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# Interaction of the Papillomavirus Transcription/Replication Factor, E2, and the Viral Capsid Protein, L2

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The minor capsid protein L2 of papillomaviruses (PVs) likely plays a role in the selective encapsidation of PV DNA in viral capsids and in the infectivity of PV virions. The L2 protein also can cause the relocalization of the PV early protein, E2TA, to nuclear subdomains known as promyelocytic leukemia oncogenic domains (PODs) in which it is localized. E2TA is a transcriptional transactivator that also plays a critical role in viral DNA replication. In this study, we investigated whether L2, in causing the relocalization of E2TA, alters the activities of E2TA. We provide evidence that L2 inhibits the transcriptional transactivation function of E2, but it does not specifically inhibit the capacity of E2 to support viral DNA replication. We also investigated whether the colocalization of E2 and L2 to PODs and the ability of L2 to inhibit the transcriptional transactivation activity of E2TA might be mediated through a direct interaction between these two proteins. Using an *in vitro* protein–protein association assay, we found that L2 binds to E2TA. Two regions in E2TA were found to mediate this interaction. One of those domains is present in an alternative E2 gene product, E2TR, which is an antagonist to E2TA. Here we show that the L2 protein also relocalizes the E2 transcriptional repressor, E2TR, to the nuclear subdomains. These data suggest that the ability of L2 to relocalize E2 proteins to PODs is mediated through a direct interaction with L2. © 2000 Academic Press

## INTRODUCTION

Papillomaviruses (PVs) are small, nonenveloped, icosahedral DNA viruses of about 55 nm in diameter that replicate in the nucleus of epithelial cells. They display strict species and tissue specificity and induce squamous epithelial or fibroepithelial tumors in their natural hosts. The size of the genome is about 8 kb, and it is divided in early and late regions. The open reading frames (ORFs) of the early region are identified as E1–E8, and those of the late region are identified as L1 and L2. The early genes are involved in the regulation of viral gene expression, viral genomic DNA replication, and cellular transformation. The late genes code for structural proteins that are required in the formation of virus particles. The long control region contains many *cis* regulatory elements involved in viral replication and transcription.

PVs cause the formation of warts and benign tumors, which in the case of some PVs can progress to carcinomas. PVs infect basal cells of epidermis at sites of wounding. The life cycle of the virus is separated into two phases: the nonproductive, plasmid maintenance and the productive, vegetative amplification phase. These two stages of the viral life cycle are linked to the differentiation stage of the epithelial cells, where the nonproductive phase is supported by undifferentiated cells and the productive phase is supported by differentiated cells. In the maintenance phase, the viral genome is replicated at low copy number, and only the early genes are expressed. As the epithelial cells differentiate, the “late” gene expression is turned on, and structural proteins are expressed, viral DNA is further amplified, and progeny virions are produced.

The E2TA protein is a transcriptional transactivator (Spalholz *et al.,* 1985) that enhances transcription from multiple viral promoters by its ability to bind as a dimer to a DNA recognition element that is found in multiple copies on the viral genome. In addition to its DNA binding and dimerization properties, the N-terminus of E2TA contributes to transactivation (Lambert *et al.,* 1989; Sakai *et al.,* 1996). In addition to the full-length E2 protein, BPV-1 encodes two shorter forms of the E2 protein, the E2 transcriptional repressors (E2TR) and E8/E2TR, which were named for their ability to inhibit the transactivation activity of E2TA (Choe *et al.,* 1989; Lambert *et al.,* 1990, 1987). They are encoded by the 3′ half of the E2 ORF. E2TR is expressed from the $p_{3080}$ promoter and is initiated at an internal initiation codon at residue 162. E8/E2TR is encoded by a spliced message so that the first 11 amino acids are encoded by E8 ORF and joined to the C-terminal 205 amino acids of E2. Both E2TR and E8/E2TR can modulate the function of E2TA in transcription. They are thought to do so either by forming heterodimers that are attenuated in transactivational activity or by compet-

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ing, as homodimers, for E2 binding sites on the viral genome (Barsoum *et al.,* 1992; McBride *et al.,* 1988).

The full-length E2 facilitates viral DNA replication through cooperative binding to the origin of replication with the E1 protein. The E1 protein is a DNA helicase that is essential for the replication of viral DNA in rodent cells and *in vitro*. For BPV DNA replication, both the E1 and E2TA proteins are needed *in trans* (Ustav and Stenlund, 1991). The *cis* element necessary and sufficient for DNA replication is referred to as the origin (*ori*) of DNA replication (Ustav *et al.,* 1991). The E2TA protein can associate with E1 protein and increases the binding E1 protein to its recognition element in *ori*. Thus E2TA likely contributes to replication by facilitating E1 binding to the viral replicon. Other studies suggest that E2TA plays a role in mediating viral DNA segregation in dividing cells (Ilves *et al.,* 1999) by binding to the metaphase chromosomes and in this way facilitating the viral genomes to segregate to daughter cells in approximately equal numbers (Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998).

As infected epithelial cells progress through terminal differentiation, the viral genome is amplified to high copy number, and all viral genes, including the "late" structural genes L1 and L2, that encode the viral capsid proteins are expressed and viral capsids are formed. The capsid is composed of 60 hexavalent and 12 pentavalent capsomeres. Each capsomere is a pentamer of the major capsid protein, L1. The L1 protein can self-assembly into virus-like particles (VLPs) (Zhou *et al.,* 1991), even in the absence of L2. These VLPs have been shown to have very similar capsid structure to virions (Heino *et al.,* 1995; Kirnbauer *et al.,* 1992, 1993). The PV capsid contains L1 and L2 proteins in a molar ratio estimated at approximately 30:1. The exact location of the minor capsid protein, L2, is not known. The ability of an antibody to L2 to neutralize PV infectivity demonstrates that a portion of the protein is exposed on the surface of the capsid (Christensen *et al.,* 1991; Heino *et al.,* 1993). Although hexavalent and pentavalent capsomeres form star-shaped pentamers of the L1 protein, they have distinct high-resolution structures. L2 protein is speculated to be located in the center of the pentavalent capsomeres (Trus *et al.,* 1997).

The exact role of the L2 protein in virus life cycle is still unclear. The viral genome has been shown to be packaged into VLPs composed of both L1 and L2 proteins but not into capsids composed of L1 only. The requirement of L2 protein for genome encapsidation and its ability to bind DNA nonspecifically *in vitro* (Zhou *et al.,* 1994) suggest that L2 contributes to the packaging of the viral DNA. Both capsid proteins are required to generate infectious virus (Volpers *et al.,* 1995; Zhou *et al.,* 1993).

