The p53 Activation Domain Binds the TATA Box-Binding Polypeptide in Holo-TFIID, and a Neighboring p53 Domain Inhibits Transcription

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Antioncogene product p53 is a transcriptional transactivator. To investigate how p53 stimulates transcription, we examined the interaction of p53 with general transcription factors in vitro. We found that p53 binds directly to the human TATA box-binding polypeptide (TBP). We also observed a direct interaction between p53 and purified holo-TFIID, a complex composed of TBP and a group of TBP-associated polypeptides known as TAFs. The p53 binding domain on TBP was mapped to the conserved region of TBP, including residues 220 to 271. The TBP binding domain on p53 was mapped to the p53 activation domain between residues 20 and 57. To analyze the significance of the p53-TBP interaction in p53 transactivation, we compared the ability of Gal4-p53 fusion proteins to bind to TBP in vitro and to activate transcription in transient transfection assays. Fusion proteins which bound to TBP activated transcription, and those that did not bind to TBP did not activate transcription to a detectable level, suggesting that a direct interaction between TBP and p53 is required for p53 transactivation. We also found that inclusion of residues 93 to 160 of p53 in a Gal4-p53 fusion repressed transcriptional activation 100-fold. Consequently, this region of p53 inhibits transcriptional activation by the minimal p53 activation domain. Highest levels of activation were observed with sequences 1 to 92 of p53 fused to Gal4, even though this construct bound to TBP in vitro with an affinity similar to that of other Gal4-p53 fusion proteins. We conclude that TBP binding is necessary for p53 transcriptional activation and that p53 sequences outside the TBP binding domain modulate the level of activation.

Wild-type p53 protein is most commonly described as a tumor suppressor or an antioncogene product. Alteration or loss of p53 is associated with a wide variety of human tumor cells (18, 23, 25). Mutations in the p53 gene are the most frequently observed genetic lesions in spontaneous human cancers (50). Considerable evidence indicating that p53 functions as a transcriptional activator to regulate cell cycling has now accumulated. p53 was first shown to activate transcription as a Gal4-p53 fusion protein from a promoter carrying Gal4 DNA binding sites (10, 34, 37). Then native p53 was shown to activate transcription from the muscle creatine kinase promoter (51, 54), and purified p53 was shown to stimulate transcription directly in in vitro transcription reactions (9). Evidence suggests that a transcriptional activation domain is present in the N terminus of p53 (10) and that p53 activates transcription by binding to specific DNA sequences in regulatory regions (22, 54). In this regard, p53 is similar to many other well-studied DNA-binding transcription factors (31).

Inhibition of p53's ability to function as a transactivator correlates with transformation in several different situations. The transforming activity of the adenovirus large early 1B protein depends on its ability to inhibit transactivation by p53 (52). A cellular protein, p90, encoded by the *mdm2* gene, inhibits transactivation by p53. A murine immortalized cell line overexpresses p90 as a consequence of amplification of the *mdm2* gene, suggesting that inhibition of p53 transactivation is associated with the transformation of this cell (32). Furthermore, several p53 mutants isolated from human

tumors are dominant negative inhibitors of p53 transactivation (9, 22, 54). These results together with the finding that wild-type p53 suppresses cell cycling (1, 6, 8, 11) have led to the following general model. Wild-type p53 activates transcription of genes encoding proteins which suppress entry into S phase. Therefore, blocking p53 transactivation promotes entry into S phase, contributing to a multistep process of oncogenic transformation. p53 may be particularly important in activating genes involved in a checkpoint which assesses the integrity of cellular DNA before entry into S phase (30, 53). Consequently, an understanding of the mechanism of p53 transactivation is directly relevant to p53 function as a tumor suppressor.

Factors involved in RNA polymerase II transcription can be classified according to their function as general transcription factors or regulatory transcription factors. The general factors are required for transcription initiation from most promoters and have been defined as TFIIA, -B, -D, -E, -F, -G, -H, and -J by purification from nuclear extracts (5, 12, 39, 44). Among the regulatory transcription factors are sequence-specific DNA-binding transactivators. These are believed to stimulate transcription through interactions with an initiation complex composed of the general transcription factors plus RNA polymerase II assembled at the start site of transcription (35, 36). The assembly of general transcription factors at a promoter containing a TATA box is initiated by the binding of TFIID to the TATA sequence (4, 49). TFIID is a complex protein composed of a TATA box-binding polypeptide (TBP; 38 kDa in human cells) and several TBP-associated polypeptides (TAFs) (7, 46, 55). Recent work has shown that some transactivators interact directly with TBP (19, 24, 26, 42). Other experiments have demon-

strated an interaction between the acidic activation domain of VP16 and TFIIB which stabilizes the binding of TFIIB to promoter DNA during assembly of a preinitiation complex (28, 29). Currently, little is known concerning the mechanism of transactivation by p53.

To investigate how p53 stimulates transcription, we examined the interaction of p53 with TBP and TFIIB. Our results show that p53 interacts directly with TBP. Furthermore, the interaction is within the p53 activation domain, leading us to suggest that p53 stimulates transcription, at least in part, through a direct interaction with TBP. A much weaker interaction between p53 and TFIIB was also detected in one of our assays. Transient transfection assays also revealed a transcriptional inhibitory domain in p53 just C terminal to the p53 activation domain.

MATERIALS AND METHODS

Plasmids. pKB104 (20), pGAL4-p53 (34), pGAL4-p53N (34), pGAL4-p53C (34), pGAL4-VP16 (38), pG5E1BCAT (27), and pET-eTBP (55) have been described previously. For p53 in vitro transcription-translation, the wild-type p53 cDNA (16) was cloned into pcDNA.1 (Invitrogen) between the HindIII and BamHI sites, creating pcDNA-p53. To create pGST-p53, p53 was amplified from pcDNA-p53 by the polymerase chain reaction (PCR), using primers which introduced a BamHI site at the 5' end and an EcoRI site at the 3' end of the protein coding region, and the PCR product was cloned between the BamHI and EcoRI sites of pGEX-2T (41) (Pharmacia). pBluescript-p53C was constructed by deletion of the NcoI fragment encoding amino acid residues 1 to 160 from p53 and ligation between pBluescript (Stratagene) HindIII and BamHI sites. Gal4-p53 deletion mutants were constructed by PCR amplification of amino acid residues 1 to 20, 1 to 57, 1 to 92, 58 to 92, and 93 to 160 from wild-type p53, using primers that introduce an EcoRI site at the 5' end and a BamHI site at the 3' end. The amplified DNA fragments were cloned between the EcoRI and BamHI sites of pGALM (27). pSRα-eGal4-p53 deletion mutants were constructed by PCR amplification of these same Gal4-p53 deletion mutants, using primers which introduce a PstI site and the amino acid sequence MYPYDVPDYA at the 5' end and a KpnI site at the 3' end. The amplified DNA fragments were cloned between the PstI and KpnI sites of pcDL-SRα296 (45). pBluescript-Gal4-p53 deletion mutants were constructed by cloning these same Gal4-p53 deletion mutants between the EcoRI and BamHI sites of pBluescript. pET-eTFIIB was constructed by PCR amplification of TFIIB cDNA (14), using primers which introduce an NdeI site and the amino acid sequence MYPYDVPDYA at the 5' end and a BamHI site at the 3' end. The amplified DNA fragments were cloned between the NdeI and BamHI sites of pET-3B (43).

