Cooperative DNA binding of p53 with TFIID (TBP): a possible mechanism for transcriptional activation

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The p53 tumor-suppressor gene product, a sequence-specific DNA-binding protein, has been shown to act both as a transcriptional activator and repressor in vivo and in vitro. Consistent with its roles in regulating transcription are recent observations that p53 binds directly to the TATA box-binding protein (TBP) subunit of the basal transcription factor TFIID. Here, we show that p53 cooperates with either recombinant TBP or partially purified TFIID in binding to a DNA fragment containing both a specific p53-binding site (RGC) and a TATA box (RGC–TATA). Surprisingly, both TBP and TFIID also stimulate p53 binding to DNA containing a specific p53-binding site but lacking a TATA box. These data are supported by the observation that p53 and Drosophila TBP combinatorially activate transcription in vivo. Our results suggest that p53 activates transcription through the formation of a more stable p53–TFIID–promoter complex. We also examined whether p53 might affect the ability of TBP or TFIID to interact with DNA containing a TATA box but lacking a p53-binding site. Although p53 strongly inhibited the interaction of TBP with such DNA, it had virtually no effect on TFIID binding. Thus, transcriptional repression by p53 may require additional functions other than inhibiting TBP binding.

[Key Words: p53; TBP and TFIID; cooperative DNA binding; transcriptional activation and repression]

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Molecular and cytogenetic studies have provided convincing evidence that accumulation of wild-type p53 inhibits the proliferation of many types of cells [Michalovitch et al. 1990; Martinez et al. 1991; Bischoff et al. 1992; Kuerbitz et al. 1992; Lin et al. 1992; Nigro et al. 1992]. This is consistent with recent evidence that p53 may serve as a checkpoint function in causing cells to arrest at G1/S [Kastan et al. 1992; Livingstone et al. 1992; Yin et al. 1992, for review, see Lane 1992; Hartwell 1992]. p53 has been shown to activate transcription of constructs bearing p53-binding sites in vivo and in vitro [Aoyama et al. 1992; Farmer et al. 1992; Funk et al. 1992; Kern et al. 1992; Mietz et al. 1992; Scharer and Iggo 1992; Zambetti et al. 1992]. However, transcription of a number of other promoters that lack p53-binding sites is inhibited by p53 [Ginsberg et al. 1991; Santhanum et al. 1991; Chin et al. 1992; Seto et al. 1992; Subler et al. 1992; Jackson et al. 1993]. Therefore, p53 may selectively activate or repress expression of certain classes of genes that are involved in control of the cell cycle. Understanding the mechanism by which p53 regulates transcription should provide insight into its ability to control cell growth.

Transcriptional initiation of class II genes requires several general or basal transcription factors, which, in addition to RNA polymerase II (Pol II), include TFIIA, TFII B, TFIID, TFII E, TFII F, and TFII H (for review, see Zawel and Reinberg 1993). Initiation, a multistep process that is the primary target of transcriptional regulation, is largely controlled by activator proteins targeted to the promoter region by sequence-specific DNA binding [Gill and Ptashne 1988; Mitchell and Tjian 1989; Zawel and Reinberg 1993]. It is assumed that such activator proteins, when bound to DNA, interact with one or more of the general transcription factors or with an intermediary factor, thus accelerating a rate-limiting step of transcriptional initiation. For example, VP16 [Stringer et al. 1990; Ingles et al. 1991], Ela [Horikoshi et al. 1991; Lee et al. 1991], and Zta [Lieberman and Berk 1991] were shown to bind directly to the TATA box-binding protein (TBP), a subunit of TFIID. Alternately, VP16 [Lin and Green 1991; Lin et al. 1991] and several members of the steroid receptor family [Ing et al. 1992] bind to TFIIB directly, and hybrid proteins GAL4–AH and GAL4–VP16 containing a GAL4 DNA-binding domain fused to either a 15-amino-acid amphipathic α-helix peptide or a VP16 activation domain recruit TFIIB to a preinitiation transcription complex [Lin and Green 1991; Lin et al. 1991]. In addition, Sp1 interacts specifically with a TBP-associated factor (TAF), TAF110 [Hoey et al. 1993], a coactivator that is required for factor-activated transcription but not basal transcription (for review, see Gill and Tjian 1992). Earlier experiments performed with partially purified human TFIID showed that transcriptional activa-
tors USF, ATF, and GAL4 extend TFIID DNase I protection over the region downstream of the TATA box (Sawadogo and Roeder 1985; Horikoshi et al. 1988a, b) and that TFIID reduces the off-rate of USF (Sawadogo and Roeder 1985).

The mechanisms by which transcriptional repressors function differ from those of activators. For examples, Id (Benezra et al. 1990) and IκB (Baeuerle and Baltimore 1988) repress specific gene transcription by sequestering site-specific DNA-binding proteins. The Drosophila homeo domain Even-skipped protein (Eve) represses all promoters that contain Eve-binding sites, including both TATA-containing and TATA-lacking minimal promoters, as well as promoters activated by several different classes of activator proteins, suggesting that the general transcription machinery is a target of Eve (Han and Manley 1993). However, the general transcriptional repressors NC1 (Meisterernst et al. 1991), NC2 (Meisterernst and Roeder 1991), and Drl (Inostroza et al. 1992) interact with TBP, precluding the association of TFIID or TFIIB, or both, with TBP and, thereby, inhibiting transcriptional initiation.

