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Establishment of the Human Papillomavirus Type 16 (HPV-16) Life Cycle in an Immortalized Human Foreskin Keratinocyte Cell Line

Elsa R. Flores,* B. Lynn Allen-Hoffmann,† Denis Lee,* Carol A. Sattler,* and Paul F. Lambert*,[1]

*McArdle Laboratory for Cancer Research and †Department of Pathology, University of Wisconsin Medical School, 1400 University Avenue, Madison, Wisconsin 53706

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The study of human papillomaviruses (HPVs) in cell culture has been hindered because of the difficulty in recreating the three-dimensional structure of the epithelium on which the virus depends to complete its life cycle. Additionally, the study of genetic mutations in the HPV genome and its effects on the viral life cycle are difficult using the current method of transfecting molecularly cloned HPV genomes into early-passage human foreskin keratinocytes (HFKs) because of the limited life span of these cells. Unless the HPV genome transfected into the early-passage HFK extends the life span of the cell, analysis of stable transfectants becomes difficult. In this study, we have used BC-1-Ep/SL cells, an immortalized human foreskin keratinocyte cell line, to recreate the HPV-16 life cycle. This cell line exhibits many characteristics of the early-passage HFKs including the ability to stratify and terminally differentiate in an organotypic raft culture system. Because of their similarity to early-passage HFKs, these cells were tested for their ability to support the HPV-16 life cycle. The BC-1-Ep/SL cells could stably maintain two HPV genotypes, HPV-16 and HPV-31b, episomally. Additionally, when the BC-1-Ep/SL cell line was stably transfected with HPV-16 and cultured using the organotypic raft culture system (rafts), it sustained the HPV-16 life cycle. Evidence for the productive stage of the HPV-16 life cycle was provided by: DNA *in situ* hybridization demonstrating HPV-16 DNA amplification in the suprabasal layers of the rafts, immunohistochemical staining for L1 showing the presence of capsid protein in the suprabasal layers of the rafts, and electron microscopy indicating the presence of virus like particles (VLPs) in nuclei from cells in the differentiated layers of the rafts. © 1999 Academic Press

INTRODUCTION

Human papillomaviruses (HPVs) are small DNA viruses that infect epithelial cells. A subset of HPVs infects the anogenital tract and can be divided into two types: the low-risk and high-risk genotypes. The low-risk genotypes lead to the production of benign lesions or warts, whereas the high-risk genotypes, in addition to producing warts, have been implicated in cervical cancer (zur Hausen, 1991). The HPV life cycle is dependent on the differentiation of the host epithelial cell and can be separated into two stages: the nonproductive and productive stages. The nonproductive stage of the HPV life cycle occurs in the basal layer of the epithelium, where the virus is believed to gain entry at a site of wounding. In the basal compartment of the epithelium, the virus establishes itself as a low-copy-number episome by utilizing the host DNA replication machinery to synthesize its DNA on average once per cell cycle via a bidirectional theta mode (Gilbert and Cohen, 1987; Flores and Lambert, 1997). The productive stage of the viral life cycle occurs in the suprabasal layers of the epithelium. There the virus switches to a rolling circle mode of DNA replication (Flores and Lambert, 1997), amplifies its DNA to high copy number, synthesizes the capsid proteins, L1 and L2, and causes viral assembly to occur. Subsequently, virions are released into the environment as the upper layer of the epithelium is shed (Howley, 1996).

The study of HPVs in tissue culture has been difficult because of the lack of a cell-culture system that stably maintains HPVs episomally and the difficulty in recreating the three-dimensional structure of the epithelium to induce the full HPV life cycle. Within the past decade, great progress has been made in facilitating the study of HPVs in tissue culture. Several cell lines have been derived from HPV-infected patients that stably maintain HPV genomes episomally, for example, W12E cells harboring HPV-16 (Stanley *et al.,* 1989; Jeon *et al.,* 1995) and CIN612–9E cells harboring HPV-31b (Bedell *et al.,* 1991; Hummel *et al.,* 1992). These cells have proven to be excellent tools for the study of the full HPV life cycle (Bedell *et al.,* 1991; Hummel *et al.,* 1992; Meyers *et al.,* 1992; Flores and Lambert, 1997; Ozbun and Meyers, 1997). Although these cells have provided a great means to study HPV in tissue culture, they cannot be used to determine the consequence of mutations in the viral genome on the HPV life cycle. To achieve this goal, a method using transfected HPV genomes in human foreskin keratinocytes (HFKs) was developed (Frattini *et al.,* 1996; Meyers *et al.,* 1997). The power of this method is

[1] To whom reprint requests should be addressed. Fax: (608) 262-2824. E-mail: lambert@oncology.wisc.edu.

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that it allows genetic manipulation of the viral genome and analysis of genetic effects on the HPV life cycle.

Working with HFKs in cell culture is problematic because of their limited life span, which makes it difficult to select stable transfectants before they senesce. Recently an immortalized HFK cell line called BC-1-Ep/SL has been isolated. These cells are primarily diploid but contain a duplication in the small arm of chromosome 8 (Allen-Hoffmann *et al.,* submitted). While these cells are immortal and contain a chromosomal abnormality, they exhibit many characteristics similar to primary or early-passage HFKs. For example, these cells have been shown to differentiate normally as demonstrated by the organotypic raft culture system (Allen-Hoffmann *et al.,* submitted). BC-1-Ep/SL cells are not tumorigenic in nude mice, and the tumor suppressor genes *p53* (Allen-Hoffmann *et al.,* submitted) and *Rb* (Lockstein and Lambert, unpublished data) are wild type in their DNA sequence. These data indicate that these cells could be suitable hosts for the HPV life cycle.

In this study, the ability of the BC-1-Ep/SL cells to support the HPV-16 life cycle was examined. We found that the BC-1-Ep/SL cells can stably maintain HPV-16 and HPV-31b episomally, as is seen in the nonproductive stage of the HPV life cycle. Additionally, these cells were able to support the productive stage of the HPV life cycle. BC-1-Ep/SL cells stably transfected with HPV-16 (BC-1-Ep/SL/HPV-16 cells) were cultured using the organotypic raft culture system to differentiate the cells. Immunohistochemical analysis was performed using antibodies for the differentiation markers, keratin 10 (K10) and filaggrin. The BC-1-Ep/SL rafts gave rise to uniform staining for K10 in the suprabasal layers and filaggrin in the granular layer of the raft, indicating that terminal differentiation in these cells occurred similar to that found in intact tissue. K10 and filaggrin also were present in the BC-1-Ep/SL/HPV-16 rafts; however, the staining was not uniform in these layers, indicating that HPV-16 in the episomal state perturbs the program of keratinocyte differentiation. Evidence for the productive stage of the HPV-16 life cycle was demonstrated by DNA *in situ* hybridization and immunohistochemistry using an antibody against the major capsid protein, L1. The BC-1-Ep/SL/HPV-16 rafts contained cells located in the suprabasal layer of the epithelium, which contained amplified HPV-16 DNA and expressed the major capsid protein, L1. These data indicate that the BC-1-Ep/SL cells provide an immortalized HFK cell line as an alternative host for the study of the HPV life cycle.