The E2TA and L2 proteins of BPV-1 have been shown to colocalize to subdomains of the nucleus when these two proteins are overexpressed in cells. These subnuclear domains, where E2TA and L2 proteins colocalize, are promyelocytic leukemia (PML) oncogenic domains (PODs) (Day *et al.,* 1998). The exact role of PODs is unknown. They were originally identified by immunostaining with antisera from patients with primary biliary cirrhosis. PML, encoded by a gene mutated in acute promyelocytic leukemia (APL), localizes to these nuclear domains. The t(15;17) chromosome translocation in APL cells fuses PML to the retinoic acid receptor-$\alpha$ (RAR$\alpha$) gene to form oncoprotein PML-RAR$\alpha$. PODs in APL cells are disrupted, and disruption of PODs has suggested to play a general role in carcinogenesis. Viral proteins from multiple virus types are associated with PODs. Several regulatory proteins encoded by DNA viruses target PODs at very early times of infection. Early steps of transcription and replication of some DNA virus families (Ad5, HSV-1, SV40) take place at the peripheries of PODs (Ahn *et al.,* 1998; Ahn and Hayward, 1997; Carvalho *et al.,* 1995; Maul *et al.,* 1996; Szekely *et al.,* 1996). Proteins of certain DNA virus families interact with PODs without changing their structure, whereas proteins from other virus families destroy or reorganize the structure of PODs. However, at this point it is not clear what role interactions between PODs and viruses play in the viral life cycle of different viruses.

In this study, we show that the L2 protein of BPV-1 modulates the transcriptional transactivation function of the E2TA protein but not E2TA's role in viral DNA replication. Using an *in vitro* protein–protein association assay, we show that E2TA binds L2. In the E2TA protein, we identified two domains, one in the amino-terminus and the other in the middle of E2TA, that independently mediate binding to L2. The L2 protein is known to recruit E2TA protein to nuclear subdomains. Here we show that in addition to relocalizing the full-length E2TA to nuclear subdomains, the L2 protein also recruits the E2 transcriptional repressor, E2TR, to nuclear subdomains.

## RESULTS

### The L2 protein of BPV-1 modulates the transcriptional transactivator, E2TA

The full-length E2 protein of BPV-1 is a transcriptional transactivator. It has been shown that L2 protein relocalizes the E2TA protein to subdomains of the nucleus. To learn whether the L2 protein modulates the transcriptional transactivation function of E2TA, we monitored the transcriptional activity of E2TA in transient transfection assays using the reporter plasmid pBS1073. In the pBS1073 plasmid, the luciferase gene is expressed from the minimal thymidine kinase promoter, of which four E2 binding sites (E2BS) are positioned upstream. The calcium phosphate precipitation method was used to cotransfect the E2TA expression plasmid pC59 into the

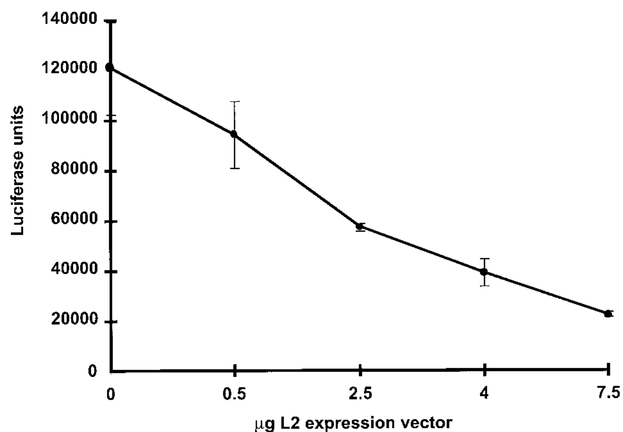


FIG. 1. Repression of E2-dependent luciferase expression from pBS1073 by increasing amounts of L2 protein. In the *y* axis are shown the relative luciferase units, and in the *x* axis are shown the amounts of L2 expression plasmid pcDNAHBL2 cotransfected into CV-1 cells. Shown are the average luciferase activities of an experiment with five different L2 concentrations. Each experiment was performed in triplicate. In each experiment, 1 μg of pBS1073, 0.1 μg of E2 expression plasmid pC59, and 0–7.5 μg of L2 expression plasmid pcDNAHBL2 were cotransfected into cells. The L2 protein is expressed from the CMV-promoter in the pcDNA3 plasmid. Within the experiment, we controlled for any effect of the CMV-promoter itself by maintaining the total amount of the parental plasmid, pcDNA3, constant throughout the experiments (e.g., 7.5 μg of pcDNA3 was cotransfected into cells when no L2 expression plasmid was used in transfections). Error bars indicate standard deviations.

CV-1, 3T3–10:3, or 293 cells with the luciferase reporter plasmid pBS1073. High levels of luciferase were expressed in these cells (Fig. 1). To determine whether the L2 protein has an effect on the activity of the E2 transcriptional transactivator, we cotransfected pC59 and pBS1073 plasmids along with the L2 expression plasmid pcDNAHBL2. In the absence of any L2 protein, a strong activation of luciferase gene expression was seen (Fig. 1). However, when increasing amounts of the L2 expression plasmid were cotransfected into the cells, the luciferase activity was decreased (Fig. 1). The luciferase activity from experiments in which no L2 protein was present (equivalent amounts of the parental vector pcDNA3 were transfected into the cells) was compared with luciferase activity from experiments in which L2 expression plasmid pcDNAHBL2 was cotransfected into cells. In the multiple cell types that were assayed, the levels of luciferase activity found in cells expressing L2 were 1–13% of the activity found in cells not expressing L2 (Table 1). In control experiments in which we used plasmids with promoters that are not responsive to the E2TA protein, the effect of the L2 protein was minimal compared with its effect on E2TA-dependent transcription (Table 1). From these experiments, we conclude that the L2 protein affects the transcriptional transactivation function of the E2TA protein.

