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The Adenovirus E1A Repression Domain Disrupts the Interaction between the TATA Binding Protein and the TATA Box in a Manner Reversible by TFIIB

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The human adenovirus E1A 243 amino acid oncoprotein possesses a transcription repression function that appears to be linked with its ability to induce cell cycle progression and to inhibit cell differentiation. The molecular mechanism of E1A repression has been poorly understood. Recently, we reported that the TATA binding protein (TBP) is a cellular target of E1A repression. Here we demonstrate that the interaction between TBP and the E1A repression domain is direct and specific. The TBP binding domain within E1A 243R maps to E1A N-terminal residues ~1 to 35 and is distinct from the TBP binding domain within conserved region 3 unique to the E1A 289R transactivator. An E1A protein fragment consisting of only the E1A N-terminal 80 amino acids (E1A 1–80) and containing the E1A repression function was found to block the interaction between TBP and the TATA box element as shown by gel mobility and DNase protection analysis. Interestingly, a preformed TBP-TATA box promoter complex can be dissociated by E1A 1–80. Further, TFIIB can prevent E1A disruption of TBP-TATA box interaction. TFIIB, like TBP, can overcome E1A repression of transcription in vitro. The ability of the E1A repression domain to block TBP interaction with the TATA box and the ability of TFIIB to reverse E1A disruption of the TBP-TATA box complex implies a mechanism for E1A repression distinct from those of known cellular repressors that target TBP.

Adenovirus (Ad) encodes two major regulatory proteins of 243 and 289 amino acid residues (E1A 243R and E1A 289R for group C Ads) synthesized from alternatively spliced RNA transcripts of 12S and 13S encoded by early region E1A. E1A proteins contain multiple independent domains involved in diverse functions, including transcriptional activation, transcriptional repression, induction of cellular DNA synthesis, cell immortalization, and cell transformation as well as inhibition of metastasis and cell differentiation (for reviews, see references 9, 13, 43, and 47). E1A 289R differs from E1A 243R by conserved region 3 (CR3), a 46-amino-acid domain unique to 289R (see Fig. 1 for location of conserved regions). CR3 is essential (18, 26, 33, 41, 46) and sufficient (20, 34) for activation of viral early genes. Domains common to E1A 243R and 289R are required for the growth-regulatory functions of E1A; these include the relatively nonconserved N terminus (amino acid residues 1 to 39), CR1 (residues 40 to 80), and CR2 (residues 120 to 139) (40).

Ad E1A 243R is a potent inducer of cellular DNA synthesis and a strong deregulator of cell cycle control. Two separate domains of E1A 243R can induce progression of quiescent cells to S-phase cellular DNA synthesis by independent pathways (24, 34). The first pathway involves binding sites within CR1 and CR2 for the retinoblastoma tumor suppressor protein, pRb. These sites sequester and dissociate pRb and related family members from complexes with the E2F family of transcription factors, whose activities can induce cell cycle progression (for a review, see reference 44). The second pathway maps within the E1A N-terminal 80 amino acids (49, 50; for a review, see reference 39). The N-terminal pathway takes on added importance because E1A growth-regulatory functions require sequences within this region. An intriguing biochemical function which maps to this region is the ability to repress transcriptionally cellular genes, some or all of which are involved in cellular proliferation or cell differentiation (8, 21, 52, 55, 58; for a review, see reference 7). Much has been learned recently about the Rb-E2F pathway. By contrast, the biochemical functions and protein-protein interactions involved in the N-terminal pathway are poorly understood.

To understand the molecular mechanism of E1A transcriptional repression, our laboratory has developed a cell-free transcription repression system that recapitulates E1A repression in vitro (49–51). In this system, an E1A recombinant protein containing only the N-terminal 80 amino acids (E1A 1–80) represses the transcription of E1A-repressible genes and is used as a prototype repressor to avoid complications from other E1A functional domains. Transcription repression in vitro is promoter specific; we have shown that the interstitial collagenase, rat insulin II, human immunodeficiency virus long terminal repeat (HIV LTR), and simian virus 40 early promoters are repressed by E1A 1–80 in vitro, whereas the Ad major late promoter (MLP), the Rous sarcoma virus LTR, and the human histone 4 promoter are not repressed (49, 50). Analysis of E1A mutant proteins shows that E1A amino acids ~1 to 35 within the relatively nonconserved N terminus are absolutely required and that amino acids 48 to 60 within CR1 are needed for full activity (49). These sequence requirements for E1A repression in vitro are consistent in the main with the results of DNA transfection studies (for a review, see reference 7).

Our recent findings provide evidence that the TATA binding protein (TBP) component of the multiprotein transcription complex TFIID is a cellular target of E1A repression (51). TFIID is central to transcriptional regulation. TFIID binds to

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the TATA box of the promoter and nucleates formation of a transcription initiation complex. Transcription in eukaryotes by RNA polymerase II involves several groups of factors. A first group, which constitutes the general transcription machinery, includes RNA polymerase II and the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. A second group consists of sequence-specific transcriptional activators that stimulate transcription, at least in part, by increasing the number of functional transcription complexes (reference 10 and references therein). A third group represents an increasing number of proteins classified by function as coactivators, positive cofactors, negative cofactors, corepressors, and general repressors of transcription (for reviews, see references 19 and 60). This third group activates or represses transcription through protein-protein interaction with components of the general transcription apparatus. The E1A transcriptional repressor fits into this third group.

In this report, we show that the E1A 243R repressor protein contains a strong binding site for TBP at its N terminus. This TBP binding site is distinct from the previously reported binding site within CR3 of the 289R transactivator protein (23, 31). We provide evidence that the interaction of the E1A N terminus with TBP is direct and specific, and we delineate the sequences within E1A required for interaction with TBP. Further, we demonstrate that the E1A N-terminal repression domain can disrupt TBP interaction with the TATA box and that TFIIB can reverse this disruption, implying a unique mechanism for E1A transcriptional repression.

