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# Mapping and Characterization of the Interaction Domains of Human Papillomavirus Type 16 E1 and E2 Proteins

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**The papillomavirus E1 and E2 proteins are both necessary and sufficient in vivo for efficient origin-dependent viral DNA replication. The ability of E1 and E2 to complex with each other appears to be essential for efficient viral DNA replication. In this study, we used the yeast two-hybrid system and in vitro binding assays to map the domains of the human papillomavirus type 16 (HPV16) E1 and E2 proteins required for complex formation. The amino-terminal 190-amino-acid domain of HPV16 E2 was both required and sufficient for E1 binding. The carboxyl-terminal 229 amino acids of E1 were essential for binding E2, and the amino-terminal 143 amino acids of HPV16 E1 were dispensable. Although the ability of the E1 minimal domain (amino acids [aa] 421 to 649) to interact with E2 was strong at 4°C, it was significantly reduced at temperatures above 25°C. A larger domain of E1 from aa 144 to 649 bound E2 efficiently at any temperature, suggesting that aa 144 to 420 of E1 may play a role in the HPV16 E1-E2 interaction at physiological temperatures.**

The papillomaviruses are small DNA viruses that infect humans and a wide range of higher vertebrate species, causing cutaneous and mucosal squamous epithelial lesions. Some human papillomaviruses (HPVs), such as HPV types 16 and 18 (HPV16 and HPV18), are involved in the pathogenesis of certain human anogenital carcinomas, most notably cervical cancer (reviewed in reference 42). Like other small DNA tumor viruses, the papillomaviruses have been studied as a model for DNA replication in eukaryotic cells, since papillomavirus DNA replication is primarily dependent on the host cell DNA replication machinery (23, 32). However, in addition to host cell factors, efficient replication of the papillomavirus genome also requires two viral proteins, the products of the E1 and E2 open reading frames, as well as the *cis*-acting papillomavirus origin of replication (ori). The E1 and E2 proteins are sequence-specific DNA binding proteins, and binding sites for E1 and E2 proteins are found in the ori sequences conserved among different papillomavirus genomes (30, 53, 54). The papillomavirus E1 and E2 gene products play important roles in viral transcription as well as in DNA replication. In general, the properties of E1 and E2 proteins have been studied most extensively for bovine papillomavirus type 1 (BPV1) (reviewed in reference 19).

In studies using BPV1, the full-length E2 protein (E2-TA) was first defined as a transcription activator of both viral and heterologous promoters containing E2 binding sites ($ACCN_6GGT$) (16, 45, 48). The HPV16 E2 protein has also been shown to be a transcriptional activator (7, 33). Depending on the context of the E2 binding sites within the promoter, E2-TA can also function as a transcriptional repressor (27). Transcriptional repression by E2-TA has been well studied for the HPV16 $P_{97}$ and HPV18 $P_{105}$ promoters, which contain E2 binding sites immediately upstream of the TATA box (2, 35, 49). Structural studies of the E2 protein have revealed that E2-TA consists of two functional domains, the transactivation domain (AD; approximately 200 amino acids [aa] at the amino terminus) and DNA binding domain (DBD; approximately 100 aa at the carboxyl terminus), linked by a nonconserved hinge region (12, 17, 28, 29).

E1 is the largest and most conserved early open reading frame of the papillomaviruses, particularly in its carboxyl terminus (6, 26). BPV1 E1 protein is a DNA binding phosphoprotein with helicase and ATPase activities (25, 41, 47, 54, 56). Previous studies have shown an absolute requirement for BPV1 E1 protein in papillomavirus DNA replication (53). BPV1 E1 protein is known to form a specific complex with the E2 transcriptional regulator protein (1, 3, 30, 55). Although biochemical studies of the E1 proteins of HPVs have been limited, their properties are likely similar to those of BPV1 E1 because of the strong sequence conservation. Recently, HPV6b E1 was shown to be a DNA helicase/ATPase (20). HPV11 E1 expressed in baculovirus also exhibits ATPase activity (5). Several studies have shown that HPV E1 proteins are able to bind E2 (5, 11, 18, 31).

Studies with BPV1 provided genetic evidence that the E1 and E2 genes are each essential for viral DNA replication, both in transformed cells (9, 34) and in transient replication assays (52). More recently, it has been demonstrated that BPV1 E1 alone can support DNA replication in vitro but not in vivo (22, 30). HPV1a E1 by itself can support measurable transient DNA replication in vivo, but this replication was greatly stimulated by E2 (14). The enhancement of DNA replication by E2 may be caused by increasing the affinity of E1 or recruitment of E1 to the origin of replication through the interaction between E1 and E2 (30, 40, 44, 50, 55). Thus, the interaction between E1 and E2 plays an important role in efficient viral DNA replication. In this study, we have characterized and mapped the interaction domains of HPV16 E1 and E2.

Two different versions of yeast two-hybrid system were used to define the interaction domains between HPV16 E1 and E2 in vivo. Furthermore, in vitro binding experiments were performed to confirm and extend these results. The interaction domains were mapped to the amino-terminal 190 aa of HPV16 E2 and to aa 421 to 649 of HPV16 E1 [E1(aa421-649)]. Although a 229-aa carboxyl-terminal domain (aa 421 to 649) was capable of binding E2 at lower temperatures, a larger domain

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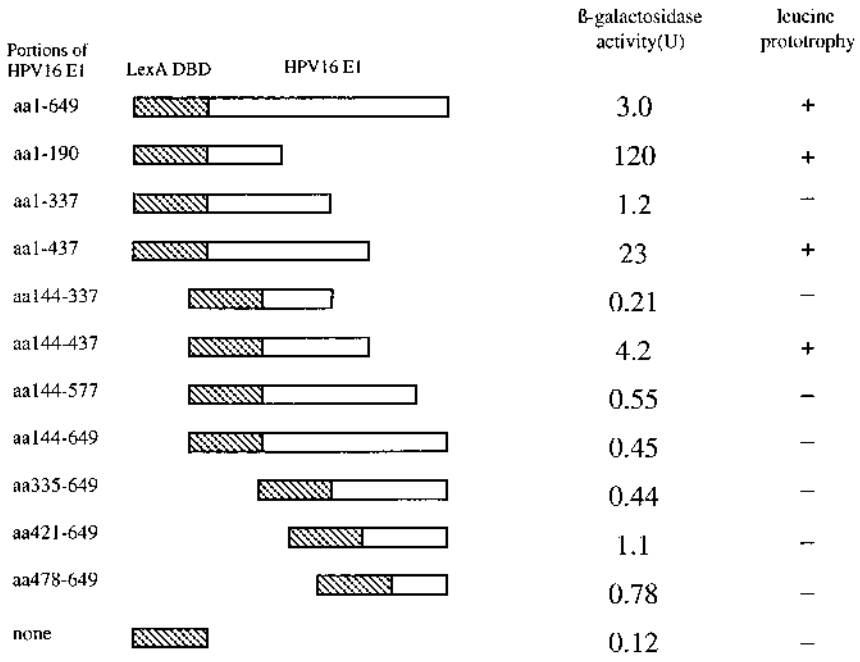


