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Wild-type p53 binds to the TATA-binding protein and represses transcription

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ABSTRACT **p53 activates transcription of genes with a p53 response element, and it can repress genes lacking the element. Here we demonstrate that wild-type but not mutant p53 inhibits transcription in a HeLa nuclear extract from minimal promoters. Wild-type but not mutant p53 binds to human TATA-binding protein (TBP). p53 does not bind to yeast TBP, and it cannot inhibit transcription in a HeLa extract where yeast TBP substitutes for human TBP. These results suggest a model in which p53 binds to TBP and interferes with transcriptional initiation.**

The p53 gene product functions as a transcription factor. It can activate transcription when bound to a promoter through a heterologous DNA-binding domain (1, 2). Wild-type p53 (p53wt) binds to DNA sequences termed p53 response elements (3, 4), and when these binding sites are adjacent to a minimal promoter, they stimulate expression in a p53-dependent fashion (5–8). p53wt also negatively regulates a variety of genes that lack a p53 response element, including the c-*fos*, c-*jun*, retinoblastoma, interleukin 6, and proliferating cell nuclear antigen genes as well as the p53 gene itself (9–12). Expression of a class I major histocompatibility complex gene (9) and the Ha-*ras1* gene (13, 14), however, are reportedly neither activated nor repressed by p53wt. Thus, it appears that p53wt may exert positive or negative effects on the expression of some but not all genes, and this may form the mechanistic basis for its ability to regulate cell proliferation.

Here we show that p53wt, but not mutant p53 (p53mt), inhibits transcription in nuclear extracts from minimal promoters. Furthermore, p53wt can bind directly to the human TATA-binding protein (TBP). These results suggest that p53 functions as part of the transcriptional machinery, regulating transcription.

MATERIALS AND METHODS

Plasmids, Chloramphenicol Acetyltransferase (CAT) Assays, and Protein Purification. p11-4 encodes a murine p53wt (m-p53wt) cDNA and pSVKH215 contains a m-p53mt cDNA with a four-amino acid insert at residue 215; both are controlled by the simian virus 40 early promoter (15). pc53-CIN encodes a human p53wt (h-p53wt) gene containing introns 2–4, under control of the cytomegalovirus immediate-early promoter, and pc53-Cx22AN is identical to pc53-CIN except that its coding region contains an Arg-175 to His substitution (16). pTICAT (17), p50-2 (8), and pMLTATA (18) have been described. h-p53wt and His-175 h-p53mt cDNAs were subcloned into pET11d DNA (19) to create pET-p53wt and pET-p53mt. Plasmids have been described for expression in *Escherichia coli* of human TBP (pKB104; ref. 20), yeast TBP (pASY2D; ref. 21), and human transcription factor IIB (TFIIB) (phIIB; ref. 22). CAT assays were performed as described (8).

m-p53wt and KH215 m-p53mt proteins were purified from mammalian cells on an immunoaffinity matrix (23). They were ≈90% pure, as determined by gel electrophoresis followed by silver staining. h-p53wt and His-175 h-p53mt (purified from baculovirus-infected Sf27 cells by immunoaffinity chromatography; ref. 6; gift of C. Prives, Columbia University, New York) were further purified by gel filtration, isolating monomeric p53 molecules. The proteins were >95% pure. h-p53wt and His-175 h-p53mt were also prepared from *E. coli* inclusion bodies. *E. coli*-produced h-p53wt and h-p53mt proteins were about 80% and 95% pure, respectively. Human TFIIB (22) as well as human and yeast TBPs (24) were prepared from *E. coli* and further purified by gel filtration.

***In Vitro* Transcription and Analysis of p53–TBP Interaction.** Nuclear extracts prepared by the method of Dignam *et al.* (25) received 100 ng of supercoiled template DNA. Where appropriate, the extract was heated at 47°C for 15 min to inactivate endogenous TBP (26). Reaction and primer-extension conditions were as described (27); experiments were repeated three times.

