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Reciprocal Interference between the Sequence-Specific Core and Nonspecific C-Terminal DNA Binding Domains of p53: Implications for Regulation

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The tumor suppressor p53 has two DNA binding domains: a central sequence-specific domain and a C-terminal sequence-independent domain. Here, we show that binding of large but not small DNAs by the C terminus of p53 negatively regulates sequence-specific DNA binding by the central domain. Four previously described mechanisms for activation of specific DNA binding operate by blocking negative regulation. Deletion of the C terminus of p53 activates specific DNA binding only in the presence of large DNA. Three activator molecules (a small nucleic acid, a monoclonal antibody against the p53 C terminus, and a C-terminal peptide of p53) stimulate sequence-specific DNA binding only in the presence of both large DNA and p53 with an intact C terminus. Our findings argue that interactions of the C terminus of p53 with genomic DNA in vivo would prevent p53 binding to specific promoters and that cellular mechanisms to block C-terminal DNA binding would be required.

The tumor suppressor p53 is a transcription factor that controls genes important in the cellular response to a variety of stresses (7, 13, 16). Aberrant growth signals by oncogenes, DNA-damaging agents, and perhaps additional environmental stimuli result in the accumulation of p53 and the transactivation of p53 responsive genes. Transactivation in turn contributes either to the arrest of the cell cycle or to apoptosis depending on the cell type and the nature and extent of cellular stress. Failure of either of these protective mechanisms increases the incidence and severity of cancer. Although p53 may have functions in addition to its transactivation function, sequence-specific DNA binding and transactivation by p53 present logical targets for the regulation of p53.

We and others showed previously that p53 has two autonomous DNA binding domains (2, 8, 18, 24). A large, central core domain of p53 binds a 20-bp recognition sequence in preference to other DNAs (6, 12). Similarly, the C terminus of p53 binds to DNA in the absence of the central DNA binding domain (24). While the C-terminal domain binds DNA without known sequence specificity, it apparently binds preferentially to certain structural features of DNA such as DNA ends (21), DNA distortions (15), and modifications of DNA caused by gamma irradiation (20). Although these preferences have suggested that the C terminus of p53 participates directly or indirectly in the response of p53 to DNA damage, the biological significance of C-terminal DNA binding remains to be demonstrated.

The interaction of the C-terminal domain of p53 with a variety of molecules has been implicated in the regulation of sequence-specific DNA binding by the central domain. Negative regulation of specific binding was first reported by Hupp et al. (9), who showed that deletion of the C terminus enhanced specific binding as quantitated by gel mobility shift assays. They further showed that monoclonal antibody PAb421, DnaK protein, and casein kinase II could activate specific DNA binding by wild-type (WT) p53. A number of investigators have shown that an isolated C terminus of p53 itself and peptides derived therefrom activate specific DNA binding by p53 (1, 10, 11). Other work has implicated nucleic acids in the regulation of specific DNA binding by the C terminus of p53. Bayle et al. (3) reported that in the presence of poly(dI-dC), WT p53 binds specific DNA only in the presence of PAb421. Jayaraman and Prives (11) reported that short single strands of DNA stimulated p53 binding while larger DNAs inhibited DNA binding. Many investigators have attributed the regulation of sequence-specific DNA binding to allosteric changes in p53 (7, 13, 16).

Although Bayle et al. presented evidence that poly(dI-dC) can interfere with specific DNA binding by p53, the mechanisms underlying interference remain to be defined. Furthermore, many investigators continue to study sequence-specific DNA binding in the presence of large nonspecific DNAs. In the present study, we show that large but not small DNAs strongly interfere with specific DNA binding. Interestingly, four distinct mechanisms previously reported to activate DNA binding do so primarily by interfering with negative regulation by large DNAs. Our findings provide a simple, unifying explanation for both negative and positive regulation of p53 by a variety of molecules.

MATERIALS AND METHODS

Expression vectors. We have described the construction of baculovirus vectors for the expression of p53 or segments of p53 in insect cells (19). The vectors used in the present study overproduce p53s with N-terminal tags encoding 27 amino acids (MAYPYDVPDYAARHHHHHHARRASVGV). The tags include the hemagglutinin epitope for immunological identification and six histidines for purification by metal affinity chromatography. The full-length p53 was derived from the wild-type human p53 described by Friedman et al. (5). All p53 segments were derived from the wild-type p53. All DNAs encoding p53s in expression plasmids were verified by sequencing.

Purification of p53. Human and mouse p53s, identified by their amino acid numbers, were expressed by infecting Sf9 insect cells with recombinant baculovirus as previously described (24). The p53s were purified by metal chromatography, dialyzed against 20 mM Tris-HCl (pH 8.0)–100 mM NaCl–50% glycerol–1 mM β-mercaptoethanol overnight at 4°C, and stored in aliquots at −70°C. Purified proteins were quantitated by Coomassie blue staining following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14). Purified p53 segments were the only bands seen.

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Nucleic acids. We prepared double-stranded DNA probes containing a single 20-bp p53 recognition sequence within a 29-bp sequence as previously described (24). Equal amounts of the complementary synthetic oligonucleotides 5′-ctaga AGACATGCCTAGACATGCCTta-3′/3′-tTCTGTACGGATCTGTACGGAat gc-5′ (lowercase letters represent nonconsensus nucleotides) were mixed in 20 mM Tris (pH 7.4)–2 mM $MgCl_2$–50 mM NaCl at 97°C for 5 min and cooled slowly to room temperature. The annealed oligonucleotides were diluted to 1 μg/μl in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA).

A variety of nucleic acids were used for competition studies. We prepared a 29-bp double-stranded DNA without a p53 recognition site by using the complementary oligonucleotides 5′-ctagaatgaattaatatgaattaatta-3′/3′-ttacttaattatactt aattaatgc-5′ as described above. Phenylalanine-tRNA purified from yeast (Sigma, St. Louis, Mo.) consisted of 70 nucleotides in a single native conformation. Circular pBluescript KS (Stratagene, La Jolla, Calif.), consisting of 2,964 bp, was purified by CsCl gradient centrifugation. Poly(dI-dC) · poly(dI-dC) had an average length of 4,900 bp (Pharmacia Biotech, Piscataway, N.J.) or of 30 bp (prepared from synthetic oligonucleotides as described above). Lambda phage DNAs were obtained from BioVentures, Inc. (Murfreesboro, Tenn.). The results of quantitative analyses of nucleic acids by UV spectroscopy and by silver staining (Pharmacia Biotech) of specific nucleic acid species in gels were in good agreement.

