JOURNAL OF VIROLOGY, July 1995, p. 4364–4372 Vol. 69, No. 7
0022-538X/95/$04.00+0

Amino-Terminal Domains of the Bovine Papillomavirus Type 1 E1 and E2 Proteins Participate in Complex Formation

JOHN D. BENSON AND PETER M. HOWLEY*

Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

Received 26 January 1995/Accepted 10 April 1995

Interaction between the E1 and E2 papillomavirus proteins appears to play an important role in viral DNA replication, although the exact domains of each protein involved in this interaction have not been identified. Using bovine papillomavirus type 1 (BPV-1) as a model for examining interactions between E1 and E2, we have used the two-hybrid and glutathione *S*-transferase (GST) fusion systems to map domains of BPV-1 E1 and E2 that interact in vivo and in vitro. In the two-hybrid system experiments, portions of BPV-1 E2 were expressed in *Saccharomyces cerevisiae* as LexA fusion proteins, which were tested for interaction with various domains of BPV-1 E1. These assays indicated that domains sufficient for E1-E2 interaction are present within the amino-terminal 250 amino acids of E1 and within the first 91 amino acids of E2. Interestingly, a LexA fusion protein that included amino acid residues 53 to 161 of BPV E2 demonstrated transcriptional activation in this system. In vitro binding assays using combinations of BPV-1 E1-GST fusion proteins and BPV-1 E2 expressed by in vitro translation confirmed the observations from the yeast system; a GST fusion protein containing the first 222 amino acids of BPV-1 E1 bound specifically to full-length BPV-1 E2 in vitro. Furthermore, E1(1-222)-GST bound to forms of E2 deleted of the carboxy-terminal DNA binding-dimerization domain, suggesting that E2 dimerization is not required for this interaction. Finally, in vitro interaction between E1-GST and E2 was observed at 22°C but not at 4°C.

The papillomavirus E1 and E2 open reading frames encode proteins important in the regulation of viral transcription and DNA replication. The bovine papillomavirus type 1 (BPV-1) E1 gene product, a 68-kDa nuclear phosphoprotein, is essential for papillomavirus DNA replication and binds directly to the viral DNA replication origin (50). The BPV-1 E1 protein, like simian virus 40 large T antigen, has ATPase activity and DNA helicase activity and associates with DNA polymerase alpha. These activities, as well as sites of threonine and serine phosphorylation on BPV-1 E1, have been mapped (24, 35, 40, 45, 53). E1-mediated, ATP hydrolysis-dependent structural distortion of the viral origin in vitro has also been recently reported (12). Although E1 has been most extensively studied in the BPV-1 system, the E1 protein of human papillomavirus type 11 (HPV-11) appears to share many of these features (3).

The transcriptional regulatory properties of the papillomavirus E2 gene product have been widely studied. E2 binding sites are clustered within the long control regions of all papillomaviruses, where they mediate E2-dependent expression of viral early gene products (17, 18, 41, 43, 44). In BPV-1-transformed cells, three E2 polypeptides have been detected. The full-length E2 (E2-TA) is 410 amino acids (aa) in length, with a carboxy-terminal DNA binding-dimerization domain and an amino-terminal domain that is required for transcriptional activation (13, 17, 31–33). Another form of BPV-1 E2, referred to as the repressor (E2-TR), consists of aa 162 to 410, containing the DNA binding-dimerization domain but not the transcriptional activation domain (7, 23). This form of E2 can form homodimers, as well as heterodimers, with full-length E2.

Initial observations that established an essential role for BPV-1 E2 in viral DNA replication attributed this requirement to its function as a transcriptional inducer of viral early gene expression, including expression of E1 (9, 36). However, transient replication assays have shown that the requirement of BPV-1 E2 for origin-dependent replication is independent of its role as a transcriptional activator of other viral genes (49, 51). Similar experiments have indicated a requirement for HPV E1 and E2 gene products in the replication of HPV origin-containing plasmids (5, 6, 8, 46). Recent studies with HPV-1 have demonstrated measurable transient DNA replication in the presence of only E1. However, this replication was greatly stimulated by E2 (15). In addition, it has been shown that the transcriptional activation and replication functions of BPV-1 E2 are genetically separable in transient assays (51).

BPV-1 E1 and E2 coimmunoprecipitate when coexpressed in insect cells (2, 28, 34, 54). In these experiments, BPV-1 E1 could associate with the full-length E2 protein but not with the repressor form of E2, suggesting that the amino-terminal domain of E2 is required for interaction with E1. Further studies have suggested that a BPV-1 E1-E2 interaction occurs between the non- or hypophosphorylated form of E2 and the carboxy terminus of E1 (28), although the specific relevant sites of phosphorylation were not identified. The E1 binding site at the viral origin of replication, as well as E2 binding sites immediately adjacent, are necessary *cis* elements for BPV-1 DNA replication in vivo (27, 42, 48). Several studies have presented data consistent with the notion that the interaction between BPV-1 E2 and E1 facilitates binding of E1 to its cognate site (39, 42, 47, 54). This cooperative binding has been assumed to play an important role in BPV-1 viral DNA replication, although the exact nature of this interaction has not been examined. E2 stimulates BPV-1 DNA replication in vitro (26, 52), and E2 can also alleviate chromatin repression of E1-dependent in vitro BPV-1 DNA replication, suggesting that E2 may play a role in configuration of the papillomavirus origin of replication (26). BPV-1 E2-dependent formation of an E1 replication complex has recently been described. Interestingly, BPV-1 E2 associates transiently with E1 during assembly of the

* Corresponding author. Mailing address: 200 Longwood Ave., Building D2, Room 629, Boston, MA 02115. Phone: (617) 432-2886. Fax: (617) 432-2882.

initiation complex and is absent in a subsequent replication-competent complex that contains multimers of DNA-associated E1 molecules (29). Indeed, the intracellular ratios of E1 and E2 may be an important controlling factor in the status of viral DNA replication.

Several systems for examining protein-protein interactions in *Saccharomyces cerevisiae* have recently become available. These systems, sometimes collectively referred to as the two-hybrid (10) and interaction trap systems, rely upon protein-protein interactions to reconstitute an active DNA binding-transcriptional activation complex, resulting in transcriptional activation in *S. cerevisiae*. This system has the benefit of great sensitivity and offers an in vivo alternative to the present in vitro approaches to the study of protein-protein interactions. Using the two-hybrid system, we have examined BPV-1 E1-E2 binding, in order to more fully document the interaction itself and to further map the domains of BPV-1 E1 and E2 that participate in this interaction. We show that a specific interaction domain resides in the amino-terminal portion of each protein. Furthermore, in vitro binding experiments indicate that the interaction properties observed in the two-hybrid system accurately reflect the properties of in vitro BPV-1 E1-E2 interaction.