Coimmunoprecipitation. ^{35}S-labeled proteins were synthesized by in vitro transcription-translation. Briefly, 10 μg of plasmid pKB104 or pcDNA-p53 was digested to completion with Asp 718 or BamHI, respectively, and transcribed with 30 U of T3 or T7 RNA polymerase, respectively. ^{35}S-labeled proteins were synthesized according to a standard in vitro translation protocol (Promega) by incubating 10 μg of cRNA with 100 μl of rabbit reticulocyte lysate in the presence of 10 μl of ^{35}S Trans label (1,017 Ci/mmol; ICN). Truncated TBP proteins (TBP/Stu and TBP/Apa) were made from cRNA synthesized from pKB104 cut with StuI and ApaLI, respectively. p53 was expressed in Escherichia coli as a glutathione S-transferase fusion protein. The fusion protein was purified

by affinity chromatography using glutathione-Sepharose 4B (Pharmacia), and p53 was recovered from the affinity resin by cleavage with thrombin (Sigma) as described previously (41). Expression and partial purification of the epitope-tagged TFIIB (eTFIIB) from E. coli were as described previously (14). For experiments with labeled TBP and TBP deletion mutants, 0.5 μg of p53 expressed in and purified from E. coli and 1 μg of β-galactosidase were mixed with 4 μl of in vitro-translated TBP or truncated TBP proteins in a 100-μl volume of 10S buffer (50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.2], 250 mM NaCl, 0.2% Nonidet P-40, 0.1% Triton X-100, 0.01% sodium dodecyl sulfate [SDS], 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) and incubated at 30°C for 30 min. Fixed Staphylococcus aureus cells (20-μl packed volume) in 400 μl of 10S buffer were mixed with this solution and centrifuged for 2 min at maximum speed in an Eppendorf microcentrifuge (preclearing). Then 80 μl of supernatant of hybridoma cells expressing anti-p53 antibody pAb421 (15) or 0.5 μg of a monoclonal antibody against β-galactosidase (Boehringer Mannheim) was added to the supernatants, which were mixed and incubated at 4°C for 2 h. Protein A-Sepharose was added and mixed for 1 h at 4°C. After centrifugation as described above, pellets were washed three times with 10S buffer, once with 0.8 M LiCl buffer (0.8 M LiCl, 0.1 M Tris [pH 7.2]), and once with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$ · 7H$_2$O, 1.4 mM KH$_2$PO$_4$). Pellets were suspended in 1× Laemmli sample buffer and loaded onto SDS–10 or 15% polyacrylamide gels. Radiolabeled proteins were visualized by fluorography after enhancement with 1 M sodium salicylate. Immunoprecipitation experiments using labeled p53 were performed as described above except that 10 μg of HeLa cell nuclear extract phosphocellulose D fraction, 1 μg of E. coli-expressed TBP (24), or 1 μg of E. coli-expressed eTFIIB plus 1 μg of β-galactosidase was incubated with 4 μl of in vitro-translated p53 in 100 μl of 10S buffer for 30 min at 30°C. Immunoprecipitation proceeded as described above, using 4 μl of rabbit antisera raised against TBP, 1 μg of monoclonal antibody 12CA5, or 0.5 μg of a monoclonal antibody against β-galactosidase.

Far-Western blotting. Extract (10 to 20 μg) either from E. coli expressing TBP or TFIIB or from HeLa cells infected with a vaccinia virus vector expressing high levels of TBP (24) was incubated at 100°C for 5 min in 1× Laemmli sample buffer, electrophoresed in SDS–10 or 15% polyacrylamide gels, and electrophoretically transferred to nitrocellulose filters (BA83; Schleicher & Schuell). The protein blots were then subjected to a protein renaturation protocol and incubated with ^{35}S-labeled probes as described previously (24). ^{35}S-labeled p53 was synthesized as described above. The N-terminal 160 residues of p53 were translated from cRNA transcribed from pBluescript-p53 cut with ThaI, creating p53N. The C-terminal p53 (p53C) and Gal4-p53 deletion mutants were translated from cRNAs transcribed from pBluescript-p53C and pBluescript-GAL4-p53 deletion mutants, respectively. The blots were then washed and autoradiographed. The protein blot was also subjected to a standard Western blotting procedure with anti-human TBP serum followed by enhanced chemiluminescence detection of bound antibody (Amersham) to visualize TBP protein.

12CA5 affinity bead binding assay. Epitope-tagged human TBP (eTBP) and eTFIIB were partially purified from E. coli as described previously (24, 14). The phosphocellulose D fraction was prepared from the human eTBP-expressing LTRα3 cell line as described previously (55). One milliliter

of the undialyzed D fraction (0.8 M KCl) from LTRα3 cell nuclear extract (1.6 mg/ml) was incubated with 0.1 ml of packed beads of protein A-Sepharose covalently coupled to monoclonal antibody 12CA5 at 4°C for 6 h with rotation. Alternatively, 1 ml 0.8 M KCl D buffer (20 mM HEPES, 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride [pH 7.9]) containing a roughly equivalent amount of eTBP or eTFIIB was incubated with the same amount of 12CA5 affinity beads. The 12CA5 affinity beads were then washed three times with 0.4 M KCl D buffer and two times with 0.1 M KCl D buffer. eTBP, eTFIIB, and holo-eTFIID immobilized on the 12CA5 affinity beads were then independently incubated at room temperature for 90 min in a 0.5-ml volume of 0.1 M KCl D buffer containing 30 μl in vitro-translated ^{35}S-labeled p53 protein as described above. The beads were then washed twice for 4 min each time with 0.25 M KCl D buffer and twice for 4 min each time with 0.1 M KCl D buffer. Subsequently, the washed beads were eluted with 60 μl of the influenza virus epitope oligopeptide at 1 mg/ml in 0.1 M KCl D buffer as described previously (55). Duplicate sets of sample aliquots from the resulting bead eluates were then resolved by electrophoresis in an SDS–10% polyacrylamide gel. One set of samples was subjected to Western blot analysis using antibody 12CA5 to ensure that approximately equivalent amounts of eTFIIB, eTBP, or holo-eTFIID were present in all samples. The remaining sample set was subjected to 1 M sodium salicylate fluorography treatment and autoradiography.

