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The Tumor Suppressor p53 Regulates Its Own Transcription

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The ability of p53 to suppress transformation correlates with its ability to activate transcription. To identify targets of p53 transactivation, we examined the p53 promoter itself. Northern (RNA) analysis and transient transfection experiments showed that p53 transcriptionally regulated itself. A functionally inactive mutant p53 could not regulate the p53 promoter. Deletion analysis of the p53 promoter delineated sequences between +22 and +67 as being critical for regulation. Electrophoretic mobility shift analysis and methylation interference pinpointed the p53 DNA responsive element. When oligomerized in front of a heterologous minimal promoter, this element was regulated by wild-type p53 and not by mutant p53. Point mutations in the DNA element that eliminated protein-DNA interactions also resulted in a nonresponsive p53 promoter. The DNA element in the p53 promoter responsive to p53 regulation is similar to the p53 consensus sequence. However, we have been unable to detect a direct interaction of p53 with its promoter.

The p53 protein is a potent tumor and growth suppressor. Addition of wild-type p53 to transformation assays dramatically decreases the number of foci formed (16, 20). The overexpression of wild-type p53 by the introduction of p53 expression plasmids into tumor cells lacking a p53 gene is incompatible with cell growth (3, 9, 14, 29). Even in studies using equal amounts of the wild-type and mutant p53 genes, inhibition of growth was comparable to that with the presence of wild-type p53 alone (9). Also indicative of a suppressor function is the fact that numerous human tumors contain mutations, alterations, and/or deletions of the p53 gene (for reviews, see references 7 and 28). The inheritance of a mutant p53 allele is seen in patients with Li-Fraumeni syndrome (33, 52). Tumors develop as a result of loss of the remaining normal p53 allele.

Additional experimental data suggest that p53 functions as a transcriptional activator to suppress transformation (31, 42, 43). Fusion proteins made with wild-type p53 and the DNA-binding domain of the yeast transcription factor GAL4 specifically transactivate a reporter plasmid (19, 40, 43). In transfection experiments, p53 by itself transactivates the muscle creatine kinase promoter (56, 58) and a DNA responsive element (21, 31). Other lines of investigation suggest that p53 may also be able to suppress transcription of some promoters (10, 23, 48). Potential DNA-binding sites for p53 have been identified previously (5, 15, 21, 30, 58).

P53 mutants, some of which are commonly found in various tumors, have lost the ability to suppress transformation, to activate transcription, and to bind DNA (5, 20, 27, 30, 42, 43, 55).

Wild-type p53 function is inactivated in many tumors by various mechanisms in addition to mutation or deletion. DNA viruses encoding proteins that bind p53 can inactivate its function. For example, the adenovirus E1b and simian virus 40 (SV40) T-antigen proteins inactivate p53 transcriptional activity (18, 28b, 57), and the human papilloma viral protein E6 quickly degrades p53 (50). A cellular gene *mdm-2*, which is amplified in many sarcomas, also inhibits p53 transactivation (36, 39). These various mechanisms have evolved to inhibit the suppression function of p53 by inhibiting its transactivation ability.

Thus, a direct correlation exists between transcriptional activation by wild-type p53 and its ability to suppress transformation. An important question that remains to be addressed in order to understand the role of p53 in tumor suppression is what targets are transactivated by p53. Numerous oncogenes that function as transcription factors autoregulate themselves. The c-Myc and Fos oncoproteins down regulate their respective promoters (41, 49), while products of the *jun* and *myb* oncogenes positively autoregulate their promoters (1, 38). Therefore, we chose to analyze the ability of p53 to affect its own promoter. In this report, we present data that wild-type p53 activates its own promoter, whereas a p53 mutant that cannot suppress transformation and cannot activate transcription as a p53/GAL4 fusion protein also cannot activate the p53 promoter. Deletion constructs at the 5′ end of the p53 promoter were made and used to localize the regulatory sequence. Electrophoretic mobility shift assays (EMSA) and methylation interference experiments were used to pinpoint the exact nucleotides involved in this regulation. Multimerization of an oligonucleotide containing this site in front of a minimal promoter was regulated by wild-type p53, while mutation of this site resulted in loss of regulation. We have been unable to detect direct interaction of p53 with its promoter.

MATERIALS AND METHODS

Plasmid constructions. Construction of plasmid p0.7CAT has been described previously (46). Plasmid p53CAT was constructed as follows. Using synthetic oligonucleotides, genomic sequences from −320 to +149 of the murine p53 gene were amplified by polymerase chain reaction (PCR) and cloned upstream of a chloramphenicol acetyltransferase (CAT) gene. The upstream p53 sequences were separated from the CAT gene by 250-bp minimal SV40 promoter/enhancer sequences (32). p53CAT was used to generate two other reporter plasmids. Plasmid pBSp53CAT was constructed by subcloning the *Afl*III-*Nde*I fragment of p53CAT into the *Eco*RV site of Bluescript KS$^+$; it thus contained a convenient restriction site for generating exonuclease III deletion digests of the p53 promoter. Plasmid Sst/Nde was