Seto et al. (1992), Truant et al. (1993), Liu et al. (1993), and Martin et al. (1993) used affinity chromatography, protein blotting, and immunoprecipitation to show that TBP interacts directly with p53. Importantly, Liu et al. (1993) showed that the TAF-associated TBP holoprotein TFIID also associates with p53. It was inferred from these studies that this interaction confers on p53 the ability to activate or repress transcription. However, experiments to follow the biochemical changes resulting from the p53–TBP interaction were not reported. Furthermore, it is important to compare the relative effects of recombinant TBP protein with the TFIID holoprotein. Here, we describe results showing that the interaction between p53 and TBP or TFIID changes their respective DNA-binding properties.

**Results**

**Cooperative binding is seen when both p53 and TBP bind directly to DNA**

We used the gel electrophoretic mobility shift assay (EMSA) to determine whether any changes in DNA-binding activities can be detected when both p53 and TBP bind to their respective DNA-binding sites (Fig. 1A). The probe used in the assay, RGC–TATA, was a 32P-labeled DNA fragment from fosIwt, a plasmid template used previously to show that p53 activates transcription in vitro (Farmer et al. 1992). p53 and TBP bound individually to RGC–TATA (Fig. 1A, lanes 2, 3). Generally, in the presence of TBP, a single protein–DNA complex was detected, whereas p53 alone resulted in two discrete complexes. The presence of two (or more) p53–DNA complexes has been observed previously in gel band-shift assays (Bargonetti et al. 1992) and is most likely related to the fact that immunopurified p53 preparations consist predominantly of tetramers and multiples of tetramers (Stenger et al. 1992; Friedman et al. 1993). When both p53 and TBP were present, the mobility and relative amounts of DNA bound (Fig. 1A, cf. both free and bound DNA in lane 4 with that in lanes 2 and 3). Previously, TFIID was shown to be incapable of supporting in vitro reconstituted transcription after heat treatment at 47°C for 15 min (Nakajima et al. 1988). When p53 and TBP were inactivated by heat treatment at 95°C for 5 min and at 47°C for 15 min, respectively, no cooperative binding was observed (Fig. 1A, cf. lanes 5 and 6 with lane 4). In addition, wild-type but not mutant TATA box-containing oligonucleotides blocked this cooperative DNA binding of p53 plus TBP (Fig. 1A, cf. lane 4 with lanes 7 and 8), further confirming

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**Figure 1.** Cooperative DNA binding of TBP with p53. EMSA was performed as described in Materials and methods with 32P-labeled RGC–TATA (A) SRE–TATA (B), and GAL4–TATA (C) DNA fragments (0.3 ng). A, TBP (1 ng) and p53 (15 ng) were used as indicated above each lane. Heat-treated p53 (95°C for 5 min) and TBP (47°C for 15 min) were added to reaction mixtures in lanes 5 and 6, respectively. Mutant (MtTATA) and wild-type (Wt-TATA) TATA box-containing double-stranded oligonucleotides (1 ng) were used as competitors in reactions in lanes 7 and 8, respectively. B, SRF (1 ng) and TBP (1 ng) were used as indicated above each lane. Mutant (MtTATA) and wild-type (Wt-TATA) TATA box-containing double-stranded oligonucleotides (1 ng) were used as competitors in reactions in lanes 5 and 6, respectively. B, SRF (1 ng) and TBP (1 ng) were used as indicated above each lane. Mutant (MtTATA) and wild-type (Wt-TATA) TATA box-containing double-stranded oligonucleotides (1 ng) were used as competitors in reactions in lanes 5 and 6, respectively.
that the increased binding is the result of a specific interaction between the two proteins.

Using the probes SRE–TATA and GAL4–TATA, which were generated by placing the serum response factor [SRF] DNA-binding site [serum response element [SRE]] and the GAL4–VP16-binding site [GAL4] upstream of the c-fos promoter [Zhu et al. 1991], respectively, no evidence for cooperative DNA binding was found between SRF and TBP, or GAL4–VP16 and TBP (Fig. 1B,C, cf. lane 3 with lanes 2 and 4, respectively). GAL4–VP16 appeared to inhibit somewhat the overall DNA-binding affinity of TBP to TATA box [Fig. 1C, cf. lanes 2 and 3]. Although GAL4–VP16 interacts with TBP [Ingles et al. 1991, Stringer et al. 1990], no cooperative DNA binding has been reported. Similarly, no evidence of a direct interaction between SRF and TBP has been found (H. Zhu, V. Joliot, and R. Prywes, in prep.).

Further characterization of the interaction between p53 and TBP is shown in Figure 2. Protein–DNA complexes formed with a range of TBP and a constant amount of p53 [Fig. 2A] or, reciprocally, with varying p53 and constant TBP [Fig. 2B] show extensive cooperation between the two proteins. For example, even when there was only marginal binding of TBP alone to the RGC–TATA fragment, there were dramatic differences in the complexes detected when p53 was present [Fig. 2A, cf. lanes 9–12 with lanes 3–6]. Quantitation by Phospho-rlming analysis was performed for several experiments, such as that shown in Figure 2, A and B, with either a constant amount of p53 and an increased amount of TBP or a constant amount of TBP and an increased amount of p53. These results showed that the DNA-binding ability of p53 and TBP together to RGC–TATA engenders increased binding by values ranging from 50% to greater than fourfold more than the additive binding of p53 and TBP individually (data not shown).