MATERIALS AND METHODS

Yeast strains and vectors. *S. cerevisiae* W3031 (*ura3 his3 trp1 ade1 leu2*) and EGY048 (*MAT***a** *ura3 his3 trp1 LEU2*::*LexAop6-LEU2*) were used in this study. EGY048, as well as the plasmid components of the interaction trap system, was kindly provided by Roger Brent (Harvard University). Portions of BPV-1 E1 and E2 were generated by PCR with the appropriate synthetic oligonucleotides and cloned as *Eco*RI-*Sal*I restriction fragments into the pLex202 vector for expression as "bait" fusion proteins to the LexA DNA binding domain (4). The pLex202 vector contains the genes for the first 202 aa of the LexA protein, as well as the yeast *HIS3* gene as a selectable marker and the 2μm origin of replication. Expression of LexA fusion proteins from this vector is driven by the constitutively active yeast ADH1 promoter.

The *lexA* operator reporter vector used in these studies, JK103 (20), contains two high-affinity ColE1 *lexA* binding sites upstream of the *GAL1* promoter, followed by the *lacZ* reporter gene. This vector expresses the yeast *URA3* selectable marker, along with the 2μm origin of replication. (*LEU2*, the auxotrophic marker reporter gene in this system, is integrated into the EGY048 host chromosome and contains six upstream *lexA* operator sites.)

Portions of the BPV-1 E1 open reading frame were cloned into the JG4-5 vector as *Eco*RI-*Xho*I fragments following PCR with the appropriate synthetic oligonucleotides. This vector expresses E1 domains as fusion proteins to the influenza virus hemagglutinin 1 epitope tag (recognized by monoclonal antibody 12CA5), the B42 acidic activation domain (33), and the nuclear localization signal of simian virus 40 large T antigen. The *GAL10* promoter in this vector renders expression of the acidic activator chimeric protein galactose responsive: expression is induced by galactose and repressed by glucose. Vectors were introduced into W3031 or EGY048 by a modification of the lithium acetate technique (11).

Yeast cultures were maintained in SD minimal medium (0.67% [wt/vol] Bacto-yeast nitrogen base, 2% [wt/vol] dextrose) containing the appropriate amino acid supplements, except under induction conditions, when SG minimal medium (0.67% [wt/vol] Bacto-yeast nitrogen base, 2% [wt/vol] galactose, 2% [wt/vol] glycerol) was used. Leucine auxotrophy was determined by assessing growth on parallel minimal SD and SG plates with or without leucine.

β-Galactosidase activation assays. For galactose induction experiments, cells from saturated overnight cultures grown in SD minimal medium were pelleted, washed once with sterile water, and resuspended in the appropriate medium containing galactose at an optical density at 600 nm of 0.2 to 0.3. The cultures were harvested at mid-log phase (optical density at 600 nm, 0.6 to 0.8), and β-galactosidase assays were performed. Assays for β-galactosidase expression in yeast cells were performed as described previously (16). The reported measurements represent three assays of at least two independent transformants.

Detection of BPV-1 E1-E2 complexes in vitro. Fragments of BPV-1 E1 DNA were generated by PCR with the appropriate oligonucleotide primers and cloned as *Eco*RI-*Sal*I fragments into the *Eco*RI-*Xho*I sites of the pGEX-4T-1 vector (Pharmacia). Glutathione *S*-transferase (GST) fusion proteins were induced and harvested as described by Kaelin et al. (19). Approximately 0.5 μg of each E1-GST fusion protein was used in each binding reaction. In E1-GST binding experiments, [^{35}S]methionine-labeled E2 was generated by using the T7-TNT coupled reticulocyte lysate system (Promega). Full-length E2 was generated with the plasmid pCMV-E2 as a template. Linear E2 templates encoding open reading frames for the amino-terminal 262, 161, and 91 aa were generated by PCR with a 5′ oligonucleotide that contained a T7 promoter and an initiation methionine codon in optimal Kozak context. Templates encoding E2 (aa 161 to 410) and E2 with aa 159 to 281 deleted [E2(Δ159-281)] were generated by linearization of plasmids p2423 and p2439, respectively (33). Unless otherwise indicated, E1-GST fusion proteins bound to glutathione Sepharose beads (Pharmacia) were preincubated with agitation for 30 min at room temperature in 0.5 ml of binding buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 2 mM dithiothreitol, 5 mM $MgCl_2$, 0.5% Nonidet P-40) with 2% nonfat dry milk. Aliquots of in vitro translation reaction mixtures containing radiolabeled BPV-1 E2 or RPA3 protein were then added, and incubation was continued at room temperature for 30 min. After three 0.5-ml washes in binding buffer without milk, the samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Radiolabeled bands were visualized by autoradiography and quantitated by phosphorimager analysis, using a GS-250 Molecular Image Analyzer (Bio-Rad). In temperature sensitivity experiments, preincubation, binding reactions, and washes were carried out at the temperatures indicated. Bound proteins were detected by autoradiography and quantitated by phosphorimager analysis (Bio-Rad).

