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Quantitative Role of the Human Papillomavirus Type 16 E5 Gene during the Productive Stage of the Viral Life Cycle

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Human papillomaviruses (HPVs) are small circular DNA viruses that cause warts. Infection with high-risk anogenital HPVs, such as HPV type 16 (HPV16), is associated with human cancers, specifically cervical cancer. The life cycle of HPVs is intimately tied to the differentiation status of the host epithelium and has two distinct stages: the nonproductive stage and the productive stage. In the nonproductive stage, which arises in the poorly differentiated basal epithelial compartment of a wart, the virus maintains itself as a low-copy-number nuclear plasmid. In the productive stage, which arises as the host cell undergoes terminal differentiation, viral DNA is amplified; the capsid genes, L1 and L2, are expressed; and progeny virions are produced. This stage of the viral life cycle relies on the ability of the virus to reprogram the differentiated cells to support DNA synthesis. Papillomaviruses encode multiple oncoproteins, E5, E6, and E7. In the present study, we analyze the role of one of these viral oncogenes, E5, in the viral life cycle. To assess the role of E5 in the HPV16 life cycle, we introduced wild-type (WT) or E5 mutant HPV16 genomes into NIKS, a keratinocyte cell line that supports the papillomavirus life cycle. By culturing these cells under conditions that allow them to remain undifferentiated, a state similar to that of basal epithelial cells, we determined that E5 does not play an essential role in the nonproductive stage of the HPV16 life cycle. To determine if E5 plays a role in the productive stage of the viral life cycle, we cultured keratinocyte populations in organotypic raft cultures, which promote the differentiation and stratification of epithelial cells. We found that cells harboring E5 mutant genomes displayed a quantitative reduction in the percentage of suprabasal cells undergoing DNA synthesis, compared to cells containing WT HPV16 DNA. This reduction in DNA synthesis, however, did not prevent amplification of viral DNA in the differentiated cellular compartment. Likewise, late viral gene expression and the perturbation of normal keratinocyte differentiation were retained in cells harboring E5 mutant genomes. These data demonstrate that E5 plays a subtle role during the productive stage of the HPV16 life cycle.

Human papillomaviruses (HPVs) are small circular DNA viruses that cause warts. Significantly, infection with high-risk anogenital HPVs, such as HPV type 16 (HPV16), is associated with human cancers, specifically cervical cancer (50). The life cycle of HPVs is intimately tied to the differentiation status of the host epithelium and has two distinct stages: the nonproductive stage and the productive stage. The nonproductive stage of the viral life cycle occurs in the basal compartment of the host epithelium where the virus gains entry, presumably at a site of wounding. In this nonproductive stage, the virus maintains itself as a low-copy-number nuclear plasmid (10). As the host cells differentiate, the productive stage of the viral life cycle occurs in the suprabasal compartment of the epithelium. In the productive stage, viral DNA is amplified; the capsid genes, L1 and L2, are expressed; and progeny virions are produced.

The 7,904-bp HPV16 genome contains eight viral genes encoding six nonstructural and two structural proteins. Three of the nonstructural proteins, E5, E6, and E7, are designated as oncoproteins because they are able to transform cells in vitro (22, 28, 29, 33, 37, 45) and, in the case of E6 and E7, induce tumors in vivo (23, 42). Two of the other three nonstructural proteins, E1 and E2, are involved in DNA replication and transcription of the viral genome. E4 is predicted to contribute indirectly to the replication of the viral DNA genome in the productive stage (J. Doorbar, unpublished data; T. Nakahara, personal communication). L1 and L2 are the major and minor capsid proteins, respectively.

Unlike E6 and E7, the major viral oncoproteins, the E5 protein of HPV16 is not commonly found in cervical carcinoma cells (3, 4). However, it is considered an oncogene given its ability to transform mouse fibroblasts and keratinocytes, cause the mitogenic stimulation of human keratinocytes, and cooperate with E7 to stimulate proliferation of human keratinocytes (5, 28, 29, 37, 45). The E5 gene of HPV16 is an 83-amino-acid hydrophobic membrane protein (8, 21) found localized to the Golgi apparatus, endoplasmic reticulum, and nuclear membrane (11).

What led researchers to study the HPV16 E5 protein was that, in contrast to the HPVs, the major transforming protein of bovine papillomavirus type 1 (BPV1) is the E5 protein, a 44-amino-acid highly hydrophobic protein that is localized predominantly to the Golgi and exists as homodimers (13, 40, 41). The BPV1 E5 protein is able to transform both murine fibroblasts and keratinocytes in transformation assays in vitro (9,

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29). The BPV1 E5 protein is able to bind to and activate the platelet-derived growth factor β receptor in the absence of exogenous ligand (35, 36), and this has been shown to correlate with cellular transformation (27). BPV1 E5 also binds to the 16-kDa pore-forming membrane component of the vacuolar proton ATPase (v-ATPase), a protein essential for the acidification of intracellular compartments such as lysosomes, endosomes, and the Golgi (19). The binding of BPV1 E5 to the 16-kDa protein is able to cause alkalization of the Golgi, and this has been shown to correlate with cellular transformation (39). Because of the structural similarities between the BPV1 E5 and HPV16 E5 proteins, and because BPV1 E5 has strong transforming potential, work was begun to determine if HPV16 E5 was also an oncogene.

Multiple studies have suggested a link between the HPV16 E5 gene and epidermal growth factor (EGF) receptor (EGFR) signaling. Studies indicate that HPV16 E5 causes an increased activation of the EGFR in the presence of ligand (5, 28, 37), and coimmunoprecipitation experiments indicate that HPV16 E5 can form a complex with growth factor receptors (25). The HPV16 E5 protein also binds to the 16-kDa membrane component of the v-ATPase (11) and delays endosomal acidification in human keratinocytes (44). It has been argued that in binding to the 16-kDa protein, E5 disrupts the 16-kDa protein-v-ATPase complex (1, 7), which results in the inhibition of endosomal acidification.

To assess the role of E5 in the HPV16 life cycle, we generated cell populations in which we had transfected wild-type (WT) or E5 mutant HPV16 genomes. We discovered that E5 was not required for the viral genome to become established as a stable nuclear plasmid in keratinocytes when these cells are maintained in the poorly differentiated state characteristic of basal epithelial cells. These data indicate that E5 is not required for the nonproductive stage of the viral life cycle. Using these cell lines, we further characterized the effect that the loss of the E5 protein had on the productive stage of the viral life cycle. For this purpose, cell populations harboring WT and E5 mutant genomes were grown in organotypic raft cultures, which permit the full differentiation of keratinocytes. Whereas loss of E5 had no effect on the nonproductive stage of the life cycle, it had a negative effect during the productive stage of the life cycle as evidenced by the quantitative reduction of DNA synthesis in the supraparabasal compartment of rafts harboring E5 mutant genomes. We had previously demonstrated that another viral oncogene, E7, is essential for cells within the differentiated cellular compartment to support DNA synthesis. Our present studies argue for a role of a second viral oncogene, E5, in reprogramming differentiated cells to support DNA synthesis, a prerequisite for amplification of the viral DNA.

MATERIALS AND METHODS

HPV DNA preparation for transfections. As a source of HPV16 DNA, plasmid pEFHPV-16W12E derived from W12E cells (GenBank accession no. AF125673), was used as described previously (17). To construct HPV16^{E5XCM-}, a single nucleotide, adenine (at nucleotide position 30 of the E5 open reading frame [ORF]) was deleted from the *Xcm*I site in the E5 gene of pEFHPV-16W12E. Transfections were performed as described previously (16). Briefly, the viral DNA sequences were excised from the pUC 18 vector by digestion with *Bam*HI. The HPV DNAs were gel purified, ethanol precipitated, quantified, and ligated at low concentrations (50 ng/μl) to avoid formation of multimers.

Cell culture. Epithelial cells were cultured as described previously (16, 18, 26). Briefly, cells were maintained at subconfluence on mitomycin C-treated m_1 3T3 feeder cells in F medium (0.66 mM Ca^{2+}) composed of 3 parts Dulbecco's modified Eagle's medium and 1 part Ham's F-12 medium and supplemented with the following components: 5% fetal bovine serum (FBS), adenine (24 μg/ml), cholera toxin (8.4 ng/ml), EGF (10 ng/ml), hydrocortisone (2.4 μg/ml), and insulin (5 μg/ml). When the epithelial cells reached subconfluence, the m_1 3T3 feeder cells were removed with 0.02% EDTA and tapping of the plate. The epithelial cells were removed from the dishes by incubation with 0.1% trypsin–0.5 mM EDTA at 37°C.