Gel shift assay for DNA binding by p53. Specific, 29-bp DNA probes were end labeled with [^{32}P]dCTP by using the Klenow fragment of DNA polymerase I and were ethanol precipitated as previously described (24). Human wild-type p53 (50 ng) or autonomous domains of p53 were mixed with specific, 29-bp radiolabeled DNA probes (2 ng) in a final volume of 20 μl of 25 mM HEPES (pH 7.4)–50 mM KCl–20% glycerol–0.1% Nonidet P-40–1 mM dithiothreitol–1 mg of bovine serum albumin per ml. In complex gel shift assays, components were added in the following order: buffer; interfering, large, nonspecific DNAs; competitor (specific or nonspecific) small DNAs; activating molecules (tRNA, PAb421, or the C terminus of p53); p53; and radiolabeled, sequence-specific, small probe DNA. After incubation at room temperature for 30 min, the samples were analyzed in 4% polyacrylamide gels electrophoresed at 200 V in 0.3× Tris-borate-EDTA buffer at room temperature for 45 min.

Electroblotting of p53 and DNA. Free p53, p53-DNA complexes, and free DNAs from each gel shift analysis were transferred by the semidry blotting method with a Graphite Electroblotter II (Millipore) onto two stacked membranes which differentially bound proteins and DNA (4). The first, a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.), bound proteins but not DNA, and the second, a DE-81 membrane (Whatman, Maidstone, England), bound DNA quantitatively. p53 oligomers were identified by enhanced chemiluminescence (Pierce, Rockford, Ill.) with 12CA5 monoclonal antibody against the hemagglutinin epitope (Santa Cruz Biochemicals, Santa Cruz, Calif.) in the p53 tags, and the DNA was detected by autoradiography. The nitrocellulose and DE-81 membranes were processed to avoid changes in size. Protein and DNA images on separate X-ray films could therefore be compared directly by superimposing the two films.

RESULTS

Purification and quantitation of p53. We wished to analyze the intrinsic DNA binding properties of human p53 by using purified reagents to avoid complications inherent to crude extracts which would contain both nucleic acids and DNA binding proteins. For this purpose, we purified histidine-tagged, human WT p53 and isolated segments of p53 (Fig. 1A) by metal affinity chromatography. WT p53 has two autonomous DNA binding domains separated by a tetramerization domain (23, 24). p53(1–355), consisting of amino acids 1 to 355, includes the sequence-specific or core DNA binding domain and the tetramerization domain but not the C-terminal, basic segment of p53 implicated in the regulation of specific DNA binding. p53(283–393) includes the tetramerization domain and the basic C terminus of p53 which binds DNA without known sequence specificity. Figure 1B shows 1, 2, and 4 μg of each of the three purified proteins analyzed by gel electrophoresis and stained with Coomassie blue. The three p53s were homogeneous, free of visible contamination, and of the appropriate size.

p53 binds DNA via two domains. We compared the binding efficiencies of WT p53, p53(1–355), and p53(283–393) to a 29-bp DNA with a single recognition site for p53 (ds1). To measure both specific and nonspecific DNA binding, we used a gel shift assay in the absence of competitor DNA. The p53s were added to DNA at molar ratios of 2 to 8 tetramers/DNA.

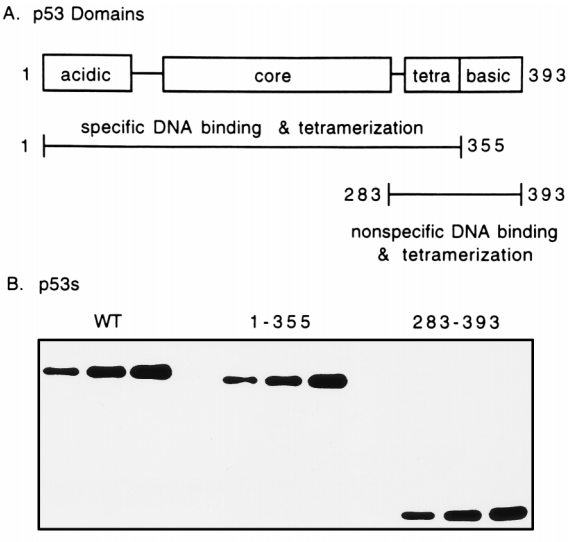

FIG. 1. Quantitation of p53. (A) Domain map of human WT p53, p53(1–355), and p53(283–393). tetra indicates the tetramerization domain. (B) Quantitation of 1, 2, and 4 μg of purified p53s by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining.

The position of p53 after native gel electrophoresis was determined by transfer of the protein to a nitrocellulose membrane and immunoblotting. We found in preliminary experiments that more than 95% of the p53s that entered the gel were transferred from the gel and bound quantitatively to a single nitrocellulose membrane. Radiolabeled DNA electrophoresed through the nitrocellulose membrane and bound quantitatively to a single DE-81 membrane.

The p53 blot in Fig. 2A shows the position of p53 after the gel shift assay. In the absence of DNA, WT p53 either remained at the top of the gel as large complexes or electrophoresed to positions that we previously identified as those of octamers and tetramers in native gels (22, 24). In the presence of DNA, a greater proportion of the WT p53 entered the gel as octamers and tetramers. p53(1–355) formed fewer large complexes than did WT p53 and migrated mostly as octamers and tetramers. p53(283–393) remained at the top of the gel. The failure of this segment to enter the gel could reflect the formation of large complexes, the basic charge of the p53 segment, or both. Because the presence of DNA influenced the entry of WT p53 into gels, we monitored the distribution of p53 in gel shift assays to aid in their interpretation.

The DNA blot in Fig. 2B demonstrates DNA bound to the p53 species shown in Fig. 2A. WT p53 bound DNA as large complexes, octamers, and tetramers. p53(1–355) bound the DNA as octamers and tetramers. WT p53 and p53(1–355) bound similar amounts of specific DNA. The small amount of p53(283–393) at the top of the gel bound little or no DNA as stable complexes. A smear of DNA in the gel and a reduction in the amount of free DNA, however, indicates that p53(283–393) bound DNA transiently and that the DNA was released during electrophoresis. Although the binding was unstable, p53(283–393) bound most of the probe DNA at an input ratio of 8 tetramers/DNA.

We interpret these findings to mean that in the absence of competitor DNA, the C terminus of WT p53 has little negative effect on the DNA binding efficiency of the core domain. That isolated C termini bound DNA at tetramer/DNA ratios only slightly higher than the ratios required for specific DNA bind-

