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Human papillomavirus type 59 immortalized keratinocytes express late viral proteins and infectious virus after calcium stimulation

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Abstract

Human papillomavirus type 59 (HPV 59) is an oncogenic type related to HPV 18. HPV 59 was recently propagated in the athymic mouse xenograft system. A continuous keratinocyte cell line infected with HPV 59 was created from a foreskin xenograft grown in an athymic mouse. Cells were cultured beyond passage 50. The cells were highly pleomorphic, containing numerous abnormally shaped nuclei and mitotic figures. HPV 59 sequences were detected in the cells by DNA in situ hybridization in a diffuse nuclear distribution. Southern blots were consistent with an episomal state of HPV 59 DNA at approximately 50 copies per cell. Analysis of the cells using a PCR/reverse blot strip assay, which amplifies a portion of the L1 open reading frame, was strongly positive. Differentiation of cells in monolayers was induced by growth in F medium containing 2 mM calcium chloride for 10 days. Cells were harvested as a single tissue-like sheet, and histologic analysis revealed a four-to-six cell-thick layer. Transcripts encoding involucrin, a cornified envelope protein, and the E1^E4 and E1^E4^L1 viral transcripts were detected after several days of growth in F medium containing 2 mM calcium chloride. The E1^E4 and L1 proteins were detected by immunohistochemical analysis, and virus particles were seen in electron micrographs in a subset of differentiated cells. An extract of differentiated cells was prepared by vigorous sonication and was used to infect foreskin fragments. These fragments were implanted into athymic mice. HPV 59 was detected in the foreskin xenografts removed 4 months later by DNA in situ hybridization and PCR/reverse blot assay. Thus, the complete viral growth cycle, including production on infectious virus, was demonstrated in the HPV 59 immortalized cells grown in a simple culture system.

Introduction

Human papillomaviruses (HPVs) infect genital epithelial tissues causing a wide spectrum of clinical states including genital warts and cervical cancer (Koutsky, 1997; Schneider and Koutsky, 1992; ZurHausen, 1996, 1999). The HPV growth cycle is completed only in differentiated keratinocytes. Development of experimental models for studies of HPV has been hampered by the lack of permissive cell-culture systems. Several immortalized cells lines including HeLa, SiHa, and CaSki cells contain integrated HPV sequences and have been extremely useful in numerous studies, including HPV transcription, early HPV protein functions, and the effects of HPV on cell-cycle parameters. However, these cells cannot be induced to differentiate with serum, calcium, methylcellulose, or in organotypic raft cultures. The integration of the viral genome, in addition to the fact that they originate from carcinomas and fail to differentiate, has limited their usefulness in studies of the HPV growth cycle.

In contrast, the W12 and CIN-612 cell lines have been valuable in the study of episomal maintenance functions of HPV 16 and HPV 31b, respectively, as these cells can be induced to differentiate in organotypic rafts (Bedell et al., 1991; Stanley et al., 1989). In addition, a few types including HPV types 11 and 16 have been propagated in the athymic or SCID mouse xenograft system (Bonnez et al., 1998; Brown and Bryan, 2000; Brown et al., 1998; Chris-

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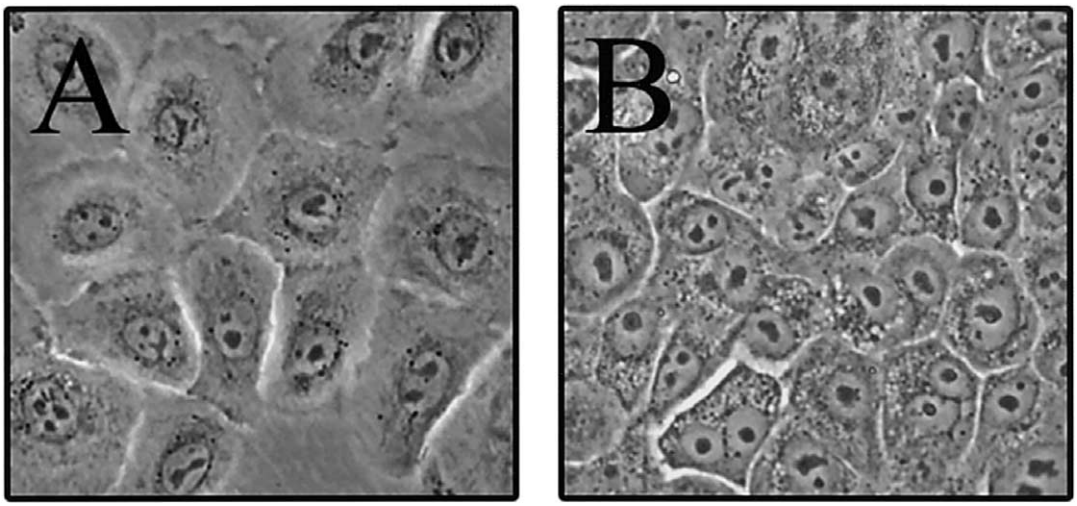

Fig. 1. Phase contrast microscopy of foreskin keratinocytes (A) and HPV 59-immortalized keratinocytes (B). HPV 59 immortalized keratinocytes have been grown in complete F medium beyond 45 passages. Original magnification: ×400.

tensen et al., 1997; Kreider et al., 1986, 1987). These systems are expensive and labor intensive. A less complex and costly system would be desirable to examine the viral growth cycle, and the effects of oncogenic HPV on cell growth and differentiation.