Stable transfections. The recircularized HPV16 DNAs were transfected into NIKS cells as previously described (16). NIKS cells (previously named BC1-Ep/SL cells) are a spontaneously immortalized cell line that differentiates normally (2) and supports the HPV16 life cycle (17). Briefly, the HPV16 DNAs (3 μg) were cotransfected into NIKS cells with pEGFPN1(1.2 μg) (Clonetics), which encodes the green fluorescent protein and confers G418 resistance. The DNA was transfected into the cells on a 6-cm-diameter dish in low-Ca^{2+} F medium supplemented with adenine (24 μg/ml), cholera toxin (8.4 ng/ml), EGF (10 ng/ml), hydrocortisone (2.4 μg/ml), and insulin (5 μg/ml) using SuperFect (Qiagen) as specified by the manufacturer. At day 1 posttransfection, the cells were trypsinized and plated in F medium (0.66 mM Ca^{2+}) supplemented with 5% FBS, adenine (24 μg/ml), cholera toxin (8.4 ng/ml), hydrocortisone (2.4 μg/ml), and insulin (5 μg/ml) on 10-cm-diameter dishes containing m_1 3T3 feeder cells. At day 2 posttransfection, 100 μg of G418 per ml was added to the medium. The level of G418 was reduced to 50 μg/ml 4 days after transfection. The cells were fed every other day until the resulting G418-resistant colonies were pooled and expanded for Southern analysis. The pool is referred to as a cell population.

Screening stable transfectants. Hirt DNA (low-molecular-weight DNA) (24) was extracted from one 15-cm-diameter dish of each HPV16^{WT} or HPV16^{E5XCM-} cell population and used for Southern analysis. A portion, 10^6 cells' worth, of the resulting DNA was linearized with *Bam*HI, while 3×10^6 cells' worth remained undigested to determine the presence of open circular and supercoiled viral DNA, indicators of extrachromosomal viral DNA. Hirt DNA extracted from W12E cells was used as a positive control. Untransfected NIKS cells were used as a negative control. The DNA was electrophoresed on a 0.8% agarose gel and transferred to nitrocellulose membrane (Schleicher & Schuell). The blot was probed with a full-length HPV16 probe generated by *Bam*HI digest of pEFHPV-16W12E and labeled with [α-^{32}P]dCTP using a random primer labeling kit (Amersham). To visualize HPV DNA, the blot was exposed to a PhosphorImager screen overnight.

Immunoprecipitation and Western blot analyses. To detect phosphorylated EGFR (P-EGFR), cells were grown to 80% confluence, washed two times with 1× phosphate-buffered saline (PBS), and lysed with RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl, leupeptin [1 μg/ml], pepstatin [1 μg/ml], 1 mM NaF, 50 mM Na β-glycerophosphate), and the lysate was scraped into Eppendorf tubes and incubated on ice for 20 min. The lysates were sheared five times with a 27-gauge needle and centrifuged at 14,000 rpm in a microcentrifuge (Eppendorf model 5415c) for 10 min at 4°C. The supernatant was recovered, and the concentration of protein was quantified using the Bradford assay (Bio-Rad). Two micrograms of anti-EGFR antibody (Ab-15; Labvision) was added to 1 mg of protein per lysate, and the samples were incubated for 3 h at 4°C with rotation. Protein G beads (15 μl) were added; the samples were rotated for an additional 1 h at 4°C; the beads were recovered by microcentrifugation and washed four times; and the bound protein was eluted in 2× SDS loading buffer, boiled for 5 min, and electrophoresed through a 5% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Resolved proteins were transferred to a polyvinylidene difluoride membrane, and the membrane was blocked with 5% bovine serum albumin (BSA) in 0.1% Tween 20 in 1× PBS (PBST) for 1 h and then probed with an antiphosphotyrosine antibody at 1:1,000 in 1% BSA-PBST (PY20; Santa Cruz) for 1 h. at room temperature. The blot was washed and then probed with an anti-mouse peroxidase-labeled secondary antibody at 1:10,000 in 1% BSA-PBST (A106PN; American Qualex Antibodies) and visualized using chemiluminescence (Boehringer Mannheim). To detect total EGFR, 50 μg of protein lysate was electrophoresed through an SDS–8% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane, and the membrane was blocked with 5% nonfat milk in PBST for 1 h and then probed with anti-EGFR antibody (Ab-15; 0.25 μg/ml; Labvision) for 2 h. The same secondary antibody and detection method was used as described above for detection of P-EGFR.

Raft cultures. Transwell inserts (24 mm in diameter and 0.4 μm in pore size; Costar) were coated with 1 ml of rat tail collagen type I (4.5 mg/ml; Upstate Biotechnology, Inc.) (2, 16, 17). Early-passage human foreskin fibroblasts (600 μl

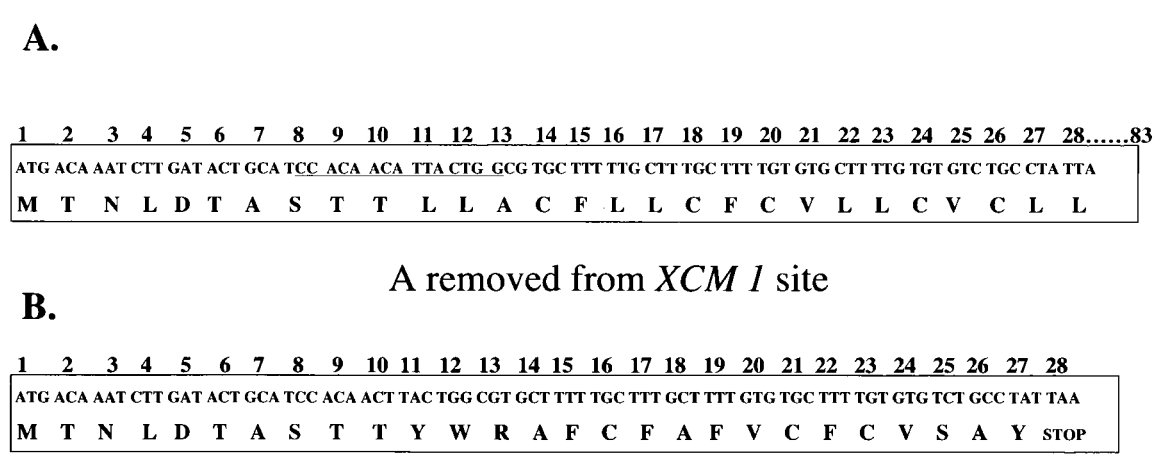

FIG. 1. Schematic displaying the DNA sequence of the first 28 amino acids of HPV16 $E5^{WT}$ ORF (A) and the corresponding 28-amino-acid $E5^{XCM-}$ mutant (B). To construct the $E5^{XCM-}$ HPV16 mutant, a single nucleotide, adenine, was deleted in codon 10 of the E5 ORF, disrupting the *Xcm*I restriction site (underlined) and leading to a frameshift with a stop codon (TAA) encountered at codon 28. Only the first 10 amino acids of $E5^{XCM-}$ are derived from the E5 ORF.

at 7.5×10^5 cells/ml) were embedded into the remaining collagen and plated on the collagen-coated Transwell inserts. The collagen was allowed to incubate for 4 days in a 5% CO_2 incubator at 37°C in F-12 medium containing 10% FBS. After the 4-day incubation, 1.5×10^6 keratinocytes per 100 μl of keratinocyte plating medium (F medium [1.88 mM Ca^{2+}]) containing 0.5% FBS, adenine (24 μg/ml), cholera toxin (8.4 ng/ml), hydrocortisone (2.4 μg/ml), and insulin (5 μg/ml) were plated onto the collagen dermal equivalent. Four days after plating, the Transwell inserts were placed onto two 1-in^2 cotton pads (Schleicher & Schuell) in a six well tray (Organogenesis). The rafts were fed from below the Transwell insert with cornification medium (keratinocyte plating medium containing 5% FBS and 10 μM C8:0) every other day. Eleven days after being lifted to the liquid-air interface, the rafts were fed for 8 h with cornification medium containing 10 μM bromodeoxyuridine (BrdU). Subsequently, the rafts were embedded in 2% agar–1% formalin, fixed in 4% formalin at 4°C overnight, embedded in paraffin, and cut into 4-μm-thick cross sections.