For analysis of interactions by affinity chromatography, TBP or TFIIB was coupled to Affi-Gel 10. p53 proteins were incubated with the coupled proteins in buffer containing 50 mM KCl, 50 mM Hepes (pH 7.5), 0.2 mM EGTA, 0.5 mM dithiothreitol (DTT), 20% (vol/vol) glycerol, and 0.2 mg of lysozyme per ml (pI similar to that of TBP) for 40 min at 4°C. Beads were pelleted and washed with buffer, proteins were eluted in the same buffer containing increasing concentrations of KCl, residual proteins were stripped from the beads by boiling in 1% SDS, and p53 proteins were assayed by Western blot. For analysis of protein–protein interactions by gel filtration, p53 and TBP were incubated for 30 min at 25°C in buffer containing 50 mM KCl, 50 mM Hepes (pH 7.5), 0.2 mM EGTA, 0.5 mM DTT, 20% glycerol, and 0.2 mg of lysozyme per ml and loaded onto a Superose 12 column; fractions were collected and analyzed by Western blot. Analysis of interactions by protein blotting used the procedure of Horikoshi *et al.* (28).

RESULTS

p53wt Inhibits Expression from a Minimal Promoter in Transfected Cells. pTICAT (17) contains a CAT gene whose expression is directed by only two elements: the initiator sequence from the terminal deoxynucleotidyltransferase gene (29) and the TATA motif from the adenovirus major late

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Abbreviations: TBP, TATA-binding protein; CAT, chloramphenicol acetyltransferase; p53wt, wild-type p53; p53mt, mutant p53; m-, murine; h-, human; TFIIB, transcription factor IIB.

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promoter. The effect of p53 on pTICAT expression was studied in human SAOS-2 cells, which do not contain endogenous p53 (30). Cotransfection of SAOS-2 cells with pTICAT plus p11-4 (m-p53wt) caused a marked decrease (≈20-fold) in CAT expression (Fig. 1*A*, compare lanes 1 and 2 to lanes 3 and 4). Cotransfection with pTICAT plus pSVKH215 (m-p53mt) caused only a modest decrease (≈2.5-fold) in CAT expression (Fig. 1*A*, compare lanes 1 and 2 to lanes 5 and 6). m-p53mt exhibited significantly less inhibitory activity than the wild-type protein. In fact, this mutant p53 and others often produced no inhibitory effect in experiments in which a strong suppression was mediated by the wild-type protein. Like the murine protein, h-p53wt repressed expression from pTICAT, while a h-p53mt did not (Fig. 1*B*). The inhibition caused by p53wt was not due to nonspecific toxicity. Cotransfection of SAOS-2 cells with p50-2 (a derivative of pTICAT with two p53 DNA-binding sites) and p11-4 (m-p53wt) led to enhanced CAT expression as compared to transfection with pTICAT alone (8). Thus, p53 can activate promoters that include a p53 response element and inhibit a minimal promoter lacking the site.

p53wt but Not p53mt Represses Transcription *in Vitro*. To demonstrate that the effect of p53 was direct and occurred at the level of transcription, we tested the ability of purified p53 to influence *in vitro* transcription. Nuclear extracts were prepared from HeLa cells, which contain low endogenous levels of p53 (31). When 50 ng of m-p53wt (a temperature-sensitive protein purified in its wild-type conformation from transformed A1 rat cells; ref. 32) was added to a reaction mixture containing pTICAT as template, the level of correctly initiated transcripts decreased (maximal repression was ≈8-fold: Fig. 2*A*, compare lane 6 to lanes 7 and 8). Neither m-p53wt that was denatured by heating (Fig. 2*A*, lane 7), p53 buffer (Fig. 2*A*, lane 8), nor m-p53mt (Fig. 2*B*) inhibited transcription. h-p53wt (purified from baculovirus-infected cells) also repressed the minimal promoter (Fig. 2*C*, lanes 3–6); 25.5 ng completely blocked detectable transcription. Heated h-p53wt (Fig. 2*C*, lane 7) and h-p53mt (Fig. 2*C*, lane 8) failed to inhibit.