FIG. 1. Intrinsic transactivation properties of HPV16 E1-LexA DBD chimeric proteins. The indicated portions of HPV E1 cloned into the Lex202PL vector were transformed into yeast strain EGY048 together with the pJK103 vector. β-Galactosidase activities and leucine prototrophy were determined as described in Materials and Methods.

consisting of HPV16 E1(aa144-649) was required for optimal E1-E2 interaction at physiologic temperatures.

## MATERIALS AND METHODS

**Yeast strains and vectors.** Two versions of the yeast two-hybrid system were used in this study.

**(i) LexA-based two-hybrid system.** *Saccharomyces cerevisiae* EGY048 (*MAT***a** *ura3 his3 trp1 LEU2*::*lexAop6-LEU2*) (provided by Roger Brent, Massachusetts General Hospital, Charlestown) (13, 58) was used as the host strain for the LexA-based two-hybrid system. This strain contains the prototrophic marker reporter gene, *LEU2*, with six upstream binding sites for LexA dimers integrated at the *LEU2* locus.

The Lex202PL vector (13, 58), containing the LexA DBD (aa 1 to 202), the yeast *HIS3* gene as a selectable marker, and the 2 μm origin of replication, was used as the bait plasmid in host strain EGY048. Portions of HPV16 E1 were expressed as bait proteins fused to the LexA DBD. For cloning HPV16 E1 into LexA202PL, DNA fragments of E1 were generated as *Eco*RI-*Sal*I restriction fragments by PCR, using appropriate synthetic primers. LexA-HPV16 E1 fusion proteins are expressed under the control of constitutive *S. cerevisiae ADC1* promoter.

Portions of HPV16 E2 were generated as *Eco*RI-*Xho*I restriction fragments by PCR, using appropriate synthetic primers, and then cloned into the pJG4-5 vector (13, 58). From this vector, E2-B42 acidic activation domain hybrid proteins were expressed in host strain EGY048. These hybrid proteins also contain a nuclear localization signal from the simian virus 40 large T antigen and an epitope tag from the influenza virus hemagglutinin which can be recognized by monoclonal antibody 12CA5. The expression of these hybrid proteins is controlled by the *GAL10* promoter, which is induced by galactose and repressed by glucose.

pJK103 (13, 22) contains the *lacZ* reporter gene with two upstream binding sites for LexA dimers, 2 μm origin of replication, and the *URA3* selectable marker.

**(ii) Gal4-based two-hybrid system.** MaV103(*MAT***a** *ura3-52 leu2-3,112 trp1-901 his3Δ200 ade2-101 gal4Δgal80ΔGAL1*:*lacZ GAL1*:*HIS3@lys2 SPAL10*:*URA3*) (39) was used as the host strain for the second two-hybrid system based on the Gal4 protein. The MaV103 strain contains three reporter genes whose expression is regulated by different Gal4-responsive promoters. In this study, we used *HIS3* and *URA3* reporter genes integrated in the genome of MaV103 to detect the E1-E2 interaction. The pPC97 vector is used as the bait plasmid in this system. This vector contains the DBD of Gal4 (aa 1 to 147) and the *LEU2* selectable marker. Full-length HPV16 E2 was cloned into the pPC97 vector (39) as a *Sal*I-*Bgl*II restriction fragment by PCR, using appropriate primers (pPC97-E2wt). For the construction of pPC97-E2E39A, pPC97-E2wt was digested with restriction enzymes *Nco*I and *Msc*I. This *Nco*I-*Msc*I fragment was replaced by the *Nco*I-*Msc*I fragment containing the E39A mutation from the pCMV4 plasmid harboring an E39A substitution mutant of HPV16 E2. The E39A mutant is defective for binding with E1 in vitro (36). The Gal4 DBD-HPV16 E2 fusion protein is expressed under the control of constitutive *S. cerevisiae ADC1* promoter.

Portions of E1 were generated as *Sal*I-*Not*I restriction fragments by PCR, using appropriate synthetic primers, and cloned into the prey centromeric plasmid (pPC86). The pPC86 vector (39) contains the AD of Gal4 (aa 768 to 881) and the *TRP1* selectable marker. HPV16 E1-Gal4 AD fusion proteins are constitutively expressed under the control of *ADC1* promoter.

Growth and manipulation of the yeast strain was done according to standard procedures (51). Yeast cultures were maintained in SD (0.67% [wt/vol] Bacto Yeast Nitrogen Base, 2% [wt/vol] dextrose [glucose]) minimal medium containing the appropriate amino acid supplements, except under induction conditions, when SG (0.67% [wt/vol] Bacto Yeast Nitrogen Base, 2% [wt/vol] galactose, 1% [wt/vol] raffinose) minimal medium was used. Leucine auxotrophy for the transformants of EGY048 was determined by assessing growth on parallel minimal SD and SG plates in which leucine was included or omitted. Interaction phenotypes of the MaV103 transformants were determined by the differences in growth on dropout plates lacking histidine containing 15 mM 3-aminotriazole (3AT) and on plates containing uracil and 0.1% (wt/vol) 5-fluoroorotic acid (5FOA).

**β-Galactosidase activation assays.** Assays for β-galactosidase expression in yeast were performed as previously described (15). For galactose induction experiments, cells from 1-ml saturated overnight cultures grown in SD minimal medium were pelleted, washed once with sterile water, and resuspended in 5 ml of SG minimal medium supplemented with appropriate amino acids. The cultures were harvested at mid-log phase (optical density at 600 nm, 0.6 to 0.8), and β-galactosidase assays were performed. The reported measurements represent three assays of three independent transformants.

**Detection of HPV16 E1-E2 complexes in vitro by using GST-E2 fusion proteins.** The glutathione *S*-transferase (GST) fusion proteins were induced and harvested by using standard methods (43). Fragments of HPV16 E2 were generated by PCR, using appropriate oligonucleotide primers, and cloned as *Eco*RI-*Xho*I fragments into pGEX4T-1 vector (Pharmacia). *Escherichia coli* DH5α was used to create GST-E2 fusion proteins.

