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The Major Transcriptional Transactivation Domain of Simian Virus 40 Large T Antigen Associates Nonconcurrently with Multiple Components of the Transcriptional Preinitiation Complex

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Simian virus 40 (SV40) large T antigen (Tag) is a promiscuous transcriptional transactivator; however, its mechanism of transactivation remains unknown. Recent studies have suggested the possible involvement of protein-protein interactions with TBP, the TATA box-binding protein of TFIID, and TEF-1, an enhancer-binding factor. We show here that (i) the Tag domain containing amino acids 133 to 249 directly interacts with the general transcription factor TFIIB, the activator protein Sp1, and the 140-kDa subunit of RNA polymerase II, as well as with TBP and TEF-1; (ii) these interactions can also occur when these transcription factors are present in their functional states in cellular extracts; (iii) binding of Tag to TBP is eliminated by preincubation of TBP either at 48°C or with the adenovirus 13S E1a protein; (iv) this domain of Tag cannot bind concurrently to more than one of these transcription factors; and (v) the substitution of Tag amino acid residues 173 and 174 inactivates the ability of this Tag domain both to associate with any of these transcription factors and to transactivate the SV40 late promoter. Thus, we conclude that SV40 Tag probably does not transactivate via the concurrent interaction with multiple components of the preinitiation complex. Rather, we hypothesize that transactivation by Tag may primarily occur by removing or preventing the binding of factors that inhibit the formation of preinitiation complexes.

Simian virus 40 (SV40) large T antigen (Tag) is a multifunctional protein that controls many aspects of the viral life cycle (for a review, see reference 15). It contains ATPase and helicase activities, binds DNA, directs viral DNA replication, represses transcription from the SV40 early promoter, and binds the tumor suppressor proteins Rb, p53, and p107. Tag is also a promiscuous transactivator of most cellular and viral promoters, including the SV40 late promoter (references 18 and 48 and references cited therein) which can be activated by both TEF-1-dependent (8, 32) and TEF-1-independent mechanisms (7). Much of Tag's transactivation of the SV40 late promoter is achieved by inducing replication of the viral genome to a high template copy number, thereby enabling the titration of cellular repressors of this promoter (60). However, Tag also activates SV40 late transcription in the absence of either viral DNA replication or sequence-specific DNA binding (5, 31, 48).

The SV40 late promoter is transcribed by RNA polymerase II (RNA pol II) with the host cell's machinery. The general transcription factor TFIID in mammals is a complex consisting of at least six proteins (54, 64). The TATA box-binding protein (TBP) is the subunit of TFIID that binds specifically to the −30 region of promoters transcribed by RNA pol II (for a review, see reference 24), including the "TATA-less" SV40 major late promoter (59). The remaining subunits of TFIID are referred to as TBP-associated factors. Binding of TFIID to the promoter is one of the earliest steps in the formation of a preinitiation complex (24).

In contrast to TFIID, TFIIB consists of a single protein (20, 39). Its binding is the rate-limiting step in transcription initiation from the SV40 late promoter (53). TFIIB contains at least two distinct functional domains and interacts in vitro with both TBP and RNA pol II-TFIIF (1, 6, 21).

Numerous cellular and viral activator proteins specifically bind TBP. VP16 of herpes simplex virus (51), the 13S E1a protein of adenovirus (25, 36), and Tag of SV40 (19, 40) are among the viral transactivators shown to associate with TBP. VP16 (37) and the cellular protein COUP-TF1 (chicken ovalbumin upstream promoter-transcription factor 1) (26) are among the transactivators shown to bind TFIIB. Direct contact between a transcriptional activator and the RNA pol II holoenzyme itself is sufficient for weak activation (2, 23). A single-amino-acid change in VP16 can inhibit VP16's ability both to activate transcription and to bind TFIID (27), TFIIB (37), TFIIH (62), and RNA pol II holoenzyme (23); however, binding these transcription factors is not sufficient for full transactivation by VP16 (57) or by E1a (17). One current hypothesis is that these proteins activate transcription by binding concurrently with general transcription factors, enhancer-binding factors, and/or the promoter, thereby increasing the stability or rate of formation of the preinitiation complex (e.g., reference 19).

SV40 Tag has been shown to associate directly with the tumor suppressor proteins p53 and Rb (reference 15 and references cited therein) and the enhancer-binding protein TEF-1 (3, 19) as well as with TBP. The amino-terminal 121 or 138 amino acid residues of Tag are sufficient for weak (i.e., two- to threefold) transactivation in vivo (51, 65). Amino acid residues 101 to 249 of Tag are sufficient to activate transcription 20-fold in a cell-free transcription system (3). The amino-terminal 383 or 249 amino acid residues are necessary for transactivation both in vivo (19) and in a cell-free transcription system (3, 12).

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Point mutants of Tag defective in either sequence-specific DNA binding or the binding of Rb or p53 transactivate promoters on nonreplicating plasmids as well as does wild-type Tag (3, 48, 65). Mutational inactivation of the nuclear localization signal of Tag also fails to eliminate Tag's ability to transactivate (58, 65). When fused to the DNA-binding domain of GAL4, the amino-terminal 383 amino acid residues of Tag fail to activate a promoter containing GAL4-binding sites (19). Taken together, these findings are inconsistent with hypotheses for primary mechanisms of transactivation by Tag that involve either (i) indirect effects via interactions with tumor suppressor proteins or (ii) direct effects via simultaneous interactions with multiple components of the preinitiation complex.

Here, we describe studies to identify other potentially meaningful protein-protein interactions that may be involved in the mechanism(s) of transactivation by SV40 Tag. We show that amino acid residues 133 to 249 of SV40 Tag can specifically associate in vitro with TFIIB, Sp1, and RNA pol II as well as with TBP; however, we also show that (i) a two-amino-acid-substitution mutant of this domain of Tag is defective in both binding to each of these four factors and transactivation and (ii) these interactions cannot occur concurrently. Thus, we conclude that the hypothesis that Tag transactivates by concurrently binding multiple transcription factors is not valid.

MATERIALS AND METHODS

Plasmids. Plasmids directing the expression of glutathione *S*-transferase (GST)-E1a and GST-TBP and for the in vitro transcription and translation of TBP, Zta, Zta$TBP_{221–271}$, and E1a were gifts of A. Berk (36). Plasmids pGEX-$Tag_{1–249}$, pGEX-$Tag_{1–230}$, pGEX-$Tag_{1–147}$, pGEX-$Tag_{45–249}$, pGEX-$Tag_{101–249}$, pGEX-$Tag_{128–249}$, pGEX-$Tag_{133–249}$, pGEX-$Tag_{101–249.E107K}$, pGEX-$Tag_{101–249.S120A.S123A}$, pGEX-$Tag_{101–249.T124A}$, pGEX-$Tag_{101–249.T124S}$, pGEX-$Tag_{101–249.N153T}$, pGEX-$Tag_{101–249.S189N}$, and pGEX–TEF-$1_{1–167}$ were generously provided by A. Wildeman (3, 12). The subscripts indicate the amino acids of Tag present in the construct, with any point mutations indicated after the period (e.g., in pGEX-$Tag_{101–249.E107K}$, K is substituted for E-107). The plasmid pCOUP-TF1, encoding the human homolog of COUP-TF1 (11), was a gift of M.-J. Tsai. pGEX–COUP-TF1 was constructed by subcloning the COUP-TF1 cDNA from pCOUP-TF1 into the *Bam*HI-to-*Eco*RI fragment of pGEX-3X (Pharmacia). Plasmids pGEX-TFIIB and phIIB (20) were gifts of D. Reinberg. pGEX-$Rb_{379–928}$ was a gift of P. Farnham (29). Plasmid pRb, containing a full-length Rb cDNA clone in pBluescript KS, was a gift of P. Lambert. Plasmids pGEX-Tag and pGEX-$Tag_{1–138}$ were constructed by PCR-based amplification of a Tag cDNA and subcloning into the *Bam*HI-to-*Eco*RI fragment of pGEX-3X. Plasmid pGEX-$Tag_{1–272}$ was constructed from pGEX-Tag by digestion with *Hin*dIII and religation. Plasmid pGEX-$Tag_{101–147}$ was constructed by replacing the *Sty*I-to-*Bst*EII fragment of pGEX-$Tag_{1–147}$ with the corresponding *Sty*I-to-*Bst*EII fragment of pGEX-$Tag_{101–249}$. Plasmid pTM-Tag, used to synthesize Tag in a reticulocyte lysate system, was constructed by the insertion of a PCR-amplified cDNA of Tag into the *Nco*I-to-*Pst*I fragment of pTM-1 (36). Plasmid pGEX-βglobin$_{1–123}$ was constructed by subcloning the *Nco*I-to-*Eco*RI fragment of plasmid pβ-βIVS(−) (63) into pGEX-2T.

The plasmids encoding pGEX-$Tag_{101–249.K165A.E166A.K167A}$, pGEX-$Tag_{101–249.K173A.K174A}$, and pGEX-$Tag_{101–249.E177A.K178A}$ were produced by a whole-plasmid PCR mutagenesis of pGEX-$Tag_{101–249}$ (22). Briefly, a mutagenic and second primer whose 5′ ends are next to each other on opposite strands were used to direct the amplification of the plasmid by Vent DNA pol (exo$^-$) (New England Biolabs). The newly generated plasmids were ligated during the PCR by the inclusion of *Taq* DNA ligase in the reaction mixture (New England Biolabs). The input DNA was digested with *Dpn*I, and the newly generated plasmids were transformed into bacteria. The entire Tag-coding regions of these mutants were sequenced to check that no additional mutations had been inadvertently introduced by this procedure.

