Amplification of Human Papillomavirus Genomes In Vitro Is Dependent on Epithelial Differentiation

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Human papillomaviruses (HPVs) infect squamous epithelium and establish their genomes as episomes in proliferating basal cells. As infected cells differentiate, the viral DNA is amplified to high copy number and infectious virus is produced. Viral production has not yet been observed in vitro due to the inability of standard culture methods to duplicate most stages of epithelial differentiation. In this study, we have examined a cell line derived from a low-grade cervical lesion and found that it contained episomal copies of an HPV-31 subtype, HPV-31b, at approximately 50 copies per cell. When allowed to stratify at the air-liquid interface of in vitro raft cultures, this cell line differentiates in a manner which histologically resembles a low-grade cervical lesion in vivo. We have observed the amplification of HPV-31b genomes in distinct foci in the upper portion of the in vitro-stratified epithelium similar to that found in productive HPV infections in vivo. Although transcripts from the late region of HPV-31b were also detected specifically in stratified raft cultures, no capsid protein was found. These studies duplicate one important aspect of a productive HPV infection in vitro, the differentiation-dependent amplification of papillomavirus genomes.

Human papillomaviruses (HPVs) are epithelium-specific viruses which are the presumed etiological agents of many anogenital cancers, particularly squamous cell carcinoma of the cervix (3, 7, 10, 19). Precursor lesions of cervical cancer are termed cervical intraepithelial neoplasia (CIN) and are graded into three categories (I to III) depending on the extent of abnormal maturation. In CIN I, undifferentiated basal-like cells are present in the lower one-third of the epithelium, with differentiation occurring in the upper two-thirds. These lesions are characterized by the presence of cells with unusually shaped nuclei surrounded by large vacuolated regions, called koilocytes, in the more superficial layers of the stratified epithelium (14). In CIN III lesions, little evidence for epithelial differentiation is seen and abnormal basal-like cells are found throughout the entire thickness of the epithelium (10). HPV types 16 (HPV-16), -18, -31, and -33 are associated with malignant lesions of the cervix (3, 7, 16, 19), while HPV-6 and -11 are generally limited to low-grade lesions which rarely progress to malignancy (6, 19). In contrast to the episomal forms of viral DNA found in low-grade (CIN I) lesions, viral sequences are often found integrated into the host chromosome in high-grade lesions (CIN III and carcinomas) (8, 17, 19, 26). Integration has been proposed as an activation mechanism for progression from low- to high-grade lesions, with the E6 and E7 transforming genes being selectively retained and expressed (21).

In a productive infection in vivo, amplification of papillomavirus genomes takes place in highly differentiated epithelial cells (4, 17, 26). Papillomaviruses infect basal epithelial cells, establishing their genomes as low-copy-number, au-

tonomously replicating episomes. In the upper portion of an infected epithelium, no replication of cellular chromosomes is observed, whereas viral genomes are amplified to high copy numbers. The expression of capsid proteins and the packaging of viral genomes are also limited to the most highly differentiated layers of the epithelium (15). Although a subtype of HPV-11 has been propagated by transplanting infected foreskin explants into nude mice (24), attempts to propagate HPVs in vitro have met with little success. Recently, two groups have reported the isolation of cell lines derived from cervical biopsies which contain episomal forms of HPV-16 DNA (5, 22). When one of these HPV-16-positive cell lines was transplanted onto the back of a mouse, synthesis of viral particles was detected (23), demonstrating that these lines are competent for differentiation-specific viral functions. No evidence for genome amplification or viral synthesis in vitro has been reported, and this most likely reflects a requirement for epithelial cell differentiation not achieved with traditional culture methods.