The ability of p53wt to influence transcription was also tested using p50-2 as template (p53 response element upstream from the minimal promoter in pTICAT). *In vitro* transcription directed by the p50-2 template was not altered by m-p53wt (Fig. 2*D*, lanes 1 and 2), arguing that the repression of pTICAT promoter activity was not due to a nonspecific toxic effect of the m-p53. In contrast to m-p53, h-p53wt stimulated transcription from the p50-2 template (Fig. 2*D*, lanes 4–7). This result fits with earlier findings that p53wt can stimulate transcription from p50-2 within transfected cells (8) and can stimulate the activity of promoters containing a p53 response element in cell-free extracts (6). The different response of p50-2 to h-p53 as compared to m-p53 might be due to the oncogene product mdm-2 (33, 34), which is present in the m-p53 but not h-p53 preparations. The mdm-2 protein forms a complex with p53 and blocks its ability to induce transcription through a p53 response element in transfected cells (23).

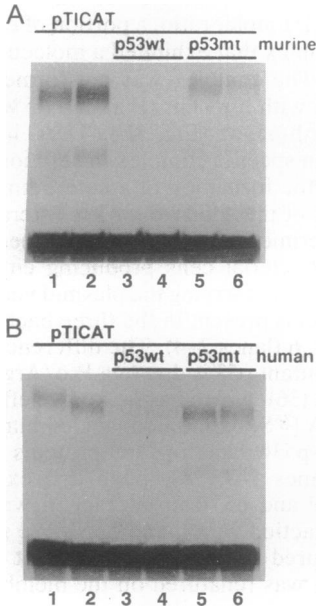

FIG. 1. Inhibition of a minimal promoter by p53. Duplicate SAOS-2 cultures were transfected with pTICAT alone or together with a plasmid expressing murine (*A*) or human (*B*) p53, and CAT activity was assayed 72 hr later.

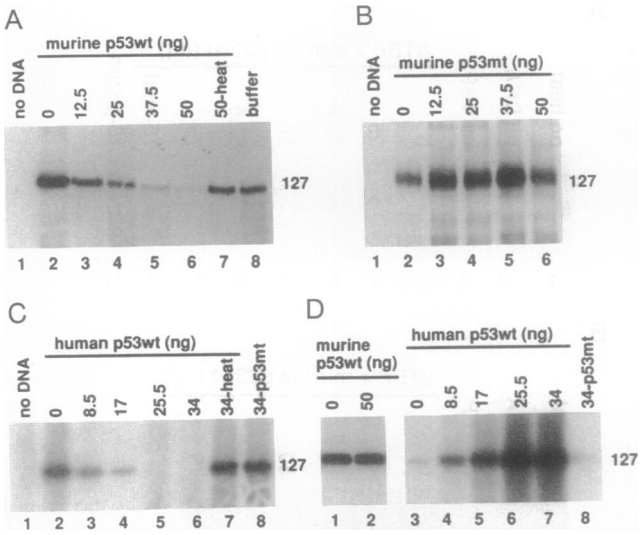

FIG. 2. Repression of transcription in HeLa extracts by p53wt. Each reaction mixture received 100 ng of supercoiled pTICAT plus the indicated amounts of p53. Primer-extension reactions generated 127-nucleotide products. Controls received denatured (100°C; 20 min) p53wt (50-heat) or buffer. (*A*) Inhibition of pTICAT transcription by m-p53wt. (*B*) No inhibition of pTICAT transcription by m-p53mt. (*C*) Inhibition of pTICAT transcription by h-p53wt but not h-p53mt. (*D*) No inhibition of p50-2 transcription by 50 ng of m-p53wt and stimulation by h-p53wt.

In sum, m-p53wt or h-p53wt inhibited transcription from a minimal promoter, while two different mutant proteins had no effect.

p53 Interferes with Reactivation of Transcription by Human but Not Yeast TBP. The ability of p53 to suppress transcription directed by a minimal promoter raised the possibility that p53 might interact with TBP. To probe this hypothesis, TFIID activity was selectively inactivated (26) in a HeLa nuclear extract by heat treatment (Fig. 3*A*, compare lanes 1 and 3). The decrease in transcription was partially rescued by the addition of recombinant human TBP (lane 4). Maximal restoration of activity was achieved by adding 20 ng of TBP. However, when human TBP was incubated with 50 ng or more of m-p53wt before addition to the heated extract, its ability to restore transcription was blocked (lanes 5–9). We also tested the ability of m-p53wt to inhibit restoration of transcription by yeast TBP. Fifteen nanograms of yeast TBP produced maximal restoration of transcriptional activity, and p53wt had no effect on transcription mediated by the yeast protein. These results indicate that human but not yeast TBP is a target of p53wt-mediated repression.