RESULTS

Cloning and analysis of HPV-16 DNA from the W12-E cells

The difficulty in studying HPV-16 in tissue culture has been confounded by the existing prototype HPV-16 clone, which contains a frameshift mutation in the E1 gene (Seedorf *et al.,* 1985). The W12E cells are a clonal cervical epithelial cell population, derived from an HPV-16-infected patient, that stably harbor HPV-16 episomally (Stanley *et al.,* 1989; Jeon *et al.,* 1995). They were chosen as the source of HPV-16 in this study because we hypothesized that HPV-16 derived from W12E cells would contain all the DNA sequences necessary for episomal maintenance. The HPV-16 DNA was cloned from the W12E cells by extracting Hirt DNA, which enriches for low-molecular-weight DNA containing the viral DNA. This DNA was digested with the restriction enzyme, *Bam*HI, and ligated to a pUC19 vector linearized with *Bam*HI. The ligation products were transformed into bacteria, and HPV-16 positive bacterial colonies were selected by colony hybridization using a full-length α^{32}P-dCTP-labeled HPV-16 DNA probe. The resulting clone, pEFHPV-16W12E was sequenced (GenBank Accession No. AF125673). pEFHPV-16W12E was found to contain no frameshift mutation in E1 as existed in the prototype HPV-16 clone (Seedorf *et al.,* 1985). Other differences in the pEFHPV-16W12E clone included missense amino acid substitution changes in E2, E6, L1, and L2; E2 had a P-to-S substitution at amino acid 219, E6 contained an L-to-V substitution at amino acid 90, and L1 contained a T-to-A substitution at amino acid 265. L2 contained four amino acid substitutions: V to I at amino acid 243, L to F at amino acid 330, P to S at amino acid 335, and S to L at amino acid 357. The changes detected in E2, E6, L1, and L2 of pEFHPV-16W12E have not been reported in any other HPV-16 isolate. Additionally, we found differences in the long control region (LCR). There was a G-to-T transversion at nucleotide 7191. This same change was detected in a HPV-16 clone derived from a human oral cancer cell line containing HPV-16 and HPV-18 (Chen *et al.,* 1997). A transversion (T to A) at nucleotide 7416 also was seen in pEFHPV-16W12E. A transition (G to A) at nucleotide 7519 changed the putative binding core for the transcriptional factor, YY1, from ATGG in the prototype to ATAG in pEFHPV-16W12E (Gualberto *et al.,* 1992). This change could be a means of escape from cellular repression by HPV-16 as previously reported (May *et al.,* 1994). This change from the prototype also was detected in an HPV-16 clone, p114/K, derived from an HPV-16 productive lesion (Brune and Dürst, 1995). Last, a missing nucleotide (A) was detected at position 7862 making the region from nts 7858 to 7869 an E2 binding site, $ACCN_6GGT$. This deletion has been found in other HPV-16 isolates (Seedorf *et al.,* 1985; Vormwald-Dogan *et al.,* 1992; Lu *et al.,* 1993). Studies of the E2 binding sites within the LCR of HPV-16 have been characterized. The E2 binding sites proximal to P_{97} at positions 35 and 50 are important for repression of the promoter, whereas the E2 binding site at position 7858 may help to mediate E2 transactivation of P_{97} in an enhancer-like fashion (Romanczuk *et al.,* 1990).

Early-passage human foreskin keratinocytes can stably maintain HPV-16 episomes

The first step in recreating the HPV life cycle in tissue culture depends on the ability for the host cell to stably maintain HPV episomally as detected in productive HPV infections. Early-passage human foreskin keratinocytes (HFKs) have been shown previously to stably maintain HPV-18 and HPV-31b episomally (Frattini *et al.,* 1996; Meyers *et al.,* 1997). To determine whether HPV-16 W12E could replicate in early-passage HFKs, the cells were cotransfected with HPV-16 DNA isolated from the W12E cells (HPV-16 W12E) and pEGFPN1, which encodes the green fluorescent protein (GFP) and confers G418 resistance. The expression of GFP by the transfected HFKs allowed the calculation of the transfection efficiency (10–25%) by FACS analysis. Stable HPV-16 transfectants were selected by the addition of G418. The resulting colonies on one 10-cm tissue culture dish (~5 colonies) were pooled and expanded. This group of cells is referred to as a cell population. Hirt DNA was extracted from each cell population and screened for episomal HPV DNA by Southern analysis. Hirt DNA was extracted from HFK cell populations, 9-4 and 24-4, which had been stably transfected with HPV-16W12E DNA. The Hirt DNA extracted from 9-4 and 24-4 cells was separated on an agarose gel. Subsequently, Southern analysis was performed using a full-length α^{32}P-dCTP-labeled HPV-16 DNA probe. Hirt DNA extracted from W12E cells, clone 20863, was used as a positive control for supercoiled (SC) and open circular (OC) episomes and linear (L) HPV-16 DNA (Fig. 1A, lanes 5 and 6). Undigested Hirt DNA from 9-4 and 24-4 cells gave rise to bands on the Southern blot corresponding to OC and supercoiled SC DNA (Fig. 1A, lanes 1 and 3). These DNA species are indicative of episomal HPV-16 DNA. The Hirt DNA from each cell population was linearized by restriction enzyme digestion with *Bam*HI (Fig. 1A, lanes 2 and 4). The multiple bands detected in Fig. 1A, lane 2, are the result of a partial restriction enzyme digestion. These results demonstrate that HPV-16W12E DNA can stably replicate in early-passage HFKs.