We have recently used an in vitro system which allows the stratification and differentiation of keratinocytes at the air-liquid interface of collagen raft cultures (1, 13) to demonstrate that transfected HPV-16 and HPV-18 sequences can alter the morphological differentiation of human keratinocytes in a manner similar to that seen in cervical lesions in vivo (12, 18). This system has also been used to demonstrate that the in vitro differentiation of cells isolated from biopsies of CIN I lesions or carcinomas morphologically resembles the lesion from which the cell lines were derived (20). In the present report, we describe the amplification of HPV genomes dependent on the state of epithelial cell differentiation in this in vitro system. A cell line derived from a CIN I lesion, CIN-612, was found to contain episomal copies of a subtype of HPV-31, HPV-31b. Upon stratification and differentiation of this cell line in the raft system, amplification of the viral genome was observed in the superficial layers. While differentiation-specific late viral transcripts were also

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FIG. 1. Genomic organization of HPV-31 (9). The HPV-31 sequences for the early (open bars) and late (solid bars) open reading frames and potential polyadenylation signals (▲) are shown. (A) Increments of 1 kb are shown; the virus is 7,900 nucleotides in length. (B) Restriction sites: H, *HincII*; S, *SphI*; E, *EcoRI*; X, *XbaI*. (C) Restriction site polymorphisms identified in subtype HPV-31b. Symbols: *, loss of the *HincII* site in the E5 open reading frame; E, additional *EcoRI* site in the L1 open reading frame. (D) Restriction fragments used as probes for Northern analysis, a 650-bp *HpaI* (Hp)-*Sau3A* (Sa) fragment in the E6-E7 region and a 1,450-bp *XbaI* (X)-*NcoI* (N) fragment in the late region.

detected in CIN-612 raft cultures, no capsid protein synthesis was detected, suggesting that a block to the generation of infectious virus in vitro may exist.

MATERIALS AND METHODS

Cell culture. The CIN-612 cell line was expanded from a biopsy specimen from a patient with a CIN I lesion (20) in KGM medium (Clonetics) and has been maintained in E medium with mitomycin-treated fibroblast feeders (12, 18). Subclones were isolated by limiting dilution of the parent line and expansion of single colonies. The CIN-612-5 line was isolated by limiting dilution of CIN-612 at passage 15. Collagen raft cultures for in vitro differentiation were performed as described previously (12, 18, 20). Cells were seeded onto a collagen plug containing fibroblast feeders and grown to confluence. The plugs were then lifted onto a stainless steel grid, and the epithelial cells were allowed to stratify and differentiate at the air-liquid interface for 2 weeks. The rafts were then fixed in 4% paraformaldehyde and embedded in paraffin, and 4- μ m cross sections were cut.

DNA and RNA analyses. Total cellular DNA was isolated and Southern blots were performed with 0.7% agarose gels as described previously (2). Hirt extracts were prepared from total cell lysates by described methods (5). Total cellular RNA was prepared and analyzed on Northern (RNA) blots as described previously (2). A 650-bp *HpaI*-*Sau3A* fragment (nucleotides 218 to 868) was used as an early-region probe, and a 1.45-kb *XbaI*-*NcoI* fragment (nucleotides 4999 to 6449) was used as a late-region probe (Fig. 1). High-stringency hybridization and wash conditions were used for both Northern and Southern blots (2).

In situ hybridization of the biopsy specimen and raft sections was performed by a modified method (Digene Diagnostics, Inc., Silver Spring, Md.). Cross sections of tissue or stratified rafts were cut onto slides treated with 3-amino-propyl triethoxysilane and baked for 2 h at 60°C. After removal of the paraffin with xylene, sections were digested with 400 to 1,200 mg of protease type XXV (Sigma Chemical Company, St. Louis, Mo.) per ml for 5 min at 37°C and, in some cases, treated with RNase (10 to 30 mg/ml) for 10 min at 37°C. Probes were prepared by Digene Diagnostics by conjugating horseradish peroxidase enzyme to HPV-31, HPV-16, pUC19 plasmid, or total human DNA. Slides were

FIG. 2. Southern blot of total cell DNA and Hirt supernatant DNA from CIN-612 cell line (passage 11). Lanes 1 through 4, Total DNA; lanes 5 through 9, Hirt supernatants. Restriction digests: lanes 1 and 5, uncut DNA; lanes 2 and 6, *Bam*HI; lanes 3 and 7, *Xba*I; lanes 4 and 8, *Sph*I; lane 9, *Mbo*I. Full-length HPV-31 viral DNA was used as a probe.

denatured, hybridized, and silver enhanced by the methods provided by the manufacturer, stained with hematoxylin and eosin, and visualized by light microscopy.

