Bovine Papillomavirus Type 1 E2 Transcriptional Regulators Directly Bind Two Cellular Transcription Factors, TFIID and TFIIB

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The bovine papillomavirus type 1 (BPV-1) E2 translational open reading frame encodes three proteins that regulate viral transcription and DNA replication: the E2 transcriptional activator (E2TA), the E2 transcriptional repressor (E2TR) and the E8/E2 transcriptional repressor (E8/E2TR). E2TA is a strong activator of papillomaviral promoters and is required for viral DNA replication. E2TR and E8/E2TR inhibit the activities of E2TA but also possess weak transactivational properties of their own. Two components of the cellular transcription apparatus, TFIID and TFIIB, have previously been shown to associate with other viral and cellular transcriptional activators. We present evidence here that E2TA, the full-length E2 open reading frame gene product, directly binds both of these transcription factors *in vitro*. Glutathione *S*-transferase E2TA (GST-E2TA) fusion protein bound *in vitro*-synthesized TATA-box-binding protein (TBP), a component of TFIID, and *in vitro*-synthesized TFIIB. Likewise, GST-E2TA bound TFIID and TFIIB present in a nuclear extract from the human cervical cancer-derived cell line, HeLa. The binding of GST-E2TA to TBP and TFIIB required no additional mammalian factors, as shown by direct binding of GST-E2TA to bacterially synthesized recombinant TBP and recombinant TFIIB. The domain of E2TA required for its interaction with both TBP and TFIIB was localized to the C terminus of E2TA, a region also present in E2TR and E8/E2TR. This domain lies within the region of E2TA previously shown to confer cooperative DNA binding by E2TA and TBP and overlaps with the region of E2TA required for DNA binding and dimerization. Our findings, taken in context with previous studies, lead us to conclude that (i) cooperative DNA binding by E2 proteins and TBP is likely mediated by the direct binding of E2 proteins to TBP, (ii) the weak transcriptional transactivation by E2TR and E8/E2TR may result as a consequence of direct TBP and TFIIB binding by these proteins, and (iii) TBP and/or TFIIB binding may be required but is not sufficient for E2TA's strong transactivational activity.

Papillomaviruses are small DNA tumor viruses that infect mammalian hosts, causing benign lesions. These benign lesions can progress, as is the case for some human papillomaviruses, to form cancer. Because they encode few viral gene products, these viruses rely heavily on the host cell machinery for both replication and transcription. These viruses therefore provide a simple model for studying the regulation of eukaryotic transcription and replication. In this study we address the mechanism by which papillomaviruses recruit the host transcriptional machinery for the synthesis of viral mRNAs.

Bovine papillomavirus type 1 (BPV-1) has become the prototype virus for the molecular analysis of papillomaviruses because of its ability to transform rodent cells in tissue culture. In the BPV-1 genome, viral genes are expressed from multiple promoters (4, 76, 88). Previous studies have shown that a family of three genetically related proteins encoded by the viral E2 translational open reading frame (ORF) regulate transcription from these promoters (Fig. 1). They do so in part by binding to the viral DNA genome at multiple sites having the conserved sequence 5'-ACCN₆GGT-3' (2, 53, 57). The multiple viral promoters are differentially regulated by the three E2 proteins, and this regulation appears to be determined at least in part by the relative position of the E2 binding sites with respect to promoter elements (27, 28, 72, 75, 82). The three

proteins synthesized from the E2 ORF (Fig. 1), a full-length product referred to as the E2 transcriptional activator (E2TA) and two C terminus-specific products referred to as the E2 transcriptional repressors, E2TR and E8/E2TR, possess antagonistic transcriptional activities as previously established and indicated by their names (11, 38, 73). All three have a common domain that mediates their ability to bind DNA specifically and to form both protein homodimers and heterodimers (Fig. 1). E2TA is capable of strongly activating transcription (73). The N terminus of E2TA likely contributes to this activity (17, 36, 51), though the domain of E2TA sufficient for transactivation has not been defined. E2TA can associate with the cellular transcription factor Sp1, and this interaction may play a role in the regulation of certain viral promoters by E2TA (44). E2TA also plays a positive role in BPV-1 DNA replication together with another viral protein, E1 (81). E2TA binds E1 protein (56), a DNA helicase (70, 87). Through this association, E2TA is thought to increase E1's occupancy at the BPV-1 origin, through E2TA's own DNA binding potential at the origin (86). Relatedly, E2TA has been found to derepress the replicational capacity of BPV-1 replicons that have been assembled into chromatin *in vitro* (43). The E2 repressors, E2TR and E8/E2TR, are thought primarily to inhibit the multiple activities of the activator (10, 38), either by competition for DNA binding sites or by subunit mixing (5, 38, 53). Recently, however, two studies demonstrated that the E2 repressors can also activate transcription, albeit poorly, from heterologous and mutated homologous promoters (14, 74). The strong phenotypes of mutant BPV-1 DNAs defective in the expression of these dif-

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FIG. 1. E2 proteins and their functional domains. Numbers indicate the amino acid residues in the BPV-1 E2 ORF. (A) Alignment of the coding regions for the genetically related BPV-1 proteins, E2TA, E2TR, and E8/E2TR (11, 37, 38, 73). The filled boxes and represent the domains within the E2 ORF that are conserved among papillomaviruses (3). The asterisks mark the positions of phosphorylation sites (50). The small filled box represents the portion of E8/E2TR derived from the E8 ORF. (B) Shown are the regions of the E2 proteins with identified biochemical activities. Domains necessary for E2 enhancer-dependent transcriptional activation are known to lie within amino acids 1 to 220 (17, 36, 51, 52); however, the domain(s) sufficient for this activity has not been determined. The domains sufficient for DNA binding and dimerization (13, 51, 53, 58, 59), TFIID and TFIIB binding (this study), and cooperative DNA binding with TBP (74) are shared by all of the E2 proteins.

ferent E2 proteins demonstrate their importance in the regulation of viral transcription, DNA replication, and cellular transformation (21, 37, 61, 63, 69, 80, 90). In this study we investigate the mechanism(s) by which the E2 proteins activate transcription.

Transactivators are believed to function, at least in part, through contacting components of cellular transcriptional machinery and affecting the formation and/or stability of the preinitiation complex (12). The preinitiation complex contains multiple components of the cellular transcriptional machinery, including RNA polymerase II and transcription factors TFIIA to -J (for a review, see reference 6). In many cases, virally encoded transcriptional activators have been found to contact directly components of the host cell's transcriptional machinery; these interactions may be important, though not necessarily sufficient, for mediating viral transactivation (16). For example, the adenovirus E1A protein has been shown to contact the TATA-box-binding protein TBP through its activation domain (16, 29, 40). The herpes simplex viral transactivator VP16 contacts TFIIB and two components of TFIID, TBP and the TBP-associated factor TAF_{II}40 (18, 47, 77). Simian virus 40 (SV40) large T antigen was shown to bind both TFIID and TFIIB (22, 31). Genetic analyses indicate that VP16's association with TFIIB is functionally significant; TFIIB mutants not defective for basal transcription but defective for VP16-activated transcription are also unable to bind VP16 directly (65). TFIIB and TBP have been separately described to be rate limiting for preinitiation complex formation (33, 47); thus, both are reasonable targets for transactivators. In this regard, the Epstein-Barr viral transcriptional activator Zta, in associating with TBP, enhances TBP's binding to the TATA box (45).

