A Functional Interaction between the Human Papillomavirus 16 Transcription/Replication Factor E2 and the DNA Damage Response Protein TopBP1*

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The human papillomavirus (HPV) transcription/replication factor E2 is essential for the life cycle of HPVs. E2 protein binds to DNA target sequences in the viral long control regions to regulate transcription of the viral genome. It also enhances viral DNA replication by interacting with the viral replication factor E1 and recruiting it to the origin of replication and may also play a more direct role in replication. The cellular proteins with which E2 interacts to carry out these functions are largely unknown. To identify these proteins a yeast two-hybrid screen was carried out with the transcription/replication domain of HPV16 E2. This screen identified several candidate interacting partners for E2 including TopBP1 (topoisomerase IIβ-binding protein 1). TopBP1 has eight BRCA1 carboxyl-terminal domains that are found in proteins regulating the DNA damage response, transcription, and replication. Here we demonstrate that HPV16 E2 and TopBP1 interact in vitro and in vivo and that TopBP1 can enhance the ability of E2 to activate transcription and replication. This is the first time that TopBP1 has been shown to function as a transcriptional coactivator and that E2 interacts with TopBP1. Removal of the amino-terminal domain of TopBP1 abolishes coactivation of transcription and replication. This interaction may have functional consequences upon the viral life cycle.

HPVs* are causative agents in a number of human diseases the most common of which is cervical cancer (1). More than 95% of cervical carcinomas harbor HPV sequences, and the most frequently detected is HPV16. The HPV16 E2 protein is a 43-kDa phosphoprotein that binds as a homodimer to 12-bp palindromic DNA sequences in the transcriptional control region of the viral genome (2). After binding E2 can either up-regulate or repress transcription from the adjacent promoter depending upon cell type and protein levels, and this regulation controls the expression of the viral oncoproteins E6 and E7 (3–6). As well as regulating transcription, E2 interacts with the viral replication factor E1 and recruits it to the origin of replication enhancing the ability of the E1 protein to interact with the origin (7–9). E2 may also have an additional role in replication by recruiting cellular proteins to the replication origin.

The E2 protein can be divided into three domains: the amino terminus, which mediates the transcription and replication properties of the protein; the carboxyl terminus, responsible for homodimerization and binding to DNA; and the hinge region between these domains, which is of indeterminate function (10). Several proteins interact with the transactivation domain of E2 including papillomavirus proteins E1 (7–9) and L2 (11, 12), as well as cellular proteins AMF1 (13), TBP (14), TFIIIB (15–17), p300/CPB (18), and SMN (19). However, to our knowledge, a systematic approach to identify cellular partners for the activation domain of HPV16 E2 has not been carried out. The transcription/replication domain of HPV16 E2 can activate transcription in yeast making a traditional yeast two-hybrid screen impossible (20, 21). To overcome this we screened several point mutants of the HPV16 E2 transactivation domain and identified a mutant, E39A (glutamic acid at position 39 mutated to an alanine) (22), which failed to activate transcription of the GAL1 promoter in yeast but retained the ability to activate transcription in mammalian cells. Using the E39A mutant activation domain of E2 a yeast two-hybrid screen was carried out, and several candidate E2 partners were identified. One of these candidates was TopBP1, first identified as interacting with topoisomerase IIβ (23). The most striking feature of TopBP1 is that it has eight BRCT domains; these were first identified in BRCA1 and have since been detected in a host of cellular proteins (24). Most of the BRCT-domain-containing proteins function in the DNA damage response pathways, although several can act as transcriptional coactivators (25–27).

TopBP1 is an attractive candidate as a functional E2 partner for several reasons. It can interact with topoisomerase IIβ (topoisomerases are essential for transcription and replication) (28), has a transcriptional activation domain (29), the Drosophila homolog is involved in DNA replication and repair (30), TopBP1 is essential for the cell cycle (31), and it can interact with single stranded and damaged double stranded DNA (32). Because E2 regulates transcription and replication of the viral genome and can regulate the cell cycle (33–35) these properties
of TopBP1 make it an excellent candidate as an in vivo functional interacting partner for E2.

