Infectious Virions Produced from a Human Papillomavirus Type 18/16 Genomic DNA Chimera

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The organotypic raft culture system has allowed the study of the differentiation-dependent aspects of the human papillomavirus (HPV) life cycle. However, genetic strategies to more completely understand the HPV life cycle are limited. The generation of chimeric viruses has been a useful tool in other virus systems to analyze infection and replication. To investigate the specificity of the interaction of nonstructural genes of one HPV type with the structural genes of another HPV type, we have replaced the L2 and L1 open reading frames (ORFs) of HPV type 18 (HPV18) with the L2 and L1 ORFs of HPV type 16 (HPV16). The resulting HPV18/16 chimeric construct was introduced into primary keratinocytes, where it was stably maintained episomally at a range of 50 to 100 copies of HPV genomic DNA, similar to that typically found in HPV-infected cells in vivo. The integrity of the HPV18/16 genomic DNA chimera was demonstrated. Upon differentiation in raft cultures, late viral functions, including viral DNA amplification, capsid gene expression, and virion morphogenesis, occurred. Chimeric HPV18/16 virions were purified from the raft cultures and were capable of infecting keratinocytes in vitro. Additionally, infection was specifically neutralized with human HPV16 virus-like particle (VLP)-specific antiserum and not with human HPV18 VLP-specific antiserum. Our data demonstrate that the nonstructural genes of HPV18 functionally interact with the structural genes of HPV16, allowing the complete HPV life cycle to occur. We believe that this is the first report of the propagation of chimeric HPV by normal life cycle pathways.

The life cycle of human papillomaviruses (HPV) is intimately connected to the differentiation program of host epithelial tissues (14, 20, 22, 36). The use of an organotypic (raft) epithelial culture system has allowed for the development of an in vitro culture system capable of reproducing the complete HPV life cycle, including the propagation of infectious viral particles (20, 22). The raft culture system has been used to describe in detail the steps in the HPV life cycle (2, 8, 11, 18, 25–30), including the kinetics and spatial patterns of HPV gene expression (18, 25–29). Flores et al. used the raft culture system to begin a genetic analysis of the HPV life cycle by using an E7-deficient HPV type 16 (HPV16) genome (7). They found that this genome, while being maintained episomally, failed to amplify its DNA and expressed reduced levels of the L1 capsid protein. That study was done by using a spontaneously immortalized keratinocyte cell line (1). Attempts have been made to use a genetic approach to study the HPV life cycle by using primary keratinocytes (15, 39). These studies found that the majority of mutations examined, both in noncoding and in coding regions, were unstable in their ability to maintain the viral DNA (vDNA) in an episomal state.

It has been reported that the interaction of the HPV nonstructural proteins, in particular, E2, with the structural capsid proteins, L1 and L2, is important for viral morphogenesis (5, 13). We designed experiments to investigate whether the nonstructural genes from one HPV type could functionally interact with the structural genes of another HPV type, allowing the complete viral life cycle to occur, with the production of infectious progeny. To explore the probability of the functional interaction and the relatedness of the nonstructural and structural genes of two different HPV types, we used the genetic approach of making a chimeric virus.

The generation of chimeric viruses has been a useful genetic tool in other virus systems to analyze viral infectivity, replication, transformation, and virulence factors (3, 4, 6, 9, 10, 12, 16, 17, 23, 24, 33, 34, 37, 38, 40, 41, 43). Chimeric viruses are commonly used to compare genes from one virus with the homologous genes from a related virus to determine the similarities and differences of these genes. A chimeric virus system can be used to assign a particular viral phenotype to a specific gene or sequence. Another use of a chimeric virus system is to ascertain the commonalities of related viral genes. A chimeric virus is typically generated by replacing a gene sequence from one virus with the similar sequence from a related but different virus. Information gained from these kinds of studies includes defining mechanisms of viral replication and pathology and identifying common mechanisms for therapeutic targeting. In addition, chimeric viruses containing altered and optimized capsid protein epitopes could be generated and used as a tool in viral vaccine development.

We have replaced the L1 and L2 capsid protein open reading frames (ORFs) from HPV type 18 (HPV18) with the L1 and L2 capsid protein ORFs from HPV16. The resulting
TABLE 1. Oligonucleotide primers used for construction of the HPV18/16 chimera