Immortalized human foreskin keratinocytes can stably maintain HPV episomes

The same protocol to transfect and analyze HPV DNA replication in HFK cells was used to study BC-1-Ep/SL cells. Uncut Hirt DNA extracted from W12E cells was used as a positive control for episomal HPV-16 DNA (Fig. 1B, lane 9). Undigested Hirt DNA from the BC-1-Ep/SL cell populations, 10C, 33C, 34C and 41A, gave rise to bands corresponding to OC and SC HPV-16 DNA (Fig. 1B, lanes 1, 3, 5, and 7). Upon digestion with *Bam*HI, the linear 7.9-kb HPV-16 DNA species resulted (Fig. 1B, lanes 2, 4, 6, and 8). To determine whether these cells could stably harbor other HPV DNA episomally, the BC-1-Ep/SL cells were stably transfected with HPV-31b. Analysis of Hirt DNA extracted from these cells: 20-3, 41-3, and 31B, gave rise to OC and SC HPV DNA (Fig. 1C, lanes 1, 3, and 5). Some minor bands were detected in some of the DNA samples derived from the epithelial cells harboring HPV-16 and HPV-31b (Figs. 1A, lanes 2 and 4, 1B, lanes 4 and 8, and 1C, lanes 4 and 6). The presence of these minor bands, which also have been detected in longer exposures of W12E DNA, may be indicative of integrated HPV DNA because Hirt DNA is only an enrichment of low-molecular-weight DNA. These bands could have also arisen from deletions in episomal HPV DNA. Taken together, the results from Fig. 1 indicate that BC-1-Ep/SL cells, similar to early-passage HFKs, can stably maintain HPV-16 and HPV-31b episomally.

Evidence for terminal differentiation using the organotypic raft culture system

Because the terminal differentiation of the epithelium is essential for the productive stage of the HPV life cycle, we monitored several markers of terminal differentiation in the untransfected BC-1-Ep/SL organotypic raft cultures, which were used as a host for HPV in this study. Additionally, HPV has been shown to perturb the differentiation of keratinocytes (McCance *et al.,* 1988). To determine what consequence HPV-16 had on the differentiation of BC-1-Ep/SL keratinocytes, BC-1-Ep/SL, BC-1-Ep/SL/HPV-16, and W12E cells were cultured using the organotypic raft culture system (rafts). The cells cultured on rafts formed stratified layers detected by hematoxylin and eosin (H&E) staining (Figs. 2A–2C). The rafts were analyzed for terminal differentiation by performing immunohistochemical staining using antibodies for the epidermal differentiation markers, keratin 10 (K10) and filaggrin. In normal epithelia, K10 is expressed in the suprabasal layers beginning in the spinous layer, and filaggrin is present in the granular layer and is associated with keratohyalin granules. The suprabasal layers of BC-1-Ep/SL (Fig. 2D), BC-1-Ep/SL/HPV-16 (Fig. 2E), and W12E rafts (Fig. 2F) stained positively for K10. The K10 staining pattern in BC-1-Ep/SL rafts (Fig. 2D) was uniform throughout the suprabasal layers. In contrast, the K10 staining pattern in BC-1-Ep/SL/HPV-16 (Fig. 2E) and W12E rafts (Fig. 2F) was disrupted by cells which do not stain positively for K10. These rafts also stained positively for filaggrin in the granular layer BC-1-Ep/SL (Fig. 2G), BC-1-Ep/SL/HPV-16 (Fig. 2H), and W12E (Fig. 2I). Again, uniform filaggrin staining was seen throughout the granular layer of the untransfected BC-1-Ep/SL raft, whereas the staining pattern in the BC-1-Ep/SL/HPV-16 raft was not uniform. Reduced staining for filaggrin was seen in the W12E rafts indicating that differentiation is incomplete.

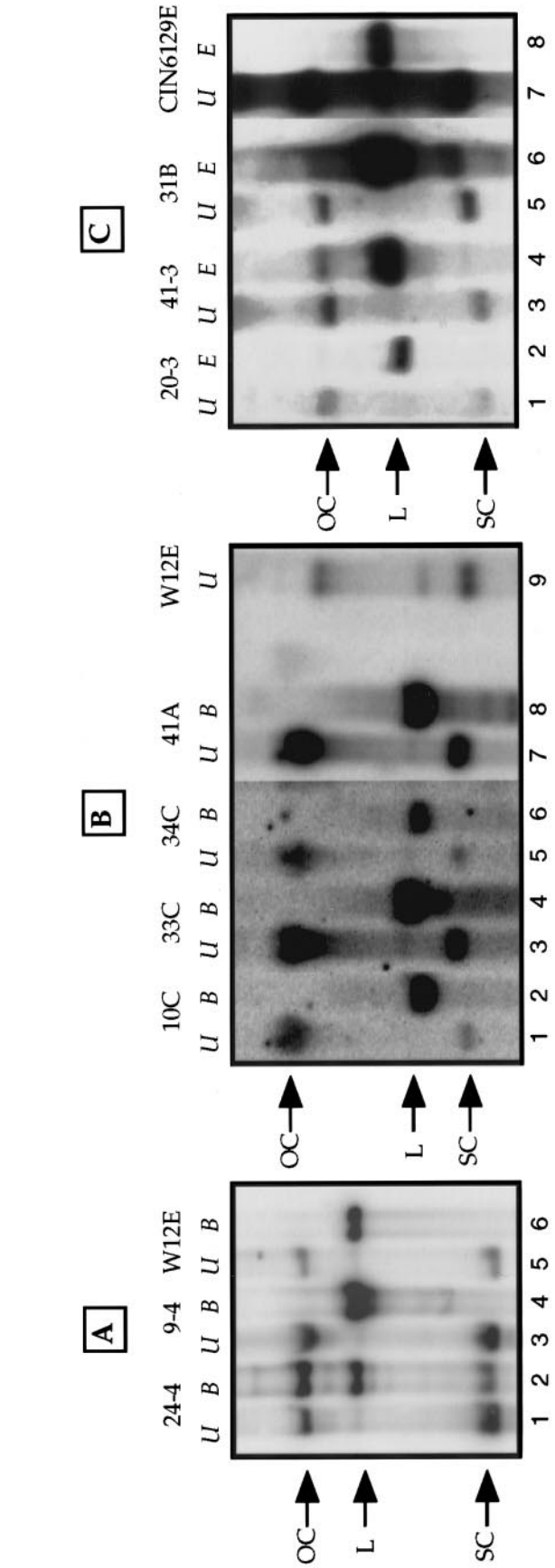
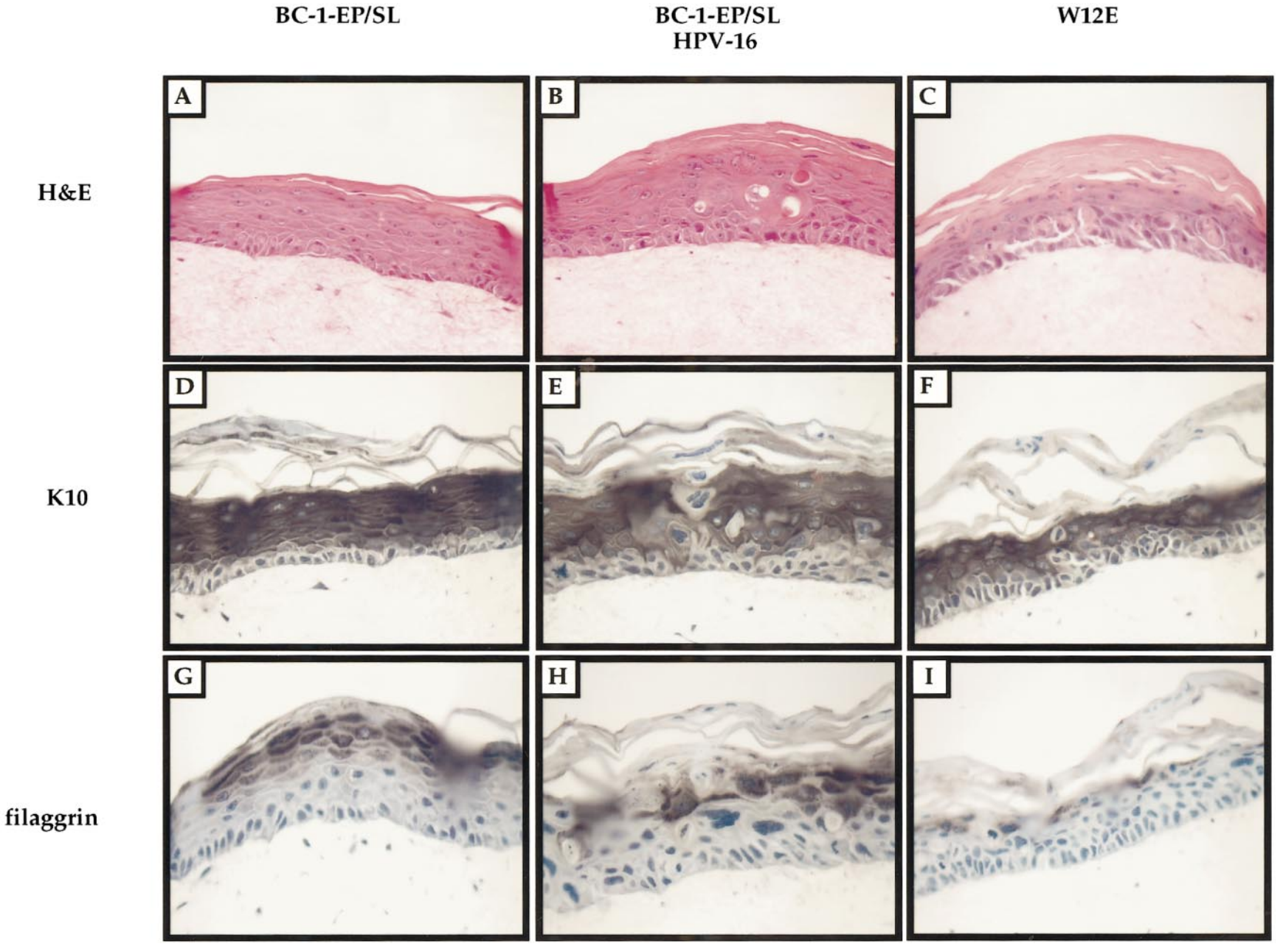