Here we show that E2 interacts with the carboxyl terminus of TopBP1 in yeast, in vitro and in vivo, and coactivates transcription with E2 while having little effect upon replication. Deletion of the transcriptional activation domain from TopBP1 abolished any enhancement of E2 transcription and replication properties but failed to act as a dominant negative to block E2 function. The results demonstrate that E2 and TopBP1 can interact functionally in vivo but suggest that TopBP1 may not be essential for mediating either the transcription or replication properties of E2. The results also demonstrate for the first time that TopBP1 can act as a transcriptional coactivator when recruited to a promoter by an interacting partner, E2 in this case. A possible role for the E2-TopBP1 interaction in the viral life cycle is discussed.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screen**—The Matchmaker 3 system from CLONTECH was used to carry out the two-hybrid screen. A DNA fragment encoding the first 229 amino acids of HPV16 E2 was PCR amplified and digested with EcoRI and BamHI, and then the appropriate enzyme sites were incorporated into the fragment using the primers. This fragment was then cloned into pGBK7 to make the bait plasmid for the two-hybrid screen encoding a fusion between the GAL4 DNA binding domain and E2. This plasmid activated transcription in yeast making it unsuitable for use in the two-hybrid screen. Several mutants of E2 were screened for their ability to activate transcription in yeast. Using this approach we identified a mutant, glutamic acid at position 39 mutated to an alanine (E39A), which failed to activate the GAL1 promoter, driving expression of LacZ in yeast strain Y187 and HIS3 in yeast strain PJ629-2A, while still activating the GAL2 promoter driving the expression of ADE2. This mutant also maintained the ability to activate transcription in mammalian cells and was therefore used in the two-hybrid screen. The pGBK7TE2–1–229(E39A) plasmid was transfected into PJ629-2A, and the resulting transformed yeast mated with Y187 cells expressing a HeLa cDNA library fused to the GAL4 transcriptional activation domain. The ability of the resultant mated cells to grow on quadruple dropout medium (lacking ADE/HIS/LEU/LTR) was monitored. The mating efficiency of this procedure was 15% and resulted in the screening of 5 × 10^6 HeLa cDNAs, more than representative of the expressed human genome. From this screen only 22 clones grew, and 6 of these clones encoded the carboxyl terminus of TopBP1.

**Cell Culture**—C33a and U2OS cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum and were passaged routinely every 3–4 days; C33a cells were split 1 in 10 and U2OS 1 in 6. In *Vivo* GST Pull Down Assay—The vector isolated from the HeLa library containing the full-length TopBP1 was pGEX4T-1TopBP1. The vector containing the TopBP1 fragment was removed from this vector as an Smal-XmnI fragment and cloned into pGADD7. As a negative control a cDNA encoding the transcriptional coactivator PC4 (36) was PCR amplified from HeLa cDNA and cloned into pGADD7 as an EcoRI-XmnI fragment. One of the other E2-interacting encoding cDNAs isolated in the two-hybrid screen, POMP (37), was removed from pGADGH as an Smal-XmnI fragment and cloned into pGADD7 and also used in these assays. All of the vectors were sequenced to confirm the correct sequence had been cloned. To express the respective proteins all of the plasmids carrying these cDNAs were transcribed in *vitro* and translated using the TNT7 system from Promega. The GST-E2 proteins were prepared in the following manner. Fragments from the wild type E2 sequence were generated using PCR and cloned into pGEX4T2 as EcoRI-BamHI fragments. The restriction sites were incorporated during the PCR amplification. In this way fragments representing E2 amino acids 2–229, 25–229, and 50–229 were cloned into pGEX4T2, and the encoded fusion proteins were expressed and purified essentially as described (38). All resulting vectors were sequenced. After purification the proteins were run on SDS-PAGE, and the gels were stained with Coomassie Blue to confirm appropriate size of GST fusion proteins. Each deletion mutant attached to beads was then mixed with 5 μl of the 50 μl in *vitro* transcription/translation mix (1 μg of the expression vectors was used in the *in vitro* transcription/translation). GST alone was used as a control. The samples were mixed in 200 μl of pull down buffer, which was 50 mm Tris, pH 7.9, 100 mm NaCl, 1 mm diithiothreitol, 0.5 mm EDTA, 0.5 mm EGTA, 0.5% Nonidet P-40, and 1 mm phenylmethylsulfonyl fluoride. The dithiothreitol and phenylmethylsulfonyl fluoride were added fresh to the buffer just before use. This mixture was rotated at 4 °C for 30 min and then the beads pelleted with a brief centrifugation. The beads were then washed four times with 500 μl of pull down buffer. They were then resuspended in 1x SDS-PAGE sample buffer and then run on a 12% SDS-polyacrylamide gel. After electrophoresis the gel was fixed in 10% methanol and 7.5% acetic acid for 1 h. The gel was then dried and exposed to film.