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Oligonucleotide sequence (5’ to 3’)</th>
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<tr>
<td>18 AatII</td>
<td>CGG CCA GAC GTC GGC TGC TAC ACG</td>
</tr>
<tr>
<td>18 BglII B</td>
<td>GCT AGC AGA TCT ACT TTT ATT ACA AAA ATA CAA AAA GC</td>
</tr>
<tr>
<td>18 AflII</td>
<td>GTA TGC AAT TAG CTT AAG TAA AAA CAA AC</td>
</tr>
<tr>
<td>18 BglII F</td>
<td>GCT AGC AGA TTT GTG TGT GTT TAT ATA TAT CAT</td>
</tr>
<tr>
<td>16 L2 BglII F</td>
<td>GCT AGC AGA TCT ATG CAG AAA CGT TCT GCA AAA CG</td>
</tr>
<tr>
<td>16 L1 BglII B</td>
<td>GCT AGC AGA TCT TCA CAG CTT ACG TTT GCG TTT AGC AG</td>
</tr>
<tr>
<td>16 L1 EcoRIM1</td>
<td>CTA TAC ATA CAT TCT ATG AAC TCT ATG ATT TGT GAG</td>
</tr>
<tr>
<td>16 L1 EcoRIM2</td>
<td>CTC CAA AAT GGA GGT CAT AGA ATG TAT GTA TG</td>
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* The EcoRI site is underlined, and the mutated nucleotide is shown in bold type.

HPV18/16 chimeric construct was introduced into primary keratinocytes and then allowed to grow and differentiate in raft cultures. The HPV18/16 chimeric genomic DNA immortalized primary keratinocytes, was maintained in an episomal state, and induced a transformed phenotype in raft cultures. The episomal HPV18/16 genomic DNA maintained the integrity of the chimeric structure. Late viral functions of vDNA amplification and capsid gene expression appeared normal in raft cultures. The expression of the HPV16-specific structural gene, L1, was demonstrated, and HPV18/16 virus was produced. Finally, early transcript expression and neutralization with HPV16 virus-like particle (VLP)-specific antisera demonstrated the propagation of infectious virus. This is the first report of the propagation of chimeric HPV virions by normal life cycle pathways.

**MATERIALS AND METHODS**

**Plasmid construction.** HPV18 DNA, a generous gift from Harold zur Hausen, was cloned into pBluescript SK(+) [pBSSK(+)]) (Stratagene, La Jolla, Calif.) at the unique HPV18 EcoRI site within the E1 ORF, a unique site within the HPV18 upstream regulatory region (URR). In order to substitute the HPV16 L2 and L1 ORFs with the HPV16 L2 and L1 ORFs, pBHHSV18 was digested at the unique AarII site within the E2 ORF, and the digested DNA was purified with a Centrisep column (Princeton Separations, Inc., Adelphia, N.J.). The purified DNA was further digested with AphiII, and the resulting 6,647-bp fragment containing only HPV18 early gene and vector sequences was gel purified by using a QIAquick gel extraction kit (Qiagen, Inc., Valencia, Calif.). Using oligonucleotides (sequences are shown in Table 1) named 18 AatII and 18 BglII B (The Midland Certified Reagent Co.), a 593-bp PCR product containing HPV18 DNA from the stop codon at nt 7198 to 7173 (3′) and nt 5426 to 5452 (5′) of the HPV18 genome was amplified by using primers corresponding to the HPV18 URR. The amplified PCR product was digested with AphiII, and the 5′ end of the PCR product contained an AphiII restriction site, and the 3′ end contained a BglII restriction site. Using oligonucleotides (Table 1) named 18 AphiII and 18 BglII F (The Midland Certified Reagent Co.), a 593-bp PCR product containing HPV18 DNA from the stop codon at nt 7198 to 7173 (3′) and nt 5426 to 5452 (5′) of the HPV18 genome was amplified by using primers corresponding to the HPV18 URR. The amplified PCR product was digested with AphiII, and the 5′ end of the PCR product contained a BglII restriction site, and the 3′ end contained an AphiII restriction site. The 720- and 593-bp products were purified by using a QIAquick PCR purification kit (Qiagen). The 720-bp product was digested with AarII, and the 593-bp product was digested with AphiII. The AarII/AphiII-digested 6,647-bp product was then ligated to the 720- and 593-bp PCR products, and the resulting ligation product was digested with BglII, purified, and religated. The resulting construct, pBHHSV18L2L1, contains HPV18 sequences on a pBSSK(+) backbone with the L2 and L1 ORFs removed and replaced with a BglII site.

Oligonucleotides (Table 1) named 16 L2 BglII F and 16 L1 BglII B were used to generate a 2.9-kb PCR product containing the HPV16 L2 and L1 ORFs. The DNA template was derived from CaSk cell HPV16 L2 and L1 DNAs previously cloned into pUC18 (Promega, Madison, Wis.). Both the 5′ and 3′ ends of the 2.9-kb PCR product contained a BglII restriction site. The 2.9-kb PCR product was digest with BglII and purified.