Transfection and CAT assay. HeLa cell monolayers were plated at 10^7 cells per 100-mm-diameter plate and transfected by the calcium phosphate method with 2 μg of pG5E1BCAT (27), 2 μg of the β-galactosidase expression plasmid pCH110 (17), and 1 μg of effector DNA (Gal4-VP16, Gal4-p53, Gal4-p53N, Gal4-p53C, or Gal4-p53 deletion mutant). Cells were harvested 48 h posttransfection. All samples were normalized for transfection efficiency by measuring β-galactosidase activity. Chloramphenicol acetyltransferase (CAT) activity was measured as described previously (13), using 20-to 30-μl extracts for all p53 constructs and 20% of this volume for Gal4-VP16. CAT activity was quantitated by scanning the entire thin-layer chromatography plate with an AMBIS β-scanning system.

Detection of Gal4-p53 fusion proteins in transfected COS cells. COS-7 cells cultured on 60-mm-diameter plates at 70% confluency were transfected by the calcium phosphate method with 5 μg of pSRα-Gal4-p53 deletion mutants. At 60 h posttransfection, the cells were incubated for 1 h at 37°C in methionine-free medium. Then each plate was incubated with 0.1 mCi of ^{35}S Trans label (1,017 Ci/mmol; ICN) in 1 ml of methionine-free medium supplemented with 2% dialyzed newborn calf serum. After 2 h at 37°C, the cells were washed with PBS at 4°C and lysed by adding 0.5 ml of 10S buffer. The lysate was clarified by centrifugation for 2 min at 4°C in a microcentrifuge. The extracts were incubated overnight at 4°C with 1.5 μl of a polyclonal antibody to Gal4 (generous gift of Ivan Sadowski). The immunoprecipitate was recovered by using protein A-Sepharose and washed with 10S wash buffer. The immunoprecipitate was then dissociated from the Sepharose by boiling in 25 μl of sample boil buffer (2.5% SDS, 100 mM Tris [pH 7.2]). The protein A-Sepharose beads were removed by centrifugation, and the supernatant was diluted 25-fold with 10S wash buffer and reimmunoprecipitated with 1 μl of anti-Gal4 antiserum. The immunoprecipitate was analysed on an SDS–12% gel and visualized by autoradiography.
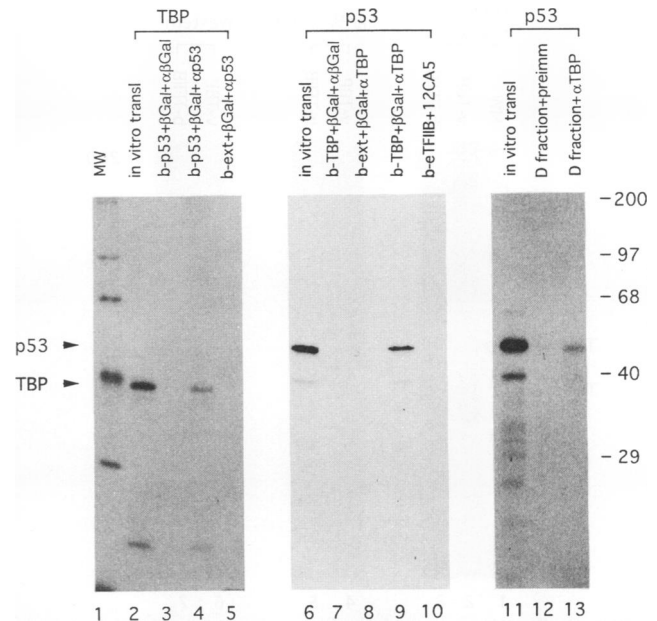

FIG. 1. Coimmunoprecipitation of p53 and TBP. Autoradiograms of SDS-polyacrylamide gels of immunoprecipitates are shown. Lanes 2, 6, and 11 show 10% of the input proteins prior to immunoprecipitation. ^{35}S-labeled in vitro-translated TBP (lanes 3 to 5) was incubated with β-galactosidase (βGal) and bacterially expressed p53 (b-p53) (lanes 3 and 4) and immunoprecipitated with either anti-β-galactosidase antibody (αβGal) (lane 3) or anti-p53 antibody (αp53) (lane 4). ^{35}S-labeled p53 (lanes 7 to 10, 12, and 13) was incubated with β-galactosidase and b-TBP (lanes 7 and 9) or with b-eTFIIB (lane 10) and immunoprecipitated with anti-β-galactosidase antibody (lane 7), anti-TBP antibody (αTBP) (lanes 9 and 13), or anti-epitope antibody (12CA5) (lane 10). The labeled TBP and p53 were also incubated with a control extract (b-ext) and immunoprecipitated with anti-p53 antibody (lane 5) or anti-TBP antibody (lane 8). ^{35}S-labeled p53 was incubated with the phosphocellulose D fraction prepared from HeLa cells and immunoprecipitated with preimmune serum (lane 12) or anti-TBP serum (lane 13). MW, molecular weight markers. Sizes are indicated in kilodaltons.

