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Biochemistry

TATA-binding protein and the retinoblastoma gene product bind to overlapping epitopes on c-Myc and adenovirus E1A protein

(retinoblastoma protein)

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ABSTRACT **Using a protein binding assay, we show that the amino-terminal 204 amino acids of the c-Myc protein interact directly with a key component of the basal transcription factor TFIID, the TATA box-binding protein (TBP). Essentially the same region of the c-Myc protein also binds the product of the retinoblastoma gene, the RB protein. c-Myc protein coimmunoprecipitates with TBP in lysates of mammalian cells, demonstrating that the proteins are also complexed *in vivo*. A short peptide that spans the RB binding site of the E7 protein of human papilloma virus type 16 interferes with the binding of c-Myc to TBP. The same peptide also blocks binding of adenovirus E1A protein to TBP, suggesting that c-Myc and E1A bind to RB and TBP through overlapping epitopes. Furthermore, we show that binding of RB to E1A prevents association of E1A with TBP. Our data suggest that one of the functions of RB and RB-like proteins is to prevent interaction of viral and cellular oncoproteins, such as c-Myc and E1A, with TBP.**

Myc proteins are sequence-specific DNA-binding proteins that control proliferation and differentiation of a variety of cell types. The carboxyl terminus of c-Myc is required for dimerization and DNA binding, whereas the amino-terminal third of c-Myc has transcription-activating ability (1–3). Gene-specific transcription regulators like c-Myc somehow mediate their response through the basal transcription machinery utilized by RNA polymerase II (4). Consistent with this is the finding that the adenovirus E1A transactivator protein binds to the TATA-binding protein (TBP), a factor required for transcription by all three nuclear RNA polymerases but originally defined as a subunit of the basal transcription factor TFIID (5–8). Other examples of transactivators which interact with the basal polymerase II factors include the herpes simplex virus VP16 and the Epstein–Barr virus Zta proteins (9–13).

The c-Myc protein is structurally and functionally related to the E1A oncogene products (14–17). Furthermore, both c-Myc and E1A can form a specific complex with the RB protein, the product of the retinoblastoma gene (18, 19). Given these similarities, we have investigated whether Myc proteins and DNA tumor virus-encoded transactivators could contact a common target within the basal polymerase II machinery. We show here that the c-Myc oncoprotein, like E1A, contacts TBP. Moreover, our findings have important implications for understanding the function of both c-Myc and RB proteins.

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MATERIALS AND METHODS

Generation of Fusion Proteins and Assay of *in Vitro* Protein Interaction. To generate ^{32}P-labeled bacterially synthesized TBP protein, a cDNA encoding human TBP was cloned into vector pET11b (Novagen) downstream of a sequence encoding a cAMP-dependent protein kinase phosphorylation site and upstream of six histidine codons. The encoded protein was purified from bacteria and labeled *in vitro* with protein kinase (Sigma catalogue no. P-2645) and [γ-^{32}P]ATP as described (20, 21). Vectors that direct the synthesis of TBP and various glutathione *S*-transferase (GST)–Myc fusion proteins were generated by PCR using human TBP and c-*myc* cDNAs as templates. All c-*myc* PCR products were cloned in the pGEX-2T vector (Pharmacia) and sequenced. GST fusion proteins were purified from *Escherichia coli* as described (19). GST–RB contains amino acids 379–729 of human RB. GST–RBΔ21 carries an internal deletion in the region of RB that is normally required for interaction with viral proteins (22).

Human TBP was made by *in vitro* translation of the human cDNA (23) with rabbit reticulocyte lysates and [^{35}S]methionine. Conditions for *in vitro* binding assays have been described (19).

Immunoprecipitation. For methionine labeling, cells were incubated with 250 μCi (1 μCi = 37 kBq) of [^{35}S]methionine per 100-mm dish for 45 min. After this, cells were lysed by sonication in ELB buffer (19). Equal amounts of radioactive lysates were incubated on ice for 1 hr with antibody. Immunoprecipitates were collected by binding to protein A-Sepharose, heated in SDS buffer, and loaded on an SDS/10% polyacrylamide gel. For phosphate labeling, cells were starved for 1 hr in phosphate-free medium. After this, 5 mCi of [^{32}P]orthophosphate was added for 4 hr.