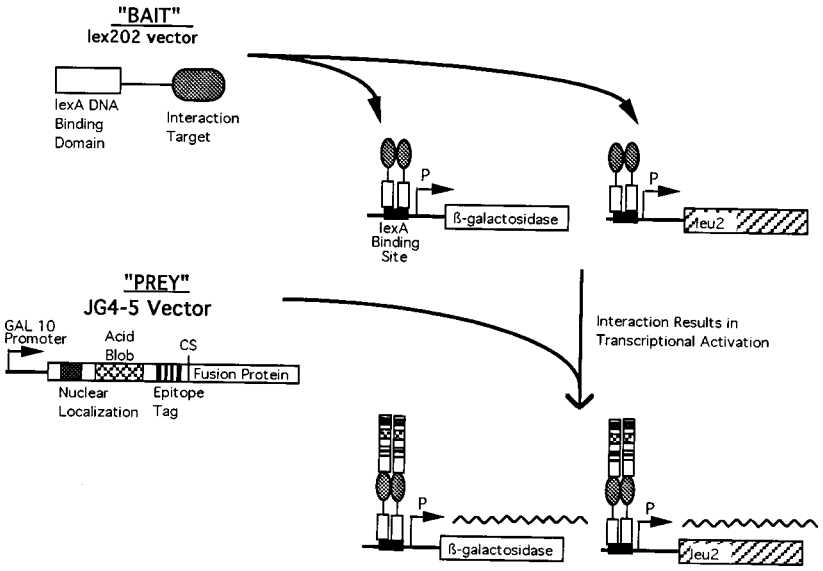
RESULTS

Transcriptional activation by E2-LexA chimeric proteins. Several yeast systems have been developed to search for and characterize intermolecular protein interactions (10, 55). The system used in our examination of BPV-1 E1-E2 interactions was developed in the Brent laboratory (20, 55). As depicted in Fig. 1, this system employs coexpression of two chimeric proteins in yeast cells, one of which is expressed from the pLex202 vector as a fusion protein to the DNA binding domain of the sequence-specific DNA-binding protein LexA (14, 20). This fusion protein, called the bait, binds as a dimer to operator sequences positioned within the promoters of two reporter genes, the *Leu2* auxotrophic marker (integrated into the yeast genome) and the β-galactosidase gene (located on the yeast vector JK103). For the two-hybrid approach to work, this LexA fusion protein must have no intrinsic transcriptional activation capacity. Transcriptional activation of the reporter genes relies upon a second chimeric protein fused to an acidic activation domain (30). This second protein, called the prey, is expressed in a galactose-dependent manner from the JG4-5 vector. Chimeric proteins from this vector also contain a nuclear localization signal and a hemagglutinin epitope tag. If the LexA and JG4-5 activation domain fusion proteins are drawn into a complex through the interaction of the two chimeric components, the reporter genes will be activated, resulting in β-galactosidase activity and leucine auxotrophy.

As stated above, any LexA fusion protein is useful as bait only if it has no intrinsic transcriptional activation activity in yeast cells. Portions of the BPV-1 E2 transcription activation domain were cloned into the pLex202 vector and evaluated for their utility as bait in the two-hybrid system. Figure 1B shows that, compared with the LexA DNA binding domain alone, LexA fusion proteins containing aa 1 to 91 or 161 to 268 of BPV-1 E2 had no transcriptional activation capacity. However, the BPV-1 E2/LexA fusion protein containing E2 (aa 53 to 161), a central portion of the previously described BPV-1 E2 transcription activation domain, exhibited significant transcriptional properties in this context, making it unsuitable as bait in this system. In addition, although BPV-1 E1 has no known transcriptional activation capacity, segments of this protein were also evaluated for transactivation activity when expressed as LexA fusion proteins. As expected, none of the segments of E1 tested exhibited any transcriptional activation activity. Comparable levels of expression of all LexA fusion proteins in yeast cells were confirmed by Western blot (immunoblot) analysis with antibodies to the LexA DNA binding domain (data not shown).

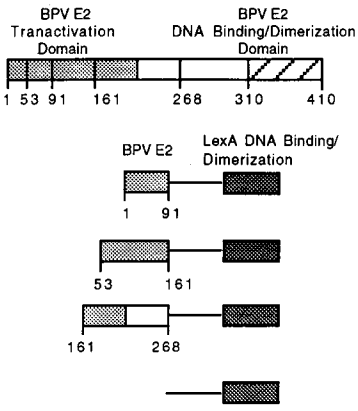
Interaction of E1 with the amino terminus of E2. The domains of E2 that did not transactivate when fused to the LexA

A.



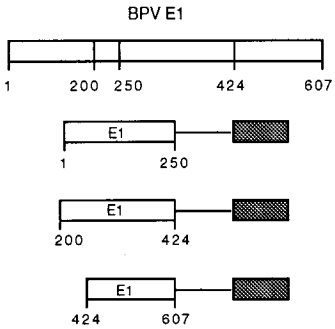
B.

E2 BAITS



	Transactivation (ß-galactosidase units)	Leucine Auxotrophy
1–91	0.556	-
53–161	72.4	+
161–268	0.738	-
	0.459	-

E1 BAITS



1–250	0.453	-
200–424	0.468	-
424–607	0.405	-

FIG. 1. (A) The yeast interaction trap system. Protein domains to be used as interaction targets, or bait, are expressed as fusions to the LexA DNA binding domain, allowing them to bind to *lexA* operator sequences located upstream of either the β-galactosidase reporter gene or the integrated yeast *LEU2* gene. The JG4-5 prey vector expresses a chimeric protein that includes the simian virus 40 T antigen nuclear localization domain, an acidic transcriptional activation domain, and the hemagglutinin 12CA5 epitope tag. Protein expression from the JG4-5 vector is induced by galactose. Interaction between LexA fusion proteins and proteins expressed from the JG4-5 vector results in transcriptional induction of the β-galactosidase and *LEU2* reporter genes. CS, cloning site; P, promoter. (B) Transcriptional activation phenotypes of E1 and E2 LexA chimeric bait constructs. The indicated regions of BPV-1 E1 and E2 were cloned into the pLex202 vector and transformed into EGY048, along with the JK103 reporter vector. EGY048 contains a genomic copy of the *LEU2* auxotrophic marker located downstream of six *lexA* binding sites. JK103 contains two *lexA* binding sites located upstream of the β-galactosidase reporter gene. Transcriptional activation by E1 or E2 LexA fusion proteins results in induction of β-galactosidase and *LEU2* expression, as determined by measurement of β-galactosidase activity and by assessment of growth on plates without leucine (leucine auxotrophy), respectively.

DNA binding domain could serve as bait for screening in the two-hybrid system. Interactions between these E2/LexA fusion proteins and portions of E1 were therefore tested (Fig. 2). Portions of BPV-1 E1 were expressed as fusion proteins in the JG4-5 vector. Interaction between the BPV-1 E2/LexA bait constructs and E1 domains expressed from the JG4-5 vector would be observed as galactose-dependent leucine auxotrophy and concomitant induced expression of the β-galactosidase reporter gene. BPV-1 E1 (aa 1 to 250) scored for interaction with the LexA fusion protein containing 1 to 91 of E2 but not for interaction with a LexA fusion containing aa 161 to 262 of E2. This activation phenotype was dependent upon growth in galactose-containing medium. No other portion of E1 scored for association with BPV-1 E2 (aa 1 to 91), BPV-1 E2 (aa 161 to 262), BPV-1 E1 (aa 200 to 424), or the LexA protein in this assay. This interaction appeared to be specific, since neither this nor any other domain of BPV-1 E1 tested appeared to interact with E1(200-424)/LexA or with a variety of other irrelevant bait constructs (data not shown). Galactose-induced expression of E1 fusion proteins from the JG4-5 vector was confirmed by Western blot analysis with the monoclonal antibody 12CA5 (data not shown). Thus, we conclude that domains sufficient to mediate intermolecular association between E1 and E2 of BPV-1 in the two-hybrid assay are contained within the first 91 aa of E2 and the first 250 aa of E1.

Interaction between portions of E1 and full-length E2. To confirm that native, full-length BPV-1 E2 protein was also capable of specific interaction with the amino-terminal 250 aa of E1 in a yeast expression system, an alternative interaction trap strategy that exploits the intrinsic transactivation activity of full-length BPV-1 E2 in yeast cells was used (21). This strategy is depicted schematically in Fig. 3A. In these experiments, portions of BPV-1 E1 expressed as LexA fusion proteins were used as bait, and the full-length E2 protein, containing its own transactivation domain, was used as the prey to assay E1-E2 interactions. Thus, E1/LexA chimeric proteins may bind to *lexA* operator sequences within the reporter vector, but β-galactosidase activity depends upon interaction between E1 molecules and E2 expressed from the separate vector pPD2 (21). The interaction phenotype was scored by measuring transcriptional activation of β-galactosidase from the target plasmid JK103. As shown in Fig. 3B, full-length E2 interacts with the E1 bait containing only the first 250 aa of E1 but not with bait constructs containing other portions of E1.

