Synthesis of Infectious Human Papillomavirus Type 18 in Differentiating Epithelium Transfected with Viral DNA

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The lack of a permissive system for the propagation of viral stocks containing abundant human papillomavirus (HPV) particles has hindered the study of infectivity and the early stages of HPV replication. The organotypic (raft) culture system has permitted the study of a number of the differentiation-specific aspects of HPV, including amplification of viral DNA, expression of late genes, and viral morphogenesis. However, these investigations have been limited to a single virus type, namely, HPV type 31 (HPV31). We have artificially introduced linearized HPV18 genomic DNA into primary keratinocytes by electroporation, followed by clonal expansion and induction of epithelial stratification and differentiation in organotypic culture. We report the synthesis of infectious HPV18 virions. Virus particles approximately 50 nm in diameter were observed by electron microscopy. HPV18 virions purified by isopycnic gradient were capable of infecting keratinocytes in vitro, as shown by the expression of multiple HPV18-specific, spliced transcripts.

Cancer of the cervix is the most common cancer in developing countries and the second most common malignancy in women worldwide (26). Papillomaviruses are associated with greater than 90% of all cases of cervical cancer (29). Over 70 human papillomavirus (HPV) types have been identified, with subsets that are associated predominately with malignant or benign neoplasias of the anogenital area, particularly the cervix (29).

Replication of HPV is intimately connected to the differentiation program of the host tissue, squamous epithelium. The ability to synthesize HPV virions in vitro has been hindered by the lack of a reproducible tissue culture system proficient in viral morphogenesis. With the organotypic (raft) culture system, our laboratory and others have been able to achieve the differentiation-specific viral DNA amplification, late gene expression, and virion morphogenic stages of HPV type 31 (HPV31) (9, 19). However, culture systems continue to be deficient in the ability to propagate stocks of HPV competent for infectivity studies. Additionally, investigations of the differentiation-dependent life cycle of HPV in vitro are limited to a single viral type, HPV31 (9, 19). These limitations continue to hinder the ability to study the life cycle of the virus, in particular, the early stages of infection.

A system whereby HPV DNA could be introduced into keratinocytes to yield virus stocks would be useful in the analysis of the early stages of infection of various HPV types. In addition, serologic and genetic analyses would also be made practical. We have investigated the probability that the artificial introduction of cloned HPV DNA into keratinocytes might yield sufficient quantities of infectious virus. Following epithelial stratification and differentiation in organotypic cultures, electron microscopy examination of raft culture tissue sections revealed virus particles approximately 50 nm in diameter. HPV18 virions were subsequently purified by isopycnic gradient and were able to infect keratinocytes. We report the expression of HPV18-specific E6*I, E6*II, and E1*E4 spliced transcripts in infected keratinocytes.

MATERIALS AND METHODS

Keratinocyte cultures, organotypic cultures, and electron microscopy. Primary human foreskin keratinocytes (HFK) and primary human ectocervical keratinocytes (HCK) were isolated from newborn circumcision and adult hysterectomy tissue specimens, respectively, as previously described (35). Keratinocytes were grown in monolayer culture by using E medium plus 5 ng of epidermal growth factor per ml in the presence of mitomycin-c-treated J2 3T3 feeder cells (18, 21, 22). Keratinocyte lines stably maintaining HPV18 DNA following electroporation were subcloned by limiting dilutions of cells and isolation of individual colonies on tissue culture plates with cloning cylinders.

Organotypic (raft) epithelial culture tissues were grown as described previously (20–22, 24, 35). Briefly, keratinocytes transfected with HPV18 DNA were seeded onto rat tail type 1 collagen matrices containing J2 3T3 feeder cells not treated with mitomycin. After epithelial attachment and growth to confluence, collagen matrices were lifted onto stainless steel grids. Once lifted to the air-liquid interface, epithelial raft cultures were fed by diffusion from underneath with E medium lacking epidermal growth factor. As viral gene expression has been shown to peak at 12 days in the raft system (25), raft cultures were allowed to stratify and differentiate for 12 days. Electron microscopy was performed on raft tissue cross sections and purified virus preparations (19).