RESULTS

Cells expanded from cervical neoplasia biopsy specimens or normal cervical epithelium can be propagated in vitro for several passages as monolayer cultures but usually senesce. Occasionally, cells derived from CIN I lesions survive crisis and become established cell lines. The cells derived from one CIN I biopsy specimen have survived over 30 passages in culture (crisis occurs at passage 4 to 6) and appear to have become a continuous cell line, which we have designated CIN-612.

Initial Southern blot analysis of the CIN-612 cell line indicated the presence of HPV-16 sequences (20); however, detailed analysis of total cellular DNA under stringent conditions demonstrated only the presence of viral sequences homologous to HPV-31. The genomic organization of all HPVs is similar, and the map of HPV-31 (9) is shown in Fig. 1. To characterize the HPV-31 sequences present in this cell line, total cellular DNA was prepared from CIN-612 cells at early passage in culture; digested with *Bam*HI (no restriction site in HPV-31), *Xba*I (a single restriction site in HPV-31), or *Sph*I (three sites in HPV-31); and subjected to Southern analysis with an HPV-31 probe. A representative analysis performed on DNA isolated from CIN-612 cells at passage 11 is shown in Fig. 2. With DNA which was either uncut or digested with *Bam*HI, a pattern of migration was observed for HPV-31 sequences which was consistent with forms I, II, and III of episomal DNA. Similar patterns of migration consistent with episomal forms were observed with DNA isolated at passages between 9 and 20. Digestion with *Xba*I resulted in all hybridizing species migrating at the position of 7.9-kb linearized HPV genome, while *Sph*I digestion gave the 5.7-, 1.9-, and 0.3-kb fragments predicted for *Sph*I digestion of full-length circular HPV-31 DNA. A detailed restriction enzyme analysis of the HPV-31 sequences of the CIN-612 cell line revealed several restriction site polymorphisms distinct from those previously reported for HPV-31 (Fig. 1). An additional *Eco*RI restriction site in the L2 open

reading frame and the absence of a *HincII* restriction site in the E2 open reading frame were found in the viral sequences in CIN-612 DNA. No differences from the published HPV-31 sequences were apparent after digestion with *BamHI*, *BglII*, *ClaI*, *HindIII*, *PvuII*, *PstI*, *SalI*, *SphI*, or *XbaI* (not shown). The presence of these restriction site polymorphisms indicates that this is a new subtype of HPV-31, and we designated this isolate HPV-31b. The copy number of HPV-31b present in CIN-612 cells was estimated to be 50 to 60 copies per nucleus by comparison with standards.

Evidence confirming the episomal nature of the HPV-31b sequences in the CIN-612 cell lines was provided by the analysis of Hirt extracts of cellular DNA. Following high-salt extraction of CIN-612 cell lysates, HPV-31b DNA was found primarily in the supernatant fraction (Fig. 2), consistent with an episomal form for viral sequences. Restriction analysis of the HPV-31b DNA isolated from Hirt extracts indicated the presence of circular genomes (Fig. 2, lanes 6 to 8), and digestion with *MboI* yielded fully digested fragments confirming the eukaryotic origin of these sequences (Fig. 2, lane 9). Further support for the extrachromosomal nature of HPV-31b sequences was obtained with two-dimensional gel electrophoresis of total cellular DNA from CIN-612 cells, which demonstrated the presence of monomeric and multimeric forms in addition to integrated copies of viral DNA (not shown). We therefore conclude that HPV-31b viral genomes are primarily present as extrachromosomal episomes in the CIN-612 cell lines at early passage.

When the CIN-612 cell line was examined in the raft system, it displayed morphological differentiation similar to that of the biopsy specimen from which it was derived (Fig. 3) (20). An identical morphology was seen in rafts of a clonal derivative, CIN-612-5, which was isolated at passage 15. The CIN-612-5 line was found to contain HPV-31b genomes at the same copy number as the parent line (data not shown). In raft cultures of both lines, an expanded parabasal layer was evident, with perinuclear clearing and abnormal nuclei reminiscent of koilocytes present in the superficial layers (Fig. 3A, CIN-612 [passage 6], and Fig. 3E, CIN-612-5 [passage 16]). No such characteristics were seen in stratified rafts of normal keratinocytes (Fig. 3B). These observations and the presence of episomal copies of HPV DNA led us to look next for evidence of differentiation-specific viral processes of genome amplification and late gene expression *in vitro*.