Plasmid pBHHSV18L2L1 was digested with BglII, dephosphorylated, and ligated to the BglII-digested HPV16 L2-L1 2.9-kb PCR product. The resulting plasmid contained the complete URR and early genes of HPV18 and the late genes, L2 and L1, of HPV16. The HPV16 L1 ORF contained a single EcoRI restriction site. The electroporation protocol requires the release of the HPV genomic DNA from the vector DNA sequences. Since the HPV18 genomic DNA was cloned into the vector DNA at an EcoRI site, it was necessary to mutate the EcoRI site contained within the HPV16 L1 ORF at nt 6819. Site-directed mutagenesis was performed by using a Quick Change site-directed mutagenesis kit (Stratagene) according to manufacturer directions. To alter the EcoRI site of the HPV16 L1 protein without altering the amino acid sequence, oligonucleotides (Table 1) named 16 L1 EcoRIM1 and 16 L1 EcoRIM2 were used. The EcoRI restriction site GAATTCA was changed to GAACCTC, altering the Asn code used from AAT to AAC. Sequence and restriction endonuclease analyses confirmed the single nucleotide change in the mutated EcoRI restriction site. The final plasmid was designated pBHHSV18/16 (Fig. 1). pCR18-L1 was produced by TA cloning (Invitrogen, San Diego, Calif.) a 1,701-bp PCR product generated by amplification with primers corresponding to nt 5426 to 5452 (5′) and nt 7198 to 7173 (3′) of the HPV18 genome. pHPV16RL1 was produced by first cloning the HPV16 genomic DNA into vector pBSSK(+) at the unique BamHI site at nt 6151 within the L1 ORF, making plasmid pBSSH18p. pBHHSV16 was then digested with EcoRI. EcoRI digested pBSSH16 in three places, once in the pBSSK(+) polylinker and twice in the HPV16 EcoRI site.

FIG. 1. HPV18/16 chimeric recombinant plasmid. Speckled areas represent HPV18 early genes and the URR. The striped area represents HPV16 late genes. The approximate positions of the HPV ORFs are shown in insets. White areas represent the pBSSK(+) vector; the position of the Amp’ gene is shown. HPV18 was cloned into pBSSH(+) at the unique EcoRI site. The HPV18 late gene ORFs were removed and replaced with a unique BglII site. The HPV16 late gene ORFs were inserted into this unique BglII site.
HPV16 sequence, at nt 6819 and 7454. The 3.6-kb fragment containing pBSK I vector sequences and HPV16 L1 ORF at nt 6151 to 6819 was gel purified and recircularized, creating pHVP16-RL1.

Cell and raft cultures. Primary human foreskin keratinocytes (HKF) were purchased from BioWhittaker (Walkersville, Md.). Keratinocytes were grown in monolayer cultures by using KGM-2 growth medium (BioWhittaker). The HCK18:1B cell line (22) and keratinocyte cell lines stably maintaining HPV18/DNA following electroporation were grown in monolayer cultures by using E medium (unless otherwise stated, our E medium lacks epidermal growth factor [EGF]) in the presence of mitomycin C-treated J2 J3T feeder cells (19, 20, 22).

Raft cultures were grown as previously described (19, 20, 22). Briefly, cell lines were seeded onto rat tail type I collagen matrices containing J2 J3T feeder cells not treated with mitomycin C. After cell attachment and growth to confluence, collagen matrices were lifted onto stainless steel grids. Once lifted to the air-liquid interface, raft cultures were fed by diffusion from below with E medium. Raft cultures were treated with 10 μM 2,3-diacetonyl-1-glycerol (CS; Sigma Chemical Co., St. Louis, Mo.) in E medium every other day. Viral gene expression has been shown to peak after 10 to 12 days in the raft system (25–29); therefore, raft cultures were allowed to stratify and differentiate for 12 days.

Electroporation of primary keratinocytes. HPV18/16 plasmid DNA was digested with EcoRI, linearizing the vDNA and separating it from the vector DNA. For each electroporation, we mixed 1 μl of EcoRI-digested pHVP18/16 plasmid DNA (1 μg/μl) and 4.25 μl of sonicated and denatured salmon sperm DNA (10 ng/μl) in a 1.5-ml Eppendorf tube. Keratinocytes (5 × 10⁶) in a volume of 250 μl of E medium containing 10% fetal bovine serum (FBS) and 5 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid were added to each DNA mixture. DNA-keratinocyte solution was transferred to an electroporation cuvette and electroporated by using a Gene Pulser (Bio-Rad Laboratories, Hercules, Calif.) set at 210 V and 960 μF. The electroporated cell solution was then layered onto 10 ml of E medium containing 10% FBS and centrifuged at 25 °C for 10 min. The medium was removed, the cell pellets were resuspended in E medium containing 10% FBS, and the suspensions were added to 10-cm tissue culture plates. Sections were stained with hematoxylin and eosin as described previously in 10% buffered formalin, and embedded in paraffin.