MATERIALS AND METHODS

Expression and purification of TBP and TFIIB. Human TBP was expressed in cell line BL21 (DE3) transformed by pARhTFIID (45) and purified through DEAE-Sepharose and heparin-Sepharose column chromatography (22). Purified human TFIIB was a generous gift of S. Roberts and M. R. Green.

Gel mobility shift and DNase footprint analysis. For gel shift analysis, an *Xho*I-*Hin*dIII fragment containing −35 to +29 of the MLP was end labeled by using the Klenow fragment of DNA polymerase with [^{32}P]dCTP. Binding reaction mixtures (10 μl each) contained 4 mM Tris (pH 7.9), 4 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 0.05 mM EDTA, 60 mM KCl, 0.1 mM dithiothreitol (DTT), 8% glycerol, 0.1% Brij 58 (polyoxyethylene 20 cetyl ether), 100 ng of poly(dG-dC) · poly(dG-dC), 1 μg of bovine serum albumin, 3 ng of labeled DNA, 5 ng of recombinant human TBP, and various levels of E1A protein as indicated. After incubation at room temperature for 40 min, the samples were run on a 6% polyacrylamide gel (40:1) as described by Auble and Hahn (3).

For DNase I footprint analysis, a fragment containing MLP sequences from −76 to +29 and 213 bp of the chloramphenicol acetyltransferase (CAT) gene was end labeled with Klenow fragment. Binding reaction mixtures (50 μl each) contained 25 mM Tris-HCl (pH 7.9), 6.25 mM $MgCl_2$, 0.5 mM EDTA, 50 mM KCl, 0.5 mM DTT, 10% glycerol, 0.5% polyvinyl alcohol, 500 ng of poly(dG-dC) · poly(dG-dC), 5 μg of bovine serum albumin, 5 ng of labeled DNA, 5 ng (or more as indicated) of recombinant human TBP, and various levels of E1A protein and/or recombinant human TFIIB as indicated. After incubation at room temperature for 40 min, the samples were digested with DNase I for 2 min at room temperature by the addition of 50 μl of 5 mM $CaCl_2$, 10 mM $MgCl_2$, and 1 ng of DNase I (Worthington Corp.).

In vitro binding assays. Recombinant E1A 243R, E1A 1–80, E1A 1–80 deletion proteins, and E1A 1–80 with amino acid residue 2, arginine, replaced with glycine (E1A 1–80,2R→G) were expressed in and purified from bacteria as previously described (49). E1A 1–80,2R→G was constructed by PCR, essentially as previously described (49), with GGGATCCATGGGACATATTATCTGC as the upstream primer. E1A 289R protein was prepared in a similar manner after the cloning of a PCR product derived from a 13S cDNA plasmid (33) into pQE-12. E1A proteins were immobilized on Affi-Gel 10 as previously described (51). ^{35}S-labeled TBP was prepared by in vitro transcription and translation with a Promega TNT reticulocyte kit, Sp6 polymerase, ^{35}S-labeled methionine, and pGEM-hTBP as a template. Affi-Gel 10 preparations containing the various E1A proteins were preincubated for 60 min at 4°C in 300 μl of Nonidet P-40 (NP-40) binding buffer (40 mM HEPES, pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.2% NP-40, and 0.5 mg of a sonicated and clarified *Escherichia coli* extract per ml) (59). Three to five microliters of in vitro-translated TBP was added, and the incubation was continued for an additional 60 min. Beads were washed four times with 1 ml of NP-40 buffer lacking *E. coli* extract and bound protein eluted with sodium dodecyl sulfate (SDS) sample buffer, resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and quantitated by phosphorimage analysis.

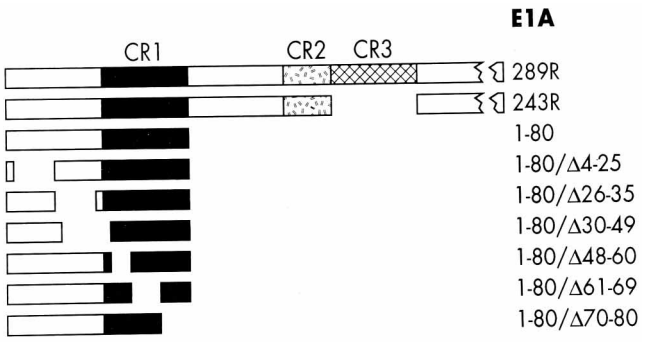

FIG. 1. Structure of the E1A 289R, E1A 243R, and E1A 1–80 deletion proteins used in these studies. CR1 (residues 40 to 80), CR2 (residues 120 to 139), and CR3 (residues 140 to 188) are indicated.

For protein-protein competition experiments, various amounts of E1A 1–80 or E1A 1–80Δ4–25 proteins were first incubated with ^{35}S-labeled TBP for 15 min, glutathione *S*-transferase (GST)-TFIIB (4 μg of ligand) immobilized on glutathione-agarose was then added, and incubation continued for 60 min. Beads were washed as described above, and bound protein was quantitated by phosphorimage analysis.

For protein-protein interaction experiments in which E1A proteins in solution were incubated with GST-TBP immobilized on glutathione-agarose, beads were washed as described above and bound protein was resolved by SDS-PAGE and electroblotted onto nitrocellulose. Immunoblot analysis was performed with a 1:500 dilution of rabbit antibody generated against a synthetic peptide, E1A 40–80. After extensive washing, the blot was probed with 1 μCi of ^{125}I-labeled protein A (low specific activity; New England Nuclear). Blots were visualized by phosphorimage analysis. The immunoblot signals for the 289R, 243R, and E1A 1–80 proteins were approximately equal on a molar basis as determined by immunoblot analysis of all three proteins on the same gel (data not shown).