In situ hybridization studies of biopsy material derived from low-grade papillomavirus-induced lesions have shown that viral DNA is amplified in the abnormal nuclei of koilocytes in the upper regions of the epithelium (26). Similar amplification was observed following hybridization of a horseradish peroxidase-labeled HPV-31 probe to sections of the biopsy specimen from which the CIN-612 cell lines were derived (Fig. 3G). A strong hybridization signal was observed in the koilocytic nuclei of this biopsy in the superficial regions of the epithelium, while no signal was seen with a control probe made with pUC19 DNA (Fig. 3H). The intensely staining HPV-31b-positive cells were found to be

clustered in two distinct foci within the biopsy specimen. After hybridization of sections of CIN-612 (passage 6) raft cultures with the HPV-31 probe, a strong signal was observed in numerous nuclei in the upper regions of the rafts (Fig. 3C), and the copy number in each strongly hybridizing nucleus was estimated to be about 10^3 . A similar pattern of hybridization was also observed with the clonal CIN-612-5 line (passage 16) (Fig. 3E).

Hybridization of serial sections of these rafts demonstrated that the DNA amplification signal was clustered in numerous distinct foci in both mass and clonal lines. This suggests that these are preferred regions of viral amplification even in raft cultures, which presumably contain a homogeneous population of cells. The weak hybridization signal observed in the basal cell nuclei of the stratified raft cultures is consistent with the presence of approximately 50 copies of HPV-31b in these cells (Fig. 3C and E). Pretreatment of the slides with RNase did not decrease the hybridization signal (data not shown), providing evidence that the signal seen in the nuclei was due to DNA amplification and not to increased RNA expression. Hybridization of cross sections of CIN-612 stratified rafts to either pUC19 (not shown) or HPV-16 (Fig. 3D) sequences failed to show any signal.

To confirm that the signal we observed in the upper portion of the raft sections was due to hybridization to amplified viral DNA and not to processing artifacts, cross sections of CIN-612 cells cultured in the raft system were hybridized with a total human DNA probe. A strong signal was observed in all nuclei throughout the section and not limited to the upper portion of the raft (Fig. 3F), confirming that the effect seen with the HPV-31 probe was not due to differential access of probe to tissue. In addition, no positive signal was observed when normal keratinocytes were hybridized with the HPV-31 probe (Fig. 3B). We therefore have been able to reproduce *in vitro* the amplification of HPV DNA in a differentiation-specific manner, which is characteristic of a productive infection *in vivo*.

We next examined the differential expression of HPV-31b early and late genes in monolayer culture and in stratified rafts. A representative Northern blot of total RNA from CIN-612 and CIN-612-5 cells (both at passage 20), probed with fragments specific for either the E6-E7 region or the late region, is shown in Fig. 4. Equal amounts of total RNA were loaded into each lane, as confirmed by ethidium bromide staining of rRNA (data not shown). Two predominant transcripts of 4.0 and 2.0 kb were observed in blots probed with an early-region probe (650-bp *HpaI*-*Sau3A* fragment from the E6-E7 region), with the smaller species being more abundant (Fig. 4A, lanes 1 to 4). No differences in E6-E7 transcripts were apparent between cells examined at passage 12 and at passage 20 in submerged cultures (data not shown) or when RNA from CIN-612 and CIN-612-5 rafts was compared with that from submerged cultures at comparable passage numbers (Fig. 4A, lanes 1 and 3 versus 2 and 4). A large hybridizing band of approximately 7 kb may be a

FIG. 3. *In situ* hybridization of stratified raft and biopsy specimen tissue sections. (A) Hematoxylin- and eosin-stained cross section of a stratified raft of the CIN-612 cell line at passage 6 in culture; (B) stratified raft of primary keratinocytes probed with HPV-31; (C and D) cross sections of rafts of the CIN-612 line probed with HPV-31 (C) and HPV-16 (D); (E) cross section of a stratified raft of CIN-612-5 at passage 16 probed with HPV-31; (F) cross section of a stratified raft of the CIN-612 line hybridized with a probe against total human DNA; (G and H) tissue sections of the biopsy specimen from which the CIN-612 cell line was derived, probed with HPV-31 (G) and pUC19 (H). Sections were hybridized with DNA probes conjugated with horseradish peroxidase, and the hybridization signal was visualized by silver precipitation. Staining was done with hematoxylin and eosin. Magnification, $\times 430$.