Whereas all of the above analyses were performed in vitro, several investigators have attempted to address the functional significance of the association of viral activators with cellular transcription factors in cells. For example, visna virus Tat and

VP16 were found to function via targeting a similar subset of cellular transcription factors in vivo (8). The addition of TBP, but not TFIIB, was found to relieve self-squelching by human T-cell leukemia virus type 1 (HTLV-1) Tax, indicating that HTLV-1 Tax targets transcription factor TFIID in vivo (7).

We were interested in addressing whether the BPV-1 E2 transcriptional activator, E2TA, directly contacts any components of the host cell's transcriptional machinery. Data from three recent studies provide a rationale for looking for such interactions. First, overexpression of TBP in vivo was found to facilitate E2TA transactivation of a minimal promoter which was otherwise refractory to E2TA activation, suggesting a functional interaction between E2TA and this basal transcription factor (25). Second, purified E2TA bound cooperatively with TBP to a DNA fragment containing both an E2TA binding site and a TATA box (15, 74). Third, the portion of E2TA required for this cooperativity (Fig. 1) was found to synergize with TBP to activate transcription in vitro (74). In this paper we provide evidence from in vivo and in vitro studies that E2TA directly associates with two components of the transcriptional machinery, TFIID and TFIIB. The domain of E2TA necessary and sufficient for these interactions was mapped to the C-terminal peptide sequence shared with E2 protein family members E2TR and E8/E2TR. Evidence for a functional interaction between E2TA and TFIIB in cells was provided in cotransfection experiments. These findings may provide insight into how this family of viral proteins regulates transcription.

MATERIALS AND METHODS

Plasmids. The full-length E2TA ORF was cloned into the *Bam*HI site of pGEX-3X (Pharmacia) to generate pGST-E2TA; this plasmid was constructed by A. Mangasarian. The E2TA insert was generated by PCR amplification of template pmh142-6 DNA, containing the entire BPV-1 genome inserted into the unique *Bam*HI site of pML2d (68) with sense (5'-GGGGGATCCA TATGGAGACAGCATGCGAA-3') and antisense (3'-GGGGGAT CCAAAGGCAATGGCAGTGAT-3') gene-specific primers containing *Bam*HI recognition sites. These primers were designed to fuse the E2TA translational ORF to that of glutathione *S*-transferase (GST). Plasmids encoding GST-E2TA mutants were generated as described above except that different template DNAs and primer combinations were used. The following previously described plasmids were used to construct the pGST-E2TA mutants: pTZE_Δ92-161, pTZE_Δ158-283 (51), pmh142-6 (68), pmh1139, pmh1141, pmh1142, and pmh1143 (38). The template DNAs and primer pairs used to create the pGST-E2TA mutant constructs were as follows: pGST-E2TA_Δ92-161 (pTZE_Δ92-161 with primers 5'-GST-E2 and 3'-GST-E2), pGST-E2TA_Δ158-283 (pTZE_Δ158-283 with primers 5'-GST-E2 and 3'-GST-E2), pGST-E2TA_Δ1-92 (pmh142-6 with primers 5'-GGGGGATCCATTCCTTGCTTGACACAAGCTG-3') and 3'-GST-E2), pGST-E2TA_Δ1-161 (pmh142-6 with primers 5'-GST-E2_Δ1-161 [5'-GGGGGATCCATGC CGGTGCTGACGCATT-3'] and 3'-GST-E2), pGST-E2TA_Δ1-283 (pmh142-6 with primers 5'-GST-E2_Δ1-283 [5'-GGGGGATCCATGTACCGTGGACTTG GCATC-3'] and 3'-GST-E2), pGST-E2TA_Δ1-310 (pmh142-6 with primers 5'-GST-E2_Δ1-310 [5'-GGGGGATCCATAGGCACCAATGATGG-3'] and 3'-GST-E2), pGST-E2TA_Δ935T (pmh1139 with primers 5'-GST-E2 and 3'-GST-E2), pGST-E2TA_Δ162ST (pmh1141 with primers 5'-GST-E2 and 3'-GST-E2), pGST-E2TA_Δ283ST (pmh1142 with primers 5'-GST-E2 and 3'-GST-E2), and pGST-E2TA_Δ378-410 (pmh1143 with primers 5'-GST-E2 and 3'-GST-E2). The plasmid pGST-βG contains a portion of the human β-globin (βG) cDNA encoding amino acids 1 to 123 inserted into the GST expression vector pGEX-3X (31).

The plasmid pTmTfIID contains the human TBP cDNA inserted into the vector, pTM1 (40). The plasmid pGemhTBP and the mutant derivatives, pGemhTBP_Δ1-201 and pGemhTBP_Δ169-220, contain complete or internally deleted copies of the human TBP cDNA inserted into the vector pGem3 (Promega) (24). The plasmid pGST-E1A contains the 13S E1A cDNA inserted into the GST expression vector, pGEX-3X, and was provided to us by Arnold Berk (University of California, Los Angeles). The plasmid pTfIIB contains the human TFIIB cDNA inserted into the vector pET11a (Novagen) (23).

The plasmid pmh407-1 contains BPV-1 nucleotides 6958 to 7945 (which includes 11 E2 binding sites) inserted into the plasmid pA₁₀CAT, which contains the chloramphenicol acetyltransferase (CAT) gene positioned downstream from the enhancerless SV40 early promoter (73). The plasmid pC59 contains a BPV-1 cDNA inserted into the Okayama-Berg cDNA vector (89). pC59 encodes the BPV-1 gene product E2TA (73). The plasmid pGAL4VP16₄₁₃₋₄₉₀ contains the SV40 early promoter positioned upstream of a chimeric GAL4-VP16 gene encoding amino acids 1 to 147 of Gal4, which is fused to amino acids 413 to 490 of

VP16 (66). The plasmid pGAL4₁₋₁₄₇ is similar to the above-mentioned plasmid, except it contains only the DNA binding domain of the *gal-4* gene (46). The plasmid p(GAL4)₅E1BTATACAT contains five copies of the Gal4 DNA binding site positioned upstream of the E1B minimal promoter which drives expression of the CAT gene (46). The plasmid pSGTFIIB, containing the human cDNA encoding TFIIB, was cloned into the plasmid pSG5 and is expressed in vivo from the SV40 early promoter. This plasmid has been previously described (7).

Antibodies and Western blotting (immunoblotting). Monoclonal antibodies that recognize TBP and TFIIB were provided by Nancy Thompson (McArdle Laboratory, Madison, Wis.) (78 and unpublished data). The polyclonal E2TA antibodies were generated by Tracy Aber (University of Wisconsin, Madison) (1). For immunoblot analyses, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore). Membranes were blocked overnight in 10 ml of blocking solution (150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 0.1% Tween, 5% nonfat dry milk) at 4°C and then incubated with primary antibody (1:2,000 dilutions) in blocking solution for 1 h with rocking at 25°C. Membranes were then washed at room temperature with the following wash solution: 10 mM Tris-HCl (pH 7.4)–150 mM NaCl–0.1% Tween. Secondary antibody (goat anti-mouse coupled to horseradish peroxidase) was added at a dilution of 1:2,000 in blocking buffer and incubated as described above. Proteins were detected by chemiluminography after exposing blots to enhanced chemiluminescence (ECL) (Amersham) reagents used according to the manufacturer's instructions.

