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Evidence for a Switch in the Mode of Human Papillomavirus Type 16 DNA Replication during the Viral Life Cycle

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The study of human papillomavirus type 16 (HPV-16) replication has been impaired because of the lack of a cell culture system that stably maintains viral replication. Recently, cervical epithelial cell populations that stably maintain HPV-16 replicons at a copy number of approximately 1,000 per cell were derived from an HPV-16-infected patient (W12 cell clone 20863 [W12-E cells]). We used neutral/neutral and neutral/alkaline two-dimensional gel electrophoretic techniques to characterize HPV-16 DNA replication in these cells. When W12-E cells were maintained in an undifferentiated state mimicking the nonproductive stage of the life cycle, HPV-16 DNA was found to replicate primarily by theta structures in a bidirectional manner. The initiation site of HPV-16 DNA replication was mapped to approximately nucleotide 100, and the termination site was mapped to between nucleotides 3398 and 5990. To study the productive stage of HPV-16 DNA replication, W12-E cells were grown under culture conditions that promote differentiation of epithelial cell types. Under these conditions, where virus-like particles were detected, the mode of viral DNA replication changed from theta structure to what is apparently a rolling circle mode. Additionally, CIN 612-9E cells, which were derived from an HPV-31-infected patient and harbor HPV-31 extrachromosomally, exhibited the same switch in the mode of DNA replication upon induction of differentiation. These data argue that a fundamental switch in the mechanism of viral DNA replication occurs during the life cycle of the papillomavirus.

Human papillomaviruses (HPVs) are small, double-stranded DNA viruses that infect epithelial cells. Over 70 different HPV genotypes have been identified. A subset of HPVs primarily cause anogenital warts and have been placed into two groups, low risk and high risk. The anogenital HPVs in the low-risk group lead only to the production of benign lesions or warts, while HPVs in the high-risk group, HPV type 16 (HPV-16), HPV-18, HPV-31, and HPV-33, are associated with 90% of cervical cancers (36). HPV-16 is the most common subtype associated with cervical cancers. The papillomaviral life cycle is tied to the differentiation of its host, epithelial cells. The virus is thought to gain access to the basal epithelial cells at a site of wounding. The life cycle can be separated into two stages, nonproductive and productive. In the nonproductive stage, the viral genome is established as a low-copy-number nuclear plasmid. This occurs in the proliferating basal layer of the epithelium, where the virus replicates its DNA to keep up with the division of basal and parabasal cells and establishes a steady-state level of viral genomes (6). As these cells undergo their normal life cycle, a subset of daughter cells become detached from the basement membrane, stratify, and differentiate. The productive stage of the viral life cycle occurs in the terminally differentiating layers of the epithelium. In these cells, the virus amplifies its genome to higher copy number, expresses late genes encoding the capsid proteins, and produces viral progeny.

The study of HPV DNA replication has been hindered by the lack of a cell culture system which stably maintains HPV replicons. Because of this limitation, many early studies of papillomavirus DNA replication were performed with bovine papillomavirus type 1 (BPV-1), because rodent cell lines exist which stably maintain BPV-1 DNA replication. Also, short-term HPV DNA replication studies were performed in transformed cells following transfection. Using these reagents and assays, the *cis* and *trans* elements required for papillomavirus DNA replication were identified. Both the viral E1 and E2 proteins are necessary for viral DNA replication. These viral proteins bind to the E1 and E2 binding sites located within the viral long control region, referred to as the origin of DNA replication (6, 8). The origin is that region of the viral genome required in *cis* for short-term replication of the papillomavirus genome. All other DNA replication machinery, DNA polymerase α/primase, DNA polymerase δ/proliferating cell nuclear antigen, replication protein A (RPA), and topoisomerases I and II (Topo I and II), is provided by the host cell (6, 27). Recently, epithelial cell populations which contain extrachromosomal HPV DNA (2, 18, 29) or which can support DNA replication subsequent to transfection (10, 23) have been isolated. By using various methods, it has been demonstrated that these cells when induced to differentiate can produce virus-like particles (10, 17, 23, 29). These cell culture systems have allowed for the study of the natural life cycle of HPVs.

DNA replication of two papillomaviruses, BPV-1 and HPV-11, has been characterized by two-dimensional gel electrophoretic techniques. In rodent cells that stably maintain BPV-1 as a replicon, viral DNA replication was found to proceed bidirectionally via theta structures (34). Density shift experiments demonstrated newly synthesized BPV-1 to be primarily present in the heavy-light fraction, consistent with semiconservative replication (12) as occurs in theta structure mode of replication. The same mode of DNA replication was seen for HPV-11 (low-risk HPV) present in laryngeal papillomas (1). In this study, we demonstrate that in undifferentiated W12-E cells, where the nonproductive stage of the viral life cycle occurs, HPV-16 DNA replicates via theta structures in a bidirectional manner initiating at approximately nucleotide (nt) 100. However, in differentiated W12-E cells, where we have shown evidence for the productive stage of the life cycle, a switch in the mode of HPV-16 DNA replication occurs. This

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switch in the mode of viral DNA replication was also apparent in HPV-31 DNA isolated from differentiated CIN612-9E cells. Our data are most consistent with a rolling circle mode of DNA replication.

MATERIALS AND METHODS

Cell culture. Cultures of HPV-16-positive cervical epithelial cells (W12 clone 20863 [W12-E cells] [18]) and cultures of HPV-31-positive cervical epithelial cells (CIN612-9E cells [2, 16]) were maintained at subconfluence on mitomycin c-treated J2 3T3 feeder cells in F medium (0.66 mM Ca^{2+}) composed of 3 parts F-12 medium and 1 part Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (FBS), insulin (5 μg/ml), cholera toxin (8.4 ng/ml), adenine (24 μg/ml), epidermal growth factor (10 ng/ml), and hydrocortisone (0.4 μg/ml). J2 3T3 feeder cells were removed from the culture dish by treatment with 0.02% EDTA. To remove the epithelial cells from the culture dish, the monolayer was rinsed with phosphate-buffered saline (PBS) followed by 0.1% trypsin–0.5 mM EDTA. Then 5×10^5 cells were plated on a 15-cm-diameter dish in low-calcium F medium (0.05 mM Ca^{2+}) composed of 1 part calcium-free F-12 medium and 3 parts calcium-free Dulbecco's modified Eagle's medium supplemented with 5% chelex-treated FBS, $CaCl_2$, and the components described above. Two methods were used to induce differentiation. In the first method, W12-E and CIN612-9E cells were grown to confluence on J2 3T3 feeder cells in F medium (0.66 mM Ca^{2+}) as described above. When cells reached confluence, they were maintained in F medium containing 1.2 mM Ca^{2+} and 20% FBS for 2 to 10 days (13, 32). Cells were removed from dishes by treatment with 0.1% trypsin–0.5 mM EDTA. In the second method, 10^7 W12-E or CIN612-9E cells were suspended in 20 ml of F medium containing 20% FBS and 1.68% methylcellulose for 2 to 10 days (13, 32). Cells were recovered from suspension by multiple dilutions with serum-free F medium and PBS followed by centrifugation.

Cornified envelope formation assay. At 2, 4, 7, and 10 days after induction of differentiation, cornified envelopes were counted (32). The total number of W12-E or CIN612-9E cells in a 1-ml aliquot of each culture was determined by counting with a hemacytometer. Subsequently, W12-E or CIN612-9E cells were pelleted and treated with 1 ml of 10 mM Tris HCl (pH 7.4)–1% sodium dodecyl sulfate (SDS)–1% β-mercaptoethanol for 10 min. Cornified envelopes were pelleted, resuspended in PBS, and counted with a hemacytometer. The percentage of cornified envelopes was determined by dividing the number of cornified envelopes by the total number of cells in the 1-ml aliquot.

Reverse-field gel electrophoresis (RFGE). Low-molecular-weight DNA was extracted from monolayers of W12-E cells maintained in low-Ca^{2+} F medium (undifferentiated) and W12-E cells maintained in high-Ca^{2+} F medium (differentiated), using the Hirt method (15). Aliquots of these DNAs were treated with Topo I to determine the migration pattern of open circular DNA. Briefly, 1.5 μg of Hirt DNA was treated with 5 U of Topo I in a 25-ml volume of 1× Topo I buffer (2 mM Tris [pH 7.4], 0.5 mM EDTA, 0.02 M KCl) at 37°C for 3 h. To terminate the reaction, the Topo I-treated DNA was incubated at 37°C for 2 h in (2% SDS, 100 mM EDTA, 0.33 μg of proteinase K per ml). The DNA was ethanol precipitated, using glycogen (0.33 μg/ml) as a carrier. The resulting DNA pellet was resuspended in Tris-EDTA (pH 8.0), and microdialysis was performed. Then 1.5 μg of Hirt DNA from undifferentiated W12-E cells, untreated and treated with Topo I, and 1.5 μg of pMMcdneo-Zta (7,996-bp plasmid) were loaded onto a 0.8% agarose (SeaKem GTG) gel in 0.5× TBE (Tris-borate-EDTA). Separately, 1.5 μg of Hirt DNA from differentiated W12-E cells was loaded onto a 0.8% agarose (SeaKem GTG) gel in 0.5× TBE. Both gels were electrophoresed at 300 V without pulse for 10 min to clear the wells. The gels were then run at 100 V with pulse for 20 h. The following settings were used on the Hoefer PC750 pulse controller: forward, 150 ms; reverse, 50 ms; and ramp factor, 0.3.

Two-dimensional gel electrophoresis. The neutral/neutral two-dimensional gel method described by Brewer and Fangman (3) was used to determine the structure of the HPV-16 and HPV-31 DNA replicative intermediates (RIs). Hirt DNA isolated from W12-E or CIN612-9E cells on one 15-cm-diameter tissue culture dish was loaded on a 0.4% agarose gel (SeaKem GTG) in 0.5× TBE. The first dimension was electrophoresed at 40 V for 24 h with recirculating buffer. To determine the migration of the viral DNA, λ-*Hin*dIII markers were loaded on the gel. The lane of interest was excised from the first dimension and turned 90°. A 1.0% agarose gel (SeaKem GTG) in 1× TBE was poured around the gel slab. The running buffer used in the second dimension was 1× TBE containing ethidium bromide (0.33 μg/ml). The second dimension was electrophoresed at 120 V for 20 h at 4°C with recirculating buffer. The neutral/alkaline two-dimensional gel electrophoresis developed by Nawotka and Huberman (26) was used to determine the mode and direction of viral DNA replication. The conditions of the first dimension were the same as described above. The second-dimension gel was a 1% agarose (SeaKem GTG) gel in water. Upon solidification, the second-dimension gel was soaked in an alkaline solution (40 mM NaOH, 2 mM EDTA) with agitation for 90 min. The second-dimension gel was electrophoresed under the following conditions: 40 V for 24 h, room temperature, and recirculation of the alkaline solution. Size markers (100-bp ladder) were used in the second dimension to determine the extent of migration of the newly synthesized DNA.