**TopBP1 Antibody Preparation**—The region encoding amino acids 861–1287 of TopBP1 was inserted into an EcoRI-Xhol-cut pGEX4T-3. The plasmid generated was pGEX4T-3TopBP1del2. Escherichia coli expressing the resultant fusion protein for TopBP1del2 and grown in 500 ml of Luria-Bertani medium, supplemented with 100 μg/ml ampicillin, at 37 °C to an A_{600} of 0.8 and induced for 5 h with 0.1 μM isopropyl-1-thio-β-D-galactopyranoside at 30 °C. Cells were harvested and the bacterial pellet resuspended in 10 ml of lysis buffer (10 mm Tris-HCl, pH 7.7, 0.5 μM NaCl, 1 mg/ml phenylmethylsulfonyl fluoride) and sonicated for 40 s. The cell extract was spun at 10,000 × g for 30 min, and the cleared extract was incubated with 1.5 ml of glutathione-Sepharose 4B beads (50% slurry, Amersham Biosciences) on a slowly rotating mixer for 1 h at 4 °C. The beads were washed once with buffer C (10 mm Tris-HCl, pH 7.7, 2 mm NaCl, 10 mm EDTA, 0.5% Nonidet P-40), once with buffer B (10 mm Tris-HCl, pH 7.7, 1 mm EDTA, 0.5% Nonidet P-40), once with buffer A (10 mm Tris-HCl, pH 7.7, 50 mm NaCl, 150 mm NaF, 0.5% Nonidet P-40) for 30 min at room temperature. The elution buffer was rotated for an additional two times. Eluted GST-TopBP1del2 was dialyzed against 4 liters of 50 mm Tris-HCl at 4 °C overnight, expression was confirmed by SDS-PAGE, and protein concentration determined by the BCA method. Antibodies to GST-TopBP1del2 were raised in rabbits by Scottish Diagnostics.

**In Vivo Immunoprecipitation and Western Blotting**—2 × 10^6 C33a cells were plated out in 100-mm^2 tissue culture plates. The following day the cells were mixed with 2.5 μg of pCMV-HPV16E2 expression vector in a calcium phosphate precipitation. 16 h later these cells were washed twice with phosphate-buffered saline and re-fed. 24 h after the wash the cells were harvested in the following manner. The cells were trypsinized and then washed twice with 10 ml of ice-cold phosphate-buffered saline. The cell pellet was then resuspended in 1 ml of ice-cold phosphate-buffered saline and transferred to a 1.5-ml Eppendorf tube and pelleted. They were then resuspended in 100 μl of lysis buffer (0.5% Nonidet P-40, 50 mm Tris, pH 7.8, 150 mm NaCl with a protease inhibitor mixture (Roche Molecular Biochemicals) dissolved in the lysis buffer). The extracts were then incubated on ice for 30 min with occasional mixing. After this time the extracts were centrifuged at 10,000 × g for 10 min at maximum speed at 4 °C. The supernatant was then removed to another tube and the cell debris discarded. 50 μl of this extract was then incubated in a total volume of 100 μl, and 1 μl of a 1:100 dilution of either rabbit α-TopBP1 serum (described above) or preimmune control serum from the same rabbit was added. This was then incubated at 4 °C with rotation for 60 min. After this, 10 μl of Protein A-Sepharose 4B (4 μl/ml of pull down buffer; Sigma) was added to the manufacturer's instructions and washed in lysis buffer) and washed and the extracts rotated for another 60 min at 4 °C. The beads were then pelleted and washed four times with 0.5 ml of lysis buffer and resuspended in SDS-polyacrylamide gel sample buffer and electrophoresed through an SDS-PAGE system. The gel was blotted onto membrane and probed for the presence of TopBP1 or E2. The primary antibody used to detect TopBP1 was a murine monoclonal purchased from BD Transduction Laboratories (T10620); TVG261, a kind gift from Merlin Hibma, was used to detect E2 (39). After incubation with the primary antibody a horseradish peroxidase-conjugated α-mouse IgG was added. The membrane was then developed using ECL-Plus (Amersham Biosciences) to detect the horseradish peroxidase conjugate and the membrane exposed to film.