RESULTS

Coimmunoprecipitation of p53 and TBP. Because p53 is a transcriptional activator, we tested the possibility that it can interact directly with TBP by using an in vitro coimmunoprecipitation assay. In vitro-translated ^{35}S-labeled TBP was incubated with 0.5 μg of p53 protein prepared from a glutathione S-transferase–p53 fusion protein expressed in and purified from E. coli. As a control for the specificity of binding, 1 μg of unlabeled E. coli β-galactosidase was also added to the binding reaction. Immunoprecipitation with an anti-p53 monoclonal antibody precipitated labeled TBP (Fig. 1, lane 4). In contrast, TBP was not coprecipitated with β-galactosidase by an anti-β-galactosidase monoclonal antibody (lane 3) or by an anti-p53 antibody when p53 was absent from the incubation (lane 5). Similarly, in vitro-translated ^{35}S-labeled p53 was immunoprecipitated by an anti-TBP serum following incubation with unlabeled TBP and β-galactosidase (lane 9). Control immunoprecipitations with an anti-β-galactosidase antibody (lane 7) and with an anti-TBP serum from a mixture lacking TBP (lane 8) failed to precipitate labeled p53. These coimmunoprecipitation results indicate that p53 binds to TBP in vitro under the conditions tested.

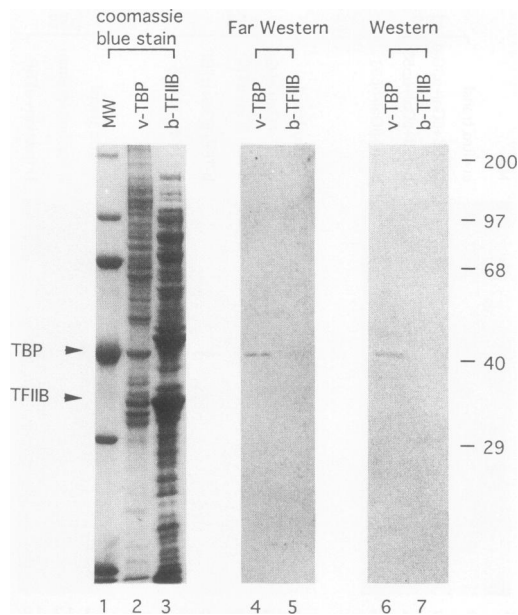Using similar methods, we sought evidence for an inter-

FIG. 2. Far-Western analysis of the p53-TBP interaction. Lanes: 1 to 3, Coomassie blue-stained lanes of an SDS-polyacrylamide gel of partially purified vaccinia virus-expressed TBP (v-TBP) (lane 2) or b-eTFIIB (lane 3); 4 and 5, autoradiogram of the far-Western blot of the vaccinia virus-expressed TBP (lane 4) and b-eTFIIB (lane 5) after incubation with ^{35}S-labeled p53; 6 and 7, Western immunoblot using anti-TBP antibody of the same nitrocellulose blot shown in lanes 4 and 5. MW, molecular weight markers. Sizes are indicated in kilodaltons.

action between p53 and TFIIB. However, no coprecipitation of labeled p53 was observed following incubation with eTFIIB and immunoprecipitation with an antiepitope monoclonal antibody (a monoclonal antibody specific for the epitope) (Fig. 1, lane 10). Control experiments demonstrated that this monoclonal antibody efficiently precipitates TFIIB (data not shown).

Far-Western analysis of the p53-TBP interaction. To confirm the specific interaction of p53 protein with TBP, we also performed protein blotting (far-Western) experiments (Fig. 2). Extracts from HeLa cells infected with a vaccinia virus vector expressing high concentrations of TBP and from *E. coli* expressing high concentrations of TFIIB were subjected to SDS-polyacrylamide gel electrophoresis. Proteins detected by Coomassie blue staining are shown in Fig. 2, lanes 2 and 3. Samples equivalent to those shown in lanes 2 and 3 were transferred to nitrocellulose paper, subjected to a renaturation protocol, and incubated with in vitro-translated ^{35}S-labeled p53 protein. TBP protein was detected on the same blot by using an anti-TBP serum (lane 6). Binding of labeled p53 to TBP, but not to TFIIB or to other HeLa, vaccinia virus, or *E. coli* proteins present on the same blot, was detected under our assay conditions (lanes 4 and 5). This far-Western blot further demonstrates the interaction of p53 with TBP and emphasizes the specificity of the in vitro interaction between p53 and TBP.

p53 directly interacts with the holo-TFIID complex. TFIID prepared from HeLa cells contains TBP and a group of tightly associated polypeptides known as TAFs (46, 47, 55). These findings raised the question of whether TBP in association with TAFs is available for interaction with p53. To address this question, we performed immunoprecipitation

studies using the phosphocellulose D fraction prepared from a HeLa cell nuclear extract. Most of the TBP present in the phosphocellulose D fraction associates with TAFs and exists in a complex with a molecular size of ~700 kDa (46, 47, 55). In vitro-translated ^{35}S-labeled p53 was incubated with the phosphocellulose D fraction, and the mixture was immunoprecipitated with an anti-TBP serum. Labeled p53 was precipitated by the anti-TBP serum (Fig. 1, lane 13) but not by preimmune serum prepared from the same rabbit (lane 12). This result shows that p53 can interact with TBP in the phosphocellulose D fraction, indicating that p53 may interact directly with the holo-TFIID complex (55), even though the TBP in the holo-TFIID complex is tightly associated with multiple TAF polypeptides. The decreased amount of p53 immunoprecipitated from the D fraction compared with the amount precipitated in experiments with bacterially expressed TBP (b-TBP) may have been due to a lower concentration of TBP in the D fraction.

To address this question directly, we analyzed whether p53 can bind purified holo-TFIID (the complex of TBP plus TAFs required for activated transcription by RNA polymerase II). A stable HeLa-derived cell line, LTRα3, which expresses eTBP was used (55). The presence of the epitope on TBP was used to facilitate the purification of holo-TFIID from the LTRα3 cell nuclear extract phosphocellulose D fraction, using monoclonal antibody 12CA5 directed against the epitope (55). Holo-TFIID purified in this way is composed of TBP and at least nine additional TAFs ranging in size from 250 to 28 kDa (53) (Fig. 3, lane 2). Bacterially expressed eTBP (b-eTBP) and bacterially expressed eTFIIB (b-eTFIIB) were also independently purified by using 12CA5 affinity beads. Immobilized b-eTFIIB, b-eTBP, and holo-eTFIID were then independently incubated with ^{35}S-labeled p53 protein translated in a rabbit reticulocyte lysate. Following extensive washing, b-eTFIIB, b-eTBP, and holo-eTFIID, together with associated proteins, were eluted from the 12CA5 affinity beads by using an excess of synthetic epitope peptide. Duplicate sets of sample aliquots from the affinity bead eluates were then subjected to SDS-polyacrylamide gel electrophoresis. One set of samples was used for quantitative Western blot analysis to ensure that equivalent amounts of eTBP or eTFIIB were present in all samples (Fig. 3, lanes 3 to 6), while the remaining sample set was analyzed by fluorography to detect the amount of ^{35}S-labeled p53 protein associated with b-eTFIIB, b-eTBP, or holo-eTFIID (lanes 7 to 10). 12CA5 affinity beads without bound epitope-tagged protein were incubated with ^{35}S-labeled p53 as a control (lanes 3 and 7). No detectable p53 eluted from the control 12CA5 beads (lane 7). At least as much p53 eluted from the holo-eTFIID beads as from the b-eTBP beads (lanes 9 and 10). This result demonstrates a direct interaction between p53 and holo-eTFIID. This direct interaction indicates that the surface on eTBP recognized by p53 is available for interaction in holo-TFIID. TAFs associated with TBP in holo-TFIID do not block this interaction. We also observed an interaction between TFIIB and p53 which was above the background level but was much weaker than the interaction with TBP and holo-TFIID (lane 8).

