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In Vitro Generation and Type-Specific Neutralization of a Human Papillomavirus Type 16 Virion Pseudotype

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We report a system for generating infectious papillomaviruses in vitro that facilitates the analysis of papillomavirus assembly, infectivity, and serologic relatedness. Cultured hamster BPHE-1 cells harboring autonomously replicating bovine papillomavirus type 1 (BPV1) genomes were infected with recombinant Semliki Forest viruses that express the structural proteins of BPV1. When plated on C127 cells, extracts from cells expressing L1 and L2 together induced numerous transformed foci that could be specifically prevented by BPV neutralizing antibodies, demonstrating that BPV infection was responsible for the focal transformation. Extracts from BPHE-1 cells expressing L1 or L2 separately were not infectious. Although Semliki Forest virus-expressed L1 self-assembled into virus-like particles (VLPs), viral DNA was detected in particles only when L2 was coexpressed with L1, indicating that genome encapsidation requires L2. Expression of human papillomavirus type 16 (HPV16) L1 and L2 together in BPHE-1 cells also yielded infectious virus. These pseudotyped virions were neutralized by antiserum to HPV16 VLPs derived from European (114/K) or African (Z-1194) HPV16 variants but not by antisera to BPV VLPs, to a poorly assembling mutant HPV16 L1 protein, or to VLPs of closely related genital HPV types. Extracts from BPHE-1 cells coexpressing BPV L1 and HPV16 L2 or HPV16 L1 and BPV L2 were not infectious. We conclude that (i) mouse C127 cells express the cell surface receptor for HPV16 and are able to uncoat HPV16 capsids; (ii) if a papillomavirus DNA packaging signal exists, then it is conserved between the BPV and HPV16 genomes; (iii) functional L1-L2 interaction exhibits type specificity; and (iv) protection by HPV virus-like particle vaccines is likely to be type specific.

Infection by certain human papillomaviruses (HPVs) is the major risk factor for the development of cervical cancer, the second leading cause of cancer deaths in women worldwide (25, 46). Approximately 95% of cervical cancer biopsies contain high-risk HPV DNA, most commonly HPV type 16 (HPV16), followed by types 18, 31, 33, and 45. Low-risk types, most often HPV6 and -11, commonly cause external genital warts but are rarely detected in genital carcinomas (4).

Basic virologic studies of high-risk genital HPVs have been hampered by the inability to produce preparative amounts of virions in vitro and by the lack of a quantitative in vitro infectivity assay (13). The former deficit has been partially overcome by the demonstration that high-level expression of the L1 major capsid protein in eukaryotic cells leads to the self-assembly of virus-like particles (VLPs) that are structurally similar to authentic virions (14, 16, 32, 35, 40, 45). The L2 minor capsid protein is incorporated into the L1 VLPs at a molar ratio similar to that of authentic virions (1:30) if it is coexpressed in the same cells (14, 18). However, the role of L2 in genome encapsidation and infection is unclear because of the lack of a procedure that permits genetic manipulation of the viral genome and subsequent generation of infectious virus.

HPV VLPs are attractive candidates for vaccines against genital papillomavirus infections because they present conformational virion surface epitopes but lack the potentially oncogenic viral genome (36). Supporting the vaccine potential of VLPs are the findings that they induce high titers of apparently type-specific neutralizing antibodies against infectious bovine papillomavirus type 1 (BPV1) (16), cottontail rabbit papillomavirus (5), and HPV11 (7). In addition, vaccination with VLPs stimulated type-specific, antibody-mediated in vivo protection against high-dose experimental infection by cottontail rabbit papillomavirus (5), BPV4 (17), and canine oral papillomavirus (39).

Although previous studies have established that the neutralizing activity and protection induced by a particular VLP type does not extend to distantly related virion types that infect another species (5), it is unclear whether this is also true for more closely related types that infect the same species (8). To make informed decisions concerning the components of a polyvalent vaccine to prevent genital HPV infection, it is necessary to evaluate the potential for cross-protection after vaccination with specific genital HPV types. Therefore, we wished to develop in vitro assays that directly measure neutralizing antibodies to high-risk genital HPVs such as HPV16. This required the in vitro generation of HPV16 virions and the development of a quantitative in vitro assay for infectivity.

To generate infectious papillomaviruses in vitro, an expression system based on Semliki Forest virus (SFV) was chosen to express the papillomavirus virion proteins (15, 21). In addition to high-level expression, the SFV system has several other theoretical advantages over previously used expression systems for in vitro production of papillomaviruses. Unlike recombinant baculoviruses, recombinant SFV can infect a wide variety of cells, including mammalian fibroblasts and keratinocytes

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which in some cases can maintain autonomously replicating papillomavirus genomes (21). Because the recombinant SFVs are defective, extracts of SFV-infected cells do not cause cytopathic effects seen with vaccinia virus expression systems which could interfere with the detection and characterization of papillomavirus infection (1). Unlike both baculovirus and vaccinia virus recombinants, SFV recombinants are positive-strand RNA viruses with no DNA intermediate. It is therefore very unlikely that papillomavirus packaging signals in the genes cloned into SFV could interfere with packaging of the full-length papillomavirus genome. Furthermore, SFV recombinants are easily generated in small bacterial plasmids, which should facilitate subsequent genetic analysis of the papillomavirus virion genes (21).

HPV genomes do not induce easily scored phenotypes when introduced in cultured cells, making it difficult to quantitatively evaluate infection by these viruses. Therefore, we first attempted to generate infectious BPV1 virus, since focal transformation of contact-inhibited mouse fibroblasts provides a sensitive and quantitative assay for infectivity by this virus (12). Specifically, cultured cells harboring autonomously replicating BPV1 genomes were infected with recombinant defective SFVs encoding the BPV capsid proteins. Successful generation of infectious BPV1 virus in this system led us to subsequently attempt the generation of pseudotyped virus through the infection of the same cells with recombinant SFV encoding the HPV16 capsid proteins. Focal transformation induced by the BPV genome pseudotyped by the HPV16 virion proteins provides the first quantitative in vitro neutralization assay for a high-risk HPV. This assay has not only permitted an analysis of the ability of antibodies raised against other HPV types to inhibit HPV16 infection but has also provided insights into the mechanism and specificity of papillomavirus genome packaging and infection.

MATERIALS AND METHODS

Reagents. BPHE-1 cells were obtained from A. Lewis (National Institutes of Health, Bethesda, Md.) (42). C127 clone C cells were obtained from W. Vass (National Institutes of Health), and BHK-21 cells were from the American Type Culture Collection. All antisera to VLPs (28) and monoclonal antibodies have been described previously (9, 30). Unless otherwise stated, all other reagents, including the SFV expression vectors, were from Life Technologies Inc., Gaithersburg, Md.