Immunohistochemistry. Immunohistochemistry was performed on 4-μm-thick cross sections of paraffin-embedded rafts using the Vectastain ABC kit (Vector). The slides were deparaffinized in xylenes and rehydrated in a graded series of alcohol washes. For keratin 10 (K10), filaggrin, and L1 immunohistochemistry, the following conditions were used. After deparaffinizing, the slides were treated to inactivate endogenous peroxidase by incubation in methanol containing 3% H_2O_2 for 30 min at room temperature, treated with 3 mg of pepsin per ml of 0.01 N HCl for 10 min (K10 and L1) or 15 min (filaggrin), blocked with 5% horse serum in 1× PBS, and incubated with primary antibodies at room temperature. For K10 staining, the K10-specific antibody (clone Ck 8.60; Sigma) was used at a dilution of 1:200 in 5% blocking serum in PBS for 3 h. For filaggrin staining, a monoclonal anti-human filaggrin antibody (BT-576; Biomedical Technologies, Inc.) was diluted 1:100 in 5% blocking serum in PBS for 3 h. L1 staining was done using the L1-specific antibody (CamVir 1; Abcam, Ltd.) at a dilution of 1:50 in 5% blocking serum in PBS for 3 h. For BrdU staining, after deparaffinizing, the slides were treated to inactivate endogenous peroxidase (3% H_2O_2 in methanol) for 10 min. After the peroxidase quenching, the BrdU antigens were unmasked in sodium citrate buffer (10 mM at pH 6.0) for 20 min rotating in the microwave (3 min power level 10 [PL-10] and 17 min at PL-7). The slides sat on the benchtop for 15 min after boiling. The slides were treated with HCl (2 N) for 20 min, blocked in 5% blocking serum in PBS, and incubated with the anti-BrdU antibody (Ab-2; Oncogene) for 3 h at room temperature. All antibodies were detected using the Vectastain ABC kit as specified by the manufacturer. All slides were counterstained with hematoxylin (Vector) for 2 min to reveal the tissue morphology and mounted with Cytoseal XYL (Richard-Allan Scientific). To quantify the percentage of BrdU-positive cells in the supraparabasal compartment of rafts, BrdU-positive cells from four sets of rafts were quantified (10 fields per slide at 40× magnification). Percent total BrdU incorporation in supraparabasal cells was calculated by quantifying the total number of BrdU-positive cells in the supraparabasal compartment per slide and dividing that number by the total number of BrdU-positive cells per slide. Relative percentage of BrdU-positive cells in the supraparabasal compartment was determined by calculating the average percent total BrdU incorporation in supraparabasal cells for the WT populations within an experiment and then setting that equal to 1.0. Each of the populations of the experiment were then divided by that average percentage to give a value relative to the WT populations (1.0). Statistical analysis was performed using the permutation test on the Mstat program.

Immunofluorescence. Immunofluorescence for the E1^E4 protein was performed on 4-μm-thick sections of paraffin-embedded raft cultures. The slides were deparaffinized with xylenes and rehydrated through a graded series of alcohols. After deparaffinizing, the antigens were unmasked in sodium citrate buffer (10 mM at pH 5.0) for 20 min, rotating the specimens in the microwave (3 min at PL-10 and 17 min at PL-7). After antigen unmasking, sections were blocked with 5% horse serum (supplied in the Vectastain kit) in 1× PBS for 30 min and then incubated in an E1^E4-specific antibody (TVG-402) (14) at a 1:50 dilution in 5% blocking serum–1× PBS for 3 h at room temperature. The antibody was detected using AlexFluor 488 goat α-mouse secondary antibody (Molecular Probes). The slides were then mounted with Vectashield mounting medium with 4′,6′-diamidino-2-phenylindole (DAPI) (Vector), which stains nuclei blue. E1^E4 cells were quantified by counting the total number of E1^E4-positive cells per slide. Statistical analysis was performed using the Wilcoxon rank sum test on the Mstat program.

FISH. Fluorescence in situ hybridization (FISH) analysis was performed as described previously (15). Briefly, a nonradioactive FISH procedure using a digoxigenin (Bioprime DNA labeling system; Gibco)-labeled HPV16 probe was used to analyze viral DNA amplification on 4-μm-thick sections of formalin-fixed, paraffin-embedded, organotypic raft cultures. Sections were deparaffinized and rehydrated, heated to 92°C in 100 mM Tris-EDTA (TE), pH 8.0, for 15 min and digested with Digest-All 3 pepsin (Zymed) for 10 s at 37°C. After dehydration in a graded series of ethanols, the genome probe was added to each slide. Codenaturation of tissue DNA and probe was performed for 3 min at 85°C. The probes were hybridized to tissue overnight at 37°C in a humidified chamber and signals were detected with a digoxigenin-specific antibody conjugated to fluorescein isothiocyanate (Boehringer Mannheim) at a 1:400 dilution for 25 min at 37°C. Nuclei were counterstained with DAPI (Vector). The percentage of FISH-positive cells was quantified by counting the total number of FISH-positive cells per slide and dividing that number by the total number of cells per slide. Statistical analysis was performed using the Wilcoxon rank sum test on the Mstat program.

RESULTS

Generation of $HPV16^{E5XCM-}$ populations. To examine the role of the E5 protein in the context of the HPV16 viral life cycle, we have analyzed a mutant HPV16 genome in which the E5 gene has been disrupted by a frameshift mutation. Shown in Fig. 1B, this mutant, $HPV16^{E5XCM-}$, has a single nucleotide

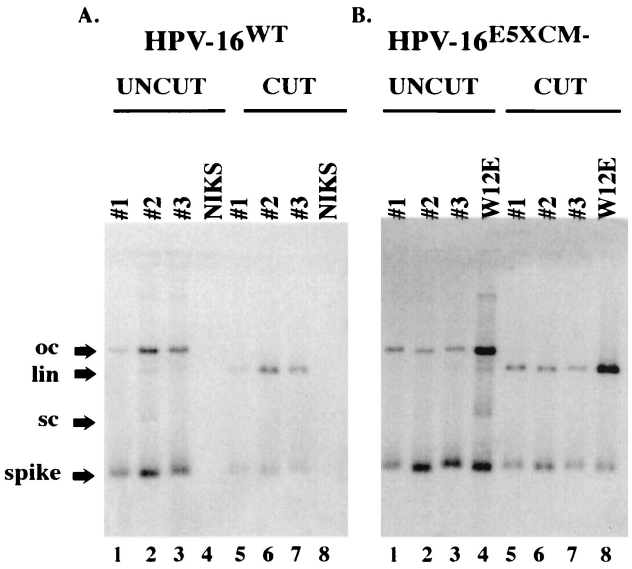

FIG. 2. Southern analysis of low-molecular-weight (Hirt) DNA extracted from three populations each of stably transfected NIKS containing the HPV16^{WT} (A) or HPV16^{E5XCM-} (B) viral DNA. Hirt DNA from passage 2 populations of NIKS cells harboring HPV16^{WT} or HPV16^{E5XCM-} genomes (lanes 1 to 3 and 5 to 7), untransfected NIKS cells and W12E cells grown on feeders were left uncut (lanes 1 to 4) or were cut with *Bam*HI (to linearize viral genomes) (lanes 5 to 8) and subjected to Southern analysis using an HPV16-specific probe. Arrows indicate the migration of open circular (oc), linear (lin), and supercoiled (sc) HPV16 genomes as well as a bacterially synthesized spike plasmid DNA that was added to the cells at the time of lysis to assess efficiency and reproducibility of recovery of low molecular weight DNAs using the Hirt extraction protocol.

deletion in codon 10 of the E5 ORF deleting the *Xcm*I restriction site. The resulting 28 amino acid gene product contains only the first 10 amino acids from E5. The E5^{XCM-} mutant is predicted to inhibit binding of the E5 protein to the 16-kDa subunit of the v-ATPase and E5-mediated EGFR activation because amino acids located 3′ of amino acid 10 have been shown to be important for these interactions. Mutagenesis of HPV16 E5 indicates that the hydrophobic amino acid sequence of amino acids 41 to 54 is critical for interaction with the 16-kDa subunit of the v-ATPase, and deletion of 5 amino acids at the carboxy terminus of the E5 protein results in a loss of E5-mediated EGFR activation (1, 38).