FIG. 1. Analysis of HPV DNA isolated from HFK and BC-1-Ep/SL stably transfected cell populations. (A) Shown is an autoradiograph of a Southern blot containing Hirt DNA extracted from HFKs stably transfected with HPV-16 (HFK/HPV-16 cells) and W12E cells. The blot was hybridized to a full-length HPV-16 DNA probe. Undigested (*U*) Hirt DNA from the HFK/HPV-16 cells, 24-4 and 9-4, and W12E cells contain open circular (OC) and supercoiled (SC) HPV-16 DNA (A, lanes 1, 3, and 5). The DNA from the HFK/HPV-16 cells, 24-4 and 9-4, and W12E cells was linearized (L) by digestion with *Bam*HI (*B*) (A, lanes 2, 4, and 6). Note: the digestion of DNA from cell population 24-4 was not complete as evidenced by the presence of OC and SC HPV-16 DNA (A, lane 2). (B): Shown is an autoradiograph of a Southern blot containing Hirt DNA extracted from BC-1-Ep/SL cells stably transfected with HPV-16 (BC-1-Ep/SL/HPV-16 cells) and W12E cells. The blot was hybridized to a full-length HPV-16 DNA probe. Undigested (*U*) Hirt DNA from the BC-1-Ep/SL/HPV-16 cells, 10C, 33C, 34C and 41A, and W12E cells contain OC and SC HPV-16 DNA (B, lanes 1, 3, 5, 7, and 9). The DNA from the BC-1-Ep/SL/HPV-16 cells, 10C, 33C, 34C, and 41A was linearized (L) by digestion with *Bam*HI (*B*) (B, lanes 2, 4, 6, and 8). (C): Shown is an autoradiograph of a Southern blot containing Hirt DNA extracted from BC-1-Ep/SL cells stably transfected with HPV-31b (BC-1-Ep/SL/HPV-31b cells) and CIN612–9E cells. The blot was hybridized to a full-length HPV-31b DNA probe. Undigested (*U*) Hirt DNA from the BC-1-Ep/SL/HPV-31b cells, 20-3, 41-3, and 31B, and CIN612–9E cells contain OC and SC HPV-31b DNA (C, lanes 1, 3, 5, and 7). The DNA from the BC-1-Ep/SL/HPV-31b cells, 20-3, 41-3, and 31B, and CIN612–9E cells was linearized (L) by digestion with *Eco*RI (*E*) (C, lanes 2, 4, 6, and 8). Arrows on the left side of each panel indicate the migration of open circular (OC), linear (L), and supercoiled (SC) DNA.