## The L2 protein of BPV-1 does not specifically modulate the replication function of E2TA

In addition to its transcriptional transactivator functions, the E2TA protein of papillomaviruses is known together with the viral replication protein, E1, to play a role in viral replication. To determine whether the L2 protein has an effect on the replication function of the E2TA protein, transient replication assays were carried out. The BPV-1 replicon pRLH89.7 contains BPV-1 long control region (LCR) (nt 7350–90) and includes the minimal origin of replication (nt 7914–27). The E1 and E2TA proteins were expressed *in trans* from cytomegalovirus (CMV) immediate-early promoter from plasmids pCGEag1235 and pC59, respectively. pCGEag1235 carries a point mutation at nt 1235 that destroys a splice donor sequence at this site, leading to more efficient production of E1 protein. The plasmids pCGEag1235 and pC59, along with the replicon pRLH89.7, were introduced into CV-1, 3T3 10:3, or 293 cells by the calcium phosphate precipitation method. The ratio of the concentration of pC59 and pcDNAHBL2 plasmids used in these replication assays was the same as the ratio used for the transcriptional activation assays described earlier. Low-molecular-weight (Hirt) DNA was isolated 3 days post-transfection, digested with *Bgl*II and *Dpn*I, and analyzed by Southern hybridization analyses with a BPV-1-specific probe. *Dpn*I cleaves the DNA sequence GATC only if the adenine residue is methylated as occurs in bacteria. Thus *Dpn*I cleaves all DNA from the bacterial origin. DNA that has undergone replication in mammalian cells is not methylated at the adenine in this sequence context and therefore is resistant to *Dpn*I cleavage. *Bgl*II linearizes the pRLH89.7 replicon. Efficient replication of the origin

TABLE 1

Inhibition of Transcriptional Transactivation Function of E2TA by L2 Protein

| Reporter plasmid | Promoter | Relative luciferase activity[a] 3T3 | CV-1 | 293 |
|---|---|---|---|---|
| pBS1073 + pC59 | HSV Tk + 4E2BS | 0.02 ± 0.02 | 0.13 ± 0.05 | 0.01 ± 0.02 |
| pBS1013 | HSV Tk | 0.19 ± 0.09 | 0.56[b] | 0.31 ± 0.13 |
| pBS1065 | CMV | 0.22 ± 0.04 | 1.21[c] | ND |
| pGLcontrol | SV40 | 0.22[d] | 0.97[e] | 0.25 ± 0.18 |

*Note.* ND, not done.

[a] Shown are the relative luciferase activities from experiments where L2 was present to the luciferase activity from experiments where no L2 was present. Unless otherwise indicated, experiments were performed three or more times. In each experiment, samples were determined in triplicate.

[b] Experiment was performed only once.

Experiments were performed twice. The relative values of each experiment are as follows: [c] 0.30 and 2.12, [d] 0.26 and 0.18, and [e] 0.41 and 1.53.

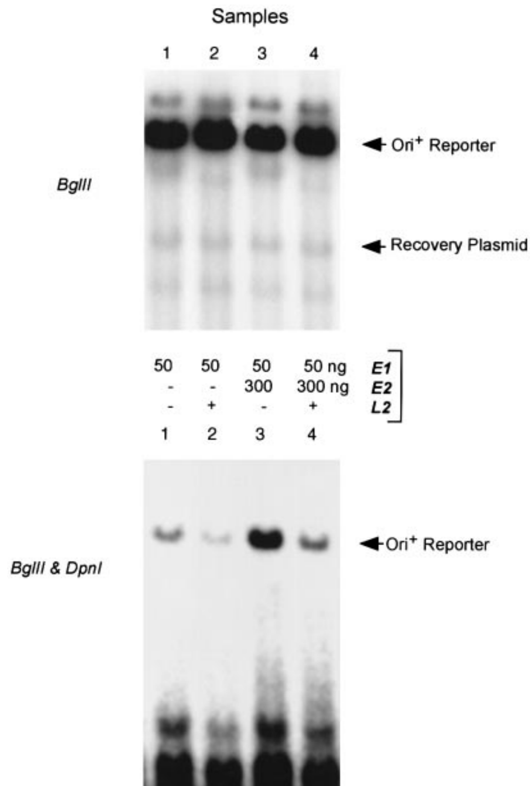


FIG. 2. Effect of L2 on the replication function of E2. Shown are phosphorimages of BPV-1-specific Southern blots in which Hirt DNA was isolated from 293 cells 3 days after transfection. Eight micrograms of the reporter plasmid pRLH89.750, 50 ng of the E1 expression plasmid pCGEag1235, and 0 or 300 ng of E2 expression plasmid pC59 were cotransfected with or without the L2 expression plasmid, pcDNAHBL2, into 293 cells as shown. *Bgl*II linearizes the reporter plasmid, pRLH89.750. Lanes 1–4 in the upper panel contain *Bgl*II-digested Hirt DNA (4 $\mu$l). The reporter and the recovery plasmids are indicated by arrows. The corresponding lanes (1–4) in the lower panel contain the same Hirt DNAs (20 $\mu$l) digested with *Dpn*I and *Bgl*II. The recovery plasmid was not detected in this lower panel, indicating complete *Dpn*I digestion (note five times more DNA was loaded in the lower panel). Therefore, the signal for reporter plasmid pRLH89.750 detected in this lower panel represents DNA that has replicated in eukaryotic cells. In the first two lanes, Hirt DNA was isolated from cells that were transfected with 50 ng of E1 expression plasmid in the presence (lane 2) or absence (lane 1) of L2 expression plasmid. The lanes 3 and 4, respectively, are Hirt DNA samples from cells in which 300 ng of E2 expression plasmid was cotransfected in addition to the plasmids used for transfections in lanes 1 and 2.

containing plasmid pRLH89.7 was dependent on the presence of both E1 and E2TA proteins (Fig. 2, lane 3). In the absence of E2, we detected low levels of replication (Fig. 2, lane 1) of pRLH89.7 consistent with prior studies *in vitro* (Bonne-Andrea *et al.,* 1995; Muller *et al.,* 1994) and in similar types of transient replication assays on HPV-1 (Gopalakrishnan and Khan, 1994). In these independent experiments, L2 led to a 1.8-fold decrease in the levels of replication in the absence of E2 (Fig. 2, lanes 1 and 2). A similar 2.7-fold decrease was observed in the presence of E2 (Fig. 2, lanes 3 and 4). The corresponding values in another experiment were 1.3- and 2.2-fold, respectively. These data indicate that L2 can inhibit slightly viral DNA replication in 293 cells, but this inhibition was not dependent on E2. The degree of inhibition of replication was small compared with the degree of inhibition of E2-mediated transcription in this same cell line (100 fold; see Table 1), suggesting that L2 preferentially inhibits the transactivation activity of E2. Similar studies carried out in 3T3 cells demonstrated an absence of any significant effect of L2 on E2-dependent or E2-independent replications (data not shown); further supportive of the conclusion that L2 is largely affecting only the transcriptional activation activities of E2TA. Here, the lack of an effect of L2 on replication was evident at varying levels of E1 and E2TA DNA tested (data not shown).