Generation of recombinant pSFV-1 plasmids. To remove an internal *Spe*I site, BPV L1 was amplified by PCR in two separate reactions from BPVpML DNA, using oligonucleotides CCGCTGGATCCCACTATTATATAGCACCATG GCGTTGTGGCAACAAGGCCAG and CAGTTGAGACTAGAGAGCCAC for one reaction and oligonucleotides GTGGCTCTCTAGTCTCAACTG and GCGGTGGATCCTTATTTTTTTTTTTTTTTTGCAGGCTTACTGGAAGTT TTTTGGC for the second. The products were gel purified and mixed, and the full-length L1 gene was reamplified by using the outside primers. The product (~1.5 kb) was gel purified, digested with *Bam*HI, and cloned into the *Bam*HI site of pSFV-1 (21). The clone was sequenced to confirm the orientation and absence of the *Spe*I site and amplification errors. BPV L2 was amplified by PCR from *Bam*HI-cut and religated BPVpML DNA, using GCGGTAGATCTAATAT GAGTGCACGAAAAAGAGTAAAACGTGCCAGT and CCGCTAGATCTA GGGAGATACAGCTTCTGGCCTTGTTGCCACAACGC for primers. The product (~1.5 kb) was gel purified, digested with *Bgl*II, cloned into the *Bam*HI site of pSFV-1, and sequenced. Wild-type (114/K) and capsid assembly-deficient mutant (pAT) HPV16 L1 were excised from pEVmod by using *Bgl*II and subcloned into pSFV-1. HPV16 L2 was subcloned from a pEVmod vector into the *Bam*HI site of pSFV-1.NruI (which is linearized by using *Nru*I rather than *Spe*I). All plasmids were purified from *Escherichia coli* HB101 by alkaline lysis and cesium chloride isopynic density centrifugation.

Generation of recombinant SFV stocks. The recombinant pSFV-1 clones and pHelper-2 (1) plasmid were linearized by using *Spe*I (or *Nru*I for pSFV-1.NruI-based clones). The DNAs were phenol-chloroform extracted and ethanol precipitated. To generate SFV RNA, 1 μg of each linearized pSFV-1 clone and 1 μg of pHelper-2 were resuspended in 100-μl reaction mixtures containing 1 mM ATP, 1 mM CTP, 1 mM UTP, 0.5 mM GTP, 1 mM RNA capping analog $m^7G(5')ppp(5')G$, 5 mM dithiothreitol, 100 U of human placental RNase inhibitor, and 75 U of SP6 RNA polymerase in 1 × SP6 reaction buffer. The reaction mixtures were incubated for 1 h at 37°C, and 2.5 μl was analyzed on a 0.7% agarose gel to assess the integrity of the SFV RNAs. The remaining RNA was diluted in 1 ml of OptiMEM medium, mixed with 100 μl of Lipofectin in 1 ml of OptiMEM, and incubated for 15 min at ambient temperature. BHK-21 cells in a T-75 tissue culture flask were washed and covered with 2 ml of OptiMEM. The RNA-Lipofectin mix was added, and the cells were incubated for 4 h at 37°C. The cells were washed once and maintained for 24 h in 13 ml of complete medium (5% fetal calf serum, 10% tryptose phosphate broth, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 1× nonessential amino acids, 100 U of penicillin per ml, and 100 μg of streptomycin per ml in Glasgow's minimal essential medium). The medium was harvested, clarified by centrifugation (1,000 × *g*, 10 min), aliquoted, and stored at −80°C.

Generation of papillomavirus in BPHE-1 cells. The recombinant SFV stock was rendered infectious by incubation with 0.5 mg of chymotrypsin A4 (Boehringer Mannheim) per ml for 30 min on ice and treatment with 0.5 mg of aprotinin (Sigma) per ml. Then 4×10^6 BPHE-1 cells maintained for 12 to 20 h in Dulbecco modified Eagle medium containing 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml in a 100-mm-diameter tissue culture plate were washed in D-PBS (phosphate-buffered saline [PBS] containing 0.9 mM calcium and 0.5 mM magnesium). The cells were incubated for 2 h at 37°C with activated recombinant SFV (titrated to give maximum expression levels, but generally 0.5 ml of each high-titer stock) diluted to 25 ml in D-PBS. The virus was aspirated, replaced with complete medium, and maintained for 30 h. The cells were scraped from the dish into the medium, which was collected and centrifuged (1,000 × *g*, 10 min), and the cell pellet was resuspended in 1 ml of D-PBS. The cells were lysed by sonication (10 s, 60% power; Fischer model 150 sonic dismembranator with a microtip).

In vitro focal transformation assay. Cell lysates were added to the medium (Dulbecco modified Eagle medium containing 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml) of monolayers of C127 clone C cells in 60-mm-diameter tissue culture plates. The cells were incubated at 37°C for 1 h, washed, and maintained in adoptive immune medium 5 (AIM-V) containing 2% fetal calf serum for 3 weeks. The cells were stained with 0.5% (wt/vol) methylene blue–0.25% (wt/vol) carbol fuschin in methanol, and the number of foci was scored (12).

Purification of particles from mammalian cells. For preparation of VLPs, BHK-21 cells were maintained for 3 days after infection with recombinant SFV. To generate full virions, BPHE-1 cells were maintained for only 30 h after infection with recombinant SFV. Ten 500-cm^2 culture dishes of cells were scraped from the plates into the medium, which was centrifuged (1,000 × *g*, 10 min, 4°C), and the cell pellet was resuspended in 5 ml of ice-cold PBS. The cells were lysed by sonication (1 min, 60% power) and treatment with 0.5% Nonidet P-40. Extracts were layered over a 30-ml 40% (wt/vol) sucrose-in-PBS cushion and centrifuged for 150 min at 80,000 × *g* at 4°C. The pellets were resuspended in 12 ml of 27% (wt/wt) cesium chloride in PBS and centrifuged for 20 h at 275,000 × *g*. The isopynic density gradient was fractionated, and the density of each fraction determined with an Abbe3L refractometer (Milton Roy, Rochester, N.Y.) (18).

Southern blot analysis. Cesium chloride gradient samples were mixed with 2.5 volumes of ethanol and stored overnight at −20°C. The samples were centrifuged (16,000 × *g*, 10 min, 4°C); the pellets were washed with 70% ethanol and resuspended in 10 mM Tris–1 mM EDTA (pH 8) (TE). Each sample was treated with proteinase K, phenol-chloroform extracted, ethanol precipitated, and resuspended in TE. Samples were separated on a 0.8% agarose gel, transferred to nylon membrane (Hybond N; Amersham), and UV cross-linked (120,000 μJ; UV Stratalinker 1800; Stratagene). BPV DNA was detected by using a ^{32}P-labeled random primed *Spe*I-*Kpn*I fragment of BPVpML under high-stringency conditions (33).