FIG. 4. Northern blot analysis of CIN-612 cell lines and normal keratinocytes. (A) HPV-31 early-gene probe (650-bp *HpaI-Sau3A* fragment); (B) HPV-31 late-gene probe (1.5-kb *XbaI-NcoI* fragment). Relative exposure times were approximately four times longer for panel B than for panel A. (A) Lanes 1 and 2, total RNA from cultures of CIN-612 (passage 20); lanes 3 and 4, cultures of CIN-612-5 (passage 20); lanes 5 and 6, cultures of normal keratinocytes; lanes 1, 3, and 5, submerged monolayers; lanes 2, 4, and 6, stratified rafts. (B) Lane 1, submerged monolayer culture of CIN-612-5 (passage 20); lanes 2 and 3, stratified raft cultures of CIN-612-5 (passage 20). Ten micrograms of RNA was loaded in each lane except lane 3 in panel B, which has 20 μ g of RNA. The relative migration positions of 28S and 18S rRNAs are indicated as markers.

precursor RNA (Fig. 4A, lane 3) and was not seen in other RNA preparations. Hybridization of the E6-E7 probe to RNA from normal keratinocytes grown in either submerged or raft cultures revealed no signal (Fig. 4A, lanes 5 and 6). When RNA prepared from stratified raft cultures of CIN-612-5 (passage 20) was hybridized to the late-region probe (1.45-kb *XbaI-NcoI* fragment), high levels of a 3.2-kb and a 1.7-kb transcript (Fig. 4B, lanes 2 and 3) were seen. While a low level of the 3.2-kb transcript was seen in monolayer cultures at comparable passage numbers (Fig. 4B, lane 1), it was much reduced (approximately 10-fold) from that seen in raft cultures (Fig. 4B, lane 2) and no evidence for a 1.7-kb late-region RNA was apparent. In addition, no evidence for these transcripts was seen in stratified rafts of primary keratinocytes (data not shown).

To determine whether capsid protein was being synthesized in raft cultures, cross sections of stratified rafts of CIN-612 adjacent to those shown to be positive for viral DNA amplification were examined by immunohistochemistry for the presence of late proteins. Preliminary staining with antiserum against bovine papillomavirus type 1 (BPV-1) capsid proteins showed weak positive perinuclear staining, but no signal could be detected with a polyclonal antiserum generated against bacterially synthesized L1 protein from HPV-16 (data not shown), which did detect antigen in the original biopsy specimen. In addition, no capsid proteins were immunoprecipitated with HPV-16 L1 polyclonal serum from raft cultures which had been labeled overnight with [35 S]methionine (data not shown). We therefore conclude that little or no capsid proteins are synthesized in this system and that the positive signal with the BPV-1 antiserum was due to cross-reactivity with koilocyte-specific cellular proteins.

The above studies were performed on CIN-612 cells at early passage (less than 25), which contained primarily episomal forms of HPV-31b. Cells containing only integrated copies of HPV-31b were also detected, and the number of such cells increased after passage in culture. At passage 8, five of six clones generated by limiting dilution of the parent line were found to contain primarily episomal copies, while

at passage 15, five of eight clones generated were found to contain primarily episomal HPV-31b sequences (data not shown). A predominance of cells containing integrated copies was apparent at passage numbers above 25 and may reflect a growth advantage for these cells. Typically, the copy number of HPV-31b in these integrated lines ranged from 3 to 50 genomes per cell, and no amplification of templates was observed following stratification in the raft system (data not shown). As previously reported for other HPV-expressing keratinocyte cell lines (12, 18, 20), the CIN-612 cell lines also lost the ability to differentiate in the raft system following passaging in culture. At passages greater than 25, the CIN-612 cell lines retain the ability to stratify but lose the capacity to differentiate with a CIN I-like morphology (not shown).