Proteins. GST fusion proteins were prepared from *Escherichia coli* DH5α cells essentially as described previously (50). An overnight culture was diluted 1:10 into Luria-Bertani broth containing ampicillin and incubated for 2 h at 37°C. Afterward, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to 0.4 mM and the incubation was continued for 2 h at 30°C. The bacteria were collected by centrifugation, resuspended in NETN buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 0.02 mg of aprotinin per ml) and disrupted by sonication. After centrifugation to remove debris, the supernatant containing the fusion protein was stored at −70°C. The concentrations of glutathione-Sepharose-purified fusion proteins were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining; all fusion proteins were judged to be at least 70% pure.

Radiolabeled proteins were synthesized with Tran^{35}S Label (ICN) and a rabbit reticulocyte lysate coupled in vitro transcription and translation system (Promega) by following the manufacturer's suggested protocol. Radiolabeled brome mosaic virus (BMV) proteins were synthesized from BMV RNA (Promega). Recombinant human TBP and TFIIB (purified from *E. coli*) and Sp1 (purified by DNA affinity chromatography from recombinant vaccinia virus-infected HeLa cells) were supplied by Promega. Immunoaffinity-purified calf thymus RNA pol II was generously provided by N. Thompson and R. Burgess (55). RNA pol II was biotinylated with D-biotinoyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester as suggested by the manufacturer (Boehringer Mannheim), dialyzed in NETN with 5% glycerol, and stored at −70°C. HeLa cell nuclear extract was prepared as previously described (14, 60).

To prepare competitor proteins, the appropriate bacterial lysate was incubated at 4°C for 30 min with glutathione-Sepharose 4B (Pharmacia). The beads were washed three times with NETN buffer and twice with thrombin digestion buffer (50 mM Tris-HCl [pH 8.5], 50 mM KCl, 2 mM $CaCl_2$) and were incubated at 25°C for 2 h with 10 μg of thrombin (Boehringer Mannheim) in 400 μl of thrombin digestion buffer. The supernatant was recovered, adjusted to 1 mM phenylmethylsulfonyl fluoride, and briefly centrifuged to remove any residual glutathione-Sepharose. The resulting protein was stored at 4°C until used.

Binding assays. Bacterial lysates containing the GST fusion proteins were thawed on ice and mixed with glutathione-Sepharose at 4°C for at least 30 min. Typically, approximately 4 pmol of fusion protein was used in each binding assay. The beads were washed twice at 4°C with 500 μl of NETN buffer, resuspended in 50 μl of NETN buffer, mixed at 4°C for at least 1 h with the test protein (typically in 0.2 to 2 μl of reticulocyte lysate), and washed three times at 4°C with NETN buffer. The bound proteins were eluted from the beads by boiling in SDS loading buffer and resolved by SDS-PAGE. To improve the signal-to-noise ratio, binding assays involving TBP contained 200 mM NaCl. Competition binding assays were performed as described above, except that (i) thrombin-cleaved protein was included as a competitor, (ii) 0.5 mM phenylmethylsulfonyl fluoride was present in the buffers, and (iii) approximately 0.4 pmol of GST fusion protein was used in each assay.

For assays involving radiolabeled proteins, the gels were stained with Coomassie brilliant blue, destained, equilibrated in water and treated with 1 M salicylic acid for 30 min before being dried and exposed to X-ray film. For immunoblot analyses, the gels were electroblotted to nitrocellulose. The membranes were first incubated with 1% nonfat dry milk and then with anti-TBP, anti-TFIIB, (IIB8 [56]), antibody 8WG16 (55) (gifts of N. Thompson and R. Burgess), and anti-Sp1 antibodies (Santa Cruz Biotech) or with streptavidin-peroxidase (Boehringer Mannheim) and later, as needed, with an appropriate secondary antibody conjugated to peroxidase. Retained antibodies were detected by enhanced chemiluminescence (Amersham). Far Western blot analysis was performed essentially as previously described (33).

Transactivation assays. Assays for transactivation of the SV40 late promoter by Tag were performed with a cell-free system as described previously (3, 12). Each reaction mixture contained 8 μl of HeLa whole-cell extract, 0.18 μg of plasmid pBEL1 (linearized at its *Ava*II sites) as the template, and 0.25 or 0.50 μg of the indicated glutathione-Sepharose-purified fusion protein.

RESULTS

Tag and TBP associate specifically in vitro. Given that transactivation by adenovirus E1a involves its direct interaction with TBP (4, 17, 25, 36), we and others (19, 40, 48) hypothesized that transactivation by SV40 Tag may involve similar direct interactions with general transcription factors. To determine whether SV40 Tag binds TBP, we synthesized in *E. coli* a variety of test and control GST fusion proteins. Radiolabeled proteins, synthesized in a rabbit reticulocyte lysate, were incubated with these GST fusion proteins bound to glutathione-Sepharose, washed, eluted, and separated by SDS-PAGE (Fig. 1A). Because the carboxy-terminal two-thirds of Tag does not appear to play a role in transcriptional transactivation (3, 12, 19, 65), we used the fusion protein GST-$Tag_{101–249}$ in most of the experiments described here instead of one containing full-length Tag. This fusion protein has been shown to contain enough of the Tag sequence to efficiently transactivate the SV40 late promoter in a cell-free transcription system (3, 12).

GST-$Tag_{101–249}$ specifically bound radiolabeled TBP (Fig. 1A, lane 2) and Rb (lane 5) but not the BMV proteins (lane 6). The amounts of the input TBP that bound GST-$Tag_{101–249}$ and GST-E1a were similar (Fig. 1A, lanes 2 and 3, respectively);