Electron microscopy. Transmission electron microscopy was performed by adsorbing 5-μl samples to carbon-coated copper grids, staining with 1% (wt/vol) uranyl acetate, and examination in a Philips EM400RT electron microscope at 100 kV. Samples for cryoelectron microscopy were spun for 15 min in an airfuge onto carbon-coated copper grids, frozen in liquid ethane, and also examined in a Philips EM400RT electron microscope at 100 kV (3).

RESULTS

BPV L1 expressed from recombinant SFV in mammalian cells binds L2 and assembles into VLPs. SFV is a simple positive-strand RNA virus. The pSFV-1 expression vector contains the gene for the SFV RNA replicase, the inserted gene, and a *cis*-acting virion packaging signal (21). In vitro-synthesized RNA from this vector is cotransfected with a helper vector (pHelper-2) RNA that encodes the SFV structural genes. Upon transfection, the replicase is translated and initiates successive rounds of RNA replication and translation, thereby amplifying the viral RNAs. Translation of the helper RNA leads to production of the SFV virion proteins and encapsidation of the expression vector RNA but not that of the

helper, which lacks the packaging signal. Therefore, the high-titer virus generated is defective because it does not encode the SFV virion proteins. Upon infection of susceptible cells (e.g., BHK-21 or BPHE-1), the replicase again amplifies the infecting RNA. Amplification of subgenomic RNAs encoding the cloned gene leads to high-level expression of the encoded protein.

Defective BPV1 L1 and BPV1 L2 recombinant SFVs (SFV-BL1 and SFV-BL2) were generated by cotransfecting BHK-21 cells with in vitro-transcribed pHelper-2 RNA (Life Technologies) (1) and a recombinant pSFV-1 RNA encoding the BPV1 L1 or BPV1 L2 gene. BHK 21 cells were infected with the recombinant SFVs and harvested 72 h later. Expression of BPV1 L1 and L2 was demonstrated by Western blot (immunoblot) analysis with monoclonal antibody 1H8 (Chemicon) (9) for L1 and rabbit antiserum to a bacterially produced glutathione *S*-transferase–BPV1 L2 fusion protein for L2 (not shown) (16). Cell fractionation studies (not shown) demonstrate that at least 80% of both L1 and L2 resided in the nuclear fraction at the time of harvest.

BHK-21 cells were infected for 3 days with either SFV-BL1 or SFV-BL2 alone or were coinfected with the two defective viruses. The cells were harvested, and VLPs were prepared by centrifugation through a 40% (wt/vol) sucrose cushion and cesium chloride isopycnic density gradient centrifugation (18). A visible band with a density of approximately 1.28 g/cm^3 was extracted from cesium chloride density gradients of the SFV-BL1 alone- and SFV-BL1-plus-SFV-BL2-infected cell extract and dialyzed into PBS containing 0.5 M NaCl. A corresponding band was not detected in the gradient containing the extract from the cells infected with only SFV-BL2. Transmission electron microscopy of the BPV1 L1 alone (Fig. 1A) and the L1 plus L2 (not shown) preparations demonstrated large numbers of 55-nm-diameter particles with a morphology similar to that of BPV virions that were absent from the L2 alone preparation. Analysis of the L1 and L1 plus L2 preparations on a 10% Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel revealed a single 55-kDa protein band corresponding to L1 (Fig. 1B). Full length (~70-kDa) L2 was detected by Western blot analysis with rabbit antiserum to bacterially expressed glutathione *S*-transferase–BPV L2 fusion protein in the L1 plus L2, but not the L1 alone, preparation (data not shown). Coimmunoprecipitation (data not shown) and copurification of L1 and L2 suggest that L2 coassembled with L1 into VLPs.

Infectious BPV1 virions are generated by coexpression of both BPV1 L1 and L2 in BPHE-1 cells. Since expression of the recombinant SFVs led to efficient assembly of VLPs in mammalian cells, we attempted to generate infectious BPV in vitro and to determine which capsid proteins were required for virion formation. To this end, the SFV recombinants were used to infect a hamster cell line, BPHE-1, that maintains 50 to 200 copies of episomal BPV1 genomes per cell (42). The BPHE-1 cells were infected with either SFV-BL1 or SFV-BL2 alone or were coinfected with the two recombinant viruses. The cells were maintained for 30 h, harvested, and lysed by sonication, and the extracts were incubated in the medium of monolayers of mouse C127 fibroblasts for 1 h at 37°C. The cells were washed and maintained for 3 weeks in complete medium and stained, and the foci were counted (12). Approximately 50 foci occurred in plates of C127 cells treated with BPHE-1 extracts expressing both BPV L1 and L2, but no foci were produced by extracts expressing only BPV L1 or only BPV L2 in multiple experiments (Fig. 2A to F).

To determine if focal transformation was due to transfer of BPV1 DNA to the mouse C127 cells, six of the foci were ring cloned and expanded for further analysis (20). A Hirt extract from each of the six clones was separated on a 0.8% agarose gel, Southern blotted, and probed with a ^{32}P-labeled fragment of the BPV genome. High-copy-number episomal BPV geno-