We transfected this E5^{XCM-} mutant HPV16 genome as well as the WT HPV16 genome into NIKS, an immortalized keratinocyte cell line that supports the papillomavirus life cycle (17). Following transfection and G418 selection, cell populations were expanded under growth conditions that maintain poorly differentiated, or basal-cell-like properties. It is this basal-cell-like state that supports the nonproductive stage of the viral life cycle. Low-molecular-weight (Hirt) DNAs from these populations were screened for the presence of HPV16 viral DNA. Figure 2 is a Southern blot of representative cell populations. Extrachromosomal viral DNA at 1 to 10 copies per cell was detected in each population of NIKS cells transfected with E5 mutant HPV16 genomes. A similar range in copy number was seen in cells transfected with WT HPV16 genomes. After serial passaging of WT and E5^{XCM-} mutant HPV16 genomes, we also determined by Southern analysis of total genomic DNA that the extrachromosomal viral DNA was maintained in the majority of populations of WT and E5 mutant transfected cells, although some populations did lose their viral DNA over time (data not shown). The loss of viral genomes from some populations of cells was observed with both WT and E5 mutant-transfected cells. These results demonstrate that E5 is not required for the establishment and maintenance of the viral genome as a nuclear plasmid in keratinocytes when the cells are cultured under conditions that maintain their poorly differentiated, basal-cell-like state, the cellular state that supports the nonproductive stage of the viral life cycle.

Cells harboring WT or E5 mutant HPV16 genomes display similar growth properties in the presence or absence of exogenous EGF. HPV16 E5 has been argued to activate the EGFR in a ligand-dependent manner (5, 28, 37). The immortalized keratinocyte cell line we used in this study, NIKS, responds normally to treatment with EGF as demonstrated by the ligand-dependent phosphorylation of the EGFR (Fig. 3A). To assess whether the presence or absence of functional E5 alters the growth properties of these cells in an EGF-dependent manner, we monitored the growth properties of multiple populations of NIKS cells harboring WT or E5 mutant HPV16 genomes in the absence or presence of exogenous EGF (10 ng/ml) in monolayer culture. In the absence of exogenous EGF we saw no differences in the growth properties of the cell populations harboring WT versus E5 mutant genomes. Addition of exogenous EGF to the culture medium led to more robust growth kinetics, but no differences were seen in the growth properties of cell populations harboring WT or E5 mutant HPV16 genomes (data not shown). Western analyses indicate that cell populations harboring WT or E5 mutant HPV16 genomes have similar levels of EGFR, with modest reductions in levels of receptor noted in those cultures grown in the presence of exogenous EGF (10 ng/ml) (Fig. 3B). These results indicate that the growth properties of keratinocytes in monolayer culture are not notably influenced by E5 in the presence or absence of exogenous EGF.

Loss of E5 does not result in gross morphological changes of organotypic raft cultures. To address the role of the HPV16 E5 protein in the productive stage of the viral life cycle, we cultured keratinocyte populations harboring WT or E5^{XCM-} mutant HPV16 genomes in organotypic raft cultures. Briefly, keratinocytes were plated onto a dermal equivalent made of collagen embedded with fibroblasts. After the keratinocytes reached confluence, the collagen raft was lifted to the liquid-air interface and the culture was maintained for an additional 11 days. During this time, the keratinocyte population continues to divide and stratify to form a three-dimensional architecture reminiscent of the epidermis. Raft cultures of NIKS harboring WT (Fig. 4A) or E5^{XCM-} mutant (Fig. 4B) extrachromosomal HPV16 DNA or untransfected NIKS (Fig. 4C) were grown; the resulting cultures were harvested, fixed, and embedded in paraffin; and 4-μm-thick sections were stained with hematoxylin and eosin. In the cases of all populations studied, terminal differentiation occurred as evidenced by the presence of squames in the most superficial layers of the cultures. No gross morphological differences were discerned between the rafts

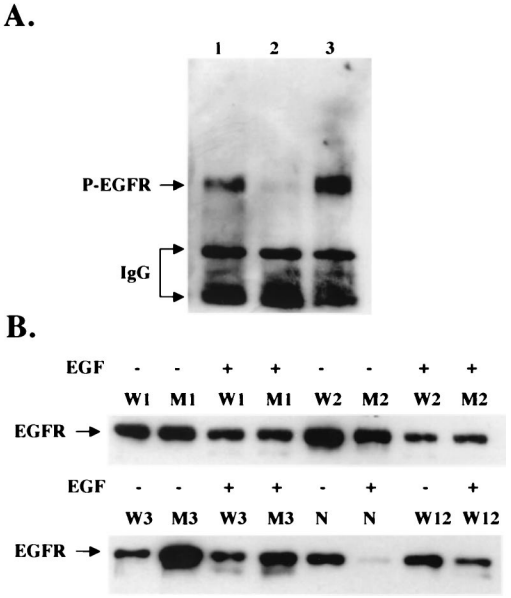

FIG. 3. Presence and phosphorylated state of EGFR in NIKS cells. (A) Levels of P-EFGR (as detected by immunoprecipitation and Western analysis; see Materials and Methods) in NIKS cells either grown on fibroblast feeders in normal F medium containing 5% FBS and EGF (10 ng/ml) (lane 1), serum-starved for 24 h in F medium containing 0.1% FBS (lane 2), or serum-starved in F medium containing 0.1% FBS for 24 h followed by the addition of EGF (10 ng/ml) for 15 min (lane 3). Note the induction of P-EGFR in serum-starved, EGF stimulated cells. Indicated by the arrows at left is the migration position of P-EGFR and immunoglobulin (IgG) from the immunoprecipitation using anti-EGFR antibody (Ab-15; Labvision). The blot was probed with an antiphosphotyrosine antibody (PY20; Santa Cruz). (B) Levels of total EGFR protein (as detected by direct Western analysis using anti-EGFR antibody) (Ab-15; Labvision) in untransfected NIKS (N); three independently derived, transfected populations of NIKS cells harboring the WT HPV16 genome (W1, W2, and W3); three independently derived, transfected populations of NIKS cells harboring the $E5^{XCM-}$ mutant HPV16 genome (M1, M2, and M3); and W12E cells (W12) grown in the absence (−) or presence (+) of EGF (10 ng/ml). Note the similar range in levels of EGFR in different populations of NIKS cells harboring the WT or $E5^{XCM-}$ mutant HPV16 genomes and the similar modest reduction in levels of EGFR in cells grown in the presence of EGF at 10 ng/ml.

generated with NIKS harboring WT or E5 mutant HPV16 genomes (Fig. 4A to C).

E5 does not contribute to the alteration of keratinocyte differentiation by HPV16 in organotypic raft cultures. Organotypic raft cultures of NIKS cells harboring HPV16 WT DNA extrachromosomally have altered differentiation programs compared with HPV16-negative NIKS raft cultures (17). The two major viral oncoproteins, E6 and E7, have been shown to contribute to this altered differentiation program in early passage human foreskin keratinocytes (31, 49), NIKS cells (16), and the mouse epidermis (42). To address the question of whether E5 also contributes to this alteration of differentiation, immunohistochemistry was performed on sections of raft cultures using antibodies for markers of keratinocyte differentiation, K10 and filaggrin. K10 is normally expressed in the spinous and granular layers of the epithelium. K10 was detected in the supraparabasal cells (those greater than two cells removed from the basement membrane) of raft cultures harboring $HPV16^{WT}$ and $HPV16^{E5XCM-}$ genomes (Fig. 4D and E), whereas it was found in all suprabasal cells (those greater than one cell removed from the basement membrane) of untransfected NIKS rafts (Fig. 4F). This result indicates that while HPV16 delays the onset of expression of K10 in raft cultures, consistent with prior studies (16), E5 is not essential for this perturbation. Filaggrin is normally expressed in the granular layer of stratified squamous epithelia. Filaggrin was detected in the superficial layers of the HPV16 WT (Fig. 4G) and E5 mutant (Fig. 4H) raft cultures as well as in the untransfected NIKS (Fig. 4I) raft cultures. However, large dysplastic cells that did not stain positive for filaggrin were found in the more superficial layers in both the HPV16 WT and E5 mutant-harboring rafts, but not in the untransfected NIKS rafts. These data indicate that, while HPV16 can perturb normal cellular differentiation, E5 does not contribute to this altered differentiation status in keratinocytes harboring HPV16.

Loss of E5 results in reduced DNA synthesis in the supraparabasal layers of raft cultures during the productive stage of the HPV16 life cycle. We and others have demonstrated that HPVs, and the HPV E7 gene alone, reprogram terminally differentiating cells to support cellular DNA synthesis (5, 16, 49). This property, referred to as unscheduled DNA synthesis (49), is thought to contribute to the ability of these terminally differentiating cells to support viral DNA amplification, which occurs during the productive stage of the viral life cycle selectively within this tissue compartment. To address whether HPV16 E5 contributes to unscheduled DNA synthesis in the terminally differentiating epithelial compartment, we monitored DNA synthesis in situ by incubating the raft cultures for a short period in media containing the nucleotide analog BrdU, and performing BrdU-specific immunohistochemistry on histology sections derived from these rafts (Fig. 5A to C). The percentages of BrdU-positive cells present in the terminally differentiating compartment were quantified (Fig. 5D).