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obtained by subcloning the *Sst*I-*Nde*I fragment of p53CAT into the *Eco*RV site of Bluescript KS$^+$; it contained an 83-bp deletion of the 5′ end of the p53 promoter. A series of plasmids containing 5′ deletions of the p53 promoter were generated by linearizing pBSp53CAT with *Spe*I, digesting it with exonuclease III, releasing the deleted promoter fragments with *Eco*RI, and subcloning the fragments into a vector derived from a *Bam*HI-*Eco*RI digest of p53CAT. The resulting deletion plasmids were identical to the p53CAT plasmid (except for the p53 promoter sequences digested by exonuclease III). In all cases, the endpoints of the exonuclease III digestions were determined by DNA sequencing. Plasmid p53BHI was constructed by digesting p53CAT with *Bam*HI, which removes the entire p53 upstream element −320 to +149 from p53CAT. pA2CAT was constructed by inserting three copies of an oligonucleotide (+49 to +68) upstream of the SV40 minimal promoter and the CAT gene. pMCAT was generated by PCR-directed mutagenesis with the mutant oligonucleotide described in the text and its complement. The PCR-amplified product was cloned into p53CAT after removal of wild-type p53 promoter sequences.

The wild-type p53 expression plasmid LTRXA has been described previously (26). LTRKH contains a linker insertion at p53 amino acid 215, which alters a Val-Pro dipeptide to Pro-Ser-Leu-Ala (53). It is identical to LTRXA except for the linker insertion mutation. These plasmids contain the SV40 3′ untranslated and poly(A) recognition sequences.

Cell culture, DNA transfection, and CAT assay. NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum at 37°C in a humidified 8% CO_2 incubator. Cells were freshly thawed from a frozen stock and grown for 3 days prior to transfection. Cells were plated at a density of 0.5×10^6 cells per 100-mm-diameter culture plate for 24 h before transfection. The effector and reporter plasmids (10 μg each) and a β-galactosidase (β-gal)-expressing plasmid (5 μg) were cotransfected by calcium phosphate precipitation for 16 h (8). At 40 h after transfection, extracts were prepared from all plates, normalized for β-gal activity, and assayed for CAT activity (24).

DP15 cells were maintained as a suspension culture in alpha minimal essential medium containing 10% fetal bovine serum. Cotransfection of effector and reporter plasmids (10 μg each) and the β-gal plasmid (5 μg) was done by electroporation. Cells were harvested 2 to 3 days after electroporation, and the extracts were prepared for β-gal and CAT assays as described above.

RNA extraction and Northern blot analysis. Total RNA was extracted from transfected cells (11). Poly(A)$^+$ enrichment was performed by batch chromatography of total RNA over oligo(dT) cellulose (34). RNA was denatured with formaldehyde-formamide and size fractionated on a 1% agarose with formaldehyde gel in borate-EDTA buffer. Blotting of RNA to a Zeta membrane (Bio-Rad) was done by standard procedures (34). Prehybridization, hybridization, and posthybridization washes were done as described previously (12). Blots were sequentially hybridized with an SV40 3′ probe, a p53 probe, and a mouse β-actin probe. The relative intensity of RNA signals on the Northern (RNA) blots was estimated by densitometry.

EMSA and methylation interference. The *Alu*I fragment (+19 to +99) of the p53 promoter was inserted into the *Sma*I site of pBluescript. For the EMSA probe, the *Xba*I-*Xho*I fragment of this plasmid was end labeled with Klenow fragment and [α-^{32}P]dCTP. EMSA were performed with 1 fmol of labeled probe, 2 μg of nuclear extract, and the following buffer: 40 mM NaCl, 10 mM MOPS (morpholinepropanesulfonic acid [pH 7.0]), 1% Nonidet P-40, 1 mM EDTA, 2.5% glycerol, and 0.5 μg of poly(dIdC). The wild-type oligonucleotide (+49 to +70), the mutant oligonucleotide 5′ TGGGATTATCACTTTAAGCTCC 3′ (mutated nucleotides are underlined), and their complements were synthesized and used in competition experiments. The p53 con oligonucleotide and gel shift conditions used with this oligonucleotide were identical to those described previously (21).

Nuclear extracts were prepared from F9 cells as described by Dignam et al. (13), with the addition of phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (5 μg/ml), and pepstatin (5 μg/ml) to all buffers.

Methylation interference was performed essentially as previously described (25), except DNA purified from a gel was prefiltered and concentrated through an Elutip D prefilter and column (Schleicher & Schuell) before piperidine treatment. The same *Alu*I fragment from +19 to +99 of the p53 promoter was used.

RESULTS

Wild-type p53 transactivates its own promoter. Since numerous transcription factors regulate their promoters, we tested the possibility that p53 could also regulate its promoter. Two cell lines were chosen for this analysis: NIH3T3 and DP15 cells. NIH3T3 cells have high levels of p53, whereas DP15 cells make no functional p53 (47). DP15 cells, which arose from a mouse erythroleukemia, are missing one p53 allele and have in the other allele a small 3-kb deletion that removes p53 exons 2 and 3 but leaves the p53 promoter intact. This deletion produces a 44-kDa p53 protein missing the first 40 amino acids. Since the first 42 amino acids encode a minimal acidic domain required for transactivation, this truncated p53 is most likely nonfunctional (55). In addition, deletion analysis has shown that deletion of amino acids 11 to 33 results in a p53/GAL4 fusion protein that cannot function as a transactivator (28a). Thus, transfections in the DP15 cell line and regulation of p53 RNA levels would be due to the transfected p53 DNA.