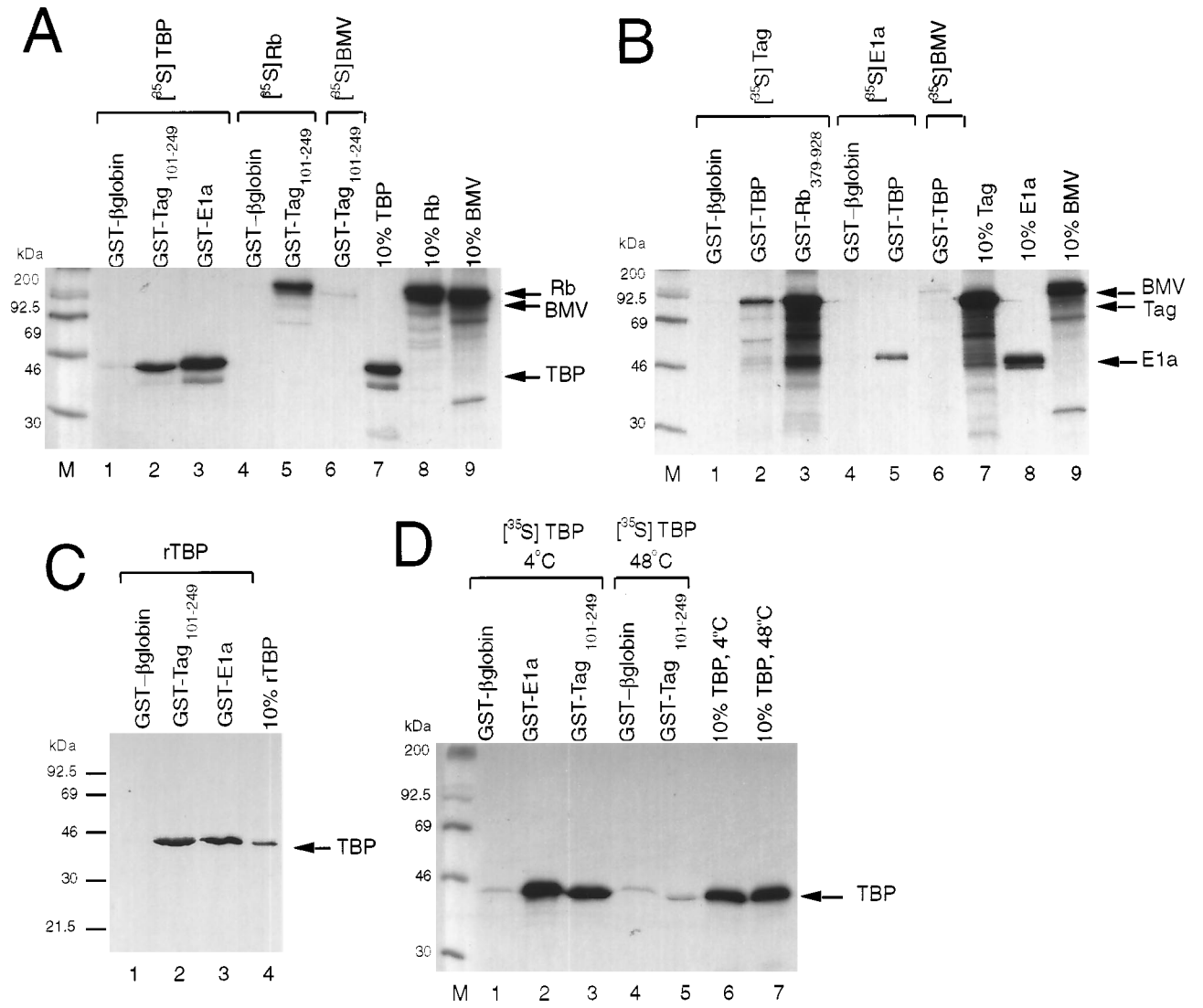


FIG. 1. SV40 $Tag_{101–249}$ binds specifically and directly with TBP in vitro. (A) Fluorogram showing specific binding of TBP with GST-$Tag_{101–249}$. Radiolabeled TBP (lanes 1 to 3), Rb (lanes 4 and 5), and BMV proteins (lane 6), synthesized in a reticulocyte lysate system in the presence of [^{35}S]methionine, were incubated with GST–β-$globin_{1–123}$ (lanes 1 and 4), GST-$Tag_{101–249}$ (lanes 2, 5, and 6), or GST-E1a (lane 3) bound to glutathione-Sepharose. After washing, the proteins were eluted from the resin by denaturation, resolved by SDS-PAGE, and detected by fluorography. Lanes 7 to 9 contained 10% the amount of the indicated radiolabeled protein that was used in each of the corresponding binding assays. Lane M contained molecular mass markers. (B) Fluorogram of full-length Tag binding specifically with GST-TBP. Radiolabeled Tag (lanes 1 to 3), 13S E1a (lanes 4 and 5), and BMV proteins (lane 6) were incubated with the indicated fusion proteins and processed as described for panel A. (C) Immunoblot showing that recombinant TBP (rTBP) and $Tag_{101–249}$ interact directly in vitro. Recombinant human TBP (4 ng) was incubated with GST–β-$globin_{1–123}$ (lane 1), GST-$Tag_{101–249}$ (lane 2), or GST-E1a (lane 3) and processed as described above, except that TBP was detected by immunoblotting. (D) Fluorogram showing inactivation by mild heat treatment of the binding of TBP with $Tag_{101–249}$. Radiolabeled TBP was diluted in NETN and incubated for 10 min either at 4°C (lanes 1 to 3) or 48°C (lanes 4 and 5) prior to incubation with the fusion proteins and processing as described for panel A.

however, fivefold more Tag was needed to obtain this level of binding. GST-$βglobin_{1–123}$ (used as a negative control) bound <1% of either the input TBP (Fig. 1A, lane 1) or Rb (lane 4). Thus, assuming similar percentages of the fusion protein in each preparation are active, we conclude that $Tag_{101–249}$ binds specifically in vitro with TBP but with a somewhat lower level of affinity than does the 13S E1a protein.

To confirm this conclusion, we performed the reciprocal experiment with immobilized GST-TBP and radiolabeled, full-length Tag (Fig. 1B). GST-TBP bound both Tag (Fig. 1B, lane 2) and E1a (lane 5) but not the BMV proteins (lane 6). However, the level of affinity of the Tag-TBP interaction was lower than that of another well-characterized interaction, that between Tag and $Rb_{379–928}$ (30) (Fig. 1B, lane 2 versus lane 3). Tag did not interact with GST-$Rb_{379–928.C706F}$, a fusion protein containing a mutant variant of Rb (30) (data not shown). Thus, we conclude that $Tag_{101–249}$ and TBP associate specifically in vitro but with only moderate levels of affinity.

Numerous controls were performed to rule out the possibility of the Tag-TBP interaction being nonspecific. First, we employed relatively low concentrations of proteins in our reaction mixtures. For example, we typically used approximately 200 ng (approximately 4 pmol) of fusion protein, an amount that is one-half to one-fifth that used in other studies (e.g., references 9, 19, 38, and 41). Second, titrations were performed to ensure that all assays were linear. Third, all binding assays were repeated at least three times with at least two different preparations of fusion protein to ensure reproducibil-

ity of the results. Fourth, the native structure of TBP and the precise sequence of $Tag_{101–249}$ were found to be crucial for this protein-protein interaction to occur (see below). Fifth, GST-$Tag_{101–249}$ was also found to associate with TBP in the form(s) in which it exists in a nuclear extract (see below). Sixth, numerous other general transcription factors and transactivator proteins failed to bind to this region of Tag in our assay (see below). Finally, when the GST fusion proteins were eluted from the glutathione-Sepharose with free, reduced glutathione instead of SDS, these associations were still detected (data not shown). Thus, we conclude that the interaction of Tag with TBP is specific and probably physiologically relevant.

Because the above assays included proteins from a rabbit reticulocyte lysate, many other eukaryotic proteins were also present. One or more of these unidentified proteins could have facilitated the binding of TBP to Tag by either (i) posttranslationally modifying TBP or Tag or (ii) simultaneously binding to both TBP and Tag. To test these hypotheses, we repeated the binding assays using only bacterially expressed proteins and immunoblotting with an anti-TBP antiserum to detect bound protein (Fig. 1C). Both GST-$Tag_{101–249}$ and GST-E1a bound recombinant TBP (Fig. 1C, lanes 2 and 3, respectively). Therefore, GST-$Tag_{101–249}$ can associate with TBP directly in the absence of either auxiliary proteins or eukaryotic-specific posttranslational modifications.

TBP is a very basic protein (45). Portions of the amino-terminal region of SV40 Tag have an acidic character (12). Thus, the association between TBP and Tag observed here and elsewhere (19, 40) might be a trivial consequence of a nonspecific electrostatic interaction. If this were the case, Tag would be expected to bind denatured TBP as well as it binds active TBP. Mild heat treatment destroys the transcriptional activity of TFIID (43), presumably by altering or inactivating functional binding interactions. Thus, we reasoned that a functional association of TBP with Tag might also be affected by mild heat treatment. To test this hypothesis, radiolabeled TBP was incubated for 10 min at either 4 or 48°C prior to incubation with the GST fusion proteins (Fig. 1D). The mild heat treatment caused TBP to lose its ability to specifically bind either GST-E1a or GST-$Tag_{101–249}$ (Fig. 1D, lanes 1 to 5) without TBP being degraded (lane 6 versus lane 7). Therefore, we conclude that this association of $Tag_{101–249}$ with TBP is not simply a nonspecific electrostatic attraction; rather, the active conformation of TBP is necessary for this specific association to occur.

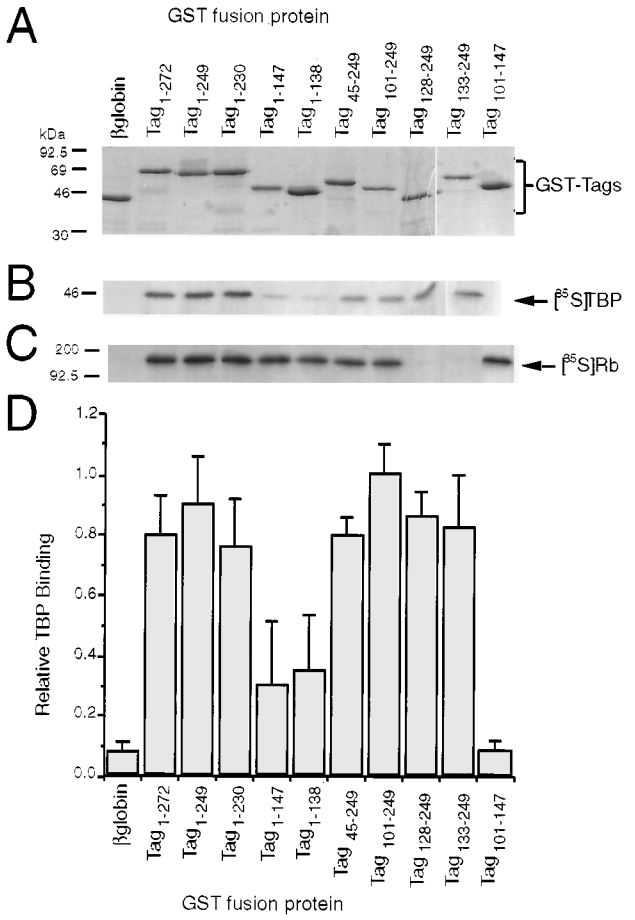


FIG. 2. Mapping the TBP-binding sites on Tag. (A) Coomassie brilliant blue-stained polyacrylamide gel of the GST-Tag fusion proteins used in the experiments shown in panels B, C, and D. Fluorogram of radiolabeled (B) TBP or (C) Rb binding to deletion variants of GST-$Tag_{1–272}$. (D) Summary of data on the binding of TBP to deletion variants of GST-$Tag_{1–272}$ relative to its binding to GST-$Tag_{101–249}$. The relative amounts of TBP retained by the fusion proteins shown in panel A were quantified by PhosphorImager analysis (Molecular Dynamics). Each bar indicates the mean plus standard error of the mean of data obtained from three or more experiments performed with two or more independent preparations of the fusion protein.

Domains involved in the interaction of Tag and TBP. To map the region of Tag required for in vitro association with TBP, plasmids encoding deleted variants of GST-Tag were constructed or obtained from A. Wildeman. Approximately equimolar amounts of each GST-Tag fusion protein (Fig. 2A) were used in binding assays with radiolabeled TBP (Fig. 2B) as described above. To ensure that the differences observed with the GST-Tag proteins were not due to artifacts of preparation, the binding assays were repeated at least three times with at least two preparations of each protein. The extent of binding was quantitated by PhosphorImager analysis (Fig. 2D). Most of the deleted variants of GST-Tag bound TBP fairly well. The 1 to 147 and 1 to 138 regions of Tag bound TBP less well than did the other deleted variants but still bound above the background level. Only the 101 to 147 region of Tag by itself failed to bind TBP. Because the 1 to 138 and 133 to 249 regions of Tag are both capable of binding TBP but the 101 to 147 region is not, we conclude that full-length Tag probably contains at least two binding sites for TBP, with the 133 to 249 region containing the higher-level-affinity site.