$^{35}$S-labeled E1 was generated by using the T7-TNT coupled rabbit reticulocyte lysate (RRL) system (Promega). The template for in vitro translation were prepared by introducing the T7 promoter sequence on the 5′ end of each template DNA, using a T7 promoter-containing primer for PCR amplification. Approximately 2 μg of each GST-E2 fusion protein was preincubated for 30 min in 0.5 ml of binding buffer (20 mM Tris [pH 7.5], 50 mM NaCl, 4 mM $MgCl_2$, 2 mM dithiothreitol, 0.5% Nonidet P-40) with 2% nonfat dry milk. Then 10 μl of a radiolabeled HPV16 E1 transcription reaction mixture was added, and incubation was continued for 60 min. After three 1-ml washes in binding buffer

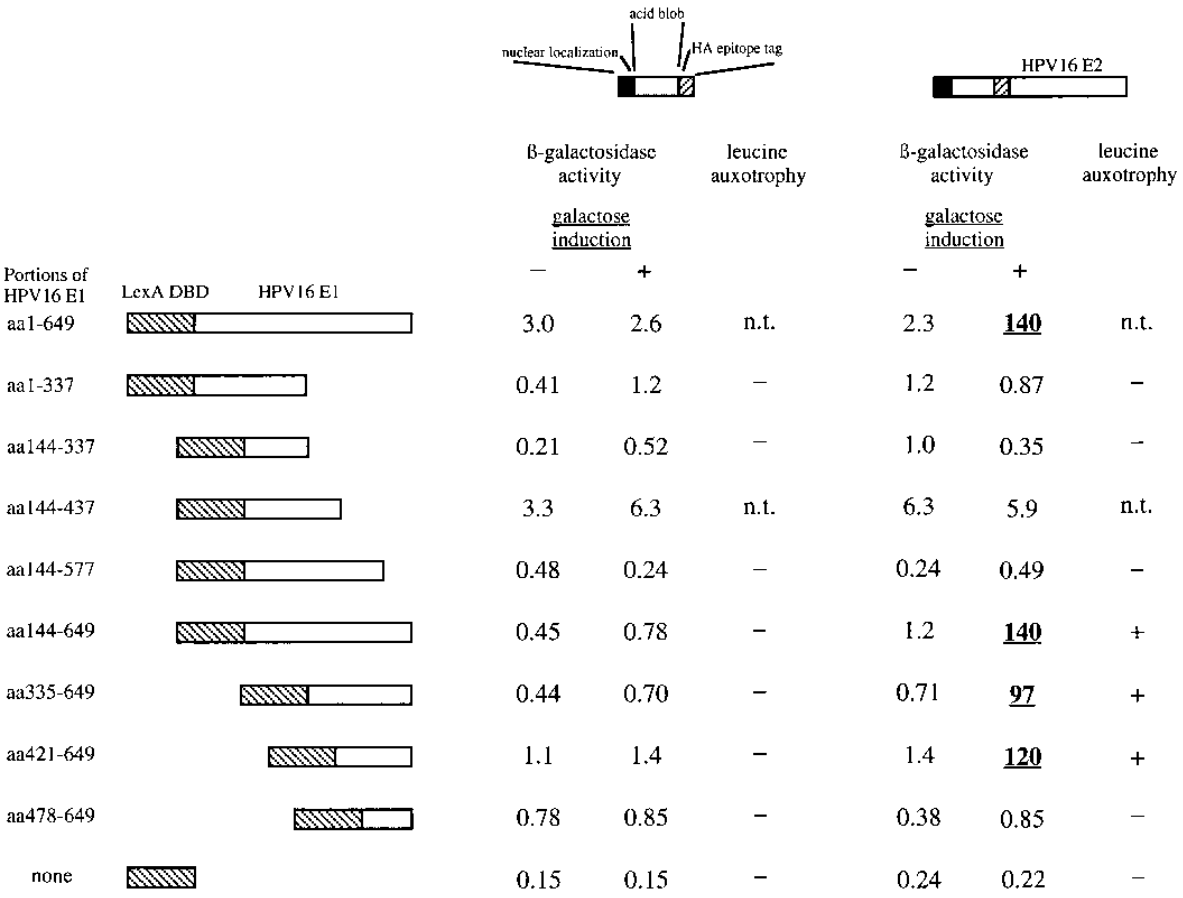


FIG. 2. Yeast two-hybrid interactions between the full-length HPV16 E2 protein and portions of HPV16 E1. The E1-LexA DBD chimeric proteins that lacked strong intrinsic transactivation activity were used as bait to score interaction with the full-length E2 protein. β-Galactosidase assays and leucine auxotrophy were determined as described in Materials and Methods. HA, hemagglutinin; n.t., not tested. Bold underlined values indicate the β-galactosidase activities of yeast in which bait-prey interaction was observed.

without milk, samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Bound E1 was detected by autoradiography.

**Coimmunoprecipitations of HPV16 E1 and E2.** Each template was prepared by PCR, using appropriate primers containing the T7 promoter sequence on the 5′ end. [$^{35}$S]methionine-labeled full-length E1 and portions of E1 were generated by using RRL. Unlabeled E2 was also generated by RRL in the absence of radioactive amino acids. Radiolabeled E6-AP was used as a negative control for binding. Typically, coimmunoprecipitations were performed as follows. Fifteen microliters of the 50-μl translation reaction containing radiolabeled E1 and 10 μl of the translation reaction of unlabeled E2 were incubated in 0.5 ml of binding buffer without milk for 1 h, then 2 μl of anti-E2(C) antibody (36) was added, and incubation was continued for 1 h. Immunocomplexes were collected by addition of 25 μl of 50% protein A-Sepharose slurry equilibrated in binding buffer. After incubation for 45 min at the indicated temperature, beads were sedimented and washed three times with 1 ml of binding buffer. The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography.

**Production of anti-HPV16 E1 rabbit antiserum.** Fragments of E1, aa 1 to 190 (E1N) and aa 335 to 649 (E1C), were cloned into the *Eco*RI-*Sal*I site of pGEX4T-1. GST-E1N and GST-E1C proteins expressed in *E. coli* HB101 were purified with glutathione-Sepharose 4B beads (Pharmacia). These purified GST fusion proteins were directly used for the immunization of rabbits. All procedure requiring handling the animals were performed by Berkeley Antibody Company, Berkeley, Calif.