p53wt Can Bind to Human but Not Yeast TBP. Since p53wt was able to interfere with the function of human TBP (Figs. 2 and 3), we tested whether the two proteins formed a

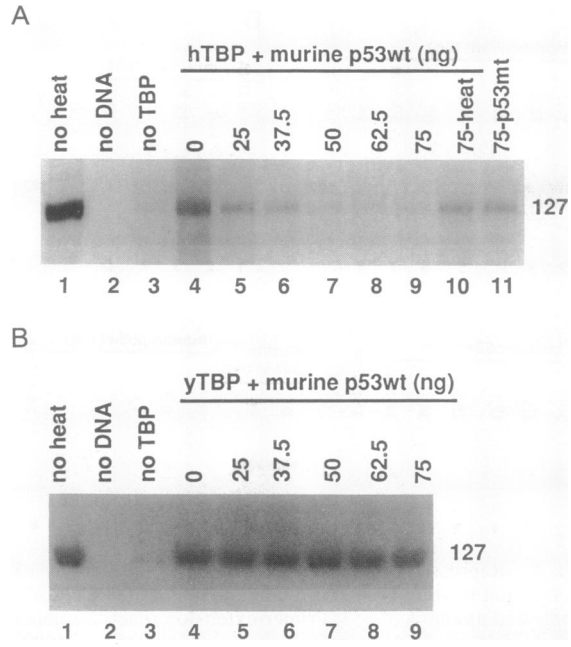

FIG. 3. Inhibition of TBP function in HeLa nuclear extracts by p53wt. Each reaction mixture received 100 ng of supercoiled pTICAT plus the indicated amounts of m-p53wt. Primer-extension reactions generated 127-nucleotide products. (*A*) m-p53wt inhibits reactivation of a heated extract by 20 ng of human TBP (hTBP). Controls received TBP plus heat-denatured p53wt (75-heat) or m-p53mt (75-p53mt). (*B*) m-p53wt does not inhibit reactivation of a heated extract by 15 ng of yeast TBP (yTBP).