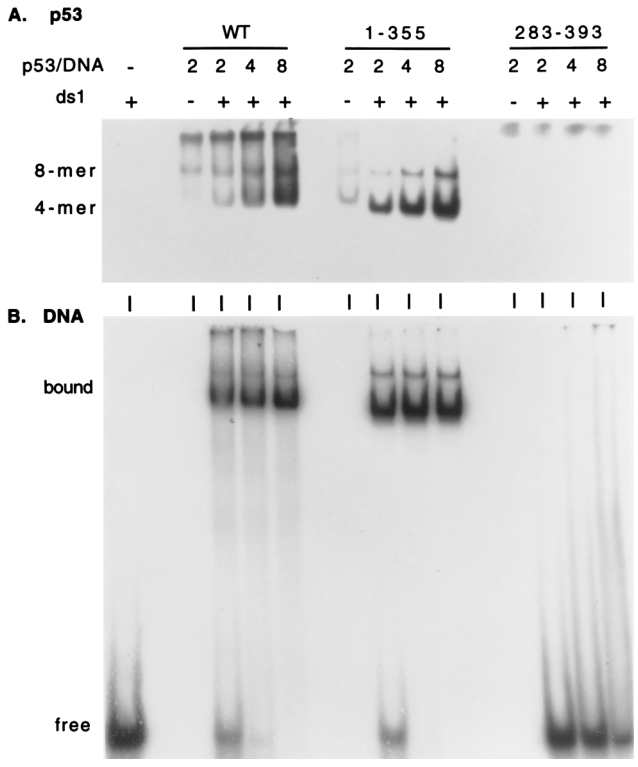

FIG. 2. DNA binding by p53. WT p53 (50, 100, and 200 ng) and equivalent numbers of tetramers of p53(1–355) and p53(283–393) were incubated in the absence and presence of a 29-bp radiolabeled DNA (2 ng) encoding a single p53 recognition sequence (ds1) for 30 min at room temperature. Tetramer/ds1 ratios were approximately 2, 4, and 8. Complexes were analyzed by gel shift analysis and double blotting. (A) p53 immunoblot. (B) DNA blot.

ing argues that DNA binding by the C termini of WT p53 might regulate sequence-specific DNA binding at somewhat higher concentrations of DNA.

Large nucleic acids interfere with specific DNA binding by WT p53. Many investigators include nonspecific nucleic acids in gel shift assays to suppress nonspecific protein-DNA interactions. Thus, we investigated the effects of a variety of nucleic acids on DNA binding by WT p53 (Fig. 3). Because p53 has two DNA binding domains that may have interrelated functions, this investigation was of particular interest.

The p53 blot in Fig. 3A shows the effects of a variety of unlabeled, competitor DNAs on the electrophoretic migration of the p53s. A 20-fold mass excess of unlabeled 29-bp DNA with a single recognition sequence (ds1), 29-bp DNA without a recognition sequence (ds0), one of the two strands in ds0 (ss0), tRNA, or 30-bp poly(dI-dC) · poly(dI-dC) (IC-S) increased the entry of WT p53 into the gel to a modest extent. In contrast, a fivefold mass excess of 2,964-bp pBluescript DNA (pBS), poly(dI-dC) · poly(dI-dC) with an average length of 4,900 bp (IC-L), and lambda phage DNA of 500 and 1,000 bp shifted WT p53 tetramers and octamers to the top of the gel. A 200-bp lambda phage DNA retarded WT p53 significantly but allowed it to enter the gel. None of the competitor nucleic acids had a significant effect on p53(1–355). The difference in the behavior of WT p53 and p53(1–355) in the presence of larger DNAs argues that large DNAs bound the C terminus of WT p53 and thereby interfered with the entry of WT p53 into the gel.

The DNA blot in Fig. 3B shows the effects of a variety of unlabeled nucleic acids on the binding of p53 to the 29-bp radiolabeled, specific DNA. A 20-fold mass excess of unlabeled, 29-bp specific DNA (ds1) competed very well with radiolabeled, specific DNA for binding to WT p53. In contrast, a 20-fold excess of nonspecific 29-bp DNA (ds0), one strand of ds0 (ss0), tRNA, or 30-bp poly(dI-dC) · poly(dI-dC) (IC-S) competed poorly with the radiolabeled, specific DNA for the binding of WT p53. Conversely, none of these nonspecific nucleic acids increased net binding of the specific DNA probe by WT p53. Strikingly, a fivefold mass excess of large pBluescript (pBS), poly(dI-dC) · poly(dI-dC) (IC-L), and the lambda phage DNAs strongly interfered with the binding of WT p53 to specific DNA, even though a 20-fold excess of the smaller nucleic acids did not interfere. In the presence of large DNAs, essentially all of the labeled specific DNA (ds1) electrophoresed in the position of free DNA and did not associate with WT p53. This result is particularly evident for the 200-bp lambda phage DNA that allowed entry of p53 into the gel. When the 200-bp DNA was radiolabeled in a separate experiment, it coelectrophoresed with p53 near the top of the gel (data not shown). These findings indicate that the large, nonspecific DNAs bound WT p53 and blocked binding to specific DNA. In contrast, only specific DNA (ds1) competed with specific DNA binding by p53(1–355). That the larger DNAs did not interfere with the binding of p53(1–355) to specific DNA indicates that the reduction of DNA binding to WT p53 in the presence of large DNAs depends on the C terminus of p53.

Importantly, a comparison of DNA bound to WT p53 and p53(1–355) demonstrates that deletion of the C terminus appeared to activate specific DNA binding strongly only in the presence of the large interfering DNAs. In the absence of large nonspecific nucleic acids, p53(1–355) appears to have bound slightly more specific DNA at the position of tetramers than did WT p53 (compare the first lanes of the two panels in Fig. 3B). Because more p53(1–355) than WT p53 entered the gel (Fig. 3A) as tetramers, the apparent increase in the amount of DNA bound by p53(1–355) tetramers could simply reflect the increased level of assayable p53 rather than an increase in the intrinsic activity of p53. That the amount of free DNA not bound by p53(1–355) and WT p53 is equivalent strongly supports this interpretation.

We conclude that unlabeled nucleic acids can reduce the binding of radiolabeled, specific DNA to p53 by two distinct mechanisms. Specific, unlabeled DNAs bind directly to the core domain of either WT p53 or p53(1–355) and compete with specific, labeled DNA for the core DNA binding domain. We refer to this mechanism as competition for core binding. In contrast, large but not small nonspecific nucleic acids block the binding of WT p53 to specific DNA. Because large DNAs have no effect on p53(1–355), they affect WT p53 via the C-terminal domain. We refer to the antagonism between C-terminal and core DNA binding as interference.

Small nucleic acids block interference by large nucleic acids. The above findings argue that large DNAs bound to the C terminus of p53 interfere strongly with both specific and nonspecific DNA binding by the core domain of WT p53. If small nucleic acids also bind the C terminus, they might be expected to block p53 interference by large nucleic acids. Very large excesses of small, nonspecific nucleic acids, however, would also compete directly with sequence-specific DNA binding by the core domain.