RESULTS

Direct Binding Between c-Myc and TBP *in Vitro*. Using an *in vitro* binding assay, we found that *in vitro* transcribed and translated TBP bound to Sepharose-linked GSTΔMyc, a fusion protein containing the GST protein linked to the amino-terminal 204 amino acids of c-Myc (19), but not to control Sepharose beads, beads loaded with GST alone, or beads loaded with two other GST fusion proteins. As expected, TBP also bound to GST fused to the 13S adenovirus E1A protein (7, 8). In contrast, *in vitro* translated basal transcription factor TFIIB did not bind any of the GST fusion proteins. E1A has also been shown to interact with other transcription factors, including Oct-4, ATF2, and AP-1 (24–

Abbreviations: TBP, TATA-binding protein; TAF, TBP-associated factor; GST, glutathione *S*-transferase.

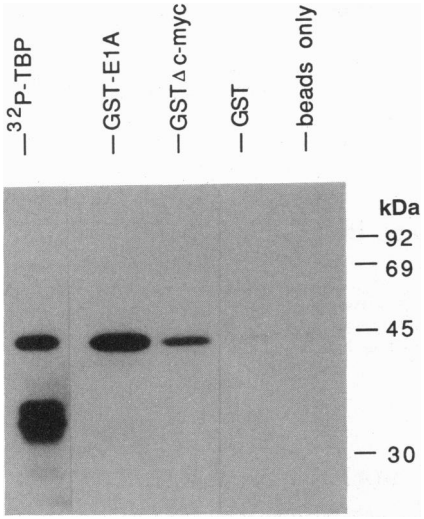

FIG. 1. *In vitro* binding of c-Myc to TBP. ^{32}P-labeled bacterially synthesized human TBP was incubated with GST fusion proteins linked to glutathione-Sepharose (Pharmacia) beads. Proteins bound to the GST fusion proteins were separated in an SDS/10% polyacrylamide gel and detected by fluorography. The fusion proteins were GST fused to amino acids 1–204 of c-Myc (GSTΔc-Myc) or GST fused to the 13S E1A protein of adenovirus type 5 (GST–E1A). Lane labeled ^{32}P-TBP contained bacterially synthesized TBP labeled *in vitro* with [γ-^{32}P]ATP.

26). We detected no binding of any of these factors to GSTΔMyc in the assay described above (data not shown).

Mammalian TFIID exists as a complex consisting of the 38-kDa TBP and several TBP-associated factors (TAFs; refs. 27 and 28). TFIID capable of directing transcription by RNA polymerase II can be purified from mammalian cells in two discrete size complexes of 300 kDa and >700 kDa (27, 29). In the experiments described above, TBP was synthesized in a rabbit reticulocyte lysate, which may have contained one or more TAFs. Therefore, to investigate whether c-Myc binds TBP directly or via associated proteins, we incubated bacterially synthesized human TBP with *E. coli*-produced GSTΔMyc protein. TBP was labeled *in vitro* with ^{32}P through an engineered kinase substrate site (21). Fig. 1 shows that bacterially synthesized TBP bound GSTΔMyc and GST–E1A. Since in this binding assay all components were bacterially expressed, these data suggest strongly that c-Myc interacts directly with 38-kDa TBP and does not require the presence of additional mammalian factors. Quantitative analysis indicated that the relative affinity of E1A for TBP is 3-fold higher than that of c-Myc for TBP.

TBP Is Complexed with c-Myc *in Vivo*. We initially performed transient transfection assays with c-Myc and TBP expression vectors in COS-7 cells. In these experiments, human TBP was fused to a 10-amino acid epitope that is recognized by the monoclonal antibody 12CA5 (30). Under mild lysis conditions, the 12CA5 antibody precipitated 38-kDa TBP and a 62-kDa protein that comigrated with c-Myc. Subsequent experiments showed that the 62-kDa protein that coprecipitated with TBP was recognized by a c-Myc monoclonal antibody, suggesting that the two proteins are complexed in transiently transfected COS-7 cells (data not shown).