Transfections into NIH3T3 and DP15 cells were performed by using a wild-type p53 expression plasmid, LTRXA, and a mutant p53 expression plasmid, LTRKH (Fig. 1A). These two plasmids contain identical regulatory signals and differ only in the p53 coding sequence. LTRKH encodes a p53 protein with a linker-scanning mutation at amino acid 215 that alters a Val-Pro dipeptide to Pro-Ser-Leu-Ala and that has a longer half-life (53). Previous data have shown that a wild-type p53/GAL4 fusion protein could specifically transactivate a CAT reporter plasmid that contained GAL4-binding sites, while the p53KH215/GAL4 fusion protein containing the linker substitution at amino acid 215 could not activate transcription (43). LTRXA or LTRKH was transfected into NIH3T3 and DP15 cells by calcium phosphate precipitation or electroporation, respectively. Approximately 48 h after transfection, the cells were harvested and RNA was extracted. The results of Northern analysis are shown in Fig. 1B for each cell line. The same Northern blot was sequentially hybridized with an SV40 3′ probe, a p53 cDNA probe, and an actin probe. The SV40 probe was used to distinguish the mRNA made from the transfected p53 expression plasmids and the endogenous p53 mRNA. The transfected p53 plasmids did not express detectable levels of p53 mRNA (data not shown). From densitometric scanning of Northern blots and normalization against actin mRNA levels, transfection of wild-type p53 in

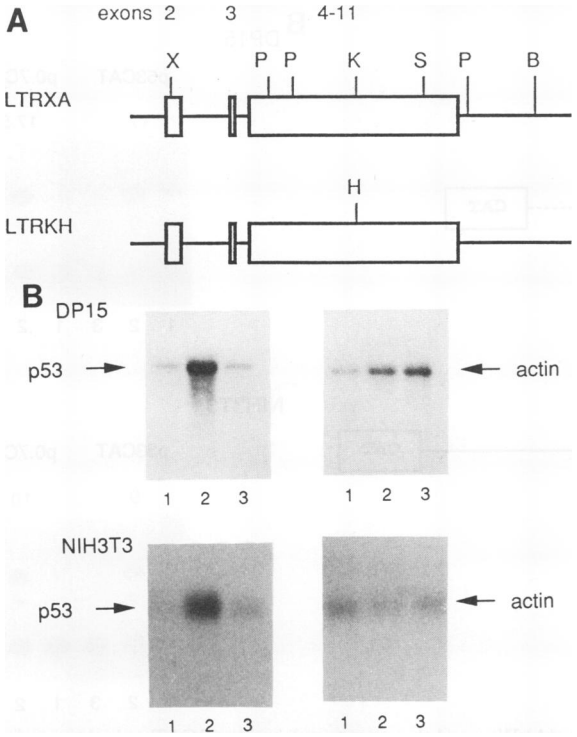


FIG. 1. Northern analysis of p53 transfected cells. (A) LTRXA and LTRKH encode wild-type and mutant p53, respectively. X, *Xho*I; P, *Pst*I; K, *Kpn*I; S, *Sac*I; B, *Bam*HI; H, *Hin*dIII. (B) DP15 and NIH3T3 cells were transfected with plasmid pBR322 (lane 1), wild-type p53 expression plasmid LTRXA (lane 2), or mutant p53 expression plasmid LTRKH (lane 3). β-Gal was used to monitor transfection efficiency. RNA was isolated 48 h after transfection, fractionated on formaldehyde gels, and blotted. Filters were hybridized sequentially with p53 and actin probes.

both NIH3T3 and DP15 cells (lane 2) transactivated the endogenous p53 gene three- to fivefold. Transfections with control plasmid DNA (lane 1) or mutant p53 (lane 3) had no effect on endogenous p53 expression.

To determine the sequence involved in p53 regulation, we established a transient transfection assay and examined the ability of wild-type p53 to regulate two different p53 promoter constructs. The plasmid p0.7CAT contains an *Eco*RI-*Hin*dIII fragment of approximately 0.7 kb, which encompasses 5′ p53 promoter elements, a noncoding exon 1, and 132 bp of intron 1 (Fig. 2A). The p53 promoter does not contain CAAT or TATA sequences (6). Three transcription start sites (+1, +67, and +105) have been mapped within the p53 promoter, the site at +105 being the most frequently used (6, 54). In addition, some *cis*-acting sequences that affect p53 expression have been identified within exon 1 (46). The second construct, p53CAT, contains the p53 promoter from −324 to −1, 150 bp of the first p53 exon, and 255 bp of an SV40 minimal promoter (Fig. 2A). Transfections were performed with either wild-type or mutant p53, either CAT reporter described above, and a β-gal plasmid to measure transfection efficiency. In control experiments, the promoter of the β-gal plasmid was not affected by wild-type or mutant p53 expression (data not shown). The results depicted in Fig. 2B show a 9- to 17-fold increase in the ability of wild-type p53 to transactivate either p53 promoter CAT construct in both NIH3T3 and DP15 cells compared with that of mutant p53.