In vitro transcription repression analysis. In vitro transcription reactions were performed as previously described (48, 49). RNA transcripts were analyzed by primer extension using the HIV LTR construct pBennCAT or the interstitial collagenase construct CL CAT3.

RESULTS

Both E1A 243R and E1A 289R contain domains that bind TBP in vitro. Figure 1 illustrates the organization of the domains of E1A 243R and E1A 289R and the E1A 1–80 deletion proteins used in these studies. E1A 243R and E1A 1–80 are active as transcriptional repressors in vitro and in vivo and can bind TBP in vitro (49–51). TBP has been previously reported to bind the E1A 289R protein (23, 31) through interaction with CR3 (17, 31), which is unique to 289R and can function independently as a transactivator (20, 34). To determine the relative strengths of binding to TBP, approximately equimolar amounts of 289R, 243R, and E1A 1–80 proteins immobilized on Affi-Gel 10 were incubated with a ^{35}S-labeled reticulocyte translate of TBP. After extensive washing, bound TBP was resolved by SDS-PAGE and quantitated by phosphorimage analysis. E1A 289R captured two to four times as much TBP as did 243R and E1A 1–80 in several experiments (see Fig. 2A for an example and Table 1 for quantitation).

To confirm these results and to rule out the possibility that E1A domains are affected differently by linkage to Affi-Gel 10, binding was measured with TBP instead of E1A as the immobilized ligand. A GST fusion of human TBP (GST-TBP) bound to glutathione-agarose was incubated with various amounts of E1A 289R, E1A 243R, or E1A 1–80. Bound protein was eluted and subjected to immunoblot analysis with antibody against an E1A CR1 peptide (E1A residues 40 to 80), followed by treatment with ^{125}I-protein A and phosphorimage analysis. The results in Fig. 2B show strong binding of TBP to all three E1A

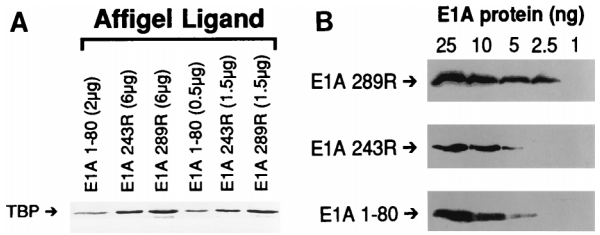

FIG. 2. TBP interacts strongly with E1A 1–80, E1A 243R, and E1A 289R in vitro. (A) Protein-protein interactions between ^{35}S-labeled in vitro-translated TBP and the indicated amounts of E1A 289R, E1A 243R, or E1A 1–80 protein ligands immobilized on Affi-Gel 10 were performed as described in Materials and Methods. (B) GST-TBP (2 μg of ligand) immobilized on glutathione-agarose was incubated with the indicated amounts of E1A proteins, and bound protein was detected by immunoblot analysis as described in Materials and Methods.

proteins. We conclude that there are at least two independent TBP binding domains within E1A proteins. The first, within CR3, is unique to 289R. The second, within E1A 1–80, is shared by E1A 243R and E1A 289R.

Sequences within E1A 1–80 required for interaction with TBP. E1A residues 4 to 25, in the context of the E1A 1–80 protein, are important for binding TBP (51). To determine whether other sequences within E1A 1–80 are required for interaction with TBP, deletion mutant proteins E1A 1–80Δ4–25, E1A 1–80Δ26–35, E1A 1–80Δ30–49, E1A 1–80Δ48–60, E1A 1–80Δ61–69, and E1A 1–80Δ70–80 were immobilized on Affi-Gel 10 and incubated with in vitro-translated ^{35}S-labeled TBP. Bound TBP was visualized after SDS-PAGE by phosphorimage analysis. Figure 3A shows that E1A 1–80Δ4–25 is completely defective in binding TBP and that deletion of residues 26 to 35 markedly reduces binding efficiency. In addition, deletion of residues 48 to 60 reduces binding somewhat (20 to 30%). We conclude that the major, if not sole, binding site within E1A 1 to 80 for TBP resides in residues 1 to 35 (see Discussion).

An E1A 243R mutant with a substitution of glycine for arginine at residue 2 (2R→G) has been reported to be defective in repression function as determined by transient-expression analysis (54). To determine whether residue 2 is required for interaction with TBP, we compared the binding efficiency of wild-type E1A 1–80 with that of E1A 1–80,2R→G. At all levels of E1A 1–80 ligand tested (5.0, 2.5, 1.0, 0.5, and 0.25 μg of protein), TBP bound efficiently (Fig. 3B). By contrast, TBP bound less efficiently when E1A 1–80,2R→G was used as a ligand. As previously reported, E1A 1–80Δ4–25 did not bind

TABLE 1. Binding of TBP to E1A 289R, E1A 243R, and E1A 1–80 proteins[a]

Immobilized ligand		Bound TBP ratio (pixels, 10^3)
E1A protein	μg (10^{-10} mol)	
289R	6.0 (2.6)	1.00 (266)
289R	1.5 (0.65)	0.95 (254)
243R	6.0 (1.9)	0.61 (162)
243R	1.5 (0.48)	0.45 (119)
1–80	2.0 (2.3)	0.23 (60)
1–80	0.5 (0.58)	0.22 (58)

[a] The amount of ^{35}S-labeled TBP bound to E1A ligands in Fig. 2A was quantitated by phosphorimage analysis on a Molecular Dynamics model 400B PhosphorImager with ImageQuant software. The highest pixel value was normalized to 1.0, and ratios were calculated. The quantity of TBP translation product bound ranged from 17.7% (ratio of 1.00) to 3.8% (ratio of 0.22).