Southern (DNA) blot hybridization. Total cellular DNA was isolated as previously described (20, 22, 25); 5-μg samples were digested with EcoRI or BglII or left undigested and then electrophoresed in an 0.8% agarose gel. Southern blot hybridization was performed as previously described (20, 22, 25). Blots were probed first with an HPV18 complete genomic probe and then were stripped and probed with an HPV16 complete genomic probe. Stripping was accomplished by placing the blots in 0.1% SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate and boiling for 1 h.

Histochemical analyses. Raft cultures were grown for 12 days, harvested, fixed in 10% buffered formalin, and embedded in paraffin. Sections were cut at 3 to 5 μm and stained with hematoxylin and eosin as described previously (18, 20, 21, 29, 30, 42). Immunostaining was done by using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif.) (18, 20, 30, 42). HPV16 VLP-specific antisera, a generous gift from John T. Schiller, was used for immunostaining (1:500) (31, 32). HCK18:1B raft cultures were raised as positive and negative controls.

RPAs. Antisense HPV16 and HPV18 L1 RNA probes were synthesized by using constructs pCR18-L1 and pHPV16-RL1. Total RNA was extracted from raft cultures by using TRZol reagent according to manufacturer instructions (Gibco BRL, Bethesda, Md.) and was treated with DNase I to remove contaminating cellular DNA. Antisense RNA probes were synthesized as previously described (35). HaCaT cells were grown to 50% confluence in 96-well flat-bottom tissue culture plates and cultured in Dulbecco’s modified Eagle’s medium containing (final concentrations) 10% fetal bovine serum, 100 U of penicillin/ml, 100 μg of streptomycin/ml, 4,500 ng of glucose/liter, L-glutamine, and pyridoxine hydrochloride but no sodium pyruvate. At the time of infection, the medium was aspirated and 50 μl of HPV18/16 diluted to 1:20 was added. For antibody–mediated neutralization, the final dilutions of polyclonal serum used were 1:200, 1:400, 1:800, and 1:1,600. Twenty-five microliters of diluted serum was added to the cells, and the plates were rocked back and forth. Virus diluted in a volume of 25 μl for a final virus concentration of 1:200, was added immediately. Dilutions of virus and serum were made with Opti-Mem (Gibco BRL) and incubation for 1 h at 37°C, with occasional rocking to mix. An additional 100 μl of Dulbecco’s modified Eagle’s medium was then added to each well, and the plates were incubated at 37°C in 5% CO₂ for 4 days.

The human sera used in this study was obtained from individuals shown to be PCR negative for HPV16 and HPV18 prior to immunization with either HPV16 VLPs or HPV16 L1 RNA, and postimmunization sera from a total of three individuals immunized with either 16 VLPs or 18 VLPs were tested.

Detection of the spliced HPV18 E1-E4 message and cellular β-actin mRNA by RT-PCR. HPV18/16 infection of HaCaT cells after 4 days of culturing was demonstrated by reverse transcriptase PCR (RT-PCR) amplification of the HPV18 E1-E4 mRNA species (35). RNA was extracted from the cells by using the Qiagen RNaseasy 96-well method as described by the manufacturer. Efficient and sensitive detection of HPV18 E1-E4 in this cell culture system required the use of nested PCR primer sets. The locations of the primers used in these studies and their nucleotide sequences are shown in Table 2. A Gibco Quantitative Platinum one-step RT-PCR kit was used with the first set of primers, H18-F1, H18-R1, BA-U1, and BA D2, and the conditions were as follows: 50°C for 30 min for the RT reaction; 95°C for 5 min, 95°C for 1 s, 65°C for 30 s, and 72°C for 30 s for 25 cycles; 72°C for 30 s for the final cycle; and a 4°C hold. A portion of this reaction (10 μl from the 50-μl reaction) was then used in a second set of PCRs with nested primers H18-F2 and H18-R2 and the following temperature profile: 95°C for 1.3 min, 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s for 35 cycles; 72°C for 7 min; and a 4°C hold. The final amplifier sizes were 733 bp for β-actin and 521 bp for HPV18 E1-E4. The final concentration of deoxyribonucleotides triphosphates during cDNA synthesis and PCR was 0.2 mM, and the final concentration of primers was 0.2 μM.

Negative control PCRs were routinely performed to confirm the absence of contamination events. The PCR products were separated by 2% agarose–ethidium gel electrophoresis and photographed with Polaroid film.