The p53 blot in Fig. 4A shows the effects of tRNA on p53. In the absence of pBluescript, the addition of tRNA increased the entry of p53 into the gel to a modest extent. In the presence of large pBluescript DNA, in a fivefold mass excess relative to the specific DNA probe ds1, p53 did not enter the gel. That in-

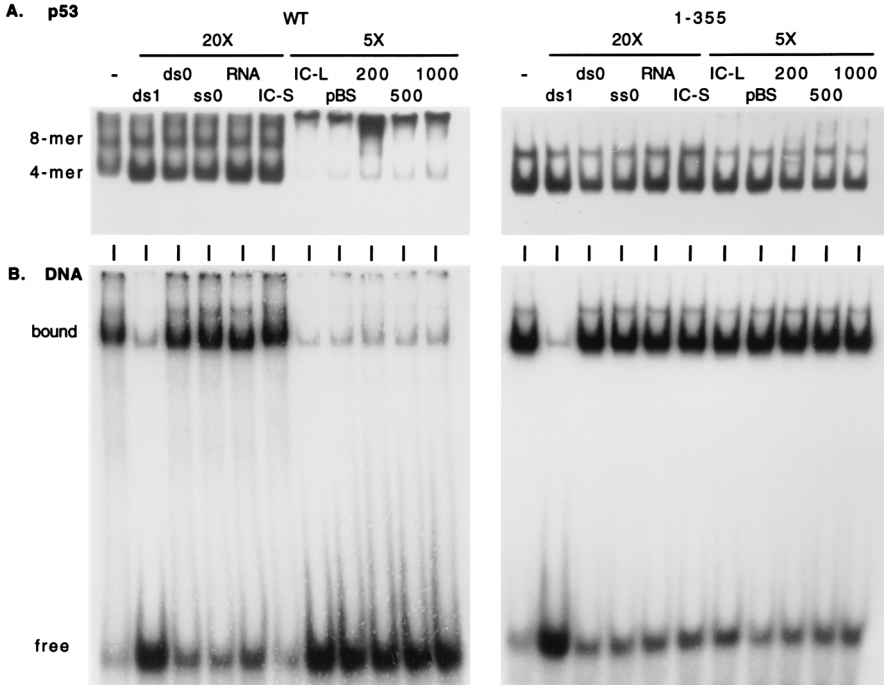

FIG. 3. Effects of nucleic acids on DNA binding by p53. WT p53 (50 ng) and p53(1–355) were incubated with a 29-bp radiolabeled DNA segment (2 ng) encoding a single p53 recognition sequence (ds1) for 30 min at room temperature. The tetramer/ds1 ratios were 2. Unlabeled nucleic acids (40 or 10 ng) were used at a 20-fold (20×) or 5-fold (5×) mass excess over ds1. Complexes were analyzed by gel shift analysis and double blotting. (A) p53 immunoblot. (B) DNA blot. ds0, a 29-bp DNA segment without a p53 recognition sequence; ss0, one strand of ds0; RNA, phenylalanine-tRNA from yeast; IC-S, 30-bp poly(dI-dC) · poly(dI-dC); pBS, 2,946-bp pBluescript plasmid; IC-L is poly(dI-dC) · poly(dI-dC) with an average length of 4,900 bp; 200, 500, and 1,000, lambda phage DNAs of 200, 500, and 1,000 bp, respectively.

creasing amounts of tRNA resulted in the entry of increasing amounts of p53 into the gel indicated that tRNA blocked interactions of pBluescript with WT p53.

The DNA blot in Fig. 4B demonstrates that in the absence of pBluescript, WT p53 bound the specific DNA probe and tRNA did not activate binding to specific probe DNA at any concentration. Indeed, mass excesses of tRNA in the range of 10- to 300-fold gradually reduced binding to the specific probe. Strikingly, a 1,000-fold mass excess of tRNA was required to reduce binding to the specific probe to the same extent as did a fivefold mass excess of 2,946-bp pBluescript DNA. In the presence of pBluescript, essentially all of the labeled specific DNA (ds1) electrophoresed in the position of free DNA and was therefore not associated with WT p53. Furthermore, increasing amounts of tRNA first increased DNA binding to the specific probe DNA and then reduced it. A mass excess of tRNA equal to 300 times ds1 and 60 times pBluescript restored specific DNA binding to the same level as seen under the same conditions in the absence of pBluescript. Thus, correcting for direct competition between tRNA and ds1 in the absence of pBluescript, a 300-fold excess of tRNA completely blocked the interference by pBluescript. Although we show only the results with tRNA and pBluescript, any combination of the small nonspecific nucleic acids and pBluescript or the 200-bp lambda phage DNAs shown in Fig. 3 gave similar results.

Importantly, small nucleic acids activated specific DNA binding only in the presence of large, interfering, nonspecific DNA. That pBluescript interfered with specific DNA binding via an interaction with the C terminus of WT p53 (Fig. 3) argues that the small nucleic acids also interact with the C terminus and thereby block the interference of WT p53 by pBluescript.

PAb421 blocks p53 interference by large nucleic acids. Hupp et al. (9) have shown that the monoclonal antibody PAb421 against the C terminus of p53 activates site-specific DNA binding. Their assays were done in the presence of pBluescript. We have shown above that pBluescript interferes with specific DNA binding via an interaction with the C terminus of p53, and we previously established that PAb421 blocks DNA binding by the C terminus (24). Together, these findings argue that PAb421 may activate specific binding by interfering with the binding of pBluescript to the C terminus rather than by direct stimulation of the protein.

The p53 blot in Fig. 5A shows the effects of pBluescript and of PAb421 on the electrophoresis of p53. In the absence of pBluescript and PAb421, WT p53 electrophoresed as large complexes, octamers, or tetramers. PAb421 reacted with WT p53, modestly enhanced its entry into the gel, and retarded the migration of tetramers and octamers. The addition of large pBluescript DNA prevented the entry of WT p53 into the gel. The addition of both pBluescript and PAb421 allowed migration of p53 to the same position in the gel seen in the presence of PAb421 alone. Thus, PAb421 blocked the effects of pBluescript on p53 electrophoresis. As expected, neither pBluescript nor PAb421 had an effect on the electrophoresis of p53(1–355), which lacks the C-terminal nonspecific DNA binding domain and the PAb421 epitope.

The DNA blot in Fig. 5B shows that WT p53 bound the radiolabeled, 29-bp probe DNA with a p53 recognition sequence. The addition of PAb421 retarded the electrophoresis of the complex and appeared to increase DNA binding modestly. Because more WT p53 tetramers entered the gel in the presence than in the absence of PAb421 (Fig. 5A), the modest increase in bound DNA at the position of tetramers could

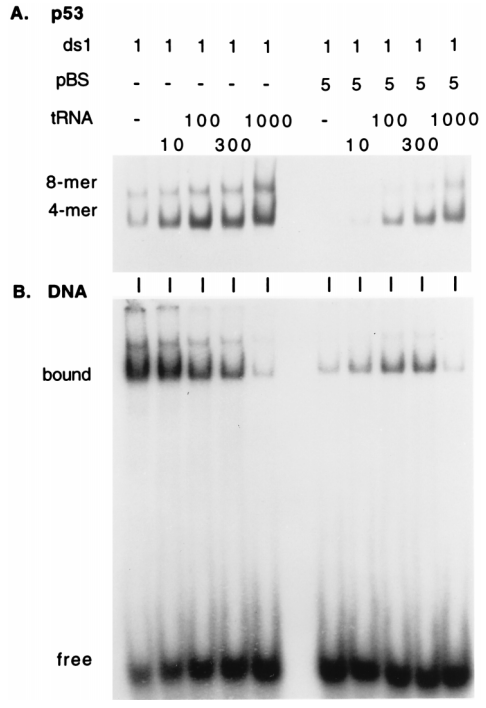

FIG. 4. Opposing effects of large and small nonspecific nucleic acids on the binding of WT p53 to specific DNA. Purified p53 (50 ng) was incubated with a 29-bp radiolabeled DNA segment (2 ng) encoding a single p53 recognition sequence (ds1) for 30 min at room temperature at a tetramer/ds1 ratio of 2. Large pBluescript (10 ng) was used at a 5-fold mass excess over ds1, and small tRNA (20 to 2,000 ng) was used at 10- to 1,000-fold mass excesses over ds1. Complexes were analyzed by gel shift analysis and double blotting. (A) p53 immunoblot. (B) DNA blot. pBS, 2,946-bp pBluescript plasmid; RNA, phenylalanine-tRNA from yeast. The numbers above the gel indicate relative rather than absolute amounts of ds1, pBS, and tRNA.