To identify further the proteins present in the complexes, purified monoclonal antibodies specific for p53 and TBP were used in "supershift" experiments. The p53-specific antibody, PAbl801, was shown previously to supershift p53–DNA complexes [Bargonetti et al. 1992]. To help us assess the presence of TBP in the complexes, several TBP monoclonal antibodies were generously provided by N. Hernandez (Cold Spring Harbor Laboratory). Of this series, one, SL-39, clearly supershifted TBP bound to DNA (Fig. 2C). When both TBP and p53 were bound to the RGC–TATA fragment, addition of the p53 and TBP antibodies each caused unique changes in the complexes tested (Fig. 2D). The p53 monoclonal antibody caused the supershifting of both the rapidly migrating [lower] and the more slowly migrating [upper] complexes, suggesting that each complex detected contained p53 and also that the antibody did not disrupt any p53-containing complexes [Fig. 2D, cf.

Figure 2. Characterization of the cooperative DNA binding of p53 and TBP. EMSA was performed as in Fig. 1. The probe used was RGC–TATA (0.3 ng). (A) p53 (15 ng) was used as indicated above each lane. The amount of TBP used was 0.125 ng [lanes 3,9], 0.25 ng [lanes 4,10], 0.5 ng [lanes 5,11], 1.0 ng [lanes 6,12], 2.0 ng [lanes 7,13], and 4.0 ng [lanes 8,14]. (B) TBP (1.0 ng) was used as indicated above each lane. The amount of p53 used was 15 ng [lanes 3,5] and 30 ng [lanes 4,6]. (C) TBP (1.0 ng) was used as indicated above each lane. Purified monoclonal anti-TBP (1 μl) was used as indicated above each lane. (D) TBP (1.0 ng) and p53 (15 ng) were used as indicated above each lane. PAbl801 or anti-TBP (1 μl) were used for lanes 4 and 5, respectively.
p53 and TBP or TFIID change each others’ interactions with DNA

To analyze further the cooperative DNA binding shown by EMSA, DNase I footprinting analysis was performed with the RGC-TATA fragment. In this experiment we analyzed the ability of both recombinant TBP and partially purified TFIID to cooperate with p53. Characterization of the TFIID used for the experiments in this study is described in Materials and methods. In contrast to the EMSA analysis where experiments were performed in probe excess, DNase I footprinting requires conditions such that the vast majority of the DNA probe is bound. Hence, larger quantities of the proteins were used, although we determined that a relatively greater quantity of recombinant TBP than of TBP in the TFIID fraction were required for efficient protection. As shown previously {Horikoshi et al. 1988a, b; Lieberman and Berk 1991}, both TBP and TFIID bound to and protected the TATA region (Fig. 3A, B, cf. lanes 1 and 2). Increasing concentrations of p53 led to increased protection of the RGC site (Fig. 3A, B, cf. lane 1 with lanes 7–10). Comparing the region of RGC protected by p53 in the presence or absence of TBP and TFIID revealed somewhat unexpected differences in that both TBP and TFIID increased the ability of p53 to protect RGC from DNase I cleavage (Fig. 3A, B, cf. lanes 3–6 with lanes 7–10). We determined that both TBP and TFIID reduced by a factor of at least four the amount of p53 required to fully protect its binding site. In addition, strikingly different effects of p53 on TATA region protection by TBP or TFIID were observed. p53, in a dose-dependent manner, decreased the affinity of TBP for the TATA box, and an area of hypersensitive DNase I cleavage occurred at a region downstream of the TATA box as indicated by the open rectangle [Fig. 3A, cf. lane 2 with lanes 3–6]. In contrast, p53 slightly increased the affinity of TFIID for the TATA box and also caused extended DNase I cleavage over the regions surrounding the TATA box as indicated by the solid bars [Fig. 3B, cf. lane 2 with lanes 3–6]. Similar results were obtained with DNase I footprinting analysis in which increasing concentrations of TBP or TFIID were used with a fixed amount of p53 (data not shown).

Binding of TBP to a p53–RGC complex

Our results, showing that TBP and TFIID both increase p53 protection of the RGC site on the RGC–TATA fragment, led us to ask whether either preparation might affect p53 binding to RGC DNA that lacks a TATA box. Using a 100-bp BamHI–KpnI DNA fragment containing the RGC sequence, DNA binding by p53 in the presence of increasing amounts of TBP was examined by EMSA (Fig. 4A). When both p53 and TBP were present, more binding was observed than with p53 alone, and an additional complex migrating slightly more slowly than the p53–RGC complex was seen. With a constant amount of p53 and an increased amount of TBP, EMSA showed that more total shifted DNA radioactivity and more slowly migrating band containing p53–TBP–DNA complex were observed (Fig. 4A, cf. lane 1 with lanes 2–4). This new complex presumably contains p53 and TBP. At the highest concentration of TBP, a second, still more slowly migrating, shifted complex could be discerned. The rapidly migrating complex apparent in all lanes that contain TBP (below the p53 band) was seen only with this preparation of TBP and thus was not considered significant. When TBP was inactivated by heat treatment at 47°C for 15 min, the upper complex disappeared (Fig. 4B, lane 4), confirming that TBP is a component of the new complex. Furthermore, as shown in Figure 4B, oligonucleotides containing wild-type, but not mutant, TATA box sequences blocked the binding of TBP to the p53–RGC complex. To confirm that both bands contained p53, the p53-specific monoclonal antibody, PAb1801, was used (Fig. 4C). PAb1801 supershifted both bands, indicating that both bands contain p53. To further confirm that the upper band contains TBP, an anti-TBP poly-
Cooperative DNA binding of p53 and TFIID