RESULTS

Construction of an HPV18/16 chimeric genomic plasmid. To investigate whether the nonstructural genes of one HPV type could functionally interact with the structural genes of a second HPV type during the complete viral life cycle, we constructed a recombinant plasmid consisting of the URR and the nonstructural early gene ORFs of HPV18 and the structural late genes ORFs of HPV16 (Fig. 1). We used a plasmid containing the complete HPV18 genome cloned at the unique EcoRI site
within the E1 ORF (22). The HPV18 L2 and L1 ORFs were removed and replaced with a novel BglII site, resulting in pBSHPV18L2/L1. The HPV16 L2-L1 ORF sequence was amplified by using PCR primers which added BglII restriction sites to both ends. The amplified HPV16 L2-L1 ORF sequence containing BglII at its ends was ligated to pBSHPV18L2/L1 at its unique BglII site. We designated the resulting chimeric plasmid pHPV18/16.

Development of cell lines maintaining episomal copies of HPV18/16 chimeric genomic DNA. In order to develop cell lines maintaining episomal copies of HPV18/16 chimeric DNA, we used an electroporation technique previously developed by our laboratory to establish cell lines maintaining episomal copies of wild-type HPV18 (22). The pHPV18/16 chimeric plasmid was digested with EcoRI, linearizing and separating the HPV18/16 genomic DNA from the vector DNA. Several different batches of primary HFK were electroporated with the linearized HPV18/16 chimeric DNA. Cells from an individual electroporation were pooled and expanded into cell lines. Recircularization and episomal maintenance of the HPV18/16 chimeric genome were confirmed by Southern blot hybridization (Fig. 2). All cell lines maintained HPV18/16 chimeric genomic DNA episomally, most averaging approximately 50 to 100 copies per cell (Fig. 2, lanes 3, 6, 9, 12, 15, and

<table>
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<th>Primer description</th>
<th>Location in nested set</th>
<th>Genomic nucleotides</th>
<th>Nucleotide sequence (5' to 3')</th>
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<td>HPV 18</td>
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<tr>
<td>H18-F1</td>
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<td>530–553</td>
<td>CAACCGAGCAGCACAGGAACGAC</td>
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<td>3583–3604</td>
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<td>554–574</td>
<td>TCCAAGCAGCACAGAAACAC</td>
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<td>3560–3580</td>
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<td>β-Actin</td>
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<td>2735–2744 and 2857–2876</td>
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FIG. 2. Southern (DNA) blot hybridization of chimeric HPV18/16 DNA-electroporated HFK cell lines grown in monolayer cultures. Six independently derived cell lines, 2A, 3A, 3B, 3C, 4C, and 5C, were analyzed for the episomal maintenance, copy number, and integrity of the chimeric HPV18/16 genomic DNA. (A) The blot was probed with an HPV18-specific probe. (B) The blot was stripped and reprobed with an HPV16-specific probe. Samples in lanes 1, 4, 7, 10, 13, and 16 were undigested. Samples in lanes 2, 5, 8, 11, 14, and 17 were digested with EcoRI, a single cutter of the HPV18/16 genome. Samples in lanes 3, 6, 9, 12, 15, and 18 were digested with BglII to separate the HPV18 sequences (early ORFs) from the HPV16 sequences (late ORFs). HPV18 genomic 100-copy-number standards (left side) and HPV16 genomic 100-copy-number standards (right side) are shown. Arrows indicate form I DNA (FI), form II DNA (FII), form III DNA (FIII), early ORFs (HPV18), and late ORFs (HPV16).
HPV18/16:5C, and HPV18/16:5E. Two general patterns were observed for four representative cell lines, HPV18/16:2A, HPV18/16:3A, and HPV18/16:5C, representing the two tissue morphology patterns observed. The HPV18 L1-specific probe protected a 420-nt fragment only when RNA from HPV16 VLPs (31, 32). In tissues displaying numerous koilocytes, parakeratosis, and a greatly disturbed cornified layer, high levels of L1 capsid protein were clearly expressed in the nuclei of cells of the intermediate layers (Fig. 3). Tissues with only occasional koilocytes had L1 capsid protein expression detected by IHC analysis to a similar magnitude across the upper strata. Raft culture tissues grown from HCK18:1Bj, an HPV18-positive cell line, were found negative for the expression of L1 capsid protein by use of HPV16 VLP-specific antisera (data not shown). These data suggest that there is a correlation between the tissue morphology and cytopathic effects (CPE) of a viral infection and the ability to express viral structural genes.

**RPAs to identify the specific expression of the HPV16 L1 capsid protein in HPV18/16 chimeric raft cultures.** Southern blot analysis definitively confirmed that the chimeric nature of the viral genomes was maintained in the cell lines that we developed. IHC analysis provided evidence that the HPV16 L1 capsid protein was efficiently expressed from the chimeric vDNA. To provide further evidence that the major HPV16 L1 capsid protein was being expressed and to compare the levels of expression between the two morphological tissue patterns observed, RPAs were performed. Two cell lines, HPV18/16:2A and HPV18/16:5C, representing the two tissue morphology patterns, were chosen for this analysis. Total RNA was harvested from HPV18/16:2A and HPV18/16:5C raft culture tissues allowed to grow and differentiate for 10 days at the air-liquid interface. For a positive control of HPV18 L1 expression, we used the HCK18:1Bj cell line, capable of undergoing the complete viral life cycle, including the synthesis of infectious HPV18 particles (22). HCK18:1Bj cells were also allowed to grow and differentiate for 10 days in raft cultures, and then total RNA was harvested.