Southern analysis. For RFGE, the HPV-16 DNA was detected by Southern transfer to a nylon membrane (GeneScreen). The blot was probed with a full-length HPV-16 probe generated by digesting pHPV-16 (18) with *Bam*HI and labeled with [α-^{32}P]dCTP, using a random primer labeling kit (Amersham). The percentage of dimeric HPV-16 replicons was determined by using a PhosphorImager and ImageQuant software (Molecular Dynamics). For neutral/neutral two-dimensional gel analysis, the HPV-16 and HPV-31 DNA RIs were detected by Southern transfer to nitrocellulose membranes (Schleicher & Schuell). The blots were hybridized with indicated subgenomic HPV-16 or HPV-31 probes and labeled as described above. The blots were placed on X-ray film for 1 week. For neutral/alkaline two-dimensional analysis, the RIs were detected by Southern transfer to a nylon membrane (GeneScreen). The Southern blot was hybridized with subgenomic HPV-16 probes labeled as described above. The blot was placed on a PhosphorImager screen for 1 to 2 days. Each probe was stripped from the blot after PhosphorImager analysis by washing with 0.4 N NaOH at 42°C for 45 min, followed by two 10-min washes at room temperature in 0.2 M Tris HCl (pH 7.4)–0.01% SDS–0.01× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Western analysis. Undifferentiated and differentiated W12-E cells and human foreskin keratinocytes (HFKs) were lysed in 2× SDS loading buffer (4% SDS, 125 mM Tris-HCl [pH 6.8]), 20% glycerol, 0.1% bromophenol blue, 0.2% β-mercaptoethanol). The protein in the cell lysates was quantified by the Bradford assay (Bio-Rad). Equal amounts of protein samples which were heated in a 100°C water bath for 5 min were electrophoresed on an SDS–8% polyacrylamide gel. The protein was transferred by electroblotting to a polyvinylidene difluoride membrane (Immobilon-P transfer membrane; Millipore). The membrane was blocked in Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 7.4], 150 mM NaCl) containing 0.2% Tween 20 (TBST 0.2%) and 5% nonfat dried milk (Carnation) overnight at 4°C. Suprabasal keratins were detected by incubation with a 1:1,000 dilution of monoclonal antibody Ck 8.60 (Sigma) in TBST 0.2% for 1 h. The blot was washed five times for 5 min each in TBST 0.2% and then once for 5 min in TBS. To detect protein bands, the membrane was incubated for 45 min with an anti-mouse immunoglobulin G secondary antibody conjugated to peroxidase (American Quallex) diluted 1:10,000 in TBST 0.2% and then washed as described above. Detection was performed with the substrate luminol, available in the BM Chemiluminescence Substrate (POD) kit (Boehringer Mannheim).

Electron microscopy (EM) analysis. W12-E cells that were maintained in high-calcium and -serum F medium for 10 days were fixed in 3% glutaraldehyde in 0.1 M cacodylate (pH 7.4) at room temperature for 45 min. Fixed sheets of cells were scraped from plastic dishes and transferred to a centrifuge tube. Cells came off of plastic dishes as intact sheets. These sheets of cells were placed in a tube and centrifuged to form a pellet. Cell pellets were resuspended in 2% OsO_4 and glutaraldehyde fixative described above for 45 min and dehydrated with a graded series of ethanol. Cell pellets were then infiltrated with 1:1 propylene oxide-Eponate overnight prior to being embedded in 100% Eponate (Ted Pella, Redding, Calif.). Cell pellets were thin sectioned with a Reichert Ultracut E3 (Reichert, Buffalo, N.Y.) equipped with a diamond knife. Sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-7000 electron microscope (Hitachi, San Jose, Calif.) at 75 kV.

RESULTS

The HPV-16 DNA replicons from W12-E cells are primarily in monomeric form. In this study, we made use of two-dimensional gel electrophoretic procedures to analyze DNA RIs. To interpret the results, we needed to learn whether the HPV-16 replicons present in the HPV-16-positive cervical epithelial cell population, W12-E cells, were monomeric or multimeric in size. To determine this, RFGE was performed with a low-molecular-weight fraction of DNA (Hirt DNA) isolated from the W12-E cells (15, 18) (Fig. 1, middle lane). An equal amount of the Hirt DNA extract was treated with Topo I to relax the supercoiled DNA molecules and indicate the migration of open circular HPV-16 molecules (Fig. 1, right lane). In addition, an 8-kb bacterial plasmid was run alongside the Hirt DNA extract as a molecular weight marker for HPV-16 monomeric molecules (Fig. 1, left lane). The RFGE analysis indicated that the majority of HPV-16 replicons from the W12-E cells are monomeric and about 3% of the replicons are dimeric.

Mapping the initiation site of HPV-16 DNA replication in undifferentiated cervical epithelial cells. To identify the initiation site of HPV-16 DNA replication, Hirt DNA was extracted from undifferentiated W12-E cells and digested with various restriction enzymes. These HPV-16 DNA RIs were analyzed by neutral/neutral two-dimensional gel electrophore-

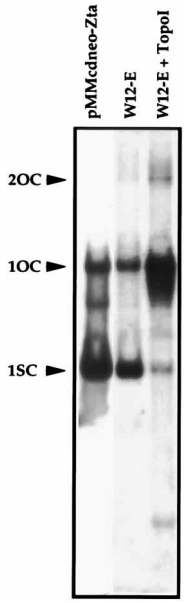

FIG. 1. Analysis of HPV-16 extrachromosomal DNA by RFGE. Shown is an autoradiograph of a Southern blot containing Hirt DNA from W12-E cells resolved by RFGE (middle lane). In the right lane, an equal amount of the Hirt DNA sample was pretreated with Topo I to relax the supercoiled forms of the DNA. The Southern blot was hybridized with a full-length HPV-16 probe. The arrows indicate monomeric supercoiled Hirt DNA (1SC), monomeric open circular Hirt DNA (1OC), and dimeric open circular Hirt DNA (2OC). This experiment was repeated four times.

sis (3). The first dimension separates DNA molecules by molecular weight, while the conditions of the second dimension allow DNA RIs to be separated by shape. Subsequently, Southern analysis was performed with subgenomic HPV-16 probes to detect the DNA RIs. Figure 2A depicts the various hybridization patterns of DNA RIs generated by this analysis. The *cis* elements required for short-term HPV-16 DNA replication are located between nt 7856 and 110 (8). To determine whether HPV-16 DNA replication initiates in or around this region, we analyzed a 5.3-kb *Msc*I fragment that spans nt 5990 to 3398 and includes the HPV-16 DNA replication *cis* elements. The hybridization pattern arising from this fragment was a discontinuous arc starting from the 5.3-kb unreplicated HPV-16 DNA spot in the lower right corner of the autoradiograph (Fig. 3A). A discontinuous arc indicates the presence of a "bubble" or initiation site asymmetrically placed within the fragment (Fig. 2A, column 3). To map more finely the initiation site, regions within the 5.3-kb *Msc*I fragment were analyzed. The hybridization pattern arising from a 4.6-kb fragment, spanning nt 5990 to 2708, was a discontinuous arc, indicating that initiation occurred within this smaller region of the genome. The left side of the discontinuous arc is indicative of Y-shaped intermediates (Fig. 2A, column 2). These intermediates appear when one replication fork reaches the restriction enzyme site before the other. This part of the arc is shorter in the 4.6-kb fragment (Fig. 3B) than in the 5.3-kb fragment (Fig. 3A), indicating that the initiation site is located more symmetrically in the 4.6-kb fragment than in the 5.3-kb fragment. An even smaller fragment within the 5.3-kb *Msc*I fragment spanning nt 5990 to 551 was analyzed. The RIs from this fragment gave rise to a partial arc emanating from the fully replicated HPV-16 DNA spot, but no pattern indicative of bubble-shaped intermediates was apparent (data not shown). Prior studies have shown that bubble-form intermediates are difficult to detect when the initiation site is not located within the central third of the restriction fragment (21). Thus, we interpret the absence of bubble-form RIs to indicate that the initiation site is not within the central third, nt 6812 to 7634, of the fragment. Analysis of a 2.5-kb fragment, spanning nt 863 to 3398, resulted in a continuous arc emanating from the 2.5-kb unreplicated HPV-16 DNA spot in the lower right corner of the autoradiograph (Fig. 3C). This pattern indicates that a single replication fork traverses that region of the viral genome. Thus, no initiation or termination sites are present between nt 863 and 3398. The region on the opposite side of the HPV-16 genome from nt 4466 to 7463 gave rise to a similar hybridization pattern, indicating that no initiation or termination site is present within this fragment (Fig. 3D). From these data, the initiation site of HPV-16 DNA replication must lie between nt 7634 and 551.

The hybridization pattern seen with the 5.3-kb *Msc*I fragment is consistent with the presence of bubble-shaped DNA RIs. This finding indicates that HPV-16 DNA replication was occurring via theta structures. If this conclusion is correct, then a termination structure in which two replication forks converge on the circular HPV-16 genome should exist. To determine whether these termination structures exist, the Hirt DNA extract was digested with *Nco*I, which cleaves at nt 863, near the initiation site of HPV-16 DNA replication. The DNA RIs formed a diagonal hybridization pattern emanating from the 7.9-kb unreplicated HPV-16 DNA spot located at the lower right corner of the autoradiograph (Fig. 3E). This diagonal line is characteristic of two replication forks converging upon each other at the termination site approximately 180° from the *Nco*I site at nt 863 (Fig. 2A, column 4). To map more closely the termination site of HPV-16 replication, the Hirt DNA was digested with *Msc*I. The 2.6-kb fragment opposite the initiation site was analyzed, because we suspected that it contained the termination site. The DNA RIs arising from this fragment formed a complex pattern composed of a diagonal line superimposed on a continuous arc. The continuous arc pattern indicates the presence of Y-shaped intermediates, which correspond to a single replication fork traversing the DNA molecule (Fig. 2A, column 2). The superimposed diagonal line is characteristic of two replication forks converging to the termination site (Fig. 2A, column 4). This result indicates that termination of HPV-16 DNA replication occurs within the 2.6-kb fragment and is asymmetrically located between nt 3398 and 5990 (Fig. 3F). Taken together, the results presented in Fig. 3 indicate that there is a single initiation site of HPV-16 DNA replication present between nt 7634 and 551 and a termination site approximately 180° away on the circular genome.

