Development of a Cellular Assay System To Study the Genome Replication of High- and Low-Risk Mucosal and Cutaneous Human Papillomaviruses

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We found that recircularized high-risk (type 16 and 18) and low-risk mucosal (type 6b and 11) and cutaneous (type 5 and 8) human papillomavirus (HPV) genomes replicate readily when delivered into U2OS cells by electroporation. The replication efficiency is dependent on the amount of input HPV DNA and can be followed for more than 3 weeks in proliferating cell culture without selection. Cotransfection of recircularized HPV genomes with a linear G418 resistance marker plasmid has allowed subcloning of cell lines, which, in a majority of cases, carry multicopy episomal HPV DNA. Analysis of the HPV DNA status in these established cell lines showed that HPV genomes exist in these cells as stable extrachromosomal oligomers. When the cell lines were cultivated as confluent cultures, a 3- to 10-fold amplification of the HPV genomes per cell was induced. Two-dimensional (2D) agarose gel electrophoresis confirmed amplification of mono- and oligomeric HPV genomes in these confluent cell cultures. Amplification occurred as a result of the initiation of semiconservative two-dimensional replication from one active origin in the HPV oligomer. Our data suggest that the system described here might be a valuable, cost-effective, and efficient tool for use in HPV DNA replication studies, as well as for the design of cell-based assays to identify potential inhibitors of all stages of HPV genome replication.

Due to their association with distinctive human cancers, human papillomaviruses (HPVs) are widely studied. Papillomaviruses have been phylogenetically grouped into genera, species, types, subtypes, and variants (11), and more than 100 HPV types have been identified thus far. The best-characterized α-genus HPVs are associated with infections of the mucosal epithelium that lead to the induction of benign tumors. These viruses are divided into high-risk types (e.g., high-risk HPV type 16 [HR-HPV-16] and -18), which have the capability of inducing anogenital malignancies, and low-risk types (e.g., LR-HPV-6 and -11), which induce hyperproliferative mucosal lesions and are rarely associated with malignancy. Mucosal HPV infections tend to clear on their own, but in some cases, latent infection could be established and may persist for years. Cutaneous β-genus HPV infections are highly prevalent in the general population and tend to persist (14). Vaccines based on virus-like particles made up of the capsid protein L1 have been developed against HPV-6b, -11, -16, and -18 (Cervarix [GlaxoSmithKline] and Gardasil/Silgard [Merck Research Laboratories]). Because these virus types are responsible for only a portion of all HPV-induced malignant and benign tumors, a clear need exists for vaccines or antivirals against a broader spectrum of pathogenic HPV types.

Despite the differences in viral pathogenesis, progeny virion production invariably depends on cell differentiation and occurs only in terminally differentiated keratinocytes. HPVs require the host’s replication machinery to reproduce their genomes, and these viruses have developed a unique replication strategy that is adapted to keratinocyte differentiation (52). HPVs infect basal proliferating epithelial cells and establish infection in the nucleus, where viral genomes are maintained as extrachromosomal, multicopy nuclear plasmids. The papillomavirus genome replication cycle can be divided into three stages: first, the amplificational replication of the viral genome after initial entry; second, stable maintenance replication in proliferating infected cells, which involves segregation and partitioning of the viral plasmids into daughter cells upon cell division; and third, amplificational viral genome replication in differentiating nondividing keratinocytes, which provides the viral genomes for packaging into virions (25).

All papillomaviruses have similar but not identical genomic buildups. The locations of the open reading frames (ORFs) and regulatory elements are conserved; however, the sequences and compositions of these regions differ considerably between the different HPV types. The bidirectional (theta-type) replication mode of the papillomavirus genome is initiated by the coordinated action of the viral replication proteins E1 and E2 (43, 48). These two viral proteins are responsible for viral genome duplication during stable replication and during multiplication in the final phase of amplification (25). The E2 protein is important for the regulation of viral gene expression and is also responsible for the segregation and partitioning functions (35, 39) of the viral genome during the latent infection stage in dividing cells. Viral oncoproteins E6 and E7 are
capable of modulating the cell cycle, which may lead to immortalization of the infected cells (42).

Immortalized and transformed cell lines were useful in studying the activities and functions of animal papillomavirus genes and genomes in the early days of papillomavirus research. The mouse fibroblast cell line C127 transfected with the bovine papillomavirus type 1 (BPV-1) genome (30) can maintain the viral DNA as a freely replicating plasmid with a constant copy number. These cells have been used to study the basic mechanisms of viral gene transcription, DNA replication, and the regulation thereof. The BPV-1 genome replicates in a highly regulated manner in synchrony with the host DNA (25), expresses viral genes, and induces the transformation of C127 cells; however, this is a heterologous system, and thus, differentiation-specific functions of the virus cannot be studied.

Although a number of human papillomavirus types were isolated more than 30 years ago, the limited availability of appropriate cell culture systems supporting HPV DNA replication has hampered progress in researching viral DNA functions and regulatory pathways. Powerful raft culture models (3) and xenograft models have been developed; these models have been useful in vitro experimental systems for HR-HPV research (6) and for studying the pathogenesis of HPVs (22). Primary keratinocytes isolated from different body sites, including the foreskin, larynx, and cervix, can be transfected with recircularized HPV genomes to generate cells carrying the HPV genome. The first organotypic raft experiments with primary keratinocytes (PHKs) immortalized by the HR-HPV DNA were successful but gave little information regarding the normal papillomavirus life cycle. Modifications to the organotypic culture conditions to induce differentiation provided the opportunity to propagate wild-type and mutant HPV genomes; thus, the ability to study papillomavirus genetics was achieved, at least for HR-HPVs (12, 15, 16, 19, 36, 37), although the yield of virions from stably transfected cells has been low. Introducing adenovirus recombinants carrying the HPV genome flanked by loxP sites and Cre recombinase into PHKs grown in raft cultures also allowed the production of infectious HPV virions (31), and an improved method for efficient production and passage of HPV-18 has been described (7, 49). Also, cell lines have been established from mild dysplasia that harbored episomal HPV genomes, and these lines allow viral DNA amplification and packaging when grown in organotypic cultures (45, 46). Stable episomal HPV DNA replication has been extensively studied in the HPV-16-containing model cell line W12 (9, 20, 40, 41). At early passages in monolayer culture, the cells retain HPV-16 episomes at 100 to 200 copies per cell (45); however, in long-term cultivation and in the absence of feeder cells, the spontaneous loss of episomes and the appearance of cells containing only the integrated HPV-16 genome occur (41).

The use of replication systems to analyze low-risk HPV life cycles has been less effective. The episomal replication of LR-HPV-11 in the tongue squamous carcinoma cell line SCC4 (10) and in primary foreskin keratinocytes (38) has been demonstrated. Additionally, collagen raft systems have been shown to function for HPV-11-positive laryngeal cells (1). Although LR-HPV-11 cannot immortalize keratinocytes, it changes the differentiation pattern of the raft cultures and increases their life span, albeit to a lesser degree than HR-HPVs (47). Viable raft systems capable of recapitulating the HPV-11 life cycle have been obtained only in cells that have already been immortalized by ectopic expression of TERT, the catalytic subunit of telomerase (13). To our best knowledge, the transformation of normal keratinocytes by skin-related HPVs has been unsuccessful, and no replication of β-genus HPV genomes has been achieved in any in vitro system.

Most established human cell lines fail to support HPV genome replication. However, the replication of papillomavirus origin-containing plasmids can be reconstituted in many cell types of different species by expressing the viral replication proteins E1 and E2 from heterologous vectors (4, 5, 43). These results suggest that the lack of regulated expression of the viral proteins from HPV genomes could be the reason for the failure that restricts HPV genome replication to certain cell types. We analyzed the abilities of different immortalized or transformed human cell lines to support E1- and E2-dependent replication of the HPV origin-containing plasmids. Although most of the human cell lines analyzed (C33A, 293, and HaCaT) supported the E1- and E2-dependent replication of the HPV origin-containing plasmids, one cell line was remarkably efficient in supporting this type of DNA replication. We observed that the human cell line U2OS, which is derived from a moderately differentiated osteosarcoma, has an adherent epithelial morphology and carries the wild-type pRb and p53 genes, very effectively supports the DNA replication of the HPV replication origin-containing plasmids, even at low concentrations of the viral replication proteins. Further experiments with recircularized HPV-18, -16, -11, -6b, -5, and -8 genomes have shown efficient viral DNA replication in U2OS cells in transient assays. Additionally, our data suggest that the stable episomal maintenance of the different HPV types, including α-genus low-risk (LR-HPV-6b and -11) and high-risk (HR-HPV-16 and -18) beta-papillomaviruses (HPV-5 and HPV-8), has all of the characteristics of the stable replication of HPV genomes in proliferating basal cells. This U2OS system also allows the reconstitution of events leading to the amplification of the viral genome, reminiscent of the viral life cycle in its late phase. We showed that the amplification of the viral genomes was triggered in these HPV-positive cell lines when they were cultivated as confluent cultures with regular feeding.