Deletion analysis of the p53 promoter. Since transactivation of the p53 promoter by wild-type but not mutant p53 was comparable to either p53 promoter CAT construct in either cell line, we continued our experiments using p53CAT and NIH3T3 cells. By deletion analysis using exonuclease III or convenient restriction sites, we were able to generate a set of nested deletions in the p53 promoter (Fig. 3A). Sequence analysis was used to determine the exact endpoints of each deletion construct. These reporter plasmids were individually transfected with β-gal and pBR322, wild-type p53 (LTRXA), or mutant p53 (LTRKH) plasmids. Equal amounts of β-gal from transfection experiments were used to determine CAT activity. The results (Fig. 3B) show that clone Exo71 contained the shortest promoter sequence still responsive to regulation by wild-type p53; clone Exo73 was no longer regulated. These constructs delineate p53 responsive sequences to be between +22 and +67. Larger deletions, including one that deleted all of the p53 promoter and left only the SV40 sequence, were not regulated by p53. Each of these deletion constructs was tested a minimum of three times in duplicate. In all cases, a nonfunctional mutant p53 could not activate this promoter. Other promoters tested that were not regulated by p53 in our system include the adenovirus major late promoter, the retinoblastoma promoter, and the α1(I) collagen promoter (data not shown). Thus, we concluded that the activation of the p53 promoter by wild-type p53 does not have a general effect on transcription.

Identification of the p53 DNA responsive element. To further analyze the sequences involved in p53 regulation, we performed EMSA followed by methylation interference experiments. For both, we purified and end labeled an *Alu*I fragment of the p53 promoter from +19 to +99, which spans promoter deletion constructs Exo71 and Exo73 (Fig. 3A). F9 embryonal carcinoma cells were used as a source for nuclear extracts since F9 cells make wild-type p53 (26). Upon gel shift, the *Alu*I fragment resulted in a shift of two specific bands, labeled complex 1 and complex 2 (Fig. 4). Both bands were subsequently subjected to methylation interference. Complex 1 (Fig. 5A, lane 1) showed protection of two G nucleotides at positions +74 and +76 compared with that with the free probe (lane 3; the sequence of the fragment run alongside is not shown). As indicated in Fig. 5B, these G nucleotides are retained within deletion construct Exo73, and since Exo73 has lost the ability to be regulated, complex 1 is probably not involved in regulation by p53. These G nucleotides also correspond to an upstream stimulatory factor (USF)-binding site previously identified (46). Complex 1 was supershifted by using a USF antibody (44, and our data not shown). This binding was not investigated further. Methylation interference analysis of complex 2 showed the protection of several G nucleotides on both strands (Fig. 5A, lanes 2 and 4). These protected G nucleotides fall between the promoter deletion constructs Exo71 and Exo73 (Fig. 5B). The methylation pattern seen is identical to that of NF-κB, and the 11-bp sequence spanned by these protected nucleotides is GGGACTTTCCC, the consensus NF-κB-binding site (2). This DNA element also fits the p53 consensus binding site defined by El-Deiry et al. (15).

Mutational analysis of the p53 DNA responsive element. Thus, the in vitro data suggested that the NF-κB motif was the p53 DNA responsive element. We next sought to establish by in vivo data that regulation of p53 does indeed occur through this element. First, we made two complementary oligonucleotides from +49 to +68, spanning the sequence of interest. Two additional complementary oligonucleotides