HPV-16 DNA replicates bidirectionally by theta structures in undifferentiated cervical epithelial cells. The results from the neutral/neutral two-dimensional gel analysis (Fig. 3) indicated that HPV-16 DNA in undifferentiated cervical epithelial cells occurs through theta structure RIs. Replication initiates in a unique region, and termination occurs at a point on the opposite side of the circular genome. From this, we infer that replication occurs bidirectionally. To confirm the mode and directionality of HPV-16 DNA replication and to map more precisely the replication initiation site, neutral/alkaline two-dimensional gel electrophoresis was performed (26). In the second dimension, this method allows newly synthesized leading-strand DNA to be resolved apart from the parental strand. Prior studies had demonstrated that a convex migration pattern of nascent strands is indicative of theta structure RIs (26). Undigested Hirt DNA was electrophoresed on a neutral/alkaline two-dimensional gel, and the HPV-16 RIs were detected by using an HPV-16-specific probe proximal to the location of the initiation site of DNA replication mapped in Fig. 3. This

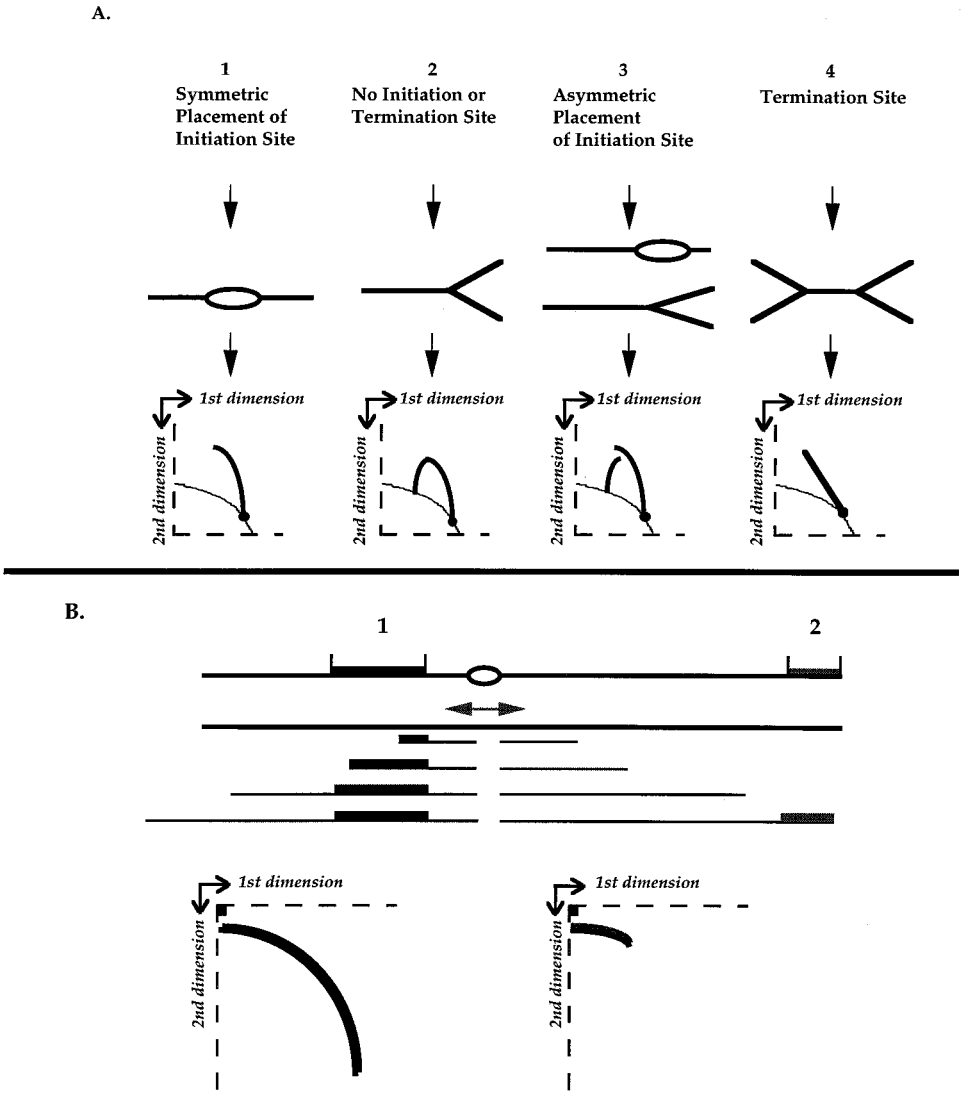

FIG. 2. Schematic of hybridization patterns of DNA RIs. (A) Pattern of migration of RIs resolved by neutral/neutral two-dimensional gel electrophoresis. The symmetric placement of the initiation site of replication within a restriction fragment gives rise to RIs containing bubbles that migrate as a partial arc (column 1). A restriction fragment containing no initiation or termination site gives rise to RIs with a single replication fork that migrate as a continuous arc (column 2). The asymmetric placement of the initiation site within the restriction fragment gives rise to RIs containing a combination of patterns (column 3) that collectively give rise to a discontinuous arc. A restriction fragment containing a termination site gives rise to RIs containing two replication forks converging to one point that migrate as a diagonal line (column 4). Pattern of migration of RIs resolved by neutral/alkaline two-dimensional gel electrophoresis. The bubble on the top line indicates the site of initiation of DNA synthesis. The double arrow under the initiation site indicates the direction of replication. The bold line under the arrows indicates the parental strand. The thin strands under the parental strand are the newly synthesized nascent-strand DNA molecules. The boxes on the nascent strands indicate the hybridizing probe. A probe near the initiation (probe 1) gives rise to a long hybridization pattern due to its detection of short and long nascent strands (shown below, left). A probe far from the initiation site (probe 2) gives rise to a short hybridization pattern due to its selective detection of only long nascent strands (shown below on the right).

gave rise to a convex arc hybridization pattern providing additional evidence that replication occurs via theta structures (Fig. 4, panel 2). To determine the direction of HPV-16 DNA replication, Southern analysis was repeated with probes specific for different regions of the HPV-16 genome. When hybridized to probe 2, spanning HPV-16 nt 6818 to 7453, a long convex hybridization pattern was detected (Fig. 4, panel 2). This result indicates hybridization to both long and short fragments of newly synthesized DNA (Fig. 2B, probe 1). The shortest fragments detected were approximately 600 bp (Fig. 4, panel 2). Only probes near an initiation site of DNA synthesis will hybridize to short nascent strands. Because this probe lies to the left side of the initiation site (Fig. 3), this result indicates that replication occurs in the leftward direction on the viral genome. Probe 3, spanning nt 863 to 1309, which is positioned to the right side of the initiation site (Fig. 3), gave rise to a similar hybridization pattern, the shortest fragment detected being approximately 800 bp (Fig. 4, panel 3). This result indicates that DNA synthesis must also occur in the rightward direction. This result also indicates that the initiation site of replication likely lies at approximately nt 100, given that the minimal lengths of nascent strands detected by probes 2 and 3 were 600 and 800 bp, respectively. Hybridization with probe 1, spanning nt 6150 to 6590, generated a shorter hybridization pattern, the smallest fragments detected being approximately 1,500 bp in length (Fig. 4, panel 1). This result indicates that probe 1 is

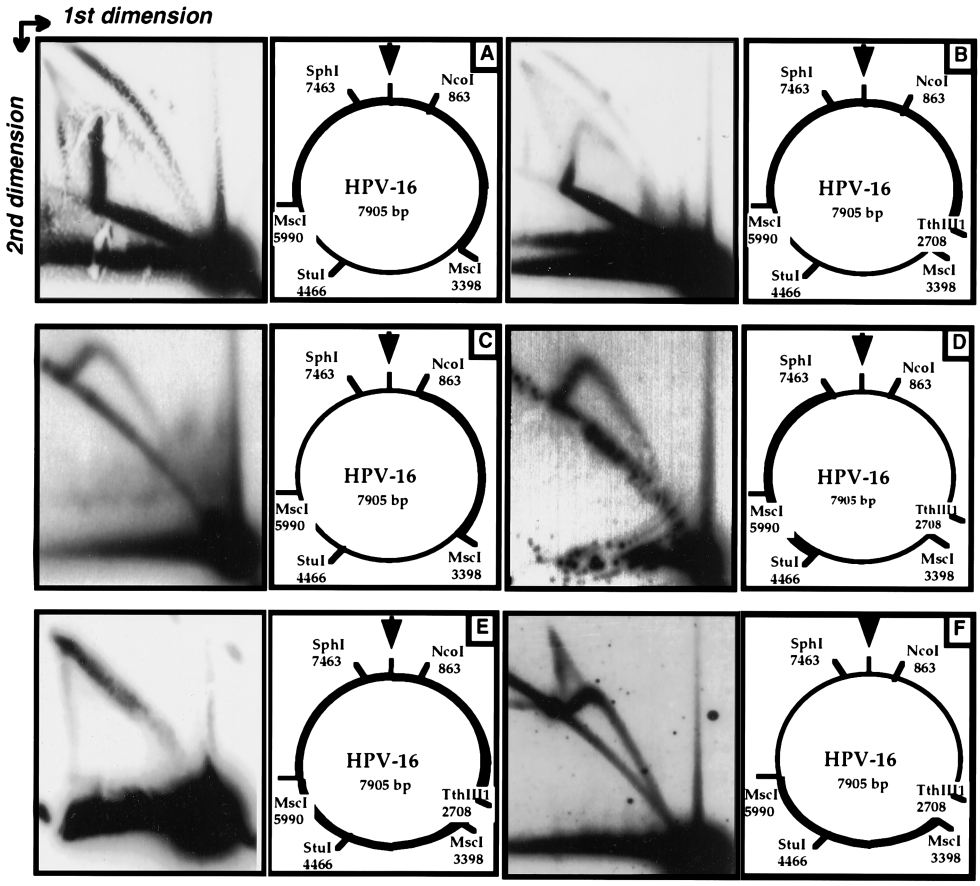

FIG. 3. Resolution of HPV-16 DNA RIs by neutral/neutral two-dimensional gel electrophoresis. Shown are autoradiographs of Southern blots containing Hirt DNA from W12-E cells digested with restriction enzymes and resolved by neutral/neutral two-dimensional gel electrophoresis. To the right of each autoradiography is a circular map of the HPV-16 genome. The large arrow above the circular map points to nt 1. Indicated is the restriction enzyme used to digest Hirt DNA prior to electrophoresis. The region of the viral genome probed is indicated by the bold segment of the circular genomic map. The regions of the HPV-16 genome analyzed were nt 5990 to 3398 (A), nt 5990 to 2708 (B), nt 863 to 3398 (C), nt 4466 to 7463 (D), the complete genome linearized at nt 863 (E), and 3398 to 5990 (F). In all panels, the strongest hybridization signal results from unreplicated HPV-16 DNA in the lower right corner. Refer to Fig. 2A to interpret migration patterns. Analyses of all fragments were repeated three or more times.

positioned farther from the initiation site than probes 2 and 3 (Fig. 2B, probe 2). Additionally, this result provides evidence that DNA synthesis was occurring in a leftward direction, from probe 2 to probe 1. Hybridization with probe 4, spanning nt 2708 to 3398, generated a short hybridization pattern (Fig. 4, panel 4), indicating that this probe is farther from the initiation site than probes 1, 2, and 3 (Fig. 2B, probe 2). Since the hybridization pattern detected by probe 4 is shorter than that detected by probe 3, this provides additional evidence that DNA synthesis proceeds in a rightward direction, from probe 3 to probe 4. Hybridization with probe 5, spanning nt 3437 to 4906, which was previously determined to contain the termination site of HPV-16 DNA replication (Fig. 3F), predictably generated the shortest hybridization pattern (Fig. 4, panel 5). This result indicated that probe 5 lies farthest from the initiation site. To ensure that the short hybridizations seen with probes 3, 4, and 5 were not due to a loss of RIs from the nylon membrane during stripping, the blot was reprobed with probe 2. The pattern was the same as the one seen in Fig. 4, panel 2 (data not shown).