Figure 4. Binding of TBP to a p53–RGC complex. EMSA was performed by using 0.3 ng of 100-bp RGC probe that contains the p53-binding site (RGCJ. (A) p53 (15 ng) was used as indicated above each lane. The amount of TBP used was 0.5 ng (lanes 2, 5), 1.0 ng (lanes 3, 6), and 2.0 ng (lanes 4, 7). (B) p53 (15 ng) and TBP (1 ng) were used as indicated above each lane. In lane 4, TBP was heat treated at 47°C for 15 min before addition to the reaction mixture. Mutant (Mt TATA) and wild-type (Wt TATA) TATA box-containing double-stranded oligonucleotides (1 ng) were used as competitors in lanes 5 and 6. Lane 7 contains DNA probe without protein. (C) p53 (15 ng) and TBP (1 ng) were used as indicated above each lane. p53-specific monoclonal antibody PAb1801 (100 ng) and polyclonal anti-TBP antibody (1 μl) were added to reaction mixture (lane 5, 6, respectively). Lane 1 contains DNA probe without protein. (D) The transcription factors used, as indicated above each lane, were TBP (1 ng), TFIIB (2.5 ng), TFIIEα (20 ng), TFIIEβ (30 ng), RAP30 (10 ng), and RAP74 (14 ng). p53 (15 ng) was used for lanes 2–8. Lane 14 contains probe DNA without protein.

Clonal antibody (clu-54) was raised and was found to supershift TBP–DNA complex and not to bind to DNA itself. However, the supershifted band was too big to enter the gel as indicated by increased retained DNA in the well [data not shown]. The anti-TBP polyclonal antibody also supershifted the p53–TBP–DNA complex although, again, no distinct new band appeared but more DNA was retained in the well (Fig. 4C, cf. lanes 4 and 6).

Quantitation of several experiments showed that TBP was capable of increasing p53 binding to RGC over a range extending from less than twofold (Fig. 4A, cf. lanes 1 and 2) to greater than fourfold (Fig. 4C, cf. lanes 2 and 4), depending on the amount of p53 used. Generally more marked effects were observed when lesser amounts and, hence, more limiting quantities, of p53 were used. It should also be mentioned here that when we set out
originally to examine the effects of TBP on p53 binding to the RGC site we used double-stranded oligonucleotides containing 35 bp. Under these conditions adding TBP actually caused a marked decrease in the amount of detectable DNA complexes (data not shown). Possibly the size or shape of the p53–TBP complex cannot associate effectively or stably with a smaller DNA fragment.

VP16, an acidic activator, interacts with TFIIB in addition to TFIID (TFIID). p53, whose activation domain is also acidic, may interact with additional general transcription factors. Therefore, EMSAs were performed with a number of additional recombinant basal transcriptional factors. These revealed that although TBP interacted with p53 producing an additional DNA–protein complex, TFIIB, TFIIE, TFIIF, RAP30 (TFIIFα), and RAP74 (TFIIFβ) did not (Fig. 4D). These results suggest further that the interaction of p53 with TBP is specific, although they do not rule out the possibility that p53 may interact with other basal factors not tested here, or that p53 interacts with those tested but such interaction does not influence the p53 DNA-binding complex, or that these interactions cannot be detected by EMSA.

**TFIID increases p53 binding to RGC**

On the basis of our observations with recombinant TBP, it was of interest to determine whether TFIID also stimulates p53 DNA binding when TFIID is not bound to the TATA box. Using EMSA, we showed that partially purified TFIID did not bind to RGC (Fig. 5A, lanes 6–8). However, TFIID markedly stimulated p53 binding to RGC (Fig. 5A, cf. lane 2 with lanes 3–5). Nevertheless, unlike the result with TBP, the addition of TFIID did not result in an additional supershifted p53–RGC complex.

Wild-type, but not mutant, RGC oligonucleotides competed away p53 binding to RGC indicating the specificity of the p53–RGC complex. Moreover, wild-type TATA oligonucleotides were significantly more effective in blocking TFIID stimulation of p53 DNA binding than were mutant TATA oligonucleotides (data not shown). Monoclonal antibodies to p53 and TBP were used to determine the content of the complex. The anti-p53 antibody supershifted the whole complex (Fig. 5C, cf. lanes 4 and 5), whereas anti-TBP did not (Fig. 5C, cf. lane 4 with lanes 6 and 7), indicating that the complex contains only p53. Thus, TFIID increases p53 DNA binding to DNA without associating with the p53–DNA complex itself. Generally, TFIID was more effective at stimulating p53 binding to RGC DNA than was recombinant TBP, even though we estimated by Western blotting that the concentration of TBP in the TFIID fraction was somewhat less than that present in the recombinant protein preparation. Depending on the concentration of p53 used, quantitation of several experiments indicated that the stimulation was on the order of fourfold (data not shown). Thus, in comparison to TBP, TFIID was both qualitatively and quantitatively different in its ability to stimulate p53 binding to the RGC DNA fragment.

**Both TBP and TFIID increase p53 DNase I protection over the RGC region**

Because EMSAs showed that both TBP and TFIID increase p53 binding to RGC, DNase I footprinting analysis was used to further confirm these observations and examine which sequences were bound when both proteins were present (Fig. 6). The RGC-containing fragment is the same as that used in the EMSA experiments.