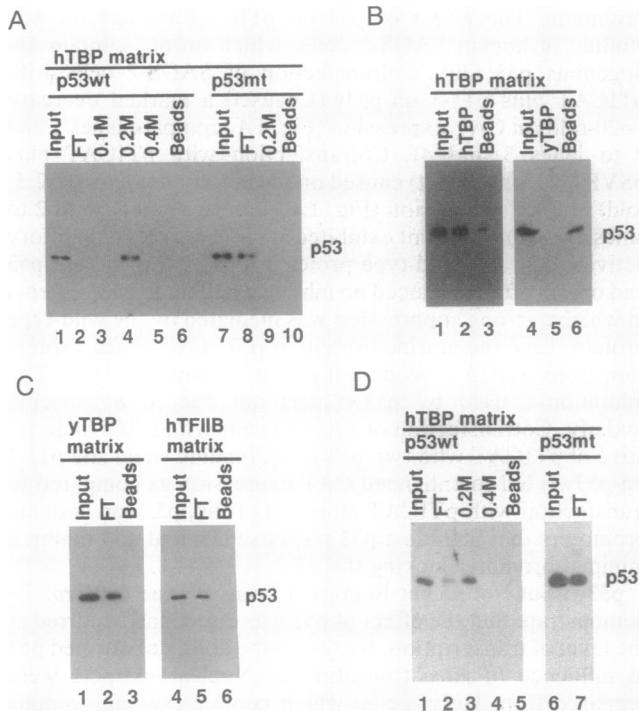

FIG. 4. h-p53wt binds to human TBP immobilized on beads. p53 was assayed by Western blot using monoclonal antibody PAB421. (*A–C*) Baculovirus-produced p53. (*D*) *E. coli*-produced p53. (*A*) h-p53wt but not h-p53mt binds to human TBP (hTBP). h-p53wt or h-p53mt (Input) (50 ng) was applied to hTBP beads [flowthrough (FT)]; sequentially eluted with buffer containing 0.1, 0.2, or 0.4 M KCl; and then the beads were boiled in 1% SDS (Beads). (*B*) h-p53wt is eluted from hTBP by soluble hTBP but not yeast TBP (yTBP). p53wt (50 ng) was applied to hTBP beads, protein was eluted by incubation with 500 ng of purified hTBP or yTBP, and then the beads were boiled in 1% SDS. (*C*) p53wt does not bind to yTBP or human TFIIB (hTFIIB). p53wt (70 ng) was applied to TBP or hTFIIB beads; the beads were washed and then boiled in 1% SDS. (*D*) p53wt but not p53mt produced in *E. coli* binds to hTBP. p53 (100 ng) was applied to TBP beads and processed as in *A*.

complex. Purified human TBP (produced in *E. coli*) was bound to Affi-Gel beads, producing an affinity matrix. When h-p53wt (purified from baculovirus-infected insect cells) was applied to the TBP matrix in buffer containing 50 mM KCl, 70–95% of it was retained in three independent experiments. It remained bound in buffer containing 0.1 M KCl and was eluted in 0.2 M KCl (Fig. 4*A*, lanes 1–6). In contrast, h-p53mt did not interact with the human TBP matrix (Fig. 4*A*, lanes 7–10). The specificity of the interaction was confirmed by first binding h-p53wt to the TBP matrix and then washing with binding buffer containing a 100-fold excess of human TBP. In three independent experiments, 75–85% of the bound h-p53wt was displaced from the TBP matrix and eluted with human TBP (Fig. 4*B*, lanes 1–3), while none was eluted using yeast TBP (Fig. 4*B*, lanes 4–6). h-p53wt did not bind at detectable levels to either yeast TBP or human TFIIB immobilized on beads (Fig. 4*C*).

The human TBP affinity matrix experiment was repeated with h-p53 produced in *E. coli* rather than in baculovirus-infected insect cells. By producing both members of the complex in *E. coli*, the possibility that a third eukaryotic protein mediated the binding was eliminated. In three independent experiments, 70–80% of the *E. coli*-produced h-p53wt was retained on the human TBP affinity matrix (Fig. 4*D*, lanes 1–3). The bound p53 was eluted by 0.2 M KCl (Fig. 4*D*, lane 3), consistent with the results obtained using protein produced in baculovirus-infected cells (Fig. 4*A*). As expected, p53mt (His-175) produced in *E. coli* was not retained on the TBP matrix (Fig. 4*D*, lanes 6 and 7). Thus, h-p53wt but not h-p53mt bound specifically and directly to human TBP on beads. The TBP–p53 interaction is weak in terms of its resistance to salt, but it was efficient in that most of the p53 was bound.

As a second test of the ability of p53wt to bind human TBP, the purified monomeric proteins, either individually or after mixing, were subjected to gel filtration on an HPLC Sepharose 12 column. Either human TBP alone or h-p53wt alone eluted from the column in the vicinity of a 43-kDa marker protein (Fig. 5 *A* and *B*). However, when the two proteins were mixed in a 1:1 molar ratio, a portion of each protein was present in a complex that exhibited a molecular mass of ≈92 kDa (Fig. 5*C*). The complex was not formed when human TBP was mixed with h-p53mt (Fig. 5*D*) or when yeast TBP was mixed with h-p53wt (Fig. 5*E*). Thus, human TBP and p53wt can form a specific complex in solution, whose size is consistent with the formation of a heterodimer.