## The E2TA protein of BPV-1 interacts directly with the minor capsid protein L2

To learn whether L2 relocalized E2TA within the nucleus and modulates the transactivation function of E2TA via a direct interaction with E2TA, we sought the potential association of E2TA and L2 proteins by using an *in vitro* protein–protein association assay. E2TA was overexpressed in *Escherichia coli* as a GST-E2TA fusion protein, batch-purified using glutathione–Sepharose, and incubated with *in vitro* translated $^{14}$C-labeled L2 protein. The level of nonspecific binding by L2 in this assay was determined by incubating L2 protein with GST-$\beta$-globin ($\beta$G). In multiple experiments, the GST-E2TA fusion protein was found to bind at approximately 10% of the input L2 protein, which was 5- to 10-fold greater than the background binding to GST-$\beta$G (Fig. 3).

To identify the domain or domains in the E2TA protein that mediate binding to the L2 protein, we performed similar *in vitro* protein–protein association assays by using derivatives of GST-E2TA deleted from various portions of E2TA. The N-terminal 52 amino acids of E2TA were sufficient for efficient binding to L2 (Fig. 4). Another independent binding site in E2TA was found in the C-terminal portion of E2TA as judged by the strong binding of GST-E2 93–410 to L2 (Fig. 4). This strong binding was also seen when this region was reduced to amino acids (aa) 93–283. When we further dissected aa 93–410 into two separate domains, aa 93–161 and aa 162–410, efficient binding to L2 was lost (Fig. 4). Thus the domain from 93–283 appears to define the second L2 association domain. However, we also saw efficient binding when we fused aa 52–91 to aa 162–410. Thus sequences from aa 52–93 or aa 93–161, each of which do not in of themselves support L2 binding, can support efficient binding to L2 when fused to the C-terminal half of the E2 protein from 162–410 and/or 162–283. One possible explanation for this result is that some peptide motif duplicated in aa 52–91 and 93–161 partially contributes to specific L2 binding. However, no obvious duplication of amino acid sequence can be found in these two regions.

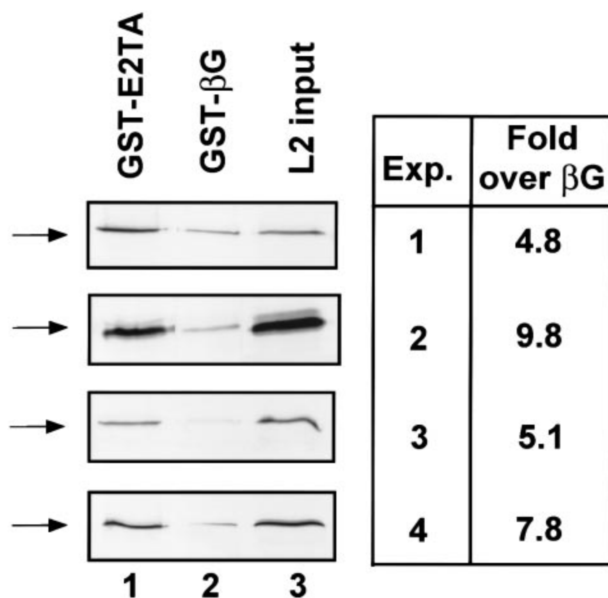


FIG. 3. L2 binds to E2TA. Binding of *in vitro* synthesized L2 to GST-E2TA fusion protein in four different reactions. The ^{14}C-labeled *in vitro* translated L2 protein was incubated with GST-E2TA (lane 1) or GST-βG (lane 2) coupled to glutathione–Sepharose beads. After the washes, proteins bound to beads were eluted, resolved on an SDS–polyacrylamide gel, and analyzed by autoradiography and phosphorimaging. *In vitro* translated ^{14}C-labeled L2 protein included in each binding reaction was loaded directly in the lane 3. In the first experiment, the L2 input was 5%, and in experiments 2–4, the input was 10%. Shown on the right is the fold of binding of L2 input protein to GST-E2TA over its binding to GST-βG, as determined by phosphorimaging analysis. Arrows indicate the site of L2.

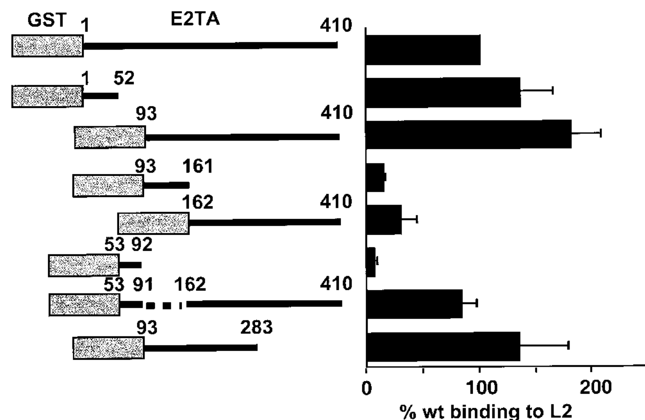


FIG. 4. Various derivatives of the E2 protein were synthesized as the GST fusion. Binding of the different fusion proteins to *in vitro* translated L2 was assessed in an *in vitro* protein association assay. Shown on the left are the amino acids of the E2 protein included in each GST fusion protein. On the right are data from corresponding GST pull-down assays. In each experiment, the percentage of input protein L2 bound to various GST-E2 derivatives was quantified by phosphorimaging analysis and compared with same amount of the input protein L2 bound to GST-E2TA. Each experiment was performed 3—10 ten times. Error bars indicate standard deviations.

Alternatively, these regions may only be providing some "spacer" function to separate GST from the C-terminus of E2TA, thereby permitting efficient binding by the C-terminus of E2TA to L2.

We further analyzed whether the capacity of the two fusion proteins harboring the two separate L2 interaction domains (GST-E2 1–52 and GST-E2 52–91/162–410) to bind L2 was specific. The capacity of these two fusion proteins to bind two irrelevant proteins, luciferase and retinoblastoma, was investigated. The ^{35}S-labeled luciferase or retinoblastoma proteins bound the GST-E2 1–52 and GST-E2 52–91/162–410 fusion proteins at about same levels as the GST-βG (Table 2). The results of this analysis indicate that these two domains in E2 display specificity for L2.