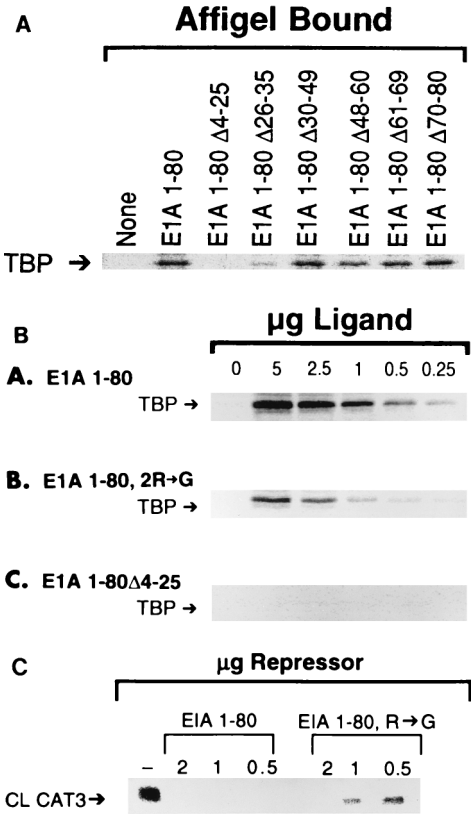

FIG. 3. Mapping E1A 1–80 sequences required for interaction with TBP. (A) A ^{35}S-labeled translation product of TBP was incubated with E1A 1–80 or E1A 1–80 deletion proteins (2 μg of ligand) immobilized on Affi-Gel 10. "None" refers to Affi-Gel 10 beads alone. Beads were washed, and bound TBP was analyzed as described in Materials and Methods. (B) A ^{35}S-labeled TBP translation product was incubated with the indicated levels of E1A 1–80, E1A 1–80,2R→G, or E1A 1–80Δ4–25 immobilized on Affi-Gel 10, and bound TBP was analyzed as described in Materials and Methods. (C) E1A 1–80 with glycine substituted for arginine at residue 2 is partially defective in repression. In vitro transcription from the interstitial collagenase promoter-CAT construct was repressed by the addition of the indicated amounts of E1A 1–80. When added to the reaction mixture, E1A 1–80,R→G repressed transcription less effectively. In vitro transcription reactions were performed as described in Materials and Methods.

TBP to a significant degree (Fig. 3B). Phosphorimage analysis revealed that the efficiency of binding of TBP to E1A 1–80, 2R→G was one-fourth that of E1A 1–80. Therefore, E1A 1–80, 2R→G is partially defective in TBP binding. To determine whether there is a correlation between TBP binding and E1A repression, we compared the ability of different levels of E1A 1–80 and E1A 1–80,2R→G to repress transcription in vitro of the interstitial collagenase promoter (CL CAT3) (49). As shown in Fig. 3C, as little as 0.5 μg of E1A 1–80 completely repressed transcription, whereas 2 μg of E1A 1–80,2R→G was required to efficiently repress transcription. Thus, the requirements for residue 2 of E1A for TBP binding and transcriptional repression correlate.

The E1A N-terminal domain can block the interaction between TBP and the TATA box DNA element in vitro. To probe the functional consequences of the interaction between the E1A N-terminal domain and TBP, we attempted to determine by gel mobility shift analysis whether E1A can block complex formation between TBP and the TATA box. A ^{32}P-, end-labeled fragment of the Ad MLP was incubated with purified recombinant human TBP in the presence or absence of E1A