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**Figure 5.** Effect of TFIID on p53 binding to RGC. EMSA was carried out as described in Fig. 3 except TFIID was used instead TBP. (A) p53 (15 ng) was used as indicated above each lane. The amount of TFIID used was 0.25 μl (lanes 3, 6), 0.5 μl (lanes 4, 7), and 1.0 μl (lanes 5, 8). Lane 1 contains probe without protein. (B) p53 (15 ng) and TFIID (0.5 μl) were used as indicated above each lane. Wild-type (Wt RGC) and mutant (Mt RGC) RGC-containing double-stranded oligonucleotides (12 ng) were used in lanes 4 and 5 as competitors. (C) p53 (15 ng) and TFIID (1.0 μl) were used as indicated above each lane. The p53-specific monoclonal antibody PAb1801 (100 ng) (lane 5) and monoclonal anti-TBP antibody (1 and 2 μl, respectively) were added to reaction mixtures (lanes 6 and 7, respectively). Lane 1 contains DNA probe without protein.

1842 GENES & DEVELOPMENT
Cooperative DNA binding of p53 and TFIIID

DNA led us to determine whether these proteins might also cooperate in activating transcription. Transfection assays have proved useful for demonstrating that p53 activates transcription from promoter-bearing reporter constructs containing a p53-binding site. We chose to examine activation by p53 using Drosophila Schneider cells because insect cells do not contain a p53-related gene [Soussi et al. 1990]; Colgan and Manley (1992) also showed that TFIIID is limiting in insect cells and that introduction of constructs expressing TBP into Schneider cells stimulates transcription from several TATA-containing basal promoters. Thus, it was possible to test whether transfection of both p53 and TBP expression vectors into insect cells would yield levels of expression greater than the sum of both constructs expressed separately. The reporter plasmid, fos1wt, the same as that employed to generate the DNA fragment that we used to demonstrate cooperative binding shown in Figures 1-3, contains a p53-binding site upstream from the c-fos TATA box and the chloramphenicol acetyltransferase (CAT) gene [Farmer et al. 1992]. fos1wt was transfected into Drosophila Schneider cells alone or with a plasmid expressing Drosophila TBP cDNA (Act-TFIIID), a plasmid expressing wild-type human p53 (Act-hp53), or both Act–TFIIID and Act–hp53 plasmids [Fig. 7]. CAT activity was determined and corrected for coexpressed β-galactosidase activity [Han et al. 1989] and expressed as counts per minute of tritium-labeled acetylated chloramphenicol. Because Drosophila Schneider cells do not express endogenous p53, basal expression of the fos1wt plasmid was low and any activated transcription was the

Figure 6. DNase I footprinting analysis of the effects of TBP or TFIIID on p53 binding to RGC DNA. The DNase I footprinting analysis was carried out as described in Fig. 2 except with 32P-labeled RGC probe (0.15 ng) instead of RGC-TATA probe. (A) The RGC probe was incubated with no protein (lane 1), 1 µl of TFIIID (lanes 5–7, 11), or 60 ng of TBP (lanes 8–10, 12). The amount of p53 used was 250 ng (lanes 2, 5, 8), 500 ng (lanes 3, 6, 9), and 750 ng (lanes 4, 7, 10). (B) The RGC probe was incubated with no protein (lane 1) or 600 ng of p53 (lanes 2–8). The amount of TFIIID used was 1 µl (lane 3), 2 µl (lanes 4, 9), and 4 µl (lane 5). The amount of TBP used was 30 ng (lane 6), 60 ng (lanes 7, 10), and 120 ng (lane 8). The RGC region is indicated at right.

in Figure 4. To determine whether TBP or TFIIID affects p53 protection of the RGC region, conditions were chosen such that a limiting amount of p53 was used and only marginal protection of RGC ensued. However, with increasing concentrations of p53, the RGC region was protected more extensively from DNase I cleavage [Fig. 6A, cf. lane 1 with lanes 2–4], whereas neither TFIIID nor TBP alone protected this region [Fig. 6A, lanes 11, 12]. In the presence of either TFIIID or TBP, the extent of protection over the RGC region by p53 was markedly increased, especially at the two higher p53 concentrations [Fig. 6A, cf. lanes 2–4 with lanes 5–7 and 8–10, respectively]. This result was confirmed in a reciprocal experiment in which the concentrations of TFIIID and TBP were varied with a constant amount of p53; both increased the ability of p53 to protect RGC from DNase I cleavage in a dose-dependent manner [Fig. 6B, cf. lanes 3–5 and 6–8 with lane 2]. Our data therefore show that TBP and TFIIID can stimulate p53 binding specifically to DNA without displaying detectable interaction with that DNA themselves.