As an additional control, the binding assays were also repeated with radiolabeled Rb protein in place of TBP (Fig. 2C). All of the GST-Tag fusion proteins that contain the Rb-binding domain (amino acid residues 102 to 115 [13]) bound the Rb protein equally well. Thus, these fusion proteins were similarly active for binding. The finding that GST-$Tag_{128–249}$ and GST-$Tag_{133–249}$ bound TBP (Fig. 2B) but not Rb demonstrates that TBP and Rb bind distinct regions of Tag.

To identify specific amino acid residues involved in the binding of TBP to Tag, nine GST-$Tag_{101–249}$ fusion proteins containing mutations of single, double, or triple clustered amino acid substitutions were assayed for their ability to bind TBP. GST-$Tag_{101–249.E107K}$, containing a glutamate-to-lysine substitution in amino acid residue 107, failed to bind Rb (13) but bound TBP as well as did GST-$Tag_{101–249}$ (data not shown). The point mutants altered in amino acid residues 120 and 123 (both of which were altered from serine to alanine), 124 (threonine to either alanine or serine), 153 (asparagine to threonine), 165, 166, and 167 (lysine or glutamate to alanine), 177 and 178 (glutamate and lysine to alanine), or 189 (serine to asparagine) were unaffected in their binding of either Rb or TBP (data not shown). However, GST-$Tag_{101–249.K173A.K174A}$

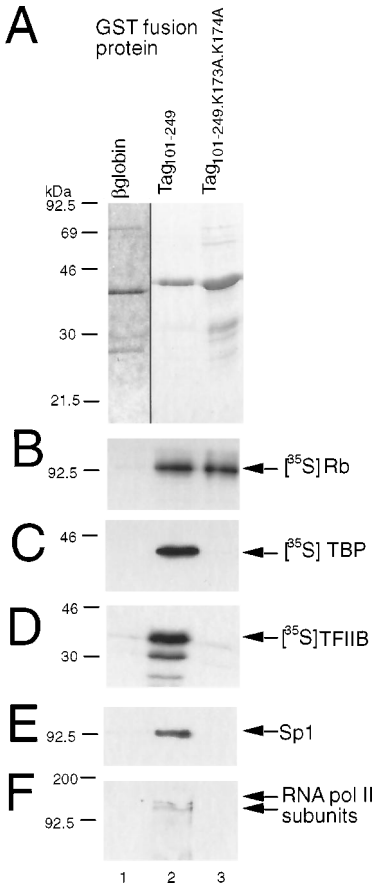


FIG. 3. Mutation of amino acid residues 173 and 174 of GST-$Tag_{101–249}$ eliminates its ability to associate with TBP, TFIIB, Sp1, and RNA pol II without altering its ability to associate with Rb. (A) Coomassie brilliant blue-stained polyacrylamide gel of the fusion proteins. Fluorograms showing (B) Rb, (C) TBP, and (D) TFIIB and immunoblots showing (E) Sp1 and (F) RNA pol II bound by each of the indicated fusion proteins.

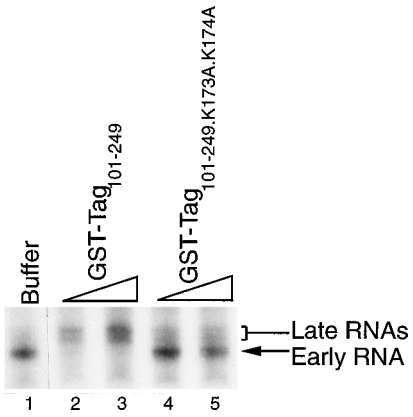


FIG. 4. Mutation of amino acid residues 173 and 174 of GST-$Tag_{101–249}$ eliminates its ability to activate the SV40 late promoter. Autoradiogram showing the inability of GST-$Tag_{101–249.K173A.K174A}$ to transactivate the SV40 late promoter in a cell-free transcription system. The indicated glutathione-eluted fusion proteins were included in cell-free reaction mixtures and transcribed as previously described (3, 12). Lane 1 contained glutathione elution buffer only as a control.

failed to bind TBP (Fig. 3C, lane 2 versus lane 3). This mutant protein still associated normally with Rb (Fig. 3B). It also retained its ability to bind nonspecifically to DNA, albeit one-sixth as well as did the corresponding wild-type protein (data not shown). Its ability to bind sequence specifically to the SV40 origin of DNA replication was only 1/50th that observed with the wild-type protein (data not shown). Thus, this mutation has multiple effects, eliminating some functions, reducing others, and leaving some functions unchanged.

GST-$Tag_{101–249.K173A.K174A}$ was also tested for its ability to activate transcription from the SV40 late promoter in a cell-free system. Wild-type GST-$Tag_{101–249}$ efficiently repressed SV40 early transcription and activated late transcription (Fig. 4, lanes 1 through 3). GST-$Tag_{101–249.K173A.K174A}$ only weakly repressed transcription from the early promoter (consistent with its weakened ability to associate specifically with SV40 DNA) and failed to activate the late promoter (Fig. 4, lanes 4 and 5). Similar results were obtained with two different preparations of each fusion protein (data not shown). Therefore, we conclude that amino acid residues 173 and 174 of Tag play crucial roles both in the association of Tag with transcription factors and in transcriptional transactivation.

Full-length Tag as present in mammalian cell extracts binds amino acid residues 203 to 275 of TBP (40). To identify the region of TBP involved in direct binding with $Tag_{101–249}$, binding assays were performed with a radiolabeled protein containing amino acid residues 221 to 271 of TBP linked to the Zta protein of Epstein-Barr virus (36). Both GST-$Tag_{101–249}$ and GST-E1a bound this protein, albeit only a fewfold above the background level (data not shown). Thus, amino acid residues 221 to 271 of TBP are sufficient for direct binding with $Tag_{101–249}$ as well as with 13S E1a; however, other regions of TBP probably enhance these interactions.

To determine whether these two viral oncoproteins bind to the same or overlapping regions of TBP, we performed a competition binding assay (Fig. 5). $Tag_{128–249}$ binds to TBP but not to Rb (Fig. 2B and C). Soluble $Tag_{128–249}$ competed efficiently with GST-E1a for binding a limiting amount of radio-

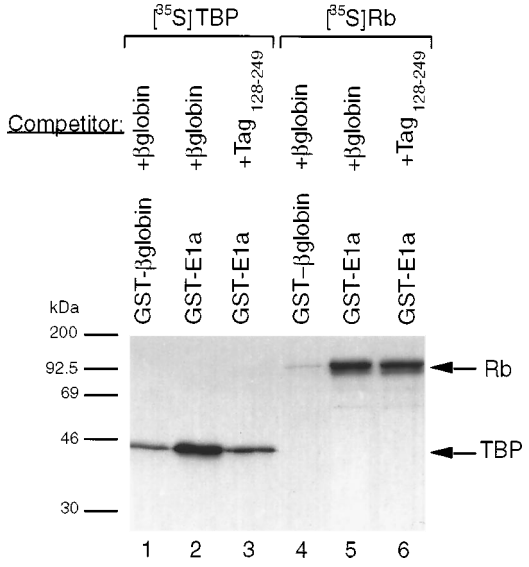


FIG. 5. Fluorogram of $Tag_{128–249}$ competing with 13S E1a for binding to TBP. Limiting amounts of radiolabeled TBP (lanes 1 to 3) and Rb (lanes 4 to 6) were incubated with either GST–β-$globin_{1–123}$ (lanes 1 and 4) or GST-E1a (lanes 2 and 3 and 5 and 6). Soluble $Tag_{128–249}$ (approximately 40 pmol) was added as a competitor in the binding reaction mixtures in lanes 3 and 6; the other lanes received 40 pmol of β-$globin_{1–123}$ as a mock competitor.

