomers tested, wt p53 was shown to decrease binding of NF-Y, but no decrease in binding was observed with mutant p53. The p53-22/23 mutant protein we have used in this study has a disruption in the transactivation domain of the protein. Previous studies in our lab with two other p53 mutants (p53-175 and p53-281) having alterations in their DNA binding domains also showed no significant inhibitory effect on topo IIα promoter activity (Wang et al., 1997). It follows that the ability to inhibit NF-Y binding to ICBs requires a p53 protein with functional transactivation and DNA binding domains. wt p53 transrepresses the human heat-shock promoter and is reported to physically interact with a CCAAT binding factor (Lum et al., 1990). The inability of mutant p53 to inhibit NF-Y binding may reflect a lack of association with such CCAAT binding factors.

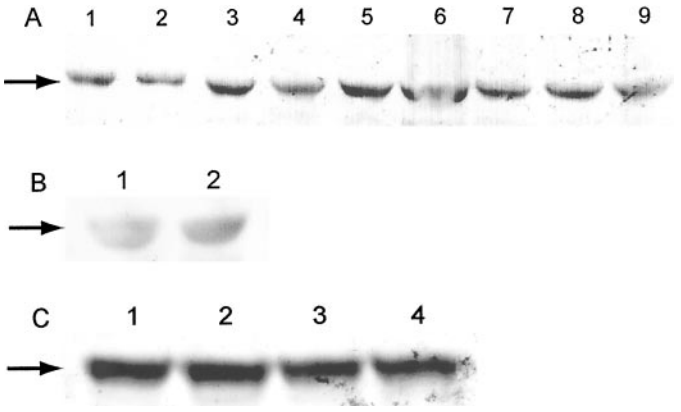

Fig. 9. A, treatment of (10)1 cells with anticancer drugs does not alter the endogenous expression levels of NF-Y protein. Cell lysates were prepared from (10)1 cells that had been serum-starved or -treated for 20 h with the indicated drug. A Western blot with antibody to NF-YA was performed as described under *Materials and Methods*. The arrow indicates the position of the NF-Y protein band. The lanes are: 1, no drug; 2, vinblastine (10 μM); 3, VP16 (10 μM); 4, mAMSA (10 μM); 5, cisplatin (10 μM); 6, aclarubicin (0.1 μM); 7, camptothecin (5 μM); 8, mitoxantrone (1 μM); and 9, ellipticine (5 μM). B, wt and mutant p53 expression does not affect the endogenous expression levels of NF-Y protein. Cell lysates were prepared from (10)1val cells that had been incubated at either 32°C (wt p53) or 39°C (mutant p53). A Western blot with antibody to NF-YA was performed as described under *Materials and Methods*. The arrow indicates the position of the NF-Y protein band. The lanes are: 1, (10)1val39; 2, (10)1val32. C, drug treatment or serum starvation of (10)1 cells does not change the endogenous expression levels of NF-Y protein. Cell lysates were prepared from (10)1 cells that had been serum-starved or -treated for 20 h with the indicated drug. A Western blot with antibody to NF-YA was performed as described under *Materials and Methods*. The arrow indicates the position of the NF-Y protein band. The lanes are: 1, untreated; 2, nocodazole (15 μM); 3, aphidicolin (5 μg/ml); 4, no serum.

The results shown in Fig. 5 confirm, through two independent means, that inhibition of NF-Y binding to the ICBs causes a decrease in topo IIα promoter activity. In this experiment we used the NF-YA plasmid, Δ4YA13m29, which expresses a dominant-negative mutant form of the NF-YA subunit containing alterations of three amino acids in the DNA binding domain. The dominant-negative NF-YA subunit forms a heterotrimer with the endogenous NF-YB and NF-YC subunits, but the resultant NF-Y complex cannot bind to the ICBs (Mantovani et al., 1994). Thus, the decrease in topo IIα promoter activity seen with coexpression of the dominant-negative NF-YA can be directly attributed to the decreased availability of viable NF-Y complex to bind the ICBs. The inhibition of topo IIα promoter activity seen with transfection of the dominant-negative NF-YA vector is strikingly similar in result, if not in precise mechanism, to the inhibition seen with transfection of wt p53. It is of note that simultaneous expression of both dominant-negative NF-YA and wt p53 does not produce significantly more promoter inhibition than either factor alone, indicating that either action can maximally reduce promoter activity to the basal promoter activity seen in the absence of NF-Y binding.

The inhibition of NF-Y binding to the ICBs of topo IIα is not caused by a decrease in the endogenous expression levels of NF-Y protein because both the anticancer drug-treated and wt p53-expressing cells exhibited no significant changes in NF-YA protein levels (Fig. 9, A and B). An earlier work by Bolognese et al. (1999) suggests that the regulation of the cyclin B2 promoter depends on cell-cycle regulated CCAAT-binding activity of NF-Y. The NF-Y protein, but not its mRNA, was found to be maximal in the mid-S phase and decreased in the G_2/M phase of the cell cycle. We used the drugs nocodazole and aphidicolin in addition to serum starvation to arrest cells in various phases of the cell cycle and found that NF-YA protein levels did not vary significantly under these conditions (Fig. 9C). Similar results recently reported by Jung et al. (2001) showed that the protein levels of all three subunits of NF-Y were unchanged by the expression of p53. These results indicate that inhibition of NF-Y binding induced by wt p53 or anticancer drugs does not occur because of a cell cycle-related decrease of NF-Y protein.