## RESULTS

**Assessment HPV16 E1 intrinsic transcriptional transactivation properties in yeast.** The yeast two-hybrid system was used to study the interaction of the HPV16 E1 and E2 proteins. A series of chimeric proteins (potential bait proteins) consisting of either the full-length E1 protein or portions thereof fused to the LexA DBD were tested for intrinsic transcriptional activation properties. Chimeric proteins that by themselves result in the significant transactivation of a reporter gene are not useful as bait proteins, since the two-hybrid system relies on transcriptional activation that occurs as a result of interaction between a bait and second fusion protein (often referred to as the prey) containing the AD (10).

Yeast strain EGY048 was transformed with Lex202PL vector plasmids containing portions of HPV16 E1 along with the pJK103 reporter plasmid. These transformants were tested for β-galactosidase activities in liquid culture and for leucine auxotrophy on solid media (Fig. 1). LexA-E1(aa1-190) and LexA-E1(aa1-437) demonstrated strong intrinsic transactivation, precluding their further analysis as bait in this system. Although EGY048 expressing LexA-E1(aa144-437) and LexA-E1(aa1-649) could grow on leucine dropout plates, these transformants showed relatively weak β-galactosidase activities (β-galactosidase activities are 4.2 and 3.0 U, respectively) (Fig. 1), making these proteins potential bait proteins using the *lacZ* reporter gene assays. Since other portions of E1 did not show significant transactivation activity when fused to the LexA DBD, these fusion proteins were used as bait for both *LEU2* and *lacZ* reporter gene assays. Equivalent expression of HPV16 E1-LexA DBD fusion proteins was confirmed by Western blot analysis with antibodies to the LexA DBD (data not shown).

**Interaction between portions of HPV16 E1 and full-length HPV16 E2 in yeast.** We next analyzed the abilities of various LexA-E1 bait proteins to interact with full-length HPV16 E2 in the LexA-based two-hybrid system. EGY048 containing the β-galactosidase reporter plasmid pJK103 and the prey plasmid pJG4-5 containing the full-length E2 [pJGE2(aa1-365)] were transformed with Lex202PL bait plasmids containing various fragments of HPV16 E1. The assays for β-galactosidase activity and leucine auxotrophy were performed by using the pJG4-5 parent plasmid as a negative control. The yeast transformants expressing LexA fusion bait proteins containing full-length E1, E1(aa144-649), E1(aa335-649), and E1(aa421-649) indicated strong β-galactosidase activity when the full-length E2 prey protein was induced with galactose (Fig. 2). Leucine auxotrophy could be assessed for all transformants except LexA-E1(aa144-437) and LexA-E1(aa1-649), which had intrinsic transactivation activity. Leucine auxotrophy correlated with the β-galactosidase phenotype in each case (Fig. 2). These results indicated that carboxyl-terminal 229 aa of HPV16 E1 are able to interact with full-length HPV16 E2 in vivo. The expression of the full-length E2 fusion protein containing the B42 acidic activation domain and the hemagglutinin epitope was confirmed by Western blot analysis using monoclonal antibody 12CA5.

To circumvent the problems encountered when using E1 amino-terminal constructs as bait in the LexA-based system, we used a second version of yeast two-hybrid system (Gal4-based system) to examine HPV16 E1-E2 interaction. In this system, Gal4 DBD-HPV16 E2 fusion proteins were expressed as bait proteins and Gal4 AD-HPV16 E1 fusion proteins were expressed as prey proteins. In these experiments, the HPV16 E2 E39A mutant was used as a negative control for E1 binding because the E39A substitution mutant does not bind HPV16 E1 in vitro (36). Neither wild-type E2 (E2wt) nor E2 mutant E39A fused to the Gal4 DBD demonstrated significant transactivation activity in the Gal4-based system (Fig. 3). However, the transformant expressing the Gal4 DBD-E2wt fusion protein and Gal4AD-E1(aa1-649) fusion protein indicated a strong interaction phenotype. In this two-hybrid system, increased HIS3 expression results as a consequence of an interacting clone. Since HIS3 expression renders yeast resistant to 3AT, 3AT at a concentration of 15 mM was used to optimize the growth difference between interaction-positive transformants and interaction-negative transformants for histidine auxotrophy determined on histidine dropout plates. The growth of the transformants containing pPC86-E1(aa1-649), pPC86-E1(aa144-649), and pPC86-E1(aa390-649) was detected when cells were cotransformed with pPC97-E2wt bait plasmid but not with the pPC86 vector or with the E39A E2 mutant controls. No growth was observed in the cotransformants expressing the E2wt bait and prey plasmids harboring the E1 amino-terminal region from aa 1 to 437 or 1 to 538. On 0.1% 5FOA plates, the growth of the transformants is inhibited when the *URA3* reporter gene is activated due to the interaction between the bait and the prey proteins (4). The cotransformants expressing the chimeric prey proteins containing the carboxyl terminus of E1 could not grow on 5FOA plates, whereas the transformants expressing E1(aa1-437) or E1(aa1-538) could grow on 5FOA plates. These results are in agreement with the observations from the LexA-based two-hybrid system that the carboxyl terminus of E1 has strong E2 binding activity and that the deletion of this carboxyl terminal region dramatically reduces E2 binding activity. The expression of Gal4 DBD fusion proteins was confirmed by using the monoclonal antibody for Gal4 DBD, and expression of Gal4 AD-E1 fusion proteins was confirmed by using polyclonal antibodies to HPV16 E1 (57).

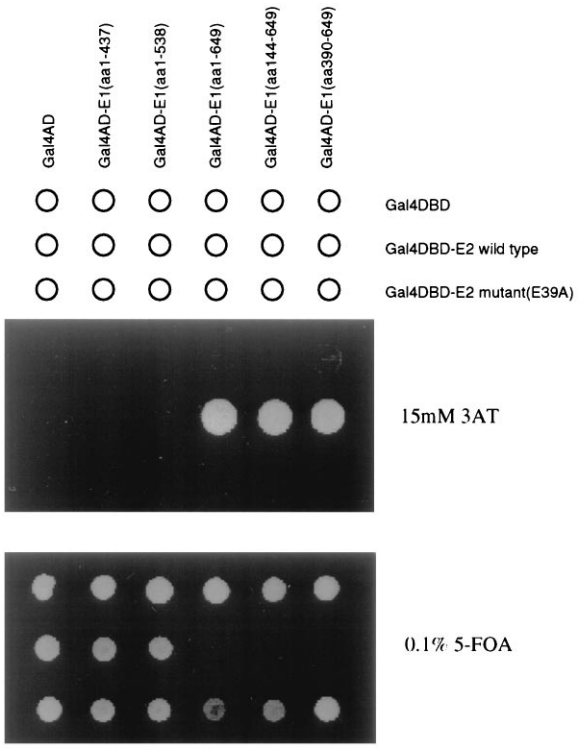


FIG. 3. Yeast two-hybrid interactions using HPV16 E2 as bait to map interacting domains of HPV16 E1. Interaction phenotypes were determined for MaV103 transformants expressing full-length E2wt or the E39A mutant form of HPV16 E2 fused to Gal4 DBD and portions of E1 fused to the Gal4 AD as indicated. Approximately equal amounts of transformed yeast cells were put on plates containing 15 mM 3AT or 0.1% 5FOA. Photographs were taken after 3 days of incubation at 30°C.