simply reflect an increased level of assayable p53 rather than an increase in the intrinsic activity of p53. That the amount of free DNA not bound by WT p53 is equivalent in the presence and absence of PAb421 strongly supports this interpretation. As shown in Fig. 3 and 4, pBluescript DNA interfered with the binding of WT p53 to the specific DNA probe; essentially all of the labeled specific DNA (ds1) electrophoresed in the position of free DNA and was therefore not associated with WT p53. The addition of PAb421 along with pBluescript restored the binding of WT p53 to the specific DNA probe. Neither pBluescript nor PAb421 affected the binding of p53(1–355) to specific DNA.

Because both pBluescript and PAb421 interact with the C terminus of p53, we conclude that PAb421 prevented the interaction of the large pBluescript DNA with the C terminus and that PAb421 evidently activates by blocking interference by pBluescript. This conclusion is supported by the finding that PAb421 does not activate specific DNA binding in the absence of pBluescript.

The C terminus of mouse p53 blocks interference by large DNAs. Other investigators (1, 10, 11) have shown that C-terminal peptides of p53 can activate site-specific DNA binding. Their assays were done in the presence of large, nonspecific DNAs and required large excesses of the C-terminal peptides relative to WT p53. Our present findings suggested to us that C termini of p53 may activate by blocking the interfering effects of large DNAs bound to the C terminus of WT p53.

The blot of human p53 with monoclonal antibody 12CA5 in Fig. 6A shows the effects of murine p53(280–390) on the electrophoresis of human WT p53 and p53(1–355). The murine C-terminal peptide does not have a 12CA5 epitope and is not visible in the blot. The C-terminal peptides, present in 12-fold molar excess over the human p53s, modestly increased the amount of WT p53 that entered the gel as octamers and tetramers but had little effect on p53(1–355). This finding suggests that there may be transient interactions between the murine C terminus and the C terminus of human WT p53. The 12CA5 blot in Fig. 6A was reprobed with PAb122. The blot of both murine and human p53s with monoclonal antibody PAb122 in Fig. 6B shows that mouse p53(280–390) enters the gel as a smear and overlaps the position of human WT p53.

The first panel of the DNA blot in Fig. 6C shows that murine p53(280–390), like human p53(283–393) (Fig. 2), bound transiently to DNA but released the DNA gradually during electrophoresis. Furthermore, the mouse C terminus alone bound 100% of the radiolabeled, 29-bp DNA even in the presence of a fivefold mass excess of pBluescript relative to radiolabeled DNA. This finding suggests that 150 ng of mouse p53(280–390) was sufficient to saturate 10 ng of pBluescript DNA which otherwise would have competed with radiolabeled DNA for binding to mouse p53(280–390) (24). The second panel in Fig. 6C shows that WT p53 bound specific DNA and that in the absence of pBluescript, the addition of mouse p53(280–390) appeared to increase modestly the amount of specific DNA bound to WT p53. Because more WT p53 tetramers entered the gel in the presence than in the absence of p53(280–390) (Fig. 6A), the modest increase in bound DNA at the position of tetramers could reflect an increased level of assayable p53 rather than an increase in the intrinsic activity of p53. That the amount of free DNA not bound by WT p53 was similar in the presence and absence of p53(280–390) strongly supports this

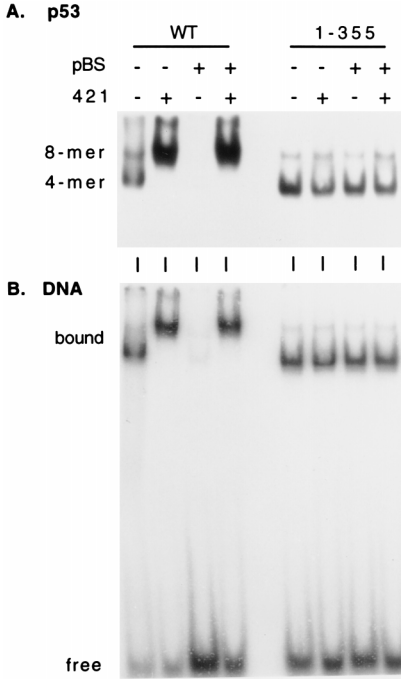

FIG. 5. Opposing effects of PAb421 and large nucleic acids on the binding of WT p53 to specific DNA. Purified WT p53 (50 ng) and p53(1–355) were incubated with a 29-bp radiolabeled DNA segment (2 ng) encoding a single p53 recognition sequence (ds1) for 30 min at room temperature. pBluescript (10 ng) and PAb421 (100 ng) were used as indicated. Complexes were analyzed by gel shift analysis and double blotting. (A) p53 immunoblot. (B) DNA blot. pBS, pBluescript plasmid; 421, monoclonal antibody PAb421.

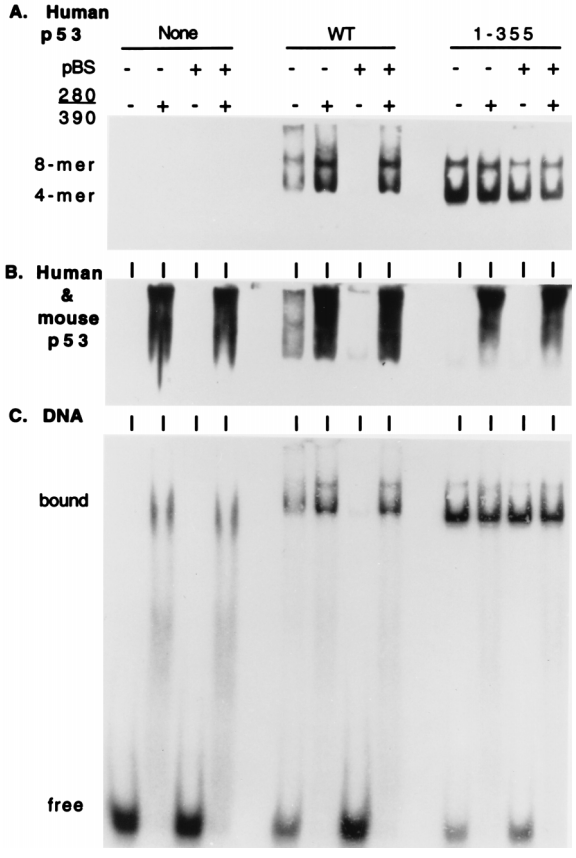

FIG. 6. Opposing effects of mouse p53(280–390) and large nucleic acids on the binding of WT p53 to specific DNA. WTp53 (50 ng) and p53(1–355) were incubated with a 29-bp radiolabeled DNA segment (2 ng) encoding a single p53 recognition sequence (ds1) for 30 min at room temperature at a tetramer/ds1 ratio of 2. pBluescript (10 ng) and mouse p53(280–390) (150 ng) were used as indicated. Complexes were analyzed by gel shift analysis and double blotting. (A) Human p53 immunoblot with 12CA5. (B) Murine p53 and human p53 immunoblot with PAb122. (C) DNA blot.