Electroporation of primary keratinocytes. HPV18 DNA, a generous gift from Harold zur Hausen, was linearized at nucleotide (nt) 2440 by restriction digestion with EcoRI, interrupting the E1 open reading frame. Viral DNA and a plasmid encoding a hygromycin B-selectable marker were electroporated into HFK or HCK (21). Cells were selected with 25 µg of hygromycin B per ml beginning 72 h after electroporation. Selection lasted 3 to 4 days, when the cells began to detach from the plate. Stacks of the electroporated cell lines were prepared and stored in liquid nitrogen until further use.

HPV particle isolation and Southern (DNA) blot hybridization. Virions were isolated by first scraping the raft culture epithelium off the collagen with a scalpel and washing the tissue with 0.15 M NaCl. Tissue was ground in a mortar with sea sand, resuspended in buffer 1 (1 M NaCl, 0.05 M Na2HPO4 [pH 8.0]), and centrifuged for 10 min at 8,000 × g. The pellet was discarded, and supernatants from both centrifugations were pooled and centrifuged for 1 h at 130,000 × g. Following centrifugation at 130,000 × g, the supernatants were discarded and the pellet was resuspended in buffer 2 (0.05 M NaCl, 0.1 M EDTA, 0.05 M Na2HPO4 [pH 7.4]) and centrifuged for 10 min at 8,000 × g. This supernatant was removed and saved on ice; the pellet was reextracted with buffer 1 and centrifuged for 10 min at 8,000 × g. The pellet was discarded, and supernatants from both centrifugations were pooled and centrifuged for 1 h at 130,000 × g. Following centrifugation at 130,000 × g, the supernatants were discarded and the pellet was resuspended in buffer 2 (0.05 M NaCl, 0.1 M EDTA, 0.05 M Na2HPO4 [pH 7.4]) and centrifuged for 10 min at 8,000 × g. This supernatant was removed and saved on ice; the pellet was reextracted with buffer 2 and centrifuged for 10 min at 8,000 × g. Supernatants from these last two centrifugations were pooled, CsCl was added to a final concentration of 1.3 g/ml, and the samples were centrifuged for 24 h at 135,000 × g. Fractions of 0.5 ml each were collected from the gradient and dialyzed against Tris-EDTA (TE) buffer (pH 8.0).

Total cellular DNA and viral DNA were isolated as previously described (19, 21), and 5 µg was electrophoresed per lane in a 0.7% agarose gel. Southern blot hybridization was performed as previously described (19).
The presence of virus particles was limited to upper suprabasal
layers of the epithelium, and the particles observed were simi-
lar to those detected in clinical biopsy material from low-grade
lesions (8, 15, 23, 27, 34). The observations of HPV18 virion
synthesis were reproducible by using several HPV18 DNA-
transfected HCK and HFK lines.

Preparation of HPV18 viral stocks for infectivity assays. We
sought to use HPV18-infected keratinocyte lines that were
shown by electron microscopy to synthesize virus particles in
differentiated raft tissue to propagate and purify virions for use
in infectivity assays. However, the yield of viral particles from
these parental cell lines was consistently low. We reasoned that
the small amounts of virus obtained would be insufficient to
pursue studies concerning infectivity and early stages of repli-
cation.