p53 and TBP function combinatorially to activate transcription in vivo

Our experiments showing that the association of p53 with TBP or TFIIID results in their cooperative binding to DNA led us to determine whether these proteins might also cooperate in activating transcription. Transfection assays have proved useful for demonstrating that p53 activates transcription from promoter-bearing reporter constructs containing a p53-binding site. We chose to examine activation by p53 using Drosophila Schneider cells because insect cells do not contain a p53-related gene [Soussi et al. 1990]; Colgan and Manley (1992) also showed that TFIIID is limiting in insect cells and that introduction of constructs expressing TBP into Schneider cells stimulates transcription from several TATA-containing basal promoters. Thus, it was possible to test whether transfection of both p53 and TBP expression vectors into insect cells would yield levels of expression greater than the sum of both constructs expressed separately. The reporter plasmid, fos1wt, the same as that employed to generate the DNA fragment that we used to demonstrate cooperative binding shown in Figures 1-3, contains a p53-binding site upstream from the c-fos TATA box and the chloramphenicol acetyltransferase (CAT) gene [Farmer et al. 1992]. fos1wt was transfected into Drosophila Schneider cells alone or with a plasmid expressing Drosophila TBP cDNA (Act-TFIIID), a plasmid expressing wild-type human p53 (Act-hp53), or both Act–TFIIID and Act–hp53 plasmids [Fig. 7]. CAT activity was determined and corrected for coexpressed β-galactosidase activity [Han et al. 1989] and expressed as counts per minute of tritium-labeled acetylated chloramphenicol. Because Drosophila Schneider cells do not express endogenous p53, basal expression of the fos1wt plasmid was low and any activated transcription was the

Figure 7. p53 and TBP activate transcription combinatorially. The plasmid fos1wt, containing a p53-binding site upstream from the c-fos TATA box, and the CAT gene [Farmer et al. 1992] were transfected into Drosophila Schneider cells alone or with 0.5 µg of a plasmid [Act-TFIIID] expressing epitope-tagged Drosophila TBP cDNA, 3 µg of a plasmid expressing wild-type human p53 [Act-hp53], or both, as indicated. The amount of each plasmid used was determined in a previous assay to provide plateau levels of activation by p53 and TBP. CAT activity is expressed as counts per minute of tritium-labeled acetylated chloramphenicol.
result of the transfected p53 expression vector. The basal level of expression of CAT activity was \(-0.1 \times 10^5\) cpm [Fig. 7]. When either TBP or p53 expression vectors were cotransfected with fos1wt, the resulting CAT activities were \(-0.3 \times 10^5\) and \(2.3 \times 10^5\) cpm, respectively. These activities were determined in previous experiments to represent the maximal or plateau level reached for either the TBP or the p53 constructs used (data not shown). However, when such amounts of both TBP and p53 expression vectors were cotransfected with fos1wt, the resulting CAT activities were \(-7.5 \times 10^5\) cpm, a value far greater than the sum of the basal and stimulated expression seen when the plamids were expressed singly. Thus, our data demonstrating that p53 and TBP activate transcription in vivo synergistically correlate well with the fact that interaction of p53 and TBP (TFIID) induces cooperative DNA binding. Two additional points should be mentioned here. First, not only Drosophila but also human TBP, when introduced into insect cells along with p53, causes combinatorial activation of fos1wt expression (G. Farmer et al., in prep.). Second, TBP stimulates activation by several other factors, such as Sp1; however, it synergizes with p53 to an extent that is equal to or greater than several other activators tested (Colgan and Manley 1992; G. Farmer, J. Colgan, J.L. Manley, and C. Prives, in prep.).

p53 inhibits TBP but not TFIID binding to a fos–TATA site

Seto et al. (1992) provided evidence suggesting that the interaction of p53 with recombinant TBP is responsible for the transcriptional repression seen with a plasmid template lacking a p53-binding site. We tested whether this transcriptional repression might be the result of the inhibition of TBP or TFIID binding to the TATA region. The probe used, fos-TATA, is a 110-bp DNA fragment that contains the c-fos TATA element but lacks any activator sequence. p53 does not activate transcription from this template [Farmer et al. 1992]. As expected, EMSA analysis showed that TBP bound to fos-TATA, whereas p53 did not (Fig. 8A, lanes 2,8–12). p53 strongly inhibited the overall affinity of TBP for the TATA box in a dose-dependent manner [Fig. 8A, cf. lane 2 with lanes 3–7]. To further confirm the inhibitory effect of p53 on TBP binding, DNase I footprinting analysis was carried out [Fig. 8B]. TBP bound to the TATA region [Fig. 8B, lane 2], as had been seen with the probe RGC–TATA, and p53 did not protect this region from DNase I [Fig. 8B, lanes 6–8]. p53 completely prevented protection of the TATA region by TBP [Fig. 8B, cf. lane 2 with lanes 3–5] confirming the data obtained by EMSA. Our results with TFIID were very different. When the ability of TFIID to protect the fos–TATA region was examined, p53 not only did not inhibit this interaction but actually slightly increased the overall affinity of TFIID to the TATA box region, as indicated by the solid bar [Fig. 8B, cf. lane 10 with lanes 11–13]. These experiments were performed with another construct in which a mutant p53-binding site was placed upstream of the fos–TATA region, and essentially similar results were obtained (data not shown). Thus, our results show that although recombinant TBP and partially purified TFIID fractions are similar in their ability to stimulate p53 DNA binding, they display dramatic differences in their ability to be inhibited by p53.