The high levels of c-Myc and TBP protein obtained in a transient expression assay may result in spurious interactions that are not found under more physiological conditions. To investigate whether c-Myc and TBP also associate in nontransfected cells, we used a monoclonal antibody to human TBP, named 14D5 (a gift of F. Holstege, University of Utrecht, The Netherlands). This antibody precipitates *in vitro* translated human TBP, but not c-Myc (data not shown). Human colon carcinoma COLO320 cells were labeled with [^{35}S]methionine, lysed under nonionic detergent conditions, and immunoprecipitated with either monoclonal antibody

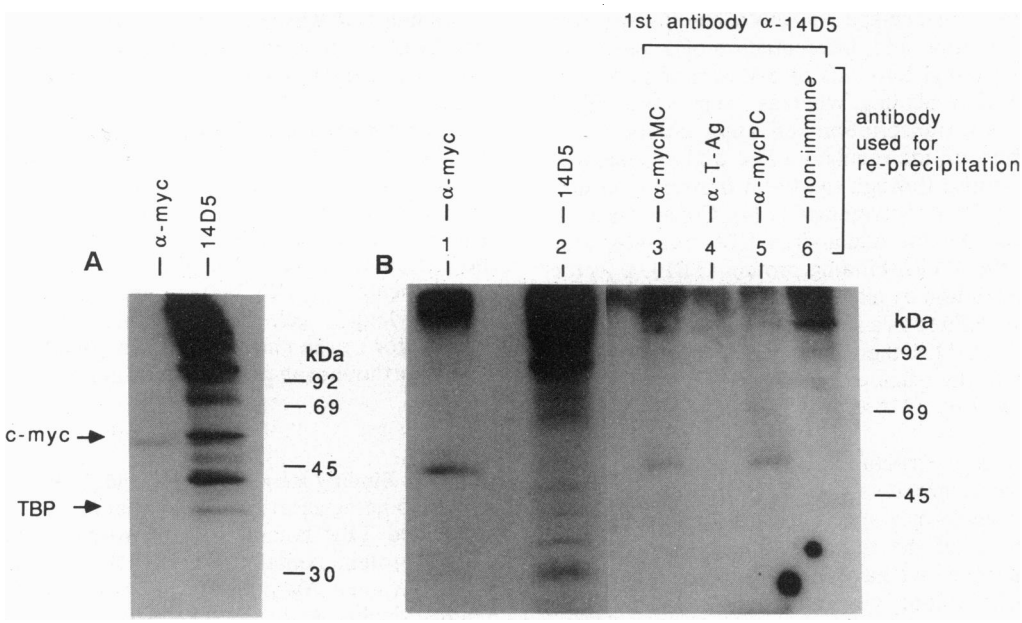

FIG. 2. c-Myc coprecipitates with TBP *in vivo*. (*A*) COLO320 cells were metabolically labeled with [^{35}S]methionine, and lysate proteins were immunoprecipitated with either c-Myc-specific monoclonal antibody or the TBP monoclonal antibody 14D5. Proteins were separated by SDS/10% PAGE and detected by fluorography. α, Anti. (*B*) COLO320 cells were metabolically labeled with [^{32}P]orthophosphate, and lysate proteins were immunoprecipitated with either monoclonal antibody to c-Myc protein (lane 1) or the TBP monoclonal antibody 14D5 (lane 2). Lanes 3–6, anti TBP immunoprecipitate was heated in SDS-containing buffer, and released proteins were reprecipitated with either a monoclonal antibody (lane 3, α-mycMC) or a rabbit polyclonal antiserum to c-Myc (lane 5, α-mycPC). As a control, equivalent amounts of radioactive protein released from the anti-TBP immunoprecipitate were reprecipitated either with a monoclonal antibody directed against the simian virus 40 large tumor (T) antigen (lane 4, α-T-Ag) or with a nonimmune rabbit serum (lane 6). Shown are a 2-hr exposure of lanes 1 and 2 and a 2-day exposure of lanes 3–6.