interpretation. As in previous experiments, pBluescript interfered with specific binding by WT p53; essentially all of the labeled specific DNA (ds1) electrophoresed in the position of free DNA and did not associate with WT p53. In the presence of pBluescript, the C terminus of murine p53 strongly activated specific DNA binding by WT p53. In contrast, the third panel in Fig. 6C shows that mouse p53(280–390) had no significant effect on specific DNA binding by human p53(1–355).

We conclude that the mouse C-terminal fragment activates specific DNA binding primarily by blocking interference by pBluescript of WT p53. That mouse p53(280–390) was present in sufficient quantity (465 tetramers/pBluescript or 1 tetramer/7 bp) to saturate pBluescript argues that pBluescript might no longer be available to bind the C terminus of WT p53 and to interfere with core DNA binding. In addition, isolated mouse C termini may have had transient interactions with intact human p53; such interactions could also have blocked interactions with large, nonspecific DNAs.

Reciprocal interference between the sequence-specific core and nonspecific C-terminal DNA binding domains of p53. The studies described above were done with minimal components to simplify interpretation. Here, we mix multiple components to gain greater insight into the interactions between the sequence-specific and the nonspecific DNA binding domains of p53. The panels in Fig. 7 are blots from a single gel and show results that are quantitatively comparable.

The 12CA5 blot of human p53 in Fig. 7A shows the effects of combinations of molecules on the electrophoresis of human WT p53. All of the molecules added to the reactions are known to interact with the C terminus of p53. In the absence of activators, a combination of a 20-fold mass excess of nonspecific 29-bp DNA (ds0) with a 5-fold mass excess of pBluescript (pBS) retarded the entry of p53 into the gel (third lanes in the first two panels). In striking contrast, a combination of specific 29-bp DNA (ds1) with pBluescript allowed p53 to enter the gel as tetramers (fourth lanes in the first two panels). This difference argues strongly that specific binding of ds1 to the core domain and binding of pBluescript to the C-terminal domain are mutually exclusive. Interference, therefore, is reciprocal. In the presence of nonspecific ds0, the addition of tRNA, PAb421, or the C terminus of murine p53 overcame the effects of pBluescript on the electrophoresis of p53 and enhanced the entry of p53 into the gel (compare the third lanes in the last three panels with the third lanes in the first two panels).

The blot in Fig. 7B represents the blot in Fig. 7A reprobed with PAb122 to detect murine p53(280–390) in addition to human p53. The PAb122 blot in the last panel of Fig. 7B shows that in the presence of a 20-fold mass excess of ds0 or ds1, murine p53(280–390) migrated to a lower position in the gel than it did in the absence of ds0 or ds1. This finding indicates that small nucleic acids, in excess, either disassemble complexes of the C terminus of murine p53, bind the C terminus and neutralize its basic charge, or both.

The DNA blot in Fig. 7C shows nonspecific and specific DNA binding by WT p53 in the presence of combinations of competitor DNAs, interfering DNA, and activator molecules. In the first panel, human WT p53 bound radiolabeled, nonspecific DNA (ds0*) as octamers, tetramers, and a faint smear of DNA released during electrophoresis. A 20-fold mass excess of unlabeled ds0 competed well with nonspecific binding to labeled ds0*. A 5-fold excess of pBluescript in combination with a 20-fold excess of unlabeled ds0 or ds1 interfered and/or competed with ds0* binding completely. In the second panel, WT p53 bound radiolabeled, specific 29-bp DNA (ds1*) as octamers and tetramers. Nonspecific DNA (ds0) did not compete with specific ds1*. pBluescript interfered with binding to ds1* in the presence of ds0, which would block nonspecific but not specific DNA binding. As expected, an excess of unlabeled ds1 competed with labeled ds1* for binding to the core domain of p53 even though ds1 blocked interference by pBluescript as determined by the p53 blot of the same lane in Fig. 7A. Each of the three activators blocked interference by pBluescript in the presence of ds0 (third lanes of the last three panels). As expected, unlabeled ds1 competed with labeled ds1* for binding to WT p53 (fourth lanes of the last three panels) in the presence of a combination of pBluescript and activator molecules and the freed probe DNA was bound by the excess of murine p53(280–390) (fourth lane of the last panel).

As shown in earlier experiments, PAb421 and the C terminus of murine p53 enhanced the entry of WT p53 into the gel. Thus, the apparent increase in DNA binding induced by these two activators in the absence of pBluescript need not represent an increase in the intrinsic activity of p53. Significant activation was evident only in the presence of pBluescript. The differential effects of nonspecific and specific DNA competitors on interference by pBluescript (third and fourth lanes in the first two panels in Fig. 7A) and on the activation of binding to ds1* in the presence of pBluescript (third and fourth lanes in the last three panels in Fig. 7C) confirm that both interference and