In summary, the convex pattern detected by this analysis confirmed that HPV-16 DNA replication occurs via theta structures. Additionally, the fact that probes 2 and 3 detected the shortest newly synthesized HPV-16 DNA fragments and probes 1, 4, and 5 detected only progressively longer HPV-16 DNA fragments indicates that HPV-16 DNA replication is bidirectional (Fig. 4). The lengths of the shortest nascent leading strands detectable by probes 2 and 3 lead to the placement of the initiation site at approximately nt 100 on the HPV-16 genome. The near absence of resolved nascent leading strands detectable with probe 5 confirms the placement of the termination site opposite the initiation site, between nt 3437 and 4906.

The mode of HPV-16 DNA replication switches in differentiated cervical epithelial cells. All analyses to this point in the study were performed on HPV-16 DNA RIs that arise in undifferentiated cervical epithelial cells, reflective of the nonproductively infective state seen in the stratum basale of the infected exocervix. To study HPV-16 DNA replication in the productive infective state, W12-E cells were induced to undergo differentiation. Prior analyses indicated that W12 cells produce virus-like particles when grown under conditions that support differentiation, such as when placed in tissue transplant chambers on nude mice (29) or cultured on fibroblast-impregnated collagen rafts (17). Because large amounts of DNA RIs must be retrieved, we grew W12-E cells by using two different methods that would generate large populations of cells undergoing differentiation. To accomplish this, W12-E

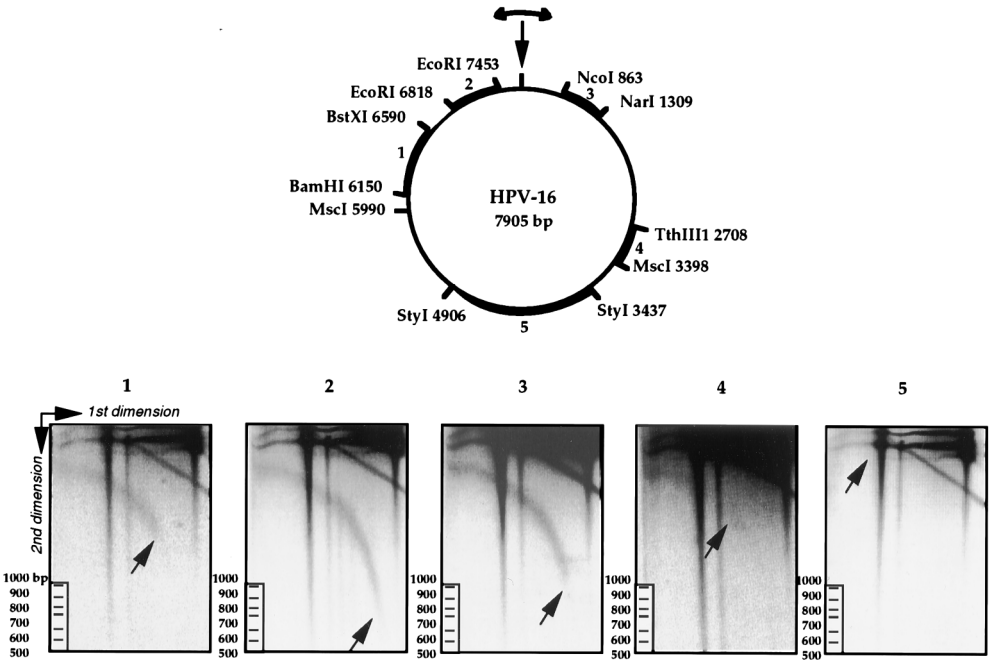

FIG. 4. Resolution of HPV-16 DNA RIs by neutral/alkaline two-dimensional gel electrophoresis. Shown are autoradiographs of a Southern blot containing uncut Hirt DNA from W12-E cells resolved by neutral/alkaline two-dimensional gel electrophoresis. The same Southern blot was hybridized with the probes, 1 through 5, shown on the HPV-16 map. The arrow above the HPV-16 map shows the approximate location of the initiation site of replication as mapped by the neutral/neutral two-dimensional gel method (Fig. 3). Below the map (panels 1 to 5) are the hybridization patterns given by the probes. The heaviest hybridization signal comes from the parental-strand DNA extending horizontally and randomly nicked nonreplicating DNA extending vertically. The arcs represent the newly synthesized nascent DNA indicated by the arrows. Shown in the lower left corner of each autoradiograph are 100-bp markers run in the second dimension to determine the lengths of the hybridization patterns. The Southern blot was hybridized sequentially with HPV-16 probes 1 (nt 6150 to 6590), 2 (nt 6818 to 7453), 3 (nt 863 to 1309), 4 (nt 2708 to 3398), and 5 (nt 3437 to 4906). The Southern blot was stripped between each hybridization. The arrows above the circular map of HPV-16 indicate the direction of replication as determined by these analyses. Refer to Fig. 2B to interpret migration patterns. Two-dimensional neutral/alkaline electrophoresis of undigested HPV-16 DNA was repeated twice. Each blot was hybridized with the relevant probe twice.

cells were grown in suspension using 1.68% methylcellulose and 20% FBS for up to 10 days. The other method used to induce differentiation was to grow the W12-E cells to confluence and then switch them into F medium containing an increased molarity (1.2 mM) of calcium and a high percentage (20%) of serum (13, 32). W12-E cells were maintained in this medium for up to 10 days. At 10 days, 35% of the cells formed cornified envelopes by both methods, a characteristic of terminal differentiation (11, 13, 32). Ultrastructural analysis of the W12-E cell maintained in the high-Ca^{2+} and -serum medium indicated that the cells exhibited characteristics of differentiation under these growth conditions. They had stratified: the lower layer contained undifferentiated cells, while the upper layers contained cells with condensed nuclei and keratin bundles. Additionally, enucleated cells were present in the uppermost layer (Fig. 5). Western analysis revealed that the suprabasal layer-specific keratins, K1 and K10, were present in epithelial cells maintained in the high-Ca^{2+} and -serum medium (Fig. 6). These results provide evidence that the W12-E cells can be induced to differentiate by the addition of high-Ca^{2+} and -serum medium. EM analysis performed at higher magnification indicated the presence of many aggregates of particles measuring between 48 and 62 nm, falling in the range of the size of HPV-16 virions, within multiple nuclei (Fig. 7) (30). Ultrastructural analysis of early-passage, uninfected HFKs induced to undergo differentiation by high calcium and serum for 10 days indicated that there were no virus-like particle aggregates present (data not shown). These data provide evidence for the productive stage of the viral life cycle in W12-E cells induced to differentiate by this alternative approach.

To analyze HPV-16 DNA replication in differentiated W12-E cells, Hirt DNA was extracted and analyzed. RFGE analysis indicated that the HPV-16 DNA isolated from differentiated W12-E cells was primarily monomeric and that only about 1% of the DNA was dimeric (Fig. 8, lane 1). By neutral/neutral two-dimensional gel electrophoresis, the 5.3-kb *Msc*I fragment, spanning nt 5990 to 3398, gave rise to a continuous arc emanating from the unreplicated 5.3-kb HPV-16 DNA spot in the lower right corner of the autoradiograph and ending at the position of fully replicated DNA (i.e., 2×5.3 kb = 10.6 kb). This hybridization pattern indicated the presence of a single replication fork traversing this region of the viral genome (Fig. 2A, column 2). Thus, DNA from differentiated cells contains no initiation site between nt 5990 and 3398 as seen in undifferentiated cells (Fig. 9A to C). To determine if the initiation site was elsewhere in the genome or if a termination site existed, the 2.6-kb *Msc*I fragment, spanning nt 3398 to 5990 and previously shown to contain a termination site in undifferentiated cells (Fig. 9D), was analyzed in differentiated cell cultures. In this case, the hybridization pattern was a continuous arc emanating from the 2.6-kb unreplicated HPV-16 DNA spot and ending at the fully replicated 5.2-kb spot (Fig. 9E and F). Again, this hybridization pattern indicated the presence of a single replication fork traversing this region of the viral genome. The absence of the diagonal line hybridization pattern in Fig. 9F indicates that there is no termination site between nt 5990 and 3398 in differentiated cells. Hirt DNA iso-