**Transcription Assays**—C33a and U2OS cells were transfected for the transcription assays using the same protocol. 2 × 10^6 cells were plated out on a 60-mm plate and transfected 24 h later using the calcium phosphate technique. The next day they were washed, and 24 h later the cells were harvested. Briefly, the cells were washed twice with phosphate-buffered saline and then lysed with 900 μl of reporter lysis buffer (Promega). After a 10-min incubation the lysate was transferred into a 1.5-ml Eppendorf tube and spun in a refrigerated microfuge for 10 min at maximum speed at 4 °C. The supernatant was then removed to a fresh tube and the pellet discarded. 80 μl of the supernatant was assayed for luciferase activity using the luciferase assay system (Promega). To standardize for cell number a protein assay was carried out, and the activities shown are expressed relative to the respective protein.
The HPV16 E2-interacting protein TopBP1. TopBP1 was first identified as interacting with topoisomerase IIβ, and the interacting domain is highlighted (23). HPV16 E2 interacts with the same region of TopBP1, although this domain is large and has the potential to interact with several proteins simultaneously. The black boxes represent domains present in TopBP1; no other protein has as many of these domains, which serve as modules for interacting with other proteins and with damaged DNA. The nuclear localization signal and the domain capable of activating transcription in yeast (29) are shown.

Concentrations of the samples. pGL3CONT, which contains the SV40 promoter and enhancer driving expression of the luciferase gene, was always included in a parallel transfection to confirm efficient transfection. The assays shown are representative of at least three independent experiments carried out in duplicate. The tk6kE2 luciferase reporter has been described previously (6) as have the E2 (3) and TopBP1 (31) expression vectors used in the transcription assays.

Replication Assays—Replication assays were carried out as a modification of a previously published technique (22). 6 × 10^5 C33a or 3 × 10^5 U2OS cells were plated out in 100-mm dishes and the next day transfected using the calcium phosphate method. 3 days post-transfection low molecular weight DNA was extracted using the Hirt method. Briefly, cells were lysed in 800 μl of Hirt solution (0.6% SDS, 10 mM EDTA) and scraped into a 1.5-ml microcentrifuge tube. 200 μl of 5 M NaCl was added, and the samples were then left at 4°C overnight. After centrifugation they were extracted once with phenol-chloroform-isoamyl alcohol and precipitated with ethanol. After centrifugation the DNA pellet was washed with 70% ethanol and dried then resuspended in H2O. A quarter of the sample was digested with XmnI for 3 h. One-tenth of this digest was removed and the rest of the sample digested with DpnI. Both the single and double digested samples were separated by 1% agarose gel electrophoresis and analyzed by Southern blotting. Blots were probed using a 700-bp HPV16 Ori containing fragment released from p16ori-m by PvuII restriction digest and 32P radiolabeled using the Stratagene Prime-it II kit. Blots were hybridized using Quikhyb solution (Stratagene). The p16ori-m plasmid is a modified version of p16ori (22). Using p16ori as a template and primers 5′-Oritaq (GTACCATTCTCTGACATGGGTTGTTGGCA) and 3′-Oritaq (GTACGATTTCTAATCTCTGGGTCTCTTCG) on EcoRI-BamHI fragment was cloned into pSkII (11). This fragment represents nucleotides 7838–139 from the HPV16 genome and contains the minimal origin of replication. Additionally there is a point mutation of base 115 from C to A to create a DpnI restriction site so that this plasmid could be used in a Taqman real time PCR-based protocol to detect viral DNA replication. Quantification of single cut (input) and double cut (replicated) 3-kbp p16ori-m bands was carried out using a Storm 870 Molecular Dynamics PhosphorImager. Strength of replication double cut (replicated) 3-kbp p16ori-m bands was carried out using a Storm 870 Molecular Dynamics PhosphorImager. Strength of replication was calculated by measuring the ratio of double cut to single cut bands. This method of measurement controls for variation in transfection efficiency.

RESULTS

A Yeast Two-hybrid Screen Identified TopBP1 as an HPV16 E2-interacting Protein—To identify cDNAs encoding cellular proteins that interact with the transcription/replication domain of E2 we carried out a yeast two-hybrid screen. Wild type E2 can activate transcription in yeast making a traditional two-hybrid screen impossible. To overcome this several point mutants of the HPV16 E2 activation domain were screened for their ability to activate transcription in yeast. These experiments identified a mutant of E2, glutamic acid at position 39 mutated to an alanine (E39A), which failed to activate the GAL1 promoter in yeast that was used in the two-hybrid screen (see “Experimental Procedures”) but retained the ability to activate transcription in mammalian cells (22). This residue of E2 is on the outer face of the proposed structure of the E2 activation domain and is important for the interaction with the viral replication protein E1 in vitro (22, 41); mutation of this residue abolishes the E2-E1 interaction and therefore abolishes viral replication mediated by E2 and E1. Using the E39A mutant we screened 5 × 10^6 cDNAs from HeLa cells in a yeast two-hybrid screen to identify E2-interacting partners. Three of the clones isolated encoded the region of HPV16 E1 which would be predicted to interact with the wild type E2 activation domain (7–9). HeLa cells are derived from a cervical carcinoma harboring HPV18 DNA, and the detection of the E1 protein therefore served as an excellent internal control to confirm that this screen was sensitive and able to identify biologically important E2-interacting partners. As stated above mutation of residue 39 from a glutamic acid to an alanine abolishes the interaction between E2 and E1 in vitro. Clearly the yeast two-hybrid screen is more sensitive at detecting the interaction between these two viral proteins than previously used methods (22). Six of the other clones isolated in the screen encoded the carboxyl terminus of TopBP1 (Fig. 1), a protein containing eight BRCT domains (23).