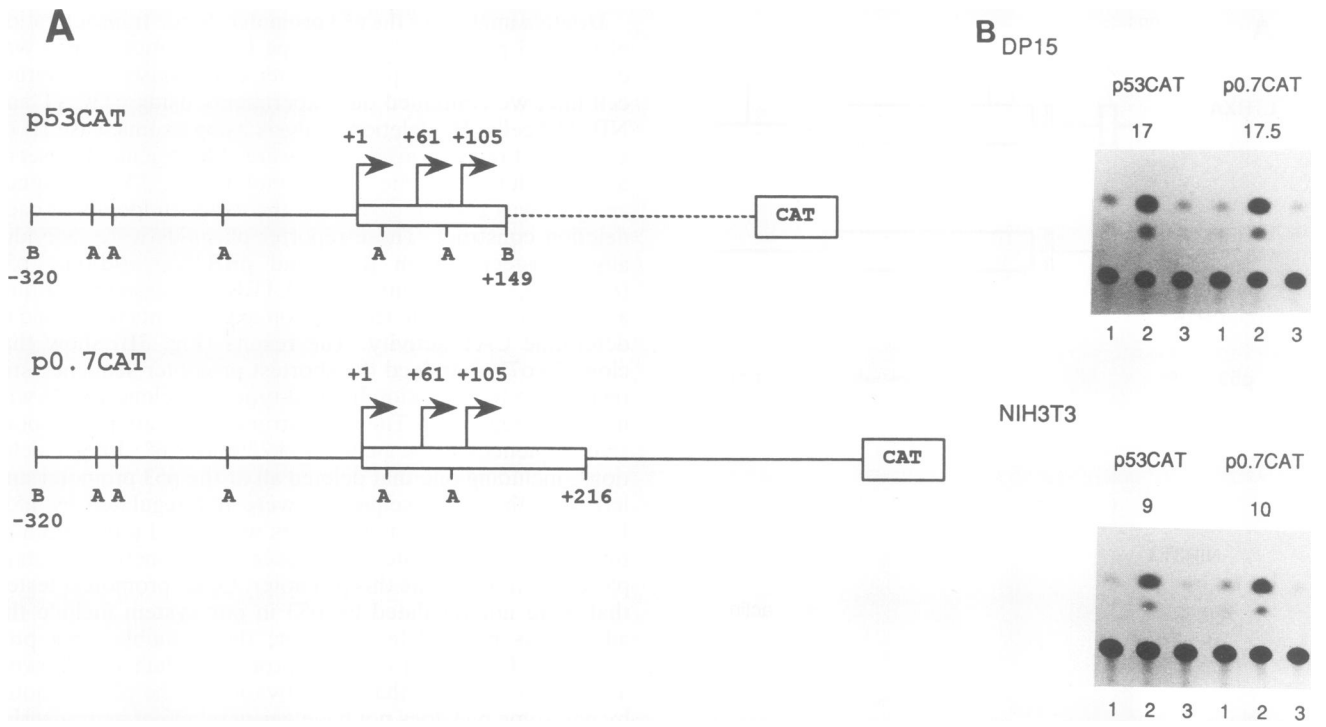


FIG. 2. Regulation of the p53 promoter by wild-type but not mutant p53. (A) We used the reporter plasmid p53CAT, which contains 320 bp of the p53 promoter and 149 bp of the first p53 noncoding exon (box) attached to a minimal SV40 promoter (dotted line). p0.7CAT contains 0.7 kb of the p53 promoter, including 320 bp of the promoter, the 216-bp first p53-noncoding exon, and 132 bp of intron 1. Arrows at positions +1, +61, +105 indicate transcription start sites. B, *Bam*HI; A, *Alu*I. (B) DP15 and NIH3T3 cells were cotransfected with effector plasmids encoding wild-type (lane 2) or mutant (lane 3) p53 and reporter plasmid p53CAT or p0.7CAT. Lane 1 was transfected with pBR322 as a control. The numbers above the CAT assays denote fold activation of wild-type p53 versus that of mutant p53.

were made with mutations in the six G nucleotides that contacted protein as determined by methylation interference. Both sets of annealed oligonucleotides were used as competitors in EMSA with F9 nuclear extracts (Fig. 6). The wild-type oligonucleotide competed well for binding of complex 2 (Fig. 6, lanes 3 and 4). The mutant oligonucleotide did not compete (Fig. 6, lanes 5 and 6). Neither the wild-type nor the mutant oligonucleotide competed for binding of complex 1. This mutant oligonucleotide also did not bind protein when labeled and subjected to EMSA (data not shown). Thus, we had made mutations that completely and specifically eliminated protein binding to the sequence of interest.

To further support our initial observation that p53 regulation occurred through the sequence GGGACTTTCCC, several in vivo experiments were performed. First, three copies of the wild-type oligonucleotide containing this sequence were inserted 5′ of the SV40 minimal promoter (plasmid pA2CAT). Transfection experiments were performed by using either p53CAT or pA2CAT and wild-type or mutant p53 expression vectors. Both CAT reporter plasmids showed comparable activation by wild-type p53 but not by mutant p53 (Fig. 7). In order to assay for the loss of regulation, the p53 DNA responsive element was mutated by using PCR-directed mutagenesis to ATCACTTTAAG, the same mutation analyzed above in EMSA, and reinserted into p53CAT. Sequence analysis of the PCR-amplified DNA showed that no additional mutations had been incorporated during the amplification reaction. The p53CAT construct containing mutations in the p53 DNA responsive element (pMCAT) was not transactivated by wild-type p53 (Fig. 7). We thus conclude that p53 regulation of the p53 promoter occurs minimally through a sequence that contains the NF-κB motif.

Analysis of p53 binding to the p53 promoter. Since the NF-κB sequence in the p53 promoter was responsive to regulation by p53, we tested whether p53 bound directly to this DNA sequence, using EMSA. As a control we used an oligonucleotide, p53con, previously shown to bind p53 in nuclear extracts (21). p53con was end labeled and used as a probe with nuclear extracts from NIH3T3 cells, from cells from p53 null mice (24a), and from F9 cells. The p53 specific monoclonal antibody PAb421 was used to detect p53 in the gel-shifted complexes. By using the p53con oligonucleotide, a specific complex was detected in extracts from NIH3T3 cells and F9 cells. Furthermore, the addition of a p53 antibody caused a supershift of the bands similar to that described in previously published results (21). Extracts made from cells from p53 null mice did not exhibit a complex. Using this same assay, we were unable to detect a direct interaction between p53 and the DNA element in the p53 promoter responsive to p53 regulation (data not shown). Comparison of the DNA sequences responsive to p53 is shown in Fig. 8. The differences seen between the p53 promoter element and the other p53 responsive elements may account for our inability to detect DNA binding.