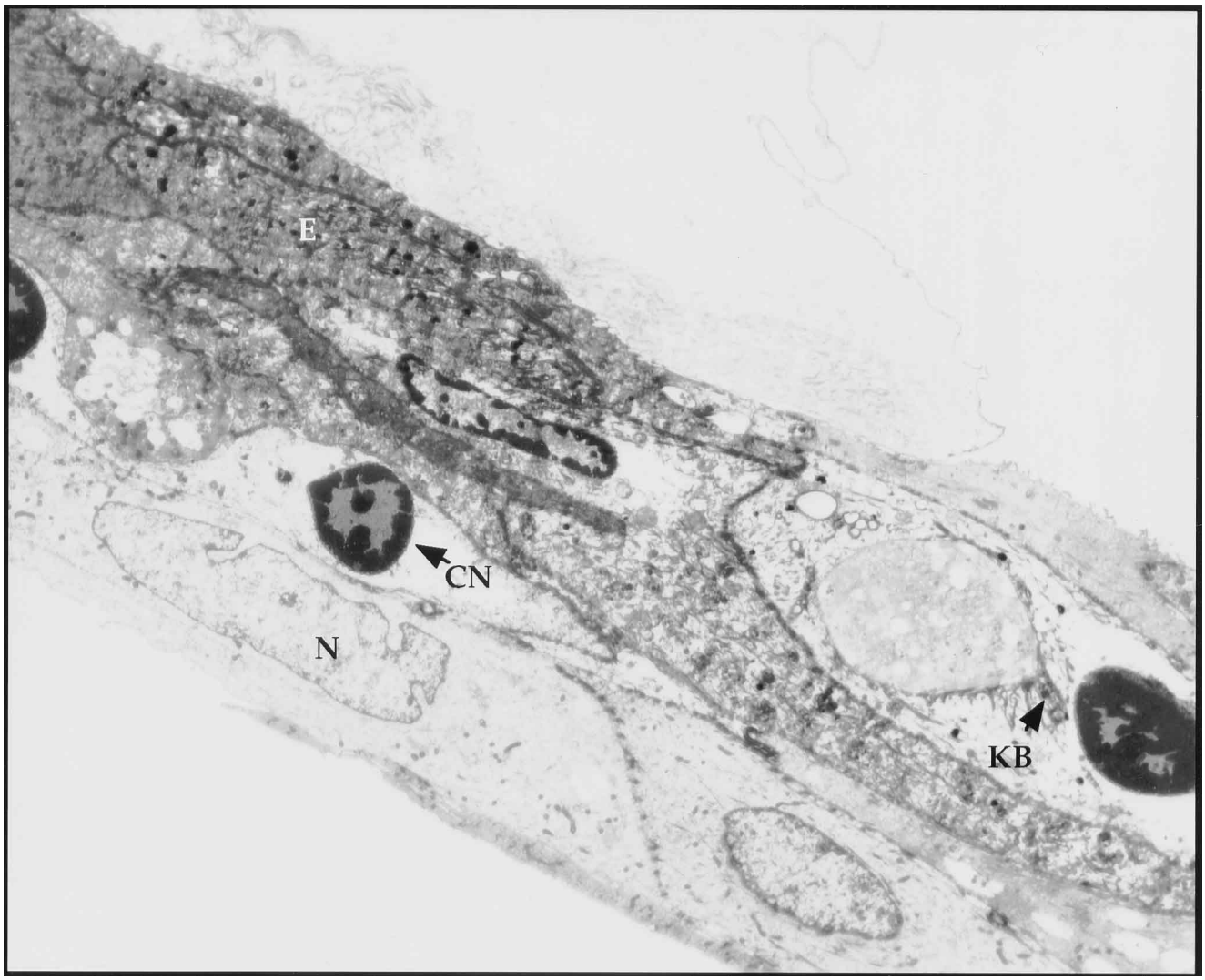

FIG. 5. Electron micrograph of W12-E cells. W12-E cells were induced to undergo differentiation by being grown to confluence and maintained in F medium containing 20% FBS and 1.2 mM Ca^{2+} for 10 days. Stratification of the epithelial cells is apparent. The lower layers of cells are undifferentiated and contain normal nuclei (N). The upper layers contain cells that appear to be differentiating. These cells have a condensed highly stained nucleus (CN) and keratin bundles (KB). The uppermost layer contains cells that are enucleated (E). Cells attached to plastic are at the bottom of the cross section.

lated from W12-E cells induced to undergo differentiation by suspension in methylcellulose gave rise to the same result shown in Fig. 9. Taken together, the results in Fig. 9 show that there are no sites of HPV-16 DNA replication initiation or termination in differentiated cervical epithelial cells. In rolling circle replication, a single nicking event on one parental DNA strand gives rise to unidirectional replication. As such no bubble form intermediates and no termination structures, resulting from the convergence of two replication forks, arise. The absence of an initiation and termination site is most consistent with a rolling circle mode of DNA replication. Neutral/alkaline two-dimensional gel analysis of undigested Hirt DNA from differentiated cell populations confirmed this conclusion. No leading strands of sizes less than genome length could be detected with any of the probes described in Fig. 4 (data not shown). These results argue for a fundamental switch in the mode of HPV-16 DNA replication in differentiated cervical epithelial cells.

In the productive stage of the papillomavirus life cycle, amplification of the viral genome occurs in the differentiated cells within the upper layers of the epidermis. We were interested in learning whether the switch in the mode of viral DNA replication precedes the point in differentiation when amplification occurs. To determine when the switch in the mode of replication occurs after the induction of differentiation by the two methods discussed, Hirt DNA was extracted at different time points: 2, 4, 7 and 10 days. Neutral/neutral two-dimensional gel analysis was performed on all samples, and similar hybridization patterns were obtained. At 2 days after induction of differentiation, only 6% of the cells formed cornified envelopes, compared to 35% at 10 days postinduction. When DNA from the 2-day time point was analyzed by neutral/neutral two-dimensional analysis, the fragment shown to contain the initiation site in undifferentiated cells gave rise to a continuous arc (Fig. 9B). This result indicates that no initiation of theta DNA RIs could be detected. When we analyzed the fragment containing the termination site in undifferentiated cells, there was still a faint diagonal hybridization pattern, indicating that theta

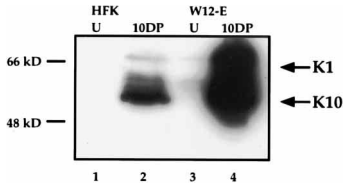

FIG. 6. Analysis of suprabasal keratin expression in HFKs and W12-E cells. Shown is a Western blot using a K10-specific antibody with weak specificity for K1. Cell lysates from HFKs and W12-E cells maintained in low-Ca^{2+} F medium (U; undifferentiated) and high-Ca^{2+} and -serum F medium (10DP; differentiated) were analyzed. Protein was quantified in each sample by the Bradford assay. Equal amounts of protein were loaded on an SDS-polyacrylamide gel. Western analysis was performed with antibody using Ck 8.60 to detect suprabasal keratins. The blot was overexposed to show that very little K10 is present in W12-E cultures maintained in the undifferentiated state whereas differentiated cells contain large amounts of K10. Western analysis using antibody Ck 8.60 was repeated three times with cell lysates from different preparations of HFK and W12-E cells.

RIs were still present; however, the major hybridization pattern was indicative of simple Y-fork RIs traversing this region, consistent with rolling circle replication (Fig. 9E). Thus, it is likely that at 2 days, there are still some cells in the undifferentiated state that are supporting theta, bidirectional viral DNA replication, but the majority of cells are already supporting the new mode of viral DNA replication. At 4 days, all RIs were of the rolling circle type (data not shown). This result indicates that the switch in replication most likely occurs early upon the induction of epithelial differentiation and therefore is likely to precede and perhaps be instrumental in the viral DNA amplification that occurs in the upper layers of the epidermis in a papilloma.

HPV-31 undergoes a similar switch in the mode of DNA replication in differentiated cervical epithelial cells. To determine whether other HPVs undergo a switch in the mode of DNA replication, CIN612-9E cells, cervical epithelial cells derived from a CIN 1 lesion that harbor HPV-31 extrachromosomally (2, 16), were analyzed. Neutral/neutral two-dimensional analysis was first performed to determine the mode of DNA replication of HPV-31 in undifferentiated cells. Hirt DNA extracted from undifferentiated CIN612-9E cells was digested with restriction enzyme *Nco*I. This led to the generation of two fragments: a 3.5-kb fragment, spanning nt 6447 to 2063, that contains the *cis* elements required for HPV-31 DNA replication, and a 4.4-kb fragment, spanning nt 2063 to 6447. Upon analysis of the 3.5-kb fragment on a neutral/neutral two-dimensional gel, a discontinuous arc pattern resulted (Fig. 10A). This indicates the presence of a bubble-shaped intermediate resulting from an initiation site. Additionally, the discontinuous arc indicates that the initiation site is asymmetrically located between nt 6447 and 2063. Analysis of the 4.4-kb fragment, spanning nt 2063 to 6447, gave rise to a complex hybridization pattern of a diagonal line superimposed on a continuous arc (Fig. 10C). The diagonal line is indicative of a