Mapping the region on TBP which binds to p53. To map the region of TBP that interacts with p53, a number of TBP C-terminal deletion mutants were translated in vitro and assayed by the immunoprecipitation protocol described above. The primary sequence of human TBP is diagrammed in Fig. 4B. The conserved region is represented by a filled bar, and the N-terminal region of TBP is represented by an open bar.
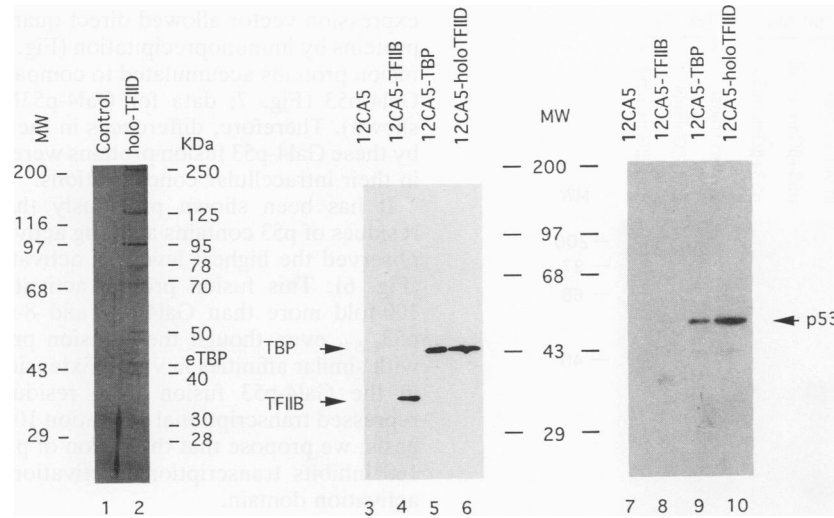
FIG. 3. Direct interaction between p53 and purified holo-TFIID. Lanes 1 and 2 are a silver-stained SDS-polyacrylamide gel of 20 µl of peptide eluate. Lane 2 shows holo-TFIID complex eluted with peptide from monoclonal antibody 12CA5 affinity beads incubated with the phosphocellulose D fraction of the eTBP-expressing LTRα3 cell line. The sizes of the individual TAFs are indicated on the right. Lane 1 shows nonspecifically bound proteins eluted with peptide from 12CA5 affinity beads incubated with the phosphocellulose D fraction of HeLa cells. Lanes 3 to 6 show a quantitative Western blot analysis using antibody 12CA5 specific for the epitope tag. Lane 3, 20 µl of peptide eluate from the control 12CA5 beads; lane 4, 4 µl of peptide eluate from 12CA5-eTFIIB beads; lane 5, 4 µl of peptide eluate from 12CA5-eTBP beads; lane 6, 20 µl of peptide eluate from 12CA5-eTFIID beads. Lanes 7 to 10 show an autoradiogram of an SDS-polyacrylamide gel of the same sample aliquots. MW, molecular weight markers. Sizes are indicated in kilodaltons.

A TBP C-terminal deletion mutant, generated by in vitro translation of RNA transcribed from the TBP vector (pKB104) cut at the StuI site (at the sequence encoding residue 271), bound to p53 as well as full-length TBP protein did (Fig. 4A, lane 6). However, a deletion mutant generated from the TBP vector cut at the ApaLI site (at the sequence encoding residue 220) did not bind to p53 (lane 9). These results indicate that TBP binding to p53 requires the conserved region of TBP between residues 220 and 271. We have not identified the ~32-kDa polypeptide generated in these in vitro translation reactions. It may represent a premature termination product which can bind to full-length TBP, since it coprecipitated with full-length TBP, whereas much less was precipitated with TBP/Stu and none was precipitated with TBP/Apa.

Mapping of the region of TBP interaction on p53. To identify the region in p53 which interacts with TBP, we performed far-Western blotting as described above. The primary sequence of p53 is diagrammed in Fig. 5C. The N-terminal 73 residues of p53 shown to contain an activation domain (10) is represented by the filled portion of the diagram. We first tested the interaction of TBP with N-terminal and C-terminal portions of p53 (Fig. 5A). Extracts of E. coli expressing TBP were fractionated on SDS-polyacrylamide gels, transferred to nitrocellulose filters, and subjected to a renaturation protocol. The filters were incubated independently with equivalent amounts of ^{35}S-labeled p53, p53N, and p53C. An N-terminal fragment of p53 (residues 1 to 160) bound to TBP as well as full-length p53 did (Fig. 5, lanes 1 and 2), whereas a C-terminal fragment (residues 161 to 393) did not (lane 3). The very minor signal observed for p53C in this experiment was not seen in repeat experiments.

To further identify the interaction domain in the p53 N-terminal region, we tested a number of fusions of N-terminal fragments of p53 to the DNA binding domain of the yeast Gal4 transcription factor (21). Below, we present results on the transcriptional activation functions of these same Gal4-p53 deletion constructs. b-TBP was fractionated on an SDS-polyacrylamide gel and transferred to nitrocellulose filters. The filters were then incubated independently with equivalent amounts of ^{35}S-labeled Gal4-p53 deletion constructs as estimated on an SDS-polyacrylamide gel (data not shown). Gal4-p53$_{1-57}$ and Gal4-p53$_{1-92}$ bound to TBP as well as full-length Gal4-p53 did (Fig. 5B, lanes 3 and 4). However, Gal4-p53$_{1-20}$, Gal4-p53$_{58-92}$, and Gal4-p53$_{93-160}$ did not bind to TBP (lanes 2, 5, and 6). Therefore residues between 20 and 57 in p53 are required for the p53-TBP interaction.