BC-1-EP/SL
BC-1-EP/SL HPV-16
W12E
H&E
K10
filaggrin
A
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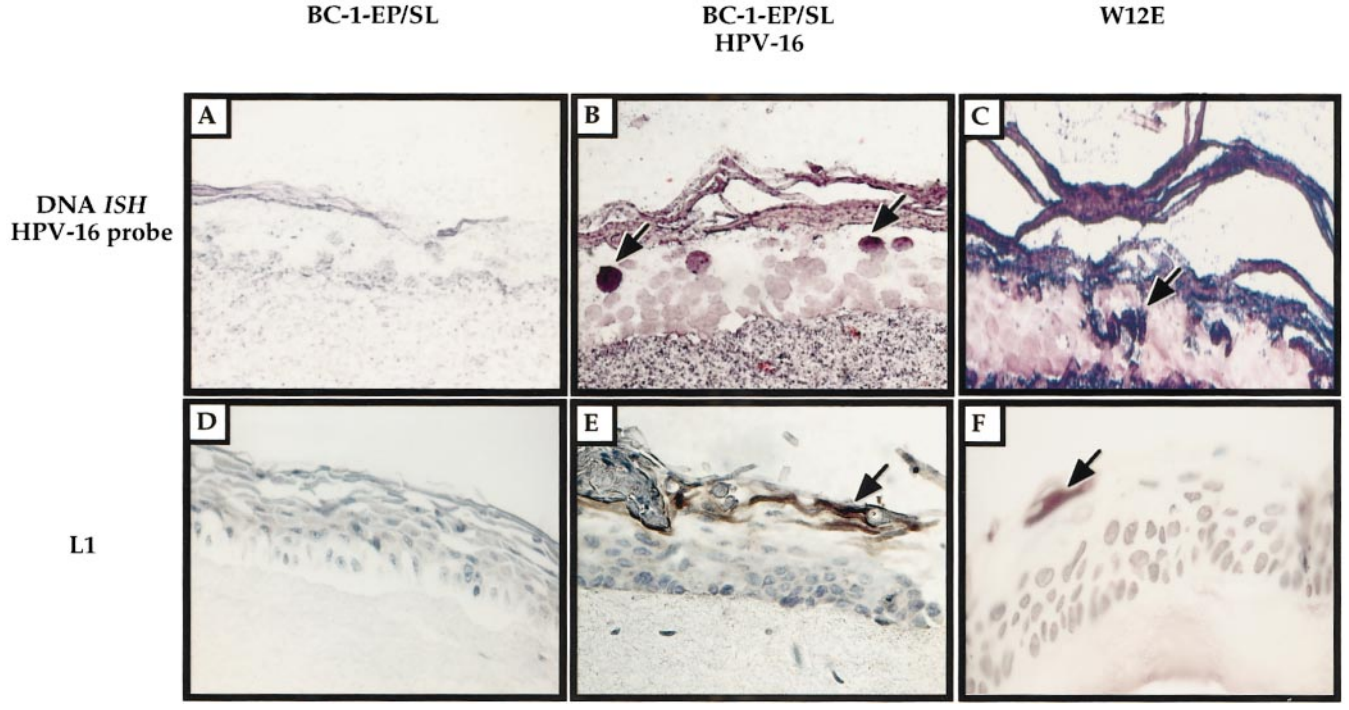

FIG. 3. HPV-16 DNA *in situ* hybridization and L1 immunohistochemical analysis of organotypic rafts cultures. Shown are epithelial organotypic raft cultures (rafts) of BC-1-Ep/SL cells, BC-1-Ep/SL/HPV-16 cells (41A), and W12E cells ([mult]40 magnification). DNA *in situ* hybridization (DNA *ISH*) was performed on cross sections from each sample using a biotin-labeled HPV-16 DNA probe. Positive nuclei are stained dark purple and are indicated by arrows. Positive nuclei are present in the BC-1-Ep/SL/HPV-16 rafts (B) and in the W12E rafts (C) and are indicated by arrows. No nuclei stained positively in the BC-1-Ep/SL rafts (A). Note: staining present in the cornified layer represents a false signal. For L1 immunohistochemistry, cross sections from each sample were incubated with an anti-L1 antibody and detected using the Vectastain ABC kit (Vector). Positive cells present in the granular layer are stained brown and are indicated by arrows. Positive cells were detected in BC-1-Ep/SL/HPV-16 rafts (E) and W12E rafts (F) but not BC-1-Ep/SL rafts (D).

Evidence for the HPV-16 life cycle

To determine whether the productive stage of the HPV-16 life cycle could be recreated in BC-1-Ep/SL cells, BC-1-Ep/SL/HPV-16 cells, population 41A, were grown using the organotypic raft culture system to recreate the three-dimensional structure of the epithelium and the environment necessary for the productive stage of the HPV-16 life cycle. Additionally, untransfected BC-1-Ep/SL cells and W12E cells were grown on rafts as a negative and positive control, respectively. The rafts were used to test for the hallmarks of the productive HPV-16 life cycle, including the evidence for HPV-16 DNA amplification, L1 synthesis, and the presence of virus like particles.

The presence of amplified HPV DNA in the suprabasal layers of the epithelium is a hallmark of the productive stage of the HPV life cycle. To determine whether the HPV-16 DNA had been amplified in the BC-1-Ep/SL/HPV-16 rafts, DNA *in situ* hybridization was performed using an HPV-16 DNA probe. Cells containing amplified HPV-16 DNA stain a dark purple color. Many positively staining cells were present in the suprabasal layers of the BC-1-Ep/SL/HPV-16 raft, 41A cells (Fig. 3B). As a comparison, the raft cultures containing untransfected BC-1-Ep/SL cells did not stain positively for HPV-16 DNA (Fig. 3A), whereas the raft cultures containing W12E cells (Fig. 3C) showed a

FIG. 2. Immunohistochemical staining for terminal differentiation markers of organotypic raft cultures. Shown are epithelial organotypic raft cultures (rafts) of BC-1-Ep/SL, BC-1-Ep/SL/HPV-16 cells, 41A, and W12E cells that were maintained on a dermal equivalent of collagen impregnated with human foreskin fibroblasts ([mult]40 magnification). The rafts were lifted to the air-liquid interface after 4 days in culture and harvested 10 days after being lifted to the air-liquid interface. The rafts were fixed in 4% Formalin, embedded in paraffin, and cut into 4-μm serial sections. Cross sections from each sample stained with hematoxylin and eosin (H&E) are shown: (A) BC-1-Ep/SL, (B) BC-1-Ep/SL/HPV-16, and (C) W12E. For keratin (K10) immunohistochemistry, cross sections from each sample were incubated with an anti-K10 antibody (CK 8.60, Sigma) and detected using the Vectastain ABC kit (Vector). Positive cells are stained brown. Positive cells were detected in the suprabasal layers of BC-1-Ep/SL rafts (D), BC-1-Ep/SL/HPV-16 rafts (E), and W12E rafts (F). For filaggrin immunohistochemistry, cross sections from each sample were incubated with an anti-filaggrin antibody (Biomedical Technologies, Inc.) and detected using the Vectastain ABC kit (Vector). Positive cells are stained brown and were detected in the granular layer of BC-1-Ep/SL rafts (G), BC-1-Ep/SL/HPV-16 rafts (H), and W12E rafts (I). Reduced filaggrin staining is evident in the (I) W12 E rafts.