As a final test of the ability of p53 to interact with TBP, a protein blot experiment (28, 35) was performed. Lysates were prepared from bacterial cells producing either h-p53wt or h-p53mt or from cells carrying the plasmid vector with no p53 insert. The proteins present in the three bacterial strains are displayed in Fig. 6 (lanes 2–4). The difference in mobility of wild-type and mutant p53 is due to a Pro/Arg polymorphism at amino acid 72 (36), which has no known effect on function of the protein. A [^{35}S]methionine-labeled human TBP probe bound to both h-p53wt and h-p53mt proteins immobilized on a membrane (lanes 5–7). Although this experiment again argues that TBP and p53 can interact, it was surprising to observe an interaction with mutant p53. We suspect that the normally denatured mutant protein, or at least its TBP-binding domain, was renatured on the membrane.

DISCUSSION

p53 inhibits expression directed by a variety of promoters within transfected cells (9–12), and we have extended this

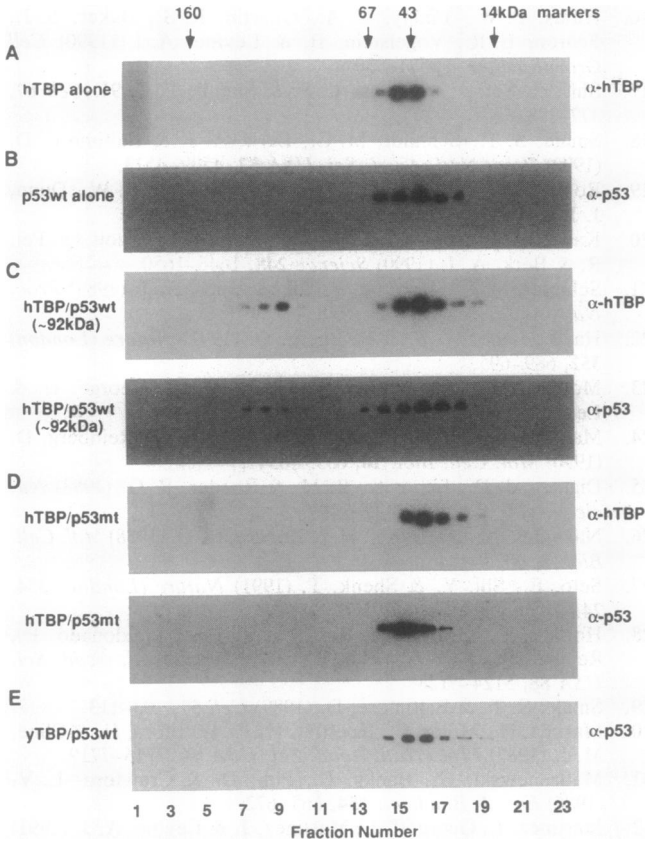

FIG. 5. h-p53wt binds to hTBP in solution. TBP and p53 monomers were incubated alone or in combination and subjected to gel filtration; fractions were assayed by Western blot using monoclonal antibody PAb421 (α-p53) or a TBP-specific polyclonal antibody (α-hTBP). IgG (160 kDa), albumin (67 kDa), ovalbumin (43 kDa), and cytochrome c (14 kDa) were markers. (A) hTBP alone. (B) p53wt alone. (C) hTBP plus p53wt. (D) hTBP plus p53mt. (E) yTBP plus p53wt.

observation to a well-defined, minimal promoter (Fig. 1). Our experiments provide several mechanistic insights to this inhibition. First, the inhibition was reproduced in cell-free extracts (Fig. 2), demonstrating that p53 acts directly to repress transcription. Both m-p53wt and h-p53wt proteins were able to mediate repression of a minimal promoter (Fig. 2 A and C), while two p53mt proteins had no effect (Fig. 2 B and C). The repression was not due to nonspecific inhibition by p53wt, since a promoter containing a p53 response element was not inhibited and could be stimulated by the human protein (Fig. 2D). Second, TBP is a target of p53-mediated repression. p53wt interfered with the ability of human TBP, but not yeast TBP, to reactivate a heat-treated extract (Fig. 3). Third, p53wt can bind to human, but not yeast, TBP (Figs. 4 and 5). The failure of p53 to bind yeast TBP is consistent with its inability to inhibit activity of the yeast protein (Fig. 3B). The interaction with human TBP was shown using three assays: binding of p53 to TBP immobilized on beads (Fig. 4), binding of p53 to TBP in solution followed by gel-filtration chromatography (Fig. 5), and binding of TBP to p53 immobilized on a membrane (Fig. 6). The binding is direct since purified p53 and TBP produced in E. coli interact (Fig. 4D), and the size of the p53–TBP complex (92 kDa) suggests that it is a heterodimer (Fig. 5).