RNA samples were analyzed by RPAs with antisense RNA probes specific to internal regions of the HPV16 and HPV18 L1 ORFs. The HPV16 and HPV18 L1-specific probes were made to protect fragments of 267 and 420 nt, respectively. In both HPV18/16 chimeric cell lines, 267-nt fragments were protected when the HPV16 L1-specific probe was used, whereas the HPV18 L1-specific probe did not produce a protected fragment when RNA from the chimeric virus tissues was used (Fig. 4). The film was overexposed to demonstrate the difference in expression between the two cell lines representing the two tissue morphology patterns observed. The HPV18 L1-specific probe protected a 420-nt fragment only when RNA from HCK18:1Bj raft culture tissue was used (Fig. 4). Like the
FIG. 3. IHC analysis of chimeric HPV18/16-infected tissues. HPV18/16-infected, fully stratified and differentiated raft culture tissues were stained with hematoxylin and eosin (A, C, E, and G) or immunostained with an HPV16 L1 VLP polyclonal antiserum (B, D, F, and H). Arrowheads indicate examples of L1 protein expression staining positive within tissue thin sections. Four independently derived cell lines, 2A, 3A, 5C, and 5E, are shown representing the two phenotypic patterns observed. The first phenotypic pattern (2A and 3A) exhibits a more disturbed differentiation program reminiscent of cervical intraepithelial neoplasia type I or II and a high level of L1 expression. The second phenotypic pattern (5C and 5E) exhibits a much less disturbed differentiation program and a low level of L1 expression.
results observed in the IHC analysis, the RPA results showed that HPV18/16:2A raft cultures produced much higher levels of HPV18 L1 transcripts. This finding was consistent with the data from Southern blot analysis, which showed that HPV18/16:2A raft cultures produced much higher levels of viral genomic DNA compared to HPV18/16:5C raft cultures. These data suggest a direct relationship between tissue CPE and the expression of viral capsid proteins.

**Amplification of viral genomic DNA in HPV18/16 cell lines grown in raft cultures.** It appeared that the tissue phenotype was directly related to the competency for expressing late viral life cycle functions, such as viral capsid protein expression. Another important step in the life cycle of HPV is the amplification of its genomic DNA in differentiating cells of the host tissue in raft cultures. Ten micrograms of DNase I-treated total RNA or yeast RNA was analyzed. The locations and sizes of RNA Century Markers (Ambion) are shown between panels A and B in nucleotides. (A) Samples were probed with an HPV16 L1-specific probe (340 nt) which was predicted to specifically protect an mRNA fragment 267 nt in length. Lane 1 contains RNA from chimeric HPV18/16:2A raft culture tissue. Lane 2 contains RNA from chimeric HPV18/16:5C raft culture tissue. Lane 3 contains RNA from HPV18-infected raft culture tissue. Lane 4 contains RNA samples digested with RNase to show probe specificity. Lane 5 contains yeast RNA samples left undigested to show the sizes of the input probes. (B) Samples were probed with an HPV18 L1-specific probe (496 nt) which was predicted to specifically protect a fragment 420 nt in length. Lane 1 contains RNA from chimeric HPV18/16:2A raft culture tissue. Lane 2 contains RNA from chimeric HPV18/16:5C raft culture tissue. Lane 3 contains RNA from HPV18-infected raft culture tissue (HCK18:1B cell line). Lane 4 contains yeast RNA samples digested with RNase to show probe specificity. Lane 5 contains yeast RNA samples left undigested to show the sizes of the input probes.

Neutralization analyses. After determining that our HPV18/16 stocks were infectious, we wanted to determine whether antisera raised against HPV16 L1 VLPs was capable of neutralizing viral infectivity. HaCaT cells were incubated in the presence of dilutions of pre- and postimmune HPV16 L1 VLP or HPV18 L1 VLP human sera and chimeric HPV18/16. Three separate sets of HPV16 L1 VLP or HPV18 VLP human sera were used. Cells were harvested after 4 days, and virus infection was detected by the presence of the HPV spliced E1-E4 transcript. Nested RT-PCR produced a 521-bp HPV18 E1-E4 product (Fig. 7). Chimeric HPV18/16:2A was infectious to a 1:400 dilution in our HaCaT cell assay, while the HPV18/16:5C preparation did not contain detectable infectious virions.