The HPV16 E2 Activation Domain Interacts with TopBP1 in Vitro and in Vivo—After the identification of interacting proteins in yeast it is important to confirm that these proteins can interact directly in vitro. To do this a GST fusion protein was prepared which encoded the wild type transcription/replication domain of HPV16 E2. The carboxyl-terminal portion of TopBP1 which interacts with E2 in yeast was translated in vitro and labeled with [35S]methionine. These two molecules were used in a GST pull down assay, and as shown in Fig. 2a there was a...
specific interaction between the GST-E2 fusion protein and the carboxyl terminus of TopBP1 (lane 6). GST alone did not interact with TopBP1 (lane 3), and the unrelated transcriptional coactivator PC4 (36) did not interact with the E2 activation domain (lane 5). Another of the E2-interacting proteins isolated in the yeast two-hybrid screen, POMP (37), also had a specific interaction with E2 (lane 4 versus lane 1). The GST-E2 fusion protein used in these pull down assays encoded the wild type E2 activation domain, demonstrating that TopBP1 interacts with wild type E2 and is not an artifact of interacting with the E39A mutant. Deletion of the amino-terminal 25 amino acids from the E2 activation domain made no difference to the interaction with TopBP1 (lane 9), whereas removal of the amino-terminal 50 amino acids substantially reduced the interaction (lane 12). This indicates that the first 50 amino acids of E2 are important for the interaction between E2 and TopBP1. However, the loss of interaction may be caused by a conformational change of E2 after the deletion of the first 50 amino acids resulting in the collapse of the TopBP1 interaction domain.

To our knowledge there are no cell lines that express detectable levels of E2 protein. Therefore, to demonstrate that E2 and TopBP1 interact in vivo protein extracts were prepared from cells transfected with an E2 expression vector, and these extracts were used in communoprecipitation experiments (Fig. 2b). TopBP1 antibodies immunoprecipitated TopBP1 and communoprecipitated E2 (lanes 7 and 9). Using preimmune serum there is no immunoprecipitation of TopBP1 or communoprecipitation of E2. The faint bands seen with the preimmune serum (lanes 8 and 10) are the result of a nonspecific interaction. Several nonspecific antibodies showed this faint band in communoprecipitation experiments (not shown).

Taken together these results demonstrate that E2 and TopBP1 can interact in yeast in vitro and in vivo in mammalian cells. The ability of TopBP1 to modulate E2 transcriptional properties was therefore tested.

TopBP1 Acts as a Transcriptional Coactivator for HPV16 E2—C33a cells are ideal for studying transcriptional activation by E2 because they are derived from a cervical carcinoma devoid of HPV sequences. Therefore there are no additional viral proteins (such as E1) expressed in these cells which might interfere with E2-mediated transcriptional regulation, and C33a cells were used to determine whether TopBP1 could regulate E2 transcriptional activity. A range of E2 plasmid concentrations was used in these experiments, and as shown in Fig. 3a, coexpression of TopBP1 resulted in an enhancement of E2 transactivation of a thymidine kinase promoter containing six E2 DNA binding sites located upstream (6). It is important to note that in these experiments TopBP1 did not activate the thymidine kinase promoter in the absence of E2, demonstrating that this is a synergistic activation of transcription. As a control, the ability of E2 and TopBP1 to activate transcription from the thymidine kinase promoter in the absence of E2 sites was tested, and although TopBP1 slightly reduced the thymidine kinase promoter function there was no cooperative effect with E2 (Fig. 3b). This demonstrates that the synergistic effect of E2 and TopBP1 on transcription depends upon E2 binding to the target promoter.