Correlation of p53-TBP interaction with p53 transactivation. To study the significance of the p53-TBP interaction for p53 transactivation, we examined the Gal4-p53 deletion mutants analyzed above for their ability to activate transcription in vivo. This was done by using a transient transfection assay in HeLa cells in which Gal4-fusion proteins are tested for their ability to stimulate expression of a CAT gene under the control of a promoter containing five Gal4 binding sites upstream of a TATA box (27). A Gal4 fusion to the strong activation domain of herpes simplex virus VP16 protein (Gal4-VP16 [39]) was used as a positive control. Results of representative CAT assays are shown in Fig. 6A and are summarized in Fig. 6B. Similar results were observed following transfection into COS and CHO cells. The TBP binding activities of these constructs are summarized at the bottom of Fig. 6B. Constructs Gal4-p53, Gal4-p53N, Gal4-p53$_{1-57}$, and Gal4-p53$_{1-92}$, which bound to TBP, all activated CAT expression. Constructs Gal4-p53C, Gal4-p53$_{1-20}$, Gal4-p53$_{58-92}$, and Gal4-p53$_{93-160}$, which were defective in binding to TBP, did not activate expression. Consequently, these results show a good correlation between the ability of the Gal4-p53 fusion proteins to bind TBP in vitro and their ability to show detectable activation function in vivo. Deletion mutants which did not activate
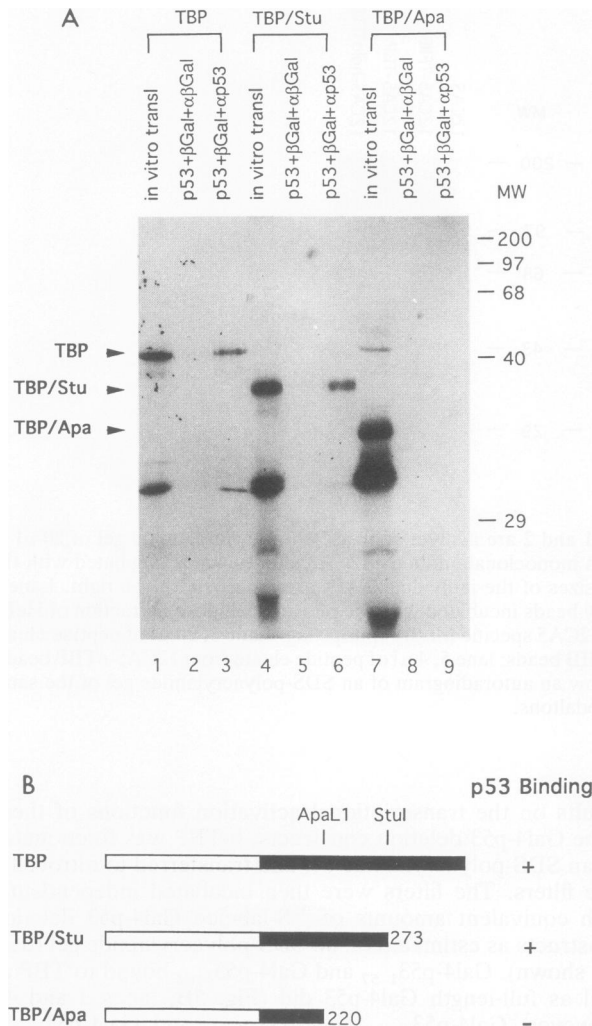
FIG. 4. Mapping the p53 binding site on TBP. (A) Autoradiogram of an SDS-polyacrylamide gel of an immunoprecipitation experiment. Lanes 1, 4, and 7 show 10% of the input protein prior to immunoprecipitation. ^{35}S-labeled TBP (lanes 2 and 3), TBP/*Stu* (lanes 5 and 6), or TBP/*Apa* (lanes 8 and 9) was incubated with β-galactosidase and bacterially expressed p53 and immunoprecipitated with an anti-β-galactosidase antibody (lanes 2, 5, and 8) or anti-p53 antibody (lanes 3, 6, and 9). For other details, see the legend to Fig. 1. (B) Diagrams of the sequence of TBP and the deletion mutants analyzed. The black area represents the conserved region of TBP. p53 binding activity is summarized on the right.

CAT expression to a detectable level did not bind to TBP in vitro. All deletion mutants which bound to TBP to a significant extent in vitro activated CAT expression in vivo. This correlation supports the model that p53 stimulates transcription through an interaction with TBP.

However, the strength of activation by the GAL4-p53 constructs did not correlate with the strength of TBP binding in vitro. Gal4-p53, Gal4-p53$_{1-57}$, Gal4-p53$_{1-92}$, and Gal4-p53N (including p53 residues 1 to 160) all bound to TBP to similar levels in vitro (Fig. 5), yet they varied widely in the degree of transcriptional activation in transfection experiments. To confirm that all Gal4-p53 deletion mutants have similar stabilities and are expressed at similar levels, we analyzed the expression of these fusion proteins in COS cells, in which the high level of expression from a replicating

expression vector allowed direct quantitation of the labeled proteins by immunoprecipitation (Fig. 7). All of the Gal4-p53 fusion proteins accumulated to comparable levels, similar to Gal4-p53 (Fig. 7; data for Gal4-p53N and Gal4-p53C not shown). Therefore, differences in the strength of activation by these Gal4-p53 fusion proteins were not due to differences in their intracellular concentrations.

It has been shown previously that the N-terminal 73 residues of p53 contains a strong activation domain (10). We observed the highest levels of activation with Gal4-p53$_{1-92}$ (Fig. 6). This fusion protein activated transcription over 100-fold more than Gal4-p53 and 8-fold more than Gal4-p53$_{1-57}$, even though these fusion proteins bound to TBP with similar affinities in vitro. Extension of the region of p53 in the Gal4-p53 fusion from residue 93 to residue 160 repressed transcriptional activation 100-fold (Fig. 6). On this basis, we propose that the region of p53 from residues 93 to 160 inhibits transcriptional activation by the minimal p53 activation domain.