**Mapping of HPV16 E2 domains that interact with HPV16 E1 in yeast.** Various portions of HPV16 E2 were tested for interaction with HPV16 E1 in the LexA-based system. In these experiments, yeast strains harboring bait plasmids containing the full-length E1 protein and the carboxyl-terminal domain from aa 421 to 469 were transformed with pJG4-5 plasmids containing various portions of E2 (Fig. 4). β-Galactosidase activity assays were performed on these transformants following growth in the presence or absence of galactose induction, and leucine auxotrophy was determined for the cotransformants containing the E1(aa421-649) bait. Figure 4 shows that the prey E2 proteins containing the amino-terminal aa 1 to 190, aa 1 to 245, or the full-length protein interacted with both full-length E1 and the carboxyl-terminal aa 421 to 649 domain of E1. No β-galactosidase activity was observed in assays using chimeric prey proteins containing E2(aa1-150) or E2(aa33-190). These results indicate that the amino-terminal 190-aa domain of E2 is sufficient and necessary for interaction with E1. The expression of the E2 chimeric proteins was confirmed by Western blot analysis using monoclonal antibody 12CA5.

**In vitro binding between full-length HPV16 E1 and GST-E2 fusion proteins.** Segments of HPV16 E2 were cloned into the pGEX4T-1 vector (Pharmacia) for expression as GST fusion proteins in *E. coli*. These proteins were purified by binding to glutathione beads, and their abilities to bind full-length E1 expressed in RRL in vitro were determined. The purified GST fusion proteins were visualized by Coomassie blue staining

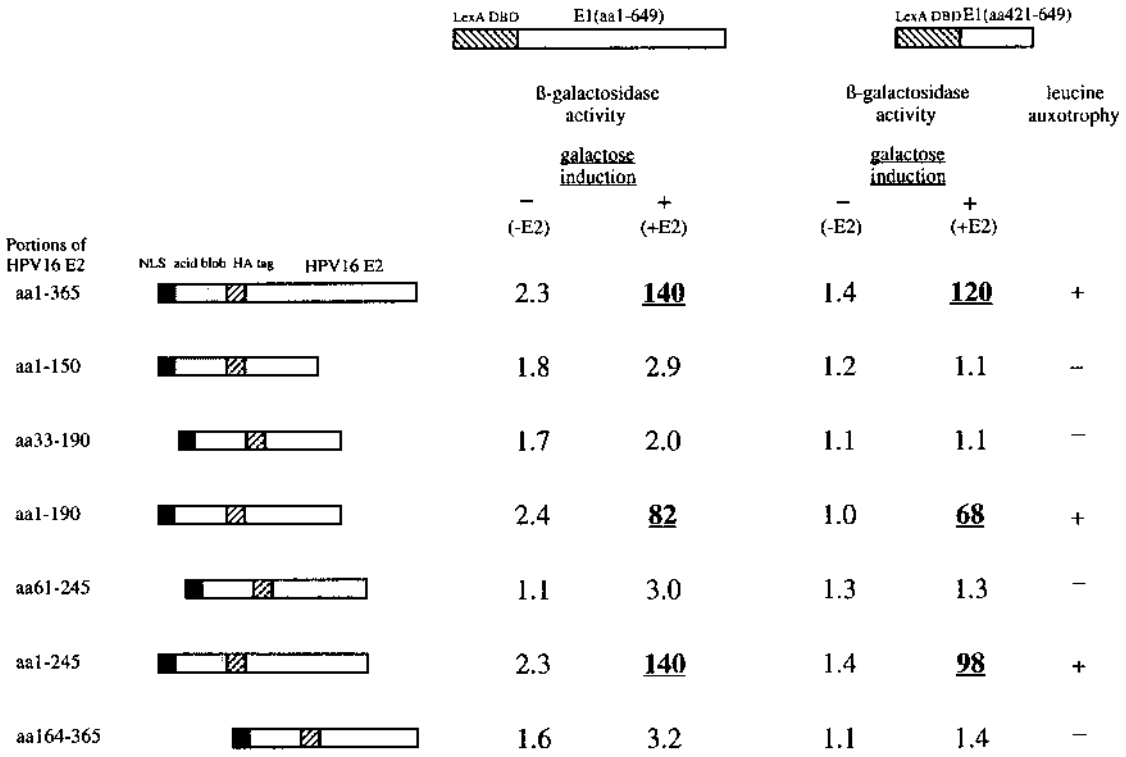


FIG. 4. Yeast two-hybrid interactions between full-length HPV16 E1 or the C terminus of HPV16 E1 as the bait and portions of HPV16 E2. The interactions between E1 and the indicated portions of E2 fused to the B42 acidic activation domain were determined by using LexA-E1(aa1-649) or LexA-E1(aa421-649) as the bait. β-Galactosidase assays were performed for all of the transformants in SD or SG medium. Leucine auxotrophy was determined for the transformants expressing the LexA-E1(aa421-649) bait protein. NLS, nuclear localization signal; HA, hemagglutinin. Bold underlined values indicate the β-galactosidase activities of yeast in which bait-prey interaction was observed.

(Fig. 5B), and an equal amount of each fusion protein was used in the in vitro binding assays. As shown in Fig. 5A, those GST fusion proteins that contained the amino-terminal 190 aa of HPV16 E2 could specifically bind full-length E1. The deletion of the first 61 aa of E2 dramatically reduced binding to E1. These in vitro binding experiments using GST fusion proteins confirm the two-hybrid results that the amino-terminal 190 aa of HPV16 E2 are necessary and sufficient for the interaction with E1 and that both hinge and DNA binding domains of HPV16 E2 are dispensable for E1-E2 interaction.