## E2TR, the transcriptional repressor, colocalizes with L2 protein in nucleus

Our protein–protein association studies led to the prediction that the relocalization by L2 of E2TA to subdomains of the nucleus may be mediated by an association between these two proteins. Based on our mutational analysis of E2TA data, the second domain of E2TA that binds to L2 may be completely contained within a second E2 gene product, called E2 transcriptional repressor, or E2TR (Fig. 5). E2TR is encoded by the 3′ half of the E2 ORF and functions as transcriptional repressor. To study whether E2TR colocalizes with L2 in the nucleus, we compared the immunofluorescent (IF) staining patterns of E2TA and E2TR in cells that did or did not express L2 protein.

A strong nuclear staining of L2 protein was seen by IF in cells transfected with the pcDNAHBL2, showing that the L2 protein was effectively expressed from the pcDNAHBL2, with "humanized" codons of L2 ORF (Fig. 6). In a large portion of the cells, the L2 protein localizes to nuclear subdomains. In COS cells expressing the full-length E2 protein only, in the absence of any L2 protein, usually a diffuse nuclear staining, sometimes with one or two darker spots, is seen with E2 antibodies by IF staining (Fig. 6d). The presence of the L2 protein causes relocalization of E2TA to nuclear subdomains, and a punctate staining pattern of both E2TA and L2 is commonly seen in the nucleus (Fig. 6a) as described previously (Day *et al.,* 1998). To study whether the shorter

TABLE 2

Relative Binding Compared with GST-βG

| | L2 | Rb | Luc |
|---|---|---|---|
| GST-E2, 1–52 | 6.8[a] | 1.3 | 1.8 |
| GST-E2, 52–91/162–410 | 8.3 | 1 | 1.1 |

Experiments were performed two times except the [a]experiment that was performed three times. Shown are average values.

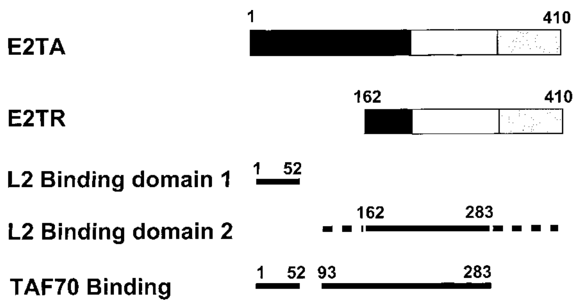


FIG. 5. Binding of the E2TA protein to the L2 protein of BPV-1. Shown at top are the alignments of the coding regions of E2TA and E2TR proteins. Numbers indicate the amino acid residues in the E2 ORF of BPV-1. The shaded boxes represent the conserved amino- and carboxyl-terminal domains among PVs. Shown are the two putative binding domains of E2 that independently bound L2. Both L2 binding domains overlap with TAF70 binding domains (N. M. Rank, H. Lu, A. J. Levine, and P. F. Lambert, unpublished observations).

form of the BPV-1 E2 protein, E2TR was colocalized with L2 in the nucleus, the E2TR expression plasmid pCGE2C was cotransfected with pcDNAHBL2 into COS cells. When E2TR protein was expressed without any L2 protein, mostly a diffuse nuclear staining of E2TR was seen. However, when E2TR was coexpressed with L2, a punctate staining pattern of E2TR was seen in the nuclei of these cells, indicating that L2 relocalizes also E2TR to nuclear subdomains (Fig. 7).

## DISCUSSION

In this study, we have shown that the minor capsid protein, L2, is able to modulate the transcriptional transactivation function of the E2TA protein of BPV-1. Using an *in vitro* protein–protein association assay, we demonstrated that L2 binds to E2TA *in vitro*. We could identify two domains in E2TA that independently bound L2. The L2 protein relocalizes E2TA to nuclear subdomain, which has been shown by others to be PODs (Day *et al.,* 1998). We have demonstrated that the L2 protein also relocalizes E2TR to nuclear subdomains and is colocalized with E2TR in these nuclear subdomains.

In the absence of L2, E2TA retains its transcriptional transactivation function, and mostly a diffuse nuclear staining, sometimes with one or two darker spots of the E2TA protein, is seen in these cells. However, in the presence of L2, the E2TA protein is colocalized with L2, often to subdomains of the nucleus. At the same time, it seems to lose at least one of its activities, transcriptional transactivation, but it retains its function in replication. The relocalization of E2TA to the nuclear subdomains may be the reason it does not function as transcriptional transactivator. Alternatively, L2 may directly interfere with the binding of E2TA to cellular transcription factors that mediate the transactivation activity of the latter. The induction of gene expression by E2TA protein of BPV-1 requires an interaction between E2TA and cellular factors that leads to the formation of an activated RNA polymerase complex. The E2TA protein of BPV-1 has been shown to directly bind to TATA binding protein (TBP) (Rank and Lambert, 1995) and to human TBP-associated factor (TAF) hTAFII70 (N. M. Rank, H. Lu, A. J. Levine, and P. F. Lambert, unpublished observations), both of which are subunits of the cellular transcription factor IID (TFIID). The basal transcription factor IIB (TAFIIB) is an essential component of the RNA polymerase II transcription apparatus. The E2TA protein of BPV-1 binds to and functionally interacts with TFIIB (Benson *et al.,* 1997; Rank and Lambert, 1995; Yao *et al.,* 1998).

Two domains in E2TA bind independently to L2 as shown in *in vitro* protein–protein association assays. The second domain that bound to L2 appears to overlap with a second E2 gene product, E2TR. Confocal microscopic studies revealed that E2TR also is colocalized with L2, whereas a diffuse nuclear staining is usually seen in the absence of the L2 protein. These data are consistent with the hypothesis that the direct association between L2 and E2 mediates their colocalization. It also raises the possibility that L2 modulates the activity of more than one E2 protein family member.

The present study shows that the L2 protein interferes with the transcriptional transactivation function of the E2TA protein but to a lesser degree or not at all with the replication function of E2TA. It is presumed that the mode of viral DNA replication in the transient replication assay used here reflects that which occurs early in the establishment of the nonproductive stage of the viral infection (i.e., a theta mode of viral DNA replication). In contrast, viral DNA replication in the productive stage of the viral life cycle, when L2 is selectively expressed, is thought to occur through a rolling circle mode (Flores and Lambert, 1997). Further studies are necessary to determine whether L2 interferes with the predicted role of E2TA in the viral DNA amplification during the productive stage of the viral life cycle (Alderborn *et al.,* 1992).