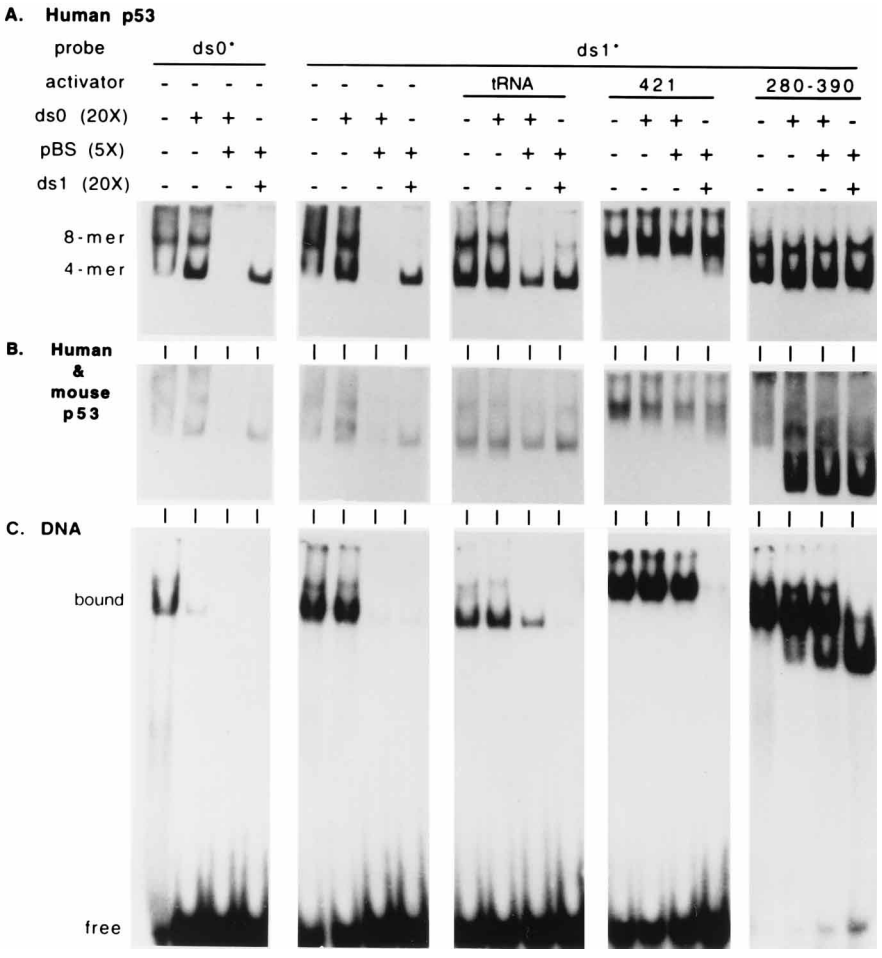

FIG. 7. Effects of combinations of competitor DNAs, interfering DNA, and activator molecules on the binding of p53 to specific DNA. Purified p53 (50 ng) was incubated with a 29-bp radiolabeled DNA segment (2 ng) encoding either a single p53 recognition sequence (ds1*) or nonspecific DNA (ds0*) for 30 min at room temperature at a tetramer/ds1 ratio of 2. Large pBluescript (pBS) was used at a 5-fold mass excess (10 ng) to ds1; nonspecific (ds0) and specific (ds1) competitor DNAs were used at 20-fold mass excesses relative to radiolabeled ds0 and ds1. tRNA (200 ng), PAb421 (100 ng), and murine p53(280–390) (150 ng) were used as in Fig. 4 to 6. Complexes were analyzed by gel shift analysis and double blotting. (A) Human p53 immunoblot with 12CA5. (B) Mouse p53 and human p53 immunoblot with PAb122. (C) DNA blot.

activation affect specific DNA binding by the core domain of p53.

DISCUSSION

Negative regulation of specific DNA binding by p53. Our findings indicate that the interaction of large, nonspecific DNA with the C terminus of p53 has a strong negative regulatory function. In our studies, five DNAs of 200 bp or larger inhibited specific DNA binding in the presence but not in the absence of an intact C terminus (Fig. 3). These DNAs differed in sequence and conformation; their common feature was their relatively large size. Our findings confirm and extend those of Bayle et al. (3), who showed that poly(dI-dC) · poly(dI-dC) inhibits specific DNA binding by p53 with a basic C-terminal region. In contrast, four small nonspecific nucleic acids did not interfere with specific DNA binding at concentrations much higher than those required for interference by large DNAs. These small nucleic acids also had a variety of sequences and structures. Competition studies confirmed that large DNA interferes with sequence-specific DNA binding (Fig. 7).

Positive regulation of specific DNA binding by p53. We show, furthermore, that activation of specific DNA binding results primarily from blocking of the negative regulation associated with the binding of large nucleic acids. Deletion of the p53 C terminus resulted in a strong increase of specific binding in the presence but not the absence of large nucleic acids (Fig. 3). Three quite different molecules (tRNA, a monoclonal antibody against the C terminus of p53, and an isolated C-terminal fragment of mouse p53) required both the presence of large nucleic acids and an intact C-terminal region for strong activation of specific DNA binding by p53 (Fig. 4 to 6). Deletion of the C terminus of human p53 or addition of activator molecules appeared to enhance DNA binding modestly in the absence of large DNA. When corrected for enhanced entry of p53 tetramers into the gel, however, there was little increase in the intrinsic DNA binding activity of WT p53. This latter observation demonstrates the importance of monitoring the effects of activating agents on the p53 protein itself. It also suggests that the C terminus of human WT p53 may contribute to the formation of larger p53 complexes. Competition studies confirmed that activators block interference with sequence-specific DNA binding (Fig. 7).

Figure 8 presents a model illustrating shared mechanisms which would account for negative and positive regulation of

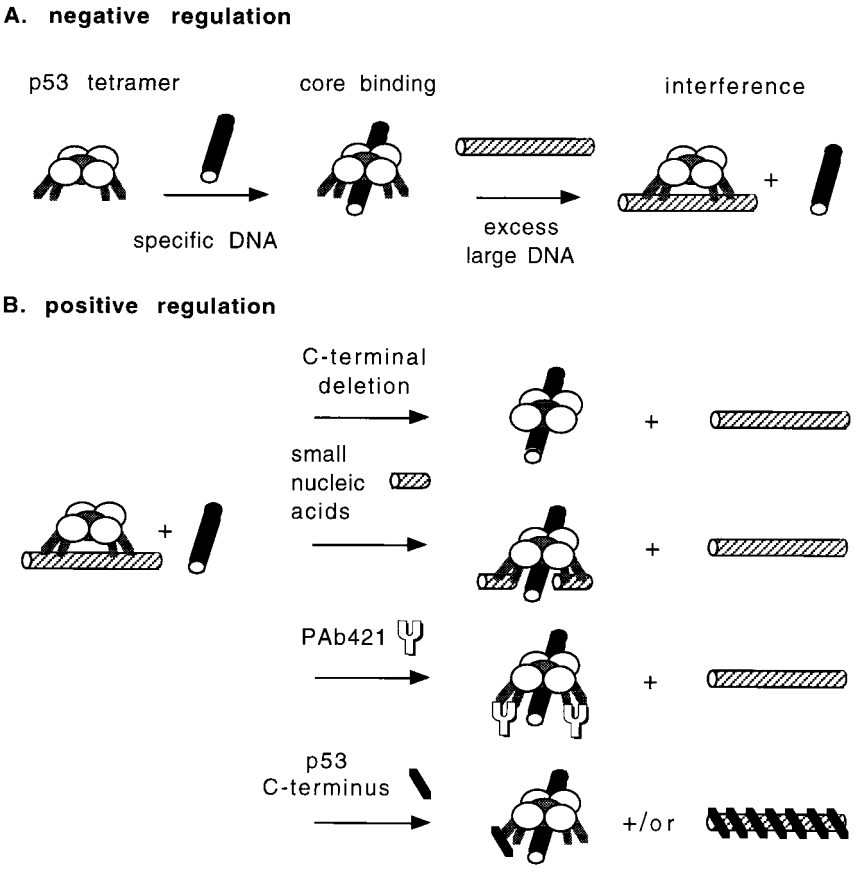

FIG. 8. Regulation of specific DNA binding by wild-type p53. (A) Negative regulation. p53 tetramers consist of N-terminal and core domains (white spheres) and tetramerization domains with basic C termini (dark areas). Specific DNA (black rod) binds to the core of p53. Large, nonspecific DNA (striped rod) binds the C termini of p53 and interferes with specific DNA binding. (B) Positive regulation of specific DNA binding by deletion of C termini, by addition of small nucleic acids or PAb421, or by an isolated mouse C terminus.