As expected, cells supporting DNA synthesis in raft cultures of untransfected NIKS cells were restricted primarily to the basal compartment, with very few supraparabasal cells supporting DNA synthesis (Fig. 5C). In contrast, raft cultures of NIKS cells harboring WT HPV16 genomes efficiently supported unscheduled DNA synthesis in the supraparabasal compartment (Fig. 5A). Raft cultures of NIKS harboring $E5^{XCM-}$ mutant HPV16 genomes also supported unscheduled DNA synthesis in the supraparabasal compartment (Fig. 5B); however, there was a statistically significant ($P = 0.0197$) twofold reduction in the frequency of BrdU-positive cells in the supraparabasal compartment of these rafts compared to the rafts of NIKS harboring WT HPV16 genomes, indicating that there is a quantitative reduction in the frequency of unscheduled DNA synthesis in rafts harboring E5 mutant HPV16 DNA (Fig. 5D). These results indicate that E5 plays a quantitative role in the capacity of HPV16 to reprogram differentiated keratinocytes to support unscheduled cellular DNA synthesis during the productive stage of the viral life cycle.

Retention of viral DNA amplification in rafts of NIKS harboring E5 mutant HPV16 genomes. The ability of differentiating HPV16-positive keratinocytes to undergo unscheduled DNA synthesis correlates with their capacity to support viral DNA amplification. Keratinocytes harboring E7 mutant HPV16 fail to undergo unscheduled DNA synthesis in the

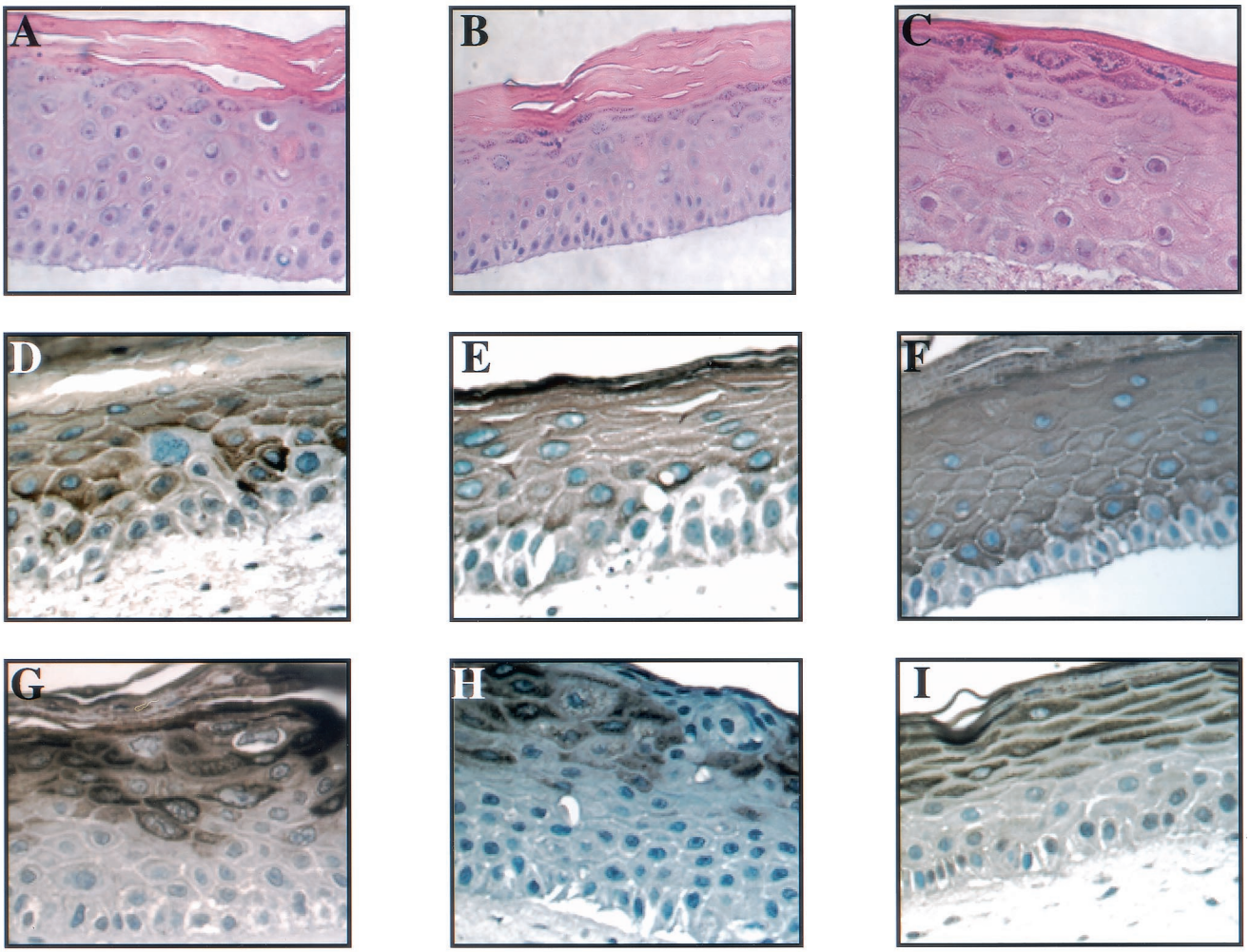

FIG. 4. Immunohistochemical analyses of the differentiation program in organotypic raft cultures. Shown are organotypic raft cultures of HPV16^{WT}-harboring NIKS cells (A, D, and G), HPV16^{E5XCM-}-harboring NIKS cells (B, E, and H), and untransfected NIKS cells (C, F, and I) that were maintained on a dermal equivalent of collagen embedded with fibroblasts. The cultures were lifted to the liquid-air interface after 4 days in culture and were harvested 11 days postlift. The rafts were fixed in 4% formalin, embedded in paraffin, and cut into 4-μm-thick serial sections. Cross sections from each sample stained with hematoxylin and eosin (A to C) reveal normal stratification of the keratinocyte cultures, with no gross morphological differences between the HPV16^{WT} (A)- and HPV16^{E5XCM-} (B)-harboring rafts. Immunohistochemical staining for terminal differentiation markers of the epithelium reveals that there is also no difference in the differentiation program between HPV16^{WT}- and HPV16^{E5XCM-}-harboring rafts. K10 was detected by immunohistochemical staining using an anti-K10 antibody (clone Ck 8.60). (D to F) Positive cells, staining brown, were localized to the suprabasal compartment of the epithelium, as expected. (G to I) Filaggrin was detected with an antifilaggrin antibody. Positive cells localized to the granular layer of the rafts, as expected.

differentiated compartment of rafts, and these cells also do not amplify viral DNA (16). To monitor viral DNA amplification in raft cultures of keratinocytes harboring WT or E5 mutant HPV16 genomes, we performed FISH using an HPV16-specific probe. FISH-positive nuclei (green), indicating amplified viral DNA, were found in both WT and E5 mutant HPV16-harboring rafts (Fig. 6A and B), but not in untransfected NIKS rafts (Fig. 6C). Although there was a twofold decrease in the average number of cells supporting viral DNA amplification in rafts harboring E5 mutant genomes, no significant differences ($P = 0.8551$) in the percentage of FISH-positive cells were observed between WT and E5 mutant-harboring rafts (Fig. 6D). These results demonstrate that, while the loss of the E5 protein does result in a quantitative reduction in unscheduled DNA synthesis, the differentiated cells retain the capacity to amplify viral DNA with no evident change in the frequency of cells supporting detectable amplification.