The level of β-galactosidase induction by E1(1-250)/LexA interaction with E2 was relatively modest. This was possibly due to limiting intracellular E2 levels. The yeast PHO5 promoter, which was used to drive BPV-1 E2 expression in these experiments, is phosphate repressed, exhibiting limited activity in normal yeast growth medium. Because the yeast pPD2 vector used for BPV-1 E2 expression in these experiments is maintained at an unusually high copy number (up to 100 copies per cell), high levels of BPV-1 E2 expression may be achieved in growth medium depleted of phosphate. The experiment whose results are shown in Fig. 3B was conducted in the presence of normal medium, under phosphate conditions that permit only limited (but detectable) expression of BPV-1 E2. Interestingly, superinduction of BPV-1 E2 expression by growth in phosphate-depleted medium appeared to be toxic when accompanied by expression of the BPV-1 E1(1-250)/LexA fusion protein, resulting in large clumps of dead cells in less than 6 h (data not shown). No severe toxicity was observed

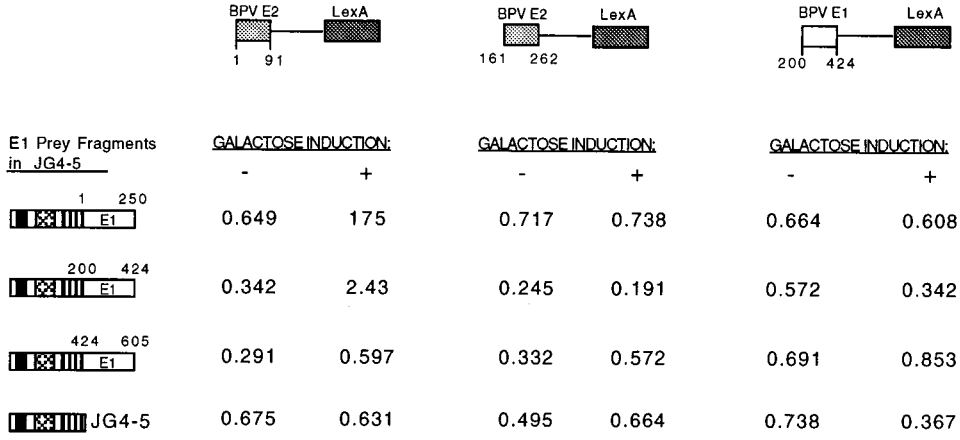
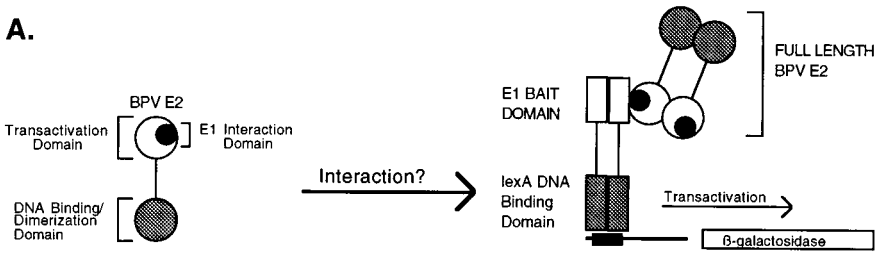


FIG. 2. Interaction between BPV-1 E1 and E2. LexA fusion proteins containing BPV-1 E2 aa 1 to 91 or 161 to 262 were used as bait to detect interaction with portions of BPV-1 E1 in EGY048. A LexA fusion bait containing aa 200 to 424 of BPV-1 E1 was used as a negative control. The indicated segments of BPV-1 E1 were expressed as prey from the JG4-5 vector. The JK103 β-galactosidase reporter vector was used in these assays. +, with galactose; −, without galactose. Galactose induces E1 domain fusion protein expression from the JG4-5 vector. β-Galactosidase activities were determined from mid-log-phase cultures under the growth conditions indicated.



B.

	lexA BAIT	E1 aa 1-250 lexA	E1 aa 200-424 lexA	E1 aa424-605 lexA
pPD2	1.31	0.908	1.01	1.19
pPD2-E2	1.17	6.40	0.854	1.11

FIG. 3. Use of E1 bait to test interaction with full-length BPV-1 E2. (A) Segments of BPV-1 E1 were expressed as LexA fusion proteins for use as bait for interaction with full-length BPV-1 E2. BPV-1 E2, which contains distinct transcriptional activation and sequence-specific DNA binding domains, was expressed from a separate vector, pPD2-E2. The JK103 *lexA* operator–β-galactosidase reporter vector, in combination with various E1/LexA bait constructs, was used to determine which portions of BPV-1 E1 could recruit full-length E2 and sponsor transcriptional activation in the yeast strain W3031. Such activation indicates interaction between the E1 fusion protein and full-length BPV-1 E2. (B) β-Galactosidase activities from yeast coexpressing various BPV-1 E1/LexA bait proteins and full-length BPV-1 E2. LexA fusion proteins including aa 1 to 250, 200 to 424, or 424 to 605 of BPV-1 E1 were tested for interaction with full-length BPV-1 E2 expressed from pPD2-E2.

with expression of either protein separately. Interestingly, this toxicity was only observed in cases of coexpression of E2 and the amino terminus of E1 with which E2 associates. Coexpression of full-length E2 with BPV-1 E1(200-424)/LexA, E1(424-607)/LexA, or LexA alone did not yield this phenotype in phosphate-depleted medium. Since the E1 fusion protein expression in these experiments is constitutively high (expression is driven off the ADH1 promoter), it is possible that E1-E2 complexes accumulate at high intracellular levels in phosphate-depleted cultures, leading to the observed toxicity. However, even with low levels of native E2 expression from the PHO5 promoter in the presence of phosphate, it is clear that full-length E2 scores for interaction only with the E1 bait including the first 250 aa of E1.