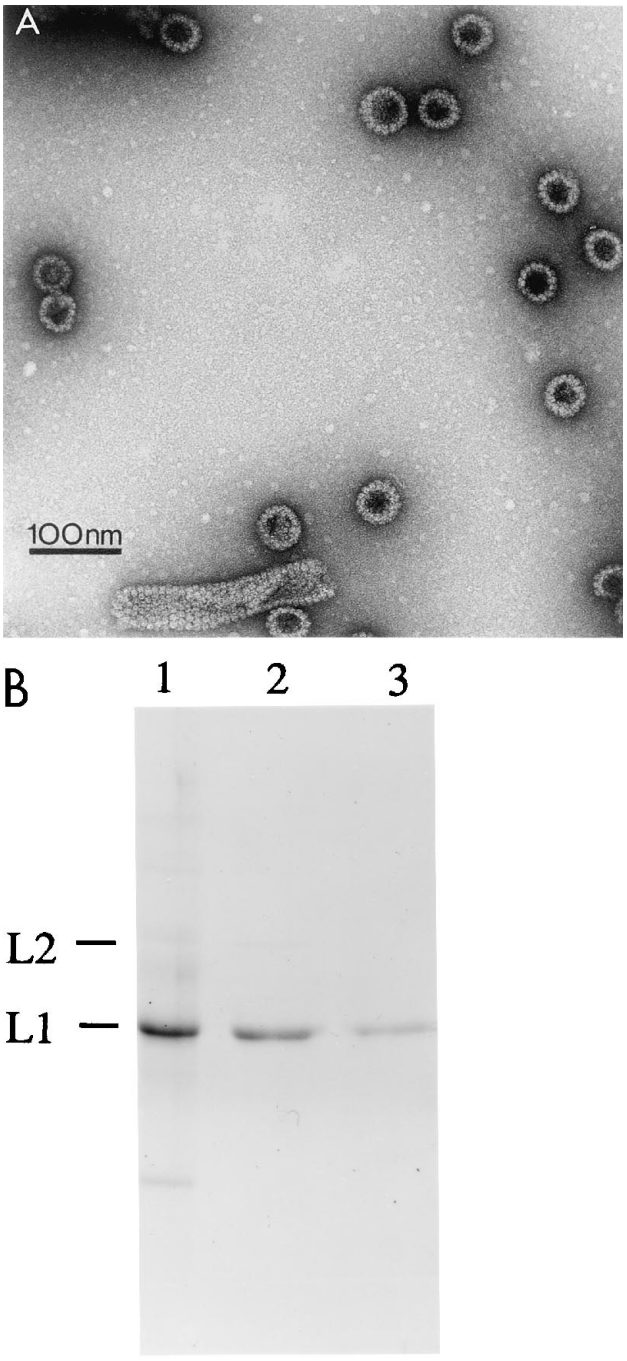


FIG. 1. Generation of BPV1 VLPs in recombinant SFV-infected BHK 21 cells. (A) BHK 21 cells were infected for 72 h with recombinant SFV that directs expression of BPV1 L1. BPV1 L1 VLPs were purified from cell lysates, mounted on carbon-coated copper grids, stained in 1% uranyl acetate, and observed by transmission electron microscopy. (B) Coomassie blue-stained SDS–10% polyacrylamide gel of purified BPV1 virions (lane 1), BPV1 VLPs purified from BHK-21 cells expressing BPV1 L1 and L2 from recombinant SFV (lane 2), and BPV1 VLPs purified from BHK-21 cells expressing BPV1 L1 alone from recombinant SFV (lane 3).

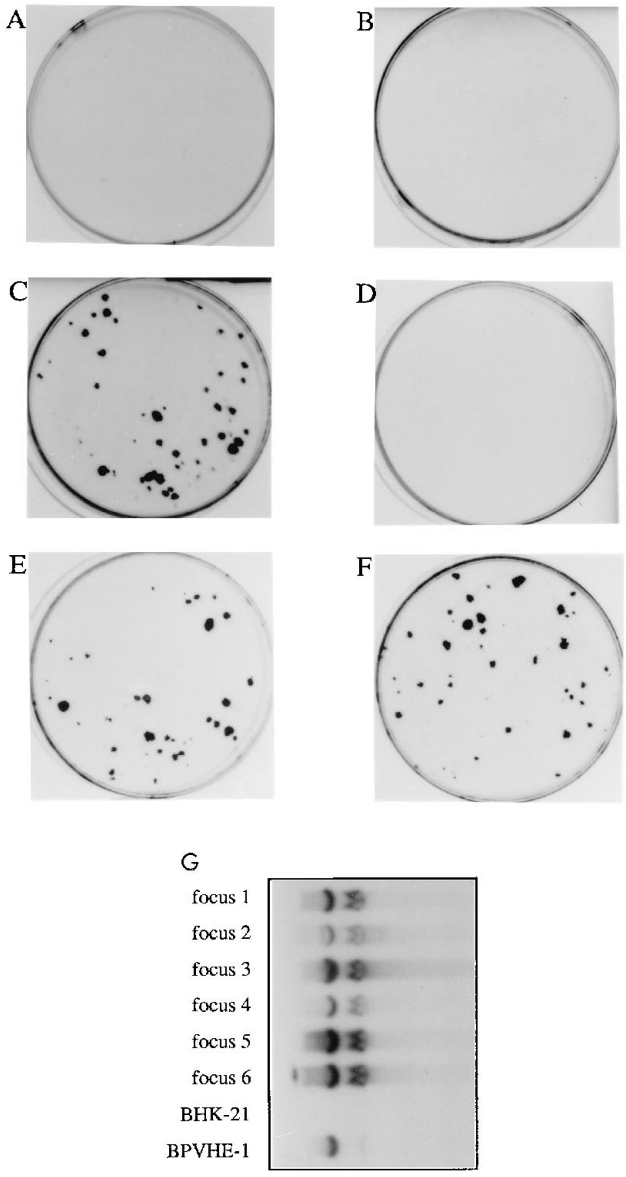


FIG. 2. Generation of infectious BPV1 virions in vitro. (A to F) Recombinant SFV-infected BPHE-1 cells (~2×10^6) expressing BPV1 L1 alone (A), BPV1 L2 alone (B), and BPV1 L1 plus L2 (C to F) for 30 h were harvested and lysed by sonication. The extracts were incubated with 10 μl of rabbit antiserum to baculovirus-expressed BPV1 L1 VLPs (D), antiserum to baculovirus-expressed HPV16 L1 VLPs (E), or antiserum to denatured BPV virions purified from bovine warts (F). The extracts were plated over monolayers of C127 fibroblasts that were washed with medium after 1 h, maintained for 3 weeks, and stained. (G) Six foci from a plate of C127 cells that had been incubated with extracts of BPHE-1 cells expressing BPV1 L1 plus L2 3 weeks earlier were ring cloned and expanded. Low-molecular-weight DNA was extracted from BPHE-1 cells, BHK-21 cells, and the cells expanded from each focus by the Hirt procedure. The presence of BPV1 DNA in the Hirt extracts was detected by Southern blot analysis.

mic DNA was detected in the extracts of all six clones (Fig. 2G).

It is possible that the BPV DNA was transferred to the C127 cells by transfection rather than infection by in vitro-generated virions. Since neutralizing antibodies should not inhibit transfection, extracts from the L1 and L2 coexpressing BPHE-1 cells were incubated for 1 h at 4°C in the presence of a 1:100 dilution (10 μl) of rabbit antiserum to either BPV1 or HPV16 L1 VLPs (purified from insect cells) or denatured BPV virions (DAKO) prior to addition to the C127 cells. The L1 plus L2 extract treated with antiserum to BPV VLPs did not produce any foci, whereas extracts treated with antiserum to HPV16 VLPs or denatured BPV virions (which do not neutralize BPV) produced similar numbers of foci as the untreated extract (Fig. 2A to F). Treatment of the same extract with monoclonal antibody 5B6, which neutralizes BPV (30), but not a control monoclonal antibody (PAb 101) of the same isotype, also inhibited focus formation (not shown). The conformationally dependent and type-specific neutralization of focal transforming activity demonstrates that infection by BPV virions and not transfection of BPV DNA was responsible for the transformation of the C127 cells.