**Coimmunoprecipitation of E1 with full-length E2.** The above-described two-hybrid system mapping indicated that the region from aa 421 to 649 is the smallest portion of HPV16 E1 capable of interaction with HPV16 E2. However, all of these experiments used fusion proteins. Therefore, coimmunoprecipitation experiments were designed to confirm and extend the results of the two-hybrid systems by using in vitro-translated portions of E1 and full-length E2 that were not fusion proteins. As shown in Fig. 6, we performed in vitro binding assays in which complex formation was assessed by coimmunoprecipitation using the anti-E2(C) antiserum (36). This antiserum, which recognizes aa 194 to 365 of HPV16 E2, should not interfere with E1-E2 interaction, since none of our experiments have implicated this portion of E2 in E1 interaction. This rabbit polyclonal anti-E2(C) antiserum could specifically precipitate in vitro-translated full-length E2 efficiently at both 25 and 4°C (Fig. 6B and C). In these binding assays, labeled 16E1 was generated by in vitro translation in the presence of [$^{35}$S]methionine, and unlabeled full-length E2 was generated in separate reactions in the absence of labeled amino acids. The full-length E1 protein, as well as fragments containing the carboxyl-terminal aa 420 to 649 of E1, were efficiently coprecipitated only in the presence of unlabeled full-length E2 at 4°C. Interestingly, when these experiments were performed at 25°C, the carboxyl-terminal aa 420 to 649 fragment of E1 was precipitated much less efficiently than at 4°C, whereas the intensity of the coimmunoprecipitated bands of full-length E1 or aa 144 to 649 of E1 did not vary according to temperature. An interaction between the amino terminus of E1 and E2 was not observed at either temperature. The specificity of binding in these experiments is established by the fact that E6-AP (21), a protein with no known HPV E2 binding activity and no observed E2 binding activity in two-hybrid experiments (57), was not coimmunoprecipitated with full-length E2 (Fig. 6, lanes 1).

**Interaction of the HPV16 E1 carboxyl terminus with E2 is temperature sensitive in vivo.** The results of coimmunoprecipitation experiments indicate that the binding ability of the carboxyl-terminal aa 420 to 649 of E1 with E2 is temperature sensitive in vitro, resulting in better binding at 4°C than at 25°C. The binding activity of this carboxyl-terminal segment was tested in the LexA two-hybrid system at three different temperatures to assess this temperature sensitivity in vivo. EGY048 was transformed with pJGE2(aa1-365) and Lex202PL-derived plasmids expressing aa 144 to 649 or 421 to 649 of E1. The transformants were tested for leucine auxotrophy at 37, 30, and 25°C. The transformant expressing aa 421 to 649 of E1 grew much more slowly at 37°C than the transformant expressing aa 144 to 649 of E1, whereas growth of these transformants was indistinguishable at 30 or 25°C (Fig. 7A). The growth of these transformants was indistinguishable at each of the temperatures when the transformants were cultured on solid SG

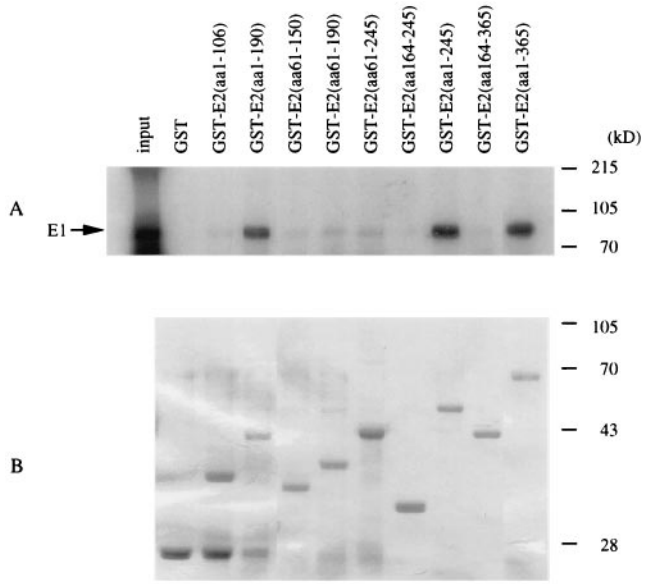


FIG. 5. In vitro mapping of the domains of HPV16 E2 which interact with full-length E1. The indicated portions of E2 were expressed as GST fusion proteins and purified from *E. coli* as described in Materials and Methods. (A) [$^{35}$S]methionine-labeled full-length E1 bound to the indicated GST fusion protein; (B) Coomassie blue stain of approximately 5 μg of the indicated GST fusion proteins. The binding reactions were performed at room temperature, and the bound E1 was visualized by autoradiography.

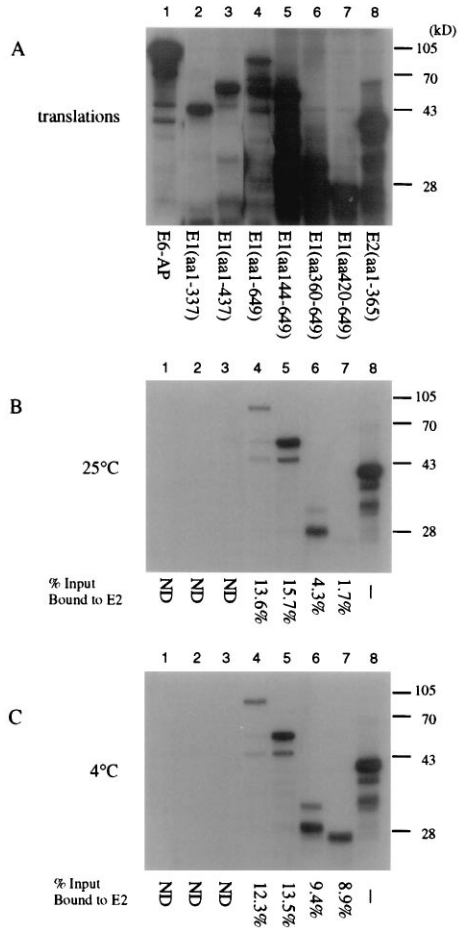


FIG. 6. In vitro mapping of the HPV16 E2 interaction domain of E1 by coimmunoprecipitation using an E2-specific antibody. The indicated truncated forms of HPV16 E1 were tested for the ability to interact with full-length E2. (A) Three microliters of each loaded input of translated lysate labeled with [$^{35}$S]methionine. Lane 1, E6-AP as a negative control; lanes 2 to 7, portions of HPV16 E1; lane 8, full-length HPV16 E2. Unlabeled full-length E2 translated in vitro was then mixed, and complexes were precipitated with a rabbit polyclonal antibody to the E2 C terminus (36). (B) Binding and precipitations performed at 25°C. (C) Binding and precipitations performed at 4°C. The precipitation of labeled full-length E2 by the anti-E2(C) antibody at each temperature is shown in lanes 8. The percent binding shown in panels B and C is the percentage of total input HPV16 E1 or E6-AP that interacted with the unlabeled E2 protein in the in vitro binding reaction. In vitro-translated products were visualized by autoradiography and quantitated by phosphorimager analysis. ND, none detected.