14D5 or a c-Myc monoclonal antibody. Antibody 14D5 precipitated 38-kDa TBP as well as a number of additional proteins, from COLO320 cells (Fig. 2*A*), in full agreement with reports that TBP is not detected as a free antigen in cell extracts but is associated with TAFs in several multiprotein complexes (27, 28, 31). One of the proteins that was coprecipitated with TBP from the COLO320 cells had an apparent molecular mass of 62 kDa and comigrated with c-Myc (Fig. 2*A*). To investigate whether the 62-kDa protein that coprecipitated with TBP was c-Myc, we performed a sequential immunoprecipitation experiment. Since Myc proteins can be labeled very efficiently with [^{32}P]orthophosphate, COLO320 cells were incubated with this isotope, lysed under mild conditions, and immunoprecipitated with monoclonal antibody 14D5. The anti-TBP immunoprecipitate from COLO320 cells was then denatured, and the released proteins were reprecipitated with various antisera. Both monoclonal antibody (Fig. 2*B*, lane 3) and polyclonal antiserum (lane 5) to c-Myc reprecipitated a 62-kDa protein from the TBP immunoprecipitate. In contrast, neither the nonimmune serum nor a control monoclonal antibody reprecipitated a protein of that mobility from the same TBP immunoprecipitate (lanes 4 and 6). No c-Myc protein was reprecipitated from an immunoprecipitate of an unrelated monoclonal antibody (data not shown). Given the inability of 14D5 to recognize c-Myc directly, these data suggest strongly that in COLO320 cells some of the c-Myc protein is associated with TBP. We estimate that ≈5% of the total amount of c-Myc protein in COLO320 cells coprecipitated with TBP. In summary, our results *in vivo* corroborate those obtained *in vitro*.

Sequences of c-Myc Required for TBP and RB Binding. To localize the region of c-Myc involved in binding to TBP, 12 deletions were introduced into the c-*myc* gene and linked to GST. These proteins were used in an *in vitro* binding assay with ^{35}S-labeled TBP (Fig. 3*A*). The observed pattern of binding appeared similar to the binding of RB to c-Myc (19), in that two regions of c-Myc could independently mediate binding to TBP. To further evaluate the similarity between RB and TBP binding to c-Myc, all 12 GST–Myc fusion proteins were also tested for RB binding. The patterns of RB and TBP binding to the GST–Myc fusion proteins are strikingly similar (Fig. 3*B*). Although these experiments did not allow an unambiguous identification of the regions of c-Myc involved in TBP and RB binding, the data do suggest that the c-Myc protein uses the same sites to bind to TBP as it uses to bind to RB.

TBP and RB Bind to Overlapping Sites on Oncoproteins. One of the GST–Myc fusion proteins (dl10, containing residues 5–41 of c-Myc) showed weak but reproducible binding to both RB and TBP (Fig. 3). Analysis of the sequence of this c-Myc domain, which is highly conserved between Myc family members, revealed similarity to the RB-binding motif of E1A, simian virus 40 T-antigen, and human papilloma virus E7 oncoproteins (32, 33). This raised the possibility that the RB-binding motif of DNA-tumor-virus oncoproteins also mediates binding to TBP. To investigate this, peptides that contained the wild-type RB binding site of the E7 protein, or a mutated peptide unable to bind RB (19), were assayed for their ability to block binding of c-Myc and E1A to TBP.

In vitro translated ^{35}S-labeled TBP was preincubated with either wild-type or mutant E7 peptide. Subsequently, binding of TBP to GSTΔMyc was assayed. Wild-type E7 peptide, but not mutant peptide, interfered with binding of TBP to GSTΔMyc (Fig. 4*A*). Similarly, wild-type E7 peptide, but not mutant peptide, interfered with binding of E1A to TBP (Fig. 4 *B* and *C*). *In vitro* translated 12S E1A protein bound weakly to GST–TBP and was also blocked by the E7 peptide (data not shown). We interpret these data as demonstrating that the E7 peptide specifies a TBP binding site that competes with the TBP binding sites of c-Myc and E1A. Since the same E7 peptide also blocks the binding of both c-Myc and E1A to RB (19), we conclude that RB and TBP have overlapping binding sites on c-Myc and E1A.