Fusion protein purification. GST-E2TA and all other fusion protein constructs were expressed and purified from *Escherichia coli* JM109 cells. Cultures (250 ml) were inoculated with 5 ml of a fresh outgrowth and grown overnight with moderate shaking at 27°C. An additional 250 ml of Luria-Bertani medium was added, and the culture was grown for an additional 3 h at the same temperature. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.2 mM to induce the expression of GST-E2TA, and cells were grown for an additional 3 h at 27°C with minimal shaking. Cells were pelleted by centrifugation at 1,380 × g for 10 min at 4°C, resuspended at 4°C in 10 ml of NET (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl [pH 8]), transferred to 14-ml conical centrifuge tubes, and respun under identical conditions. Cells were resuspended in NETN-complete buffer (NET buffer, 0.5% Nonidet P-40 [Sigma], 1 mM dithiothreitol, 1× protease inhibitor cocktail [50× protease inhibitor cocktail is 2.5 mM tosyl-lysine chloromethyl ketone, 50 mM amino caproic acid, 50 mM benzamide, 0.1 mM leupeptin, 0.1 mM pepstatin, 7% aprotinin, and 5% trypsin-chymotrypsin inhibitor in a buffer of 1 mM EDTA of pH 8, 5 mM EGTA {ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid} of pH 8, 0.02% sodium azide, 0.1% glycerol, and 25 mM MOPS {morpholinopropane-sulfonic acid} of pH 8]) and lysed by sonication at 4°C in 800-μl aliquots. Cell debris was pelleted by centrifugation at 4°C for 5 min at 12,800 × g. Aliquots of the cell lysates were stored at -70°C. E2TA fusion proteins so generated were characterized by E2-specific immunoblots.

In vitro protein association assay. Cell lysates containing fusion proteins (2 to 200 μl) were incubated with 50 μl of a 50% glutathione-Sepharose slurry, prepared according to the manufacturer's instructions (Pharmacia), in a final volume of 200 μl of NETN (NET, 0.5% Nonidet P-40) at 4°C for 30 min with rocking. Within each assay, equimolar amounts of the different fusion proteins were used. Depending on the assay, the actual amount of each fusion protein used was between 300 ng and 2 μg. The concentration of fusion protein in each bacterial lysate was assessed by Coomassie blue staining of glutathione-Sepharose-bound proteins resolved by SDS-PAGE. The glutathione-Sepharose pellets were washed three times with 300 μl of NETN. Fusion proteins coupled to glutathione-Sepharose were then either incubated with a target protein directly or eluted off of the beads. Elution was performed at room temperature in the following buffer: 10 mM glutathione–50 mM Tris-HCl (pH 8)–100 mM NaCl. GST-fusion protein was eluted off of the glutathione-Sepharose for 5 min with rocking. Glutathione-Sepharose was pelleted by a brief spin at 12,800 × g, and the supernatant was saved. Elution of fusion protein was repeated, and then the pooled supernatants were spun and transferred three times. Eluted protein was then dialyzed overnight into NETN-complete buffer plus 1 mM phenylmethylsulfonyl fluoride and 5% glycerol. Eluted fusions were stored at -20°C. In the in vitro protein association assay, fusion proteins coupled to glutathione-Sepharose were then incubated 30 min at 4°C with radiolabeled, in vitro-translated protein (4 to 12 ng), purified recombinant protein (4 ng), or HeLa nuclear extract (HNE) (10 μl of an extract with a total protein concentration of 18 mg/ml) in final reaction volumes of 60 to 200 μl of NETN, depending upon the assay. Beads were washed as described above. Bound proteins were eluted by boiling for 5 min in 15 μl of 2× SDS loading dye (2× SDS loading dye is 125 mM Tris-HCl [pH 6.8], 0.2% SDS, 20% glycerol, 10 μg of bromophenol blue per ml plus β-mercaptoethanol added to 0.2% prior to use) and resolved by SDS-PAGE. Proteins bound to the fusion proteins were then visualized either by fluorography, PhosphorImagery, or by immunoblot using chemiluminography.

Radioimmunoprecipitation assay. Immunoprecipitation assays were performed with eluted GST-fusion protein and in vitro-translated, radiolabeled TBP. A 1:10 dilution of E2TA-specific antisera was precleared with 10 mg of protein A-Sepharose slurry (Pharmacia) per ml, which contained bovine serum albumin (BSA) to a final concentration of 100 μg/ml in NETN buffer. Eluted fusion protein and TBP were incubated for 30 min on ice in NETN buffer plus

1 mM phenylmethylsulfonyl fluoride, and then 5 μl of a 1:10 dilution of the precleared E2TA-specific antisera was added and the reaction mixtures were incubated 1 h longer on ice. Fifty microliters of a 10-mg/ml protein A-Sepharose slurry containing BSA at a final concentration of 100 μg/ml was then added, and the reaction mixtures were incubated with rocking at 4°C. The protein A-Sepharose beads were washed, and bound proteins were eluted and resolved by SDS-PAGE as described above for the protein association assay.

In vitro transcription and translation. TBP and TFIIB were synthesized from the plasmids pTMhTFIID and pGemhTBP or pFIIB, respectively, using the coupled in vitro-transcription and -translation system (T_NT) from Promega according to the manufacturer's instructions and Trans³⁵S-label (ICN). TBP amino-terminal and internal deletion mutants pGemhTBP_{Δ1-201} and pGemhTBP_{Δ169-220} were also synthesized by the coupled reaction. TBP carboxy-terminal mutants were produced according to the manufacturer's instructions in an uncoupled in vitro-transcription reaction (Promega) with RNA transcribed in vitro from the mutant TBP DNA templates per the manufacturer's instructions by the Stratagene in vitro-transcription system. Mutant TBP DNA templates were prepared by cleaving pGemhTBP DNA with the appropriate enzyme to generate the effective carboxy-terminal truncations: *Ssp*I to generate the TBP_{Δ168-339} template and *Bsp*HI to generate the TBP_{Δ201-339} template. Brome mosaic virus (BMV) protein was translated in vitro with BMV RNA (Promega).

Purified recombinant proteins and HeLa nuclear extract. Recombinant TBP (rTBP) was purchased from Promega. Recombinant TFIIB (rTFIIB) was purchased from Santa Cruz Biotech. HeLa nuclear extract was prepared as described previously (84).