Loss of E5 does not inhibit expression of late viral proteins. Expression of the major capsid protein, L1, and the E1^E4 fusion protein are hallmarks of the productive stage of the HPV16 viral life cycle. Immunohistochemistry was performed using an antibody against HPV16 L1 (CamVir 1), and immunofluorescence was performed using an antibody to E1^E4 (TVG-402). The L1 capsid protein was detected in both WT and E5 mutant HPV16-harboring rafts (Fig. 7A and B) but not in NIKS cells (Fig. 7C). Similarly, the E1^E4 protein was detected in both WT (13.25 ± 12.42 E1^E4-positive cells per slide) and E5 mutant (25.33 ± 22.47 E1^E4-positive cells per slide) HPV16-harboring rafts (Fig. 7D and E) but not in NIKS cells (Fig. 7F) (mean ± standard deviation). No significant

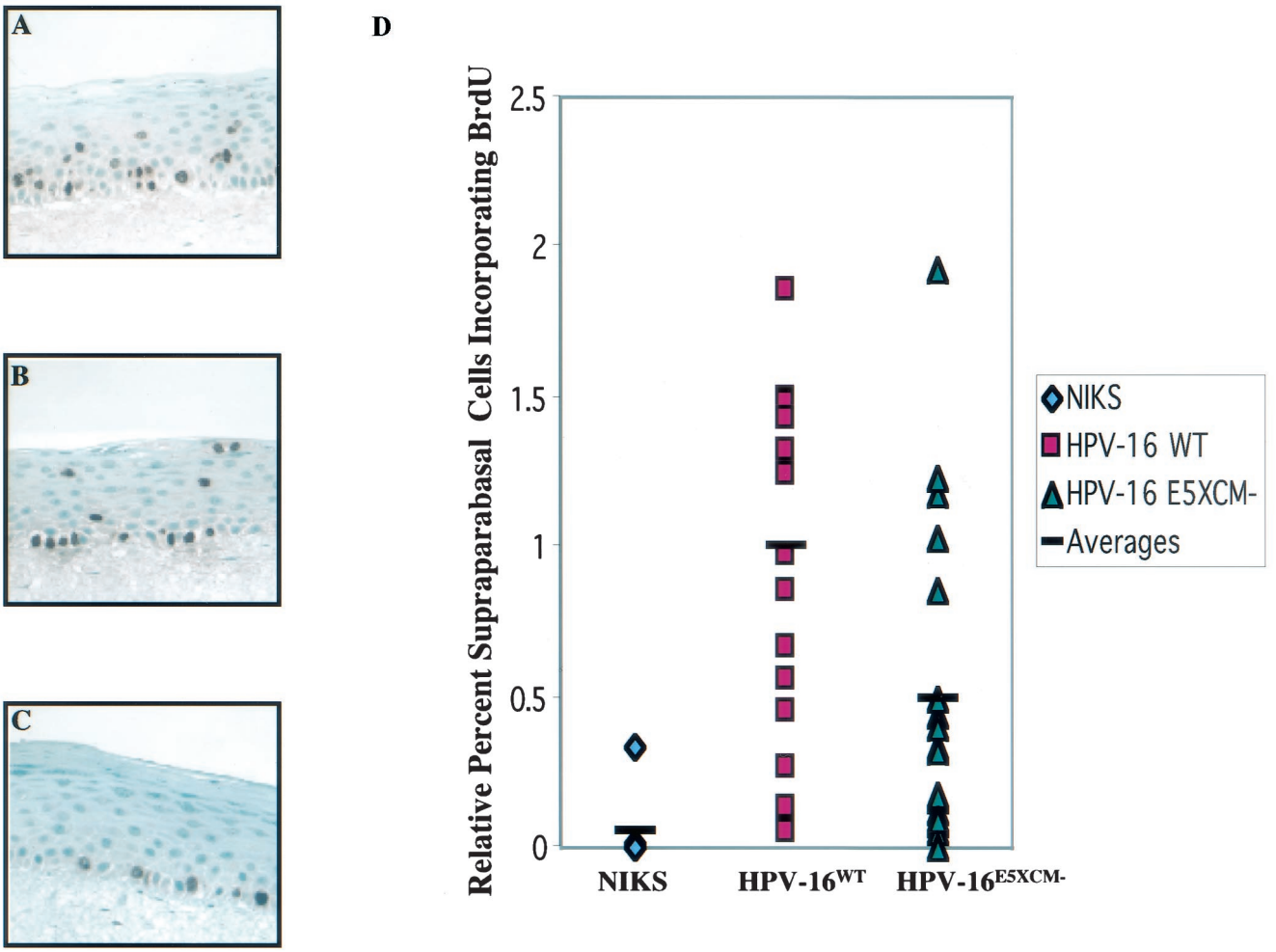

FIG. 5. Analysis of DNA synthesis in organotypic raft cultures. HPV16 reprograms terminally differentiating cells to undergo unscheduled DNA synthesis. Shown are organotypic raft cultures of HPV16^{WT}-harboring NIKS cells (A), HPV16 E5^{XCM-}-harboring NIKS cells (B), and untransfected NIKS cells (C). BrdU was added to culture media 8 h prior to harvest. BrdU incorporation was detected by immunohistochemical staining using an antibody to BrdU. Shown is BrdU-specific immunohistochemical staining (brown nuclei) with hematoxylin counterstain (blue nuclei). BrdU was detected only in the basal compartment of untransfected NIKS cells (C). In contrast, BrdU was detected in both the basal and suprabasal compartment of HPV16^{WT}- and HPV16^{E5XCM-}-harboring NIKS (A and B). However, the graph (D) demonstrates that there is a quantitative reduction (approximately twofold) in the percentage of BrdU-positive cells in the supraparabasal compartment of HPV16^{E5XCM-} mutant-harboring rafts compared with that of HPV16^{WT} rafts (D). To obtain the data graphed, BrdU-positive cells from 7 populations of untransfected NIKS cells, 8 populations of independently derived populations of NIKS cells harboring HPV16^{WT} genomes, and 10 independently derived populations of NIKS cells harboring HPV16 E5^{XCM-} genomes were counted from four independent experiments (10 fields per slide at a magnification of ×40) as described in Materials and Methods.

differences ($P = 0.3114$) in the levels of L1 or E1^E4 expression were discerned between WT and E5 mutant HPV16-harboring rafts. These data indicate that the E5 protein is not required for the expression of late viral gene products.

Loss of E5 does not affect the formation of virus-like particles (VLPs). Historically, electron microscopy has been used to demonstrate the presence of HPV16 viral particles in infected epithelium as well as in HPV16-harboring organotypic raft cultures (17, 32, 43). NIKS cells harboring WT and E5 mutant HPV16 as well as untransfected NIKS cells were subjected to this ultrastructural analysis to determine if particles of 55 nm in diameter, the size of the HPV16 viral particle, were present. Nuclei in both WT (Fig. 8A inset) and E5 mutant (Fig. 8B to D) HPV16 populations contained particles measuring ~55 nm in diameter. Particles of this diameter were not present in untransfected NIKS rafts (data not shown). These data indicate that the E5 protein is not required for the formation of VLPs.

DISCUSSION

Role of E5 during the HPV16 life cycle. In this study we have demonstrated that E5 acts during the productive stage of the HPV16 life cycle. We found that cells harboring E5 mutant HPV16 genomes have a quantitative reduction in the percentage of supraparabasal cells undergoing DNA synthesis compared with cells harboring WT HPV16 genomes (Fig. 5). Previously, it has been reported that E7 plays a critical role in the