L2 is required for efficient encapsidation of the BPV genome. L1 assembles into VLPs when expressed in eukaryotic cells, but the function of L2 in generating infectious virus is less clear (16). L2 may be necessary for some step during the infectious process and/or necessary for encapsidation of the genome (43). To explore the latter possibility further, 10 500-cm^2 plates of BPHE-1 cells were infected with SFV-L1 or SFV-L2 alone or were coinfected with SFV-L1 and SFV-L2. The cells were harvested 30 h postinfection, sonicated, and treated with DNase I (2,000 U) for 1 h at 37°C, and particles were purified. The cesium chloride gradients were fractionated, and the density of each fraction was measured. Nucleic acid was purified from 200 μl of each fraction, and BPV DNA was detected by Southern blot analysis (Fig. 3A); 0.1 ng of BPV-pML plasmid DNA was run as a size standard for uncut, DNase I-resistant BPV genomes (34). Only fraction 4 from the BPHE-1 extracts expressing both L1 and L2 demonstrated significant accumulation of DNase I-resistant BPV DNA. This fraction had a density (1.31 g/ml) consistent with that of infectious BPV virions obtained from warts under the same conditions (1.32 g/ml) (unpublished data).

Fraction 4 was examined by cryoelectron microscopy (Fig. 3B). Unlike transmission electron microscopy of negatively stained particles, cryoelectron microscopy allows the DNA inside the full capsids to be visualized directly as an electron-dense core, as opposed to the lower-density core of empty particles. Many well-formed particles were observed with electron-dense cores, as well as a smaller fraction that had a lower density core or were damaged or rod shaped (Fig. 3B). It was not possible to estimate the number or percentage of full versus empty particles, as the L1 was spread over a large number of fractions as determined by Western blot analysis. However, comparative Southern blot analysis using the cloned BPV genome as a standard indicated that approximately 1 ng of full-length DNase I-resistant DNA was observed in these extracts, which corresponds to approximately 10^8 DNA molecules. In contrast, only 10^4 infectious units were isolated from this preparation, suggesting that the particle-to-infectivity ratio is high, approximately 10^4. Using the same procedures, the number of infectious units and the amount of DNase I-resistant BPV genomic DNA present in a BPV virion preparation purified from BPVs were measured (data not shown). The values for the particle-to-infectivity (as measured by in vitro transformation of C127 cells) ratio obtained were very similar for BPV virions isolated from warts (2×10^4) or generated in BPHE-1 cells (10^4).

Generation and neutralization of infectious HPV16{BPV1} pseudotyped virions. Having demonstrated that coexpression of BPV1 L1 and L2 can result in encapsidation of BPV genomes, we wished to determine if genome encapsidation was type specific. L1 and L2 derived from HPV16 were therefore

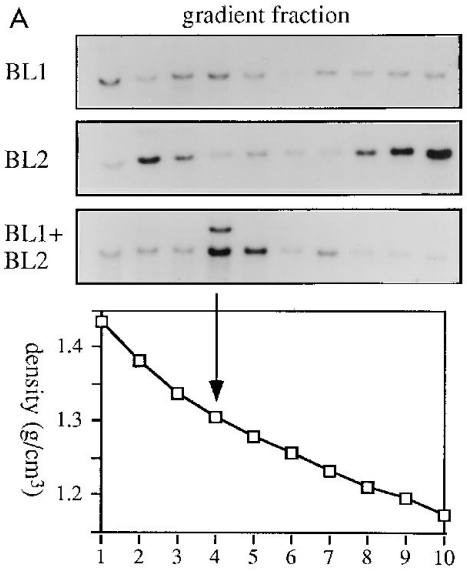
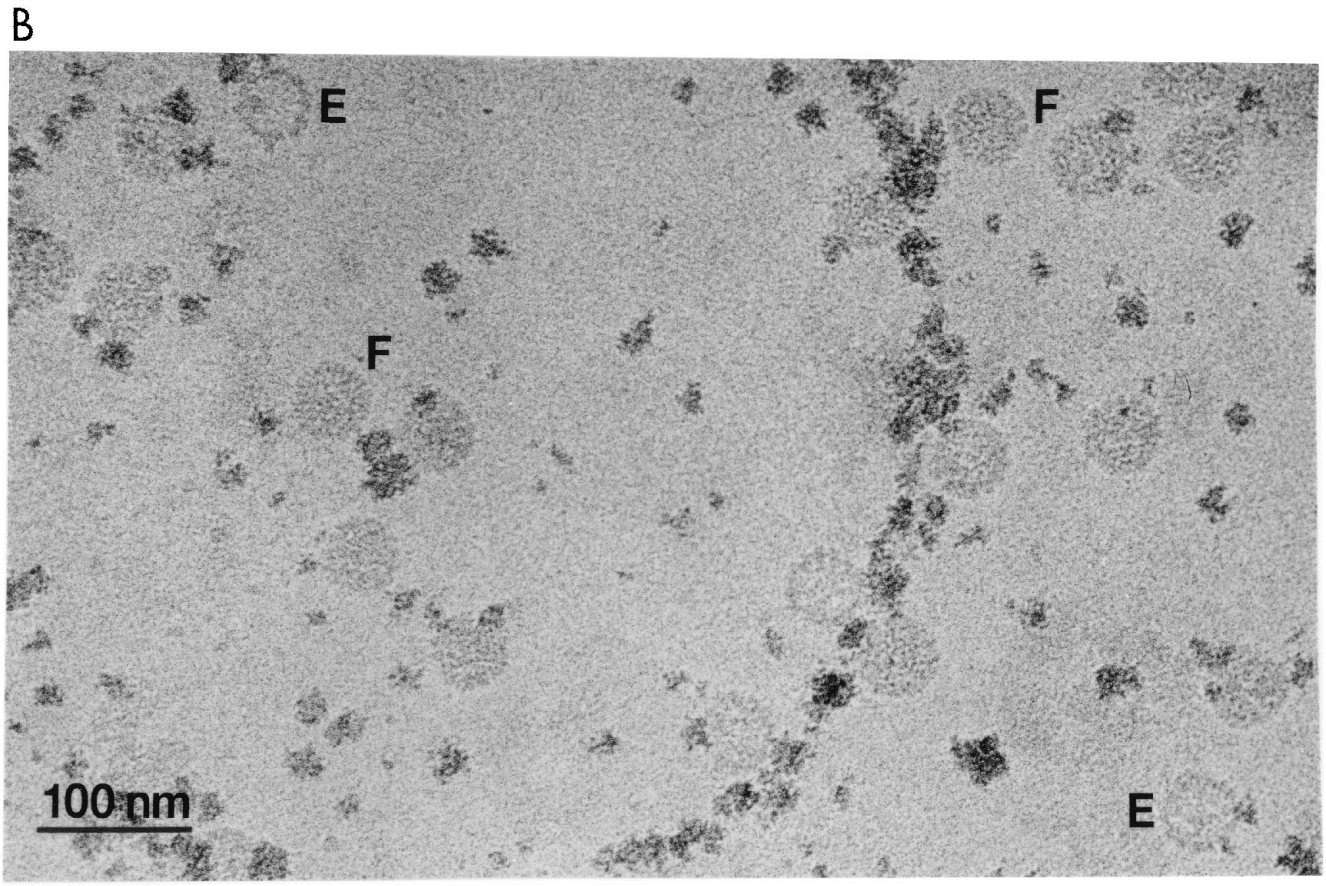