Transient transfections and CAT assays. African green monkey kidney CV-1 cells were transfected at approximately 75% confluence with plasmid DNAs by the calcium phosphate method (20). In all transfections, the total amount of DNA transfected was brought to 10 μg by the addition of salmon sperm DNA. Two micrograms of the reporter plasmid pmh407-1 was used in each transfection described in Fig. 7A. Transfections shown in Fig. 7A were performed with 50 ng of pC59 in each pC59 transfection and various amounts (50 ng to 2 μg) of either pGAL4VP16₄₁₃₋₄₉₀ or pGAL4₁₋₁₄₇. pGAL4VP16₄₁₃₋₄₉₀ transactivation was monitored in parallel transfections with 50 ng to 2 μg of pGAL4VP16₄₁₃₋₄₉₀ and 2 μg of p(GAL4)₅E1BTATACAT. Transfections shown in Fig. 7B were performed with increasing amounts of pC59 (0 ng to 8 μg) and a constant amount, 2 μg, of the reporter plasmid, pmh407-1, in each transfection set. Within each of four sets of pC59 transfections either 0 ng, 500 ng, 2 μg, or 8 μg of pSGTFIIB was cotransfected. Cells were harvested 48 h after the transfection and lysed by freeze-thaw. CAT assays were performed as described previously (19) with 5 μl of cell extract per reaction.

Assaying for DNA-mediated protein-protein interactions. Ethidium bromide (EtBr) challenge experiments were performed essentially as described previously (34). Briefly, EtBr was added to a final concentration of 200 μg/ml both to the bacterial cell lysates containing GST fusion proteins and to recombinant protein (in the case of TFIIB) or in vitro-translated protein (in the case of TBP). The same concentrations of TBP and TFIIB were used in these assays that were normally used in the protein association assays (see above). The different protein-EtBr mixtures were incubated on ice for 30 min and then cleared by centrifugation at 12,800 × g in a microfuge for 5 min at 4°C. The EtBr-treated bacterial lysate was then incubated with glutathione-Sepharose beads as described for the in vitro protein association assay, and the beads containing bound GST fusion proteins were washed and then incubated with the EtBr-treated TBP or TFIIB. Binding of the TBP and TFIIB to the immobilized GST fusion proteins was monitored by PhosphorImage analysis or immunoblot as described above.

Experiments to monitor the effect of DNA containing E2 and TBP binding sites on E2TA-TBP association were performed as follows. A twofold molar excess (over GST-E2TA protein) of a DNA oligonucleotide, E2N₅TATA, containing an E2 binding site and a TATA element separated by 5 bp (15), was added to immobilized GST fusion protein and incubated for 20 min with rocking at 4°C. In vitro-translated TBP was then incubated with fusion protein, and the standard in vitro protein association assay followed. Binding of TBP to GST fusion proteins was monitored by PhosphorImage analysis.

RESULTS

Association between E2TA and TFIID. Many transactivators have been demonstrated to interact with components of the basal transcriptional machinery. Prior studies had provided support for the hypothesis that E2TA interacts at some level with TBP, given the cooperative DNA binding properties of E2TA and TBP and their synergistic activities on certain promoters in vivo and in vitro (15, 25, 74). We were therefore interested in testing whether E2TA could interact directly with TBP. The ability of E2TA to associate with TBP was assessed by an in vitro protein association assay described in detail in Materials and Methods. Briefly, E2TA was overproduced in bacteria as a GST fusion protein and batch purified with glu-

FIG. 2. E2TA binds TFIID. (A) Binding of in vitro-synthesized TBP to GST fusion proteins. 35 S-labeled in vitro-translated TBP was incubated with GST-E2TA, GST-E1A, or GST coupled to glutathione-Sepharose beads with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) the addition of cold HNE. Following three washes, proteins bound to beads were eluted, resolved by SDS-PAGE, and visualized by fluorography. Different percentages of labeled TBP included in each binding reaction were loaded in lanes 7 and 8. (B) Binding of TBP present in HNE to GST fusion proteins. GST-E2TA, GST-E1A, or GST coupled to glutathione-Sepharose beads was incubated with cold HNE. Following three washes, proteins bound to beads were eluted, resolved by SDS-PAGE, and subjected to TBP-specific immunoblotting. TBP-specific immune complexes were detected by chemiluminescence. Ten percent of the HNE included in each binding reaction assay was loaded in lane 4. (C) Binding of bacterially synthesized recombinant TBP to GST fusion proteins. GST-E2TA, GST-E1A, or GST coupled to glutathione-Sepharose beads was incubated with rTBP in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of HNE. Following three washes, proteins bound to beads were eluted, resolved by SDS-PAGE, and visualized as described for panel B. Different percentages of rTBP included in each binding reaction were loaded in lanes 7 and 8.

tathione-Sepharose. Radiolabeled TBP was synthesized in a coupled in vitro-transcription and -translation reaction. The ability to detect functionally relevant TBP binding in this assay was confirmed with a GST-adenovirus E1A fusion protein, known to bind TBP directly, as a positive control (16, 29, 40). The level of nonspecific binding by TBP was determined with GST alone or GST- β G as negative controls. GST-E2TA was found to bind TBP at levels 10-fold over that of the background binding of GST alone (Fig. 2A, lanes 1 and 2 versus lanes 5 and 6). This binding was reproducible among greater than 20 independent experiments at levels between 5- and 10-fold above those of the binding of GST alone (Fig. 2) and was similar to the levels bound by GST-E1A (Fig. 2A, lanes 3 and 4).

We were interested in whether factors present in HNE, which contains all factors required for basal and E2-activated transcription (15), could affect the E2TA-TBP interaction. There was a small augmentation in TBP binding to both E2TA and E1A upon the addition of HNE (Fig. 2A, lanes 1 to 4). This slight effect was found in two independent experiments; however, its significance is unsubstantiated given that a similar augmentation in the low level of TBP binding to GST alone was also seen with the addition of HNE (Fig. 2A, lane 5 versus lane 6).

The radiolabeled TBP synthesized in vitro in a rabbit reticulocyte lysate may not be assembled into a TFIID complex. However, TBP is known to exist primarily in the TFIID complex in nuclear extracts (9); thus, other members of the TFIID complex could alter the interaction between TBP and E2TA. We therefore measured the interaction between GST-E2TA and TBP present in HNE (HNE-TBP). In five independent experiments, GST-E2TA was found reproducibly to bind HNE-TBP at levels 10- to 20-fold over that of GST alone (Fig. 2B, lane 1 versus lane 2) and at levels similar to those seen with GST-E1A (Fig. 2B, lane 3). These results indicate that E2TA is able to associate with TBP in the context of TFIID in vitro.

To address whether E2TA can directly associate with TBP, we assessed binding of TBP to E2TA using rTBP expressed in and purified from bacteria. Any potential contaminating polypeptides in this TBP source are of prokaryotic origin and, therefore, are less likely than contaminating proteins of eukaryotic origin to mediate associations between eukaryotic polypeptides. GST-E2TA was incubated with purified TBP in the presence or absence of HNE in an in vitro protein association assay, and bound TBP was detected by a TBP-specific

FIG. 3. E2TA binds TBP amino acids 201 to 339. Various derivatives of TBP were translated in vitro, and binding to GST-E2TA was assessed in an in vitro protein association assay. Shown on the left are amino acids of TBP present in each derivative. On the right are data from a representative experiment. For each TBP derivative, the percentage of input protein bound to GST-E2TA was quantified by PhosphorImage analysis and compared with that of wild-type TBP bound to GST-E2TA. The differences in binding efficiency of these TBP derivatives to GST-E2TA were reproduced in three independent experiments. aa, amino acids; WT, wild-type.

