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Display of Complete Life Cycle of Human Papillomavirus Type 16 in Cultured Placental Trophoblasts

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Human papillomavirus (HPV) infection is threefold more prevalent in spontaneous abortion specimens compared to elective abortions, preferentially targeting the placental trophoblasts in these specimens. Here, by using infectious center and Southern blot analysis, we demonstrate that the transfected HPV-16 genome *de novo* replicates in 3A trophoblasts in culture. Peak DNA replication occurred 9–24 days posttransfection, showing classic DNA forms I, II, and III and an 8-kb monomer band upon *Dpn*I/*Bam*HI digestion. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA expression revealed that E6 and E2 were significantly expressed by day 9, coinciding with HPV-16 DNA replication. However, significant L1 expression was delayed until day 18. L1 protein expression on day 18, but not day 9, was also confirmed by Western blot analysis. The production of HPV-16 virions was demonstrated by three techniques: the appearance of HPV-16 infectious units coinciding with L1 expression, the neutralization of these infectious units with known neutralizing anti-HPV-16 antibodies, and the appearance of spliced E1^E4 and E6^E7 transcripts (RT-PCR) in normal keratinocyte rafts infected with these trophoblast-produced HPV-16 infectious units. These data suggest that HPV-16 is carrying out its complete life cycle in trophoblasts. Previously, HPVs were known to productively replicate only in differentiating keratinocytes of skin. These findings expand HPV biology, support the hypothesis of a possible link between HPV and some spontaneous abortions, and present a new technology for studying HPV.

INTRODUCTION

There are a number of studies that suggest that human papillomavirus (HPV) infection may be linked with spontaneous abortions or commonly infect placental material (Armbruster-Moraes *et al.,* 1994; Favre *et al.,* 1998; Hermonat *et al.,* 1997, 1998; Malhomme *et al.,* 1997; Manavi *et al.,* 1992; Pao *et al.,* 1995; Rabreau and Saurel, 1997; Sikstrom *et al.,* 1995). For example, in earlier studies our laboratory found that HPV infection is threefold more prevalent in spontaneous aborted products of conception compared to elective abortions (60% vs 20%) and that the HPVs were preferentially infecting the trophoblasts within the placenta (Hermonat *et al.,* 1997, 1998). Malhomme *et al.* (1997) have also found that 70% of spontaneous abortion specimens contain HPV by polymerase chain reaction (PCR) analysis. In addition, Sikstrom *et al.* (1995) found that women with a history of HPV infection had a higher risk of spontaneous abortion (odds ratio of 3). Pao *et al.* (1995) found that HPV-18 was present in 50% of choriocarcinomas (malignant trophoblasts) and in some placentas. Also related to this issue, HPV DNA has been detected by PCR amplification of DNA from cells taken from amniotic fluids of pregnant women with evidence of HPV genital (60% HPV positive) or cutaneous infection, indicating that HPV has the capacity to cross the placental barrier when present in the cervix (Armbruster-Moraes *et al.,* 1994; Favre *et al.,* 1998). However, others do not see HPV in spontaneous abortion materials or in normal placentas (Genest *et al.,* 1999; Eppel *et al.,* 2000).

Trophoblasts are the cells of the placenta that are in direct contact with the maternal tissues. These cells are critical for anchoring the placenta to the maternal tissues. Furthermore, all nutrient exchange and waste exchange occur through the trophoblasts. Thus, the disruption of the trophoblastic layer, by HPV or any other infectious or chemical agent, could likely result in abnormal plantation or expulsion of the gestation (Clark *et al.,* 1993).

RESULTS

Long-term HPV-16 DNA replication in 3A trophoblasts

The finding that HPVs are present in trophoblasts prompted the analysis of HPV type 16 (a common genital HPV) biological activity in this newly identified target cell type. We chose to study HPV-16 biological activity in the 3A trophoblast cell line, which has been altered to contain a temperature-sensitive SV40 large T antigen. These