Analysis of BPV-1 E1-E2 interactions in vitro. To confirm and extend the results described for experiments using the two-hybrid system, as well as the previously described systems, domains of BPV-1 E1 were cloned into the pGEX-4T-1 vector for expression as GST fusion proteins in *Escherichia coli*. These proteins were purified by binding to glutathione beads, and their abilities to bind in vitro-translated, radiolabeled BPV-1 E2 were determined. Figure 4 shows that GST fusion proteins containing either the first 220 or 250 aa of BPV-1 E1 were able to bind BPV-1 E2. In contrast, the GST fusion proteins containing aa 1 to 128, 200 to 424, or 424 to 607 of BPV-1 E1 had only a background level of binding to E2. The specificity of binding in these experiments is documented by the fact that RPA3, a protein with no known E1 binding activity and with no such activity in two-hybrid experiments (data not shown), did not bind any of the E1-GST fusion proteins tested. Since E1(1-128)-GST did not bind BPV-1 E2 in vitro, it would appear that this portion of E1 is not sufficient for E2 association. However, the domain of BPV-1 E1 between aa 120 and 350 could not be directly tested, since a GST fusion protein expressing this portion of E1 was not soluble.

The BPV-1 E1-GST fusion proteins were also used to confirm the location of E1 interaction domains within BPV-1 E2 (Fig. 5). E1(1-222)-GST bound to truncated forms of BPV-1 E2 that encompassed the first 262, 161, or 91 aa of E2, confirming that the amino-terminal 91-aa domain of E2 is sufficient for E1 interaction. In contrast, E1(1-222)-GST did not bind to BPV-1 E2 proteins bearing deletions of the amino-terminal 52 or 161 aa. Deletion of the hinge region of BPV-1 E2(Δ159-281) did not abolish its ability to bind E1(1-222)-GST. Indeed, the level of binding by this form of E2 appeared to be significantly higher than that of full-length E2.

We have observed that several forms of E2 are generated by in vitro transcription and translation of the BPV-1 E2 templates used in these experiments. The E2-TR form is indicated in Fig. 4 and 6; an intermediate form that migrates between full-length E2 and E2-TR is the result of internal initiation at Met-71 of BPV-1 E2. Data from both the yeast two-hybrid and in vitro binding experiments indicate that the domain of BPV-1 E2 sufficient for interaction with BPV-1 E1 lies within the E2 amino terminus. This domain is not present in the E2-TR protein. Nevertheless, E2-TR is coexpressed and forms heterodimers with full-length E2 when generated by in vitro translation reactions. Therefore, we believe that this apparent ability of E1(1-222)-GST to bind E2-TR is a consequence of interaction between E1 (aa 1-222) and heterodimers of E2-TR and full-length E2. It is possible that the E2 (aa 71-410) species binds E1-GST either because it too is represented as a heterodimer with full-length E2 or because it contains a region downstream of residue 71 (and presumably upstream of residue 91) that is capable of E1 interaction. We have not carried out experiments to distinguish between these possibilities.

Interaction between the amino termini of BPV-1 E1 and E2 is cold sensitive. In previous assays examining the ability of BPV-1 E2 to enhance E1 binding to the BPV-1 origin of replication in vitro, Thorner et al. observed that such enhancement is cold sensitive (47). That study also suggested that the domain of E1 that interacts with E2, as well as the DNA

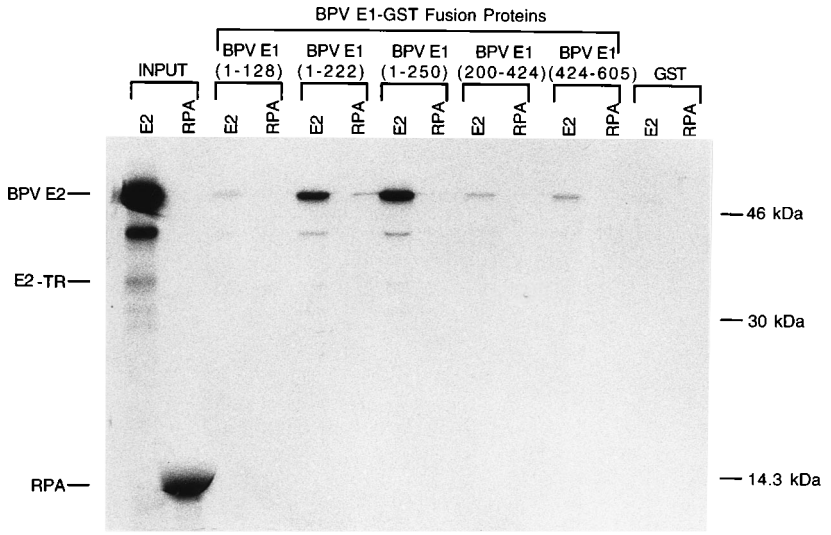


FIG. 4. Mapping the BPV-1 E2 interaction domain of E1 in vitro. The indicated domains of BPV-1 E1 were expressed and purified from *E. coli* as GST fusion proteins. [^{35}S]methionine-labeled full-length E2 was generated in vitro from linear T7 promoter-containing E2 templates by transcription and translation, using the T7-TNT reticulocyte lysate system. The full-length labeled BPV-1 E2 product, along with the E2-TR product (due to internal initiation), is indicated. Binding reactions were conducted at room temperature as described in Materials and Methods. The labeled 14-kDa subunit of human RPA, generated by T7-mediated transcription and translation, was used as a negative control in these experiments. BPV-1 E2 and RPA bands were visualized by autoradiography and quantitated by phosphorimager analysis.

binding domain of E1, is located within the amino-terminal two-thirds of E1, consistent with the mapping data presented here. However, these assays did not directly address whether E1-E2 interaction per se was affected by low temperature or whether the ability of BPV-1 E1 to bind DNA was somehow altered or impaired at the lower temperature. In order to address these issues, we examined the ability of the BPV-1 E1(1-222)-GST fusion protein to interact with full-length BPV-1 E2 at room temperature and at 4°C (Fig. 6). The results of this experiment show that E1-E2 interaction is significantly decreased at 4°C, relative to the level of interaction observed at room temperature. We conclude that cold sensitivity of E1-E2 interaction may have contributed to previously observed temperature phenotypes with respect to E2 enhancement of E1 DNA binding. Furthermore, this result suggests that the activities of the E1-GST fusion protein used in these experiments at least partially reflect the properties of the full-length native E1 protein.

DISCUSSION

We have used the yeast interaction trap system to characterize interactions between domains of the BPV-1 E1 and E2 proteins. Using a combination of BPV-1 E1-GST fusion proteins and various forms of radiolabeled BPV-1 E2 generated by in vitro translation, we have also examined this interaction in vitro. Results of experiments using both systems indicate that domains sufficient to mediate interaction between BPV-1 E1 and E2 lie within the first 91 aa of E2 and within the first 222 to 250 aa of E1. The BPV-1 E1 domain from 1 to 222 falls within a region of E1 previously shown to be required for E2-dependent enhancement of E1 DNA binding (47). In fact, it would appear that the E2 interaction domain of E1 overlaps or is closely adjacent to the DNA binding, DNA polymerase alpha binding, and nuclear localization domains of BPV-1 E1 (24, 35). Furthermore, since interaction was observed between BPV-1 E1 and forms of BPV-1 E2 encompassing only its first 91 aa, it appears that E2 dimerization is not a prerequisite for interaction with E1. Indeed, BPV-1 E1 may be capable of interaction with heterodimers of full-length BPV-1 E2 and E2-TR.