DISCUSSION

The p53 DNA responsive element. In a series of experiments using deletion analysis, EMSA, and methylation interference, we identified a sequence in the p53 promoter that is responsive to expression of wild-type but not mutant

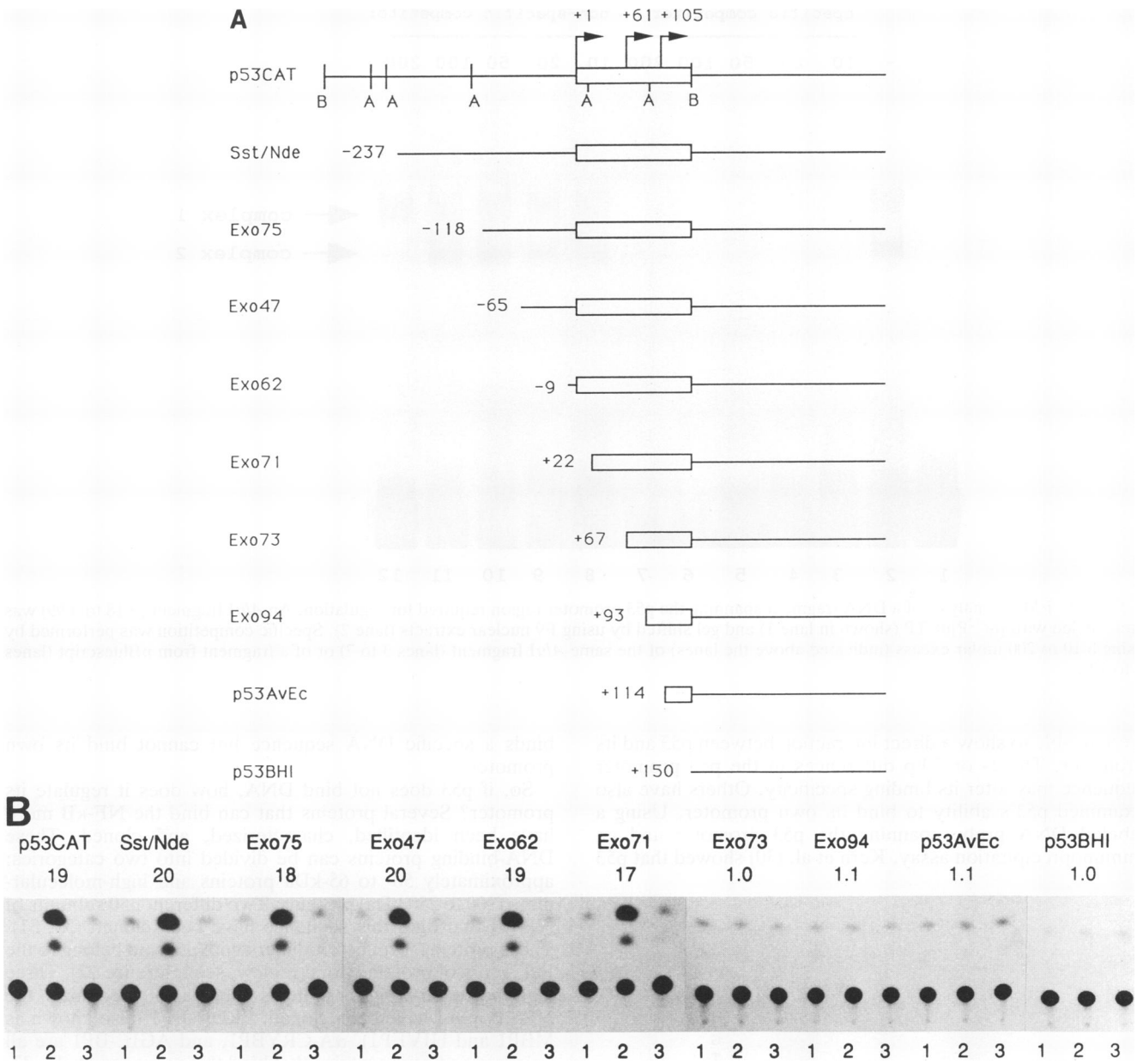


FIG. 3. Analysis of p53 promoter deletion constructs for regulation by p53. (A) Deletion constructs were made by using exonuclease III or restriction endonuclease digestion and sequenced to determine the endpoints. Numbers denote the positions of nucleotides with respect to +1, the first transcriptional start site. (B) p53 promoter deletion CAT constructs were cotransfected with pBR322 (lane 1), wild-type p53 (lane 2), or mutant p53 (lane 3) into NIH3T3 cells. Transfected cells were harvested, and the CAT assays were performed. The numbers above the CAT assays denote fold activation of wild-type p53 (lane 2) versus that of mutant p53 (lane 3).

p53. In vivo and in vitro data suggested that the NF-κB motif was the p53 DNA responsive element. Initial experiments using deletion analysis of the p53 promoter mapped the p53 responsive sequence between +22 and +67, which includes the NF-κB motif. EMSA and methylation interference with nuclear extract from F9 cells indicated that a protein-DNA interaction that spanned the NF-κB motif existed. Further, in vivo experiments proved that the NF-κB sequence was the p53 DNA responsive element. Concatamerization of a 20-bp oligonucleotide containing the NF-κB sequence in front of a heterologous promoter resulted in p53 responsiveness. In addition, mutation of several nucleotides in the NF-κB sequence yielded a DNA fragment that could not compete for binding in EMSA and, when inserted into the p53 promoter, led to a p53 nonresponsive regulatory sequence.