We therefore attempted to create an HPV-immortalized cell line that would differentiate in a simple culture system and permit such studies. HPV 59, an oncogenic type closely related to HPV 18, was recently propagated in the athymic mouse xenograft system (Brown and Bryan, 2000). Histological analysis of the HPV 59-infected xenografts revealed dysplastic features including basal hyperplasia, abundant mitotic figures, and cellular morphologic abnormalities. Epithelium from a xenograft was used to create a continuous cell line that differentiated in culture upon stimulation with calcium chloride. Differentiated cells were examined for expression of late viral proteins and infectious HPV 59 virus.

Results

HPV 59 immortalized keratinocytes

HPV 59 immortalized keratinocytes were derived from an HPV 59 infected foreskin xenograft grown in an athymic mouse. These cells have been grown in complete F medium with mitomycin-treated J2 cells beyond 50 passages. Compared to primary human keratinocytes (Fig. 1A), these cells were smaller and pleomorphic, often containing more than one nucleus (Fig. 1B).

DNA extracted from cells was used in a PCR/reverse blot strip assay (Gravitt et al., 2000). The PCR/reverse blot strip assay indicated that HPV 59 was present in high abundance (Fig. 2A). The PCR/reverse blot strip assay amplifies a portion of the L1 open reading frame. Southern blots were performed to determine if episomes of HPV 59 were present and to approximate the viral copy number in the immortalized cells. The Southern blot was consistent with HPV 59 sequences predominately as episomes (Fig. 2B). Hybridization of 4 μg of cellular DNA (not digested with restriction enzymes) with the whole genomic probe HPV 59 probe revealed two bands (Fig. 2B, Lane 1). The predominant band migrated at approximately 16 kb, and a weaker, faster migrating band was detected at approximately 4 kb, consistent with supercoiled episomes. A single band at approximately 8 kb was seen in the 4 μg sample of cellular DNA digested with *Bam*HI, which was expected to cut HPV 59 at a single location within the E6 open reading frame (Fig. 2B, Lane 2). Digestion of 4 μg of cellular DNA with *Hin*dIII (not expected to cut HPV 59) produced bands at 16 kb, 4 kb, and a faint, very high molecular weight band migrating higher than the 23.1 kb marker (Fig. 2B, Lane 3). Digestion of 4 μg of cellular DNA with *Alw*NI resulted in two bands of 4394 and 1865 bp, the predicted sizes based on the published sequence of HPV 59 (Fig. 2B, Lane 4). The smaller *Alw*NI digestion products, predicted to be less than 1000 bp, were not resolved on the 1.0% agarose gel. *Alw*NI digestion of 4 μg of cellular DNA from an HPV 59 infected foreskin xenograft grown in an athymic mouse resulted in bands of similar size, 4394 and 1865 bp (Fig. 2B, Lane 5).

The copy number of the HPV 59 sequences in the immortalized cells was estimated by comparing 1.2 μg of DNA digested with either *Bam*HI (Fig. 2C, Lane 1), or *Hin*dIII (Fig. 2C, Lane 2) from the immortalized cells with linearized, whole genomic HPV 59 DNA (Lanes 3 through 6, containing 3.2, 32, 320, 3200 pg of DNA representing approximately 1, 10, 100, or 1000 viral copies per cell). The viral copy number in the immortalized cells was estimated to be 50 copies per cell. The band hybridized to the HPV 59 probe in the *Bam*HI-digested cellular DNA sample (Fig. 2C, Lane 1) migrated slightly slower than the linearized

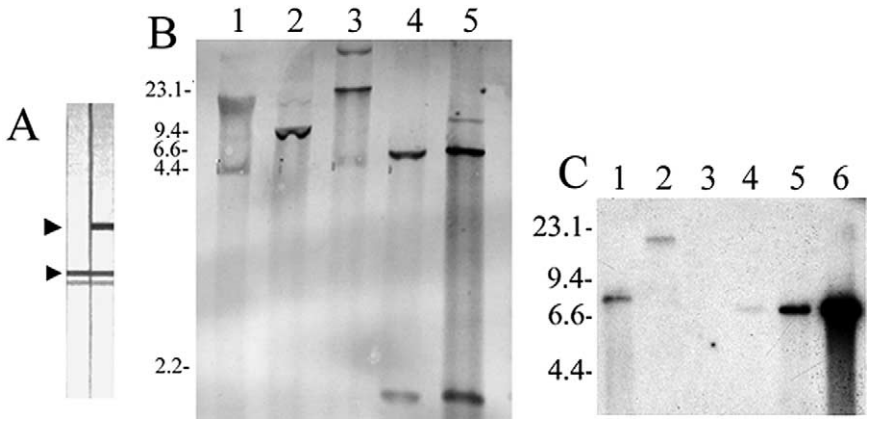

Fig. 2. (A) PCR/reverse blot strip assay indicating that HPV 59 was present in high abundance. Upper arrowhead indicates the position of the signal for HPV 59. Lower arrowhead indicates the position of the β-globin control. (B) Southern blot analysis of DNA extracted from cells at passage 25 using an HPV 59 whole genomic probe labeled with digoxigenin-11-dUTP. Lane 1: cellular DNA (4 μg), no restriction enzyme. Lane 2: cellular DNA (4 μg) DNA digested with *Bam*HI. Lane 3: cellular DNA (4 μg) DNA digested with *Hin*dIII. Lane 4: cellular DNA (4 μg) DNA digested with *Alw*NI. Lane 5: *Alw*NI digestion of 4 μg of cellular DNA from an HPV 59 infected foreskin xenograft grown in an athymic mouse. *Bam*HI is expected to cut HPV 59 once; *Hin*dIII is not expected to cut HPV 59. C: Southern blot analysis of DNA extracted from cells at passage 25 using an HPV 59 whole genomic probe labeled with [^{32}P]-dCTP. Lane 1: cellular DNA (1.2, μg) digested with *Bam*HI. Lane 2: cellular DNA (1.2, μg) DNA digested with *Hin*dIII. Lanes 3 through 6: linear genomic HPV 59 DNA added at the equivalent of 1, 10, 100, and 1000 copies per cell (3.2 pg, 32 pg, 320 pg, 3.2 ng linearized HPV 59 whole genomic DNA). Molecular markers (kb) are indicated on the left of (B) and (C).

genomic HPV 59 DNA controls (Fig. 2C, Lanes 4, 5, and 6). We cannot explain this observation, but a similar migration pattern occurred in one other published Southern blot performed to estimate HPV copy number in cells (Meyers et al., 1997).