DISCUSSION

In this study we have examined the ability of cell lines derived from a low-grade cervical neoplasia (CIN I) to duplicate features of a productive HPV infection in vitro. Unlike many cell lines derived from genital lesions which contain integrated copies of HPV sequences, the cell lines examined in this investigation contain autonomously replicating viral genomes. The CIN-612 cell line was derived from a low-grade CIN I lesion (20), and monolayer cultures have been shown to maintain episomal copies of HPV-31b DNA at approximately 50 copies per cell for more than 20 passages in culture.

We have previously shown that the morphological changes seen in cells derived from low-grade cervical lesions following stratification in the raft system resemble those seen in cross sections of the original biopsy specimens (20). When early passage cells from the CIN-612 and CIN-612-5 cell lines were allowed to stratify in vitro in the raft system, an expanded parabasal region was evident, with distinct polarity of differentiation. In addition, cells at the top of the stratified epithelium exhibited vacuolated regions around abnormally shaped nuclei, reminiscent of koilocytes seen in vivo. Cross sections of stratified rafts were examined by in situ hybridization for HPV-31b sequences, and amplification of templates was seen in the upper regions of the raft in the abnormally shaped nuclei of the koilocytic cells. As all the cells in the stratified epithelium contain HPV-31b sequences, it seems clear that the signals directing amplification of HPV-31b DNA are controlled by the differentiation state of the cell. It is intriguing that not all koilocytelike cells in the stratified rafts of the clonal CIN-612-5 line were positive for genome amplification, but this is similar to what was observed in vivo. This suggests that a high level of differentiation-specific control is used in regulating viral amplification in a subset of cells and is consistent with in situ studies of HPV-11 xenografts (24).

Examination of RNA from stratified rafts for late-gene expression revealed two major transcripts which were not present or present at much reduced levels in submerged monolayer cultures. The observation that significant late-gene expression occurs only in raft cultures is consistent with the hypothesis that a distinct differentiation state controls viral late-gene expression. However, no capsid proteins were detected by either immunohistochemistry or immunoprecipitation, suggesting that a block to translation of late transcripts exists in vitro. Alternatively, the transcripts we observed by Northern analysis may not be capable of encoding functional capsid proteins. Mutations in the L1 open reading frame have been reported in clones of HPV-16

isolated from episomal molecules (5) and may also be present in this isolate of HPV-31b. Since no information is available regarding the nature of HPV-31 late transcripts in vivo, we also cannot be sure that the RNAs we observed in vitro are identical to those seen in a productive infection.

After passaging in culture, integration of HPV-31b episomes into the host chromosome was observed. The number of cells containing integrated copies increased with time in culture, and we suspect this may result from a growth advantage for cells containing integrated copies. Cells containing episomal copies of HPV-31b were found to grow very slowly compared with other HPV-transformed keratinocytes (25a). Integration has been suggested as a mechanism involved in the progression of HPV-induced lesions in vivo through the removal of E2 autoregulation (21, 25). Interestingly, in two independent CIN-612 clonal derivatives, the integration site was mapped to the E2 open reading frame (1a). The genome amplification studies described in this report were performed on cell lines at early passage, when predominantly episomal forms of HPV-31b are present. Consistent with previous reports of HPV-transformed keratinocytes, CIN-612 cells lost the ability to differentiate as a function of passaging in culture, even in cells containing episomal copies of HPV-31b (12a). These cell lines will allow a detailed examination of the role of integration of HPV genomes as an event required for the progression of papillomavirus-induced disease.

A major hindrance to papillomavirus research has been the lack of a permissive tissue culture system for propagating virus. We have made substantial progress in the development of such an in vitro system, as we can now observe differentiation-specific viral template amplification as well as late transcripts in vitro. These observations suggest that the raft system mimics those differentiation-specific processes seen in vivo, and they provide support for the ability of the raft system to duplicate many aspects of the viral life cycle in vitro. The mechanism by which epithelial differentiation controls papillomavirus replication can now be addressed with this in vitro system. These viral processes could be controlled either by the differential activation of cellular transcription factors, such as occurs during keratin gene expression, or as a result of changes in the intracellular structure during squamous differentiation. As is the case with Epstein-Barr virus (11), it is possible that two different HPV origins of replication exist which have distinct roles in basal and suprabasal cells. The eventual generation of virions in vitro will allow detailed study of the entire life cycle of the virus from infection and establishment to virus production.

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