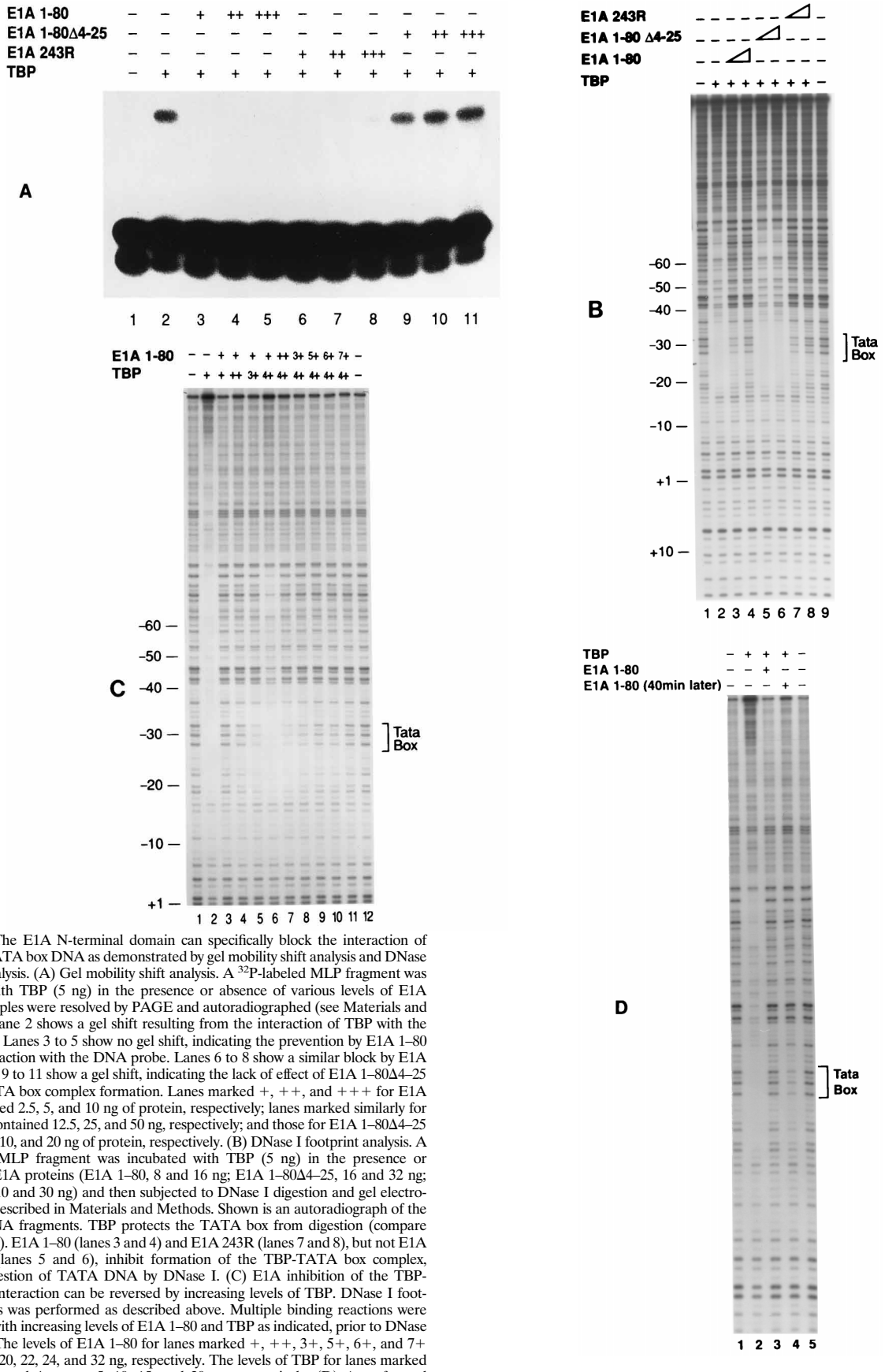

FIG. 4. The E1A N-terminal domain can specifically block the interaction of TBP with TATA box DNA as demonstrated by gel mobility shift analysis and DNase footprint analysis. (A) Gel mobility shift analysis. A ^{32}P-labeled MLP fragment was incubated with TBP (5 ng) in the presence or absence of various levels of E1A protein. Samples were resolved by PAGE and autoradiographed (see Materials and Methods). Lane 2 shows a gel shift resulting from the interaction of TBP with the DNA probe. Lanes 3 to 5 show no gel shift, indicating the prevention by E1A 1–80 of TBP interaction with the DNA probe. Lanes 6 to 8 show a similar block by E1A 243R. Lanes 9 to 11 show a gel shift, indicating the lack of effect of E1A 1–80Δ4–25 on TBP-TATA box complex formation. Lanes marked +, ++, and +++ for E1A 1–80 contained 2.5, 5, and 10 ng of protein, respectively; lanes marked similarly for E1A 243R contained 12.5, 25, and 50 ng, respectively; and those for E1A 1–80Δ4–25 contained 5, 10, and 20 ng of protein, respectively. (B) DNase I footprint analysis. A ^{32}P-labeled MLP fragment was incubated with TBP (5 ng) in the presence or absence of E1A proteins (E1A 1–80, 8 and 16 ng; E1A 1–80Δ4–25, 16 and 32 ng; E1A 243R, 10 and 30 ng) and then subjected to DNase I digestion and gel electrophoresis as described in Materials and Methods. Shown is an autoradiograph of the resulting DNA fragments. TBP protects the TATA box from digestion (compare lanes 1 and 2). E1A 1–80 (lanes 3 and 4) and E1A 243R (lanes 7 and 8), but not E1A 1–80Δ4–25 (lanes 5 and 6), inhibit formation of the TBP-TATA box complex, allowing digestion of TATA DNA by DNase I. (C) E1A inhibition of the TBP-TATA box interaction can be reversed by increasing levels of TBP. DNase I footprint analysis was performed as described above. Multiple binding reactions were carried out with increasing levels of E1A 1–80 and TBP as indicated, prior to DNase I digestion. The levels of E1A 1–80 for lanes marked +, ++, 3+, 5+, 6+, and 7+ were 16, 18, 20, 22, 24, and 32 ng, respectively. The levels of TBP for lanes marked +, ++, 3+, and 4+ were 5, 10, 15, and 20 ng, respectively. (D) A preformed TBP-TATA box complex can be disrupted by E1A 1–80. DNase I footprint analysis was performed as described above, except that for lane 4, TBP was preincubated with the TATA probe for 40 min prior to the addition of E1A 1–80, as indicated.

protein. A TBP-TATA element complex was formed in the absence of E1A (Fig. 4A, lane 2). Both E1A 243R and E1A 1–80 inhibited the interaction of TBP with the TATA box probe (Fig. 4A, lanes 3 to 5 and 6 to 8). Evidence for the specificity of this interaction is provided by the inability of E1A 1–80Δ4–25, at even higher concentrations than that of E1A 1–80, to prevent TBP-TATA interaction (Fig. 4A, lanes 9 to 11).

To rule out the possibility that the effect of E1A was specific to the gel shift assay, the ability of E1A to block TBP-TATA box interaction was confirmed by DNase footprinting experiments. TBP protected from nuclease digestion the bases at positions −18 to −37 of the promoter (Fig. 4B, lane 2). The addition of E1A 1–80 or E1A 243R, but not E1A 1–80Δ4–25, abrogated the TBP footprint, indicating that TBP was prevented from binding to the TATA box (Fig. 4B, compare lanes 3 to 4 and lanes 7 to 8 with lanes 5 to 6). We have previously reported that addition of TBP overcomes E1A repression in a transcription reaction (51). We therefore tested whether increasing the concentration of TBP would restore a TBP-protected footprint in the presence of E1A 1–80. Figure 4C shows that increasing the level of TBP indeed restored complex formation and, further, that increasing the level of E1A 1–80 again prevented complex formation. We conclude that the E1A N-terminal sequence can interact with TBP in a manner which prevents complex formation with the TATA box element.