The human osteosarcoma cell line U2OS has a deletion at position q21 on chromosome 3 (American Tissue Culture Collection) where the TopBP1 gene is located (29). Western blotting revealed that U2OS cells express reduced levels of TopBP1 compared with C33a cells (not shown). The ability of E2 and TopBP1 to regulate transcription in this cell line was tested, and the results of these experiments are summarized in Fig. 3c. The levels of E2 used were suboptimal for maximum transcriptional activation by E2 in U2OS cells, and coexpression of TopBP1 enhanced the level of transcriptional activation mediated by E2. TopBP1 had very little effect on the thymidine kinase promoter activity in the absence of E2, demonstrating again that this was a synergistic interaction. The ability of E2 and TopBP1 to regulate transcription from the thymidine kinase promoter without the E2 DNA binding sites was assayed (Fig. 3d), and E2 and TopBP1, either individually or together, did not affect the levels of transcription from the thymidine kinase promoter. The levels of transcriptional cooperation between E2 and TopBP1 were greater than that observed in C33a cells perhaps because of the reduced levels of endogenous TopBP1 protein in U2OS cells.

The Effect of TopBP1 Overexpression on E2-mediated Replication—Expression of HPV16 E2 and E1 allows for efficient activation of replication from a plasmid containing the HPV16 origin of replication. To determine whether TopBP1 can regu-

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2 W. Boner and I. M. Morgan, in preparation.
late the ability of E2 to activate DNA replication, levels of E2 which induce submaximal replication were established. A representative figure from one such experiment carried out in C33a cells is shown in Fig. 4. This experiment was carried out three times, and on each occasion 10 ng and 100 ng of E2 were added with 5 μg of E1 expression vector (where indicated with a /H1001/) and 1 μg of p16ori-m. Odd numbered lanes represent samples that were digested with Xmn I only; even numbered lanes were digested with both Xmn I and Dpn I. The single digested lanes act as an internal control confirming efficient transfection and rescue of DNA upon cell harvest. An arrow indicates the expected 3-kbp ori plasmid detected in the Southern blot; the lower bands represent digested input plasmids. This experiment was carried out three times with essentially the same results.

b, TopBP1 overexpression does not enhance the ability of E2 to activate replication in C33a cells. The amount of E2 and TopBP1 expression vectors used in each sample is indicated below the graph, and these were added with 5 μg of E1 expression vector (where indicated with a +) and 1 μg of p16ori-m. The graph represents a summary of three independent experiments, and the ratio is determined as the activity obtained with Xmn I-Dpn I samples divided by the activity obtained with Xmn I alone; for an explanation of the quantitation, see "Experimental Procedures." c, TopBP1 overexpression enhances the ability of E2 to activate replication in U2OS cells. The amount of E2 and TopBP1 expression vectors used in each sample is indicated below the graph, and these were added with 5 μg of E1 expression vector (where indicated with a +) and 1 μg of p16ori-m. The graph represents a summary of three independent experiments, and the ratio is determined as the activity obtained with Xmn I-Dpn I samples divided by the activity obtained with Xmn I alone; for an explanation of the quantitation, see "Experimental Procedures." E2 or E1 expressed by itself did not activate replication in U2OS cells (not shown).
expression vector gave submaximal levels of replication compared with the levels of replication obtained with 1 μg of E2 expression vector. At 5 μg of E2 expression vector the system was clearly saturated as replication efficiency was reduced compared with the levels obtained with 1 μg of E2 (compare lane 16 with lane 14). The ability of TopBP1 to regulate replication mediated by 10 ng and 100 ng of E2 expression vector was determined. These experiments were carried out three times and quantitated, and the results obtained with 10 ng of E2 are shown in Fig. 4b (quantitation is described under “Experimental Procedures”). Clearly overexpression of the TopBP1 protein at a variety of concentrations had very little effect on replication induced by 10 ng of E2 in C33a cells. Decreasing or increasing the E2 expression levels did not result in any effect on TopBP1 on the ability of E2 to regulate replication. Because the cooperative effect of E2 on transcription is greater in U2OS cells than in C33a it was of interest to determine the effects of TopBP1 overexpression on E2-mediated DNA replication in this cell line. The replication by E2 in U2OS cells was submaximal at 100 ng of E2 and maximal at 1 μg of E2; the results obtained with both of these levels of E2 are shown in Fig. 4c. The results demonstrate that at 100 ng of E2 there is a 2–3-fold enhancement of replication upon expression of TopBP1. At increased levels of E2 expression vector, 1 μg, this enhancement with added TopBP1 is not detected.