We believe that the described HPV replication system can be useful for studying the fundamental properties of papillomavirus DNA replication and for the development of inhibitors of HPV genome replication in human cells.

MATERIALS AND METHODS

Cell lines and transfection. U2OS cells from the American Type Culture Collection (ATCC) (number HTB-96) were grown in Iscove’s modified Dulbecco’s medium (IMDM) that was supplemented with 10% fetal calf serum (FCS). Cells were transfected by electroporation (48) using a Bio-Rad Gene Pulser II apparatus supplied with a capacitance extender (Bio-Rad Laboratories). The capacitance was set to 975 μF, and the voltage was set to 220 V in all experiments.

Plasmids. HPV DNA sequences were cleaved out of the following vector backbones: the HPV-18 genome from pBR322 with EcoRI, HPV-6b from pBR322 with BamHI, HPV-16 and HPV-11 genomes from pUC19 with BamHI, HPV-8 DNA from pUC19 with BamHI, and HPV-5 from pBR322 with SacI. Linear HPV fragments (ca. 8 kb) were religated at low DNA concentrations (30 μg/ml) for 16 h at 4°C.

Generation of mutant genomes (see Fig. 3H, line 1). The region of the genome encoding late proteins (L1 and L2 ORFs) of HPV-18 was removed by cleavage
of the HPV genome with Apal and BpiI (Fermentas). HindIII linkers were added at the ends, and a subgenomic fragment of HPV-18 was cloned into the HindIII site in the pUC18 cloning vector, generating plasmid pUCeHPV18, which carries the early region of HPV-18. The E1 protein expression mutant pUCeHPV18/E1/OilI was generated by digestion of the subgenomic plasmid with OilI (a single site in the E1 ORF), and a 22-bp oligonucleotide, 5'-TCGGTGATGCAAACCUGAGG-3', was inserted into the OilI site with T4 ligase. The same approach was used for generating the HPV-18 full-genome E1 mutant in the OilI site (pBRHPV1-18/EoOilI). The E1 protonation and expression mutant pUCeHPV18/E1/BcuI was generated by digesting the HPV-18 sub-genome construct with BcuI (Fermentas) and filling the BcuI site with a Klentaq fragment of DNA polymerase (Fermentas) in the presence of deoxynucleoside triphosphates (dNTPs), followed by religation of the DNA. The E2 protein expression mutant pUCeHPV18/E2/StuI was generated in the subgenomic fragment of HPV-18 at the StuI site by insertion of a 22-bp oligonucleotide, 5'-TCCGACCTGCGACGCTTTC3'.

Expression vectors for the HPV-18 E1 and E2 proteins were engineered, and their capability to express the respective proteins was verified in our previous publication (20).

Subcloning, maintenance, and amplification. Religated circular HPV plasmids (5 µg of HPV-6b, -11, -16, -18, or -8), with T4 ligase as described above (see Materials and Methods), were transfected into 293T cells as described previously (21). The transfected cells were cultured by treating the gel with Sol A (0.5 M NaOH and 1.5 M NaCl) for 30 min. Ethidium bromide (0.3 g/ml denatured carrier DNA) was added every second day, and the medium was changed at the last two time points to ensure the availability of nutrients to the cells. Total or episomal DNA was extracted after 2, 4, 6, 8, 10, and 12 days.

DNA extraction. Extrachromosomal DNA was extracted from exponentially growing cells or from dense cultures by the modified Hirt method (21, 24). Cells were lysed in 0.8 ml of lysis buffer (0.5% SDS, 50 mM Tris, pH 8.0, and 10 mM EDTA) per 10-cm dish at room temperature for 15 min, the NaCl concentration of the lysates was brought to 1 M with 5 M NaCl and the lysates were stored at -20°C overnight on ice. The lysates were centrifuged at 12,000 rpm and 4°C for 20 min. The Hirt pellet was dissolved in TE (10 mM Tris, pH 8.0, and 1 mM EDTA) at 4°C for 1 h and the solution was used as the second dimension. The separated DNA fragments were transferred onto a membrane (Hybond-N+, Amersham Pharmacia Biotech) using the Trans-Blot Semi-dry Transfer system (Bio-Rad) and stored at -20°C.

DNA digestion with restriction enzymes. DNA cleavage with restriction enzymes (Fermentas) was carried out as recommended by the manufacturer. Extracted DNA from transient-replication assays was digested with the appropriate enzymes and with DpnI for fragmentation of the bacterially produced input plasmids.

1D gel electrophoresis. For one-dimensional (1D) replication product analysis, digested DNA was resolved in a 0.8% agarose gel in 1× Tris-acetate-EDTA buffer (TAE). The uncut DNA samples were loaded into the wells of standard horizontal 0.4% agarose gels, and the gels were submersed in 1× TAE containing 0.3 µg/ml of ethidium bromide. Electrophoresis was carried out at room temperature at 6.0 V/cm for 4 to 5 h for restriction analyses and at 0.2 V/cm for 72 h for uncut-DNA analysis.

Two-dimensional (2D) gel electrophoresis. For 2D replication product analysis, the first dimension was run on a 0.4% agarose gel in 0.5× TBE (Tris-borate-EDTA) without ethidium bromide at 0.4 V/cm for 72 h at room temperature. The second dimension was run on a 1% agarose gel in 0.5× TBE at 6 V/cm for 5 h at 4°C. Ethidium bromide (0.5 µg/ml) was added to the gel and buffer of the second dimension. The separated DNA fragments were transferred onto a membrane and hybridized with the appropriate 32P-labeled probe generated by random priming.

Southern transfer and hybridization. The DNA in the agarose gel was denatured by treating the gel with Sol A (0.5 M NaOH and 1.5 M NaCl) for 30 min. The gel was then rinsed with double-distilled water (ddH2O) and with Sol B (1 M Tris, pH 7.4, 1.5 M NaCl). DNA was transferred by capillary transfer to a Hybond-N+ filter (Amersham Pharmacia Biotech) using 10× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate) buffer (1.5 M NaCl and 150 mM Na2C6H4O6) for 6 to 18 h. The DNA was cross-linked to the filter using a UV Stratalinker 1800 apparatus (Stratagene). To avoid nonspecific binding to the filter, it was treated with prehybridization solution at 65°C for at least 45 min (30% 20× SSC, 10% SDS, and 200 µg/ml denatured carrier DNA). An HPV genomic DNA fragment was used as a specific probe and labeled using a DecaLabel DNA Labeling Kit (Fermentas) and radioactive [γ-32P]dCTP (Du Pont NEN). The filter was hybridized for 2 to 3 h at 65°C. Posthybridization, the filter was washed three times for 15 min each time with washing buffer I (2× SSC and 0.1% SDS) and three times for 15 min each time with washing buffer II (0.2× SSC and 0.1% SDS). Signals were detected by exposure to X-ray film.

FISH. To perform fluorescence in situ hybridization (FISH) analysis on interphase cells, the cells were grown on polylysine-coated coverslips (Thermo Scientific). The cells were fixed in ice-cold methanol-glacial acetic acid (3:1). FISH probes were generated by nick translation, using biotin-16-dUTP as a label and a corresponding HPV genome as a template. The final sizes of the probe fragments were adjusted to 200 to 500 bp by DNase I digestion. Cell preparations were denatured at 75°C in 70% formamide for 3 min, immediately dehydrated in a series of ethanol washes (70%, 80%, and 100%), and air dried. The hybridization mixture (10 µl per slide) was composed of 50% formamide in 2× SSC, 10% dextran sulfate, 100 ng of a denatured probe DNA, and 5 µg of denatured herring sperm carrier DNA. Hybridization was performed overnight at 37°C in a moist chamber. The following FISH procedures were performed according to the manufacturer’s protocol (Invitrogen Corporation; TSA Kit 22). Nuclei were counterstained with DAPI (4’,6-diamidino-2-phenylindole) and mounted in phosphate-buffered saline (PBS) with 50% glycerol. The slides were analyzed with an Olympus IX81 fluorescence microscope equipped with the appropriate filter set.