The NF-κB sequence in the p53 promoter regulated by p53 and identified in this study is also a consensus p53-binding site at 10 of 11 nucleotides, as defined by El-Deiry et al. (15) using random oligonucleotide binding to baculovirus-purified p53 and PCR amplification (Fig. 8). In addition, Funk et al. (21) isolated a similar p53 consensus sequence which was shown to bind p53 in nuclear extracts. We, therefore, examined whether p53 could directly bind this sequence but

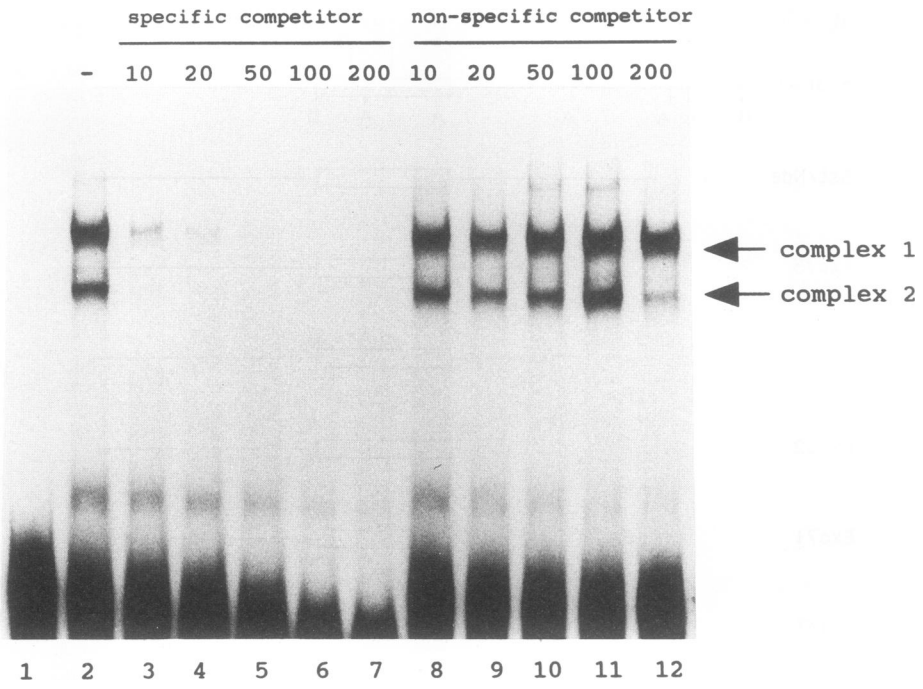
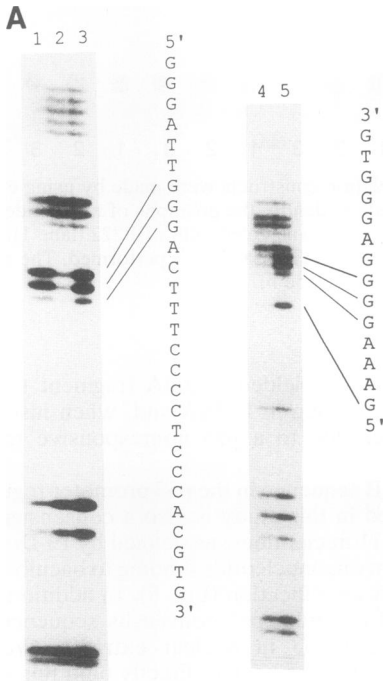


FIG. 4. EMSA analysis of a DNA fragment spanning the p53 promoter region required for regulation. An *Alu*I fragment (+18 to +99) was end labeled with [α-^{32}P]dCTP (shown in lane 1) and gel shifted by using F9 nuclear extracts (lane 2). Specific competition was performed by using a 10 to 200 molar excess (indicated above the lanes) of the same *Alu*I fragment (lanes 3 to 7) or of a fragment from pBluescript (lanes 8 to 12).

were unable to show a direct interaction between p53 and its promoter. The 1- or 2-bp differences in the p53 promoter sequence may alter its binding specificity. Others have also examined p53's ability to bind its own promoter. Using a labeled DNA probe spanning the p53 promoter and an immunoprecipitation assay, Kern et al. (30) showed that p53 binds a specific DNA sequence but cannot bind its own promoter.