HPV 59 immortalized keratinocytes differentiate with calcium chloride stimulation

The HPV 59 infected keratinocytes were grown to near confluence in complete F medium (Flores and Lambert, 1997) with mitomycin-treated J2 cells, and 2 mM calcium chloride was added to induce differentiation. Cells began to pile onto one another after reaching confluence in this medium. Cells were harvested and embedded in paraffin for preparation of sections. Histologically, the cells were present as a layer approximately four to six cells in thickness (Fig. 3A). No definite ordered differentiation pattern was present, and cells were markedly varied in shape and size. Nuclei were also variable in shape and size. BC-1-Ep/S1 (NIKS) cells (HPV-negative, immortalized human keratinocytes) were used as control cells (Allen-Hoffmann et al., 2000). NIKS cells induced to differentiate in the same high-calcium growth medium were somewhat more ordered, and similar to each other in shape and size (Fig. 3B).

DNA in situ hybridization assays were performed on the HPV 59 immortalized cells and the NIKS cells grown in high calcium medium (Fig. 4). Many HPV 59 immortalized cells were positive diffusely throughout nuclei for HPV 59 in DNA in situ hybridization assays (Fig. 4B). Previous studies have shown that DNA in situ hybridization assays performed on cells or tissues containing predominately episomal HPV sequences results in a diffuse nuclear staining pattern (Cooper et al., 1991; Evans et al., 2002). In contrast, integrated HPV sequences resulted in a punctuate pattern of staining, resulting from the presence of HPV in specific integration sites. The diffuse nuclear pattern of hybridization in the HPV 59 immortalized cells was consistent with an episomal state of the viral genome in the cells. NIKS cells were completely negative for hybridization with the HPV 59 probe (Fig. 4D). Additional controls for the DNA in situ hybridization assay were performed, including human foreskin tissue (two specimens) and HPV-negative foreskin implants grown as xenografts in athymic mice. These controls were completely negative for fluorescence (data not shown).

Late viral and cellular transcripts are expressed in differentiated cells

In cells induced to differentiate with calcium chloride (see below), the E1^E4 and E1^E4^L1 transcripts were detected in reverse transcriptase–polymerase chain reaction (RT-PCR) assays (Fig. 5). The E1^E4 transcript was weakly detected at Day 0, prior to adding calcium, and increased markedly after 3–5 days of growth in medium containing 2 mM calcium chloride. The E1^E4^L1 transcript was not detected initially, but was detected after 5–7 days of differentiation. L1 sequences were also amplified in the RT-PCR after 7 days of growth in the medium containing 2 mM calcium chloride.

Transcripts encoding involucrin, a cornified cell envelope scaffold protein, were also detected in cells grown in the medium containing 2 mM calcium chloride, providing confirmation of cellular differentiation. Transcripts for both β-actin and GAPDH were used to determine adequacy and

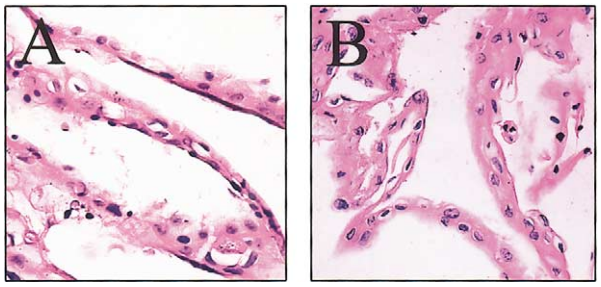

Fig. 3. Histologic analysis of HPV 59 immortalized cells (A) or NIKS cells (B) induced to differentiate with 2 mM calcium chloride. Original magnification: ×400.

relative abundance of the transcripts isolated from cells. Both of these human transcripts were assayed because there is evidence that β-actin may be disregulated in HPV-infected epithelium (Steele et al., 2002). In our analysis, β-actin and GAPDH transcripts were detected in relatively equal abundance in the cells induced to differentiate with calcium chloride.

The E1^E4^L1 transcript was cloned and sequenced (Fig. 6). As was previously determined for the HPV 11 E1^E4^L1 transcript, the E1^E4 splice was in-frame. Also similar to the HPV 11 transcript was a 12-nucleotide untranslated region present between the end of the E4 open reading frame and the L1 start codon.