Reverse transcription (RT)-PCR. Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. The total RNA was treated with DNase I (Fermentas), followed by heat inactivation of the enzyme. cDNA was synthesized with a First Strand cDNA Synthesis kit (Fermentas) using 1 µg of total RNA as a template and oligo(dT) primers in a 20-µl reaction volume. cDNA was diluted to 160 µl; 2.5 µl of the dilution was used in a single PCR, along with 300 nM forward and reverse primers and 2 µl commercial master mix, 5× Hot FirePol EvaGreen qPCR Mix (Solis Biodyne), in 10 µl of total reaction volume. Amplification was performed on a 7900HT Real-Time PCR System (Applied Biosystems) and analyzed using a comparative threshold cycle (ΔCt) method, comparing HPV transcript specific signals to the signals of a housekeeping β-actin gene. The signals were normalized to time point zero.

The primers used were as follows (see Fig. 3H, line 2, for a scheme). For HPV18 E6, the forward primer was 5’TGGCGTGTGCAGGAAACGTTG3’ and the reverse primer was 5’GTCGCGTGTCAGCAGACGAGT3’. For HPV18 E7, the forward primer was 5’CATTTACCAGCCCGGACAG3’ and the reverse primer was 5’GTCGCGTCGTGCGATGGT3’. For HPV18 E1, the forward primer was 5’GATGTTGCTGTTGTGCGATG3’ and the reverse primer was 5’GCCTGGTTGTGCTGCTGCTGCTG3’. For HPV18 L1, the forward primer was 5’GTTGACACTGTCACCTAAC3’ and the reverse primer was 5’AACATTGGGATGCGAGGTAAC3’. For beta-actin, the forward primer was 5’TGGCGTGTGCAGGAAACGTTG3’ and the reverse primer was 5’GTCGCGTGTCAGCAGACGAGT3’.
ward primer was 5’CTGGACCGTGAAGTGAC3’, and the reverse primer was 5’CGGCCACATTGTGAAC3’.

RESULTS

Transient replication of six different types of HPV genomes in the human U2OS cell line. Preliminary experiments had shown that the human osteosarcoma U2OS cell line is very efficient in supporting HPV E1 and E2 protein-dependent replication of viral-origin-containing plasmids (data not shown). This effect is due to the highly efficient transfection of the U2OS cells by electroporation and the high level of E1 and E2 protein expression in these cells. The results raised the question as to whether viral trans factors could be expressed at sufficient levels by viral promoters from their native genomic context. The successful expression of these factors would support the viral functions of replication, segregation, and partitioning of the HPV genomes in U2OS monolayer cultures. A set of six different papillomavirus genomes (four alphapapillomaviruses, HPV-6b, HPV-11, HPV-16, and HPV-18, and two betapapillomaviruses, HPV-5 and HPV-8) were cleaved from their cloning plasmids and recircularized at low DNA concentrations as described previously (18). The U2OS cells were transfected by electroporation with different amounts of recircularized HPV genomes in the presence of carrier DNA. Extrachromosomal DNA was extracted from the transfected cells at different time points using the Hirt method. DNA was then purified and analyzed by Southern blotting after linearization with specific endonucleases and digestion with DpnI to remove bacterially produced input DNA. The HPV-16 (Fig. 1A), HPV-6b, HPV-11, HPV-18 (Fig. 1B), HPV-5 (Fig. 1C), and HPV-8 (Fig. 1D) genomes replicated efficiently in these cells. After the introduction of increasing amounts of the recircularized HPV-16 genome (1, 2, or 5 μg) into the U2OS cells, the viral DNA replication signal (indicated by the arrowhead) accumulated in a time- and input-dependent fashion (Fig. 1A, lanes 1 to 4, 5 to 8, and 9 to 12). Similar types of viral genome replication patterns were obtained with the five other HPV types studied. As shown in the figures, the intensity of the linear DpnI-resistant signal increases with time, which is consistent with viral genome replication in these cells (Fig. 1B, lanes 1 to 4 with 5 μg of inserted HPV-6b plasmid DNA, lanes 7 to 10 with HPV-11, and lanes 11 to 14 with HPV-18 DNA [5 μg]; C and D, HPV-5 and HPV-8, respectively). The transient-replication assays for the HPV genomes measure not only DNA replication, but also the transcription properties of the viral genomes. Therefore, we can conclude that all studied recircularized HPV genomes can initiate the expression of the viral replication factors from the viral promoters at levels sufficient for the initiation of viral genome replication in U2OS cells. This conclusion is important for the HPV-5 and -8 genomes, because U2OS cells provide an opportunity to study viral genome replication and viral gene expression for these HPV in the cellular environment for the first time.

In order to confirm that the observed initiation of replication of the viral genomes in U2OS cells is dependent on the E1 and E2 proteins, we generated several mutant genomes of the HPV-18 defective for expression of the E1 or E2 protein (described in Materials and Methods; see Fig. 3H, line 1, for a scheme). First, we generated the subgenomic fragment of HPV-18 lacking part of the genome encoding the L1 and L2 proteins. A related subgenomic fragment contained the viral origin of replication and coding sequences for the early proteins of HPV-18. In the transient-replication assay, such truncated viral genomes replicated effectively (Fig. 1E, lanes 1 and 2) at a level comparable to that of the recircularized HPV-18 genome (Fig. 1E, lanes 3 and 4). A defect in the E2 ORF (the Stul site) eliminated the capability of the truncated HPV-18 genome to replicate (Fig. 1E, lanes 5 and 6), while cotransfection of this recircularized subgenomic fragment with the HPV-18 E2 protein expression vector restored the replication function to the truncated viral genome (Fig. 1E, lanes 7 and 8). Elimination of E1 protein function by insertion of the oligonucleotide linker at the Olil site eliminated the capability of the truncated genome (Fig. 1E, lanes 9 and 10) and full-size (Fig. 1E, lanes 21 and 22) HPV-18 genome to initiate replication of the viral origin. Complementation of these truncated or full-size E1-defective genomes with a truncated HPV-18 genome (pStuI) defective in E2 expression restored the transient-replication function to the truncated and full-size E1 mutants (Fig. 1E, lanes 11 and 12, and 23 and 24, respectively), which indicates that the E1 and E2 proteins are both necessary for the initiation of replication of the viral genome in U2OS cells and that the two defective viral genomes complement each other’s defects. An identical result was obtained with the E1 protein mutation at the BcuI site, where a frameshift in the E1 ORF was generated by Klenow fill in. The truncated genome was unable to initiate replication in a transient assay (Fig. 1E, lanes 15 and 16). This defect was complemented by the E2 mutant producing E1 protein (Fig. 1E, lanes 17 and 18). For a reason that is unclear, the E2 mutant genome replicates less effectively than E1 mutant genomes in complementation assays (Fig. 1E, lanes 23 and 24). All E1 mutant genomes were effectively complemented by the expression vector producing E1 protein (Fig. 1E, lanes 13 and 14, lanes 19 and 20, and lanes 25 and 26).

We believe that these data provide evidence that replication of the studied HPV genomes in U2OS cells is dependent on the E1 and E2 proteins, as described previously for the replication of the BPV-1 genome (48) or for the HPV genomes in primary keratinocytes (23, 29).