The nuclear subdomains where E2TA and L2 proteins colocalize are PODs, which may play a role in cellular antiviral defense because infection, such as with HSV-1, adenovirus, and human CMV, disrupts PODs (Ahn *et al.,* 1998; Ahn and Hayward, 1997; Carvalho *et al.,* 1995; Szekely *et al.,* 1996). Association with PODs has also been speculated to play a positive role in viral replication (e.g., the localization to PODs may increase the local concentration of viral products and promote virus assembly, or it may interfere with normal differentiation and/or apoptotic responses to the viruses and promote processing of viral products) (Day *et al.,* 1998). Association with PODs may also represent a switch in virus life cycle from the nonproductive to the virus-producing, productive phase. The POD-binding proteins HSV-1 ICPO (Maul *et al.,* 1996) and Epstein–Barr virus EBNA-5 (Szekely *et*

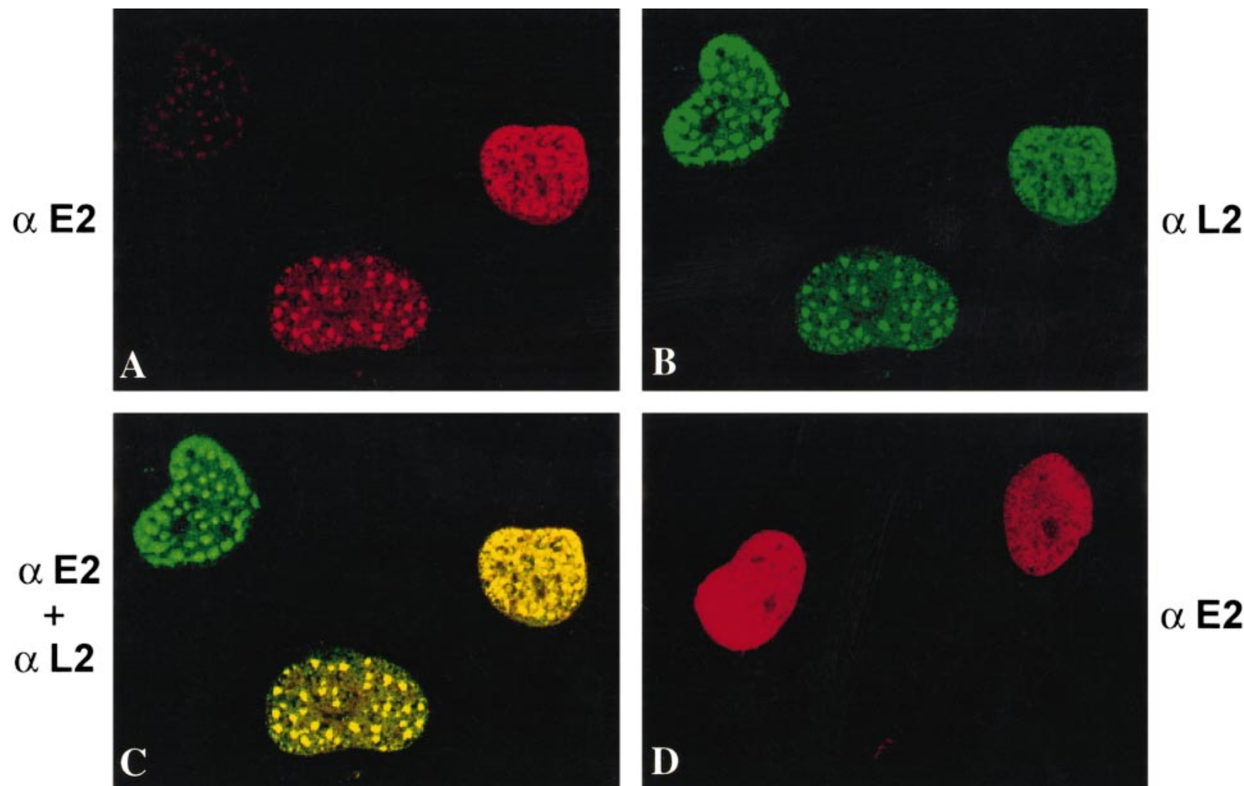


FIG. 6. Colocalization of the E2 and L2 proteins. The 35-mm plates of COS cells were cotransfected with 0.5 μg of pcDNAHBL2 and 0.5 μg of pCGE2 (A–C) or with 1 μg of pCGE2 only (D). After fixing, the cells were double-stained for immunolocalization of the E2 and L2 proteins. The E2 protein was detected with mouse monoclonal anti-E2 antibody, B201, and Texas Red-linked sheep anti-mouse secondary antibody (A and D). The L2 protein was detected with rabbit polyclonal anti-L2 antiserum and with FITC-conjugated goat anti-rabbit IgG (B). The double immunofluorescence staining was analyzed by laser scanning confocal system. The same field is shown in A–C. In panel C is a superimposition of images in panels A and B. The yellow staining in the merged panel, C, indicates coincidence of green and red staining.

*al.,* 1996), which have been implicated in the escape from latency, have been speculated to serve an analogous function for their respective viruses (Day *et al.,* 1998).

Human PVs are one of the most frequently found viruses in human cancers, yet little is known about the life cycle of PVs. Our findings that the minor capsid protein L2 and the transcriptional transactivator E2TA of BPV-1 bind to each other *in vitro* and that the L2 protein modulates the transcriptional transactivation function of E2TA offer important information about the gap in our knowledge concerning the productive phase in the infected cell. The E2TA protein may play a role in the nonproductive as well as in the productive phase of PV life cycle. The L2 gene gets turned on in the productive phase of viral life cycle in the differentiated layers of the epithelium. We have shown that L2 modulates one of the known functions of E2TA: its role as transcriptional transactivator. Therefore, we hypothesize that L2 can modulate the function of E2TA during the productive phase of the viral life cycle. The interaction of E2TA and L2 proteins may also allow E2TA to contribute to other activities postulated to be mediated by L2, such as encapsidation.