FIG. 3. Viral genomes are packaged into L1 plus L2, but not L1 alone, capsids. (A) BPHE-1 cells were infected with recombinant SFV expressing BPV1 L1 or BPV1 L2 alone or BPV1 L1 plus L2. Virions were purified from the cell extracts by centrifugation through a 40% (wt/vol) sucrose cushion and then cesium chloride isopycnic density centrifugation. Ten fractions were harvested from each cesium chloride gradient, and the density of each was measured with a refractometer (the density of infectious BPV virions purified from bovine warts is indicated with an arrow). DNA was obtained from each fraction, and the presence of BPV1 DNA was detected by Southern blot analysis. (B) The cesium chloride gradient fraction obtained from BPHE-1 cells expressing BPV1 L1 plus L2 with a density of 1.31 g/cm^3 and containing full-length DNase I-resistant BPV1 genomes (lane 4) was examined by cryoelectron microscopy. BPV1 particles containing correctly packaged DNA and those that are empty are indicated (F and E, respectively).



tested for the ability to encapsidate the BPV genome and thereby generate infectious pseudotyped virions. L1 and L2 derived from a wild-type HPV16 isolate (114K) were cloned into SFV vectors and expressed in BPHE-1 cells (15, 18). Expression was confirmed by Western blot analysis using monoclonal antibody CamVir-1 (Pharmingen) for L1 and rabbit antiserum to bacterially expressed glutathione *S*-transferase–HPV16 L2 fusion protein for L2 (not shown). Production of infectious virions was assessed by using the C127 focus-forming assay, as described for the experiment discussed above. Expression of the L1 and L2 derived from HPV16 in BPHE-1 cells consistently produced infectious virions (Fig. 4F), hereafter referred to as HPV16{BPV1} virus, although approximately 5- to 10-fold less efficiently than BPV L1 and L2

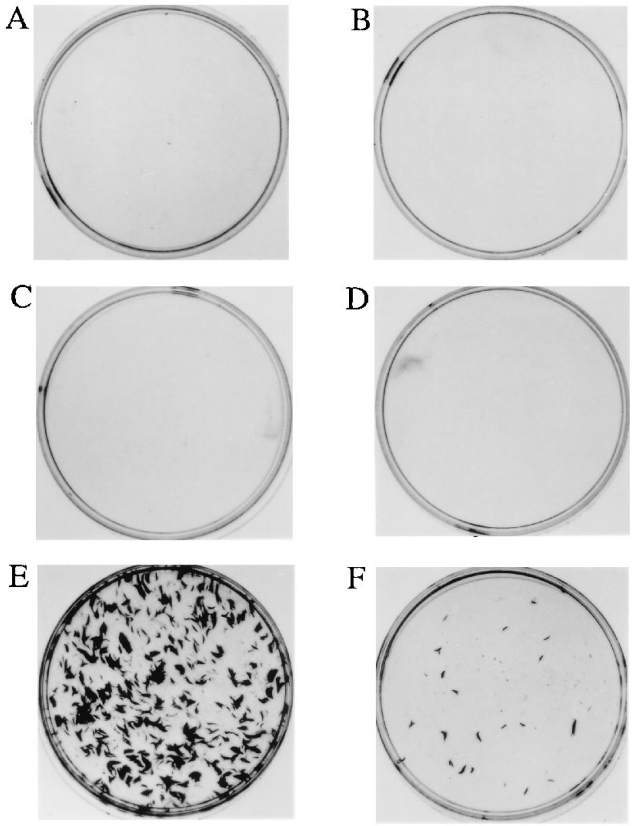


FIG. 4. Encapsidation of BPV1 genomes by HPV16 capsids. BPHE-1 cells ($\sim 4 \times 10^6$) were infected with recombinant SFV expressing HPV16K L1 alone (A), HPV16 L2 alone (B), HPV16 D202H mutant L1 plus HPV16 L2 (C), BPV1 L1 plus HPV16 L2 (D), BPV1 L1 plus BPV1 L2 (E), or HPV16K L1 plus HPV16 L2 (F). Thirty hours postinfection, the cells were lysed and plated over monolayers of C127 cells. The C127 cells were maintained for 3 weeks and stained.

(Fig. 4E). No foci were observed when BPV L1 and HPV16 L2 (Fig. 4D) or HPV16 L1 and BPV L2 (not shown) were coexpressed, suggesting a functional interaction between L1 and L2 that is type specific. However, low-efficiency encapsidation by heterologous pairs of capsid proteins cannot be discounted (Fig. 4). Furthermore, expression in BPHE-1 cells of L1 and L2 derived from a capsid assembly-deficient mutant of HPV16 did not produce any foci (Fig. 4) (18, 37).

Type-specific neutralization of pseudotyped virions. Treatment of the HPV16{BPV1} extracts with 5 or 50 μl of rabbit antiserum to 114K HPV16 VLPs prevented focus formation, whereas addition of antiserum to BPV1 VLPs, denatured BPV virions, or assembly-deficient HPV16 L1 of the prototype strain did not prevent focus formation (Fig. 5). Both antiserum to HPV16 L1 alone and antiserum to L1/L2 VLPs were neutralizing. Antiserum generated to the L1 VLPs of a divergent Zairian isolate of HPV16 also neutralized the HPV16{BPV1} virions (6). This finding demonstrates that infectious virus with HPV16 capsids, not BPV capsids, was produced and that infection of the C127 cells and not transfection by the BPV DNA had occurred.

The ability of antisera raised against VLPs derived from low-risk HPV6b or -11 and high-risk HPV18, -31, -33, or -45 to prevent infection by HPV16{BPV1} virions was also tested. All of these sera contain high titers of antibodies ($\geq 10^4$ [described in reference 28]) that recognize their corresponding VLPs in enzyme-linked immunosorbent assays (ELISA) and hemagglutination inhibition assays (28). However, none of the sera were able to prevent infection of the HPV16{BPV1} virions when 50 μl (or 5 μl not shown) was added to the pseudovirion extract (Fig. 5).