Maintenance of the HPV genomes in the U2OS cell line. In these transient assays, the strong, time-dependent HPV DNA replication signals in U2OS cells suggested further evaluation of the HPV genomes’ capacity to undergo stable episomal replication. To evaluate stable episomal replication, 5 μg of recircularized HPV-6b, -11, -16, -18, -5, or -8 genome was cotransfected by electroporation with 5 μg carrier plasmid (AraD) and 2 μg of linearized pNeo-EGFP or pBabeNeo plasmid. These plasmids encode G418 resistance and allow the selection of transfected cells. A dilution series of cells was made 48 h posttransfection, and G418 selection (400 μg/ml) was applied. The medium was changed twice per week. Two to 3 weeks later, the G418-resistant cell clones were chosen separately or left over cells were pooled and cultivated for analysis. In a parallel experiment, the cells were maintained after transfection with recircularized viral genomes without the addition of G418 to the medium. The low-molecular-weight (LMW) Hirt extracts from the entire antibiotic-resistant cell population (“pool” DNA) or from cells grown without G418 were analyzed by Southern blotting using radioactively labeled probes.
FIG. 1. Southern blot analysis of the transient DNA replication of high- and low-risk mucosal HPV and cutaneous HPV genomes in U2OS cells. (A) Analysis of HPV-16 genome replication in U2OS cells. Episomal DNA was extracted at the indicated time points from U2OS cells that had been transfected with 1 μg (lanes 1 to 4), 2 μg (lanes 5 to 8), or 5 μg (lanes 9 to 12) of recircularized HPV-16 genomes. The DNA was then purified and treated with a linearizing enzyme (BamHI) and DpnI to eliminate the input DNA. The samples were loaded onto a 0.8% agarose gel, separated, transferred to nylon membranes, and hybridized with radiolabeled HPV-16 genome-specific probe as described in Materials and Methods. The episomal DNA extracted from the carrier DNA-transfected U2OS cells is in lane 13. The marker (lane 14) for the replication of the linearized 8-kb fragment of the HPV-16 genome is also indicated by an arrowhead. The DpnI-cleaved input HPV-16 DNA is in lane 15. fr., fragmentation, or cleaving. (B) Analysis of the transient DNA replication of recircularized HPV-6b, -11, and -18 genomes. Religated genomic DNAs of HPV-6b, -11, and -18 (5 μg) were introduced into U2OS cells by electroporation. The episomal DNA was isolated and treated as described above with the exception that subtype-specific hybridization probes were used for each virus. Lanes 1 to 4 represent analyses of HPV-6b DNA replication at the indicated time points. Lane 5 is the marker for the replicating unit size of the HPV-6b genome. Lanes 6 to 9 represent the analyses of HPV-11 genome replication. Lane 10 represents the linear marker for HPV-11 replication. Lanes 11 to 14 represent the analyses of HPV-18 genome replication. Lane 15 is the linear marker for HPV-18 replication. Lane 16 represents the EcoRI- and DpnI-cleaved marker for the input HPV-18 DNA. (C) Transient replication of the HPV-5 genome in U2OS cells. Shown is analysis of replication by restriction with the linearizing enzyme SacI. Lanes 1 to 4, 5 to 8, and 9 to 12 represent cells transfected with 2 μg, 5 μg, or 10 μg, respectively, of recircularized HPV-5 genomes. Lane 13 shows the analysis of the carrier-transfected cells. Lane 14 is the linear marker for HPV-5 replication. Lane 15 is the SacI- and DpnI-cleaved marker for the input HPV-5 DNA. (D) Transient replication of the HPV-8 genome in U2OS cells. Analysis of replication by restriction with the linearizing enzyme BamHI. Lanes 1 to 4, 5 to 8, and 9 to 12 represent cells transfected with 2 μg, 5 μg, or 10 μg, respectively, of recircularized HPV-8 genomes. Lane 13 shows the analysis of the carrier-transfected cells. Lane 14 shows the linear marker for HPV-8 replication. Lane 15 is the BamHI- and DpnI-cleaved marker for the input HPV-8 DNA. (E) Mutants in two of the early open reading frames, ORFs for E1 and E2, are deficient for transient replication for HPV-18 genomes in U2OS cells. Two micrograms of the viral genome carrying mutations in the E1 or E2 ORF (described in Materials and Methods; a scheme is shown in Fig. 3H, with the names of plasmids shortened) was tested in transient-replication assays separately, in combination with each other, or in complementation with expression vectors for E1 or E2. The mutants alone failed to replicate (lanes 5 and 6, 9 and 10, 15 and 16, and 21 and 22). Replication competence can be restored to E1 and E2 mutant genomes through complementation with each other (lanes 11 and 12, 17 and 18, and 23 and 24) or by addition of appropriate expression vectors (lanes 7 and 8 for the E2 mutant and 13 and 14, 19 and 20, and 25 and 26 for E1 mutants).
against the appropriate HPV types (Fig. 2A). The analysis showed that all tested pool samples contained HPV genomes at comparable levels, which indicates that the selected cells carry the HPV genome despite the fact that selection was for the transfected cells and not for the viral genomes (Fig. 2A, lanes 2, 4, 6, and 8). The HPV-transfected cells were surprisingly efficient at maintaining the HPV genomes even under nonselective conditions (Fig. 2A, lanes 1, 3, 5, and 7). This result confirms the transfection efficiency of U2OS cells by electroporation and the compatibility of the different viral genomes with replication and maintenance in these cells. It seems that the replication and maintenance of the viral epigenomes is not a burden to the cells and does not slow the growth of the HPV-containing cells compared with the parental U2OS cells.

Subcloning of the G418-resistant cells allowed isolation of cell lines that carry stably persisting HPV genomes. The single-cell colonies were picked, expanded, and cultivated under G418 selection. Total genomic DNA was extracted from these clones. Southern blot analysis was performed with 10 µg of EcoRI-linearized HPV-18 DNA; BamHI-linearized HPV-16, -11, -6b, or -8 DNA; or SacI-linearized HPV-5 DNA from total cellular DNA using the appropriate radiolabeled full-length HPV type-specific probes (Fig. 2C). Sets of single-cell subclones from the parental U2OS cell line for every HPV type were isolated. The results are grouped and marked above the blocks in Fig. 2C. Series of positive examples of high-risk HPV-18 and HPV-16 subclones that carry different copies of the HPV genome per cell line are shown. U2OS cell clones carrying the low-risk types HPV-11 and HPV-6b were also isolated, as well as subclones for betapapillomavirus types 5 and 8. The viral-DNA copy numbers in different cell lines varied from very low to very high, as indicated by Southern blotting. Interestingly, we noticed that the yield of isolation of the HPV-positive cases was dependent on the selection marker used (Fig. 2B). In the case of the mucosal HPV genomes, we used the commercial pNeo-EGFP plasmid (Clontech), which, in addition to the selection marker, also expressed enhanced green fluorescent protein (EGFP). The high-risk HPV-positive subclones were isolated with approximately 45% efficiency, whereas the low-risk HPV-11 and HPV-6b were identified in 29% and 15% of cases. In the case of cutaneous HPV5 and HPV8, we used two different plasmids for G418 selection, linearized pNeo-EGFP and pBabeNeo. In both viruses, expression of EGFP reduced the frequency of HPV-positive subclones.

Long-term follow-up of HPV-positive subclones. Experiments were performed to assess the persistence of HPV DNA in HPV-positive cell lines. Isolated HPV-positive subclones were passaged in cell culture to assess the long-term stable persistence of HPV genomes in U2OS cells. The majority of the tested cell lines were stable in monolayer cultures with regularpassaging (see Materials and Methods) during at least 2 months of follow-up. The stability of the extrachromosomal HPV DNA over the time course was determined by Southern blot analysis of equal amounts of linearized total cellular DNA (2 µg) (Fig. 2D) or from Hirt lysates (data not shown) extracted from the regularly passaged subclones. Figure 2D shows examples of stable HR-HPV-18 subclones 18#1.13 and 18#1.4. The restriction pattern was unchanged during the time course. One HPV-18 subclone, 18#1.10, with an irregular restriction pattern, apparently contained HPV-18 sequences integrated into the host genome. The majority of subclones with high-risk HPV-16 also maintained viral DNA quite stably, as shown for the HPV-16 subclones 16#2.5 and 16#3.16; however, there were some lines characterized by an immediate and stable integration pattern analogous to that of 18#1.10. Subclone 16#3.3 showed a slow decrease in the HPV-positive signal. When analyzing the low-risk HPV-11 and -6b genomes, we observed a rapid loss of the viral genome in some subclones upon continuous passage (Fig. 2D, subclone 6b#41); however, in other LR-HPV subclones, the viral genome was stably maintained (e.g., 6b#11). With subclone 11#3.13, we were able to follow the appearance of an HPV-11 genome integration event during the passage of the cells, accompanied by a parallel drop in the plasmid copy number. The summary of the analysis of the isolated G418-resistant subclones (Fig. 2B) suggests that not every isolated subclone carries an HPV genome; however, high-risk viruses established maintenance in the U2OS cells more effectively. This result may reflect the actual situation during infection with these viruses in vivo.