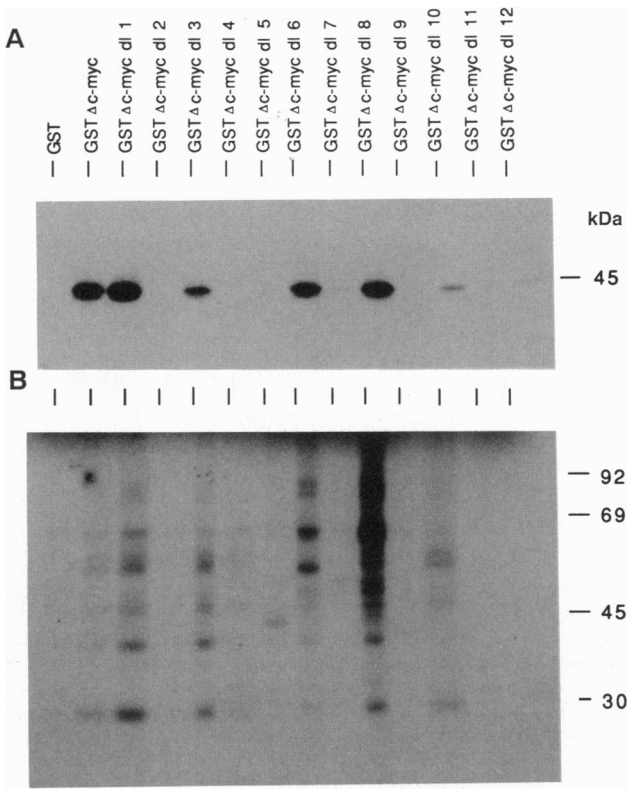

FIG. 3. TBP and RB bind to similar sites on c-Myc. Twelve deletions were introduced into the c-*myc* gene and cloned downstream of the GST gene to produce 12 different GST–c-Myc deletion proteins. Each of the GST fusion proteins was incubated with 10 μl of *in vitro* translated TBP (*A*) or with 10 μl *in vitro* translated human RB (*B*). Synthetic human RB mRNA yields several *in vitro* translation products that are generated by initiation of translation on internal AUG triplets. Only translation products of >28 kDa are bound by E1A and c-Myc (19). The residues of c-Myc that are *present* in the twelve GST–Myc proteins are as follows: dl 1, amino acids (aa) 1–108; dl 2, aa 1–39 and 55–108; dl 3, aa 75–204; dl 4, aa 75–105 and 144–204; dl 5, aa 1–5 and 91–204; dl 6, aa 1–39 and 55–204; dl 7, aa 1–108 and 144–204; dl 8, aa 1–55 and 105–204; dl 9, aa 1–39 and 178–204; dl 10, aa 5–41; dl 11, aa 35–72; dl 12, aa 122–146.

Binding Interactions of RB and TBP with E1A Are Mutually Exclusive. The data presented above are consistent with a model in which TBP and RB bind to overlapping sites on c-Myc and on E1A. To further test this model, *in vitro* translated nonradioactive 13S E1A protein was incubated with either GST–RB or a mutant GST–RB protein that does not bind to E1A (GST–RBΔ21; ref. 34; Fig. 5*A*). After this, ^{35}S-labeled *in vitro* translated TBP was added. Proteins bound to E1A were immunoprecipitated with an E1A antibody, separated by SDS/PAGE, and detected by fluorography. Preincubation of wild-type RB protein with E1A completely blocked binding of TBP to E1A, whereas preincubation of E1A with mutant RBΔ21 protein did not interfere with binding of E1A to TBP (Fig. 5*B*). Binding of the RB-related protein p107 (35) to E1A also blocked binding of E1A to TBP (Fig. 5*B*). These data are in agreement with the peptide competition experiment shown above and indicate that binding of RB or p107 to E1A can prevent association of E1A with TBP.