medium containing leucine (Fig. 7A), indicating that the poor growth exhibited by the transformant expressing aa 421 to 649 of E1 when tested for leucine auxotrophy was not due to toxicity from expression of the protein. The growth curves of these transformants cultured in the SG medium lacking leucine at 37°C or at 30°C are depicted in Fig. 7B. The doubling times at 30°C of the transformants expressing LexA-E1(aa144-649) and LexA-E1(aa421-649) were 6.9 and 7.0, respectively, whereas at 37°C, the doubling times were 8.4 and 11.1 h, respectively. This result indicates that although the carboxyl-terminal 229 aa of HPV16 E1 are sufficient for E2 interaction, domains within aa 144 to 420 may act to stabilize this interaction at higher temperatures both in vivo and in vitro.

## DISCUSSION

In this study, we have used two different versions of the yeast two-hybrid system to identify and study interaction domains of HPV16 E1 and E2 in vivo. The interaction domains were confirmed in vitro by using GST fusion proteins and by coimmunoprecipitation assays. The cumulative results of these mapping experiments indicate that the carboxyl-terminal 229 aa of E1 and the amino-terminal 190 aa of E2 are required and sufficient for HPV16 E1-E2 interaction. This amino-terminal 190-aa domain includes almost the entire E2 AD (12, 28, 36). However, no interaction between smaller domains of the E2 amino terminus and E1 was detected either in two-hybrid experiments or in vitro. Hence, the results of both two-hybrid analyses and in vitro binding experiments indicated that a domain encompassing aa 1 to 190 of HPV16 E2 is the smallest portion which can interact with HPV16 E1. This finding is consistent with several other studies that have indicated the importance of the N terminus of HPV16 E2 in binding to E1. Mutation of glutamic acid to alanine in codon 39 of HPV16 E2 has been shown to disrupt E1-E2 interaction and to abrogate the ability of E2 to stimulate E1-dependent replication in vivo (36). Hibma et al. have also suggested that the domain of HPV16 E2 interacting with E1 lies in this region, since HPV16 E1-E2 interaction could be blocked in vitro by a monoclonal antibody that recognized E2 in the region of aa 18 to 41 (18). These data are also consistent with the findings of Storey et al., who have shown that HPV16 E1 can bind the full-length form of HPV16 E2 but not E2(aa1-140) or a shortened form of E2 deleted of its amino-terminal AD (46). Studies with BPV1 E2 have also implicated the N terminus in E1 binding, where it has been shown that aa 1 to 91 of BPV1 E2 are sufficient for interaction with E1 (1). Thus, in comparison to BPV1 E2, it would appear that a relatively large domain of HPV16 E2 is required for optimal HPV16 E1-E2 interaction. Alternatively, there may be a smaller domain of HPV16 E2 involved in the binding that has not been revealed by our deletion analysis,

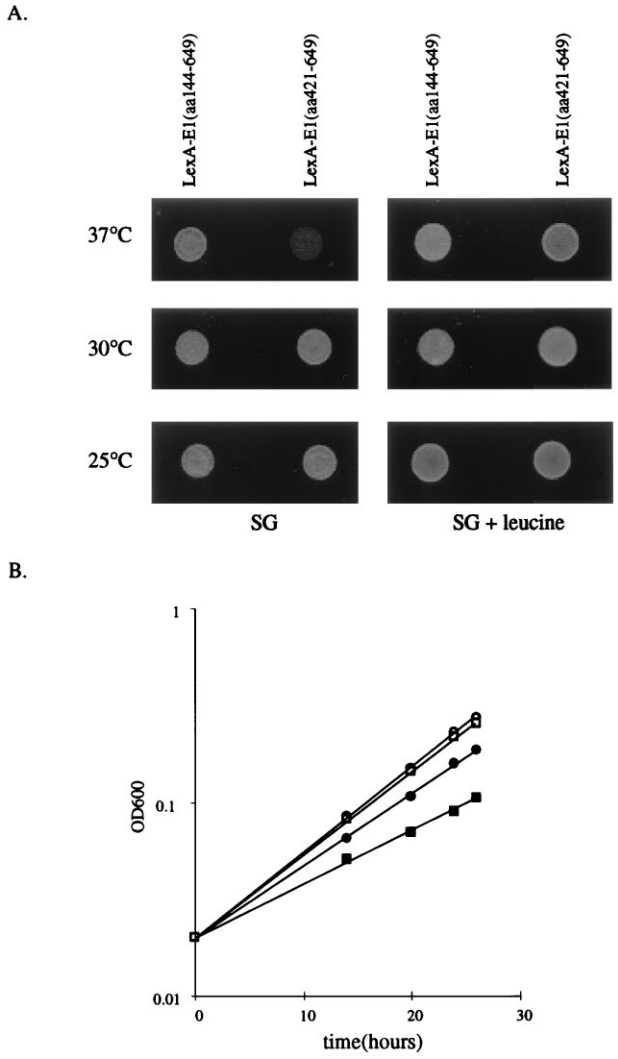


FIG. 7. The interaction between the E1 C terminus (aa 421 to 649) and E2 is temperature sensitive in vivo. Levels of growth of EGY048 transformants expressing LexA-E1(aa144-649) and LexA-E1(aa421-649) with the induction of full-length E2 prey chimeric protein were compared at the indicated temperatures. (A) The transformants expressing the LexA-E1 fusion proteins together with full-length E2 fused to B42 acidic activation domain were incubated on solid SG medium lacking or containing leucine at 25, 30, or 37°C. Photographs were taken after 4 to 6 days incubation. (B) Growth curves of these transformants at 37 and 30°C in liquid SG medium lacking leucine. The optical density at 600 nm (OD600) was measured at several points in log phase, and the data are plotted. Open circles, LexA-E1(aa144-649) at 30°C; closed circles, LexA-E1(aa144-649) at 37°C; open squares, LexA-E1(aa421-649) at 30°C; closed squares, LexA-E1(aa421-649) at 30°C.

perhaps because of conformational constraints of the specific constructs used.