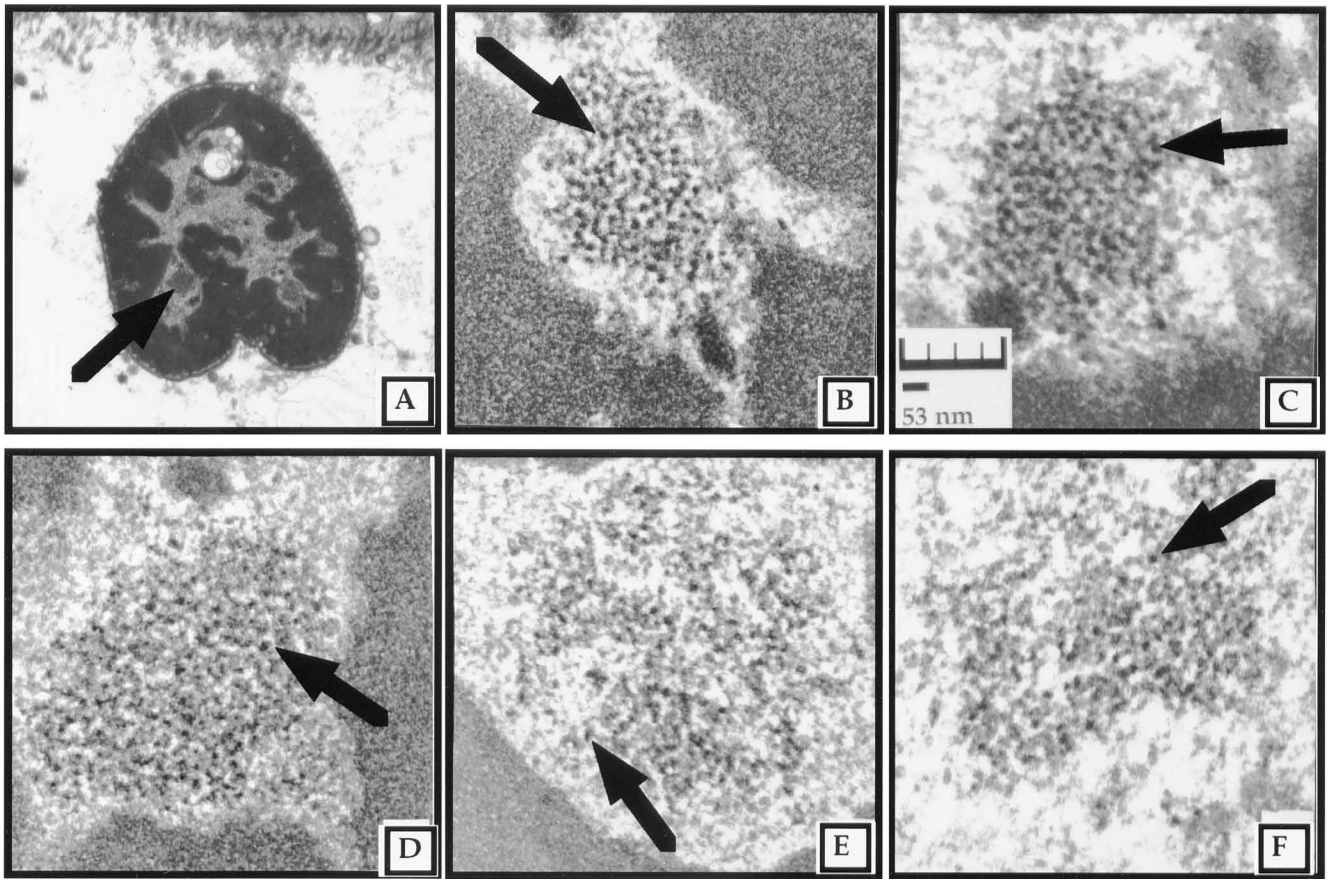

FIG. 7. High-magnification electron micrographs of many nuclei from upper layers of cervical epithelial cells (W12-E cells) induced to undergo differentiation (Fig. 5). (A) The arrow points to an aggregate of virus-like particles in a nucleus from differentiated cervical epithelial cells at high magnification (×5,400). (B to F) Higher magnifications (×49,500) of virus-like particle aggregates in many nuclei of differentiated cells. Individual virus-like particles were measured, and the sizes ranged from 50 to 62 nm. The arrow point to specific virions of 50 nm, (B), 62 nm (C), 62 nm (D), 55 nm (E), and 53 nm (F).

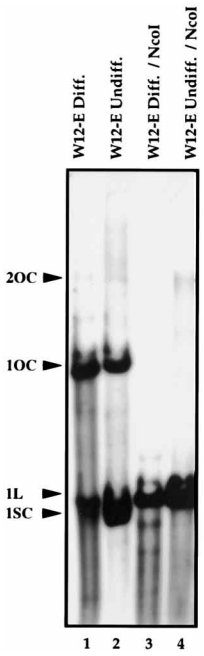

FIG. 8. Analysis of HPV-16 extrachromosomal DNA isolated from differentiated W12-E cells by RFGE. Shown is an autoradiograph of a Southern blot containing Hirt DNA from undifferentiated and differentiated W12-E cells and resolved by RFGE. Equal amounts of DNA (1.5 μg) were loaded in each lane. Lanes 1 and 2, undigested Hirt DNA from differentiated and undifferentiated W12-E cells, respectively; lanes 3 and 4, Hirt DNA, linearized with the restriction enzyme *Nco*I, from differentiated and undifferentiated cells. Southern analysis was done with a full-length HPV-16 probe. The arrows indicate monomeric supercoiled Hirt DNA (1SC), monomeric linear Hirt DNA (1L), monomeric open circular Hirt DNA (1OC), and dimeric open circular Hirt DNA (2OC). This experiment was repeated three times.

termination site. Because of the mixture of hybridization patterns indicative of simple-Y- and double-Y-shaped intermediates, it was determined that the termination site of HPV-31 DNA replication exists asymmetrically within the fragment spanning nt 2063 and 6447. This result provides evidence that HPV-31 DNA replication occurs bidirectionally via theta structures in undifferentiated cervical epithelial cells.

To determine the mode of HPV-31 DNA replication in differentiated cervical epithelial cells, the CIN612-9E cells were induced to differentiate. CIN612-9E cells differ from W12-E cells in that they do not stratify when maintained in our high-Ca^{2+} and high-serum medium. The upper layers of the cells slough off (data not shown). After 10 days in high-Ca^{2+} medium, only 5% of the cells had formed cornified envelopes. Hirt DNA extracted from these cells and analyzed by a neutral/neutral two-dimensional gel gave rise to patterns similar to those in Fig. 10A and C (data not shown). Thus, the alternative method was used to differentiate the CIN612-9E cells. Cells were grown suspended in semisolid F medium containing 1.68% methylcellulose and 20% FBS for 10 days. At the end of the 10-day period, 25% of the cells formed cornified envelopes comparable to the number in W12-E cells both in high-Ca^{2+} and -serum medium and methylcellulose. To analyze the HPV-31 DNA RIs from differentiated CIN612-9E cells, Hirt DNA was isolated from cells suspended in semisolid medium for 10 days and digested with *Nco*I, which generated the 3.5- and 4.4-kb fragments described above. These fragments were then analyzed on a neutral/neutral two-dimensional gel. The 3.5-kb fragment gave rise to a continuous arc (Fig. 10B). This arc is indicative of simple-Y-shaped intermediates resulting from a single replication fork traversing the molecule. The absence of a bubble arc indicates that no initiation site exists between nt 6447 and 2063. The 4.4-kb fragment also gave rise to a continuous arc (Fig. 10D). The absence of the diagonal hybridization pattern indicates that no termination site is present within this fragment in HPV-31 in differentiated cells. These data are most consistent with a rolling circle mode of DNA replication where a single replication fork traverses the molecule in a single direction. Collectively, these data indicate that the switch in the mode of DNA replication is not specific to HPV-16 and also occurs in HPV-31.

DISCUSSION

Using two-dimensional gel analyses, we have shown that there is a switch in the mode of HPV-16 DNA replication during its life cycle. In the nonproductive stage of the life cycle, HPV-16 DNA replicates bidirectionally via theta structures. HPV-16 DNA replication initiates within a bubble located at approximately nt 100. Termination of replication occurs between nt 3437 and 4906, where two replication forks converge to a common site. In the productive stage of the life cycle, the mode of HPV-16 DNA replication changed. The absence of bubble and double-Y-shaped DNA RIs indicates that replication did not occur via theta structures. The presence of only Y-shaped HPV-16 DNA RIs from differentiated cells is most consistent with a rolling circle mode of replication (Fig. 11). This switch in the mode of viral DNA replication occurs in another HPV high-risk genotype, HPV-31; in undifferentiated cervical epithelial cells, HPV-31 replicates bidirectionally via theta structures, while in differentiated cells, the mode changes to what appears to be a rolling circle mode.

We were able to map with relatively high precision the initiation site of HPV-16 DNA replication by using a combination of neutral/neutral and neutral/alkaline two-dimensional gel methods. The approximate location of the initiation site of DNA replication was mapped by the neutral/neutral two-dimensional gel method to between nt 7463 and 551. To map more precisely the initiation site by this method, analysis of small fragments is required. However, fragments smaller than 1,000 bp are difficult to resolve on these gels. Thus, we made use of the neutral/alkaline two-dimensional gel method to map more precisely the initiation site. By making use of hybridization probes that flanked either side of the region where initiation occurs (nt 7463 to 551) and determining the shortest nascent leading strand detectable by each probe, we were able to locate the 5′ ends of leading strands to approximately nt 100 on the HPV-16 DNA genome. This finding is significant in that it locates the initiation of DNA replication to a site proximal to the *cis* elements that comprise the minimal origin of DNA replication (8). These *cis* elements include an E1 binding site at nt 7896 to 8 and E2 binding sites at nt 7859 to 7870, nt 34 to 45, and nt 49 to 61 (6, 8). The finding that replication initiates proximal to the origin and not randomly on the circular genome is consistent with the predictions that *cis* elements present in the origin direct primer synthesis and/or that E1, which is thought to assemble the cellular replicative machinery including DNA polymerase α, does not begin unwinding the double-stranded viral genome extensively until primer synthesis occurs or elongation begins. The initiation site of DNA replication of HPV-11 replicons extracted from laryngeal papillomas mapped to a similar region in the genome between nt 7833 and 33 (1). This places the initiation site of HPV-11 DNA replication within its known minimal origin sequences, nt 7888 to 110 (8). In murine C127 cells, BPV-1 DNA replication initiates within an E2-responsive enhancer element (E2RE1)

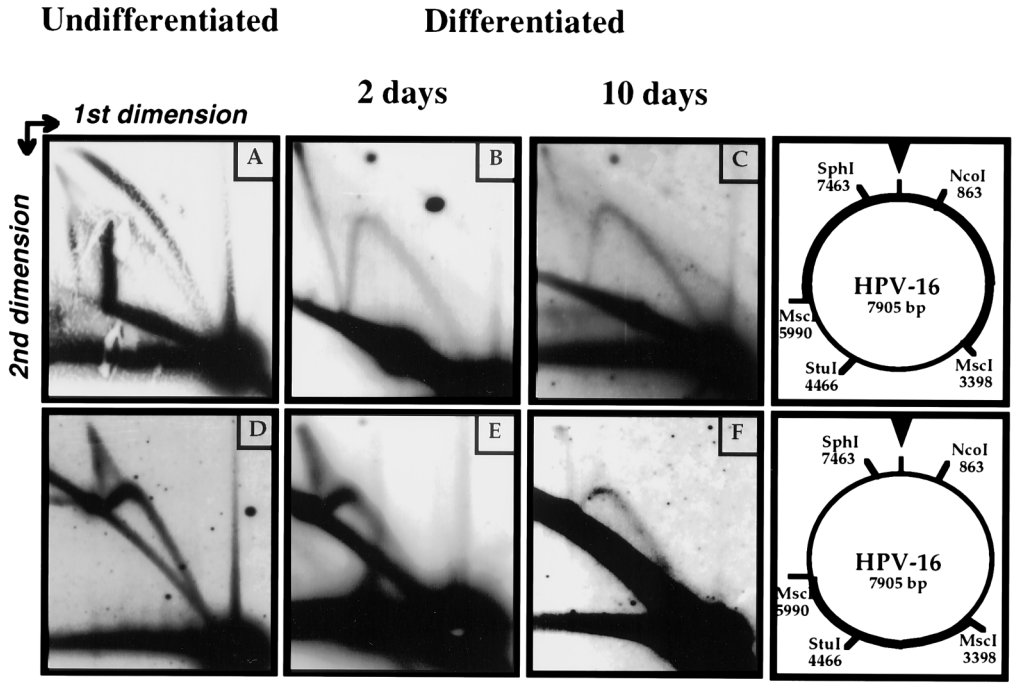

FIG. 9. Resolution of HPV-16 DNA RIs from undifferentiated and differentiated cervical epithelial cells by neutral/neutral two-dimensional gel electrophoresis. Shown is a comparison of autoradiographs of Southern blots containing Hirt DNA from undifferentiated and differentiated W12-E cells digested with restriction enzymes and resolved by neutral/neutral two-dimensional gel electrophoresis. To the right of each pair of autoradiographs is a circular map of the HPV-16 genome. The region of the viral genome analyzed is indicated by the bold segment of the circular genomic map. (A) Region spanning nt 5990 to 3398 in undifferentiated cells; (B) region spanning nt 5990 to 3398 in cells 2 days after induction of differentiation; (C) region spanning nt 5990 to 3398 in cells 10 days after induction of differentiation; (D) region spanning nt 3398 to 5990 in undifferentiated cells; (E) region spanning nt 3398 to 5990 in cells 2 days after induction of differentiation; (F) region spanning nt 3398 to 5990 in cells 10 days after induction of differentiation. Panels A and D are the same as Fig. 3A and F. Refer to Fig. 2A to interpret migration patterns. Analyses of all fragments were repeated three or more times.

at bp 7730 ± 100 (34) positioned approximately 150 bp 5′ of the BPV-1 minimal origin sequences, nt 7894 to 33 (33). The E2RE1 contains four of the strongest E2 binding sites in BPV-1, while the E2 binding sites within the minimal origin have weaker affinity for E2 (20). In contrast, the E2 binding sites within the origin in HPV-16 and HPV-11 are strong E2 binding sites (6). Thus, it is possible that the location of strong E2 binding sites determines the initiation site of papillomavirus DNA replication. This role of E2 may be limited to its tethering the E1 protein to the viral genome, which recruits host cell DNA polymerase α (6, 24). Alternatively, the E2 protein may contribute to the assembly of cellular replication proteins. E2 is argued to bind RPA (24), a single-stranded DNA binding protein that is required in vitro for papillomavirus replication and is thought to contribute to the capacity for polymerase α to synthesize DNA (22, 25).