It is obvious from a comparison of Figs. 2 and 3 that there is a distinct difference in the strong binding affinity of NF-Y for ICB-1, -3, and -4, and the very weak affinity of NF-Y for ICB-2. For the detection of NF-Y binding to ICB-2 in Fig. 3, the autoradiogram was exposed for more than five times longer then was necessary for detection of binding to ICB-1. It is interesting to note that studies of the hamster topo IIα promoter show NF-Y to have a weaker binding affinity for ICB-2 and ICB-4 than the other ICBs, and mutations in ICB-2 cause only minimal decreases in promoter activity (Ng et al., 1995). This is not to say that ICB-2 is of lesser importance in the regulation of topo IIα. To the contrary, Isaacs et al. (1996) presented evidence that decreased binding of NF-Y to ICB-2 may play a key role in mediating down-regulation of topo IIα transcription in confluence-arrested cells, which is relieved in proliferating cells through the binding of NF-Y to the ICB-2. Thus, the relative contribution of an ICB to the regulation of topo IIα may depend on factors other than its

binding affinity for NF-Y. The possibility of distinct roles for the ICBs is also supported by Falck et al. (1999), who report that ICB-1 may play an important role in the S phase-specific induction of topo IIα expression, and by Morgan and Beck (2001), who report that ICB-3 may play a specific role in topo IIα up-regulation in ICRF-187-resistant CEM leukemic cells.

The protein factor binding to ICB-5 is unknown at the present time. Despite having the required ATTGG sequence, NF-Y does not bind to the ICB-5 oligomer. However, the ICB-5-binding factor responds similarly to NF-Y in that its binding is reduced by the expression of wt p53 but not mutant p53. The presence of an AT-rich flanking sequence decreases the ability of NF-Y to bind to an ICB (Dr. Mantovani, personal communication). The ICB-5 of the human topo IIα promoter has such an AT-rich flanking sequence. ICB-5 is also unique with respect to its position in the promoter sequence. A sequence alignment of the human and hamster topo IIα promoters will show no comparable ATTGG site in the hamster to match the ICB-5 site in the human. The fifth ICB in the hamster coincides with ICB-4 in the human, whereas the positions of the first three ICBs are equivalent in both sequences (Ng et al., 1995).

To examine the possibility that treatment of cells with anticancer drugs could induce the expression of p53 and thus down-regulate topo IIα expression, we analyzed the effect of a panel of drugs on the binding of NF-Y to ICB-1 (Fig. 6). Nuclear extracts from NIH3T3 cells treated with the topo II-targeting drugs VM-26 or VP-16 exhibited decreased binding of NF-Y to the ICB. Treatment with cisplatin, which does not induce DNA breaks, did not result in any change in NF-Y binding to the ICB. Treatment with vinblastine, a mitotic spindle inhibitor, or aclarubicin, an inhibitor of topo IIα catalytic activity and not a cleavable complex-forming drug, caused inhibition of NF-Y binding. Aclarubicin has been shown to stabilize topo I cleavage resulting in single strand breaks (Nitiss et al., 1997). It has also been reported that vincristine, a mitotic spindle inhibitor similar to vinblastine, induces p53 in MCF7 cells (Vayssade et al., 2002). We would have expected that p53 would be expressed after treatment with the topo II-targeted drugs because of double strand breaks resulting from stabilization of the cleaved topo II-DNA complex. Western blot analysis of lysates from drug-treated cells confirmed that vinblastine and aclarubicin could induce the expression of p53 protein in the NIH3T3 cells. However, it was unexpected that p53 was not expressed at detectable levels after treatment with the topo II-targeted drugs under the conditions of this experiment.

To follow-up this observation of drug-induced inhibition of NF-Y binding, we treated the p53-null (10)1 cells with these same anticancer drugs. Even though the (10)1 cells are completely deficient for p53, we saw similar inhibition of NF-Y binding to ICBs of topo IIα when the cells were treated with the anticancer agents. These results are substantiated in an earlier study by Goldwasser et al. (1999) suggesting that down-regulation of topo IIα expression by ionizing radiation can occur independent of the p53 status of the cell. Thus, it seems that the inhibition of topo IIα by anticancer drugs could occur either by a p53-dependent and/or -independent mechanism. We are presently exploring the possibility that p53 transcriptional targets, such as $p21^{Waf1/Cip}$ and 14-3-3σ, which function in DNA damage-induced G_2/M arrest, may be activated by alternative means in the drug-treated cells.

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