FIG. 4. E2TA binds TFIIB. (A) Binding of in vitro-translated TFIIB to GST fusion proteins. 35 S-labeled in vitro-translated TFIIB was incubated with GST-E2TA or GST coupled to glutathione-Sepharose beads with (lanes 2 and 4) or without (lanes 1 and 3) the addition of cold HNE. Following three washes, proteins bound to beads were eluted, resolved by SDS-PAGE, and visualized by fluorography. Different percentages of the labeled TFIIB that was included in each binding reaction were loaded in lanes 5 and 6. (B) Binding of TFIIB present in HNE to GST fusion proteins. GST-E2TA or GST coupled to glutathione-Sepharose beads was incubated with (lanes 1 and 3) or without (lane 2) cold HNE. Following three washes, proteins bound to beads were eluted, resolved by SDS-PAGE, and subjected to TFIIB-specific immunoblotting. TFIIB-specific immune complexes were detected by chemiluminescence. Ten percent of the HNE included in each binding reaction assay was loaded in lane 4. (C) Binding of bacterially synthesized rTFIIB to GST fusion proteins. GST-E2TA or GST coupled to glutathione-Sepharose beads was incubated with rTFIIB. Following three washes, proteins bound to beads were eluted, resolved by SDS-PAGE, and visualized as described in the legend to panel B. Five percent of the rTFIIB included in each binding reaction was loaded in lane 3.

immunoblot. Similar to E1A, E2TA was found reproducibly to associate directly with TBP at levels 10-fold higher than that of binding to GST alone in three independent experiments (Fig. 2C). Thus, E2TA and TBP interact directly.

Mapping the domains of TBP important for the E2TA-TBP association. To characterize further the associations between TBP and E2TA, a panel of deletion mutants of TBP was used to localize the domain on TBP important for its association with E2TA. Mutant TBP proteins were produced and radiolabeled in either coupled or uncoupled in vitro-transcription and -translation reactions. These mutant, radiolabeled TBP proteins were incubated with GST-E2TA, and interactions were monitored in in vitro protein association assays (Fig. 3). Mutants that lack the C-terminal portion of TBP (TBP $_{\Delta 168-339}$ and TBP $_{\Delta 201-339}$) were unable to bind E2TA, while a mutant lacking the N-terminal portion of TBP (TBP $_{\Delta 1-201}$) was fully competent in E2TA binding. An internal deletion removing amino acids 169 to 220 had only 30% of the binding activity of wild-type TBP, suggesting that at least some portion of this region is likely to be important for optimal binding. Taken together, these results, reproduced in three independent experiments, indicate that a domain on TBP important for associating with E2TA lies between amino acids 201 and 339. This domain includes a portion of the conserved region of TBP to which other transactivators have been shown to bind, including adenovirus E1A (40).

Association between E2TA and TFIIB. Transactivators have been found to bind multiple components of the cellular transcriptional machinery. This has been demonstrated most clearly for VP16, which can associate with TBP, TFIIB, TFIID, and TAF $_{40}$. To test whether E2TA can bind TFIIB in vitro, TFIIB synthesized and radiolabeled in rabbit reticulocyte lysate was incubated with GST-E2TA in the protein association

assay. In more than five independent experiments, TFIIB reproducibly bound to GST-E2TA fusion protein at levels 10-fold over that of its binding to GST alone (Fig. 4A, lane 1 versus lane 3). When unlabeled HNE was added, the amount of radiolabeled TFIIB bound by GST-E2TA decreased by threefold (Fig. 4A, lane 1 versus lane 2); however, a similar decrease was seen with GST alone (Fig. 4A, lane 3 versus lane 4).

It is possible that TFIIB, in the context of other transcription factors, may be altered in its ability to interact with activators such as E2TA. To test this possibility, GST-E2TA was incubated with HNE in the in vitro protein association assay, and interactions between GST-E2TA and endogenous TFIIB were subsequently monitored by immunoblot with monoclonal TFIIB antibodies. The results from such assays (Fig. 4B) reproduced in three independent experiments demonstrated that E2TA binds TFIIB in the context of other nuclear proteins.

To address whether E2TA directly associates with TFIIB, we assessed binding of TFIIB to E2TA using bacterially produced purified rTFIIB. Any potential contaminating polypeptides in this TFIIB source are of prokaryotic origin and therefore less likely than contaminating proteins of eukaryotic origin to mediate associations between eukaryotic polypeptides. To test for a direct association, GST-E2TA was incubated with purified TFIIB in an in vitro protein association assay, and the bound TFIIB was detected by a TFIIB-specific immunoblot. The results show that E2TA indeed directly binds TFIIB (Fig. 4C).

Domains of E2TA involved in binding TBP and TFIIB. To map the domain(s) on E2TA important for interacting with TBP, E2TA deletion mutants were constructed as fusions with GST. We determined by Coomassie blue staining of the GST-E2TA derivatives that the major protein products were of the predicted size for the fusion protein (Fig. 5B). The ability of

these proteins to associate with radiolabeled, in vitro-transcribed and -translated TBP was tested in protein association assays. The deletion of the C-terminal 127 amino acids led to the complete loss of E2TA's capacity to bind TBP (Fig. 5A, GST-E2TA_{283ST}), indicating that this region is necessary for binding. A GST-E2TA fusion protein containing the C-terminal 99 amino acids of E2TA was capable of binding TBP (Fig. 5A, GST-E2TA_{Δ1-310}), indicating that this region is sufficient for binding TBP. Importantly, this domain of E2TA is shared with the two transcriptional repressor proteins, E2TR and E8/E2TR. Indeed, the GST-E2TA_{Δ1-161} fusion protein which was also competent for binding TBP (Fig. 5A) contains the exact E2TR coding region. Furthermore, the C-terminal 99 amino acids of E2TA sufficient for binding TBP lie within the region required for the cooperative DNA binding of E2TA and TBP (Fig. 1) (74).

The N terminus of E2TA, the region not shared with the E2 repressors, has been shown to be required for transactivation. We therefore anticipated that this region might be required for TBP binding. Consistent with this expectation, deletion of the N-terminal 92 amino acids of E2TA led to a diminution in TBP binding activity (Fig. 5A, GST-E2TA_{Δ1-92}). However, other GST-E2TA fusions containing alternative N-terminal truncations (GST-E2TA_{Δ1-161} and GST-E2TA_{Δ1-283}) or internal deletions extending into the N terminus of E2TA (GST-E2TA_{Δ158-282} and GST-E2TA_{Δ92-161}) were capable of binding TBP at levels equivalent to or greater than those of full-length E2TA (Fig. 5A and data not shown). Furthermore, GST-E2TA fusion proteins containing only the E2TA N-terminal 93, 162, or 283 amino acids failed to bind TBP (Fig. 5A, GST-E2TA_{93ST}, GST-E2TA_{162ST}, and GST-E2TA_{283ST}). These data were inconsistent with the N terminus containing a TBP binding domain. If E2TA had two independent TBP binding sites, one in its N terminus and one in its C terminus, then it would have been anticipated that deletion mutants containing just one TBP binding domain would bind at levels measurably above that of the control protein, GST-βG, but below that of the full-length E2TA protein. In titration experiments designed to monitor minor differences in binding of different E2 proteins for TBP, none of the N-terminal or C-terminal mutants were found to possess intermediate binding properties (62). Taken together, the simplest interpretation of these data is that certain N-terminal deletions in E2TA (those present in GST-E2TA_{Δ1-92} and GST-E2TA_{Δ1-283}) induce altered conformations in the resulting fusion proteins that negatively affect their capacity to bind TBP. While not necessarily indicative of a second binding site, this interpretation raises the possibility that the N terminus may modulate the strength of interactions between E2TA and this transcription factor.