DISCUSSION

Previous experiments have shown that p53 binds to DNA and activates transcription both in vivo and in vitro (9, 10, 22). Our results suggest that p53, like several other transcription factors (19, 24, 26), stimulates transcription through a direct interaction with the general transcriptional machinery. Furthermore, our results show that TBP is one of the specific targets in the general transcriptional machinery which interacts with p53. Under our assay conditions, the p53 protein binds TBP both specifically and stably. The p53-TBP complex can form in vitro under relatively stringent conditions, i.e., in a buffer containing 0.25 M NaCl and nonionic detergents (Fig. 1 and 4) or in a buffer containing up to 0.75 M KCl without detergents (data not shown). Furthermore, in vitro-translated p53 bound with high specificity to TBP immobilized on a nitrocellulose membrane (Fig. 2). Although there was a large amount of TFIIB on the same blot (Fig. 2), no binding of p53 to TFIIB was detected under our assay conditions. A similar result was observed in immunoprecipitation experiments, demonstrating that p53 bound to TBP but not to TFIIB under these conditions (Fig. 1). However, in our 12CA5 affinity bead binding experiment, a very weak interaction between p53 and b-eTFIIB was detected (Fig. 3, lane 8). Therefore, we conclude that p53 binds to TBP to a much greater extent than to TFIIB.

A 53-residue region of TBP mapping from residues 220 to 273 was required for p53 binding (Fig. 4). This region contains a repeat of basic residues and has been shown to interact with the adenovirus E1A activation domain (24). TBP has also been shown to associate with a number of additional polypeptides called TAFs in the TFIID complex isolated from HeLa cells (7, 47, 55). This finding raised the question of whether p53 could interact with TBP when it is associated with multiple TAFs. We addressed this question by assaying the interaction between p53 and the TBP present in the phosphocellulose D fraction prepared from a HeLa cell nuclear extract (Fig. 1, lanes 11 to 13). Because of its tight association with TAFs (46, 47, 55), most of the TBP present in such a phosphocellulose D fraction is found in a complex that has a molecular mass of ~700 kDa. p53 was able to bind to the TBP present in the D fraction, indicating that p53 might directly interact with the holo-TFIID complex. To demonstrate this directly, we analyzed the interaction of p53 with purified holo-TFIID complex (Fig. 3). On a per-TBP-molecule basis, p53 bound to holo-TFIID at least as
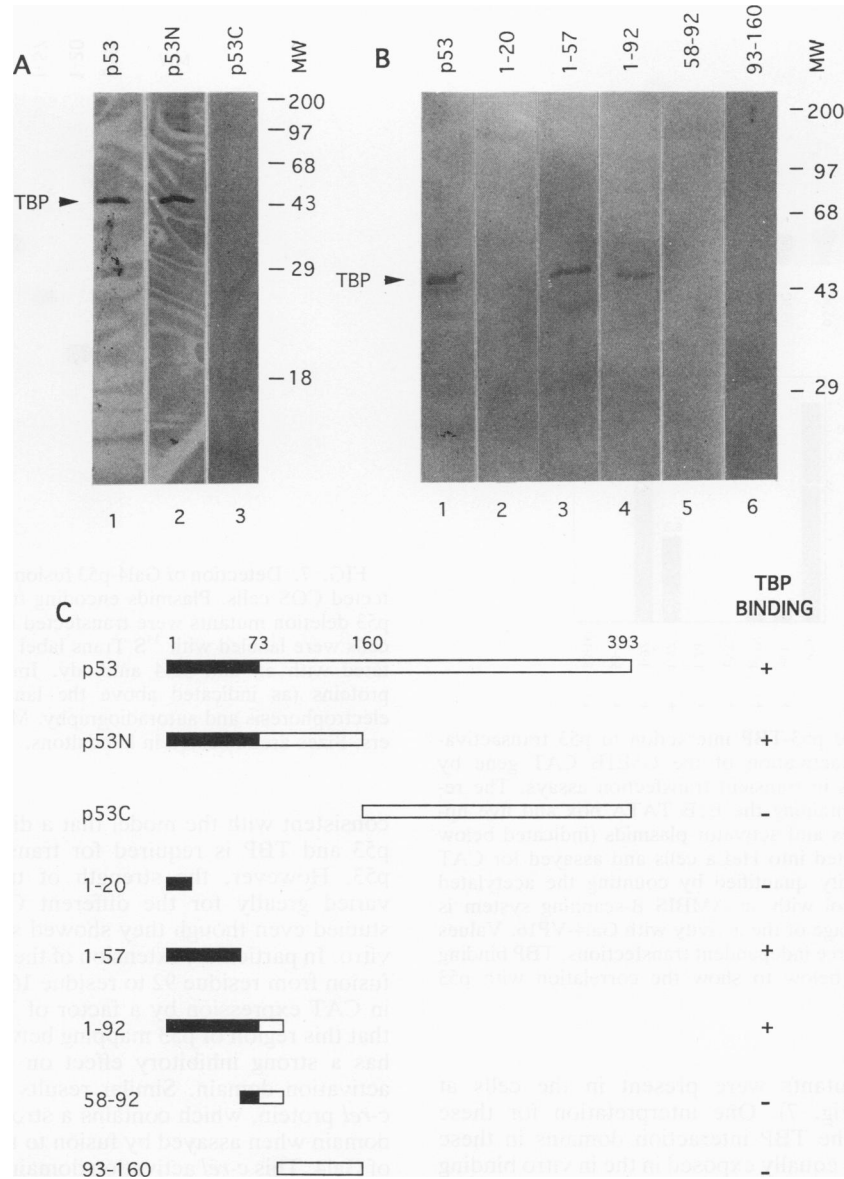
FIG. 5. Mapping the TBP binding site on p53. (A) Far-Western autoradiograms using p53 deletion mutants. b-TBP was subjected to SDS-gel electrophoresis and transferred to nitrocellulose filters. After renaturation, the filters were incubated with ^{35}S-labeled full-length p53 (lane 1), p53N (lane 2), and p53C (lane 3) and exposed to film. MW, molecular weight markers. Sizes are indicated in kilodaltons. (B) Far-Western autoradiograms using Gal4-p53 fusion proteins. Nitrocellulose filters with renatured b-TBP were incubated with ^{35}S-labeled full-length p53 (lane 1), Gal4-p53$_{1-20}$ (lane 2), Gal4-p53$_{1-57}$ (lane 3), Gal4-p53$_{1-97}$ (lane 4), Gal4-p53$_{61-97}$ (lane 5), and Gal4-p53$_{91-160}$ (lane 6) and exposed to film. (C) Diagrams of p53 deletions used in the far-Western analyses. Black bars represent the previously mapped activation domain of p53 (residues 1 to 73 [3]). Numbers above the bar represent p53 residues. TBP binding is summarized on the right.

well as to isolated TBP. This observation indicates that the surface on TBP recognized by p53 must be available for interaction in the holo-TFIID complex, even though TBP in the complex is tightly associated with at least nine TAFs.