In adapting the two-hybrid system for examination of E1-E2 interactions, it was necessary to determine which domains of these proteins could be used as bait. These evaluations revealed that a LexA fusion protein containing aa 53 to 161 of BPV-1 E2 had an intrinsic transcriptional activation capacity in yeast cells, suggesting the possible presence of a discrete transcription activation function within this region of BPV-1 E2. In contrast, previous deletion studies that removed aa 1 to 52, 53 to 161, or 157 to 263 of BPV-1 E2 all abolished its transcriptional activation activity both in yeast cells and in higher eukaryotes (13, 21, 31, 32), leading to the suggestion that the BPV-1 E2 activation domain is very large and/or very complex. Whether the domain from aa 53 to 161 represents a heretofore unappreciated discrete transactivation domain of BPV-1 E2 or provides spurious transactivation in the context of a LexA DNA binding moiety will need to be investigated further. To that end, it will also be interesting to evaluate the transcriptional activation activity of these BPV-1 E2/LexA fusion proteins in mammalian cells, in order to address the possibility that the three BPV-1 E2 domains used in these yeast assays might have different functional properties in higher and lower eukaryotes.

Previous coimmunoprecipitation studies have implied that interaction between BPV-1 E1 and E2 is mediated through the carboxy terminus of E1 (28). These experiments were carried out using baculovirus expression systems to coexpress E1 and E2 in insect cells, and interactions were detected by coimmunoprecipitation of BPV-1 E1 and E2. In contrast, mapping studies by Thorner et al. have suggested that the E2 interaction domain of E1, vis-à-vis the ability of E2 to stimulate E1 origin binding, does not require this carboxy-terminal portion of BPV-1 E1 (47). It is possible that expression of the E1 carboxy terminus as a fusion protein in our assays altered its activity. Since we were unable to express full-length BPV-1 E1 in yeast cells, it was not possible to compare the relative E2 binding by

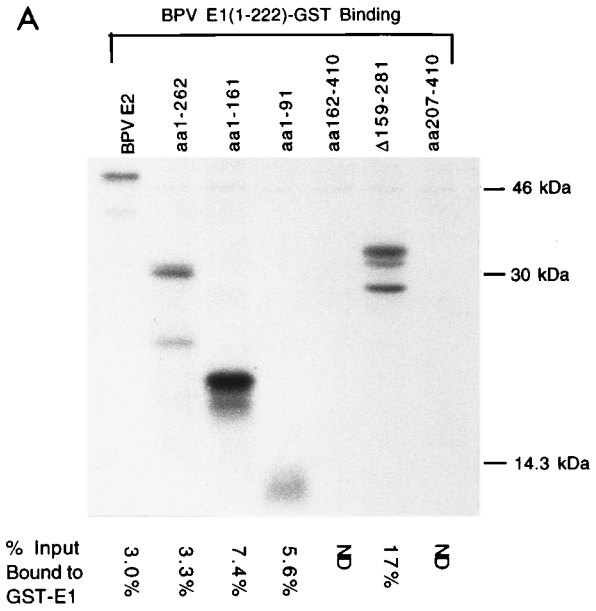


FIG. 5. Mapping of the BPV-1 E2 domain that interacts with E1 in vitro. A BPV-1 E1-GST fusion protein containing aa 1 to 222 was tested for binding to various deleted and truncated forms of BPV-1 E2 expressed as [^{35}S]methionine-labeled proteins in reticulocyte lysates. (A) E1(1-222)-GST-associated E2 species; (B) aliquots of the E2 in vitro translation products. Percent binding (A) is the percentage of total input E2 that associated with E1(1-222)-GST in the in vitro binding reaction. All binding reactions were performed at room temperature. BPV-1 E2 bands were visualized by autoradiography and quantitated by phosphorimager analysis. ND, not determined.

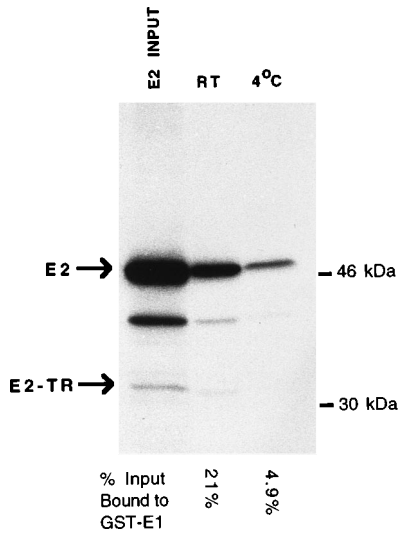


FIG. 6. In vitro interaction of BPV-1 E1 and E2 is cold sensitive. Binding reactions of BPV-1 E1(1-222)-GST to [^{35}S]methionine-labeled full-length BPV-1 E2 were conducted as described in the legends to Fig. 4 and 5, except that preincubation, binding reactions, and washes were performed at the temperatures indicated. BPV-1 E2 bands were visualized by autoradiography and quantitated by phosphorimager analysis. RT, room temperature.

full-length E1 with that of E1 subfragments, using the two-hybrid system. Furthermore, we observed nonspecific protein binding by the carboxy terminus of BPV-1 E1 in vitro in experiments conducted at 4°C (1). On the basis of these previously published results, we cannot rule out the possibility that BPV-1 E1-E2 interactions involve the carboxy terminus of BPV-1 E1. However, in our assays, interaction at physiologically relevant temperatures (i.e., 30°C or above) required only the BPV-1 E1 amino terminus. The carboxy terminus of E1 may also play a contributory role in E1-E2 complex formation in vivo, in the context of the papillomavirus origin of replication, or in other types of assays.

The observed temperature modulation of BPV-1 E1-E2 interaction may be an indication of important conformational changes in these proteins. Such temperature-mediated protein-protein interactions may modulate certain physiologic aspects of the viral life cycle, perhaps regulating a switch from nonvegetative DNA replication at warmer lower layers of epithelium to vegetative DNA replication at the cooler surface of skin. A number of studies have suggested a role for BPV-1 E1 in the control of viral transcription (22, 37, 38), suggesting the possibility that modulation of E1-E2 interaction plays a role in controlling expression of papillomavirus gene products. Thus, the status of E1-E2 complexes in the cell may modulate both viral gene expression and DNA replication.