p53 by diverse molecules. In the model in Fig. 8A, purified WT p53 exists as tetramers and multiples of tetramers; for simplicity, we show only tetramers here. The core domains of each tetramer bind a single p53 recognition sequence in preference to other DNAs. The presence of excess large DNAs, however, favors nonspecific interactions of the C terminus over specific DNA binding by the core domain and leads to negative regulation of the p53 core DNA binding function. That excess specific DNA can overcome the effects of the interaction of large DNA with the C terminus indicates that interference between specific DNA bound to the core domain and large DNA bound to the C terminus is reciprocal. In the model in Fig. 8B, the four activation mechanisms analyzed in this study operate primarily by blocking interference by large DNAs. Deletion of the p53 C terminus precluded any binding of large DNAs to the C terminus. Two activator molecules, tRNA and a monoclonal antibody, interfered with the binding of large DNAs to the C terminus of p53 by direct competition for the same or overlapping binding sites. The third activator molecule, the isolated C terminus of mouse p53, operated in part by saturating the large DNAs and thereby blocking their binding to the C termini of WT p53. In addition, the isolated C termini may have undergone transient interactions with intact p53 that could have blocked interaction with large, nonspecific DNAs. A decapeptide corresponding to the PAb421 epitope, like mouse p53(280–390), reduces the formation of large complexes by WT p53 (data not shown).

Interference with sequence-specific DNA binding by interactions of nucleic acids with the C terminus of p53 is related to the size of the nucleic acids. The importance of size is particularly compelling in the case of large and small poly(dI-dC) · poly(dI-dC)s, which share the same sequence. Simultaneous binding of large DNAs to the C terminus and of specific DNA to the core domain of p53 is apparently not possible under the conditions of our assay. The ability of small nucleic acids to block interference by large nucleic acids implies that small nucleic acids also bind the C terminus of p53 but do not block the binding of specific DNA to the core domain. This conclusion is consistent with recent results of others (17, 21), who have presented direct evidence for simultaneous binding of small nucleic acids to the two domains of p53. The different effects of large and small DNAs on specific DNA binding suggest that large DNAs may interfere, at least in part, through steric hindrance. Interestingly, one or more 200-bp DNAs (132 kDa) bound to the C terminus block the core domain while one or more monoclonal antibodies (160 kDa) do not. Possibly, the elongated shape of DNA, which has a persistence length of 150 to 200 bp, allows it to engage all four C termini simultaneously. Two globular antibodies, in contrast, would have different steric relationships with p53. Alternatively, it is possible that long DNAs bound to the C terminus bind simultaneously to the core domain whereas antibodies do not.

Our findings that three distinct activators of p53 operate by blocking interference by large DNAs suggest that there is no

need to invoke allosteric mechanisms to account for the positive regulation of p53 by these particular molecules. The simplest explanation for the marked difference in the electrophoretic positions of p53 bound to large and small nucleic acids is the large difference in the size of the bound nucleic acids rather than conformational differences in p53 tetramers. Our findings, however, do not exclude the possible importance of allosteric changes in p53 function.

Comparisons with the findings of others. Previous descriptions of p53 activators by other investigators are consistent with our present findings. Interpretations of past studies were complicated by the presence of two DNA binding domains within p53, the presence of large nucleic acids in assays, and the lack of information on the effects of activator molecules on p53 itself during gel shift assays. Hupp et al. (9) showed that deletion of the C terminus of p53 or the addition of PAb421 to WT p53 could activate specific DNA binding in the presence of pBluescript. No results related to activation in the absence of pBluescript were reported. Jayaraman and Prives (11) used DNase footprinting of a p53 recognition sequence in intact pBluescript DNA to show that small nucleic acids activate specific DNA binding by p53. The same group used a gel shift assay to show that the C terminus of mouse p53 activated human p53 to a greater extent in the presence than in the absence of poly(dI-dC). This result is similar to our findings. However, under our conditions, we showed that the low levels of activation by the C terminus in the absence of large nucleic acids could be accounted for by increased entry of p53 into the gel during the gel shift assay rather than by an increase in the intrinsic activity of p53 (Fig. 6).

Our findings may explain a number of puzzling phenomena related to specific DNA binding by p53 in crude or partially purified cell extracts. The efficiency of specific DNA binding by WT p53 purified from insect cells argues that p53 has a high level of activity after purification. In contrast, WT p53 in crude extracts binds specific DNA poorly and requires the addition of activators; indeed, some investigators add PAb421 to assays routinely. Clearly, the presence of large nucleic acids in extracts could negatively regulate p53. Conversely, Funk et al. (6) found that nuclear extracts could promote DNA binding by p53 in crude preparations of animal cells. In this case, either small nucleic acids or nonspecific DNA binding proteins might have activated p53 in the presence of large DNAs. There have been frequent reports of latent p53s that can be activated by a variety of molecules (7, 13, 16). We are aware of no published results that demonstrate direct activation of documented amounts of p53 in the absence of either unidentified contaminating molecules or of added large nucleic acids. We suggest that small rather than large DNAs should be used to suppress background DNA binding proteins in crude extracts containing p53 and that the amount of p53 entering the gel in gel shift assays should be monitored for maximal information.

Biological implications. The intrinsic DNA binding properties of p53 described here and by Bayle et al. (3) argue that interactions of the C terminus of p53 with genomic DNA in vivo would ordinarily inhibit the binding of p53 to specific response elements in promoters. It is striking that small mass excesses of large DNAs block specific DNA binding so efficiently in vitro. The mass excess of large genomic DNA in cells relative to specific recognition elements is thousands of times larger than the excesses used in our in vitro experiments. Even if much of the genomic DNA in cells were not available for nonspecific DNA binding by the C termini of p53, we would still expect that p53 would be inactive in normal cells. Thus, in response to stress or other situations requiring transactivation by p53, cells would need mechanisms to inhibit the binding of p53 C termini to genomic DNA and thereby to activate specific transcription. In addition to posttranslational modifications of p53, cellular proteins or small nucleic acids might activate p53. It seems unlikely, however, that small single-stranded segments of DNA released during repair of damaged DNA would be sufficiently abundant to compete with the binding of genomic DNA to the C terminus of p53. Our findings argue that cells may have very specific activator molecules that are amplified in response to appropriate signals. The mechanisms described here have implications for the design of practical therapeutic approaches to the activation of p53 in tumor cells.

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