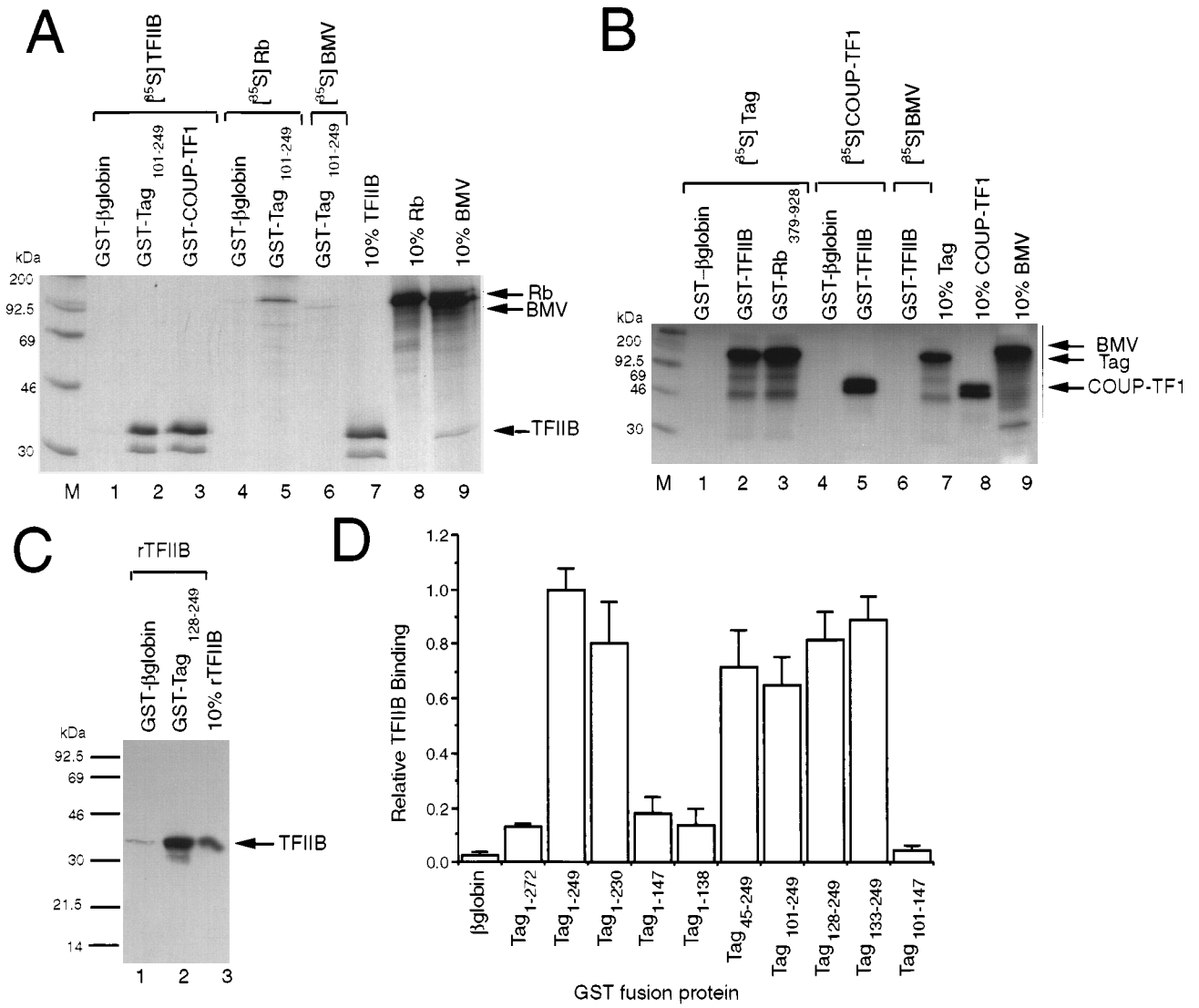


FIG. 6. Tag associates specifically with TFIIB in vitro. (A) Fluorogram showing specific binding of TFIIB with GST-Tag$_{101–249}$. Radiolabeled TFIIB (lanes 1 to 3), Rb (lanes 4 and 5), and BMV proteins (lane 6) were incubated with GST–β-globin$_{1–123}$ (lanes 1 and 4), GST-Tag$_{101–249}$ (lanes 2, 5, and 6), or GST–COUP-TF1 (lane 3) and processed as described in the legend to Fig. 1A. (B) Fluorogram showing specific binding of full-length Tag with GST-TFIIB. Radiolabeled Tag (lanes 1 to 3), COUP-TF1 (lanes 4 and 5), and BMV proteins (lane 6) were incubated with GST–β-globin$_{1–123}$ (lanes 1 and 4), GST-TFIIB (lanes 2, 5, and 6), or GST-Rb$_{379–928}$ (lane 3) and processed as described in the legend to Fig. 1A. (C) Immunoblot showing specific binding of *E. coli*-produced TFIIB with GST-Tag$_{128–249}$. Two hundred nanograms of recombinant human TFIIB (rTFIIB) was incubated with GST (lane 1) or GST-Tag$_{128–249}$ (lane 2) and processed as described in the legend to Fig. 1C, except for the use of an anti-human TFIIB antiserum. (D) Mapping of the TFIIB-binding domain of Tag. Radiolabeled TFIIB was incubated with equimolar amounts of each of the indicated deletion variants of GST-Tag$_{1–272}$ and processed as described in the legend to Fig. 2, except for normalization to GST-Tag$_{1–249}$.

labeled TBP (Fig. 5, lane 3 versus lane 2) but not for binding Rb (lane 6 versus lane 5). Thus, the in vitro bindings of Tag and 13S E1a to TBP are mutually exclusive and, probably, involve identical or overlapping sites located at least in part within amino acid residues 221 to 271 of TBP. The similarities observed here between Tag and E1a in their associations with TBP likely reflect functional similarities of these associations in vivo. Because the E1a-TBP interaction is important for transactivation by E1a (4, 17), we conclude that the Tag-TBP interaction probably plays a physiological role in transactivation by Tag as well.

Tag$_{133–249}$ also associates with TFIIB in vitro. To determine whether Tag associates in vitro with TFIIB as well as with TBP, we performed binding assays with radiolabeled TFIIB synthesized with a rabbit reticulocyte lysate (Fig. 6). TFIIB bound to GST-Tag$_{101–249}$ at a level similar to that of GST–COUP-TF1 (Fig. 6A, lane 2 versus lane 3). Conversely, GST-TFIIB bound to radiolabeled, full-length Tag and COUP-TF1 (Fig. 6B, lanes 2 and 5) and Tag bound to GST-TFIIB and GST-Rb$_{379–928}$ at similar levels (Fig. 6B, lane 2 versus lane 3). Thus, we conclude that Tag associates in vitro with TFIIB as well as with TBP.

To determine whether this association between TFIIB and Tag is by direct protein-protein interaction, binding assays were also performed with recombinant TFIIB produced in *E. coli* (Fig. 6C). GST-Tag$_{128–249}$ was found to associate efficiently and specifically with the *E. coli*-produced TFIIB (Fig. 6C, lane 2 versus lanes 1 and 3). Therefore, Tag and TFIIB directly bind each other without the need for associated factors or eukaryotic-specific posttranslational modifications of either protein.

To map the domain of Tag responsible for binding of TFIIB,

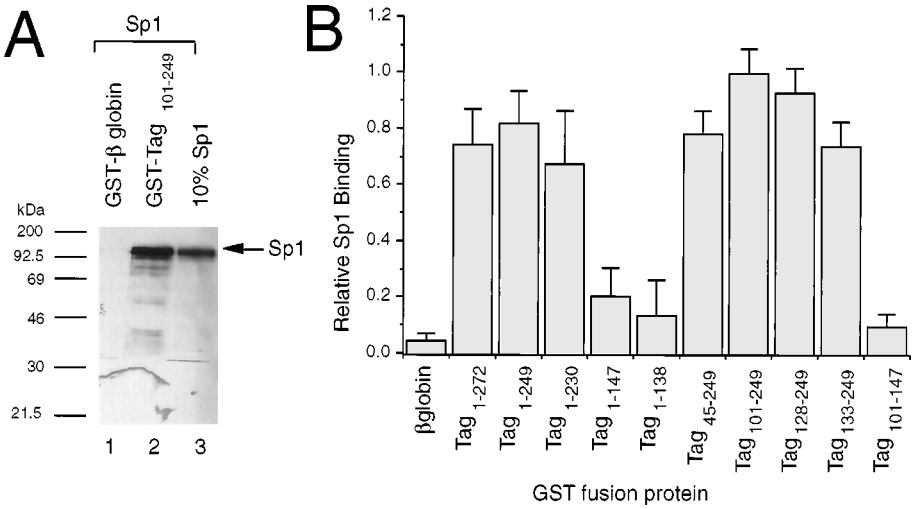


FIG. 7. Tag associates with Sp1 in vitro. (A) Immunoblot showing specific binding of Sp1 with GST-$Tag_{101–249}$. Sp1 (15 ng) was incubated with GST–β-$globin_{1–123}$ (lane 1) or GST-$Tag_{101–249}$ (lane 2) and processed as described in the legend to Fig. 1C, except for the use of an anti-Sp1 antiserum. (B) Mapping of the Sp1-binding domain of Tag. Sp1 was incubated with equimolar amounts of each of the indicated deletion variants of GST-$Tag_{1–272}$ and processed as described above. The resulting immunoblots were quantitated with a laser densitometer. Each bar indicates the mean plus standard error of the mean of data obtained from three or more experiments using two or more independent preparations of fusion protein.

deletion variants of GST-Tag were used in binding assays as described above but with radiolabeled TFIIB (Fig. 6D). Whereas GST-$Tag_{1–147}$ bound TFIIB only marginally above background levels, GST-$Tag_{133–249}$ bound TFIIB essentially as well as did GST-$Tag_{1–249}$. Therefore, as with TBP, the primary TFIIB-binding domain resides within amino acid residues 133 to 249 of Tag. However, GST-$Tag_{1–272}$ failed to associate significantly with TFIIB. Likely, amino acid residues 250 to 272, in the absence of more carboxy-terminal residues, interfere with the TFIIB-Tag interaction because of improper protein folding. The GST-$Tag_{101–249}$ fusion proteins with the point mutations described above were also tested for their ability to bind TFIIB in vitro: each associated with TFIIB as efficiently as did the wild-type GST-$Tag_{101–249}$ (data not shown) except for GST-$Tag_{101–249.K173A.K174A}$, which failed to bind TFIIB (Fig. 3D) and failed to transactivate the SV40 late promoter in vitro (Fig. 4). Thus, as with TBP, the primary TFIIB-binding domain lies within amino acid residues 133 to 249 of Tag, with amino acid residues 173 and 174 playing a crucial role in this protein-protein interaction.