DISCUSSION

Despite some progress, difficulties in generating infectious papillomavirus virions in vitro and manipulating them genetically continue to limit studies of this tumor virus (13). Use of a mouse xenograft system has led to the limited production of HPV11 and an in vivo infectivity assay (8, 19). As an alternative approach, raft cultures of human keratinocytes can undergo relatively normal terminal differentiation, thereby permitting expression of the late proteins and virion biosynthesis (11, 23). Small quantities of morphologically correct HPV31b virions have been produced by this method, but no quantitative infectivity assay has been developed by using this system (23). Furthermore, neither the xenografts nor raft cultures are readily amenable to genetic manipulation.

In this study, we produced infectious papillomavirus by expressing the virion capsid proteins in *trans*, via defective SFV vectors, in cells that contain an intact viral genome. Production of infectious BPV was monitored by a standard, quantitative, in vitro BPV infectivity assay (12). BPV-induced focal transformation of C127 cells was specifically inhibited by incubating infectious preparations with neutralizing BPV antisera, which confirmed that the transformation resulted from BPV infection and not from transfection of viral DNA.

This method of virus production provides the opportunity to determine the functions of the virion proteins in virion formation and to generate virions with specific mutations. The presence of DNase I-resistant full-length BPV DNA in the extracts expressing L1 plus L2, but not either L1 alone or L2 alone, implies that L2 is required for encapsidation of the BPV genome.

The ability of L1 and L2 derived from HPV16 to encapsidate the BPV genome suggests either that this process is sequence independent or that if a viral DNA packaging signal exists for papillomavirus genomes, it must be conserved between the highly evolutionarily divergent BPV1 and HPV16. The apparent inability to generate infectious virus with BPV1 L1 and HPV16 L2 or HPV16 L1 and BPV L2 implies that either functional L1-L2 interactions are not conserved or virions with chimeric capsids are produced with efficiencies too low to be measured in our assay.

Using recombinant vaccinia virus as a vector for BPV1 L1 and L2, Zhou and colleagues previously concluded that both L1 and L2 were necessary to encapsidate viral DNA and to generate infectious BPV virions (43). Because their BPV preparations contained infectious vaccinia virus, which is cytotoxic for many cell types, including C127, they used transient expression of viral RNA as their marker for infectivity. One notable difference between the results reported in that study and those obtained here was that their infectivity marker was neutralized by antiserum to denatured BPV1 virions (DAKO). In contrast, our SFV-derived or cattle papilloma-derived virions induced focal transformation that was not inhibited by any of the several lots of this sera that were tested (Fig. 2 and data not shown), in agreement with previous reports that DAKO sera or other sera to denatured virions are nonneutralizing. Perhaps differences in the endpoint measure of infectivity in the two studies are responsible for these discordant results.

Our examination of particles purified from the BPV L1 plus L2 extracts by cryoelectron microscopy revealed that a large fraction of the particles have electron-dense cores, indicating