DISCUSSION

Using HPV chimeric genomic DNA as a genetic tool, we have investigated the effect on the viral life cycle of exchanging either HPV18 or HPV16 demonstrated a linear band approximately 7,800 nt in length, consistent with linearized HPV18/16 genomic DNA (Fig. 5, lanes 2, 5, 8, and 11). HPV16-probed blots showed a band approximately 2,900 nt in length, the size of the HPV16 L2 and L1 ORFs following digestion with BglII (Fig. 5, lanes 3 and 6). HPV18-probed blots showed a band approximately 4,900 nt in length, a size consistent with the HPV18 URR and early ORFs following digestion with BglII (Fig. 5, lanes 9 and 12). Whether probed with HPV18 or HPV16, blots containing undigested DNA exhibited supercoiled (F1) and nicked (FII) genomic DNA (Fig. 5, lanes 1, 4, 7, and 10).

While both cell lines had equivalent viral genomic copy numbers in monolayer cultures, it was observed that HPV18/16:2A cells amplified their vDNA during differentiation of the host tissue in raft cultures, whereas HPV18/16:5C cells did not amplify their vDNA during host tissue differentiation (Fig. 5, compare lanes 2 and 5 or lanes 8 and 11). These data suggest that the tissue phenotype directly relates to the efficiency with which late viral life cycle functions occur. They also suggest that a viral genome dosage effect dependent on the level of amplified viral genomes in differentiating tissues and not the maintenance copy number seen in proliferating monolayer cell cultures may be associated with CPE and structural gene expression.

Infectious chimeric HPV18/16 biosynthesis. The final step in the HPV life cycle is the biosynthesis of infectious viral particles. To investigate the possibility that chimeric HPV18/16 raft culture tissues allow for the morphogenesis of virions, putative viral stocks were prepared and examined by electron microscopy. Electron microscopic examination showed the presence of viral particles of the proper shape and approximately 50 to 55 nm in diameter (Fig. 6).

We next wanted to know whether our HPV18/16 stocks were infectious in an HaCaT cell infection assay (35). HaCaT cells were infected with dilutions of chimeric HPV18/16 stocks. Cells were harvested after 4 days, and virus infection was detected by the presence of the HPV spliced E1-E4 transcript. Nested RT-PCR produced a 521-bp HPV18 E1-E4 product (Fig. 7). Chimeric HPV18/16:2A was infectious to a 1:400 dilution in our HaCaT cell assay, while the HPV18/16:5C preparation did not contain detectable infectious virions.

The results observed in the IHC analysis, the RPA results showed that HPV18/16:2A raft cultures produced much higher levels of HPV16 L1 than did HPV18/16:5C raft cultures. These data suggest a direct relationship between tissue CPE and the expression of viral capsid proteins.
the structural genes between two different HPV types while maintaining the nonstructural genes. We created a genomic construct containing the URR and early (nonstructural) genes of HPV18 combined with the late (structural) genes of HPV16. When this HPV chimeric genomic DNA was introduced into keratinocytes, it was maintained in an episomal state at approximately 50 to 100 copies per cell. Southern blot analysis demonstrated that the chimeric genotype of the vDNA was stable. When HPV18/16-infected cells were allowed to fully stratify and differentiate, they behaved in a manner similar to that of wild-type HPV18-infected cells in our raft culture system (22). When HPV18/16-containing cells were allowed to fully stratify and differentiate in raft cultures, chimeric genomic DNA was amplified, capsid genes were expressed, and complete virion morphogenesis occurred. Diagnostic restriction digestion and Southern blotting demonstrated that the cells contained vDNA composed of the URR and early genes of HPV18 and the late genes of HPV16. Chimeric HPV18/16 raft cultures specifically expressed HPV16 L1 capsid genes. The virions produced in raft cultures were shown to specifically express HPV18 early genes and HPV16 late genes. The infectivity of the viral stocks was neutralized by antibodies raised against HPV16 VLPs and not by antibodies raised against HPV18 VLPs. These data demonstrate that the nonstructural genes of HPV18 can interact with the structural genes of HPV16 to efficiently reproduce the complete HPV life cycle, including the production of infectious virions.