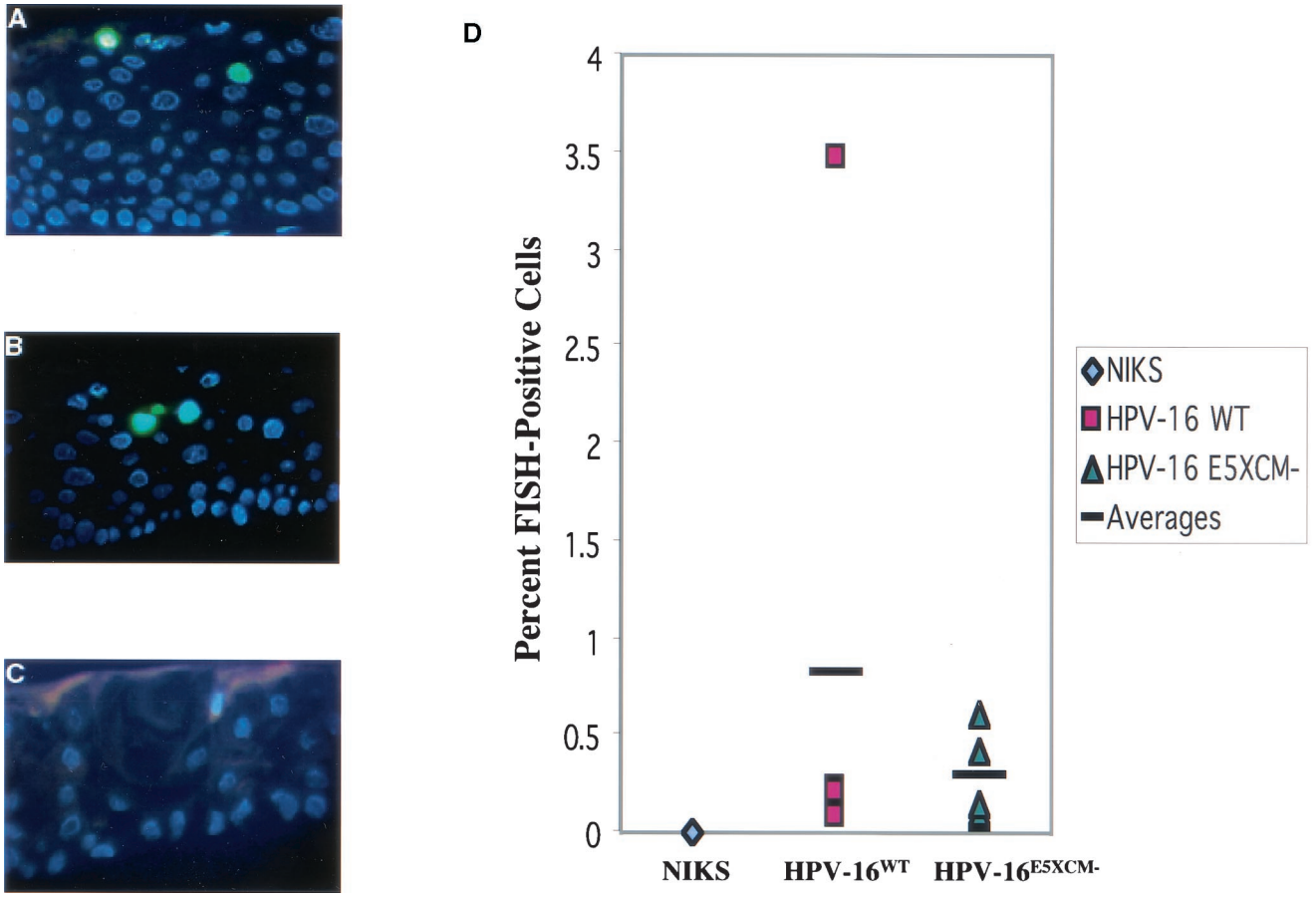

FIG. 6. FISH analysis on sections of organotypic raft cultures of NIKS cells harboring HPV 16 WT (A) and E5 mutant (B) genomes as well as untransfected (C) NIKS cells, using an HPV16-specific probe. The sections were counterstained with DAPI (blue nuclei). FISH-positive nuclei (green), indicating amplification of viral DNA, were found in the terminally differentiating compartment of rafts generated with NIKS cells harboring either $HPV16^{WT}$ (A) or $HPV16^{E5XCM-}$ genomes (B), but not in rafts generated with untransfected NIKS (C). (D) Histogram in which the frequency of cells harboring amplified copies of HPV16 were quantified in raft cultures subjected to HPV16-specific FISH analysis, as described in Materials and Methods. No significant difference in the frequency of cells with amplified copies of HPV16 genomes was evident between the cell populations harboring WT or E5 mutant viral genomes ($P = 0.8551$).

productive stage of the HPV16 life cycle (16). In that study, it was demonstrated that E7 mutant HPV16 genomes do not reprogram supraparabasal cells to support DNA synthesis, and this defect correlates with an absence of viral DNA amplification. That study also established that cells harboring E7 mutant HPV16 genomes failed to perturb the differentiation program of raft cultures of NIKS cells and displayed reduced expression of L1, the major viral capsid protein expressed in the productive stage of the HPV16 life cycle. In contrast, the E5 mutant HPV16 genomes did perturb the differentiation program of raft cultures of NIKS cells, as was seen in NIKS harboring the WT HPV16 genome (Fig. 4); expressed late viral proteins in the productive stage of the HPV16 life cycle at the same levels as seen in HPV16 WT harboring raft sections (Fig. 7); and retained viral DNA amplification as analyzed by FISH (Fig. 6). Thus, E5 plays a more subtle role during the productive stage of the viral life cycle than does E7.

A parallel study of E5 in the context of the HPV31 life cycle by Fehrmann et al. (15a) also has shown a subtle effect of E5 during the productive stage of the viral life cycle. In both studies, the disruption of E5 had no observable effect on the nonproductive stage of the viral life cycle. Specifically, both E5 mutant HPV16 and HPV31 genomes could be established and maintained as nuclear plasmids, and cells harboring these E5 mutant genomes displayed similar growth kinetics in the absence or presence of EGF in monolayer culture compared to cells harboring WT HPV genomes. In contrast, both studies noted defects during the productive stage of the viral life cycle. In accordance with our data demonstrating that cells harboring E5 mutant HPV16 genomes display a quantitative reduction in the percent of suprabasal cells undergoing DNA synthesis in raft culture (Fig. 5), the Laimins group found a reduced induction of cyclins A and B and reduced retention of proliferative potential in cells harboring E5 mutant HPV31 genomes compared to those harboring WT genomes, upon suspension of those cell populations in semisolid medium. In both studies cells harboring E5 mutant HPV genomes retained the ability to amplify viral DNA upon induction of cellular differentiation. The Laimins group discerned a twofold decrease in the degree of amplification in cells harboring E5 mutant HPV31 genomes compared to those harboring WT HPV31 genomes based upon quantitative Southern analysis of cells induced to differentiate

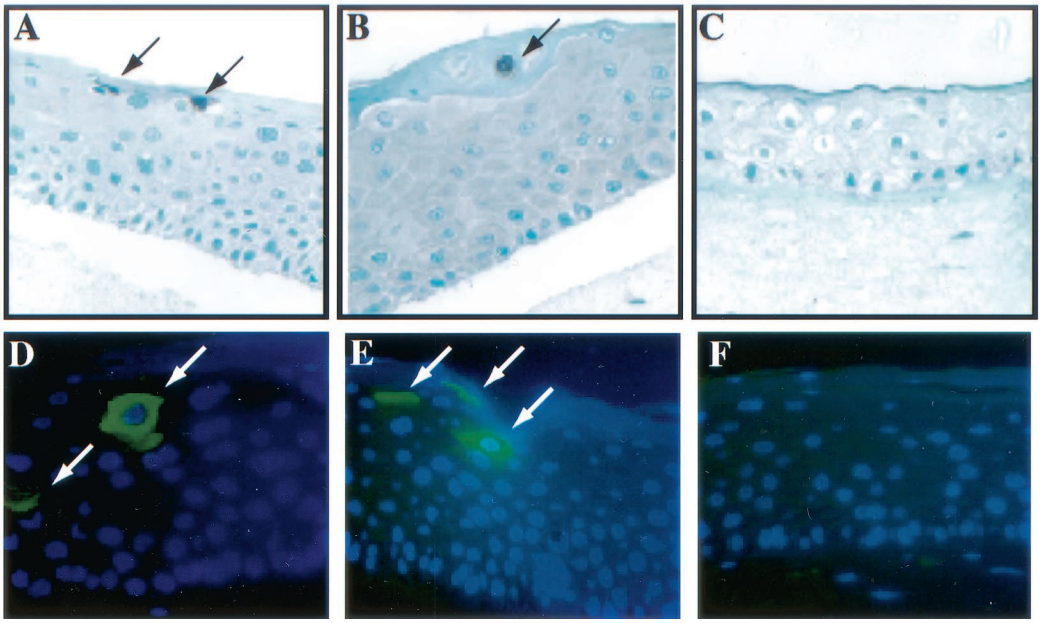

FIG. 7. Immunohistochemical and immunofluorescent staining for late viral gene products in organotypic raft cultures. Shown are histological sections of organotypic raft cultures generated with WT and E5 mutant-harboring NIKS cells, or untransfected NIKS cells. For L1 immunohistochemistry, cross sections from each sample were incubated with an anti-L1 antibody (CamVir 1) and detected using the Vectastain ABC Kit. L1-positive cells, staining brown, are indicated by arrows. The sections were counterstained with hematoxylin, which stains nuclei blue. L1 was detected in both the WT (A) and E5 mutant (B) HPV16-positive raft cultures, but not in the untransfected NIKS raft cultures (C). For E1^E4 immunofluorescence, cross sections from each sample were incubated with an anti-E1^E4 antibody (TVG-402) and detected using a fluorescent-conjugated secondary antibody (AlexaFluor 488; Molecular Probes). E1^E4-positive cells are indicated by arrows. The sections were counterstained with DAPI (blue nuclei). The E1^E4 protein also was detected in both the WT (D) and E5 mutant (E) HPV16-positive NIKS raft cultures but not in untransfected NIKS raft cultures (F).