A carboxyl-terminal domain encompassing aa 421 to 649 is the smallest portion of HPV16 E1 capable of interaction with E2. Deletion of this carboxyl-terminal domain of HPV16 E1 disrupts efficient binding to HPV16 E2. No measurable interaction with HPV16 E2 was found to be mediated through the amino terminus of HPV16 E1 in either two-hybrid or in vitro GST-E2 binding experiments. This is consistent with the data of Hibma et al., who reported that aa 143 to 649 of HPV16 E1 could interact with E2 and that aa 1 to 102 of E1 could not interact (18). These results are in marked contrast to a previous characterization of BPV1 E1-E2 interaction by our laboratory, in which the amino-terminal 250 aa of BPV1 E1 were shown to be capable of binding BPV1 E2 and in vitro and in yeast two-hybrid assays (1). Thorner et al. have also suggested an E2 interaction domain exists in the amino terminus of BPV1 E1, using assays that measured the ability of E2 to stimulate of E1 origin binding in vitro (50).

Although the amino terminus of HPV16 E1 may not be sufficient for HPV16 E2 binding, this domain may contribute to E2 binding under certain conditions. In vitro binding experiments at various temperatures using HPV16 E2 indicated that interaction between E2 and the carboxyl terminus of E1 (aa 420 to 649) is much weaker at 25°C than at 4°C. In contrast, interaction of either full-length E1 or E1(aa144-649) with HPV16 E2 did not exhibit such sensitivity to higher temperatures in vitro. This phenomenon was also demonstrated in the yeast two-hybrid experiments, where interaction of E2 with E1(aa144-649) was readily detected at 37°C, but E2 interaction with aa 421 to 649 of E1 was not detected at that temperature. These results suggest that the carboxy-terminal 229 aa of HPV16 E1 may not be sufficient for optimal interaction with E2 at physiologic temperatures. Since E1(aa144-649) does not show the temperature sensitivity exhibited by E1(aa421-649), it is possible that the amino terminus of HPV16 E1 participates in complex formation with E2 but that this interaction is much weaker than that of BPV1 E1. It is also possible that a domain of HPV16 E1 encompassing aa 144 to 420 exerts an allosteric effect on the carboxy terminus of E1 that is modulated by temperature. Several studies with different HPV types have implicated amino-terminal regions of other HPV E1 proteins in the binding of E2 (5, 11). Although E1 is highly conserved among the different papillomaviruses, a comparison of predicted polypeptide sequences of the various animal and human papillomaviruses reveals that the amino-terminal regions are relatively divergent (8, 38). This divergence could account for differences observed in these various studies.

In our assessment of various E1 bait proteins, LexA DBD proteins containing the amino-terminal 190 or 437 aa of HPV16 E1 exhibited strong intrinsic transactivation. This activation by the amino terminus of HPV16 E1 was unexpected from our studies with BPV1 E1, in which the LexA-BPV1 E1(aa1-250) fusion protein did not exhibit any transactivation activity (1). These results further support the notion that there may be important functional differences between the E1 proteins of HPV16 E1 and BPV1. It is also interesting that LexA-E1(aa1-649) transactivates only weakly compared to LexA-E1(aa1-190) or LexA-E1(aa1-437). This difference may reflect different conformations of the E1 amino terminus when expressed in the presence or absence of the carboxyl terminus. Alternatively, these differences in activity may simply be artifacts of chimeric protein expression. To date there are no studies that establish a direct role for the HPV E1 proteins in transcriptional regulation, although some studies with BPV1 suggest that the E1 protein may have an indirect role in regulating viral transcription, perhaps through E2 (24, 37).

Temperature modulation effects on E1-E2 interactions similar to those observed here have also been demonstrated elsewhere. Initial studies by Lusky and Fontane (26) suggested that a domain including aa 384 through 516 of BPV1 E1 is essential for interaction with E2. These experiments were carried out at 4°C (26). However, Thorner et al. subsequently showed a complex between BPV1 E1(aa1-450) and E2 at 30°C but not at 4°C (50). Since BPV GST-E1(aa1-250) interaction with E2 has been observed at room temperature but not at 4°C, it would appear that at least in the case of BPV1 E1, carboxy-terminally mediated E2 interactions may exist at lower temperatures, whereas amino-terminally mediated E2 interactions may be

observed only at more physiologically relevant temperatures. Recent analysis of HPV33 E1-E2 interaction suggests a somewhat analogous situation. Muller and Sapp demonstrated that whereas the carboxy terminus of HPV33 E1(aa312-644) could bind E2 in experiments carried out at 4°C, the amino terminus of HPV33 E1(aa1-450) could not (31). In contrast, in experiments conducted at room temperature, HPV33 E2 was able to bind a minimal domain of E1(aa312-450) (31). Other mapping studies have shown that the carboxyl-terminal aa 162 to 605 of BPV1 E1 are required for cooperation with E2 in binding to origin DNA and that aa 200 to 605 of BPV1 E1 are required for direct complex formation with E2 (38).

The data suggest that more than one domain of E1 may be involved in E2 interaction. Alternatively, multiple E1 conformational states may exist, each with its own E2 binding characteristics and contributory domains. Moreover, such potential alternative conformational forms of E1 could be modulated by E2 interaction itself, phosphorylation, DNA binding, ATP binding and hydrolysis, interaction with cellular proteins, ambient temperature, E1 multimerization, or any combination of the above. Furthermore, some of the differences observed in the various published studies which have mapped the E1 and E2 interaction domains may reflect intrinsic differences in the E1 and E2 proteins of the various papillomaviruses. Direct comparison of many of these results may be difficult since different studies have used a variety of E1-E2 interaction assays and expression contexts. Perhaps only through the use of experimental strategies that permit the study of full-length native E1 interaction with E2 will it be possible to comprehensively examine these issues. Such an understanding will be an important step in clarifying the exact nature of E1-E2 interaction. A more detailed definition of the HPV16 E1 and E2 domains involved in protein-protein interaction should prove useful in designing additional studies to explore the functional consequences of their binding. Our mutational analysis of HPV16 E2 has suggested that E1-E2 binding is required for efficient viral DNA replication in vivo (36). The disruption of E1-E2 interaction may therefore be a target for antiviral drug screening. Indeed, the two-hybrid systems described here could serve as the basis for an in vivo screen for inhibitors of E1-E2 binding.

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