During our studies, we observed an interesting correlation between the raft culture tissue phenotype and the ability of the tissue to produce infectious viral stocks. Raft culture tissues grown from cell lines represented by HPV18/16:2A and HPV18/16:3A exhibited numerous koilocytic cells in the intermediate layers. This finding correlated with a high level of expression of L1 transcripts, as determined by RPA (Fig. 4), a high level of expression of L1 capsid protein, as determined by IHC analysis (Fig. 3), a high level of genomic amplification (Fig. 5), and the production of infectious progeny virus (Fig. 6 and 8). Cell lines HPV18/16:5C and HPV18/16:5E represented a second tissue phenotype. In raft cultures, this tissue phenotype was only slightly disturbed (Fig. 3), exhibiting only a rare koilocytic cell in the intermediate layers. This finding correlated with a low level of expression of L1 capsid protein, as determined by IHC analysis (Fig. 3), a low level of genomic amplification (Fig. 5), and no detectable production of infectious progeny virus (Fig. 7). Interestingly, in the undifferentiated state, all of the cell lines maintained approximately the same copy number of genomic vDNA molecules episomally per cell (Fig. 2), regardless of their phenotype in raft cultures.
or their ability to express late viral life cycle functions. This result indicates that neither vDNA copy number nor episomal maintenance directly correlates with the ability of a cell line to support the complete viral life cycle in stratifying and differentiating tissues. We hypothesize that the differences between the cell lines could be due to quantitative or qualitative differences in viral gene expression and/or an undefined selection process of the cells during the electroporation and immortalization process.

It has been proposed that the papillomavirus E2 protein plays a role in the selective encapsidation of papillomavirus DNA. Using immunofluorescence staining, it was observed that in the absence of other viral components, the E2 and L1 proteins were relocalized into promonocytic leukemia protein oncogenic domains when L2 was expressed (5). This L2-dependent colocalization suggests a mechanism whereby the assembly of viral particles is augmented by an increase in the local concentrations of viral gene products involved in virion morphogenesis, raising the potential of interactions with necessary viral components. Recently, a direct protein-protein interaction between E2 and L2 was demonstrated (13). Numerous studies have shown E2 binding to vDNA (14). Additionally, a direct interaction between L2 and vDNA has been shown (44). These findings suggest that papillomavirus morphogenesis occurs via the recruitment of L1 and E2 to defined regions of the nucleus by L2. E2 and/or L2 also recruit vDNA to these same defined nuclear regions. Once the concentrations of the necessary viral components are sufficient, virion morphogenesis occurs. We have shown that to induce efficient virion production in the raft culture system, the addition of a protein kinase C (PKC) activator, such as synthetic diacylglycerol, is necessary (20, 22, 29). The addition of a PKC activator allows for the production of infectious viral stocks. The mechanism whereby PKC activation stimulates virion synthesis is unresolved, although we have demonstrated that raft cultures treated with a PKC activator express higher levels of capsid proteins L1 and L2 (29). This result suggests that PKC signaling pathways stimulate capsid gene expression, increasing the internal pool of capsid proteins available for virion morphogenesis.

In this study, we examined whether an interaction between

FIG. 6. Electron micrograph of chimeric HPV18/16 virions, approximately 50 nm in diameter. Viral stocks were prepared as described in Materials and Methods. Bar, 50 nm.

FIG. 7. Infectious titers of chimeric HPV18/16:5C and HPV18/16:2A. Shown is a 2% agarose gel of nested RT-PCR-amplified HPV18 E1-E4 and β-actin. Lane 1, negative control (no virus). Lanes 2 to 6, HPV18/16:5C at 1:10, 1:50, 1:100, 1:200, and 1:400, respectively. Lanes 7 to 11, HPV18/16:2A at 1:10, 1:50, 1:100, 1:200, and 1:400, respectively. β-Actin is indicated by the arrowhead, and HPV18 E1-E4 is indicated by the arrow. Molecular size markers in base pairs are indicated on the left.
structural and nonstructural viral genes, required to carry out the complete viral life cycle, needed to be type specific. With HPV18 and HPV16, there was no type-specific constraint. The nonstructural genes of HPV18 were able to functionally interact with the structural genes of HPV16 to carry out all necessary functions of the viral life cycle leading to the production of chimeric progeny virus. As mentioned above, previously published studies suggested that an interaction between the L2 capsid protein and the E2 nonstructural protein is necessary for virion morphogenesis (4, 19, 20, 41). Our study shows that whether an interaction between L2 and E2 is required for virion morphogenesis (4, 19, 20, 41). We present here the first successful propagation of a viable chimeric virus system to study HPV genetics. We constructed an HPV18/16 chimeric plasmid containing the nonstructural genes of HPV18 and the structural genes of HPV16. Following electroporation of the chimeric vDNA into primary keratinocytes, HPV18/16 chimeric genomes were maintained episomally. HPV late functions were active in the differentiating tissues of raft cultures, including the synthesis of HPV18/16 chimeric virions. Our study establishes the ability of the nonstructural genes of HPV18 to functionally interact with the structural genes of HPV16. Antibodies directed against HPV16 VLPs specific to HPV18 VLPs specifically neutralized the virions. Future studies of chimeric HPVs will allow increased examination of HPV genetics and assessment of HPV vaccine development.

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