in methylcellulose. We likewise observed a twofold decrease in the frequency of cells supporting amplification of the E5 mutant genome, based on FISH analysis of organotypic raft cultures; however, this difference was not statistically significant. We have performed Southern analysis of DNA extracted from HPV16 WT and HPV16 E5 mutant rafts as described by Ozbun and Meyers with HPV31 harboring rafts (34). We cannot detect viral DNA amplification in the HPV16 WT rafts or in rafts generated with a clone of cervical epithelial cells (W12E cells) that harbors the HPV16 DNA extrachromosomally. We believe that this absence of detectable amplification is due to the very low percentage of cells within a raft supporting viral DNA amplification (0.09 to 3.48%) as shown by our FISH analysis (Fig. 6D). Unfortunately, this precludes us from making a direct comparison with the Southern analysis performed by Fehrmann et al. on cells suspended in methylcellulose. In our studies we did not discern any difference in the frequency of cells supporting expression of two late proteins, L1 and E1^E4, when we analyzed by immunohistochemistry rafts from 8 independently derived populations harboring WT and 10 independently derived populations of E5 mutant HPV16 genomes. In contrast, a decrease in level of expression of E1^E4, as quantified by immunofluorescence of cells harboring WT or E5 mutant HPV31 genomes suspended in methylcellulose, was noted by the Laimins group. At this point it remains unclear whether the differences between the results obtained with HPV16 versus HPV31 reflect different degrees to which these two genotypes depend upon E5 function, the variation in behavior of individual cell populations harboring viral genomes, or a difference in the methods used to assess the productive stage of the viral life cycle (organotypic culture in the case of HPV16 studies reported herein versus suspension in methylcellulose in the case of the HPV31 studies reported by Fehrmann et al.).

In a separate study of E5 in the context of cottontail rabbit papillomavirus (CRPV), DNA was injected into the epithelium of domestic rabbits, an animal model for papillomavirus infection (6). While papillomas formed upon injection of E5 mutant CRPV genomes, there was a reduction in the frequency of papillomas formed compared to that seen with the WT CRPV genomes. It is not clear whether this reduction in frequency reflects the defects that have been discerned in our study or in the study by Fehrmann et al.

Studies have indicated that HPV16 E5 is able to cooperate with E7 to induce proliferation, enhance immortalization, and promote anchorage-independent growth of baby rat kidney cells (5, 48). In these studies, it was found that transfection of E5 alone into primary rodent cells had little effect on proliferation of these cells and that E7 alone was able to increase the levels of proliferation over control transfections, as expected. However, cotransfection of E5 and E7 resulted in a significant increase in the amount of proliferating colonies over that of E7 alone. Given our own observations that E5 contributes to the capacity of HPV16 to reprogram differentiating cells to support DNA synthesis, a property also reliant on E7 (16), we

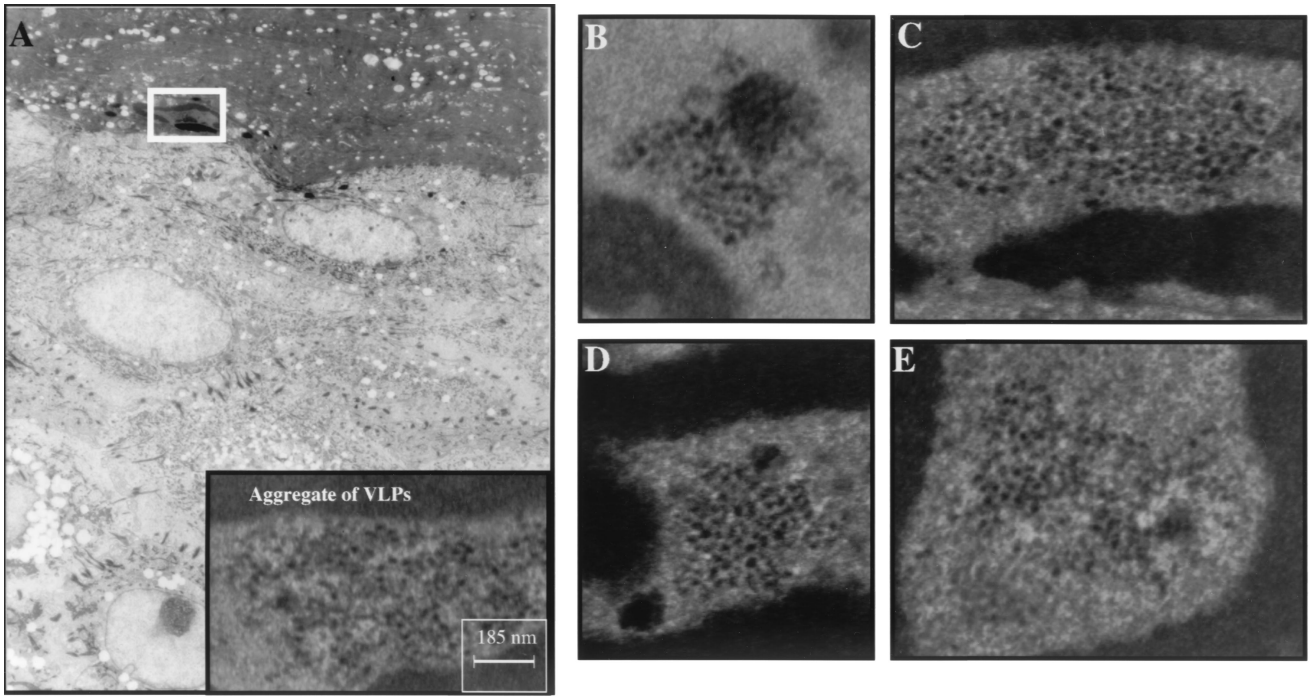

FIG. 8. Ultrastructural analysis of HPV16 WT and E5 mutant HPV16 rafts. Shown are electron micrographs of thin sections taken from rafts generated with NIKS cells harboring WT (A) or E5 mutant (B to E) HPV16 genomes. (A) Low magnification (×2,700) of an entire cross section of a raft generated with NIKS harboring WT HPV16 genomes. The white box in panel A indicates the region shown at high magnification (×54,000) in the inset. An aggregate of VLPs measuring ~55 nm were observed. A 1-cm ruler representing 185 nm is shown. Particles similar in size to these were also observed in multiple nuclei in E5 mutant HPV16-harboring rafts. (B to E) High magnification of examples of aggregates of VLPs measuring ~55 nm from different rafts of two independent populations of E5 mutant HPV16 harboring NIKS cells (magnification, ×54,000[B] or ×55,000 [C to E]).

hypothesize that E5 plays a cooperative role with E7 in the productive stage of the viral life cycle.

The mechanism by which HPV16 E5 is contributing to the productive stage of the viral life cycle is not yet clear. Many studies have suggested a link between the HPV16 E5 protein and the EGFR signaling pathway. These studies suggest that when treated with EGF, E5-expressing cells display anchorage-independent growth (37), increased mitogenic effects (5, 45), and increased growth factor receptor signaling (with or without EGF) (12, 20). Whereas the Laimins group found little, if any, EGFR present in keratinocytes following suspension in semi-solid medium, we (data not shown) and others (47) clearly find EGFR present in the superficial layers of raft cultures of early-passage human foreskin keratinocytes or NIKS, albeit at lower levels than those observed in the basal layers. A similar expression pattern of the EGFR in basal as well as suprabasal compartments of rafts was seen in the context of an HPV31-positive CIN 1 lesion-derived population, CIN 612 9E cells (30). For that study, the authors also monitored expression of HPV31 E5 in the context of raft cultures and found E5 protein levels were induced in a time-dependent manner, suggesting that its expression is tied to the differentiation and stratification of epithelial cells. Consistent with this observation, they detected E5-positive cells within the more superficial layers of their CIN 612 9E raft cultures. These data indicate that both E5 and one of its known targets, the EGFR, are expressed within the terminally differentiating cell compartment in which we have observed an effect of E5 during the productive stage of the viral life cycle.

The binding of HPV16 E5 to the 16-kDa component of the v-ATPase (11) may also be important in E5's contribution during the productive stage of the viral life cycle. It has been shown that endosomal acidification of HPV16 E5-expressing cells is inhibited and that this can lead to increased receptor recycling to the cell surface (44). Another study has suggested that HPV16 E5 affects trafficking from endocytic compartment rather than endosomal acidification (46). Studies under way will allow us to determine which of these mechanisms are involved in E5's contribution during the productive stage of the viral life cycle.

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