$Tag_{133–249}$ also associates with Sp1 in vitro. The ability of $Tag_{101–249}$ to associate with the cellular activator protein Sp1 was investigated with recombinant human Sp1. GST-$Tag_{101–249}$ efficiently and specifically retained Sp1 in vitro (Fig. 7A). The Sp1-binding domain of Tag was localized with the GST-Tag fusion proteins described above. Sp1 associated efficiently with GST-$Tag_{133–249}$ but poorly with GST-$Tag_{1–147}$ (Fig. 7B). Its binding with Tag was not affected in any of the point mutants described above (data not shown), except for GST-$Tag_{101–249.K173A.K174A}$ (Fig. 3E, lane 2 versus lane 3). Thus, Sp1 associates with the same domain of Tag as do TBP and TFIIB.

$Tag_{133–249}$ also associates with RNA pol II in vitro. We also investigated whether Tag interacts directly with RNA pol II. Immunoaffinity-purified calf thymus RNA pol II was biotinylated and used in binding assays with GST fusion proteins (Fig. 8A). The two largest subunits of RNA pol II were clearly and specifically retained by GST-$Tag_{101–249}$ and GST-$Tag_{133–249}$ as well as by GST-TFIIB (Fig. 8A, lanes 2 to 4) but not by GST-$Tag_{1–147}$ (lane 5). Therefore, $Tag_{133–249}$ binds RNA pol II in vitro. RNA pol II was also bound by all of the point mutants of Tag studied above (data not shown), except GST-$Tag_{101–249.K173A.K174A}$ (Fig. 3F). Thus, RNA pol II binds the same domain of Tag as do TBP, TFIIB, and Sp1.

To examine whether Tag might interact with a specific subunit of RNA pol II in isolation, full-length, radiolabeled Tag was used to probe a nitrocellulose filter containing the subunits of RNA pol II separated by SDS-PAGE (Fig. 8B). Tag was found to bind specifically to the second largest (i.e., 140-kDa) subunit of RNA pol II (Fig. 8B, lane 2). A second band, approximately 50 kDa in size, weakly bound Tag in some experiments and may represent a second subunit of RNA pol II that is capable of interacting with Tag, albeit with a low level

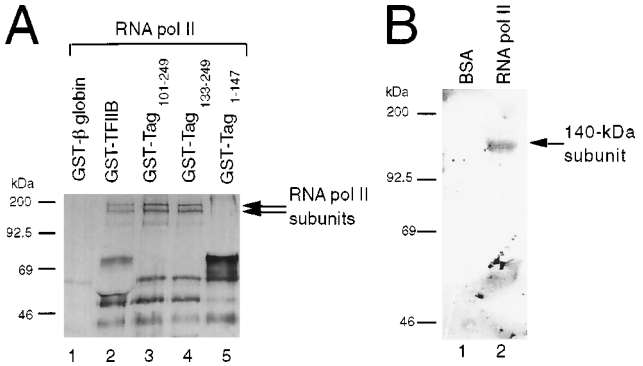
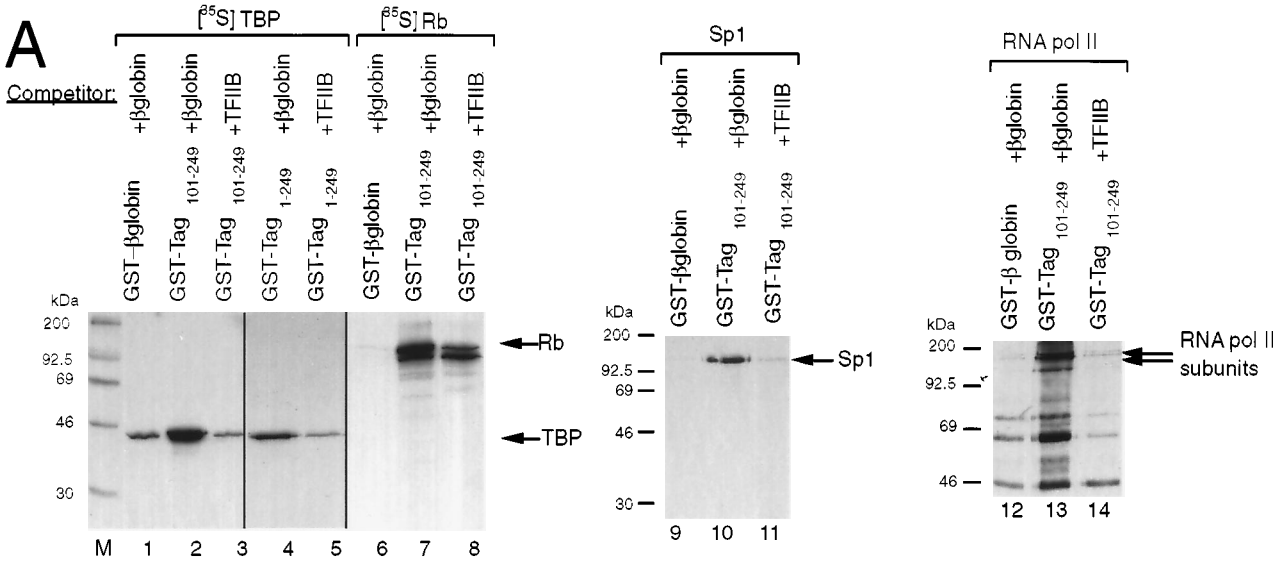


FIG. 8. Tag interacts with RNA polymerase II in vitro. (A) Autoradiograph showing specific binding of GST-$Tag_{133–249}$ with RNA pol II. Biotinylated RNA pol II (800 ng) was incubated with each of the indicated GST fusion proteins and processed as described in the legend to Fig. 1C, except for the use of streptavidin-peroxidase instead of antisera. The two indicated bands comigrated with the largest and second largest subunits of RNA pol II. The biotinylated proteins migrating between 40 and 70 kDa are artifacts introduced during the biotinylation process, as they are not seen in a silver-stained gel of the original preparation of RNA pol II (data not shown). (B) Fluorogram showing Tag binding to the second-largest subunit of RNA polymerase II. Fifteen micrograms of bovine serum albumin (BSA; lane 1) and 15 μg of RNA pol II (lane 2) were separated by SDS-PAGE, transferred to nitrocellulose, renatured, and probed with full-length [^{35}S]Tag.



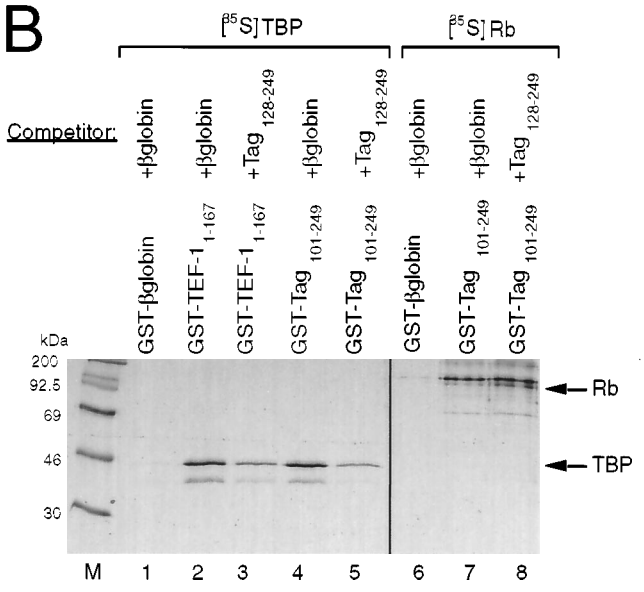


FIG. 9. $Tag_{101–249}$ cannot simultaneously associate with TBP and TFIIB, Sp1 and TFIIB, RNA pol II and TFIIB, or TEF-1 and TBP. (A) Fluorograms and immunoblots showing that TFIIB and TBP (lanes 1 to 5), Sp1 and TFIIB (lanes 9 to 11), and RNA pol II and TFIIB (lanes 12 to 14), but not TFIIB and Rb (lanes 6 to 8), bind to Tag in a mutually exclusive manner. [^{35}S]TBP (lanes 1 to 5), [^{35}S]Rb (lanes 6 to 8), 100 fmol of Sp1 (lanes 9 to 11), and 30 ng of RNA pol II (lanes 12 to 14) were incubated with GST–β-$globin_{1–123}$ (lanes 1, 6, 9, and 12), GST-$Tag_{101–249}$ (lanes 2, 3, 7, 8, 10, 11, 13, and 14), or GST-$Tag_{1–249}$ (lanes 4 and 5) and processed as described in the legend to Fig. 1A. Seventy-five picomoles of unlabeled TFIIB (lanes 3, 5, 8, 11, and 14) or unlabeled β-$globin_{1–123}$ (lanes 1, 2, 4, 6, 7, 9, 10, 12, and 13) was included in the binding reaction mixtures. (B) Fluorogram of competition binding assays performed with the indicated fusion proteins, [^{35}S]TBP or [^{35}S]Rb, and 80 pmol of $Tag_{128–249}$ or β-$globin_{1–123}$ as a competitor.

of affinity. Possibly, Tag interacts with more than one subunit of RNA pol II when the enzyme is in its native conformation. These findings indicate that Tag interacts specifically in vitro with at least one of the subunits of RNA pol II.