The full papillomavirus life cycle is dependent on the differentiation of the epithelium. Thus, in the undifferentiated basal layer of the epithelium, the nonproductive stage of the life cycle takes place, while the differentiating suprabasal layer supports the productive stage of the life cycle. The clonal cell populations harboring HPV-16 DNA extrachromosomally, W12-E cells, and those harboring HPV-31 DNA extrachromosomally, CIN612-9E cells, used in this study were derived from CIN 1 lesions and are competent to support both the nonproductive and productive stages of the HPV-16 and HPV-31 life cycles, respectively (2, 10, 16–18, 29, 30). When these cells were maintained in the undifferentiated state (i.e., nonproductive infective state), HPV-16 and HPV-31 DNA replicated bidirectionally via theta-structure RIs. In murine C127 cells, BPV-1 DNA replication also occurs bidirectionally via theta-structure RIs. The similarity in the mode of BPV-1 DNA replication in C127 cells and HPV-16 and HPV-31 DNA replication in the naturally infected cervical epithelial cells seen in this study supports the notion that the C127 cells are an appropriate cell type for studying the nonproductive stage of the viral life cycle.

To study the productive stage of the viral life cycle in tissue culture, the three-dimensional architecture of the epithelium must be reproduced. Organotypic or raft cultures have been used to recreate the three-dimensional architecture of the epithelium and reproduce the productive stage of the HPV-31 life cycle (2, 10, 23). However, raft cultures are small and insufficient at producing the large quantities of differentiated cells needed to analyze DNA RIs by two-dimensional gel electrophoretic methods. Thus, to study the mode of HPV-16 DNA replication in the productive stage of the life cycle, two alternative approaches were used to induce epithelial cell differentiation. One approach was to maintain confluent epithelial cell monolayers in a high-calcium and -serum medium for up to 10 days (13, 32). The other was to suspend the cells in methylcellulose containing a high percentage of serum for up to 10 days (13, 32). Differentiation was demonstrated by the formation of cornified envelopes, the presence of suprabasal keratins, and by ultrastructural analysis. Both methods of inducing differentiation resulted in ~35% cornified envelopes after 10 days. Induction of differentiation by high-calcium and -serum in W12-E cells gave rise to aggregates of virus-like particles measuring ~55 nm in diameter (Fig. 7) in many nuclei, which were not apparent in differentiated normal HFKs (data not shown). This is a strong indication that the HPV-16 life cycle can be reproduced when cells are induced to differentiate by high calcium and serum. This method of differenti-

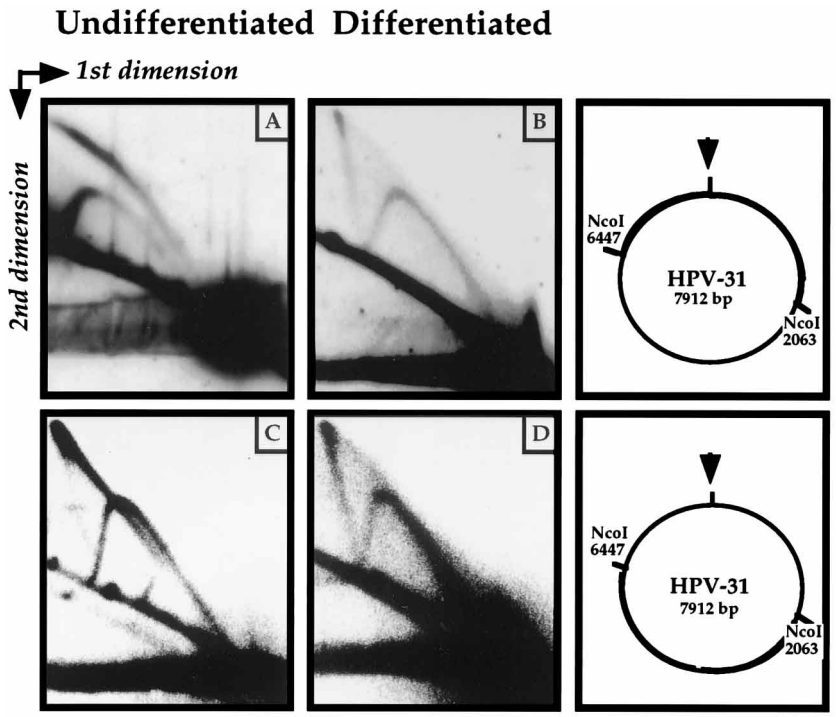

FIG. 10. Resolution of HPV-31 DNA RIs from undifferentiated and differentiated cervical epithelial cells by neutral/neutral two-dimensional gel electrophoresis. Shown is a comparison of autoradiographs of Southern blots containing Hirt DNA from undifferentiated and differentiated CIN612-9E cells digested with restriction enzymes and resolved by neutral/neutral two-dimensional gel electrophoresis. To the right of each pair of autoradiographs is a circular map of the HPV-31 genome. The region of the viral genome analyzed is indicated by the bold segment of the circular genomic map. (A) Region spanning nt 6447 to 2063 in undifferentiated cells; (B) region spanning nt 6447 to 2073 in cells 10 days after induction of differentiation by methylcellulose; (C) region spanning nt 2063 to 6447 in undifferentiated cells; (D) region spanning nt 2063 to 6447 in cells 10 days after induction of differentiation by methylcellulose. Refer to Fig. 2A to interpret migration patterns. Analyses of all fragments were repeated three or more times.

ation is advantageous for biochemical assays requiring large amounts of differentiated epithelial cells.

To study HPV-31 DNA RIs in differentiated cells, CIN612-9E cells were induced to differentiate by high Ca^{2+} and serum and by suspension in 1.68% methylcellulose containing 20% FBS. CIN612-9E cells were found to behave differently from W12-E cells in the high-Ca^{2+} and -serum medium. The CIN612-9E cells did not stratify and had only 5% cornified envelopes, compared to 35% in W12-E cells under the same conditions. Additionally, Hirt DNA from these cells analyzed on a neutral/neutral two-dimensional gel gave rise to hybridization patterns consistent with a theta mode of replication (data not shown). When CIN612-9E cells were induced to undergo differentiation by suspension in methylcellulose for 10 days, the amount of cornified envelopes increased to 25%, which is comparable to the percentage found in W12-E cells under the same condition. Hirt DNA isolated from CIN612-9E cells induced to undergo differentiation by suspension in methylcellulose gave rise to hybridization patterns most consistent with a rolling circle mode of DNA replication. The fact that only the epithelial cells with a large percentage of cornified envelopes gave rise to patterns consistent with a rolling circle mode of replication provides further evidence that differentiation of the epithelial cells is necessary for the switch in the mode of replication to occur.

Rolling circle DNA replication exhibits certain characteristics: it is unidirectional, and one initiation event leads to the generation of multiple copies of the genome. These characteristics result in distinct hybridization patterns by neutral/neutral two-dimensional gel electrophoresis. Hirt DNA extracted from W12-E cells induced to undergo differentiation by high calcium and serum or methylcellulose gave rise to continuous arc hybridization patterns indicative of Y-shaped RIs (Fig. 9). The difference in shape of the DNA RIs in undifferentiated and differentiated cells indicates that a switch in the mode of DNA replication has occurred. The nature of the DNA RIs in differentiated cells provides evidence that is consistent with a rolling circle mode of DNA replication. RFGE analyses performed on DNA from undifferentiated and differentiated populations of W12-E cells indicated no gross change in the nature of the HPV DNA (Fig. 8). This result indicates the Y forks detected by neutral/neutral two-dimensional analysis in the differentiated cells did not arise due to theta-mode replication of multimeric forms of HPV-16 DNA. This result also indicates that any large RIs must be short lived. Neutral/alkaline two-dimensional gel electrophoresis was also performed with undigested Hirt DNA extracted from differentiated W12-E cells. Southern analysis indicated an absence of resolvable nascent strands from the newly synthesized leading-strand DNA (data not shown), similar to that seen in Fig. 4, panel 5, and indicative that leading strands are genome length or longer. This result is also consistent with a rolling circle mode of DNA replication. BPV-1 has also been shown to have the ability to undergo rolling circle DNA replication. In rodent cells, a BPV-1 mutant replicates its DNA to a very high copy number by a rolling circle mechanism. This mutant is thought to mimic events in the productive stage of the viral life cycle (7). Additionally, lariat structures of BPV-1 DNA were detected by EM analysis of DNA isolated from purified BPV-1 virions consistent with a rolling circle mode of DNA replication in the