## MATERIALS AND METHODS

### Plasmids

Plasmid pBS1073 is the luciferase reporter construct. It contains luciferase gene downstream and four E2 binding sites upstream of thymidine kinase promoter of HSV-1 (T. Gahn and B. Sugden, unpublished data). pBS1013 is the same plasmid construct without the E2 binding sites (Middleton and Sugden, 1992). Plasmid p1065 contains the luciferase gene downstream of the CMV promoter. pC59 expresses the full-length E2 protein (Yang *et al.,* 1985). pRLH89.7 plasmid contains BPV-1 nt 7350–90 inserted into the backbone of $pGL_2$ basic vector (Promega, Madison, WI) in sense orientation. It contains the intact origin of replication with 12 E2 binding sites. The pWHBPV101 plasmid (Hubert and Lambert, 1993) contains the luciferase gene in p142-6 (Sarver *et al.,* 1982) background. pCGEag1235 is an E1 expression plasmid that contains a silent point mutation at BPV-1 nt 1235 that disrupts a 5′ splice signal within E1 gene (Ustav and Stenlund, 1991). The pCGE2 expresses the full-length E2 protein of BPV-1, and pCGE2C and pCGE8/E2 express the E2 transcriptional repressors (E2TR and E8/E2TR) (Ustav and Stenlund, 1991). The pCG

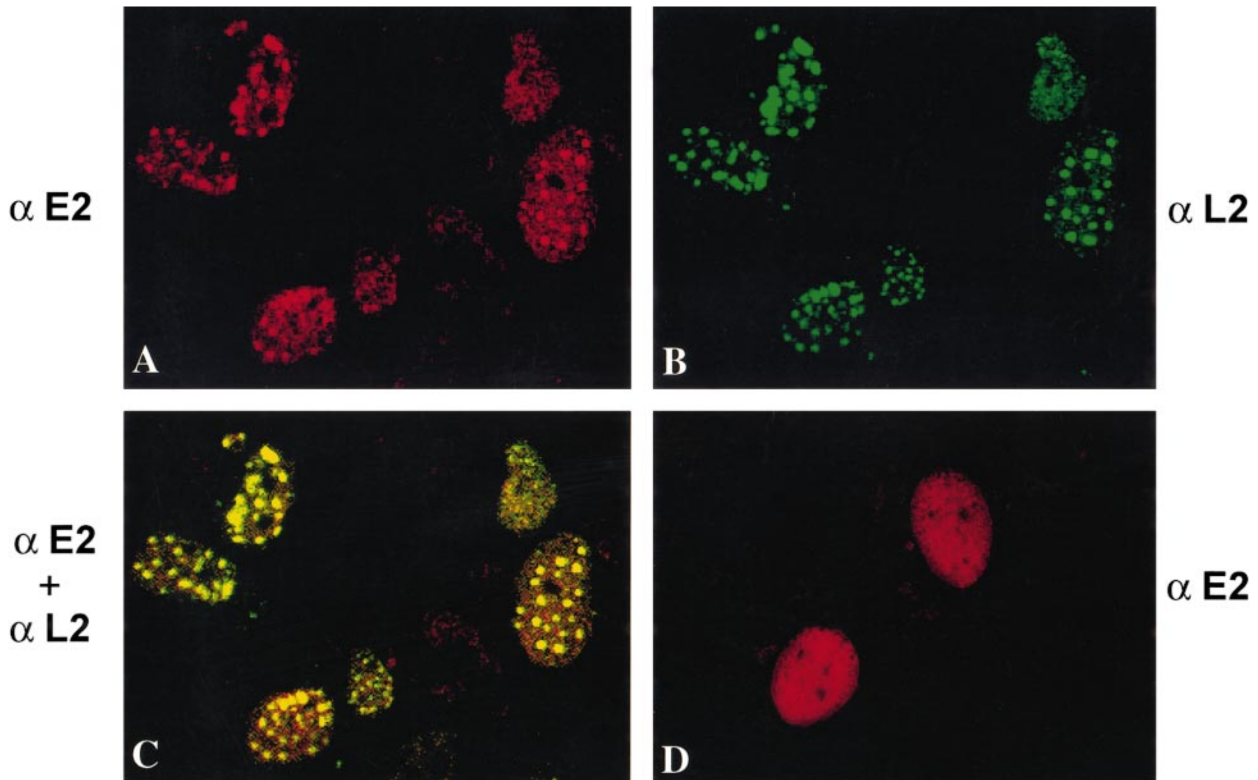


FIG. 7. Colocalization of E2TR and L2 proteins. COS cells were cotransfected with 0.5 μg of pcDNAHBL2 and 0.5 μg of pCGE2C plasmids (A–C) or with 1 μg of pCGE2 only (D). The L2 protein was detected with rabbit polyclonal anti-L2 antiserum and with FITC-conjugated goat anti-rabbit IgG (A). The E2TR protein was detected with mouse monoclonal anti-E2 antibody, B201, and Texas Red-linked sheep anti-mouse secondary antibody (B and D). The same field is shown in all panels. Panel C is a superimposition of images in panels A and B. Yellow staining in the merged panel, C, indicates coincidence of green and red staining.

vector directs expression of foreign genes from the strong immediate-early CMV promoter and contains an SV40 origin that allows for amplification in the presence of SV40 T-ag, which is expressed in COS cells. The pGL3-control vector contains the luciferase gene under the control of SV40 promoter and enhancer (Promega). The pcDNAHBL1 and pcDNAHBL2 plasmids express the viral capsid proteins L1 and L2 of BPV-1, respectively (Zhou *et al.,* 1999). These plasmids are able to efficiently produce BPV-1 late proteins in undifferentiated cells. Because the natural PV late genes frequently include codons that are rarely used in mammalian cells, which Dr. J. Zhou hypothesizes helps to restrict PV late gene expression to terminally differentiated cells, the infrequently used codons in BPV-1 L1 and L2 genes were replaced by conservative codons in pCDNAHBL1 and pCDNAHBL2. This results in efficient expression of PV late genes in most cultured mammalian cells. The pBSBPV1 L2 has the L2 gene of BPV1 inserted into pBluescript II KS (Stratagene, La Jolla, CA) background (a gift from Martin Müller). Luciferase T7 control DNA (Promega) and p565RB (under T7 control) were used to express luciferase or retinoblastoma proteins *in vitro*. Plasmids pGST-E2TA, pGST-E2TA Δ1–161 (pGST-E2TA 162–410), pGST-E2TA Δ1–92 (pGST-E2TA 93–410), and pGST-βG have been described previously (Rank and Lambert, 1995). Plasmids pGST-E2TA 1–52, pGST-E2TA Δ1–92 (pGST-E2TA 93–410), pGST-E2TA 93–161, pGST-E2TA 53–92, pGST-E2TA 93–283 were generated as described (Rank *et al.,* in preparation). The plasmid pGST-E2TA Δ1–52,Δ92–161 (pGST-E2TA 53–91,162–410) was constructed by PCR amplification of the appropriate coding region of pGST-E2TA Δ92–161 (N. M. Rank, H. Lu, A. J. Levine, and P. F. Lambert, unpublished observations) from primers designed to contain the glutathione *S*-transferase translational ORF and to contain *Bam*HI ends. The PCR-amplified E2Δ1–52,Δ92–161 DNA was cloned into the *Bam*HI site of the plasmid pGex-3X (Amersham, Arlington Heights, IL).