DISCUSSION

Several viral transactivators can interact directly with TBP, the TATA-binding component of the basal transcription

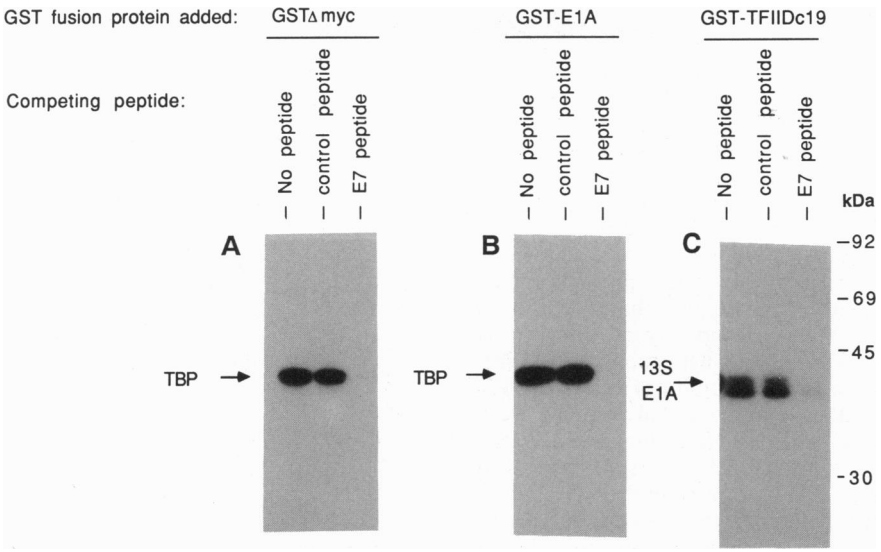

FIG. 4. Blocking of TBP binding by peptides of human papilloma virus type 16 E7 protein. (*A* and *B*) *In vitro* translated TBP was diluted with 0.5 ml of ELB buffer (19) and incubated for 30 min on ice with competitor polypeptides (200 μg of wild-type E7 peptide, amino acids 2–32 of E7, or 200 μg of a mutated version of this peptide that does not bind to RB; ref. 19). After this, 1 μg of GST fusion protein was added to each mix and incubation was allowed to proceed for 2 hr on ice. Glutathione-Sepharose beads were added for 15 min, beads were washed with ELB buffer, and bound proteins were separated by SDS/10% PAGE and detected by fluorography. (*C*) One microgram of GST–TBP (GST-TFIIDc19) was diluted with ELB buffer to 0.5 ml and incubated on ice for 30 min with competitor peptide. After this, 10 μl of *in vitro* translated ^{35}S-labeled 13S E1A protein was added to each incubation and binding was allowed to proceed for 2 hr on ice. After this, GST fusion proteins and associated proteins were collected and analyzed as described above.

factor TFIID (7–9, 13, 36). We present here three lines of evidence to indicate that the c-Myc protein can also form a specific complex with TBP. In an *in vitro* binding assay, TBP bound specifically to the amino-terminal 204 amino acids of c-Myc. Furthermore, c-Myc was coprecipitated with TBP from mammalian cells in a transient transfection experiment using a monoclonal antibody that recognized an epitope-tagged TBP. In nontransfected cells, c-Myc was again coprecipitated by using a monoclonal antibody that recognized TBP directly. In this latter experiment, apart from c-Myc, several other cellular proteins were coprecipitated with TBP, which is not unexpected since TBP is found in cell extracts