Discussion

Our experiments have extended recent observations that p53 associates with TBP or TFIID and have provided insight as to the consequences of the physical interaction

Figure 8. Effects of p53 on TBP or TFIID binding to fos–TATA. EMSA and DNase I footprinting analyses were performed as described in Figs. 1 and 3, respectively. The probe used was a 110-bp fos–TATA fragment. (A) TBP [1 ng] was used as indicated above each lane. The amount of p53 used was 5 ng [lanes 3,8], 10 ng [lanes 4,9], 15 ng [lanes 5,10], 30 ng [lanes 6,11], and 40 ng [lanes 7,12]. Lane 1 contains DNA probe without protein. (B) TBP [60 ng] and TFIID (4 \(\mu\)l) were used as indicated above each lane. The amount of p53 used was 40 ng [lanes 3,6,11,14], 80 ng [lanes 4,7,12,15], and 160 ng [lanes 5,8,13,16]. Lanes 1 and 9 contain probe DNA without TBP, TFIID, or p53. The TATA region is indicated at right. The extended protected region is indicated at right by the solid bar.
between these proteins. We have shown that p53 and either recombinant TBP or partially purified TFIID cooperates in binding to a p53-responsive promoter. These results obtained with in vitro DNA-binding assays are supported by the observation in vivo that p53 and TBP cooperate to activate transcription in insect Schneider cells. Because both the p53 and TBP used in this study were highly purified proteins expressed from a baculovirus expression system and from *Escherichia coli*, respectively, the cooperative DNA binding that we observed resulting from the interaction of p53 with partially purified TFIID from HeLa cell nuclear extracts effectively rules out the possibility that other cell proteins mediate the interaction. It has been well established that TFIID, the only general transcription factor that contains a sequence-specific DNA-binding activity, initiates a transcription-competent complex by binding to the TATA box. This step has been shown to be a potential rate-limiting step for complex assembly (for review, see Mitchell and Tijan 1989; Zawel and Reinberg 1993). By interacting with TFIID and the resulting cooperative DNA binding to the promoter, p53 most likely accelerates complex formation.

We were surprised to observe that a major component of the cooperative binding observed is the ability of TFIID or TBP to stimulate binding by p53 to DNA containing a p53-binding site but lacking a TATA box. Several cellular or viral p53 DNA-binding sites have now been identified, located within the human ribosomal gene cluster (Kern et al. 1991), SV40 DNA (Bargonetti et al. 1991), muscle creatine kinase (Zambetti et al. 1992), and the GADD45 gene (Kastan et al. 1992), as well as a number of as yet unidentified sites from human genomic DNA (Dierry et al. 1992). p53 binding to these cellular or viral DNA-binding sites varies in strength, as demonstrated by filter-binding studies, competition with non-specific DNAs, and immunobinding (Bargonetti et al. 1992; El-Dierry et al. 1992). Furthermore, the efficiency of p53 binding to DNA can be altered by a number of cellular or viral proteins. For example, wild-type p53 in complex with either SV40 T antigen or several tumor-derived mutant p53 proteins cannot bind specifically to DNA (Bargonetti et al. 1992; Kern et al. 1992). Conversely, the p53 carboxyl terminus apparently contains a regulatory domain as deduced from experiments showing that incubation with the p53-specific antibody PAb421 (Hupp et al. 1992) and mutant-specific antibodies (e.g., see Milner and Medcalf 1991) have been correlated with its DNA-binding function. TFIID or TBP may change the conformation of p53 in a manner similar to the p53-specific antibodies PAb421 (Hupp et al. 1992; Helazonetis et al. 1993) or PAb1801 (L. Jayaraman, J. Bargonetti, and C. Prives, unpub.) such that the DNA-binding domain is more favorably exposed. Second, sizing analyses (Friedman et al. 1993) and cross-linking experiments (Stenger et al. 1992, Sturzbecher et al. 1992, Friedman et al. 1993) have indicated that tetramers and multiples of tetramers are the predominant forms of immunopurified p53. TBP or TFIID may facilitate assembly of p53 larger oligomers, which may be more potent DNA-binding forms of the protein. We have determined that TBP facilitates the conversion of the p53 tetramer into a larger complex structure (data not shown). Whether this new form of p53 is a more potent DNA-binding protein remains to be determined. Because the EMSA data did not reveal the existence of significantly larger p53-DNA complexes in the presence of TBP or TFIID, at present, we tend to favor the conformational hypothesis.

During the writing of this paper, Ragimov et al. (1993) reported that wild-type, but not mutant, p53 interfered with the stable binding of TBP and TFIID to the TATA motif and proposed that upon binding to TBP, wild-type, but not mutant, p53 specifically blocks the ability of TBP to engage in interactions required for efficient transcriptional initiation. Our data from both EMSA and DNase I footprinting analyses showing that p53 inhibits TBP binding to the TATA box also suggested that p53 may repress transcription by inhibiting TBP DNA binding. Nevertheless, because we also found that p53 does not inhibit TFIID binding to the TATA box, questions must be raised as to the interpretation of experiments with TBP. To explain the result that p53 inhibits TBP but not TFIID DNA-binding ability, two suggestions are offered: First, the holoprotein TFIID used in our experiments binds DNA more tightly than does recombinant TBP, as shown by the fact that a markedly greater amount (~25-fold) of wild-type TATA box-containing oligonucleotides was needed to compete away TFIID than TBP from binding to the TATA box (data not shown). This relatively greater affinity of TFIID may...
well explain the inability of p53 to inhibit the binding of TFII D to the TATA box region. Second, the TAFs that are tightly associated with TBP may allow p53 to interact with the TBP subunit of TFII D to cooperate in DNA binding but may also prevent p53 from inhibiting TFII D DNA binding. Several models have been proposed for transcriptional repression by sequence-specific DNA-binding proteins in eukaryotes (for review, see Levine and Manley 1989). Although no specific p53 DNA-binding site has yet been identified for transcriptional repression by p53 and p53 clearly represses transcription when not bound to DNA, the squelching model proposed by Gill and Ptashne [1988] might explain p53 repression. Thus, p53 may interact with a class of transcrip-
tional factors are likely to be identified as well. Such speculations notwithstanding, because p53 did not in-
hibit TFII D binding to a p53-unresponsive promoter, we suggest that p53 transcriptional repression is not the result of decreasing TFII D binding to the TATA box, but instead may involve another mechanism.