Deletion of the Amino Terminus from TopBP1 Abolishes the Ability of this Protein to Regulate Transcription and Replication Mediated by HPV16 E2—The amino terminus of TopBP1 contains a region that activates transcription in yeast (29). We predicted that this same domain is involved in mediating the affects of TopBP1 on E2 function. To study this an aminoterminal deletion mutant of TopBP1 was generated which retained the E2 interacting domain (Fig. 5a). This TopBP1 deletion was cloned into a vector containing an hemagglutinin tag (42), and this vector produced a protein of the expected size when transfected into cells (Fig. 5b). This deleted TopBP1 protein was also expressed in the nucleus (not shown) as would be predicted from previous studies (31). The ability of the deleted TopBP1, ΔTopBP1, to regulate the transcription and replication functions of HPV16 E2 was determined. In U2OS cells deletion of the amino terminus from TopBP1 dramatically reduced any synergistic activation of transcription (Fig. 5c) or replication (Fig. 5d). Essentially identical results were obtained in C33a cells. However, ΔTopBP1 did not result in a blocking of E2 transcription or replication functions in either C33a or U2OS cells as might be expected if interaction with the endogenous TopBP1 is essential for the ability of E2 to carry out these functions. The results of these experiments demonstrated that TopBP1 can act as a transcriptional coactivator in mammalian cells and that the amino terminus mediates this property. They also demonstrate that under certain circumstances TopBP1 can enhance the ability of E2 to activate replication, and the amino terminus domain of TopBP1 also mediates this.

DISCUSSION

The HPV16 E2 protein regulates transcription from, and replication of, the HPV genome (2). For the latter function E2 must interact with the viral E1 protein (7–9) and possibly cellular proteins, and for the former E2 must interact with cellular proteins. To identify cellular proteins that mediate E2 function we carried out a yeast two-hybrid screen resulting in the isolation of TopBP1 (23) as a cellular protein that interacts with the E2 transcription/replication domain. TopBP1 has eight BRCT domains capable of interaction with other proteins (24) and with single stranded and damaged double stranded DNA (32). TopBP1 is an attractive candidate as a functional E2 partner for several reasons. First, this protein interacts with topoisomerase IIβ, and topoisomerases are involved in transcription and DNA replication (28). Second, BRCT domains 1 and 2 of TopBP1 can activate transcription in yeast when tethered to DNA, demonstrating the potential of TopBP1 to act as a transcriptional coactivator (29). Third, mutations of the Drosophila homolog of TopBP1, mus101, result in defects in DNA replication and repair as well as chromosome condensation (30). Fourth, TopBP1 is essential for the cell cycle; removal of this protein results in increased apoptosis and reduced colony survival (31). Fifth, TopBP1 can interact with single stranded and double stranded DNA breaks, suggesting possible roles in replication and repair (32). Because E2 regulates transcription and replication of the viral genome and can induce apoptosis in certain cell types (33–35) these properties of TopBP1 make it an excellent candidate as an in vivo functional interacting partner for E2.

Fig. 2 demonstrates that wild type E2 and TopBP1 can interact both in vitro and in vivo. Overexpression of TopBP1 in C33a cells resulted in the enhancement of transcriptional activation by the E2 protein (Fig. 3a), and this property of TopBP1 was also observed in U2OS cells (Fig. 3c). The synergistic activation of transcription is dependent upon the E2 DNA binding sites (Fig. 3, b and d), and these results provide further evidence that there is an interaction between E2 and TopBP1 in vivo. Removal of the amino-terminal portion of TopBP1 abolishes any significant synergistic activation of transcription with E2 (Fig. 5), demonstrating that the activation domain of TopBP1 identified in yeast cells is probably also responsible for mediating the activation of transcription in mammalian cells. This is the first time it has been shown that TopBP1 has a transcriptional activation domain that functions in mammalian cells, raising the possibility that TopBP1 can interact with cellular DNA-binding transcription factors to regulate transcription.

Overexpression of TopBP1 had no effect on the ability of E2 to activate replication in C33a cells and very little effect on the ability of E2 to activate replication in U2OS cells. The difference between the results obtained in the transcription and replication assays may be the result of a chromatin effect. In the transcription assays the thymidine kinase promoter is being activated by E2, and this activation requires chromatin modification. Although E2 can recruit p300/CBP (18) to carry out this function it seems likely that the recruitment of TopBP1 can enhance chromatin remodeling, perhaps by recruitment of alternative proteins or more p300/CBP, through its amino-terminal activation domain. In the replication assays a very simple construct containing the origin of replication was used to monitor replication mediated by E2, and perhaps this construct requires less recruitment of chromatin modifying activity because of the simplicity of the sequences around the replication origin. However, the difference between the transcription and replication functions may represent a genuine difference in the ability of TopBP1 to regulate E2 function.