The TBP-TATA box interaction is reported to be extremely stable (28). We therefore asked whether a preformed TBP-TATA box complex could be disrupted by E1A 1–80. TBP was incubated with the TATA box probe for 40 min, E1A 1–80 was then added, and the incubation was continued for 20 min. As shown in Fig. 4D, E1A 1–80 inhibited the formation of a TBP-TATA box complex, both when added to the TATA box probe at the same time as TBP (compare lanes 3 and 2) and when added 40 min later (compare lanes 4 and 2). These findings indicate that E1A can actively dissociate prebound TBP from the TATA box rather than merely blocking the interaction of the TATA box binding domain with the TATA element.

TFIIB can protect against E1A disruption of TBP-TATA box interaction. TFIIB can stabilize the interaction of TFIID with the TATA box (for a review, see reference 60). It was therefore of interest to determine whether TFIIB would affect the inhibition of TBP-TATA box complex formation by E1A. This possibility was tested by DNase I footprint analysis. Under conditions where E1A 1–80 prevented TBP interaction with the TATA box, the addition of TFIIB was able to restore TBP-TATA box interaction in a dose-dependent manner (Fig. 5A). Under similar circumstances, TFIIE and TFIIF had no effect on E1A inhibition of TBP-TATA box interaction (data not shown).

The finding that TFIIB can overcome E1A-mediated inhibition of TBP-TATA box complex formation suggested that E1A 1–80 might inhibit TFIIB interaction with TBP. This possibility was explored by protein-protein competition experiments. GST-TFIIB immobilized on glutathione-agarose was incubated with ^{35}S-labeled TBP in the presence of increasing amounts of E1A 1–80 or E1A 1–80Δ4–25, and the extent of TBP binding was analyzed. E1A 1–80 did not bind directly to TFIIB under these conditions (data not shown). As shown in Fig. 5B, E1A 1–80 prevented TBP from binding to GST-TFIIB; 60 ng of E1A 1–80 (lane 4) gave ~90% inhibition, and 120 ng (lane 5) completely abolished binding. The competition by E1A 1–80 appears to be specific, since E1A 1–80Δ4–25 did not reduce binding of TBP to GST-TFIIB (Fig. 5B, lanes 6 to 9). These experiments do not distinguish between the possibilities that (i) E1A and TFIIB bind to the same or overlapping binding sites on TBP or (ii) an E1A-TBP complex is defective in binding TFIIB, i.e., a conformational change has occurred.

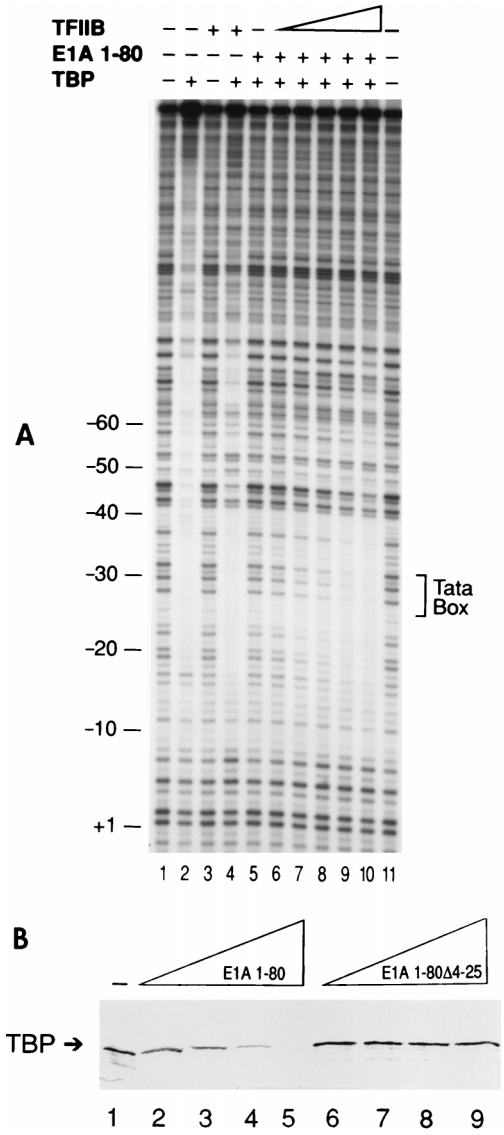

FIG. 5. (A) TFIIB can protect against E1A disruption of the TBP-TATA box complex. DNase I footprint analysis was carried out as described in the legend to Fig. 4B except that various levels of TFIIB were added as indicated. TBP (5 ng) protected the probe from DNase I digestion (compare lanes 1 and 2), whereas TFIIB (100 ng, lane 3) had no effect. E1A 1–80 (16 ng) disrupted the protection of TATA box DNA conferred by TBP (lane 5). Increasing levels of TFIIB reversed E1A 1–80 disruption in a dose-dependent manner (lanes 6 to 10 contained 25, 50, 100, 200, and 400 ng of TFIIB, respectively). (B) E1A 1–80, but not E1A 1–80Δ4–25, inhibits the binding of TBP to TFIIB. ^{35}S-labeled TBP was incubated with increasing levels of E1A 1–80 or E1A 1–80Δ4–25 for 15 min. GST-TFIIB immobilized on glutathione-agarose was then added, and incubation was continued for 60 min. TBP bound to GST-TFIIB was measured by phosphorimage analysis as described in Materials and Methods. Lane 1 has no added E1A protein; lanes 2 to 5 and lanes 6 to 9 contain 6, 30, 60, and 120 ng of E1A 1–80 or E1A 1–80Δ4–25, respectively.