HPV genome amplification in confluent cell culture. In the productive stage of the HPV life cycle, the amplification of the viral genome is triggered by the initiation of keratinocyte differentiation. Induction of this productive stage of the viral life cycle in HR-HPV-positive cells has been generated by reconstructing the three-dimensional architecture of the differentiating epithelium in organotypic and raft cultures (15, 16, 18, 19, 37) or in confluent keratinocyte cultures or by suspending cells in methylcellulose under regulated culture and growth conditions (33, 50). It has been shown with HR-HPV-transfected keratinocytes that HPV genome amplification in these cells can be induced in all these cases.

We maintained U2OS-based HPV-positive subclones as confluent cell cultures with regular medium to mimic the conditions of viral DNA amplification induction in keratinocytes. Equal numbers of the appropriate HPV-positive cell clones (1 × 10^6 cells per 10-cm culture dish) were seeded and maintained as confluent cultures with regular feeding (see Materials and Methods). The cells were cultivated to high densities, and total or low-molecular-weight DNA was isolated and purified at days 2, 4, 6, 8, 10, and 12 postseeding for analysis. Using the HPV-18-positive cell line 18#1.13 as an example, we observed that HPV-positive cells grew to a density twice that of untransfected U2OS cells, as shown by cell counts (Fig. 3A). This result was confirmed by measuring the amount of total DNA extracted from the cells in HPV-18 (Fig. 3B) and HPV-16 (data not shown) cell clones. These data suggest that the presence of a high-risk HPV genome may change the growth properties of the cells. The HPV DNA amplification in confluent cell culture is shown in Fig. 3C with the HPV-18 subclone 18#1.13. Equal amounts (2 µg) of total DNA extracted from the cells were linearized with EcoRI and analyzed by Southern blotting using a virus-specific probe. Quantification of the HPV-18 DNA showed an approximately 10-fold increase in the amount of HPV DNA per cell in the confluent cultures (Fig. 3D). The amplification of the different HPV DNA subtypes was also achieved under the confluent culture conditions (Fig. 3G).

We used this amplification induction method to further ex-
FIG. 2. Establishment and maintenance of stable replication of HPV genomes in U2OS cells. HPV-6b, -11, -16, -18, -5, and -8 recircularized genomes (5 μg), carrier plasmid (AraD; 5 μg), and Eco0109I-linearized pNeo-EGFP or EcoRI-linearized pBabeNeo (2 μg) were cotransfected into U2OS cells by electroporation as described in Materials and Methods. Single-cell colonies were picked, expanded, and analyzed using Southern blotting. (A) Analysis of the episomal DNA extracted from HPV-18-, -16-, -11-, and -6b-transfected U2OS cells after 3 weeks of cultivation in the absence (−) or presence (+) of G418. The extracted DNA was purified and cleaved with EcoRI and DpnI for HPV-18 (lanes 1 and 2) and with BamHI and DpnI for HPV-16, -11, and -6b (lanes 3 to 8). DNA from cells transfected with pNeo-EGFP as negative control lanes are also shown (Neo, lanes 9 and 10). M. Lin., position of linear 8-kb HPV fragment. Mixed radiolabeled HPV genomes were used for probes. (B) Stable transfectants and isolated HPV-positive subclones. Two types of linearized selection markers were used, pNeo-EGFP and pBabeNeo (Neo). The different HPV-positive cell lines were isolated from each of the HPV transfection series, the total number of HPV-positive subclones and the yields of HPV-positive subclones are indicated for each HPV type. Collections of these cell lines have been generated. (C) Screening of stable transfectants by Southern blot analysis. Series of identified HPV-positive (Pos.) cell lines for HR-HPV-18 and -16, LR-HPV-11 and -6b, and skin papillomavirus HPV-8 and -5 genomes are grouped and marked above the blocks. Total DNA (10 μg) was used for Southern blotting with appropriate HPV-specific probes. DNA was linearized with EcoRI for the HPV-18 transfection series; with BamHI for the HPV-16, -11, -6b, and -8 transfection series; and with SacI for HPV-5. Subclone numbers for the appropriate HPV types are indicated above the lanes in every series and markers. Calculated viral genome copy numbers per haploid host genome are indicated on the right. Variability in the HPV copy numbers was noticeable in every group. (D) Stability assay for the HR- and LR-HPV subgenomes. Selected cell lines were cultivated for an additional 6 to 11 weeks (indicated by the numbers above the lanes) under regular monolayer culture conditions after the cells were determined to be HPV positive. Total-DNA samples were collected after every week of cultivation and loaded in equal amounts (2 μg for high-copy-number HPV plasmids and 10 μg for low-copy-number HPVs). The linearized total DNA (enzymes are indicated in the previous figure) was analyzed by Southern blotting using appropriate HPV-specific probes. Examples of stable HPV-18-positive cell lines are shown: HPV-18 subclones 18#1.13 and 18#1.4, HPV-16 subclones 16#2.5 and 16#3.16, and HPV-6b subclone 6b#11. Most of the other tested HR-HPV subclones possessed the same type of stability during the tested time period. Examples of clear off for HPV DNA (unstable) were more characteristic in the case of LR-HPVs, as demonstrated for HPV-6b subclone 6b#41, but HPV DNA was also lost from one HPV-16 subclone, 16#3.3. Subclones with stable nonspecific bands (such as 18#1.10), indicating early integration events, were rare. Integration, which is not characteristic of LR-HPVs, was detected for one LR-HPV subclone at later time points (11#3.13). Skin-related subclones are being examined further.
FIG. 3. Induction of DNA amplification in HPV-positive cell lines maintained under dense culture conditions. (A to E) The HPV-18-positive cell line 18#1.13 was grown in a regular monolayer until the cells were equally distributed (1 × 10^6 cells were seeded onto each of six 100-mm culture dishes) for additional cultivation under dense culture conditions. The medium (IMDM) was changed after every second day (or 2 ml of fresh culture medium was added after every 2 days). The time points for analyses were taken the day after the medium was changed or added during the 12-day growth period, with 2-day intervals. (A) Growth curves of untransfected U2OS cells and the HPV-18-positive cell line 18#1.13. The time-dependent growth of the cells during establishment of the dense cell cultures was analyzed at 2-day intervals for 12 days. The cells were counted with an Invitrogen Countess cell counter. (B) Amounts of total DNA in the time series. Total DNA was isolated by standard procedures, and DNA concentrations were measured using an ND-1000 NanoDrop spectrophotometer. The amounts of total DNA isolated per sample collected every 4 days during one of the 12-day growth periods from U2OS cells and 18#1.13 cells are shown in the bar graph. (C) Induction of DNA amplification under dense culture conditions demonstrated by Southern blot analysis of the HPV-18-positive cell line 18#1.13. Total cellular DNA was isolated at 2-day intervals during the 12-day growth period and analyzed. Equal amounts of DNA (2 μg) were digested with EcoRI and analyzed by Southern blotting with an HPV-18 genome-specific probe. The induction of DNA amplification was demonstrated by the increase of the replication signal over time. A standard series of marker lanes to calculate the copy number per haploid host genome is on the right. (D) Relative HPV-18 DNA copy numbers during amplification. The intensities of the amplification signals from two assays of the 18#1.13 cell line grown in dense cultures were measured using a PhosphorImager and ImageQuant software. The HPV-18 genome copy number was estimated by the standard curve of the marker lanes. The values of two different series were averaged, and the standard deviations are shown by error bars. (E) Increase of the cytokeratin 10 level in confluent HPV-containing U2OS cells. One million of the untreated and HPV18-positive U2OS cells (subclone 18#1.13) were plated on a 10-cm petri dish. The cells were grown with regular feeding of the culture and lysed 1, 4, 8, and 12 days later using Laemmli loading buffer, and the cytokeratin 10 level was measured using Western blot analysis. The lysate from 100,000 cells for every time point was loaded onto an SDS-PAGE gel in sample buffer containing 8 M urea. After separation, the proteins were transferred to a polyvinylidene
amino acid sequence of the HPV-positive U2OS cell lines are for differentiation. Keratin K10 is a cytokeratin that is used as an early differentiation marker for keratinocytes. Cell lysates were prepared from cells of HPV-18 subclone 18#1.13 and from U2OS cells at days 1, 4, 8, and 12 grown in amplification series to dense cultures and subjected to Western blot analysis using anti-keratin K10 antibodies. K10 expression is triggered in parallel with the induction of HPV-18 DNA amplification in subclone analysis (Fig. 3E); the density factor of the parental U2OS cell line was less effective.