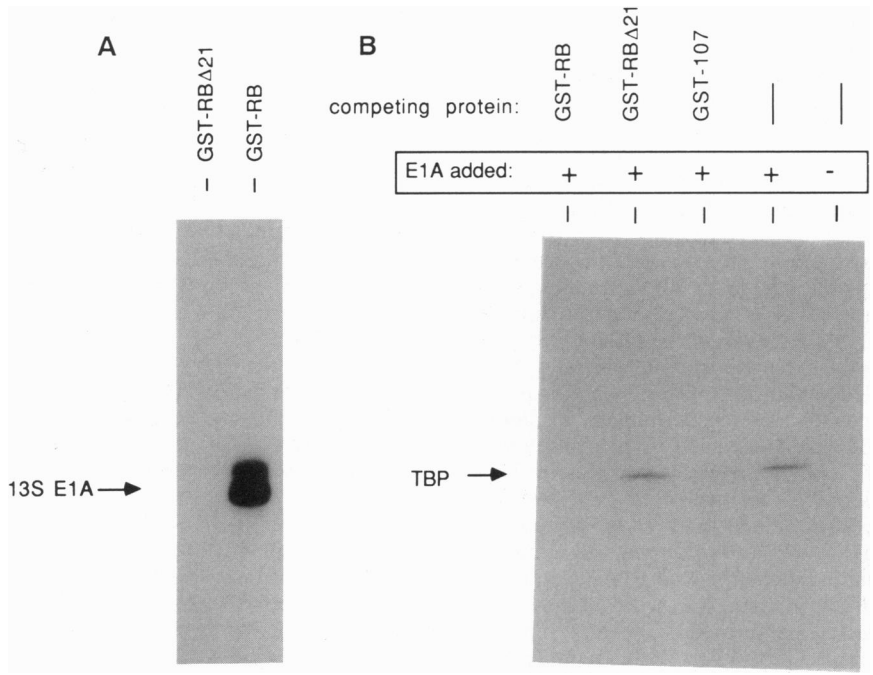

FIG. 5. Mutually exclusive binding of RB and TBP to E1A. (*A*) ^{35}S-labeled 13S E1A protein was generated by *in vitro* transcription and translation of the 13S E1A cDNA. Radioactive E1A protein was then incubated with either GST–RB (34) or a GST–mutant RB protein (GST-RBΔ21). Proteins bound to the GST fusion proteins were collected by binding to glutathione-Sepharose, separated by SDS/PAGE, and detected by fluorography. (*B*) Nonradioactive 13S E1A protein was generated by *in vitro* transcription and translation of the 13S E1A cDNA and incubated with 1 μg of GST–RB or an equimolar amount of mutant GST–RBΔ21 or GST–p107 as indicated in 100 μl of ELB buffer for 1 hr on ice. After this, ^{35}S-labeled *in vitro* translated TBP was added and incubated for 2 hr on ice. The E1A monoclonal antibody M73 was then added and allowed to bind E1A protein for 30 min on ice. Immune complexes were collected with protein A-Sepharose. Bound proteins were separated by SDS/PAGE and detected by fluorography.

in association with a number of other proteins (31). It is important to note, however, that an abundantly expressed nuclear protein, p107 (35), was not found to coprecipitate with TBP from these extracts (data not shown). The finding that c-Myc coprecipitates with TBP from mammalian cell extracts suggests that c-Myc exerts its effects on cellular physiology, at least in part, through direct binding to TBP.

Under the conditions used, only 5% of c-Myc protein was coprecipitated with TBP from mammalian cell lysates. This is not unexpected because, unlike TAFs, upstream activator proteins are thought to have predominantly transient interactions with the basal transcription machinery.

Most interesting was the finding that a peptide that spans the RB-binding motif of the E7 protein of human papilloma virus type 16 prevented the binding of c-Myc to TBP. The same peptide also blocked binding of E1A to TBP. Since this peptide is known to block E1A and c-Myc binding to RB (19), these data suggest that TBP and RB bind to overlapping motifs on c-Myc and E1A. In agreement with this is the finding that RB and TBP appear to bind to very similar sites on the c-Myc protein.

Lee *et al.* (8) have shown that the E1A activation domain (which is unique to the 13S E1A protein) mediates binding to TBP, although weak binding of 12S E1A protein (which lacks the activation domain) to TBP was also seen. Our data differ from those of Lee *et al.* (8) in that we find that the RB-binding motif of E1A (which is shared by the 12S and 13S E1A proteins) is critical for binding of TBP to E1A. One possible explanation is that the RB-binding motif of E1A is a low-affinity binding site for TBP, and that the 13S unique region stabilizes this interaction. The finding that the E7 RB-binding-site peptide blocks TBP binding to 13S E1A protein suggests that the RB-binding motif is nevertheless essential for binding of E1A to TBP. In agreement with this is the finding that binding of RB to E1A can prevent association of TBP with E1A.

Taken together, our data suggest a model in which binding of RB to viral and cellular transcription factors impairs the ability of these factors to modulate cellular gene expression by preventing their interaction with a key component of the basal transcription factor TFIID.

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