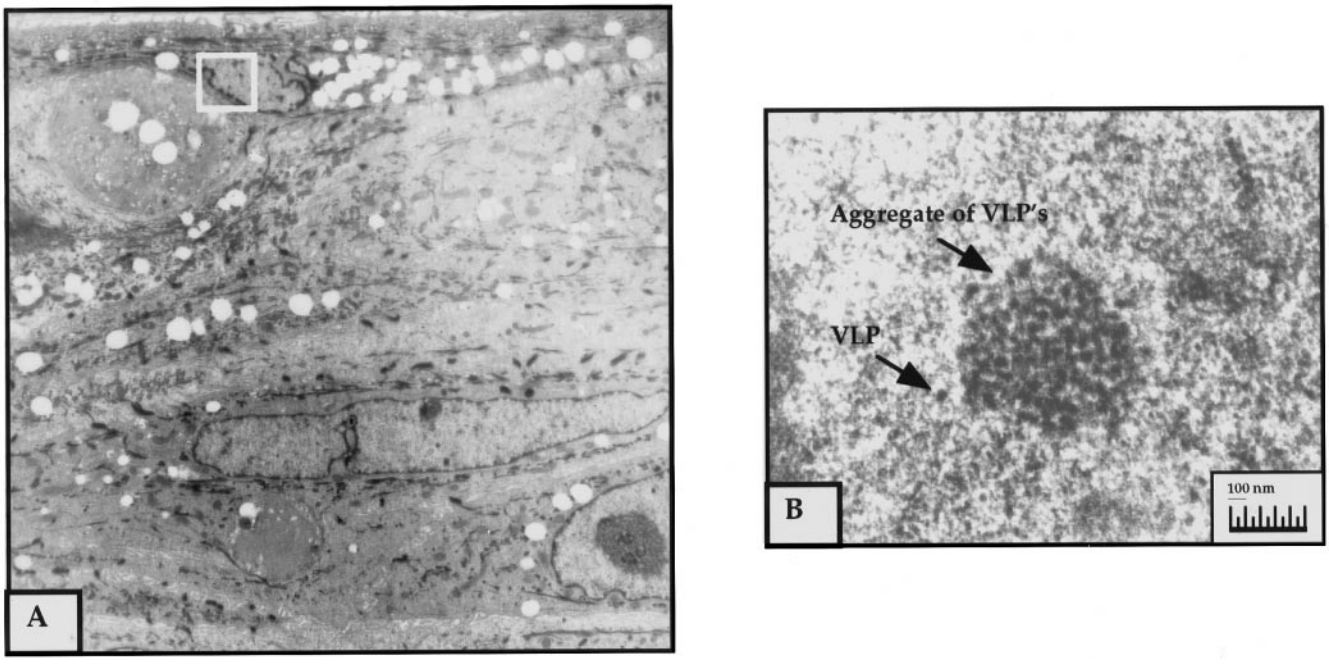

FIG. 4. Ultrastructural analysis of BC-1-Ep/SL/HPV-16 rafts. Shown are electron micrographs of BC-1-Ep/SL/HPV-16 rafts at low magnification (A) ([mult]2500) and high magnification (B) ([mult]25,000). Apparently (A) is stratified, differentiated tissue. The white box in (A) was observed under higher magnification. The presence of virus like particles (VLPs) measuring ~55 nm was apparent (B). These particles were present in multiple nuclei from cells in the suprabasal layer. The upper arrow in (B) points to an aggregate of VLPs and the lower arrow points to a single VLP. A ruler with divisions representing 100 nm is present in (B).

similar pattern of staining as the BC-1-Ep/SL/HPV-16 cells, population 41A (Fig. 3B).

Another hallmark of the productive stage of the HPV-16 life cycle is the synthesis of the late capsid protein, L1, in the suprabasal layers of the epithelium. In rafts that are productively infected, L1 can be detected by immunohistochemistry. To determine whether this aspect of the life cycle was recreated in the BC-1-Ep/SL/HPV-16 rafts cultures, immunohistochemistry was performed on the paraffin-embedded raft cultures using an antibody against L1. Cells staining positively for L1 are brown in color. Many L1-positive cells were present in the granular layer of the BC-1-Ep/SL/HPV-16 rafts (Fig. 3E). These L1-positive cells were also evident in the W12E rafts (Fig. 3F) but not in the untransfected BC-1-Ep/SL rafts (Fig. 3D). The L1 staining appears to be diffuse in these samples. This staining pattern is similar to what was seen for L1 immunostaining in rafts containing cells harboring HPV-31b and HPV-18 episomally (Frattini *et al.,* 1996; Frattini *et al.,* 1997). This pattern also may be due to the fact that the cells that stain positively for L1 are those that are in the process of undergoing nuclear dissolution. The number of L1-positive cells was lower in the W12E rafts than in the BC-1-Ep/SL/HPV-16 rafts. This result is likely due to the incomplete differentiation observed in the W12E rafts.

Ultrastructural analysis using the electron microscope has been used previously to demonstrate the presence of HPV-16 viral particles, which have a diameter of 55 nm (Sterling *et al.,* 1990; Meyers *et al.,* 1997). HPV-16-containing BC-1-Ep/SL raft cultures were processed and examined by electron microscopy. Nuclei from cells in the raft culture were examined under high magnification (Fig. 4A). The presence of particles measuring ~55 nm were detected only in the nuclei of cells in the suprabasal layers (Fig. 4B). These particles were not present in BC-1-Ep/SL untransfected rafts (data not shown).

DISCUSSION

We used an immortalized human foreskin keratinocyte (HFK) cell line, BC-1-Ep/SL, to recreate the HPV-16 life cycle. Using molecularly cloned HPV-16 derived from the W12E cells, we have demonstrated that the BC-1-Ep/SL cells can stably maintain HPV-16 episomes. We also have shown that the BC-1-Ep/SL cells can stably maintain other HPVs episomally, such as HPV-31b. Additionally, we have provided evidence for the productive stage of the HPV-16 life cycle in these cells using the organotypic raft culture system. Upon differentiation, BC-1-Ep/SL/HPV-16 cells exhibited many characteristics of the productive HPV-16 life cycle including viral DNA amplification, L1 capsid protein expression, and the formation of virus-like particles (VLPs).