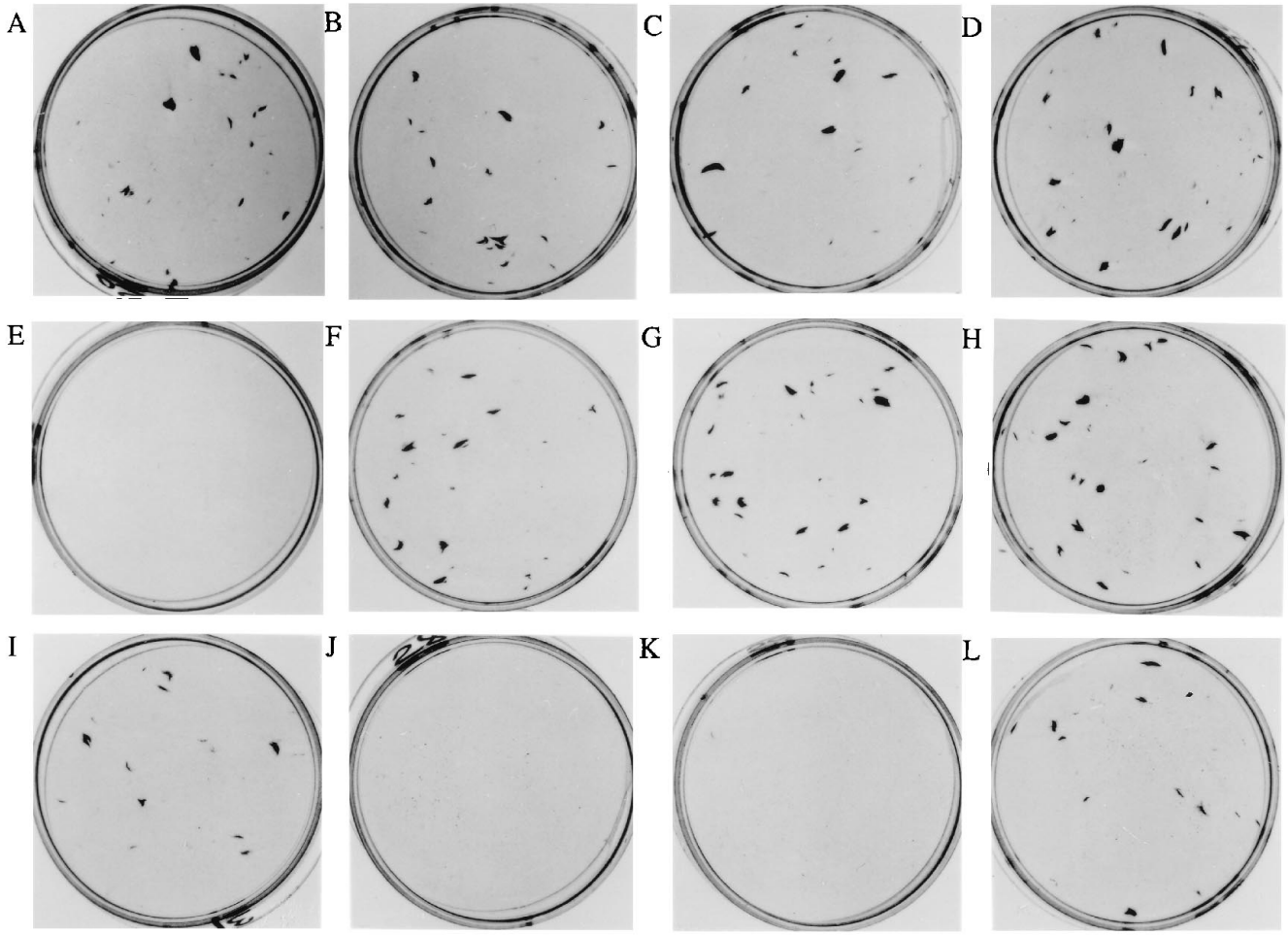


FIG. 5. Type-specific neutralization of HPV16{BPV1} virions. BPHE-1 cells expressing HPV16K L1 plus HPV16 L2 were harvested, lysed by sonication, and aliquoted. Aliquots were incubated with 50 μl of preimmune serum (A) or rabbit antiserum to purified baculovirus-expressed VLPs derived from BPV1 L1/L2 (B), HPV6b L1/L2 (C), HPV11 L1/L2 (D), HPV16 114/K L1/L2 (E), HPV18 L1/L2 (F), HPV31 L1 (G), HPV33 L1/L2 (H), HPV45 L1/L2 (I), HPV16 114/K L1 (J), HPV16 Zaire 1194 L1 (K), and reference HPV16 (D202H mutant L1) (L) for 1 h and then plated over monolayers of C127 cells. The C127 cells were maintained for 3 weeks and stained.

that they had encapsidated DNA (3). This finding suggests that the viral structural proteins efficiently packaged the viral genome. However, we estimated that the particle-to-infectivity ratio for in vitro-generated BPV virions was 10^4, suggesting that most of the full virions may be defective. This estimate for the particle-to-infectivity ratio is consistent with the high (0.5×10^3 to 1×10^3 virions per cell) multiplicity of HPV11 infection of human foreskin keratinocytes required to detect spliced viral RNA by reverse transcriptase PCR (38).

This high estimate of the particle-to-infectivity ratio, which is 1 to 2 orders of magnitude greater than that observed for simian virus 40 and polyomavirus (2, 10), does not simply reflect inefficient maturation in vitro, since a similar particle-to-infectivity was estimated for virions purified from bovine papillomas (2×10^4). Both L1 and L2 have been shown to bind nonspecifically to DNA in vitro, raising the possibility that the high particle-to-infectivity ratio could be due to packaging of cellular rather than BPV DNA (22, 44). However, restriction enzyme digestion analysis of the DNA encapsidated by wart-extracted virions suggests that most of the DNA packaged is viral (data not shown).

Expression of HPV16 L1 and L2 in cells containing the BPV genome produced infectious pseudotyped virions with HPV16 capsids. They induced typical BPV-type foci, and their infectivity was neutralized by HPV16 antisera and not by BPV antisera. Since HPV16 is not more closely related to BPV1 than are other high-risk HPV types, it is likely that a strategy similar to the one reported here for HPV16 can be used to generate infectious pseudotypes for other high-risk HPVs, and presumably for any papillomavirus. Although the focal transformation assay requires 2 to 3 weeks, this problem should in principle be circumvented by incorporation of a rapid and easily detectable marker into the BPV genome.

Results from a number of laboratories have suggested that despite their strict host range, papillomaviruses bind to a wide variety of cell types derived from diverse species (24, 29, 41). The ability of HPV16{BPV1} virions to induce focal transformation of C127 cells implies that C127 cells express the cell surface receptor for HPV16 virions and are competent to perform the subsequent steps of internalization and uncoating that are required for initiating viral infection. The simplest interpretation of these observations is that BPV and HPV16 share a common intracellular pathway of infection as well as a common cell surface receptor.

The titer of HPV16{BPV1} pseudotypes was 5- to 10-fold lower than for BPV virions produced in vitro. The basis for this

less efficient production remains to be determined. It may have resulted from the lower expression of HPV16 L1 than of BPV L1 observed in the SFV recombinant-infected BPHE-1 cells (data not shown). Alternatively, divergence in the putative packaging signals of the BPV and HPV16 genomes may have resulted in a less efficient encapsidation of the BPV DNA by the HPV16 capsid proteins.

The in vitro generation of HPV16{BPV1} pseudotyped virus has allowed us, for the first time, to develop an antibody neutralization assay for HPV16, since there is no source of infectious HPV16 or an easily scored quantitative assay for the genome of HPV16 or other high-risk genital HPVs. Titers of neutralizing antibodies induced by vaccination are the best correlate of protection for most previously developed prophylactic vaccines (26), as also seems true for the animal papillomavirus protection studies (5, 39). It is therefore important to investigate whether the HPV16 VLPs induce high titers of neutralizing antibodies and to determine the degree of cross-protection between various genital HPV types. Until now, it has been necessary to rely on surrogate assays for neutralization, such as ELISA and hemagglutination inhibition (27, 28, 31). Compared with neutralization, the VLP ELISA is relatively nonstringent because it may recognize nonneutralizing antibodies, while hemagglutination may be overly stringent because a class of neutralizing antibodies (defined for BPV, cottontail rabbit papillomavirus, and HPV11) does not score in that assay (28).

The assembly-deficient mutant L1 of the reference HPV16 strain did not induce detectable neutralizing antibodies, reinforcing the concept that most neutralizing epitopes are displayed only on intact particles. The observation that antibodies to a divergent assembly-competent variant (Zaire 1194 [6]), which differs from the 114/K HPV16 isolate at seven L1 amino acids, can efficiently neutralize the HPV16{BPV1} virions made with the 114/K isolate further suggests that VLPs of a single HPV16 variant will induce protection against divergent HPV16 variants (6). However, the pseudotyped virions were not neutralized by antisera to VLPs derived from six other genital HPV types or BPV1. This was true even though two of the VLP types tested, HPV31 and HPV33, are among those most closely related to HPV16, with 84 and 81% L1 amino acid sequence identity, respectively. These anti-VLP sera had titers in ELISA and hemagglutination assays based on the homologous VLP type of at least 10,000 (28); therefore, the negative results in the HPV16{BPV1} neutralization assay were not due to a poor antibody response to these VLPs. The data support the concept that HPV16 is a single serotype, distinct from other genotypes.

Our finding that antibodies elicited by assembled HPV16 VLPs can efficiently inhibit infection by the HPV16{BPV1} virions supports the potential utility of these VLPs as prophylactic vaccine candidates. To make an informed decision for the components of a multivalent VLP-based vaccine to prevent genital HPV infection, it will be necessary to evaluate to what extent antibodies generated against one type of HPV VLP will neutralize infection by other types. Our data that rabbit antibodies raised against VLPs derived from other genital HPV types did not neutralize HPV16{BPV1} infection suggest that protection obtained by neutralizing antibodies in humans against these genital HPVs will be type specific. The development of pseudotyped virions of other HPV types, along with HPV16{BPV1}, could be used to more broadly examine the question of cross-neutralization in animal studies and in early phases of human vaccine trials.

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