TFIIB can overcome E1A transcription repression in vitro. Inasmuch as TFIIB can prevent E1A 1–80 from interacting with TBP, as deduced from DNase I footprint analysis (Fig. 5A), it was of interest to determine whether TFIIB can over-

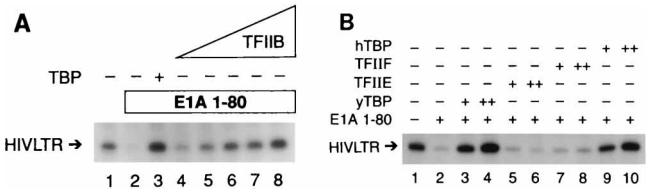

FIG. 6. TFIIB can overcome E1A repression in vitro. (A) In vitro transcription from the HIV LTR was repressed by the addition of 400 ng of E1A 1–80 (compare lanes 1 and 2). Addition to the reaction mixture of 5 ng of recombinant human TBP effectively overcomes E1A repression (lane 3). Addition of increasing levels of TFIIB can also overcome E1A repression in a dose-dependent manner (lanes 4 to 8 contained 25, 50, 75, 100, and 200 ng of TFIIB, respectively). (B) Human or yeast TBP (hTBP and yTBP), but not recombinant TFIIE or recombinant TFIIF, can overcome E1A repression in vitro. Concentrations of E1A 1–80 and TBP are as described above. TFIIE and TFIIF were used at concentrations sufficient for (+) and twice the concentration sufficient for (++) reconstituted transcription in vitro. In vitro transcription reactions were performed as described in Materials and Methods (B).

come E1A repression in an in vitro transcription reaction. As shown in Fig. 6A, HIV LTR CAT is strongly repressed by E1A 1–80 (compare lanes 1 and 2). E1A repression is reversed by addition of recombinant human TBP to the reaction mixture (Fig. 6A, lane 3), as reported previously (51). Importantly, the addition of purified recombinant human TFIIB can reverse E1A repression in a dose-dependent manner (Fig. 6A, lanes 4 to 8). In similar transcription repression experiments, it was shown that the addition of TFIIE or TFIIF had no effect on E1A repression, whereas the addition of human TBP or yeast TBP overcame E1A repression in a dose-dependent manner (Fig. 6B). We conclude that TFIIB, as well as TBP, can overcome E1A repression in vitro.

DISCUSSION

TBP is a direct functional target of the E1A N-terminal repression domain. Our previous studies and work presented here provide several lines of evidence that the TBP moiety of TFIID is a functional target of E1A repression. First, E1A repression of transcription in vitro can be overcome by TBP or a phosphocellulose-purified TFIID fraction in a dose-dependent manner (51). Second, TBP can restore transcriptional activity to a nuclear extract depleted of TFIID by E1A 1–80 affinity chromatography (51). Third, E1A 1–80 interacts specifically with TBP as shown by mutational analysis with E1A 1–80 with a large deletion or with a single amino acid substitution (Fig. 3A and B). Fourth, E1A 1–80 can block TBP interaction with the TATA box as shown by gel mobility shift (Fig. 4A) and DNase footprint analysis (Fig. 4B and C).

E1A proteins contain two independent domains that target TBP—one can serve as a repressor and the other as an activator. E1A 289R is a strong transcriptional activator of early viral gene expression, whereas E1A 243R behaves as a transcriptional repressor of some cellular genes (33) and as an activator of other cellular genes (for a review, see reference 47). The comparative binding studies described here are consistent with 289R containing two strong binding sites and 243R containing a single strong binding site for TBP. The two binding sites for TBP in 289R are within CR3 and within the N-terminal ~1 to 35 amino acids. E1A 243R, which lacks CR3, possesses only the N-terminal TBP binding site. The existence of two potentially competing domains within E1A 289R for binding TBP may offer a structural explanation for the higher repressor activity of 243R compared to that of 289R (33).

Through protein-protein interactions, both the CR3 activation domain and the N-terminal repression domain act as modular transcription factors containing two essential regions, only one of which interacts with TBP. The CR3 zinc finger has been reported to bind TBP, whereas the CR3 C-terminal sequence can interact with several transcription factors and TBP-associated factors (16, 17, 31, 32, 36–38). In contrast to CR3, which is a transcriptional activator, the E1A N-terminal domain functions primarily as a repressor. Mutational analysis indicates that E1A ~1–35 is sufficient to bind TBP strongly in vitro. Deletion of residues 48 to 60 in an E1A 1–80 background appears to slightly reduce (20 to 30%) the ability of E1A 1–80 to bind TBP. The possibility that E1A residues 48 to 60 contain a second weak binding site for TBP cannot be totally excluded. Of interest, E1A ~48–60 is required for full repression activity of E1A 1–80 in vitro (49).

Involvement of TFIIB in E1A repression. TFIIB functions as a bridging molecule in the formation of a transcription initiation complex in which it binds to the TBP-TATA box complex and recruits RNA polymerase II and other general transcription factors (for a review, see reference 60). TFIIB binding to the transcription complex is a rate-limiting step which can be enhanced by both acidic (10, 35) and nonacidic (10) transcriptional activators. Like activators, the E1A repressor may target the recruitment of TFIIB. The E1A N-terminal domain does not interact directly with TFIIB (unpublished data). However, several findings suggest the possibility that the E1A repression domain may inhibit TFIIB interaction with TFIID. First, TFIIB can overcome E1A inhibition of TBP-TATA box interaction in vitro (Fig. 5A). Second, competitive binding analysis suggests that E1A can inhibit TBP binding to TFIIB (Fig. 5B). Third, the addition of increased levels of TFIIB can overcome E1A repression of transcription in vitro (Fig. 6).