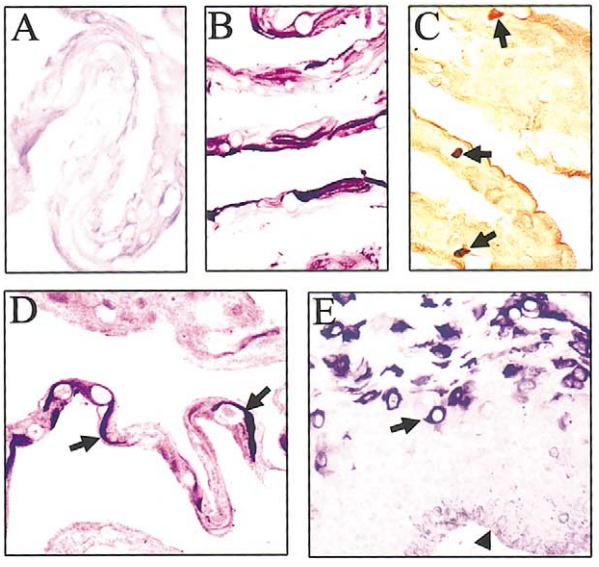

Fig. 7. Expression of involucrin and late viral proteins in HPV 59 immortalized cells induced to differentiate with calcium chloride. (A) preimmune rabbit serum. (B) antiserum against involucrin, a cornified cell envelope protein. (C) antiserum against L1 protein. Arrows indicate three nuclei positive for L1 protein. (D) affinity-purified antibodies against the HPV 59 E1^E4 protein. Arrows indicate two cells containing E1^E4 protein. (E) Affinity-purified antibodies against the HPV 59 E1^E4 protein, paraffin-embedded sections of HPV 59 infected foreskin xenografts grown in athymic mice. Original magnification: ×400.

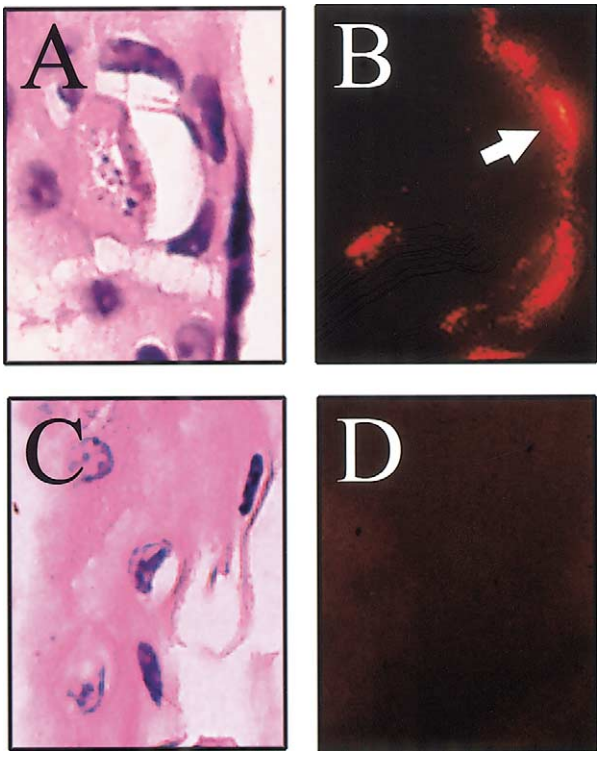

Fig. 4. Detection of HPV 59 sequences by DNA in situ hybridization. (A) Hematoxylin and eosin stain, HPV 59 immortalized cells induced to differentiate with 2 mM calcium chloride. (B) DNA in situ hybridization analysis of HPV 59 immortalized cells induced to differentiate with 2 mM calcium chloride. Arrow indicates the nucleus of a cell that contains abundant HPV 59 DNA in a diffuse pattern. (C) Hematoxylin and eosin stain, NIKS cells induced to differentiate with 2 mM calcium chloride. (D) DNA *in situ* hybridization analysis of NIKS cells induced to differentiate with 2 mM calcium chloride. Original magnification: ×400.

L1 and E1^E4 proteins are detected in differentiated cells

In immunohistochemical assays of cells grown in complete F medium supplemented with 2 mM calcium chloride, preimmune rabbit serum did not produce significant staining of cells (Fig. 7A). Involucrin was detected in many HPV 59

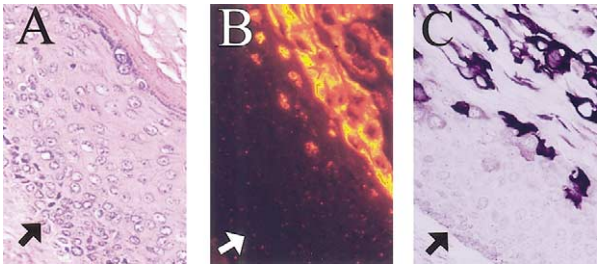

Fig. 9. A foreskin xenograft resulting from infectivity experiment using sonicated, differentiated, HPV 59 immortalized cells as the source of HPV 59 virus. (A) Hematoxylin and eosin stained sections of an athymic mouse xenograft removed 4 months after implantation. (B) DNA in situ hybridization analysis of a xenograft for detection of HPV 59 DNA. (C) Immunohistochemical detection of the HPV 59 E1^E4 protein. Arrows in each panel indicate the basal layer of cells. Original magnification: ×400.

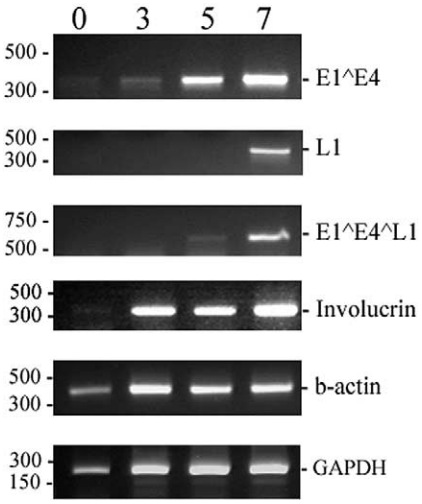

Fig. 5. RT-PCR analysis of cells induced to differentiate with calcium chloride. Transcripts encoding HPV 59 late genes (as described in the text) and involucrin are indicated. β-Actin and GAPDH were used as internal controls. Numbers at top of each lane indicate days of growth in complete F media plus 2 mM calcium chloride.

immortalized cells grown in high calcium medium, again confirming cellular differentiation (Fig. 7B). Involucrin staining was concentrated on one side of the differentiated cells.