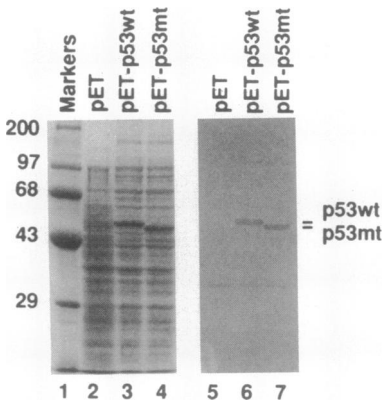

FIG. 6. Human TBP binds to p53 immobilized on a membrane. Extracts were prepared from E. coli containing vector alone (pET), vector expressing h-p53wt (pET-p53wt), or h-p53mt (pET-p53mt). After electrophoresis, proteins were visualized by Coomassie blue stain (lanes 2–4) or transferred to a membrane and probed with [^{35}S]methionine-labeled TBP (lanes 5–7). Size markers are in kDa.

p53 contains an acidic activating domain (1, 2). The acidic activating domain of VP16 can inhibit transcription through an apparently nonphysiological process termed squelching (37). High levels of VP16 are believed to bind and sequester one or more basal transcription factors, blocking their ability to enter into transcription complexes and inhibiting transcription of promoters that lack a VP16 binding site. It is unlikely that squelching can account for p53-mediated repression. If the acidic domain of p53 squelched by a mechanism similar to that documented for the acidic domain of VP16, then the two proteins might be expected to inhibit transcription at roughly similar concentrations. While 25–50 ng of wild-type p53 markedly inhibited transcription (Fig. 2), a 75-fold molar excess (3 μg per reaction mixture) of GAL4-VP16 protein (contains the DNA-binding domain of the yeast GAL4 activator fused to the VP16 acidic activating domain; ref. 38) had no effect on transcription of a template lacking a GAL4 DNA-binding site in the HeLa nuclear extract (data not shown). Furthermore, if p53 squelched, it would likely inhibit all promoters that lack a p53 response element. This is not the case; a class I major histocompatibility complex promoter (9) and the Ha-ras1 promoter (13, 14) are neither activated nor repressed by p53.

Taken together, our results suggest that p53 binds to TBP and reduces the efficiency of transcriptional initiation. p53 might contain a domain that actively interferes with some aspect of initiation when it is bound to TBP, or it could block another, positive acting factor from binding to TBP.

How might the ability of p53 to repress transcription by binding to TBP lead to specific and useful regulation of gene expression? Perhaps the extent of repression is modulated by additional factors that have DNA-binding sites within the promoter and the ability to bind to TBP. Such a protein could bridge from its DNA-binding site to TBP and conceivably exclude p53 from the transcription complex. A promoter would be more or less sensitive to p53 repression, depending on the factors that bind to it. Alternatively, p53 repression may be global. p53 functions to suppress cell growth under certain conditions, and part of the mechanism by which it suppresses growth could be the repression of a wide variety of promoters that lack p53 response elements.

Two p53mt proteins (murine KH215 and human His-175) with transforming activity (16, 39) failed to repress (Fig. 2) or interact with TBP (Figs. 4 and 5; data not shown). We do not know whether all mutant p53s with transforming activity will behave in a similar fashion. Nevertheless, our experiments raise the possibility that the transforming activity of mutant p53s results in part from loss of the ability to bind TBP and repress transcription. In fact, given earlier results showing these mutants fail to stimulate through p53 response elements (6–8), transformation by p53mt might involve reduced expression of genes normally stimulated by p53wt as well as enhanced expression of other genes normally repressed by

p53wt. It is conceivable that the primary function of p53 is to regulate transcription, and the direct consequence of p53 mutations is the abnormal expression of a variety of cellular genes.

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