To check for changes in viral transcription levels, total RNA was extracted from the HPV-18 subclone 18#1.13 at different time points during amplification, and RT-PCR using ORF-specific primers was performed (Fig. 3H). The signals obtained with primer pairs designed against five different viral ORFs (E1, E2, E6, E7, and L1) showed upregulation from 5 to 20 times compared to the signal of the housekeeping gene (beta-actin) mRNA (Fig. 3F). It is important to emphasize that the signals seen in this assay cannot be directly extrapolated to corresponding protein expression levels, because papillomaviruses harbor very complex protein expression systems in which the same protein could be produced from several alternative mRNA species and none of the mRNAs are monocistronic (51). Still, the data presented here clearly indicate that HPV genomes are transcriptionally active in these cell lines and that the activities of viral promoters increase during cultivation as a confluent culture. RT-PCR analysis using the ORF-specific primers showed a clear increase in the levels of viral transcripts for the mRNAs encoding proteins E1, E2, E6, E7, and L1 compared to the levels of mRNAs for housekeeping genes (Fig. 3F). The E2, E6, and E7 mRNA levels increased approximately 5 times, whereas the E1 and L1 mRNA levels increased 15 and 20 times, respectively, in this HPV-18-containing subclone. It is interesting that the kinetics of these mRNA level increases were different, with the E1 mRNA accumulating the fastest, which could be in agreement with the induction of viral genome amplification. The detection of HPV genome amplification in HPV-positive confluent cells is a clear indication of the episomal state of the viral genomes. The relative and absolute increases of the HPV-specific mRNA in confluent cells suggest that either the gene expression from the viral genomes or the stability of the mRNAs for these proteins was considerably increased, setting the stage for an accumulation of virus-specific mRNAs and proteins to induce amplification of viral DNA.

Analysis of HPV genome replication using FISH. To further characterize HPV DNA amplification, FISH analysis was performed on interphase cells using a commercial kit (Invitrogen Corporation; TSA kit 22). Examples of FISH analyses of interphase cells for HPV-18 subclone 18#1.13 under nonconfluent conditions before amplification are shown in Fig. 4A. The 18#1.13 cells exhibited the HPV-18 signal as a single or double dot in the nucleus of the interphase cell. This was a typical appearance of HPV genomes in interphase cells for all studied subclones (HPV-5, -8, -16, -6b, and -11) (data not shown). Two weeks after the cells were cultivated in a confluent culture, the HPV-18-positive signal in 18#1.13 cells increased due to the amplification of the viral genomes (Fig. 4B). Again, a similar robust increase in the HPV-specific signal in the nuclei of the interphase cells of all studied subclones was observed (data not shown). As shown by the examples, HPV genomes seem to localize to a few fixed locations in the nuclei of proliferating cells. Maintenance of the cells under confluent culture conditions induces the amplification of the HPV DNA at these few sites (larger dots), as well as the appearance of several dots all over the nucleus, bringing the viral copy number to several thousand per cell.

The physical state of HPV DNA in U2OS cells. To determine the physical states of the HPV DNA during different replication stages in U2OS cells, replication intermediates were analyzed in an undigested form starting from transient assays. Extracted plasmid DNA fractions and total DNA from the HPV-18-transfected cells were analyzed after DpnI treatment using Southern blotting. Analysis of low-molecular-weight DNA prepared by Hirt extraction revealed a series of discrete bands with increasing replication signals for the monomeric HPV genomic forms; however, at later time points, dimeric and higher forms of the HPV genome also appeared (Fig. 5A, lanes 1 to 3). This analysis for total DNA showed a similar appearance of the HPV genomes in the cells up to 7 days after transfection (Fig. 5A, lanes 4 to 6). An important difference between the episomal- and total-DNA samples was a clearer appearance of higher forms, presumably oligomeric forms, of the HPV genome replication products at later time points. The mechanism of oligomerization is unclear, and it could be caused by different processes. Three weeks (Fig. 5A, lane 7) after transfection, the majority of the HPV signal could be detected in dimeric and higher oligomeric forms.

In studying the physical states of HPV-positive subclones, larger-than-monomeric HPV DNA molecules from undigested...
DNA samples were the prominent signals in the gel. Analogous signals could be monitored using 1D analysis of uncut DNA from HPV-18 subclone 18#1.13 (Fig. 5B). Such oligomeric forms are characteristic of the physical states of all the HPV genomes in the different HPV-specific subclones, and oligomeric states have been effectively maintained over long passages. We speculate that oligomeric forms of the HPV genome are maintained most effectively in proliferating cells because the maintenance, segregation, and partitioning functions of the viral genomes have considerable impact on HPV DNA replication in these cells.

When the HPV-containing 18#1.13 cells were kept confluent, the robust amplification of monomeric and oligomeric forms was apparent (Fig. 5B, lanes 1 to 6). This result suggests that most of the signal came from extrachromosomal oligomeric forms of the HPV DNA capable of initiating DNA replication.

When extrachromosomal low-molecular-weight DNA and samples enriched with genomic DNA obtained by Hirt fractionation from 18#1.13 cells from nonconfluent (Fig. 5C) and confluent cultures (Fig. 5D) were analyzed in an uncut form (Fig. 5C and D, lanes 1 to 2 and 6 to 7), most of the amplified DNA remained in the low-molecular-weight fraction after induction of amplification (Fig. 5D, lanes 1 and 2). When Hirt supernatant and pellet samples were digested with three linearizing single-cutter enzymes (EcoRI, XmaJI, and BglII) for the HPV-18 genome, we found that even the highest-molecular-weight circular DNA species seen in undigested DNA could be completely eliminated, and prominent unit size bands could appear, migrating as linear 8-kb DNA fragments (Fig. 5C and D, lanes 3, 4, and 5 and 8, 9, and 10). Based on the restriction analyses of this experiment, we can conclude that the different oligomeric HPV genomes must be organized in episomal molecules as head-to-tail concatamers.

**Topology of the HPV DNA in U2OS cells.** Two-dimensional gels have been used to analyze undigested circular molecules, allowing the identification of covalently closed circles, open circles (OC), linear DNA molecules, and different forms of concatamers (Fig. 5E). Undigested HPV DNA samples were analyzed by 2D electrophoresis to identify the topological forms of the HPV DNA replication intermediates in U2OS cells. The first dimension was run on relatively low-percentage agarose gels (0.4 to 0.5%) at a low voltage (0.2 V/cm) and without ethidium bromide for 72 h to separate the molecules according to size. In the second dimension, the molecules were separated using a higher percentage of agarose (1.0%) at a higher voltage (6 V/cm) in the presence of ethidium bromide. Under these conditions, the separation occurred on the basis of the molecules’ topology. Molecular size standards were used in the first and second dimensions. Figure 5E helps to interpret the uncut DNA forms, and Fig. 5I shows the scheme for interpreting the different signals of the restriction analysis using 2D gels.

HPV DNA samples from Hirt lysates containing episomal molecules were isolated from HPV-18 18#1.13 cells from regular and dense cultures and were analyzed by 2D electrophoresis. The results presented here for 18#1.13 are representative of the results for all of the tested HPVs. Figure 5F and Fig. 5G and H represent the patterns for HPV DNA replication intermediates in the stable assay and during the amplification, respectively, using the undigested DNA samples. The complex pattern detected by the HPV-18 DNA probe during the stable-maintenance period in continuously passaged cells corresponds to different populations of plasmid forms that existed in vivo in the 18#1.13 cells (Fig. 5F). We saw most of the signal coming from the oligomeric forms of the genome, although weak signals from the monomeric episomes in both covalently closed circular (CCC) forms (shown by the arrowhead) and

![Figure 4](http://jvi.asm.org/ on August 29, 2017 by guest)
OC were detected. DNA extracted from the confluent culture contained elevated levels of HPV extrachromosomal molecules, including monomers (arrowheads) and oligomers (Fig. 5G and H). The signal present on the arc of the linear molecules is indicative of DNA linearization upon extraction and purification; however, some linearization can originate from the integrated HPV genomes.