The L1 and E1^E4 viral proteins were detected in approximately 10% of cells induced to differentiate. L1 protein was detected in nuclei (Fig. 7C). The E1^E4 protein was cytoplasmic and was distributed in the region of the cornified cell envelope (Fig. 7D). No E1^E4 protein was detected in nuclei. Paraffin-embedded sections of HPV 59 infected foreskin xenografts grown in athymic mice were used as a positive control for detection and distribution of E1^E4 protein (Fig. 7E). The pattern of E1^E4 protein in the differentiated HPV 59 immortalized cells was similar to the distribution in the xenografts. Differentiated NIKS cells were used as a negative control for the anti-E1^E4 affinity-purified antibodies. No staining of differentiated NIKS cells was observed using anti-E1^E4 antibodies (data not shown).

Virus particles detected in nuclei of differentiated cells

Cells induced to differentiate with 2 mM calcium chloride were scraped from flasks and fixed in 3% glutaraldehyde. Sections of the tissue-like mass of cells were prepared and examined by transmission electron microscopy. Virus particles of approximately 50 nm in diameter were detected in the nuclei of these cells (Fig. 8).

Infectious HPV 59 virus is produced in differentiated cells

Cells induced to differentiate with 2 mM calcium chloride were used to prepare an extract for infectivity experiments. Cells were harvested and subjected to vigorous sonication to rupture cells. Phase contrast microscopy of the sonicated preparation was performed as previously described (Brown and Bryan, 2000) and no intact cells were seen (data not shown). The athymic mouse xenograft system was used to test infectivity (Kreider et al., 1986, 1987).

Human foreskin fragments were incubated in the extract and implanted under the renal capsules of athymic mice. Xenografts were removed 4 months after implantation and examined for histologic evidence of infection and for HPV 59 sequences (Fig. 9). Histologically, three of nine xenografts removed from mice displayed features consistent with HPV 59 infection, including acanthosis, parakeratosis, mitotic figures, and a minimal degree of koilocytosis (Fig. 9A). These xenografts were positive for HPV 59 sequences in DNA in situ hybridization assays (Fig. 9B). Numerous nuclei in cells near or in the stratum corneum were positive for HPV 59 DNA, and desquamated cells were positive for HPV 59 DNA both in nuclei of cells and throughout the cell in anuclear cells. The same positivity and distribution occur in anuclear, desquamated cells in HPV 11 infected foreskin xenografts grown in athymic mice (Bryan and Brown, 2001). DNA in situ signals concentrated near the periphery of desquamated cells in those studies correlated with intact virus particles outside nuclear borders (Bryan and Brown, 2001). As indicated above, controls for the DNA in situ assay, including foreskin tissue (two individual tissues) and HPV-negative foreskin xenografts grown in athymic mice, were all completely negative for hybridization with the HPV 59 probe (data not shown). DNA extracted from the xenograft was strongly positive for HPV 59 sequences in the PCR/reverse blot strip assays, providing additional evidence of infection in the infectivity assay (not shown). Further evidence of infection in the assay was provided by a strongly positive immunohistochemical assay for the HPV 59 E1∧E4 protein in the same xenograft (Fig. 9C).

Discussion

HPV 59 is a high-risk genital type that is frequently detected in genital specimens (Becker et al., 1994; Bosch et al., 1995; Brown et al., 1999; Schiffman et al., 1993). HPV 59 was initially cloned from a vulvar intraepithelial neoplasia (Rho et al., 1994). A comparative analysis of the HPV 59 genome with other HPVs showed close homology with

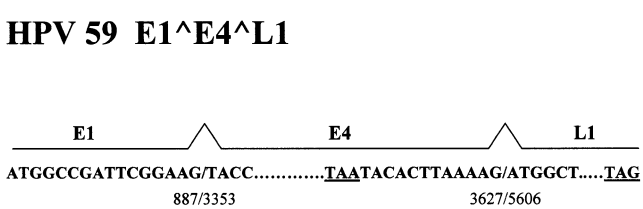

Fig. 6. Diagram representing the HPV 59 E1^E4^L1 transcript. Numbers under the sequence indicate the positions of the splice donor/accepter nucleotides. Termination codons for E4 and L1 are underlined.

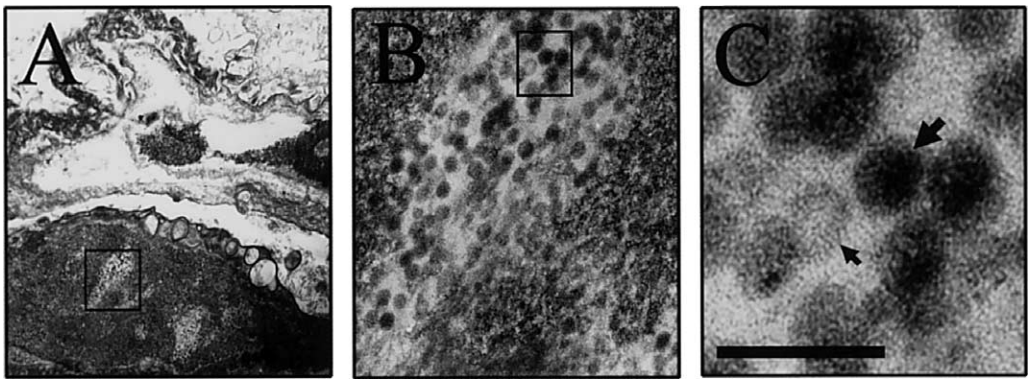

Fig. 8. Electron photomicrograph of HPV 59 immortalized cells induced to differentiate with 2 mM calcium chloride. (A) Electron photomicrograph of cells at original magnification of ×16,500. The box is in a nucleus, and is shown in (B) in the electron photomicrograph of cells at original magnification ×130,000. (C) Enlargement of the boxed portion of (B) to illustrate details of virus particles. Larger arrow indicates an electron-dense particle, likely representing a DNA-containing, or "full particle." The smaller arrow likely indicates an empty capsid. The bar in (C) represents approximately 100 nm.