Although the BC-1-Ep/SL cells are a spontaneously immortalized human foreskin keratinocyte cell line, they display features that are similar to primary or early-passage HFKs (Allen-Hoffmann *et al.,* submitted). When

these cells are grown using an organotypic raft culture system, they exhibit the features of terminal differentiation: stratification, the formation of structures including desmosomes, tonofilaments, keratohyalin granules, and enucleated cells. Additional evidence for terminal differentiation was demonstrated by immunohistochemical staining. The BC-1-Ep/SL cells stain positively for keratin 10 in the suprabasal layers of the raft culture and filaggrin in the granular layer (Fig. 2). Neither BC-1-Ep/SL cells nor primary or early-passage HFKs form tumors upon injection into nude mice (Allen-Hoffmann *et al.,* submitted). BC-1-Ep/SL cells are primarily diploid; karyotypic analysis performed on them indicates only one detectable chromosomal abnormality, a duplication in the small arm of chromosome 8 (Allen-Hoffmann *et al.,* submitted). Lastly, two tumor suppressor genes, *p53* (Allen-Hoffmann *et al.,* submitted) and *Rb* (Lockstein and Lambert, unpublished data), which play a role in the pathogenesis of HPV were sequenced; both were found to be wild type. Given that the characteristics of the BC-1-Ep/SL cells closely mimic those of primary or early-passage HFKs, these cells provide a suitable host for studying the HPV life cycle.

In other studies involving the biosynthesis of HPVs in tissue culture, activators of protein kinase C (PKC) are necessary for efficient virion production (Meyers *et al.,* 1992; Meyers and Laimins, 1994). These studies involved the use of the CIN612 cell line, which was derived from a cervical intraepithelial lesion I (CIN I). Without the phorbol ester, 12-*O*- tetradecanoyl phorbol-13-acetate (TPA), this cell line exhibited incomplete differentiation in raft cultures (Meyers *et al.,* 1992). Similarly, in our study, the W12E cells, a clonal cervical epithelial cell line harboring HPV-16 episomally, which was derived from a CIN I lesion, exhibited incomplete differentiation. While keratin 10 (K10) was normally expressed in the suprabasal layers of the raft, filaggrin staining was weak in the granular layer. This incomplete differentiation correlated with weak L1 staining in the W12E cells. Although we did not add TPA to the W12E cells in the raft system, it may be necessary to obtain a higher yield of virion production. Later studies involving early-passage HFKs transfected with cloned HPV-31b indicated that these activators of protein kinase C were not necessary to induce the productive stage of the HPV-31b life cycle but may increase the yield of virions produced (Frattini *et al.,* 1996). The addition of TPA was not necessary to induce complete differentiation in the BC-1-Ep/SL/HPV-16 cells or the productive stage of the HPV-16 life cycle (Figs. 2E, 2H, 3B, and 3E). Although the BC-1-Ep/SL/HPV-16 rafts exhibited strong staining for K10 in the suprabasal layers and filaggrin in the granular layer, the staining was not completely uniform as is seen in the untransfected BC-1-Ep/SL rafts (compare Figs. 2D with 2E and 2G with 2H); yet, the BC-1-Ep/SL/HPV-16 rafts also exhibited strong L1 staining in the suprabasal layers of the raft. Thus similar to the early-passage HFKs, the BC-1-Ep/SL/HPV-16 rafts exhibited many features of the HPV life cycle without the addition of TPA.

One of the steps in recreating the HPV life cycle in tissue culture is the establishment of stable transfectants containing episomal HPV DNA. To achieve this, the host keratinocyte must be transfected with both the HPV DNA and a plasmid conferring drug resistance and subsequently selected with a drug, such as G418. Because only a portion of keratinocytes acquire DNA and survive the selection procedure, the resulting resistant colonies are usually few in number. These colonies then must be expanded to sufficient numbers for analysis. Because of the limited life span of HFKs, unless the transfected DNA extends the life span of the cell, the cells may senesce before the analysis for episomal HPV genomes can be completed. HPVs that do not efficiently inactivate the function of the tumor suppressor genes, *p53* and *Rb,* are not predicted to extend the life span of HFKs. HPVs in this category include the low-risk HPV genomes and high-risk HPV genomes, which contain mutations in genes, such as E6 and E7. Because of the difficulty in studying HPV genomes that do not extend the life span of early-assage HFKs, previous studies had tested the ability of an immortalized keratinocyte cell line, HaCaT cells, to support the HPV-16 life cycle (Brune and Dürst, 1995). These cells were unable to support HPV-16 DNA replication. In the study conducted by Brune and Dürst, the HPV-16W12E isolate was not used, which could contribute to the failure to reproduce the HPV-16 life cycle. Additionally, the HaCaT cells, isolated from the periphery of a melanoma, are able to stratify in an organotypic raft culture system but exhibit abnormalities not present in the BC-1-Ep/SL cells (Boukamp *et al.,* 1988; Allen-Hoffmann *et al.,* submitted). The fact that the HaCaT cells are aneuploid have lowered expression of filaggrin in the granular layer and contain occasional nuclei in the cornified layer could contribute to their inability to support HPV-16 DNA replication. The BC-1-Ep/SL cells have been shown to support the replication of E7-deficient HPV-16 episomes (Flores *et al.,* manuscript in preparation). Given that the BC-1-Ep/SL cells are an immortalized HFK cell line that closely mimics the characteristics of early-passage HFKs, they may prove advantageous in the study of all HPV types and in their genetic analyses.

MATERIALS AND METHODS

HPV DNA preparation for transfections

The full-length HPV-16 genome was cloned from the W12E cells, clone 20863 (Jean *et al.,* 1995) into the *Bam*HI site of a pUC19 vector. Positive clones were identified by colony hybridization using an α^{32}P-dCTP-labeled HPV-16 DNA probe. The resulting plasmid, pEFHPV-16W12E, was propagated in bacteria and the HPV-16 insert sequenced using an ABI automated sequencer (GenBank

Accession No. AF125673). For transfections into HFKs or BC-1-Ep/SL cells, the viral genome was excised from the pUC19 vector by digestion with *Bam*HI. The HPV-16 DNA and HPV-31b DNA were gel purified by electroelution. The resulting product was ethanol precipitated, quantified, and ligated at dilute concentrations (50 ng/μl) to avoid the formation of multimers.

Stable transfections

The ligated HPV-16 or HPV-31b DNA (2–3.2 μg) was cotransfected with 1.2–1.8 μg of pEGFPN1 (Clonetics), which encodes the green fluorescent protein (GFP) and confers G418 resistance, into early-passage human foreskin keratinocytes (HFK) or immortalized human foreskin keratinocytes (BC-1-Ep/SL cells). The DNA was transfected using LipofectACE, LipofectAMINE (Gibco-BRL), or Superfect (Qiagen) following the manufacturer's instructions into keratinocytes on a 6-cm dish in low-Ca^{2+} F media supplemented with 5% chelex-treated FBS, insulin (5 μg/ml), cholera toxin (8.4 ng/ml), adenine (24 μg/ml), and hydrocortisone (0.4 μg/ml). One day posttransfection, the cells were trypsinized and plated in F media containing 5% fetal bovine serum (FBS) and the components listed for the low-Ca^{2+} F media on 10-cm dishes containing m_1 3T3 feeder cells. The cells were treated with G418 for 5–7 days until the untransfected control cells died in the following manner. Two days posttransfection, 100 μg/ml G418 was added to the media. The level of G418 was reduced to 50 μg/ml 4 days after transfection. The resulting G418 colonies (2–10 colonies per 10-cm dish) were pooled and expanded for Southern analysis. This pool was referred to as a cell population.