Molecular mechanism of E1A repression. The dual ability of the E1A N-terminal repression domain to disrupt a preformed TBP-TATA box complex and to inhibit the interaction of TBP with TFIIB appears to be unique. It is of interest that the N-terminal 80-amino-acid fragment of the TBP-associated factor p230, like E1A 1–80, has been shown to disrupt a TATA box-TBP complex (28). Several cellular cofactors have been reported recently to function as transcriptional repressors that interact with TBP, including Dr1, topoisomerase I, and MOT1 (for a review, see reference 60). Like E1A, Dr1 appears to block TFIIB interaction with TBP, and Dr1-mediated repression can be overcome by TBP (25, 57). However, unlike E1A, Dr1 does not affect the binding of TBP to the TATA box. On the other hand, topoisomerase I can interfere with the ability of TBP to bind to the TATA box motif. In addition, MOT1 prevents TBP binding to the TATA element in an ATP-dependent way (3, 4). Recently, the *Drosophila* homeodomain protein Even-skipped (Eve) was reported to inhibit transcription by interaction with TBP and to prevent TFIID binding to the TATA box (5, 53). However, neither topoisomerase I, MOT1, nor Eve interferes with TFIIB-TBP interaction.

The E1A N-terminal repression domain, like the transcriptional repressors mentioned above, is a direct transcriptional repressor which appears to contain two distinct subdomains, a binding region which tethers the protein to the general transcription machinery and a repression region of unknown function (reference 57 and references therein). The primary sequence of the TBP binding region at the E1A N terminus is relatively nonconserved among different Ad serotypes. However, it has been noted that the predicted secondary structure of the first 30 amino acids is a conserved alpha helix which contains a conserved ILE sequence at residues 18 to 20 (15). Therefore the E1A N-terminal TBP binding region may be functionally conserved. The second region required for complete E1A repression activity, which includes residues 48 to 60,

has a preponderance of acidic amino acids. This is unlike the repression subdomains of other direct repressors, such as Dr1, which contain few acidic amino acids and many alanine residues (57).

What could be the role of the second region, which includes E1A ~48 to 60? This region is not needed for strong binding of E1A 1–80 to TBP in vitro but appears to be necessary for full repression activity. It is possible that TBP in its natural conformation within TFIID requires both E1A regions for strong interaction. It is also possible that sequences within E1A 48–60 are important for specific interaction with a cellular regulatory protein. A likely candidate for a cellular regulatory protein is p300, whose association with E1A in extracts of Ad-infected cells requires E1A regions similar to those needed for E1A repression in vitro (49) and in vivo (for reviews, see references 7 and 39). p300 is closely related to the CREB binding protein CBP (11, 14). p300 and CBP may belong to a family of coactivators involved in the efficient transcription of E1A-repressible genes (1). E1A repression of a cotransfected simian virus 40 promoter was previously reported to be partially reversed by overexpression of p300 (14). Thus, it is possible that E1A represses "activated transcription," at least in part, by interaction with a putative p300 or CBP coactivator. Two findings suggest that interaction with p300 or CBP is not obligatory for E1A repression in vitro. First, E1A 1–80 affinity-depleted transcription extracts are depleted of both TBP (TFIID) and p300 and do not support transcription (51; unpublished data). Yet in vitro transcription is restored by the addition of TBP or TFIID alone, and the restored activity is repressible by added E1A without added p300 (51). Second, E1A can repress basal transcription in a reconstituted in vitro transcription system that does not contain added p300 or CBP (unpublished data). Although p300 and CBP are not obligatory for E1A repression in vitro, they may be required for efficient repression in vivo.

Recent findings indicate that p300 and CBP can interact with several sequence-specific transcriptional activators, including CREB (1, 11, 29), c-Jun (2), YY1 (30), c-Fos (6), c-Myb (12), and nuclear receptors (27), as well as with P/CAF (a histone acetylase) (56) and pp90^{RSK} (an S6 kinase involved in the Ras pathway) (42). Of particular interest, c-Fos, P/CAF, and pp90^{RSK} bind to the region within p300 or CBP, which is also recognized by the E1A N-terminal repression domain. Furthermore, like E1A, pp90^{RSK} is able to repress transcription of cyclic AMP-responsive genes (42). Mechanistically, it was suggested that pp90^{RSK} may interfere with the recruitment of a general transcription factor to the p300 or CBP complex (42). Thus, it is possible that E1A and pp90^{RSK} repress transcription by a similar mechanism.

To summarize, our results suggest that the E1A N-terminal repression domain can interact directly with TBP (TFIID) and that this interaction can uniquely disrupt a TBP-TATA box complex in a manner which is reversible by TFIIB. We can speculate that in vivo, this interaction is rendered more efficient by the recruitment of E1A to specific promoters containing bound p300 or CBP. Upon recruitment of E1A to the promoter, several possible mechanisms that may lead to the disruption of the TBP-TATA interaction and/or the inhibition of TFIIB recruitment to the promoter can be imagined. For example, E1A could compete with TFIIB for an overlapping binding site on the exposed surface of the TBP (TFIID) bound to the TATA box. This interaction could alter the conformation of TBP so that it no longer binds efficiently to the TATA box. Further studies are needed to define the precise interactions among the E1A N-terminal domain, TBP, TFIIB, and p300 or CBP that lead to transcriptional repression.

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