The exact roles of E2 and E1 in replication, as well as that of the complex in which these proteins participate, are not yet clear. Sufficient amounts of E1 alleviate the requirement of E2 for in vitro BPV-1 DNA replication. However, E2 greatly stimulates replication in such experiments (26). Although a variety of experiments have suggested a role for E1-E2 interaction in papillomavirus DNA replication, this hypothesis has not been formally tested. Furthermore, E2 may have replication functions independent of its ability to interact with E1, such as recruitment of replication accessory proteins like RPA (25). Alternatively, the physiologic role of E2 may be to properly configure chromatin in the origin region into an E1-binding or replication-competent state (26). E2 may also act to ensure origin-specific viral DNA replication by properly positioning E1 at the origin. The identification of specific interaction domains within each protein may aid in the design of discrete mutations in each protein that effect only E1-E2 interaction, an important step in allowing such questions to be addressed. Once the actual role of E1-E2 interaction in papillomavirus replication is better understood, it may be possible to adapt interaction detection systems like the two-hybrid screen to test compounds for their ability to disrupt this complex in vivo.

ACKNOWLEDGMENTS

We thank Toshiharu Yasugi for helpful discussions. Plasmids pPD2-E2, p2423, and p2439 were provided by Alison McBride. We thank J.-M. Gauthier for the pCMV-E2 plasmid. RPA3 was a gift of Anindya Dutta. Components of the two-hybrid system and antibodies to the DNA binding domain of LexA were generously provided by Roger Brent. We also thank Paul Lambert and Alison McBride for comments on the manuscript.

Portions of this research were supported by a postdoctoral fellowship from the Damon Runyon-Walter Winchell Cancer Research Fund (DRG 1065) to J.D.B. and by a Sponsored Research Agreement to Harvard University from the Terumo Corporation of Japan.