$Tag_{101–249}$ cannot simultaneously and directly bind multiple transcription factors. One hypothesis for the mechanism of transcriptional activation by Tag is that Tag increases the kinetics of formation and/or the stability of the preinitiation complex by simultaneously binding multiple components of the transcription machinery (19). Our observation that the region containing amino acid residues 133 to 249 of Tag binds to multiple general and specific transcription factors is consistent with this hypothesis. To examine whether TBP and TFIIB can simultaneously associate with $Tag_{101–249}$, we performed competition binding assays as described for Fig. 5 with radiolabeled Tag and unlabeled TFIIB as the competitor (Fig. 9A). β-$Globin_{1–123}$, prepared in parallel with TFIIB, was included as a control to ensure that the effects were competitor specific. The presence of TFIIB had little, if any, effect on the binding of Rb to GST-$Tag_{101–249}$ (Fig. 9A, lane 7 versus lane 8); however, it prevented the binding of TBP to $Tag_{101–249}$ (lane 2 versus lane 3). This finding was not a consequence of degradation of TBP caused by incubation with this preparation of TFIIB (data not shown). Thus, the Tag domain containing amino acid residues 133 to 249 cannot concurrently bind TBP and TFIIB. Taken together with the evidence showing that amino acid residues 173 and 174 of Tag are critical for its association with either TBP or TFIIB (Fig. 3C and D), it is likely that a single site is responsible for binding either TBP or TFIIB.

Tag may contain a second functional TBP-binding site in its amino-terminal domain (19, 51, 65) (Fig. 2D). If so, a fragment of Tag containing both of these domains might be able to concurrently bind TBP and TFIIB. To test this possibility, we repeated the competition binding experiment using GST-$Tag_{1–249}$. TFIIB disrupted the association of TBP with $Tag_{1–249}$ as well (Fig. 9A, lane 4 versus lane 5). Thus, the in vitro binding of TBP and TFIIB with Tag are mutually exclusive events.

We likewise investigated whether $Tag_{101–249}$ can simultaneously bind Sp1 and TFIIB. Inclusion of TFIIB in the binding reaction mixture essentially eliminated the ability of GST-$Tag_{101–249}$ to bind Sp1 (Fig. 9A, lane 10 versus lane 11). TFIIB was also found to interrupt the association of RNA pol II with GST-$Tag_{101–249}$ (Fig. 9A, lane 13 versus lane 14). Therefore, the binding to $Tag_{101–249}$ of (i) TFIIB and Sp1 and (ii) TFIIB and RNA pol II are mutually exclusive events.

One proposed model for Tag transactivation of the SV40 late promoter (19) predicts that Tag increases the formation of

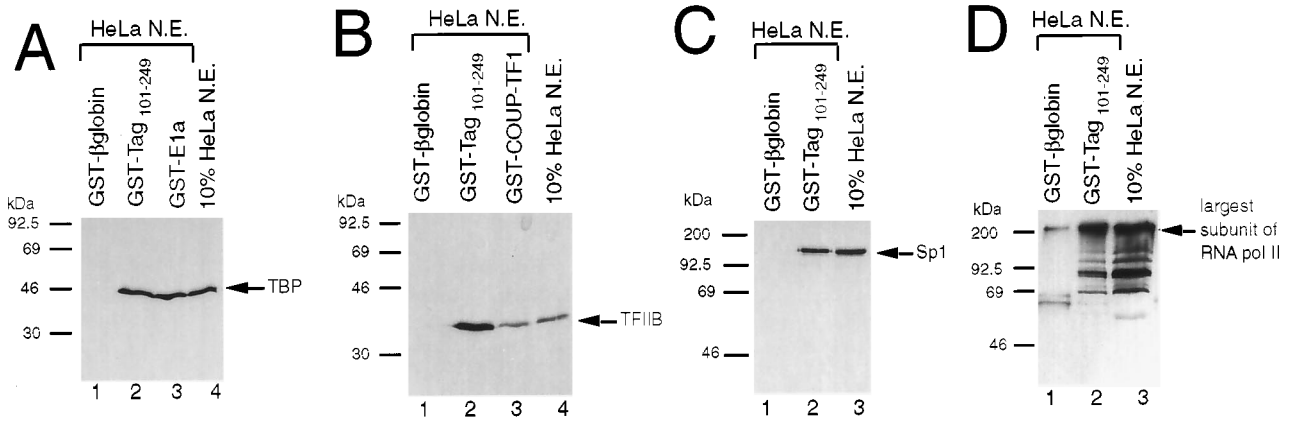


FIG. 10. $Tag_{101–249}$ can associate with (A) TBP, (B) TFIIB, (C) Sp1, and (D) RNA pol II as they exist in a HeLa cell nuclear extract (N.E.). Immunoblots showing binding of the indicated fusion proteins with 75 μg of nuclear extract. The blots were processed as described in the legends to Fig. 1C, 6C, 7, and 1C, respectively, except for the use in panel D of an antiserum, 8WG16, that recognizes the carboxy-terminal repeat domain of the largest subunit of RNA pol II.

preinitiation complexes by simultaneously binding TBP and TEF-1. We tested this prediction by a competition binding assay involving GST–$TEF\text{-}1_{1–167}$ (Fig. 9B). $Tag_{128–249}$ can bind either TEF-1 (3) or TBP (Fig. 2D). $TEF\text{-}1_{1–167}$ can also bind TBP (19) (Fig. 9B, lane 2). However, when $Tag_{128–249}$ is included in the binding reaction mixture, the amount of TBP remaining bound to GST–$TEF\text{-}1_{1–167}$ is comparable to that remaining bound to GST-$Tag_{101–249}$ (Fig. 9B, lanes 2 and 3 versus lanes 4 and 5). The control experiments shown in Fig. 9B, lanes 6 to 8, demonstrate that the presence of $Tag_{128–249}$ did not nonspecifically interfere with these binding reactions. Thus, $Tag_{128–249}$ cannot simultaneously associate with $TEF\text{-}1_{1–167}$ and TBP.

Tag can associate with TBP, TFIIB, Sp1, and RNA pol II in the forms in which they exist in a nuclear extract. TBP normally exists in vivo in a complex with numerous other proteins (for a review, see reference 24). To determine whether $Tag_{101–249}$ can bind to TBP in its physiological state, we also performed binding assays using a HeLa cell nuclear extract that is active in the transcription of genes transcribed by RNA pol II (e.g., reference 60), rather than recombinant proteins, as the source of transcription factors (Fig. 10). Both GST-$Tag_{101–249}$ and GST-E1a were found to bind TBP in the form(s) in which it exists in the nuclear extract (Fig. 10A, lanes 2 and 3). Similarly, GST-$Tag_{101–249}$ was also found to efficiently associate with TFIIB (Fig. 10B), Sp1 (Fig. 10C), and RNA pol II (Fig. 10D) provided from a nuclear extract. We conclude that Tag can bind transcriptionally active TFIID, TFIIB, Sp1, and RNA pol II in the presence of the other nuclear proteins of a mammalian cell.

DISCUSSION

We have demonstrated here that TBP binds specifically to SV40 large Tag in vitro (Fig. 1A and B), confirming previous observations (19, 40). We have gone on to amplify this conclusion by showing the following. (i) This association occurs by direct protein-protein interaction (Fig. 1C) and, thus, does not require adapter molecules or eukaryotic-specific posttranslational modifications. (ii) The native structure of TBP is required for this interaction to occur (Fig. 1D). (iii) Amino acid residues 133 to 249 of Tag are sufficient to bind with TBP (Fig. 2D). (iv) Tag and 13S E1a compete for binding to TBP (Fig. 5). (v) $Tag_{133–249}$ can also specifically bind TFIIB, Sp1, and RNA pol II (Fig. 6D, 7B, and 8A); however, these transcription factors cannot bind concurrently to Tag (Fig. 9). (vi) The same mutation in Tag at amino acids 173 and 174 disrupts the ability of Tag to bind each of these four factors (Fig. 3); however, the mutation does not eliminate binding to DNA (data not shown) or Rb (Fig. 3B). Additionally, this mutation blocks the ability of Tag to activate transcription from the SV40 late promoter in vitro (Fig. 4). (vii) Tag can specifically recognize TBP, TFIIB, Sp1, and RNA pol II as they exist in a HeLa cell nuclear extract (Fig. 10); thus, these associations are not blocked by TBP-associated factors or other eukaryotic proteins. Tag retains the ability to bind these four transcription factors after the lysates are treated with 200 μg of ethidium bromide per ml (data not shown); therefore, these interactions can occur independently of binding to DNA (35).

$Tag_{101–249}$ contains the transcription factor-binding domain and is sufficient for efficient transactivation. Previous studies by others have localized in the Tag sequence the binding domains of a number of transcription factors, including p53 (amino acid residues 259 to 517) and Rb (amino acid residues 102 to 115) (for a review, see reference 15). However, these domains of Tag are not necessary for transcriptional transactivation by Tag in vitro (3). Rather, the Tag domain containing amino acid residues 101 to 249 (with a glutamate-to-lysine substitution at position 107 preventing binding of Rb) is sufficient for efficient transactivation (3). We showed here that this domain of Tag can bind TBP, TFIIB, Sp1, and RNA pol II. Furthermore, the relative levels of affinity of these deletion variants of Tag for TBP or TFIIB are unaffected by their source, i.e., whether these transcription factors are provided from a reticulocyte lysate, purified recombinant protein, or HeLa cell nuclear extract (data not shown).