To define the domain on E2TA important for the association with TFIIB, the same panel of GST-E2TA mutants was incubated with in vitro-translated, radiolabeled TFIIB (Fig. 5A). The same 99-amino-acid carboxy-terminal domain of E2TA identified as sufficient for TBP binding was found to be

sufficient for binding TFIIB. The GST-E2TA mutants lacking the 92 or 283 amino-terminal amino acids did not bind TFIIB or bound it weakly. Other deletions in the N terminus of E2TA did not affect the binding to TFIIB (Fig. 5A and data not shown). These data are consistent with the interpretation that both GST-E2TA_{Δ1-92} and GST-E2TA_{Δ1-283} exist in conformations unable to bind TFIIB. Importantly, our mapping data failed to separate the domains of E2TA responsible for interactions with TBP and TFIIB.

Specificity of the E2TA-TBP and E2TA-TFIIB interactions.

The above-described experiments relied upon the use of glutathione-Sepharose beads to capture complexes between E2TA and TBP. We investigated whether such complexes could be detected by other means. Eluted GST-fusion proteins were incubated with ³⁵S-radiolabeled TBP, and the eluted GST-fusion proteins were retrieved by immunoprecipitation with E2TA-specific antisera. We again saw the specific interaction between TBP and E2TA derivatives (Fig. 6A). GST-E2TA and a derivative thereof, GST-E2TA_{Δ1-310}, bound TBP at levels fivefold greater than that of GST-βG.

To assess whether E2TA binding to TBP and TFIIB is a consequence of a nonspecific binding property, we also measured the binding of GST-E2TA fusion protein to ³⁵S-radiolabeled BMV proteins (Fig. 6B). GST-E2TA was found to bind less than 1% of the input BMV protein (Fig. 6B, lane 1) in two independent experiments, similar in amount to that bound by GST-βG (Fig. 6B, lane 2). In contrast, GST-E2TA bound 5 to 10% of ³⁵S-radiolabeled TBP (Fig. 2A, lane 1), an amount 5- to 10-fold greater than the amount of TBP bound by GST alone (Fig. 2A, lane 5) or by GST-βG (Fig. 5 and 6). Further evidence for the specific affinity of E2TA for TBP was demonstrated in our experiments mapping domains of TBP necessary for GST-E2TA binding (Fig. 3) in which certain derivatives of TBP were not efficiently bound by E2TA. These experiments further substantiate the specificity of the E2TA-TBP association.

It was previously demonstrated that E2TA and TBP cooperatively bind DNA fragments that contain both an E2 DNA binding site and a TATA box (15). To address whether cooperative DNA binding by these factors affected the level of interaction between the two proteins, the same oligonucleotide used in the prior cooperative DNA binding experiments, E2N₅TATA (15), was added to our in vitro protein association assay. No effect in the level or specificity of binding by TBP to E2TA was discerned by the addition of this DNA (Fig. 6C). To address the possibility that an E2TA-TBP or E2TA-TFIIB interaction was mediated by bacterially derived or input plasmid DNA bound to these proteins, we measured the effect of EtBr on complex formation. Treatment with EtBr disrupts at least some specific and nonspecific protein-DNA interactions (34). The addition of EtBr to samples in the in vitro protein association assay did not affect E2TA-TBP or E2TA-TFIIB binding (Fig. 6D and E). These results suggest that the E2TA-TBP and E2TA-TFIIB interactions are not mediated by DNA.

FIG. 5. The C-terminal 99 amino acids of E2TA are sufficient for binding TBP and TFIIB. (A) Shown are the results from protein association assays in which the efficiencies of binding of different GST-E2TA fusion proteins to in vitro-translated, radiolabeled TBP and TFIIB were measured. The box at the top represents the 410-amino-acid E2TA protein. The filled portions represent the N- and C-terminal conserved regions as described in the text and in the legend to Fig. 1. The solid lines indicate the regions of the E2TA protein present in each GST-fusion protein. At the right are shown PhosphorImages from experiments in which ³⁵S-labeled in vitro-translated TBP (³⁵S TBP) or TFIIB (³⁵S TFIIB) was incubated with the indicated GST fusion protein coupled to glutathione-Sepharose. Bound TBP or TFIIB protein (Bound) was eluted, resolved by SDS-PAGE, and visualized by PhosphorImagery. The right lane in each panel is a percentage input load of labeled ³⁵S TBP or ³⁵S TFIIB included in each binding reaction. Below each Bound lane is the percentage of input protein bound by the GST fusion protein, as determined by PhosphorImage analysis. The results shown in this figure were reproduced in multiple experiments (*n* = 2 to 5). (B) GST-E2TA, GST-E2TA derivatives, and GST-βG were separated by SDS-PAGE and stained with Coomassie blue to assess the size of the fusion proteins with respect to the predicted size of each GST fusion and to determine the levels of GST fusion protein degradation products. Shown is a composite of multiple SDS-PAGE gels. Molecular mass markers are indicated on the right. Fusion proteins in this composite have not been normalized to equimolar amounts.

FIG. 6. E2TA binds TBP and TFIIB specifically and protein-protein interactions are not mediated by DNA. (A) GST fusion proteins that were purified by batch elution from glutathione-Sepharose were incubated with 35 S-labeled in vitro-translated TBP protein, and GST fusion proteins were immunoprecipitated with E2TA antibodies. 35 S-labeled in vitro-translated TBP bound to eluted fusion protein was pelleted with protein A-Sepharose, eluted, resolved by SDS-PAGE, and visualized by autoradiography. Five percent of labeled input TBP protein was loaded in lane 4. E2TA antibodies coimmunoprecipitated fivefold higher levels of 35 S-labeled TBP in association with GST-E2TA (lane 1) and GST-E2TA $_{\Delta 1-310}$ (lane 2) than with GST- β G (lane 3). (B) Binding of in vitro-translated BMV proteins to GST fusion proteins. Nonspecific binding by GST-E2TA was tested with 35 S-labeled BMV protein. 35 S-labeled in vitro-translated BMV protein was incubated with GST-E2TA or GST- β G coupled to glutathione-Sepharose beads. Following three washes, proteins bound to beads were eluted, resolved by SDS-PAGE, and visualized by fluorography. Five percent of labeled BMV protein included in each binding reaction was loaded in lane 3. (C) Shown are protein association assays in which E2N₅TATA DNA was included to assess the effect of DNA on the E2TA-TBP interaction in vitro. GST-E2TA binding to 35 S-labeled TBP was not significantly augmented by the addition of E2N₅TATA DNA in in vitro protein association assays (lane 1 versus lane 3). GST- β G was likewise unaffected by E2N₅TATA DNA (lane 2 versus lane 4). A 5% input of 35 S-labeled TBP included in each assay was loaded into lane 5. (D) EtBr treatment of GST-E2TA and 35 S-labeled TBP was used to determine if the E2TA-TBP association was mediated by DNA. TBP binding by GST-E2TA was not affected by EtBr treatment (lane 1 versus lane 3). Similarly, nonspecific TBP binding by GST- β G was not affected by EtBr treatment (lane 2 versus lane 4). A 5% input of 35 S-labeled TBP included in each assay was loaded into lane 5. (E) EtBr treatment of GST-E2TA and rTFIIB was used to determine if the E2TA-TFIIB association was mediated by DNA. TFIIB binding by GST-E2TA was not