## Fusion protein purification

GST-E2TA and other GST fusion proteins were expressed and purified as described previously (Rank and Lambert, 1995). Briefly, cultures (250 ml) were grown at 27°C overnight. An additional 250 ml of Luria-Bertani medium was added to the overnight cultures, and the cultures were grown for an additional 3 h at 27°C. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.2 mM to induce the expression of

the fusion protein, and cells were grown for an additional 3 h at 27°C with minimal shaking. All of the following steps were performed at +4°C. Cells were pelleted by centrifugation, resuspended in 10 ml of NET buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl, pH 8.0) and centrifuged again. Cell pellets were resuspended in NETN-complete buffer [NET buffer, 0.1% Igepal (Sigma Chemical Co., St. Louis, MO), 1 mM DTT, 1× Protease inhibitor cocktail) and sonicated in 800-μl aliquots. Cell debris was pelleted by centrifugation, and aliquots of the cell lysates were stored at −70°C.

## *In vitro* protein association assay

The *in vitro* protein–protein binding assay was performed as described previously (Rank and Lambert, 1995). Briefly, cell lysates containing fusion proteins (4–200 μl) were incubated with 20 μl of a 50% glutathione–Sepharose slurry (Pharmacia, Piscataway, NJ), in a final volume of 200 μl of NETN (NET, 0.5% Igepal) at 4°C for 30 min with rocking. Equimolar amounts of different fusion proteins were used for each assay. The concentration of each fusion protein in each bacterial lysate was assessed by Coomassie blue staining of glutathione–Sepharose-bound proteins resolved by SDS–PAGE. The glutathione–Sepharose pellets were washed three times with NETN. Fusion proteins coupled to glutathione–Sepharose were then incubated for 45 min to 1 h with *in vitro* translated, $^{14}$C-labeled L2 protein or with *in vitro* translated $^{35}$S-labeled luciferase (Promega) or retinoblastoma proteins in a final reaction volume of 200 μl of NETN. Beads were washed as described earlier. Bound proteins were eluted by boiling for 5 min in 15 μl of 2× SDS loading dye and resolved by SDS–PAGE. Radiolabeled protein that was bound to E2-fusion proteins was detected by phosphorimaging and autoradiography.

## *In vitro* transcription and translation

The L2 mRNA was transcribed *in vitro* from the *Not*I linearized plasmid, pBSBPV-1 L2, using the Stratagene *in vitro* transcription system according to the manufacturer's instructions. Flexi Rabbit Reticulocyte Lysate system (Promega) was used to translate L2 protein *in vitro* using $^{14}$C-lysine. TNT-coupled Reticulocyte Lysate system (Promega) was used to express luciferase and retinoblastoma protein *in vitro* using $^{35}$S-methionine.

## Transfection of cells

*Transfection of cells for immunofluorescent staining.* Cells were incubated on coverslips in 35-mm tissue culture plates until the cells were 50–70% confluent. The plasmids used in transfections were pcDNAHBL1, pcDNAHBL2, and pCGE2 and/or pCGE2C. For each transfection, 0.5 μg of each plasmid was diluted into 100 μl of serum-free medium. The plasmid solution was then combined with 10 μl of LipoFECTAMINE in 100 μl of serum-free medium. The plasmid–LipoFECTAMINE mixture was incubated at room temperature for 20 min. The cells were rinsed once with 2 ml of serum-free medium. For each transfection, 0.8 ml of serum- and antibiotic-free medium was added to the tube containing the complexes, and the complex solution was then overlaid onto the cells. The cells were incubated from 5 h to overnight at 37°C. After incubation, the transfection mixture was removed and replaced with 2 ml of complete medium. At 24 or 36 h after the start of transfection, the cells were fixed with ethanol or 4% paraformaldehyde. Cells fixed with paraformaldehyde were permeabilized with 0.5% Triton X-100.

*Transfection of cells for luciferase assay.* Transfections for luciferase assays were performed according to the calcium phosphate precipitation method (Graham and van der Eb, 1973) on 50–70% confluent CV-1, 3T3–10:3, and 293 cells in 6-cm-diameter dishes. Then 1 μg of pBS1073, 100 ng of pC59, and varying amounts of pcDNAHBL2 and pcDNA3 were cotransfected into cells. In control experiments, 1 μg of PBS1013, pBS1065, pGL3control, or pGL2basic was cotransfected with varying amounts of pcDNAHBL2 and pcDNA3 into cells. The total amount of pcDNA derivative DNA was adjusted to 7.5 μg with pcDNA3. Cells were harvested 2 days posttransfection, and luciferase assays were performed according to the manufacturer's directions (Promega). Total cellular protein was quantified by a Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of the total cellular protein were used in each luciferase assay.

*Transient replication assay.* For transient replication assays, cells were transfected with the varying amounts of pCGEag1235 and pC59, with 8 μg of pRLH89.7 and 22.5 μg of either pcDNAHBL2 or pcDNA3 into cells by calcium phosphate transfection. Replication was scored using the pRLH89.7 plasmid that contains BPV-1 LCR and includes the minimal origin of replication. In each experiment, 2 ng of pWHBPV101 plasmid was added at the time of DNA extraction as spike DNA to control the recovery of low-molecular-weight DNA. Hirt DNA was isolated 3 days posttransfection, digested with *Bgl*II with and without *Dpn*I, and analyzed by Southern hybridization analyses with a BPV-1-specific probe. *Dpn*I cleaves the DNA sequences of bacterial origin; only in eukaryotic cells will replicated plasmids be present in *Dpn*I-treated target cells. *Bgl*II linearizes the pRLH89.7 replicon. The digested Hirt DNA was analyzed with Southern blotting, and replicated DNA was quantified by phosphorimaging analysis.

*Immunostaining of cells.* After rinsing with PBS and blocking with 5% milk in PBS for 10 min at room temperature, the cells fixed in ethanol were incubated with a mixture of a rabbit anti-L2 and a mouse anti-E2, B201, antibody in a dilution of 1:2000 and 1:100, respectively, in 5% milk at +4°C overnight. After three PBS washes, the cells were incubated for 2 h at room temperature with a

mixture of FITC-conjugated goat anti-rabbit immunoglobulins (Sigma), diluted 1:50, and Texas Red-linked sheep anti-mouse antibody (Amersham), diluted 1:50. After three washes in PBS, coverslips with cells were mounted onto microscope slides using Fluoromount-G mounting solution (Southern Biotechnology Associates, Inc.). The double immunofluorescent staining was analyzed by a laser scanning confocal system (Bio-Rad MRC 1024 Laser Scanning Confocal Microscope).

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