Deletion of the amino-terminal activation domain from TopBP1 resulted in the loss of significant synergistic activation with E2 of transcription or replication in U2OS cells (Fig. 5) and had no effect on transcription or replication by E2 in C33a cells (not shown). However, this deleted TopBP1 did not block the ability of E2 to activate transcription or replication. This suggests two things. First, TopBP1 is not essential for either the transcription or replication properties of E2. If endogenous TopBP1 were essential for these functions it would be expected that the deleted TopBP1 would interact with the E2 activation domain blocking interaction with endogenous TopBP1 and therefore preventing activation of transcription or replication. Second, the interaction between TopBP1 does not interrupt the interaction between E2 and E1; if it did it would be expected
that the deleted TopBP1 could interfere with E2-E1 interaction, resulting in a down-regulation of replication. The first 50 amino acids of E2 which are essential for efficient interaction with TopBP1 (see Fig. 2) have conserved residues that are not essential for activation of transcription and interaction with E1 and therefore replication (22). It is currently under investigation whether these residues are important for the ability of E2 to interact with TopBP1.

The question therefore remains as to what is the likely role of the interaction between E2 and TopBP1. Although we cannot at the moment eliminate the possibility that this protein is required for E2 transcription and replication, the results obtained with the deleted TopBP1 suggest that this protein is not essential for either of these functions of E2, as discussed above. It is possible that under certain conditions the ability of TopBP1 to activate transcription in conjunction with E2 may be important. For instance, treatment of cells with DNA-damaging agents may require the virus to produce additional E6 protein to combat the growth inhibitory effects of an enhanced expression of p53 in response to the damage. TopBP1 could mediate the enhanced transcriptional activity of E2, resulting in enhanced E6 expression; this is a particularly attractive possibility given the role that TopBP1 has in response to DNA damage. Another possible role for the TopBP1-E2 interaction in the viral life cycle may be in protection of the viral genome. In response to a host of DNA-damaging agents TopBP1 relocalizes from sites adjacent to replication forks to the replication forks themselves (29). This happens in S phase of the cell cycle, and it has been proposed that under these conditions TopBP1 can sense the damage at these replication forks and recruit some of the proteins required to repair the damaged replication forks and therefore allow the cell to progress through S phase to mitosis. It has been suggested that TopBP1 acts as an S phase checkpoint protein, and removal of TopBP1 expression using a targeted antisense oligonucleotide strategy results in the loss of cell viability (31). Upon DNA damage, E2 could interact with TopBP1 and act as a bridge to recruit damaged genomes to sites of repair in the cell ensuring quick and efficient repair. It is essential that HPV genomes be afforded as much protection as possible to ensure that their encoded genes are expressed correctly allowing for efficient execution of the viral life cycle.

Another possible role for the TopBP1-E2 interaction is mediating the ability of E2 to regulate apoptosis and the cell cycle. Expression of E2 in a number of cell lines results in the induction of apoptosis and the arrest of the cells in the G1 phase of the cell cycle (33–35). The ability of E2 to induce apoptosis requires an intact amino terminus; replacement of this domain with that of p53 is essential. E2 can physically interact with p53 (43), and TopBP1 can interact directly with p53 (31, 44); it has been suggested that p53 is essential. E2 can physically interact with p53 (43), and TopBP1 can interact directly with p53 (31, 44); the interaction of E2 and TopBP1 could result in the formation of a complex that may enhance the interaction between p53 and p53E2 and, therefore, alter the ability of this complex to regulate apoptosis and the cell cycle.

One other possible role for the E2-TopBP1 interaction may be in regulating the ability of E2 to interact with mitotic chromatin. Although it has not been shown with HPV16 E2, BPV1 E2 interacts with mitotic chromatin via the amino-terminal activation/replication domain (40, 45, 46) It has been proposed that this is responsible for the efficient spread of viral genomes into daughter cells because interaction with the mitotic chromatin would allow E2 to recruit the viral genome to these sites via the carboxyl-terminal DNA binding domain. In Drosophila, mutations in the TopBP1 homolog result in the failure to condense mitotic chromatin properly, and perhaps this property is conserved in TopBP1 (30). The E2-TopBP1 interaction could therefore be responsible for the recruitment of the viral genome to mitotic chromatin in mammalian cells.

All of the possibilities discussed above deserve further investigation to determine the role of the E2-TopBP1 interaction in regulation of E2 function and therefore the viral life cycle.

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A Functional Interaction between the Human Papillomavirus 16 Transcription/Replication Factor E2 and the DNA Damage Response Protein TopBP1
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