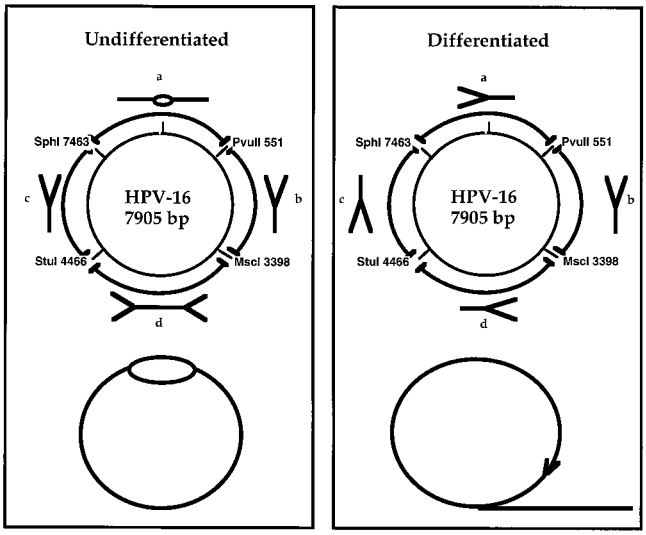

FIG. 11. Interpretation of results from two-dimensional gel analyses of HPV-16 DNA RIs from undifferentiated and differentiated W12-E cells. Shown are the types of structures detected by two-dimensional gel analysis of regions of the HPV-16 genome. Shown are the DNA RIs found in undifferentiated W12-E cells (left). The region between nt 7463 and 551 contains a bubble-shaped RI indicative of an initiation site (a). The flanking regions between nt 551 and 3398 (b) and nt 4466 and 7463 (c) contained Y-shaped intermediates indicative of a single replication fork traversing the molecule. The region between nt 3398 and 4466 (d) contained double Y-shaped intermediates which are indicative of a termination site. These results indicate that HPV-16 replicates by theta structures in these cells. Shown are RIs found in differentiated W12-E cells (right). All regions contained Y-shaped intermediates indicating that no initiation or termination of theta structures was present (right). These intermediates are most consistent with a rolling circle mode of replication.

productive stage of the life cycle (4). Others have analyzed HPV-11 DNA RIs extracted from laryngeal papillomas, which tend to contain mature virions and highly amplified viral DNA (1, 19, 31). Thus, it was argued that the RIs analyzed in that study were primarily from the productive stage of the life cycle (1). Neutral/neutral two-dimensional gel analyses indicated that the majority of RIs were theta structured and only a minority were possibly of the rolling circle type (1). This finding argues that there is a difference in the mode of DNA replication in low- versus high-risk HPV genotypes during the productive stage of the viral life cycle. Further studies must be carried out to confirm this difference and understand the underlying basis.

The cell environment of a differentiated cell is different than that of an undifferentiated cell. Undifferentiated epithelial cells, site of the nonproductive stage of the viral life cycle, are mitotically active and produce factors, such as DNA polymerase α/primase, DNA polymerase δ/proliferating cell nuclear antigen, RPA, and Topo I and II, which are necessary for DNA replication (6). The environment of the differentiated epithelial cell is not favorable for DNA replication, yet this is where HPV-16 amplifies its DNA. Differentiated epithelial cells are no longer cycling or producing the factors necessary for DNA replication (5, 9). Since the papillomavirus is dependent on these factors for its DNA replication, the virus must overcome these barriers in differentiated cells. It is possible that the papillomavirus switches to a rolling circle mode of replication as a way of escaping the unfavorable conditions of the differentiated cell. A theta mode of replication requires initiation with every round of replication. This includes E2 tethering E1 to the origin and allowing the recruitment of DNA polymerase α, which is limiting in differentiated cells. By switching to a rolling circle mode, one initiation event leads to the generation of multiple daughter DNA molecules, thus facilitating the generation of large amounts of DNA. It will also be important to understand what controls HPV-16 DNA replication in the late stage of the life cycle and to determine the *cis* and *trans* elements necessary for the switch in the mode of DNA replication in the productive stage of the life cycle. It is possible that HPV-16 DNA replicates by using different *cis* and *trans* elements to replicate its DNA throughout the life cycle as has been demonstrated in Epstein-Barr virus, which undergoes DNA replication via theta structures in the latent stage of its life cycle and via rolling circles in the lytic stage (14, 28, 35).

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REFERENCES

1. **Auborn, K. J., R. D. Little, T. H. Platt, M. A. Vaccariello, and C. L. Schildkraut.** 1994. Replicative intermediates of human papillomavirus type 11 in laryngeal papillomas: site of replication initiation and direction of replication. Proc. Natl. Acad. Sci. USA **91:**7340–7344.
2. **Bedell, M. A., J. B. Hudson, T. R. Golub, M. E. Turyk, M. Hosken, G. D. Wilbanks, and L. A. Laimins.** 1991. Amplification of human papillomavirus genomes in vitro is dependent on epithelial differentiation. J. Virol. **65:**2254–2260.
3. **Brewer, B. J., and W. L. Fangman.** 1987. The localization of replication origins on ARS plasmids in S. cerevisiae. Cell **51:**463–471.
4. **Burnett, S., J. Zabielski, L. J. Moreno, and U. Pettersson.** 1989. Evidence for multiple vegetative DNA replication origins and alternative replication mechanisms of bovine papillomavirus type 1. J. Mol. Biol. **206:**239–244.
5. **Cheng, S., D. C. S. Grimminger, T. Murant, T. R. Broker, and L. T. Chow.** 1995. Differentiation-dependent up-regulation of the human papillomavirus E7 gene reactivates cellular DNA replication in suprabasal differentiated keratinocytes. Genes Dev. **9:**2335–2349.
6. **Chow, L. T., and T. R. Broker.** 1994. Papillomavirus DNA replication. Intervirology **37:**150–158.
7. **Dasgupta, S., J. Zabielski, M. Simonsson, and S. Burnett.** 1992. Rolling-circle replication of a high-copy BPV-1 plasmid. J. Mol. Biol. **228:**1–6.
8. **Del Vecchio, A., H. Romanczuk, P. M. Howley, and C. C. Baker.** 1992. Transient replication of human papillomavirus DNAs. J. Virol. **66:**5949–5958.
9. **Demeter, L. M., M. H. Stoler, T. R. Broker, and L. T. Chow.** 1994. Induction of proliferating cell nuclear antigen in differentiated keratinocytes of human papillomavirus-infected lesions. Hum. Pathol. **25:**343–348.
10. **Frattini, M. G., H. B. Lim, and L. A. Laimins.** 1996. In vitro synthesis of oncogenic human papillomaviruses requires episomal genomes for differentiation-dependent late expression. Proc. Natl. Acad. Sci. USA **93:**3062–3067.
11. **Fuchs, E.** 1990. Epidermal differentiation: the bare essentials. J. Cell Biol. **111:**2807–2814.
12. **Gilbert, D. M., and S. N. Cohen.** 1987. Bovine papillomavirus plasmids replicate randomly in mouse fibroblasts throughout S phase of the cell cycle. Cell **50:**59–68.
13. **Green, H.** 1977. Terminal differentiation of cultured human epidermal cells. Cell **11:**405–416.
14. **Hammerschmidt, W., and B. Sugden.** 1988. Identification and characterization of oriLyt, a lytic origin of DNA replication of Epstein-Barr virus. Cell **55:**427–433.
15. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected cell cultures. J. Mol. Biol. **36:**365–369.
16. **Hummel, M., J. B. Hudson, and L. A. Laimins.** 1992. Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. J. Virol. **66:**6070–6080.
17. **Jeon, S.** 1995. Ph.D. University of Wisconsin—Madison.
18. **Jeon, S., H. B. Allen, and P. F. Lambert.** 1995. Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. J. Virol. **69:**2989–2997.
19. **Kreider, J. W., M. K. Howett, A. E. Leure-Dupree, R. J. Zaino, and J. A. Weber.** 1987. Laboratory production in vivo of infectious human papillomavirus type 11. J. Virol. **70:**590–593.
20. **Li, R., J. Knight, G. Bream, A. Stenlund, and M. Botchan.** 1989. Specific recognition nucleotides and their DNA context determine the affinity of E2 protein for 17 binding sites in the BPV-1 genome. Genes Dev. **3:**510–526.

21. **Linskens, M. H. K., and J. A. Huberman.** 1990. Ambiguities in results obtained with 2D gel replicon mapping techniques. Nucleic Acids Res. **18:** 647–652.
22. **Melendy, T., J. Sedman, and A. Stenlund.** 1995. Cellular factors required for papillomavirus DNA replication. J. Virol. **69:**7857–7867.
23. **Meyers, C., M. G. Frattini, J. B. Hudson, and L. A. Laimins.** 1992. Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. Science **257:**971–973.
24. **Mohr, I. J., R. Clark, S. Sun, E. J. Androphy, P. MacPherson, and M. R. Botchan.** 1990. Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. Science **250:**1694–1699.
25. **Muller, F., Y. S. Seo, and J. Hurwitz.** 1994. Replication of bovine papillomavirus type 1 origin-containing DNA in crude extracts and with purified proteins. J. Biol. Chem. **269:**17086–17094.
26. **Nawotka, K. A., and J. A. Huberman.** 1988. Two-dimensional gel electrophoretic method for mapping DNA replicons. Mol. Cell. Biol. **8:** 1408–1413.
27. **Park, P., W. Copeland, L. Yang, T. Wang, M. R. Botchan, and I. J. Mohr.** 1994. The cellular DNA polymerase alpha-primase is required for papillomavirus DNA replication and associates with the viral E1 helicase. Proc. Natl. Acad. Sci. USA **91:**8700–8704.
28. **Pfuller, R., and W. Hammerschmidt.** 1996. Plasmid-like replicative intermediates of the Epstein-Barr virus lytic origin of DNA replication. J. Virol. **70:**3423–3431.
29. **Stanley, M. A., H. M. Browne, M. Appleby, and A. C. Minson.** 1989. Properties of a non-tumorgenic human cervical keratinocyte cell line. Int. J. Cancer **43:**672–676.
30. **Sterling, J., M. Stanley, G. Gatward, and T. Minson.** 1990. Production of human papillomavirus type 16 virions in a keratinocyte cell line. J. Virol. **64:**6305–6307.
31. **Stoler, M. H., and T. R. Broker.** 1986. In situ hybridization detection of human papillomavirus DNAs and messenger RNAs in genital condylomas and a cervical carcinoma. Hum. Pathol. **17:**1250–1258.
32. **Sun, T. T., and H. Green.** 1976. Differentiation of the epidermal keratinocyte in cell culture: formation of the cornified envelope. Cell **9:**511–521.
33. **Ustav, M., E. Ustav, P. Szymanski, and A. Stenlund.** 1991. Identification of the origin of replication of bovine papillomavirus and characterization of the viral origin recognition factor E1. EMBO J. **10:**4321–4329.
34. **Yang, L., and M. Botchan.** 1990. Replication of bovine papillomavirus type 1 DNA initiates within an E2-responsive enhancer element. J. Virol. **64:** 5903–5911.
35. **Yates, J., N. Warren, D. Reisman, and B. Sugden.** 1984. A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. USA **81:**3806–3810.
36. **zur Hausen, H.** 1991. Human papillomaviruses in the pathogenesis of anogenital cancer. Virology **184:**9–13.