How is this possible? It has been proposed that TBP may directly contact only a subset of TAFs rather than all of the TAFs. Protein blotting experiments have revealed an interaction between TBP and only two large TAFs (TAF250 and TAF125) (56). The other TAFs may associate with TBP through indirect interactions with TAF250 and TAF125. Thus, TBP may lie not in the middle of the holo-TFIID complex but rather toward one edge of the complex. This would allow a surface of TBP to remain available for an

interaction with regulatory proteins. The crystal structure of an *Arabidopsis* TBP has shown that the p53 binding region on TBP (residues 220 to 273) does in fact lies on one side of the TBP surface (33). Adenovirus large E1A protein has also been shown to interact directly with holo-eTFIID (2a).

It has been shown previously that the N-terminal region of p53 (residues 1 to 73) contains a strong activation domain (10). Our results show that Gal4-p53$_{1-92}$ is also a very strong activator, comparable in strength to Gal4-VP16 (Fig. 6). The TBP binding domain has been mapped to the region between residues 20 and 57 (Fig. 5). Gal4-p53$_{1-57}$ bound to TBP in vitro similarly to Gal4-p53$_{1-92}$ in a protein blotting assay (Fig. 5B) but was a much weaker activator (Fig. 6). All
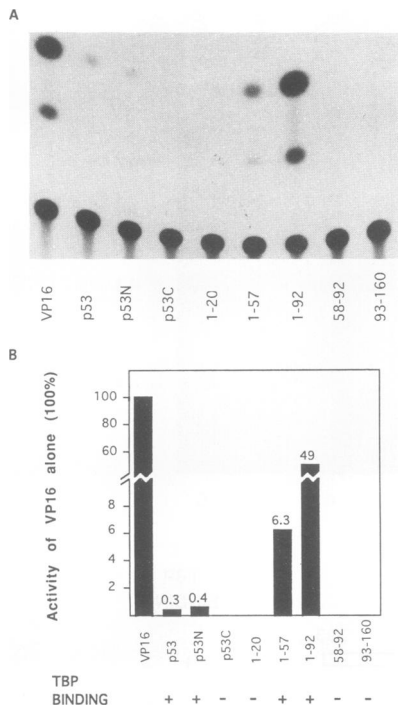
FIG. 6. Correlation of p53-TBP interaction to p53 transactivation. (A) In vivo transactivation of the G5E1B CAT gene by Gal4-p53 fusion proteins in transient transfection assays. The reporter CAT plasmid containing the E1B TATA box and five upstream Gal4 binding sites and activator plasmids (indicated below the lanes) were transfected into HeLa cells and assayed for CAT activity. (B) CAT activity quantified by counting the acetylated forms of chloramphenicol with an AMBIS β-scanning system is represented as a percentage of the activity with Gal4-VP16. Values shown are averages of three independent transfections. TBP binding activity is summarized below to show the correlation with p53 transactivation.
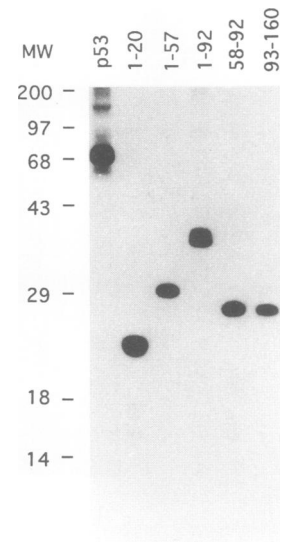

FIG. 7. Detection of Gal4-p53 fusion proteins expressed in transfected COS cells. Plasmids encoding full-length Gal4-p53 or Gal4-p53 deletion mutants were transfected into COS cells. Transfected cells were labeled with ^{35}S Trans label and double immunoprecipitated with an anti-Gal4 antibody. Immunoprecipitated Gal4-p53 proteins (as indicated above the lanes) were analyzed by gel electrophoresis and autoradiography. MW, molecular weight markers. Sizes are indicated in kilodaltons.

Gal4-p53 deletion mutants were present in the cells at comparable levels (Fig. 7). One interpretation for these observations is that the TBP interaction domains in these p53 constructs may be equally exposed in the in vitro binding assay but may have different conformations in vivo, resulting in differences in the strength of their interactions with TBP in vivo. An alternative interpretation is that the p53 activation domain may interact with other cellular proteins in addition to TBP during the activation process. The difference in activity (Fig. 6) between Gal4-p53$_{1-57}$ and Gal4-p53$_{1-92}$ suggests that the residues of p53 between 57 and 92 may play an additional role further stimulating transcription, possibly by allowing p53 to interact with these hypothetical proteins.

While this report was under review, two papers which also report the observation that p53 binds to human TBP appeared (40, 48). Using different methods, Truant et al. (48) also mapped the region of p53 which binds TBP to the amino-terminal activation domain of p53. Yet both papers report that mutations C terminal to the activation domain can inhibit TBP binding. This is probably because these mutations alter the conformation of the entire p53 protein, including the activation domain.

Our results show that the ability of Gal4-p53 proteins to activate transcription correlates well with their ability to interact with TBP in vitro (Fig. 6). This correlation is

consistent with the model that a direct interaction between p53 and TBP is required for transcriptional activation by p53. However, the strength of transcriptional activation varied greatly for the different Gal4-p53 fusion proteins studied even though they showed similar binding to TBP in vitro. In particular, extension of the region of p53 in the Gal4 fusion from residue 92 to residue 160 resulted in a reduction in CAT expression by a factor of 100. This result suggests that this region of p53 mapping between residues 92 and 160 has a strong inhibitory effect on the function of the p53 activation domain. Similar results have been seen for the c-rel protein, which contains a strong C-terminal activation domain when assayed by fusion to the DNA binding domain of Gal4. This c-rel activation domain is repressed by the c-rel N-terminal domain (3). c-rel is closely related to the Drosophila dorsal protein and the mammalian NF-κB protein, regulatory transcription factors known to be regulated by alterations in their subcellular localization and transcriptional activities by protein-protein interactions (2). The region of p53 between residues 92 and 160 may also function in the wild-type p53 protein to regulate the strong transcriptional activation domain of residues 20 to 92.

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