Analysis of the replication intermediates of the HPV genome by 2D gel electrophoresis. Cleavage of HPV genomes with appropriate restriction enzymes and analysis by 2D electrophoresis allows the assessment of HPV genome replication competence and may help explain the mode of late HPV genome amplification. The HPV-18 DNA from 18#1.13 cells grown under dense culture conditions was extracted by the Hirt method, and the low-molecular-weight fraction was digested with NcoI and Bpu11021 (enzyme locations are shown in the scheme in Fig. 5L) and analyzed by 2D gel electrophoresis using hybridization of the Southern blots with an HPV-18 genomic probe. A double digestion of the HPV genome with these enzymes generated two fragments 2.5 and 5.4 kb in length. The first carries the origin of DNA replication, and the second carries the termination site of the 2-way replication of the monomeric genome. Therefore, the 2.5-kb fragment should contain the bubble and replication fork structures if only monomeric molecules are competent for replication. The strict arc arising from the 2.5-kb spot on the curve of linear molecules could correspond to the pattern expected for replicative intermediates whose migration in the second dimension is retarded; the replication products arising from the origin increase quickly in size (Fig. 5J). The appearance of a strict bubble arc when the same fragment was used as a hybridization probe indicates that the DNA replication origin must be active inside this 2.5-kb NcoI/Bpu11021 fragment. The other arc arising from the 2.5-kb linear fragment (1N) returned to the curve of linear molecules at a 5.0-kb location (Fig. 5J and I), the position to which almost completely replicated molecules would be expected to migrate. This pattern demonstrates that the 2.5-kb fragment is replicated by a double fork that progresses from one end to the other and generates a series of Y-shaped replicative intermediates. A straight signal that started near the inflection point of the arc of the Y-shaped replicative intermediates and extended diagonally upward was interpreted as replicative intermediates generated by two converging forks. This result also indicates that termination must occur within the studied fragment. Therefore, 2D electrophoresis of this 2.5-kb fragment showed that it had all of the hallmarks of replication intermediates, including the bubble, the replication fork, and the termination fragment of replication (Fig. 5J). These data strongly suggest that not only monomeric viral genomes, but also the oligomers of the HPV genome, are replicationally active (Fig. 5L, dimeric head-to-tail HPV multimer and restriction patterns calculated from the scheme). The detection of the bubble, the replication fork, and the termination signal in the origin fragment shows that, in head-to-tail oligomers, only one origin is active for the initiation of replication. The appearance of the termination signal in the origin fragment suggests the other origins are silenced. This conclusion is supported by a similar observation for BPV-1 genome replication in C127 cells, where formation, replication, and maintenance of the oligomeric BPV-1 genome has been studied (44). For example, head-to-tail-linked dimers have a termination site 180° opposite the active origin where another potential origin is located (Fig. 5L) but is inactive. A series of nonreplicative recombinants or signals from partially digested DNA molecules may complicate the interpretation of these data. In conclusion, the HPV monomeric and multimeric genomes are replication competent in the studied model system.

By probing the Southern blots with a radiolabeled probe located on the opposite side of the monomeric HPV-18 genome (the XmnI-AatII fragment), another 5.4-kb fragment from the NcoI/Bpu11021 cleavage of the HPV-18 genomes can be analyzed (Fig. 5K). Two-dimensional electrophoresis showed two major arcs for HPV-18 monomers: one characteristic of the Y-shaped replication fork structures and one characteristic of the termination of double Y structures (Fig. 5I and K).

**DISCUSSION**

Replication of HPV genomes in the U2OS cell line. Primary keratinocytes have been successfully used to reconstitute the HPV life cycle from infection to stable maintenance and to generate viral particles of high-risk HPVs. The oncoproteins of HR-HPVs immortalize, or prevent or delay senescence of, primary keratinocytes; therefore, these proteins aid in generating cells carrying extrachromosomal viral genomes, similar to a latent infection. This approach has been less effective in the establishment of experimental systems for low-risk HPVs and betapapillomaviruses, perhaps due to the lower ability of the viral oncoproteins of these subtypes to immortalize or prevent senescence of primary cells. To establish cell lines that carry episomes of low-risk HPVs and betapapillomaviruses for the purpose of studying the mechanisms of viral DNA replication in these cells, one alternative could be to use already immortalized, stable cells with an indefinite life span.

We searched for immortalized human cell lines capable of supporting HPV genome replication and found that the human osteosarcoma cell line U2OS, which has a flat epithelial-cell-like morphology and carries wild-type p53 and pRb genes, could effectively support extrachromosomal papillomavirus DNA replication. These cells could support the replication of high-risk HPV-16 and -18, low-risk HPV-6b and -11, and betapapillomaviruses HPV-5 and -8. Additionally, subcloning the transfected cells allowed us to isolate cell lines that carry extrachromosomal HPV genomes for all studied HPV types. The most effective maintenance of the viral genomes was with HPV-16 and -18, but stable subclones carrying HPV-6b, -11, -5, and -8 were isolated without problems. Most of the isolated subclones showed long-term maintenance of the HPV genomes for all the different virus types studied; however, some subclones behaved differently (examples are shown in Fig. 2D). With HPV-16, some isolated subclones showed a stable, specific multifragment restriction pattern, an indication that integration had occurred. During the long-term cultivation of low-risk HPV subclones, one single-cell clone was isolated that exhibited a reduction in the HPV-11 episome copy number and showed the appearance of integration. The rapid reduction in the copy numbers of viral episomes was apparent upon passing some HPV-6b- and -11-containing cell lines. At the
FIG. 5. Southern blot analysis for determining the structure of DNA replication intermediates. Abbreviations: mw, ladder of linear fragments; sc, ladder of supercoiled molecules; mc, multimeric circles; L, linear molecules. (A) Detection of HPV-18 DNA species during the first amplification. U2OS cells were cotransfected with 2 μg of religated HPV-18 DNA and carrier DNA. The uncut DNA from Hirt lysates (lanes 1 to 3) or 2 μg of total DNA (lanes 4 to 7) extracted 72, 96, and 168 h and 3 weeks (3w) posttransfection were separated on a 1D agarose gel and analyzed by Southern blotting using an HPV-18 genomic probe. A 7.1-kb circular (circ) HPV-18 E1 expression construct and an 11-kb circular pBR-HPV-18 DNA were used as plasmid DNA markers. (B) DNA status of HPV-18-positive subclone 18#1.13 during the second amplification. Two micrograms of HindIII (noncutter)-treated total DNA from HPV-18-positive cell line 18#1.13 was loaded, and replication intermediates were investigated during the 12-day incubation period in the amplification assay. The 7.1-kb plasmid DNA (HPV-18 E1 expression vector) and 11-kb pUC/HPV-18 plasmid DNA were used as molecular markers. (C) Head-to-tail oligomeric forms of the HPV DNA from HPV-18-positive subclone 18#1.13. The Hirt supernatant and pellet were extracted from cells cultivated under regular growth conditions allowing the stable maintenance of viral genomic material and were subjected to 1D analysis. The Hirt supernatant (whole DNA from a 10-cm plate per lane) and pellet samples (5 μg per lane) were digested with two noncutter enzymes for HPV-18 (HindIII and BglII) (lanes 1 and 2 and 6 and 7) and with three linearizing (lin) single-cutter enzymes for HPV-18 (EcoRI, XmaJI, and BglII) (lanes 3 to 5 and 8 to 10). Samples were analyzed by Southern blotting using an 8-kb linear HPV-18 genomic probe. Size markers for the linear DNA are on the right. All three linearizing enzymes gave the same 8-kb band, indicating that the observed DNA forms had been organized into units by the head-to-tail tandems. (D) The amplification signal comes from extrachromosomal oligomeric forms of HPV DNA. The Hirt supernatant and pellet were prepared from the HPV-18-positive subclone 18#1.13 cultivated under conditions inducing amplification of the viral genomic material (see Materials and Methods). The Hirt supernatant (DNA from 1/5 of the 10-cm plate per lane) and pellet samples (2 μg DNA per lane) were digested with two noncutter enzymes for HPV-18 (HindIII and BglII) (lanes 1 and 2 and 6 and 7) and with three linearizing single-cutter enzymes (EcoRI, XmaJI, and BglII) (lanes 3 to 5 and 8 to 10). Samples were analyzed by Southern blotting using an 8-kb linear HPV-18 genomic probe. Size markers for linear DNA are on the left. Most of the generating HPV DNA structures are in extrachromosomal form or in monomeric and most likely in head-to-tail multimeric forms during passaging of the HPV-positive cells under dense culture conditions. (E) Schematic presentation of the migration of undigested DNA. L, linear molecules; CCC, covalently closed circles; and OC, open circles. The numbers refer to the multimeric state (1, monomers; 2, dimers; etc.). CCC/OC are DNA molecules that migrate as covalently closed circles in the first dimension and that were subsequently nicked and migrated as relaxed circles in the second dimension. catA represents concatamers formed by relaxed circles that are linked in a noncovalent manner. (F to H) Neutral/neutral two-dimensional gel analysis of structures of DNA replication products. The episomal DNA extracted by Hirt lysis from 18#1.13 cells grown as regular (F) or dense (G and H) monolayer cultures is shown. The undigested DNA was separated by 2D electrophoresis (see Materials and Methods) and analyzed by Southern blotting using an HPV-18 genome-specific probe. The size markers of supercoiled DNA are shown in both directions (sc). The presence of an 8-kb circular plasmid is shown by the arrowhead. The generation of additional high-molecular-weight plasmid multimers was also detected during amplification (G and H). The samples shown in panel H were allowed to run for a longer time in the second dimension than the samples shown in panel G for better separation of the molecules in this dimension. (I) Schematic presentation of the migration...
same time, many subclones of these types maintained viral episomes over several months of cultivation (Fig. 2D). The viral DNA copy numbers in different subclones varied from very low to very high, as indicated by Southern blotting (Fig. 2C). There was no clear explanation for such copy number variation in the beginning. However, we observed that, under confluent culture conditions, viral genome amplification could occur, and perhaps this phenomenon is one factor influencing the copy numbers of the viral genomes in the isolated subclones. Further study relating to the mechanism of oligomerization and the mode of stable maintenance is needed for different viral genomes.