Evidence for E2TA-TBP and E2TA-TFIIB interactions affecting E2TA activity in vivo. We were interested in whether the interactions of E2TA with TBP and TFIIB might have some biological role in E2TA-dependent transcriptional transactivation in vivo. The potential for different transactivators to interact with identical components of the cellular transcriptional machinery has been studied by in vivo and/or in vitro competition or squelching experiments (8, 55, 71). Here, the capacity for one transactivator molecule, when overexpressed, to suppress the activity of another transactivator is interpreted to reflect competition by both transactivators for the same, limiting component of the cellular machinery. Multiple components of the basal transcriptional machinery have been found to associate with VP16, including TBP and TFIIB, and these contacts have been implicated in potential mechanisms of VP16-mediated transcriptional activation (18, 47, 77). To address whether E2TA and VP16 have similar cellular targets for transactivation, a BPV-1 E2TA expression plasmid, pC59, was cotransfected into CV-1 cells with an E2-responsive reporter plasmid, pmh407-1, in the absence or presence of a plasmid that directs expression of the chimeric protein GAL4VP16₄₁₃₋₄₉₀. This chimeric protein contains the domain of VP16 sufficient for transactivation and necessary for binding to transcription factors TBP, TFIIB, TFIID, and TAF_{II}40 (18, 30, 47, 85). As previously shown (73), the CAT gene in pmh407-1 was specifically transactivated by E2TA (Fig. 7A). This activation was squelched by the expression in *trans* of GAL4VP16₄₁₃₋₄₉₀. This suppression of E2TA activity was not observed when a nonchimeric GAL4 protein, GAL4₁₋₁₄₇, was expressed in *trans*. GAL4VP16₄₁₃₋₄₉₀ alone had no negative effect on transcription when its activity was measured in parallel assays with the p(GAL4)₅E1BTATACAT reporter plasmid. One interpretation of the results of these experiments, consistent with our in vitro evidence for the E2TA-TBP and E2TA-TFIIB associations, is that E2TA and VP16 bind the same or overlapping sets of cellular targets and that certain of these targets are necessary for E2TA transcriptional activity.

To investigate further whether the E2TA-TFIIB interaction occurs in vivo and is important for E2TA function in transcriptional activation, we asked what effect the addition of exogenous TFIIB had on E2TA activity in CV-1 cells. The levels of E2TA-dependent transactivation in CV-1 cells varied depending upon the amount of the pC59 plasmid transfected. Optimal levels of transactivation were seen with 500 ng of pC59, with a fivefold decrease in levels of transactivation noted when higher amounts of pC59 were transfected (Fig. 7B). This decrease in transactivation likely represents self-squelching, the process in which excess activator titrates out a limiting factor targeted during the process of transactivation (7, 60). The readdition of such a limiting factor would be predicted to augment transactivation at low levels of activator and to relieve self-squelching at high levels of activator. The expression of exogenous TFIIB from the plasmid pSGTFIIB led to this predicted effect on E2TA activity (Fig. 7B). At low levels of activator, exogenous TFIIB augmented transactivation by E2TA, while at high levels of activator, TFIIB relieved self-squelching. As expected, the latter effect was dependent upon high levels of TFIIB expression to offset the high levels of activator. Thus TFIIB and E2TA acted cooperatively in vivo to activate transcription. These data, taken together with prior data showing in vivo

affected by EtBr treatment (lane 1 versus lane 3). Similarly, nonspecific TFIIB binding by GST- β G was not affected by EtBr treatment (lane 2 versus lane 4). A 5% input of rTFIIB included in each assay was loaded into lane 5.

FIG. 7. In vivo activity of E2TA. (A) In vivo competition between VP16 and E2TA. CV-1 cells were cotransfected with 2 μ g of the E2-responsive CAT reporter plasmid pmh407-1, 50 ng of the BPV-1 E2TA expression plasmid pC59, and the indicated amounts of either of two competitor plasmids, pGAL4VP16₄₁₃₋₄₉₀ (■) or pGAL4₁₋₁₄₇ (▲). Levels of CAT enzymatic activity present in cell lysates 48 h posttransfection were assayed, and the results are indicated as percent chloramphenicol (CAM) acetylated. Additional details of the experimental protocol are provided in Materials and Methods. Each transfection condition was performed in duplicate; error bars are indicated. The specific inhibition of E2TA activity by the Gal4:VP16 fusion protein, but not by Gal4 alone, was reproduced in three independent experiments. (B) Relief of E2TA self-squelching by TFIIB. CV-1 cells were cotransfected with 2 μ g of the E2-responsive CAT reporter plasmid pmh407-1 and 0 ng, 500 ng, 2 μ g, 4 μ g, and 8 μ g of the BPV-1 E2TA expression plasmid pC59 (■). Optimal levels of E2TA transactivation were seen with 500 ng of pC59. Self-squelching by E2TA was observed at pC59 levels ranging from 2 to 8 μ g. Cotransfection of 0 ng, 500 ng (◇), 2 μ g (●), or 8 μ g (△) of pSGTFIIB with pC59 was performed to assess whether TFIIB could relieve E2TA self-squelching. Low levels of pSGTFIIB (500 ng; ◇) did not result in a relief of E2TA self-squelching; rather, pSGTFIIB cooperated with E2TA to activate transcription at low levels of E2TA (500 ng). In contrast, higher amounts of pSGTFIIB (2 μ g [●] and 8 μ g [△]) resulted in a relief of E2TA self-squelching.

cooperativity between TBP and E2TA (25), implicate TFIIB and TBP interactions with E2TA in E2TA-dependent transactivation in vivo.

DISCUSSION

In this communication we demonstrate that the BPV-1 E2 family of proteins, E2TA, E2TR, and E8/E2TR, can associate directly with two components of the cellular transcriptional machinery, TFIID and TFIIB. In the case of the multiprotein complex TFIID, we found that E2TA could bind directly to one of its components, TBP. We mapped these interactions to the C-terminal portion of E2TA, a region also present in the other E2 protein family members, E2TR and E8/E2TR. These findings lead us to propose that E2-mediated transactivation occurs at least in part via direct contacts with the cellular transcriptional machinery. The direct interaction between TBP and E2 proteins provides a simple mechanism to explain how E2 proteins cooperate with TBP to bind DNA containing a TATA box and an E2 recognition site (74), why E2TA and TBP act synergistically to activate transcription from certain promoters (25), why TFIIB can augment E2TA-dependent transactivation and relieve self-squelching (this study), and why the E2 repressor, E2TR, can activate transcription from certain promoters (14).