So, if p53 does not bind DNA, how does it regulate its promoter? Several proteins that can bind the NF-κB motif have been identified, characterized, and cloned. These DNA-binding proteins can be divided into two categories: approximately 50- to 65-kDa proteins and high-molecular-mass (200 to 250 kDa) proteins. Two different p50 subunits of NF-κB that bind this sequence have been cloned (35, 51). These proteins form heterodimers with p65 and belong to the Rel family of proteins (for a review, see reference 22). Three high-molecular-weight proteins that also recognize the NF-κB motif have been cloned: PRDII-BF1 (also known as MBP1 and HIVEP1), αA-CRYBP1, and AGIE-BP1 are all unique zinc finger proteins that bind this sequence (4, 17, 37,



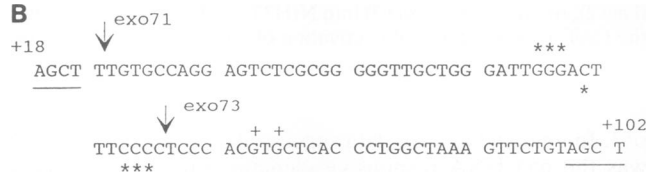


FIG. 5. Methylation interference of the *Alu*I fragment of the p53 promoter. (A) Methylation interference was performed by using complex 1 (lane 1)- and complex 2 (lanes 2 and 4)-shifted bands (from Fig. 4) and free probe (lanes 3 and 5). Lanes: 1 to 3, 3′ end labeled; 4 and 5, 5′ end labeled. Sequence reactions of the same fragment were run alongside (data not shown). (B) Sequence of the *Alu*I fragment used in gel shift and methylation interference experiments. Underlined sequences represent the *Alu*I restriction sites. Arrows indicate the 5′ ends of promoter deletion constructs Exo71 and Exo73. Asterisks and plus signs denote the protected nucleotides on each strand.

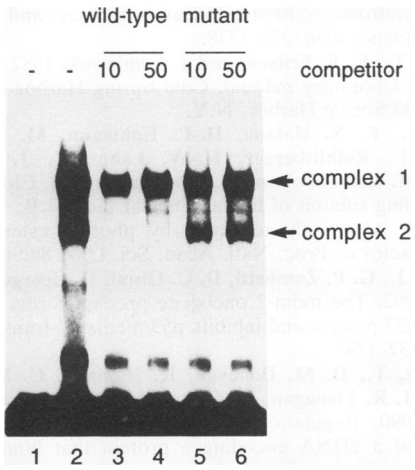


FIG. 6. Mutant oligonucleotides cannot compete for binding. Nuclear extracts were prepared from F9 embryonal carcinoma cells and mixed with the *Alu*I probe from +19 to +99. Lanes: 1, probe alone; 2, probe and F9 nuclear extract; 3 and 4, competition with wild-type oligonucleotide at 10- or 50-fold molar excess (as indicated above each lane); 5 and 6, competition with mutant oligonucleotide at 10- or 50-fold molar excess (as indicated above the lane).

45). Thus, it is possible that p53 transactivates one or more of these transcription factors to regulate its promoter. In addition, it is plausible that p53 interacts with one of these factors to bind the NF-κB sequence, but we have been unable to detect that interaction. p53 is already known to bind another zinc finger protein, mdm-2 (36).

Although very useful in identifying the p53 DNA responsive element, the protein in complex 2 in F9 nuclear extracts may not be the protein involved in regulation by p53. The formation of a complex in p53-negative cells upon transfection with wild-type p53 would be a better indicator of the proteins involved in regulation by p53. The intriguing possibility that p53 activates any one of the NF-κB-binding proteins or that p53 interacts with various proteins to activate transcription would help to explain its global role in tumor suppression. Further experiments will be performed to identify the proteins involved in p53 regulation of its own promoter.

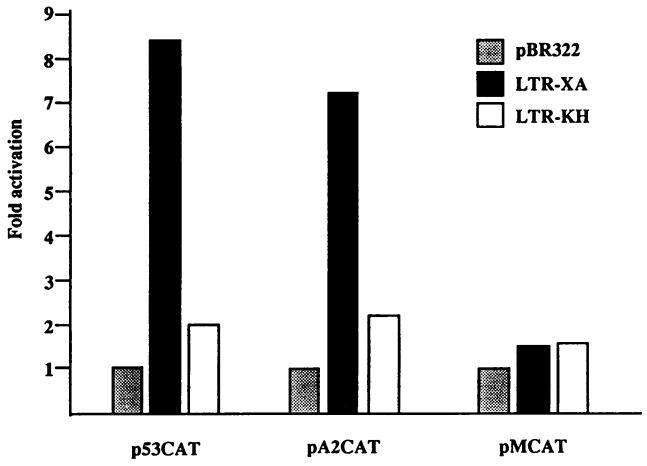


FIG. 7. Mutational analysis of the p53 DNA responsive element. NIH3T3 cells were cotransfected with pBR322, LTRXA (wild-type p53), or LTRKH (mutant p53) and one of the following plasmids: p53CAT, pA2CAT (contains three copies of the p53 DNA responsive element), or pMCAT (contains a mutation of the p53 DNA responsive element). The CAT activity of the control pBR22 transfections was arbitrarily set at 1.0. These data are a compilation of the results of three experiments performed in duplicate.

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p53con          GGACATGCCCGGGCATGTC
                ||||  |  |||
p53promoter     GGGACTTTCCCCTCCCACGT
                |||||||  |||
El-Deiry        RRRRCATGYYY
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FIG. 8. Comparison of p53 responsive elements. The sequences of two oligonucleotides identified as p53 responsive elements (15, 21) were compared with the DNA sequence in the p53 promoter responsive to p53 regulation. Vertical bars denote sequence identity.

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