Southern analysis

Hirt DNA (low-molecular-weight DNA) (Hirt, 1967) was extracted from one 10-cm dish of each HPV-16 or HPV-31b stably transfected cell population. One-half of the resulting DNA from each cell population was linearized, whereas the other half was left uncut to determine the presence of open circular and supercoiled viral DNA. Hirt DNA extracted from W12E cells was used as a positive control and a marker for open circular, linear, and supercoiled HPV-16 DNA. Similarly, Hirt DNA extracted from CIN612–9E cells was used as a positive control and a marker for open circular, linear, and supercoiled HPV-31b DNA. The DNA was electrophoresed on a 0.8% agarose gel and transferred to a nitrocellulose membrane (Schleicher and Schuell). To detect HPV-16 DNA, the blot was probed with a full-length HPV-16 probe generated by digesting pEFHPV-16W12E with *Bam*HI and labeled with α^{32}P-dCTP using a random primer labeling kit (Amersham). To detect HPV-31b, the blot was hybridized with a full-length HPV-31b probe labeled with α^{32}P-dCTP.

Raft cultures

Before being placed on the raft, epithelial cells were cultured on mitomycin c-treated m_1 3T3 feeders in F media (0.66 mM Ca^{2+}) supplemented with 5% fetal bovine serum (FBS), insulin (5 μg/ml), cholera toxin (8.4 ng/ml), adenine (24 μg/ml), hydrocortisone (0.4 μg/ml), and epidermal growth factor (10 ng/ml) as described previously (Flores and Lambert, 1997). To construct the raft, bovine tendon collagen type I (1 mg/ml) (Upstate Biotechnology, Inc.) was used to coat Transwell inserts (24-mm diameter and 0.4-μm pore size, Costar) (Allen-Hoffmann *et al.,* submitted). The remaining collagen was impregnated with early-passage human foreskin fibroblasts (7.5×10^5 cells/ml) and plated on the collagen coated Transwell inserts. The collagen was allowed to contract in F12 media containing 10% FBS for 5 days in a 37°C, 5% CO_2 incubator. After the collagen had contracted, 7×10^5 keratinocytes per 50 μl of keratinocyte plating media [F media (1.88 mM Ca^{2+}) containing 0.5% FBS, insulin (5 μg/ml), cholera toxin (8.4 ng/ml), adenine (24 μg/ml), and hydrocortisone (0.4 μg/ml)] were plated on the collagen plug. Four days after plating the keratinocytes, the rafts were placed on two 1-in^2 cotton pads (Schleicher and Schuell) in a six-well tray (Organogenesis) to lift to the air-liquid interface. The rafts were fed from below the Transwell insert with cornification media [F media (1.88 mM Ca^{2+}) supplemented with 5% FBS, insulin (5 μg/ml), cholera toxin (8.4 ng/ml), adenine (24 μg/ml), and hydrocortisone (0.4 μg/ml)] every third day. Ten days after being lifted to the air-liquid interface, the rafts were fixed in 4% Formalin, embedded in 2% agar in 1% Formalin followed by paraffin, and cut into 4-μm cross sections.

DNA *in situ* hybridization

Four-micrometer cross sections of paraffin embedded rafts were hybridized with a biotin-labeled full-length HPV-16 DNA probe. DNA *in situ* hybridization was performed using the Microprobe system (Fisher) (Chengiss and Unger, 1993). Briefly, slides were dewaxed in xylene/HemoDe (1:3 ratio). The slides then were hydrated in a graded series of alcohol washes, digested with 3 mg/ml pepsin for 20 min, neutralized with Tris/Saline Brij pH 7.5, and dehydrated with a graded series of alcohol washes. HPV-16 DNA probe (1.5 μg/ml) was added to the slides. A human placental DNA (HPD) probe was used as a positive control and a pBR322 DNA probe was used as a negative control. To denature the probe and target DNA, the slides were heated to 105°C for 18 min. Hybridization was carried out at 37°C for 2 h. Hybrids were detected by treatment with avidin alkaline phosphatase conjugate (1:300) for 20 min. For color development, the slides were incubated with McGadey reagent [nitro blue tetrazolium chloride (0.33 mg/ml) and 5-bromo-4-chloro-3-indoylphosphate *p*-toluidine salt (0.16 mg/ml)] for 1 h at 37°C. The sections were coun-

terstained with nuclear fast red, mounted with Crystal Mount (Biomeda Corp., Foster City, CA), and postmounted with Permount (Fisher).

Immunohistochemistry

Four micrometer cross sections of paraffin-embedded rafts were dewaxed in xylene and rehydrated in a graded series of alcohol washes. The slides were treated with 3 mg/ml pepsin for 10 min. The slides were incubated with the primary antibodies as follows. For L1 staining, the anti-L1 antibody (camvir-1) was used at a dilution of 1:50 for 3 h. For keratin 10 staining, the K10-specific antibody (CK 8.60) (Sigma) was used at a dilution of 1:200 for 2.5 h. For filaggrin staining, a monoclonal anti-human filaggrin antibody (Biomedical Technologies Inc.) was diluted 1:100 for 3 h. Detection was performed using the Vectastain ABC kit (Vector). Slides were counterstained with hematoxylin (Vector) and mounted with Cytoseal 60 (Stephens Scientific).

EM analysis

Raft cultures were immersed in Karnovsky's fixative (3% glutaraldehyde in 0.1 M cacodylate, pH 7.4) at room temperature for 3 h. The raft cultures then were fixed in 6% glutaraldehyde and 2% paraformaldehyde at 4°C overnight. The rafts were postfixed in OsO_4 for 2 h on ice, dehydrated in a graded series of alcohols, infiltrated with 1:1 propylene oxide:Eponate overnight before being embedded in 100% Eponate (Ted Pella, Redding, CA). Rafts were thin sectioned with a Reichert Ultracut E3 (Reichert, Buffalo, NY) equipped with a diamond knife. Sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-7000 electron microscope (Hitachi, San Jose, CA) at 75 kV.

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