We considered the hypothesis that the parental cell popula-
tions might lack homogeneity with respect to copy numbers of
episomal HPV18 DNA. To address this, we subcloned the
parental cell lines. Southern blot hybridization analysis was
used to determine the copy number and maintenance of epi-
sonal HPV18 DNA in the subcloned lines. Results obtained
with representative subcloned lines of the HCK 18:1B parental
line are shown in Fig. 2. Nearly half of the subcloned lines
contained no detectable amounts of HPV18 DNA (Fig. 2,
lanes t and k, and data not shown). Each subcloned cell line
with detectable levels of HPV18 DNA maintained the viral
dNA episomally. The subcloned cell lines maintaining HPV18
varied in viral genomic DNA copy number (Fig. 2). This illus-
trated that parental cell lines consisted of a heterogeneous
population of cells with respect to HPV18 DNA content.
The parental line HCK 18:1B and the subcloned lines HCK
18:1Bj and HCK 18:1Bs (Fig. 2) were allowed to stratify and
differentiate in the raft culture system. Viral particles were
extracted from raft tissues and purified by isopycnic gradient
centrifugation as described in Materials and Methods. With
the parental HCK 18:1B cell line, it was difficult to visualize a
band on an isopycnic gradient corresponding to the density
expected for virus particles. However, the subcloned lines
HCK 18:1Bj and HCK 18:1Bs formed readily observable bands at the expected density following isopycnic gradient centrifugation (Fig. 3). Gradient fractions were collected and analyzed by Southern blot hybridization, confirming the presence of HPV18 DNA (Fig. 4). Gradient fractions 7, 8, and 9 were found to contain HPV18 DNA at high levels (Fig. 4). Fractions positive for HPV18 DNA correlated with fractions where the banded virus was observed in the gradient (Fig. 3 and 4) and had the buoyant density reported for papillomaviruses (29). Gradient fractions positive for HPV18 DNA were investigated for the presence of viral particles. As demonstrated by electron microscopy, both the parental HCK 18:1B cell line (Fig. 5D) and the subcloned HCK 18:1Bj and HCK 18:1Bs cell lines (Fig. 5A to C, E, and F) were able to support the production of HPV18 virions, albeit in vastly different quantities. Data from Southern blot hybridization analysis indicated that viral inocula contained between $1 \times 10^6$ and $2 \times 10^9$ particles per ml for the subclonal cell lines HCK 18:1Bj and HCK 18:1Bs and greater than 1 order of magnitude fewer particles for the parental line HCK 18:1B. The levels of virions produced in the subcloned lines resulted in clumping of the particles (Fig. 5A and B), as has been seen with higher concentrations of cottontail rabbit papillomavirus and bovine papillomavirus purified from infected tissues (4). To obtain electron micrographs of individual HPV18 virions, virus preparations from subcloned cell lines were diluted (Fig. 5C and E to F). The cosedimentation of both HPV18 DNA and viral particles within the same fractions suggested that these were complete HPV18 virions.

**HPV infectivity analyses.** We next investigated whether our HPV18 viral stocks were capable of infection, the final step in the viral life cycle. Aliquots of HPV18 stocks were allowed to attach to and infect monolayer cultures of HFK. Due to the lack of an infectivity assay for HPV, we assayed for the expression of spliced viral transcripts in infected cells. Following 24 or 72 h or four passages in culture, total RNA was isolated from HPV18-infected and mock-infected HFK monolayer cultures (Fig. 6). Little is known concerning the structures of HPV18-specific mRNAs (2). By using as a model the various spliced E6 transcripts (designated E6*I, E6*II, E6*III, and E6*IV) identified for HPV16 (2, 32), we assayed for similarly spliced transcripts in our HPV18-infected cells. HPV18-specific E6*-spliced transcripts were demonstrated by RT-PCR in the subcloned HCK18:1Bj producer line and in HPV18-infected HFK lines but not in mock-infected cells (Fig. 6). The splice donor and splice acceptor sites for HPV18 E6* and E1*E4 were previously reported (30), and our results are in agreement (Fig. 6). Additionally, we detected E6*-spliced transcripts corresponding in size to E6*I and E6*II that were previously observed in HPV18-associated cervical cancer-derived cell lines but were not sequenced or otherwise characterized (13). Interestingly, RT-PCR products with sizes corresponding to four spliced transcripts (E6,E7,E1*E4, E6*E7,E1*E4, E6*II,E7,E1*E4, and E6*IV,E2) were observable in the HPV18:1Bj producer line (Fig. 6, lanes 1 and 2) but only E6* E7,E1*E4 and E6*II,E7,E1*E4 were detected in HPV18-infected lines (Fig. 6, lanes 5, 9, and 13). This implies a difference between the splice acceptors used early in infection and those used in more established HPV18-associated lines. Furthermore, sequencing of cDNAs from the HCK18:1Bj producer line verified transcripts corresponding to HPV18 E6*E7,E1*E4 and E6*III. The E2 splice acceptor (illustrated in Fig. 6, transcript E6*IV,E2) was also identified by sequenc-
ing a cDNA corresponding to E6*, E7, E1*E2 (data not shown). In addition, we detected an amplification product of approximately 430 bp in one of the infected cell lines 24 h postinfection (Fig. 6, lane 7) and in the HPV18:1Bj cell line (Fig. 6, lanes 1 and 2). The structure of the transcript corresponding to this product is currently under investigation.