**Amplification of the viral genome under confluent cell culture conditions.** We observed for many subclones that the viral genome copy number was considerably higher in cells maintained as confluent cultures. It has been shown that in HPV-positive keratinocytes, amplification of the HPV genome is initiated as a result of triggering cell differentiation, either by adding high-calcium medium or by seeding cells into semisolid agar or into Methocel (33, 50), or under confluent tissue culture conditions (15, 16, 18, 19, 37). We studied the effects of the density of the cell culture on the viral genome copy number for different HPV-containing cell clones and found that the amplification of all studied viral genomes could be detected, similar to what has been described for keratinocytes. Quantification of the replication signals on the Southern blots revealed greater than 10-fold increase in the HPV-18 copy number. 2- to 6-fold amplification was repeatedly observed for other subtypes. This level of amplification was confirmed by FISH in interphase cells. The mechanism of amplification of the viral genome in cells of confluent cultures is unclear. We have no clear data suggesting that the differentiation program in HPV-positive U2OS cells is triggered under these conditions; however, the increase in expression of cytokeratin K10 and the L1 mRNA level may be indicative of physiological changes reminiscent of cell differentiation. We can also speculate that one possible reason for the increase in the viral genome copy number is the slowing down of the cell cycle in confluent cells, as shown by the growth curves of the confluent cells (Fig. 3A). Therefore, viral genome replication, which is not restricted to a once-per-cell-cycle replication mode, can generate more viral genome copies per cell in slow-growing cells than in actively dividing cells. Further study of the mechanism of viral genome amplification is clearly needed, especially looking at the possibilities of cell differentiation inducing viral genome amplification and the potential induction of the late phase of the viral life cycle.

**Molecular status of the HPV genome in cells.** Episomally replicating circular DNA molecules can adopt many configurations and conformations, including monomers, multimers, concatemers, and knotted forms with different topologies. The conditions used for conventional 1D agarose gel electrophoresis are sufficient to analyze linear DNA fragments and to separate supercoiled circular monomeric DNA molecules from their nicked counterparts; however, this method does not allow a detailed analysis of larger DNA molecules with different topologies, such as oligomers, replication intermediates, knotted molecules, or concatamers. Two-dimensional electrophoresis allows more accurate interpretation of the migration behavior of molecules with complex topologies, such as those used in this study. Using this approach, we identified linear HPV molecules, covalently closed circles, open circles, and different oligomeric forms of HPV DNA that are organized in a head-to-tail configuration. Analysis of the transient replication of the recircularized HPV genomes in the proliferating U2OS cells showed that, in addition to the monomeric HPV genomes, dimeric and oligomeric forms of the viral genome also appeared. At later time points, the larger oligomeric forms, probably related to the confluent cultures, were the forms from which most of the replication signal was derived. These larger oligomeric forms seem to be capable of amplification under confluent cell culture conditions, indicative of the episomal state of the viral genomes. It is not entirely clear how and why oligomeric forms of the HPV genome are formed in these cells and if this phenomenon reflects the processes that take place in vivo. The physical state of HPV DNA has been extensively studied in tumors and cervix dysplasia biopsy specimens and has been considered to be an important marker of advanced carcinoma progression in latent infection with high-risk HPVs. HPV-18 is always found in the integrated form in patient samples, whereas episomal maintenance of HPV-16 is common in carcinoma cells. In patient samples, HPV-16 episomes are often multimeric (2, 8, 27, 28, 32). These data suggest that oligomerization of HPV genomes is common during viral infections in vivo and that oligomeric genomes are better maintained in the proliferating cells. Two-dimensional gel electrophoresis analysis revealed that HPV signals were frequently found in the linear DNA arc. This result may indicate that some HPV DNA integration events took place during transfection or in later phases. The simultaneous existence of episomal and integrated HPV DNA forms is also commonly found in clinical biopsy samples of cervical tumors.

**DNA replication mode of the viral genome.** Previous studies have shown that BPV-1 and HPVs replicate their DNA using a conventional two-dimensional, semiconservative replication mode that is initiated from the specific sequence of the viral replication origin. Analysis of the HPV-16 replication mechanism in proliferating versus differentiating keratinocytes indicated that there is a shift from the theta replication mode in proliferating keratinocytes to the rolling-circle replication...
mode after the cells are induced to differentiate (17). Thus, when we observed that the amplification of the HPV genome occurs in confluent U2OS cell cultures, we studied the potential switch in the replication mechanism during amplification of the HPV-18 genome. Unlike the HPV-16 genome in keratinocytes, we were unable to detect rolling-circle replication of the HPV-18 genome. Instead, the replication of the viral genome initiated from a single replication origin of monomers and multimers, and two-dimensional progression of the replication forks was clearly demonstrated. These results suggest a conventional theta mode replication pattern for the amplification of HPV-18 in U2OS cells and present good evidence that the viral origin of replication is used in these cells. Similar results have been obtained in BPV-1-transformed C127 cell lines carrying viral genome oligomers (44).

We have demonstrated that transient replication of the HPV-18 genome in U2OS cells is strictly dependent on the E1 and E2 proteins and the viral replication origin. Since HPV-6b had the poorest replication of all the viral genomes used, determining whether this genome carries certain mutations that reduce viral gene expression or have a cis effect for replication could be possible in U2OS cells.

Conclusions. The fact that the different circular HPV genomes from HPV-6b, -11, -16, -18, -5, and -8 are capable of initiating and establishing stable viral genome replication in U2OS cells suggests that the viral regulatory elements are adequately functional for supporting DNA replication of these virus types and that viral and cellular transcription and replication factors are functionally expressed. U2OS cells maintaining viral genomes as autonomously replicating extrachromosomal elements provide a useful model system to study the regulation of viral DNA replication during different phases of the viral life cycle. We believe that this labor- and cost-effective system will allow the development of cell-based assays to identify small molecules and biological therapeutics capable of specific inhibition of HPV DNA replication in different phases of the viral life cycle.

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REFERENCES

34. Reference deleted.