Role of E2TA-TFIID and E2TA-TFIIB interactions in transcription. We predict that binding by E2 proteins to TFIID and TFIIB will have markedly different outcomes on transcription, depending upon the target promoter. In chromatin-containing promoter DNA, TBP entry might be expected to be rate limiting because of the inability of TBP to bind nucleosome-harboring promoter template (33). In vivo, such a condition may exist at an E2-responsive promoter bearing a weak TATA box or lacking binding sites for other factors that augment TFIID binding. Here the capacity for E2TA to bind TFIID and alter its DNA binding properties may be more critical than

E2TA's capacity to bind TFIIB. Of likely relevance is the finding that exogenous TBP and E2TA act synergistically to facilitate high-level transcription from a minimal promoter (25). In contrast, E2TA's ability to associate with TFIIB might have a greater biological consequence at an E2-regulated promoter bearing a strong TATA box and/or containing binding sites for factors that independently augment TFIID binding. Indeed in the case of activation mediated by the viral transactivator VP16 and the cellular activator CTF1 (CAAT-box-binding transcription factor 1), the entry of TFIIB was found to be rate limiting in preinitiation complex formation (32). The functional relevance of TFIIB binding by transactivators has also been implicated in experiments that show the transactivator VP16 induces a conformational change in TFIIB upon TFIIB binding (64). In our in vivo experiments (Fig. 7B), we found TFIIB to augment E2TA-dependent transactivation of CAT expression from the plasmid pmh407-1, in which E2 DNA binding sites are positioned upstream of the partially enhancer-defective SV40 early promoter containing a strong TATA box. We also observed a pronounced relief of E2TA self-squelching when high concentrations of TFIIB-expressing plasmid were included in the transfections. These effects of TFIIB on E2TA activity in vivo suggest that TFIIB binding to E2TA is biologically relevant.

It is unlikely that E2TA's interaction with TFIID and/or TFIIB is sufficient to account for all of E2TA's transcriptional activation properties at viral promoters. In our hands, the N-terminal half of E2TA could be deleted without destroying E2TA's capacity to bind TFIID and TFIIB, yet this region of E2TA is clearly important for transcriptional activation (17, 36, 51). Thus, binding to TFIID and TFIIB, while potentially necessary, is likely not sufficient for E2TA's function in regulating viral transcription. Similarly, TBP binding by the viral transactivator VP16 and the cellular transactivator Fos was not sufficient for transactivation by these proteins (54, 83).

E2TA binds multiple proteins, including the cellular transcription factor Sp1 (44), the large subunit of the cellular replication protein A, RPA (42), and the papillomaviral protein E1 (56). Of these, Sp1 and E1 are known to bind the N terminus of E2TA, potentially providing a role for the N terminus of E2TA in activation (44, 56). It is unknown whether these interactions induce conformational changes in E2TA that affect the potential of E2TA to bind TFIID and/or TFIIB; however, it is interesting that E1 protein inhibits the transactivational activity of E2TA (39, 67). Alternatively, local conformation of the TBP-binding domain on E2TA might be altered through posttranslational modification of the E2TA protein. Of potential importance, two phosphorylation sites have been mapped to the hinge region of E2TA (50), just N terminal to the domain we mapped to be sufficient for TBP binding.

We predict that the ability of E2TA and TBP to bind cooperatively E2-TATA-containing DNA (15, 74) is dependent upon our observed direct association between E2TA and TBP. Yet, the smallest portion of E2TA sufficient for cooperation with TBP lies between amino acids 204 and 410 of E2TA (74), a region larger than that sufficient for E2TA's binding to TBP (Fig. 1). This finding indicates that additional sequences in E2TA are required for the cooperative DNA binding by E2TA and TBP compared with those required for the direct association of E2TA and TBP. In addition, we found that certain deletions within the N terminus of E2TA affected binding to TBP and TFIIB (Fig. 5). Together, these results indicate that multiple domains of E2TA, including multiple domains in the N terminus and the hinge region, may mediate effects involved in E2TA's association with these transcription factors.

It remains to be determined whether the interactions between E2TA and TFIID and/or TFIIB are necessary for E2TA's function in regulating viral transcription. The ability of E2TA to transactivate viral promoters is dependent upon its binding to the multiple copies of its cognate DNA binding site on the viral genome. For this reason, the identification of E2TA mutants defective in E2TA-TBP and/or E2TA-TFIIB interactions may prove difficult since the domain of E2TA required for these interactions appears to overlap with those required for DNA binding and dimerization (Fig. 1).

Data presented in Fig. 7A suggest that E2TA and VP16 contact similar functional targets during transactivation. This similarity supports the hypothesis that E2TA functionally interacts with TAF_{II}40 and/or TFIIB in addition to TBP and TFIIB. Interactions of E2TA with TBP-associated factors, also described as coactivators, may prove to be pivotal in the process of activation as has been recently described for p53, which was shown to mediate activation via TAF_{II}40 and TAF_{II}60 (48, 79). We have not discounted the possibility that E2TA may interact directly with other components of TFIID.

Functional significance of the E2 repressors binding TFIID and TFIIB. The domain of E2TA sufficient to support TFIID and TFIIB binding was mapped to the C terminus of E2TA, a domain shared with the E2 repressors, E2TR and E8/E2TR. This TFIID and TFIIB binding may play a role in E2TR- and E8/E2TR-mediated repression of E2TA activity (11, 37, 38) or a role in E2TR- or E8/E2TR-specific transcriptional activation (14, 74). The E2TR and E8/E2TR proteins could repress E2TA by sequestering basal factors in nonfunctional complexes at a given promoter. This mechanism has been evoked to explain the role of the cellular oncoprotein, c-Myc, in cellular transformation (41). Similar to E2 proteins, c-Myc binds TBP (26, 49). The domain of c-Myc that mediates this binding is also required for c-Myc-mediated transcriptional repression and cellular transformation (41). Alternatively, the E2 repres-

sors could activate transcription through direct contacts with cellular transcriptional machinery. In this regard, Steger et al. have demonstrated that E2 repressor protein can act synergistically with TBP to activate transcription *in vitro* from a minimal promoter (74). This raises the possibility that, at least for certain E2 responsive promoters, the E2 repressors in addition to inhibiting the activity of E2TA might activate the target promoter to low levels.

E2 proteins: multiple partners, multiple roles. Ultimately, it is necessary to consider our findings in the context of the multiple roles of E2 proteins in both viral transcription and DNA replication. The ability of E2 proteins to bind TBP and TFIIB could play a role in mediating a switch from DNA replication to viral transcription (35). As mentioned above, E2TA also binds BPV-1 E1, a protein required for viral replication, and RPA, the cellular replication protein A. It remains to be determined whether these interactions are affected by E2TA's interaction with transcription factor TFIID or TFIIB. Clearly, the choice among these different interactions may influence the replicational and the transcriptional states of the virus.

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