HPV types 18 (71%), 45 (70%), and 39 (69%), types associated with a high risk of dysplastic cervical lesions (Rho et al., 1994). The most conserved open reading frame, encoding the L1 major capsid protein, shares a 73–75% homology with HPV types 18, 39, and 45.

In the current study, an immortalized foreskin keratinocyte cell line containing episomal HPV 59 was established using epithelium from an infected foreskin xenograft grown in an athymic mouse. These cells are morphologically dysplastic. Induction of differentiation of the HPV 59 immortalized cells occurred in culture after addition of 2 mM calcium chloride to the growth medium. The E1^E4 and E1^E4^L1 transcripts were detected within 3–5 days after calcium chloride stimulation. For HPV 11, the L1 major capsid protein is expressed from an E1^E4^L1 bicistronic transcript that also encodes the E1^E4 protein (Brown et al., 1996; Nasseri et al., 1987).

The HPV 59 E1^E4 and L1 proteins were detected in differentiated cells. The detection of L1 protein suggests that virus was being produced in these cells. Virus particles were seen in numerous nuclei of differentiated cells in transmission electron microscopy studies. We would therefore argue that the detection of L1 protein in the immunohistochemical assays, detection of virus particles by electron microscopy, and the positive result of the infectivity assay strongly suggest that infectious virus was produced in the cells induced to differentiate with calcium chloride.

It is also possible that intact, differentiated HPV 59 immortalized cells were directly implanted under the renal capsules of the athymic mice in the infectivity assay, and that these cells expanded into the xenografts we recovered from the athymic mice. However, the vigorous sonication done to prepare the extract makes this very unlikely. In fact, no intact cells were seen following the sonication process. It could also be argued that naked DNA, not incorporated into virus particles, transformed the foreskin keratinocytes during the incubation period of the infectivity assay, leading to the results we present. However, this also seems unlikely, because we have tried many times to establish infections in the athymic mouse xenograft system using extracts of genital lesions that contain abundant HPV DNA but little or no detectable L1 protein (our unpublished data). This observation suggests that intact virus is required to establish infection in the athymic mouse xenograft system.

The HPV 59 immortalized cell line presented here adds to the growing number of systems that can replicate the HPV growth cycle in vitro. As indicated above, a few types including HPV types 11, 16, 40, 59, and 83 have been propagated in the athymic or SCID mouse xenograft system (Bonnez et al., 1998; Brown et al., 1998; Bryan et al., 2000; Christensen et al., 1997; Kreider et al., 1986, 1987). For HPV 16, episomal forms of the genome are present in W12 cells, a continuous cell line derived from a cervical lesion (Stanley et al., 1989). These cells were grafted onto the skin of athymic mice, and the cells stratified over a several month period (Sterling et al., 1990). Virion production was demonstrated in the differentiated cells in electron micrographs. W12 cells were also shown to differentiate and produce virus particles when grown in F medium containing 1.2 mM calcium chloride (Flores and Lambert, 1997). In that study, the mode of DNA replication was analyzed in cells grown under basal-like conditions (low calcium) and was shown to change from θ-structure to a rolling circle mode following induction of differentiation with calcium chloride. An infectivity assay was not performed to prove that HPV 16 infection of new cells or tissue could be accomplished.

The complete growth cycle of HPV 16 was also demonstrated in organotypic raft cultures derived from BC-1-Ep/S1 (NIKS) cells transfected with genomic HPV 16 DNA (Flores et al., 1999). In this case, evidence of virion production included positive staining for the L1 major capsid protein, and identification of virions in electron micrographs. In another study, HPV 16 genomic sequences were transfected into cultured placental trophoblasts (Liu et al., 2001). The L1 major capsid protein was detected after 18

days of growth, and lysed trophoblasts were used to generate an infectious viral stock. Human keratinocytes were then grown as an organotypic raft. HPV 16 DNA was detected in the rafts, indicating that infection had occurred.

A continuous cell line containing episomal HPV 31b sequences (CIN-612 cells) was shown to differentiate in organotypic raft cultures (Bedell et al., 1991). Virion production occurred in the rafts after addition of TPA, and a protein kinase C activator was added to growth medium (Meyers et al., 1992). Infectious HPV 31b virus has been isolated from organotypic rafts from the CIN-612 cell line (Ozbun, 2002a, b). In this case, virus was shown to transiently infect HaCat cells grown in culture. The viral growth cycle of a third oncogenic type, HPV 18, was demonstrated using primary human keratinocytes transfected with viral DNA and induced to differentiate in organotypic rafts (Meyers et al., 1997). Virions were seen by electron microscopic analysis of rafts, and purified virus was shown to transiently infect keratinocytes in culture.

For HPV 11, a low-risk genital type, the viral growth cycle was established by explantation of condylomata acuminata fragments onto a dermal equivalent and growing cells as organotypic rafts (Dollard et al., 1992). In that study, late gene expression was demonstrated in immunohistochemical assay (E1^E4 and L1), and virions were seen in electron microscopic analysis.