Amino acid residues 131 to 281 of Tag make up an independent, protease-resistant domain (49, 61). Variants of a protein, especially proteins with deletions, often fail to fold properly when a complete domain is not present and, thus, are frequently unstable in vivo. However, all of the variants studied here could be isolated from *E. coli* (Fig. 2A and 3A), although preparations of some of the mutants contained significant quantities of breakdown products accompanying the full-length protein (e.g., GST-$Tag_{101–249.K173A.K174A}$ [Fig. 3A, lane 3]). Nevertheless, even this protein partially retained its ability to bind double-stranded DNA (data not shown) and fully retained its ability to associate with Rb (Fig. 3B), indicating that it at least partially retains its native conformation. Thus, these

mutations do not grossly compromise the structure of this region of Tag.

Our finding that $Tag_{133–249}$ can bind multiple transcription factors might suggest that these bindings are nonspecific. Four lines of evidence are inconsistent this hypothesis. First, many other mammalian transcription factors, including E2F-1, COUP-TF1, COUP-TF2, hERR1, Dr1, TFIIE, TFIIF, and the carboxy-terminal domain of the largest subunit of RNA pol II fail to bind Tag in this in vitro system (data not shown). Second, the interaction of $Tag_{101–249}$ with TBP requires TBP to be in its native, functional conformation (Fig. 1D) and involves the same or overlapping domain of TBP as does E1a (Fig. 5). Third, the Tag S189N point mutant can bind TBP, TFIIB, Sp1, and RNA pol II (data not shown) but not TEF-1 (3), indicating that at least some of these interactions are genetically separable even though they are mutually exclusive (Fig. 9B). Fourth, GST-$Tag_{101–249.K173A.K174A}$ lacks the ability to bind to all of the four transcription factors studied here but retains the ability to associate with Rb (Fig. 3). Thus, we conclude that these protein-protein interactions with Tag are specific.

The amino-terminal domain of Tag, amino acid residues 1 to 138, also both binds each of these transcription factors (Fig. 2D, 6D, 7B, and 8A) (19) and transactivates (51, 65). However, both transactivation by and binding of these transcription factors to $Tag_{1–138}$ are much weaker than they are with $Tag_{101–249}$ (Fig. 2D, 6D, 7B, and 8A) (3). Therefore, Tag contains two transcriptional transactivation and transcription factor-binding domains; however, the $Tag_{101–249}$ domain is the primary domain.

SV40 large Tag binds multiple components of the preinitiation complex, albeit nonconcurrently. Tag is a promiscuous transactivator. It can activate transcription from a variety of different promoters, including ones with either strong or weak TATA box sequences and a variety of upstream activator-binding sites (18, 48). Our finding that Tag can interact with numerous transcription factors, any of which may be limiting for a given promoter, is consistent with Tag's promiscuity.

We have shown here that TBP and TFIIB bind to the same domain of Tag (Fig. 2D, 3C and D, and 6D); however, they cannot simultaneously bind to GST-$Tag_{101–249}$ (Fig. 9A). Both are highly basic proteins with some overall structural similarity (20, 39). Thus, one trivial explanation for this finding is that these two basic proteins are simply interacting with an acidic region of Tag (12) in a nonphysiologically relevant manner. This possibility was excluded by our finding that mild heat treatment of TBP, which inactivates its functional activity (43) presumably by disrupting the conformation of the protein, eliminates its ability to bind $Tag_{101–249}$ (Fig. 1D). Alternative hypotheses for this finding are (i) TFIIB and TBP together form a complex which is then incapable of binding $Tag_{101–249}$ and (ii) TFIIB directly competes with TBP for binding to Tag. We have been unsuccessful in our attempts to detect a complex containing TBP, TFIIB, and Tag (data not shown). We also found that $Tag_{101–249}$ cannot simultaneously bind in vitro to Sp1 and TFIIB, RNA pol II and TFIIB, and TEF-1 and TBP (Fig. 9). The inability of GST-$Tag_{101–249.K173A.K174A}$ to bind to TBP, TFIIB, Sp1, or RNA pol II (Fig. 3) indicates that an overlapping, if not identical, subregion of Tag is essential for binding to each of these factors. The combination of the mutually exclusive binding and point mutant data presented here indicate that the hypothesis that Tag activates transcription by concurrently binding to multiple components of the preinitiation complex is unlikely to be valid.

Tag naturally exists as oligomers in vivo (15). Thus, although only one transcription factor can associate with a single molecule of Tag at a given time (Fig. 9), it is possible that multiple factors can associate with oligomerized Tag to enable concurrent binding and, consequently, transactivation. However, the carboxy-terminal 118 amino acid residues are essential for oligomerization (42) yet are fully dispensable for transcriptional transactivation both in vitro and in vivo (3, 12, 19, 51, 65). Therefore, Tag can maximally transactivate transcription as a monomer. For example, while GST-$Tag_{101–249}$ can activate transcription 10- to 20-fold (3, 12) (Fig. 4), it can associate with only one transcription factor at a time (Fig. 9) and cannot oligomerize with other molecules of Tag. Thus, we conclude that SV40 large Tag does not transactivate via concurrent association with multiple components of the preinitiation complex increasing the rate of formation and/or the stability of preinitiation complexes.

Most DNA tumor viruses encode transcriptional activators. Many of these activators have been shown to interact with multiple components of the preinitiation complex. We have shown here that SV40 Tag binds at least four transcription factors. Work from other laboratories demonstrates that adenovirus E1a binds to TFIID (25, 36) and that this binding is important (4), but not sufficient (17), for transactivation. VP16 associates with TFIID (52), TFIIB (37), TFIIH (62), and RNA pol II holoenzyme (23), and a mutation altering a single amino acid in the protein inactivates both transactivation and its binding to these numerous transcription factors. Bovine papillomavirus type 1 E2 proteins both bind directly to TFIID and TFIIB and compete with VP16 for factors important for transactivation in vivo (47). The human cytomegalovirus 86K immediate-early 2 protein binds to TBP, TFIIB (9), and at least two other transcription factors (38). The HBx protein of human hepatitis B virus has been shown to interact with both TBP (46) and a subunit of RNA polymerases (10). Many of these proteins are promiscuous transactivators, weakly activating most eukaryotic promoters. All of these associations have been implicated in transactivation. We, therefore, propose that transactivation by many of these viral proteins may occur via a similar mechanism involving a single binding site on these proteins that can interact with any one of numerous cellular transcription factors. One prediction of this hypothesis is that these viral transactivators cannot concurrently bind more than one of these cellular transcription factors.

Mechanism(s) of transcriptional transactivation by SV40 Tag. As an alternative to the multiple interaction hypothesis, we hypothesize that Tag transactivates transcription primarily by competitively removing factors that inhibit the formation of preinitiation complexes. Known examples of viral proteins inactivating transcriptional repressors include the following. (i) The binding of the transcriptional repressor Dr1 to TBP can be disrupted by either adenovirus 12S E1a protein (34) or SV40 Tag (28). (ii) The association of Rb with the transcription factor E2F can be interrupted by SV40 Tag, adenovirus E1a, or human papillomavirus E7 protein (for a review, see reference 44). (iii) The binding of Tag to TEF-1 can inactivate TEF-1's ability to repress transcription from the SV40 late promoter (3).

Our hypothesis that transcriptional transactivation by SV40 Tag is actually relief of repression by Tag is consistent with numerous previous observations, some of which cannot be explained by the hypothesis of concurrent interactions with multiple transcription factors. These observations include (i) the ability of Tag to transactivate even when restricted to the cytoplasm (58, 65), (ii) the fairly weak, promiscuous transactivation by Tag (18, 48), (iii) the inability of Tag to simultaneously bind two or more transcription factors (Fig. 9), and (iv) that transactivation is independent of Tag's DNA-binding function (16, 48).

A double-substitution mutation in Tag at amino acid residues 173 and 174 both disrupts the association of Tag with all of these transcription factors (Fig. 3) and interferes with its ability to activate transcription from the SV40 late promoter (Fig. 4). This finding suggests that Tag's ability to bind to one or more of these factors is necessary for transactivation. GST-$Tag_{101–249}$ and GST-$Tag_{128–249}$ are very similar in their abilities to bind all of the transcription factors tested here (Fig. 2D, 6D, 7B, and 8A); however, only GST-$Tag_{101–249}$ activates transcription from the SV40 late promoter in vitro (3). Thus, the ability of Tag to associate with these transcription factors is not sufficient for transcriptional activation in vitro. Similarly, association with transcription factors is insufficient for full transactivation by VP16 (57) and TBP binding is necessary, but not sufficient, for transactivation by E1a (17) or c-*fos* (41). Thus, it is not surprising that the ability of Tag to associate with transcription factors is insufficient for transactivation.

In summary, we conclude that the domain of SV40 Tag containing amino acid residues 133 to 249 binds specifically and directly, but not concurrently, to TBP, TFIIB, Sp1, and RNA pol II in vitro. Tag's ability to associate with these transcription factors is necessary, but not sufficient, for transcriptional transactivation of the SV40 late promoter in vitro.

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