REFERENCES

1. **Benson, J.** Unpublished results.
2. **Blitz, I. L., and L. A. Laimins.** 1991. The 68-kilodalton E1 protein of bovine papillomavirus is a DNA-binding phosphoprotein which associates with the E2 transcriptional activator in vitro. J. Virol. **65:**649–656.
3. **Bream, G. L., C.-A. Ohmstede, and W. C. Phelps.** 1993. Characterization of human papillomavirus type 11 E1 and E2 proteins expressed in insect cells. J. Virol. **67:**2655–2663.
4. **Brent, R., and M. Ptashne.** 1985. A eukaryotic transcriptional activator bearing the DNA specificity of a procaryotic repressor. Cell **43:**729–736.
5. **Chiang, C.-M., G. Dong, T. M. Broker, and L. T. Chow.** 1992. Control of human papillomavirus type 11 origin of replication by the E2 family of transcription regulatory proteins. J. Virol. **66:**5224–5231.
6. **Chiang, C.-M., M. Ustav, A. Stenlund, T. F. Ho, T. R. Broker, and L. T. Chow.** 1992. Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. Proc. Natl. Acad. Sci. USA **89:**5799–5803.
7. **Choe, J., P. Vaillancourt, A. Stenlund, and M. Botchan.** 1989. Bovine papillomavirus type 1 encodes a transcriptional repressor: structural and functional analysis of new viral cDNAs. J. Virol. **63:**1743–1755.
8. **DelVecchio, A., H. Romanczuk, P. Howley, and C. Baker.** 1992. Transient DNA replication of human papillomavirus. J. Virol. **66:**5949–5958.
9. **DiMaio, D., and J. Settleman.** 1988. Bovine papillomavirus mutant temperature sensitive for transformation, replication, and transactivation. EMBO J. **7:**1197–1204.
10. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature (London) **340:**245–246.
11. **Gietz, D., A. St. Jean, R. A. Woods, and R. H. Schietstl.** 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20:**1425.
12. **Gillette, T. G., M. Lusky, and J. A. Boroweic.** 1994. Induction of structural changes in the bovine papillomavirus type 1 origin of replication by the viral E1 and E2 proteins. Proc. Natl. Acad. Sci. USA **91:**8846–8850.
13. **Giri, I., and M. Yaniv.** 1988. Structural and mutational analysis of E2 transacting proteins of papillomaviruses reveals three distinct functional domains. EMBO J. **7:**2823–2829.
14. **Golemis, E. A., and R. Brent.** 1992. Fused protein domains inhibit DNA binding by LexA. Mol. Cell. Biol. **12:**3006–3014.
15. **Gopalakrishnan, V., and S. A. Khan.** 1994. E1 protein of human papillomavirus type 1a is sufficient for initiation of viral DNA replication. Proc. Natl. Acad. Sci. USA **91:**9597–9601.
16. **Guarante, L.** 1983. Yeast promoters and lacZ fusions designed to study expression of cloned genes in yeast. Methods Enzymol. **101:**181–193.
17. **Haugen, T. H., L. P. Turek, F. M. Mercurio, T. P. Cripe, B. J. Olson, R. D. Anderson, D. Seidl, M. Karin, and J. Schiller.** 1988. Sequence specific and general transcriptional activation by the bovine papillomavirus E2-transactivator require an N-terminal amphipathic helix-containing E2 domain. EMBO J. **7:**4245–4253.
18. **Hermonat, P. L., B. A. Spalholz, and P. M. Howley.** 1988. The bovine papillomavirus P_{2443} promoter is E2 trans-responsive: evidence for E2 autoregulation. EMBO J. **7:**2815–2822.
19. **Kaelin, W. G., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, and M. A. Blanar.** 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell **70:**351–364.
20. **Kamens, J., and R. Brent.** 1991. A yeast transcription assay defines distinct rel and dorsal DNA recognition sequences. New Biol. **3:**1005–1013.
21. **Lambert, P. F., N. Dostatni, A. A. McBride, M. Yaniv, and P. M. Howley.** 1989. Functional analysis of the papilloma virus E2 transactivator in Saccharomyces cerevisiae. Genes Dev. **3:**38–48.
22. **Lambert, P. F., and P. M. Howley.** 1988. Bovine papillomavirus type 1 E1 replication-defective mutants are altered in their transcriptional regulation. J. Virol. **62:**4009–4015.
23. **Lambert, P. F., B. A. Spalholz, and P. M. Howley.** 1987. A transcriptional repressor encoded by BPV-1 shares a common carboxy-terminal domain with the E2 transactivator. Cell **50:**69–78.
24. **Lentz, M. R., D. Pak, I. Mohr, and M. R. Botchan.** 1993. The E1 replication protein of bovine papillomavirus type 1 contains an extended nuclear localization signal that includes a $p34^{cdc2}$ phosphorylation site. J. Virol. **67:**1414–1423.
25. **Li, R., and M. R. Botchan.** 1993. The acidic transcriptional activator domains of VP16 and p53 bind the cellular replication protein A and stimulate in vitro BPV-1 DNA replication. Cell **73:**1207–1221.
26. **Li, R., and M. R. Botchan.** 1994. Acidic transcription factors alleviate nucleosome-mediated repression of DNA replication of bovine papillomavirus type 1. Proc. Natl. Acad. Sci. USA **91:**7051–7055.
27. **Lu, J. Z.-J., Y.-N. Sun, R. C. Rose, W. Bonnez, and D. J. McCance.** 1993. Two E2 binding sites (E2BS) alone or one E2BS plus an A/T-rich region are minimal requirements for the replication of the human papillomavirus type 11 origin. J. Virol. **67:**7131–7139.
28. **Lusky, M., and E. Fontane.** 1991. Formation of the complex of bovine papillomavirus E1 and E2 proteins is modulated by E2 phosphorylation and depends upon sequences within the carboxyl terminus of E1. Proc. Natl. Acad. Sci. USA **88:**6363–6367.
29. **Lusky, M., J. Hurwitz, and Y.-S. Seo.** 1994. The bovine papillomavirus E2 protein mediates the assembly of but is not maintained in a replication-competent multimeric E1-replication origin complex. Proc. Natl. Acad. Sci. USA **91:**8895–8899.
30. **Ma, J., and M. Ptashne.** 1987. A new class of yeast transcriptional activators. Cell **51:**113–119.
31. **McBride, A. A., J. C. Byrne, and P. M. Howley.** 1989. E2 polypeptides encoded by bovine papillomavirus type 1 form dimers through the common carboxyl-terminal domain: transactivation is mediated by the conserved amino-terminal domain. Proc. Natl. Acad. Sci. USA **86:**510–514.
32. **McBride, A. A., H. Romanczuk, and P. M. Howley.** 1991. The papillomavirus E2 regulatory proteins. J. Biol. Chem. **266:**18411–18414.
33. **McBride, A. A., R. Schlegel, and P. M. Howley.** 1988. The carboxy-terminal domain shared by the bovine papillomavirus transactivator and repressor proteins contains a specific DNA binding activity. EMBO J. **7:**533–539.
34. **Mohr, I. J., R. Clark, S. Sun, E. J. Androphy, P. MacPherson, and M. R. Botchan.** 1990. Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. Science **250:**1694–1699.
35. **Park, P., W. Copeland, L. Yang, T. Wang, M. R. Botchan, and I. J. Mohr.** 1994. The cellular DNA polymerase α-primase is required for papillomavirus DNA replication and associates with the viral E1 helicase. Proc. Natl. Acad. Sci. USA **91:**8700–8704.
36. **Rabson, M. S., C. Yee, Y. C. Yang, and P. M. Howley.** 1986. Bovine papillomavirus type 1 3′ early region transformation and plasmid maintenance functions. J. Virol. **60:**626–634.
37. **Sandler, A. B., S. B. Vande Pol, and B. A. Spalholz.** 1993. Repression of bovine papillomavirus type 1 transcription by the E1 replication protein. J. Virol. **67:**5079–5087.
38. **Schiller, J., E. Kleiner, E. Androphy, D. Lowy, and H. Pfister.** 1989. Identification of bovine papillomavirus E1 mutants with increased transforming and transcriptional activity. J. Virol. **63:**1775–1782.
39. **Seo, Y. S., F. Müller, M. Lusky, E. Gibbs, H. Y. Kim, B. Phillips, and J. Hurwitz.** 1993. Bovine papilloma virus (BPV)-encoded E2 protein enhances binding of E1 protein to the BPV replication origin. Proc. Natl. Acad. Sci. USA **90:**2865–2869.
40. **Seo, Y.-S., F. Müller, M. Lusky, and J. Hurwitz.** 1993. Bovine papilloma virus (BPV)-encoded E1 protein contains multiple activities required for DNA replication. Proc. Natl. Acad. Sci. USA **90:**702–706.
41. **Spalholz, B. A., P. F. Lambert, C. L. Lee, and P. M. Howley.** 1987. Bovine papillomavirus transcriptional regulation: localization of the E2-responsive elements of the long control region. J. Virol. **61:**2128–2137.
42. **Spalholz, B. A., A. A. McBride, T. Sarafi, and J. Quintero.** 1993. Binding of bovine papillomavirus E1 to the origin is not sufficient for DNA replication. Virology **193:**201–212.
43. **Spalholz, B. A., Y.-C. Yang, and P. M. Howley.** 1985. Transactivation of a bovine papillomavirus transcriptional regulatory element by the E2 gene product. Cell **42:**183–191.
44. **Stenlund, A., G. L. Bream, and M. R. Botchan.** 1987. A promoter with an internal regulatory domain is part of the origin of replication in BPV-1. Science **236:**1666–1671.
45. **Sun, S., L. Thorner, M. Lentz, P. MacPherson, and M. Botchan.** 1990. Identification of a 68-kilodalton nuclear ATP-binding phosphoprotein encoded by bovine papillomavirus type 1. J. Virol. **64:**5093–5105.
46. **Sverdrup, F., and S. A. Khan.** 1994. Replication of human papillomavirus (HPV) DNAs supported by the HPV type 18 E1 and E2 proteins. J. Virol. **68:**505–509.
47. **Thorner, L. K., D. A. Lim, and M. R. Botchan.** 1993. DNA-binding domain of bovine papillomavirus type 1 E1 helicase: structural and functional aspects. J. Virol. **67:**6000–6014.
48. **Ustav, E., M. Ustav, P. Szymanski, and A. Stenlund.** 1993. The bovine papillomavirus origin of replication requires a binding site for the E2 tran-

scriptional activator. Proc. Natl. Acad. Sci. USA **90:**898–902.
49. **Ustav, M., and A. Stenlund.** 1991. Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. EMBO J. **10:**449–457.
50. **Wilson, V. G., and J. Ludes-Meyers.** 1991. A bovine papillomavirus E1-related protein binds specifically to bovine papillomavirus DNA. J. Virol. **65:**5314–5322.
51. **Winokur, P., and A. A. McBride.** 1992. Separation of the transcriptional activation and replication functions of the bovine papillomavirus-1 E2 protein. EMBO J. **11:**4111–4118.
52. **Yang, L., R. Li, I. J. Mohr, R. Clark, and M. R. Botchan.** 1991. Activation of BPV-1 replication in vitro by the transcription factor E2. Nature (London) **353:**628–633.
53. **Yang, L., I. Mohr, E. Fouts, D. A. Lim, M. Nohaile, and M. Botchan.** 1993. The E1 protein of the papillomavirus BPV-1 is an ATP dependent DNA helicase. Proc. Natl. Acad. Sci. USA **90:**5086–5090.
54. **Yang, L., I. Mohr, R. Li, T. Nottoli, S. Sun, and M. R. Botchan.** 1991. Transcription factor E2 regulates BPV-1 DNA replication in vitro by direct protein-protein interaction. Cold Spring Harbor Symp. Quant. Biol. **56:**335–346.
55. **Zervos, A. S., J. Gyuris, and R. Brent.** 1993. Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell **72:**223–232.