The HPV 59 immortalized cell line does not require organotypic raft culture methods to differentiate and complete the viral growth cycle. This feature will facilitate studies of oncogenic HPV infection on keratinocyte differentiation. In addition, studies of antiviral compounds will be less cumbersome with these cells than systems requiring athymic mice or organotypic raft cultures. The growth, proliferative nature, and characteristics of viral transcription of the HPV 59 immortalized cells are under investigation.

Materials and methods

Human foreskin xenografts and HPV 59 immortalized keratinocytes

HPV 59 was originally detected in a genital lesion from an immunosuppressed individual (Bryan et al., 2000). An extract of this lesion was used to infect human foreskin fragments, which were implanted under the renal capsules of athymic mice as previously described (Bryan et al., 2000). HPV 59 infected xenografts were removed from mice 6 months after implantation, and an extract of a large xenograft was prepared and used to infect additional foreskin tissue for repeated implantation into athymic mice.

A large xenograft was removed from a mouse after 4 weeks of growth. The capsular tissue of the xenograft was minced and treated with trypsin to make a single cell suspension. Cells were plated onto J2 cells (treated with mitomycin C at 4 μg per mL for 16 h) in incomplete F medium (Flores and Lambert, 1997). Medium was changed to complete F media after 24 h (Flores and Lambert, 1997). Cells were divided every 4–5 days onto J2 cells as above.

Detection of HPV 59 by PCR and Southern blot

Confluent cells (passage 25) were trypsinized and collected in a buffer containing 0.1% SDS and 10 mM EDTA. Proteinase K was added at 25 μg/mL at 55°C for 60 min. DNA was collected using the High Viral Pure Kit as recommended by the manufacturer (Roche Molecular Diagnostics, Indianapolis, IN). The Roche PCR/reverse blot strip assay was used to detect specific HPV types (Gravitt et al., 2000).

The entire HPV 59 sequence was amplified by PCR using the primers GAGGATCCTACACAACGACCATACAAACTG (forward) and TAGGATCCTCAAAGCGTGCCATGCCGTTGC (reverse). Total DNA from an HPV 59 infected foreskin xenograft was used as template DNA. The Expand Long PCR Kit was used to amplify HPV 59, using Buffer 3 as directed by the manufacturer (Roche Molecular Diagnostics). Conditions for amplification were 35 cycles of 93°C for 10 s, 60°C for 30 s, and 68°C for 8 min, followed by a single hold cycle of 68°C for 8 min. The resulting 8-kb amplimer was gel purified and cloned into pCR-XL-TOPO (Invitrogen, Carlsbad, CA).

Southern blots were performed by labeling the whole genomic probe by nick translation using either digoxigenin-11-dUTP or P^{32}-labeled dCTP. To determine the physical status of the HPV 59 sequences in the cells, the 8-kb genomic HPV 59 sequence was excised from the pCR-XL-TOPO vector by endonuclease digestion using *Bam*HI and nick translated using digoxigenin-11-dUTP using the DIG-Nick Translation kit as directed by the manufacture (Roche Molecular Diagnostics). The Southern blot to determine the physical state of HPV 59 in the cells was performed using 4 μg of cellular DNA not digested or digested with either *Bam*HI, *Hin*dIII, or *Alw*NI restriction endonucleases. Digested DNA samples were applied to a 1.0% agarose gel, separated by electrophoreses, and transferred to a nylon membrane as previously described (REF). Hybridization of digoxigenin-11-dUTP-labeled probe, posthybridization washes, and detection of hybridized probe were performed using the DIG Nucleic acid Detection Kit as recommended by the manufacturer (Roche Molecular Diagnostics).

For estimation of viral copy number, the 8-kb genomic HPV 59 sequence was excised from the vector by endonuclease digestion using *Bam*HI and nick translated using P^{32}-labeled dCTP using the Nick Translation System Kit as directed by the manufacturer (Life Technologies, Gaithersburg, MD). The Southern blot to estimate viral copy number was performed using 1.2 μg of cellular DNA digested with either *Bam*HI or *Hin*dIII. Whole genomic HPV 59 DNA was added to lanes for comparison of signal strength, in amounts of 3.2, 32, 320, or 3200 pg, corresponding to 1, 10, 100, or 1000 viral copies per cell. DNA samples were

applied to a 1.2% agarose gel, separated by electrophoreses, and transferred to a nylon membrane as previously described (Brown et al., 1993). Hybridization of radiolabeled probe, posthybridization washes, and detection of hybridized probe were performed as previously described (Brown et al., 1993).

RT-PCR to detect cellular and viral transcripts

For RT-PCR, one flask of cells was harvested at 70% confluence in RNA Later (Ambion, Austin, TX). Additional flasks of cells were permitted to reach confluence in complete F medium containing 2 mM calcium chloride. Cells were harvested in RNA Later at intervals of 3, 5, 7, and 10 days after addition of calcium chloride to induce differentiation. RNA was extracted as directed by the manufacturer using the RNaqueous Midi System (Ambion). RNA was treated with DNA-Free (Ambion) to eliminate contaminating DNA.