Materials and methods

Purification of p53 proteins

Recombinant baculoviruses expressing human wild-type and mutant p53 have been described [Friedman et al. 1990, Bargon-
etti et al. 1992]. Extracts of infected sf21 insect cells were pre-
pared, and p53 was purified from lysates by immunoaffinity procedures [Friedman et al. 1990].

Purification of transcription factors

Recombinant TFII B, TBP, TFII E, RAP30, and RAP74 were expressed in bacteria and purified as described [Ha et al. 1991, Hoffmann and Roeder 1991, Sumimoto et al. 1991, Finkelstein et al. 1992]. The TFII Eα-coding sequence from Ndel to BamHI [Ohkuma et al. 1991] was inserted into Ndel—BamHI sites of 6HisT—pET11 vector [Hoffmann and Roeder 1991]. Recombi-

DNase I protection assays

DNase I protection assays were performed as described [Hoey et al. 1988]. The 32P-labeled DNA fragments from 10d subclone of 772CAβ fos1wt, fos1mt, were the same as those used in EMSA. The probes (0.15 ng) were incubated with p53, TBP or TFII D, or a combination of p53 and TBP or TFII D at the concentrations indicated in the figure legends for 40 min at room temperature, followed by DNase I digestion. The amount of DNase I used was pretested empirically to produce an even pattern of partial cleavage products. The DNase I digestions were performed on ice for 1 min and stopped by DNase I stop buffer (1% SDS, 20

Gel EMSAs

EMSA was carried out as described [Peterson et al. 1990]. The RGC probe was a 100-bp DNA fragment generated by digestion of the 10d subclone of 772CAβ with BamHI and KpnI [Kern et al. 1991]. The TATA probe was a 110-bp DNA fragment generated by digestion with HindIII and Xba I of pFC53 containing the fos promoter sequence from -53 to +42 [Zhu and Prywes 1992]. The RGC—TATA, mRGC—TATA, SRE—TATA, and GAL4—TATA probes were derived from transcription templates fos1 wt [Farmer et al. 1992], fos1mt [Farmer et al. 1992], pFC53X [Zhu and Prywes 1992], and pFC53G1 [F. Johansen and R. Prywes, unpubl], containing the fos promoter sequence from -53 to +4 and one protein-binding site, respectively. RGC—TATA was isolated as a 155-bp EcoRI—XbaI fragment of fos1 wt. mRGC—TATA, SRE—TATA, and GAL4—TATA were isolated as 140-bp HindIII—XbaI fragments of the plasmids fos1 mt, pFC53X, and pFC53G1, respectively. The probes were 32P-labeled by the Klenow fragment of E. coli DNA polymerase. The reactions contained 4 μl of 5× EMSA buffer [100 mM HEPES (pH 7.9), 125 mM KCl, 0.5 mM EDTA, 50% glycerol, 10 mM MgCl2, 1 μM 40 mM spermidine, 1 μM 10 mM DTT, 1 μM 0.5% NP-40, 1 μM of 100 μg/ml of double-stranded poly[d(G-C)], 1 μM of 2 mg/ml BSA, 0.3 ng of 32P-labeled probe DNA, 3 μl of protein samples (in BC100), and H2O to 20 μl. The amounts of proteins used are indicated in the figure legends. The reactions were carried out at room temperature for 40 min. For antibody supershift assays, 1 μl of antibody was added directly to the reaction mixtures and incubated for 30 min. Mixtures were then loaded on a native 4% polyacrylamide gel containing 0.5× Tris-borate—EDTA (TBE) buffer, 1 mM EDTA, and 0.05% NP-40 and electrophoresed in 0.5× TBE at 4°C at 200—250 V (not to exceed 40 mA current) for 2 hr. Pab1801 has been described [Banks et al. 1986]. The polyclonal anti-TBP antibody (clu—54) was generated in rabbits using bacterially expressed human TBP protein. The monoclo-
al anti-TBP antibody (SL-39) has been described [Sadowski et al. 1991]. All antibodies were purified using protein A—Sepha-

The SRF-coding region was cloned into 6HisT—pET11, and recombinant histidine-tagged SRF was then made in bacteria and purified as described [Hoffmann and Roeder 1991]. GAL4— VP16 was purified from bacteria as described [Chasman et al. 1989]. All transcription factors were stored in buffer BC100 [100 mM KCI, 20% glycerol, 20 mM Tris—HCl (pH 7.9), 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dith-

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mm EDTA, 200 mm KCl, and 250 μg/ml of yeast tRNA. The DNA was extracted with phenol and chloroform and precipitated by ethanol, and DNA fragments were then electrophoresed on a 10% polyacrylamide/7 m urea gel.

**DNA transfection and CAT assays**

The procedures were followed as described previously (Colgan and Manley 1992). Schneider insect cells (2 x 10⁶ to 4 x 10⁶) grown at 25°C were plated the evening before transfection in Shelds and Sang M3 media (Sigma) and 10% heat-inactivated fetal calf serum. The next day the DNA was prepared by calcium phosphate precipitation and added to the media. Transfection efficiencies were determined by CAT assays for promoter activity were also performed as described in Han et al. (1989). Variations in transfection efficiencies within a given experiment were almost always less than twofold. CAT assays for promoter activity were also performed as described in Han et al. (1989).

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