As the introduction of HPV18 early genes into keratinocytes has been shown to increase their life span (10, 12), we assessed the life span of our HPV18-infected cell lines. Mock-infected keratinocytes grew in culture for 6 to 8 weeks and then senesced. By using separate viral stocks from the parental HCK 18:1B line and the subcloned lines HCK 18:1Bj and HCK 18:1Bs, we found that six of six infected lines were able to grow a month longer in culture than the mock-infected and normal keratinocyte cell lines. Infected cell lines with extended life spans also expressed HPV18 spliced transcripts (Fig. 6, lanes 9 to 11).

**DISCUSSION**

The ability to study the complete life cycle of HPVs has been limited by the lack of an efficient system yielding infectious viral stocks. We have used DNA electroporation in combination with the organotypic (raft) culture system to propagate sizable quantities of virions of a preselected HPV type in vitro. By using this system, we produced HPV18 virions, as demonstrated by isopycnic gradient sedimentation, Southern blot analysis, electron microscopy, and infectivity. Our data show that cell populations homogeneous in the maintenance of episomal viral DNA were efficient at in vitro synthesis of infectious HPV. Interestingly, the two subcloned cell lines described in this report differed 10-fold in HPV18 DNA content (Fig. 2) but produced equivalent amounts of viral particles.

To overcome the lack of an in vitro infectivity assay for HPVs, we investigated the expression of HPV18-specific, spliced transcripts. The presence of these spliced transcripts in HPV18-infected cells confirms entry of the virus into the cell and the initiation of early viral transcription. Furthermore, our data suggest changes in viral transcript expression during the first 3 days postinfection (Fig. 6, 24 h postinfection versus 72 h postinfection). An in vitro raft culture system capable of complete vegetative replication of HPV18 will permit the description of HPV18 transcripts and their temporal expression pattern, similar to what we have done with HPV31 (25).

The present work also supports the concept that epithelial differentiation is required for HPV virion production. The requirement of host tissue differentiation for efficient virus replication is not unusual and has been described for other viral systems, such as cytomegalovirus (36), Epstein-Barr virus (5, 7, 17), Friend virus (11), human immunodeficiency virus (6,
Identification of HPV18-specific, spliced transcripts in HPV18-infected keratinocytes. (A) Southern blot of RT-PCR products. Lanes: 1, 3 μl of the PCR product of HCK 18:1Bj cell line cDNA; 2, 0.3 μl of the PCR product of HCK 18:1Bj cell line cDNA; 3 to 12, 30 μl of each indicated PCR sample; 3 to 5, keratinocytes 72 h postinfection; 6 to 8, keratinocytes 24 h postinfection; 9 to 11, keratinocytes four passages postinfection; 12, control with no RNA added to RT-PCR mixture. j and P represent viruses isolated from subclone HCK 18:1Bj and parental HCK 18:1Bj raft culture tissues, respectively. At the left, DNA size markers of (from the top) 2,322, 2,027, 1,353, 1,078, 872, 603, and 310 bp are indicated. (B) Splicing patterns of HPV18 transcripts shown schematically, along with the corresponding HPV18 nucleotide numbers of the splice sites and the predicted sizes of the RT-PCR products. HPV18-specific, spliced transcripts identified in the HCK 18:1Bj cell line and in the infected keratinocytes are represented by corresponding symbols in A and B. P105 is the major early promoter of HPV18. The placement and orientation of PCR primers are illustrated by open arrows.

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