RT-PCR was performed using 100 ng total RNA using the Titan One-tube RT-PCR System (Roche Molecular Diagnostics) to amplify the human involucrin (forward TCCTCCAGTCAATACCCATC, reverse AGTTGCTCATCTCTCTTGACT) and loricrin (forward ATTGGCAGCGGCTGCATCA, reverse CTATTTGGACGGCCAGGT) transcripts. RT-PCR was also performed to amplify the E1^E4 (forward AGATCTGCAGCAAACAGTAACCTGCAATGG, reverse GCGGCCGCTTATAGGCGTAGTGTTACTGCA), and E1^E4^L1 (forward AGATCTGCAGCAAACAGTAACCTGCAATGG, reverse TACTCTGTATTGATATGCAGA) viral transcripts. In addition, primers were designed to amplify any other L1-encoding transcripts, using primers within the L1 open reading frame (forward CAGATGGCTCTATGGCGTTCT, reverse TACTCTGTATTGATATGCAGA). Human β-actin primers (forward ATGTACGTTGCTATCCAGGC, reverse CGCTCATTGCCAATGGTGAT) were used for each RT-PCR reaction to verify the integrity of RNA samples.

Reverse transcription was performed at 55°C for 30 min. Thermal cycling parameters were as follows: 94°C for 1 min, 55°C for 1 min, 68°C for 1 min for a total of 30 cycles, followed by a 6-min incubation at 68°C. The PCR products were applied to a 1.5% agarose gel and visualized by ethidium bromide staining. The E1^E4 and E1^E4^L1 RT-PCR products were cloned into the pCRII-TOPO cloning vector (Invitrogen) and sequenced.

Detection of HPV 59 by DNA in situ analysis

Paraffin-embedded tissue sections of differentiated cells or control tissues were fixed by heating at 80°C. Sections were then deparaffinized in xylene followed by ethanol. Sections were then treated with 25 μg/mL proteinase K for 5 min at 37°C followed by 3% H_2O_2 in methanol for 30 min. Sections were washed in phosphate-buffered saline, pH 7.4, dehydrated by incubation in ethanol, and dried. The entire 8-kb HPV 59 genome was nick translated using the Nick Translation System Kit as directed by the manufacturer (Life Technologies), using biotin-dCTP.

The HPV 59 DNA probe was added at 5 ng/μL to a DNA in situ hybridization buffer (Dako, Carpenteria, CA). Sections were covered with a HybriSlip (Research Products International, Mount Prospect, IL) and heated at 95°C for 10 min. Probe and target DNA were allowed to hybridize overnight at 37°C. Sections were washed in 20% formamide in 2× SSC (1× = 0.15 M NaCl, 15 mM sodium citrate) plus 0.05% Tween 20 for 10 min at 37°C, followed by an additional wash in 2× SSC plus 0.05% Tween 20 for 10 min at 37°C. Sections were blocked and probes were detected as directed by the manufacturer using the Tyramide Signal Amplification Cyanine 3 System (NEN Life Sciences, Boston, MA). Sections were dehydrated in ethanol and mounted using Vectashield (Vector Laboratories, Burlingame, CA). Slides were inspected using a fluorescent microscope at 590-nm wavelength.

Detection of the E1^E4 and L1 proteins

Cells were grown in complete F medium containing 2 mM calcium chloride for 10 days. Medium was removed, and the adherent cell mass was scraped from the flask. Cells were fixed in zinc-formalin, and paraffin-embedded tissue sections were prepared. One section was stained with hematoxylin and eosin for histologic evaluation. To detect L1 major capsid protein and involucrin in the differentiated cells, sections were deparaffinized with xylene and ethanol, treated with 3% H_2O_2 in methanol to reduce endogenous peroxidase activity, and blocked with nonspecific rabbit serum. For detection of the E1^E4 protein, sections were treated as above with the additional steps of citrate buffer unmasking followed by trypsin digestion as previously described (Brown et al., 1994).

To detect the L1 major capsid protein, anti-L1 serum (from rabbits immunized with a bacterially-expressed HPV 11 trpE-L1 fusion protein) was added at a 1:500 dilution (Brown et al., 1993). To detect the HPV 59 E1^E4 protein, a polyclonal serum was prepared by immunizing rabbits with a synthetic peptide within the E4 open reading frame: SVDTHSTLSLPACQ in single letter amino acid code (CovalAb, Lyon, France). Immune serum was then affinity purified against this peptide. Anti-E1^E4 antibodies were used at 1:100 dilution to detect E1^E4 protein in the differentiated cells. Preimmune rabbit serum was used as a control. To test the anti-E1^E4 antibodies for specificity, immunohistochemical assays were performed using paraffin-embedded sections of HPV 59-infected foreskin xenografts grown in athymic mice, and NIKS cells were induced to differentiate with 2 mM calcium chloride. Antibody binding was detected using the Vectastain ABC detection system (Vector Laboratories) to yield a purple precipitate. Slides were examined by light microscopy.

Electron microscopy

For transmission electron microscopy, cells induced to differentiate for 18 days in complete F medium containing 2 mM calcium chloride were scraped from flasks and fixed in 3% glutaraldehyde. The tissue-like mass of cells was embedded and sectioned. Sections were stained with uranyl acetate.

Infectivity assay

Cells were grown in medium containing 2 mM calcium chloride for 12 days and harvested by scraping the cells from the plate as indicated above. Cells were placed in phosphate-buffered saline, pH 7.4, and sonicated with a Fisher Sonic Dismembrator (Fisher, Pittsburgh, PA) for 60 s at high power in a covered tube to prevent aerosolization. The preparation was examined by phase contrast microscopy to assure complete lysis of all cells. Foreskin fragments were added to the sonicated cell preparation with an equal volume of KGM (Clonetics, Walkersville, MD) and incubated at 37°C for 90 min.

Foreskin fragments were then implanted under the renal capsules of six athymic mice as previously described (Bryan et al., 2000). Xenografts were removed after 4 months of growth and examined for histologic evidence of infection and for HPV 59 sequences by DNA in situ hybridization, PCR/reverse blot strip assay, and immunohistochemistry for the E1^E4 protein.

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