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cells have a normal, nontransformed phenotype at 39.5–40°C, are mortal, and have normal trophoblast traits [e.g., synthesize human chorionic gondotropin (HCG) and alkaline phosphatase] (Chou, 1978). For all of our experiments the 3A cells were maintained at 39.5–40°C. Under these conditions we found that our 3A cells secreted HCG by enzyme-linked immunosorbant assay (ELISA) (data not shown). Our 3A cultures were able to actively divide at 39.5–40°C and were heterogeneous in nature, containing both cytotrophoblasts (small precursor cells) and syncytiotrophoblasts (large, mature, multinucleated cells). We first observed whether HPV-16 might replicate in these cells using the infectious center assay, which is intended to identify individual cells with high DNA copy numbers. One million 3A cells were transfected with HPV-16 genomic DNA and at various times posttransfection, 10^4 cells were applied to a nylon membrane, lysed, and probed for HPV-16 DNA. The results (Fig. 1A) show that the HPV-positive signal and copy number in the 3A cells increase to day 15, become slightly reduced at day 21, and decline thereafter. As can be seen, there appears to be a number of high signal spots at day 15. CaSki cells (1×10^3), used as positive controls, contain about 600 copies of HPV-16 (Baker *et al.,* 1987) and the strong spots seen are likely due to cell clumping. The appearance of a limited number of strong individual spots suggests that high-level HPV-16 replication is limited to a only a subset of the 3A cells.

The HPV-16 replication levels were then analyzed in another, more exhaustive, time course experiment by Southern blot, shown in Fig. 1B. Again, 1 μg of HPV-16 reconstituted genomic DNA was transfected into a series of identical 3A cultures. The cells were fed every 3 days and total cellular DNA was harvested at the indicated times. The DNA was digested with *Dpn*I, to remove input bacterial DNA, and *Bam*HI, to drive all of the HPV-16 DNA into one size. The DNA was then size-separated, Southern blotted, and probed for HPV-16 DNA. As seen, a significant 8-kb band is visible in many of the lanes, suggesting that the HPV-16 DNA is likely to be present episomally. The DNA levels rise (replication) significantly on day 9, increase further on day 12, plateau until day 24, and then steadily decline afterward. In the early stages, significant DNA levels are not seen until day 9 and increase to the maximum at day 12. It is interesting to note that these are roughly the same time kinetics for HPV-16 replication in the organotypic epithelial raft culture system, the only other tissue culture system known to be fully productive for HPV (Meyers, 1996). There was no significant observable change in the morphology of the cells after transfection or during the complete course of the experiment. The presence of a strong 8-kb band in these specimens is consistent with episomal HPV-16 genomes or, less likely, head-to-tail integrated concatemers. To more clearly indicate that the majority of the DNA was episomal, we analyzed the

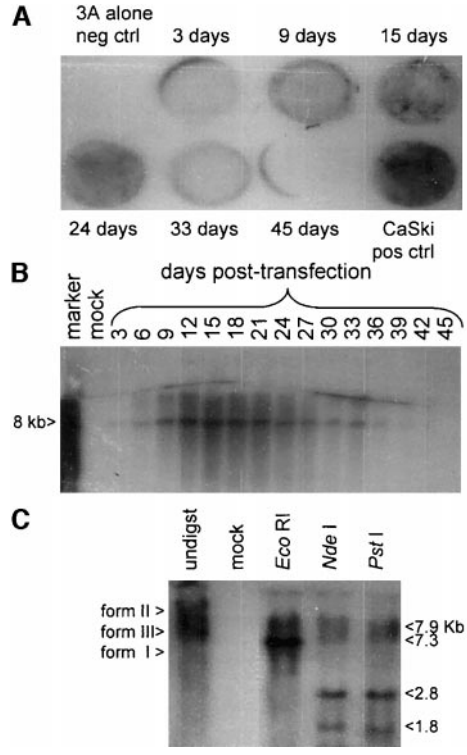

FIG. 1. HPV-16 DNA replication in 3A trophoblasts. (A) Analysis by infectious center assay. 3A trophoblasts, grown at 39.5°C, were transfected with 1 μg of recircularized HPV-16 genomic DNA at day 0. At the indicated times, cells were applied to membranes and analyzed by hybridization for HPV-16 sequences. Note positive signals at days 9–24. (B) Analysis by Southern blot assay. Cells were treated as above except that 20 μg of the total cellular DNA was analyzed by Southern blot for HPV-16 sequences. Note high positive signals at days 9–24. The marker lane was 0.5 ng of pHPV-16 plasmid cut with *Bam*HI. (C) Another analysis by Southern blot assay. This experiment was similar to B, with harvesting at day 14 and digestion with the indicated restriction enzymes. Note that DNA forms I, II, and III are seen in the undigested lane and appropriate sized fragments are seen in the *Eco*RI, *Nde*I, and *Pst*I digested lanes. *Eco*RI digestion results in fragments of 7268 and 635 bases. *Nde*I digestion results in fragments of 2847, 1825, 1357, 1225, 635, and 15 bases. *Pst*I digestion results in fragments of 2817, 1776, 1549, 1063, 483, and 216 bases.

HPV-16 DNA without digestion and with multiple zero-cut restriction enzymes. Finally, the basic structure of the replicated HPV-16 genome was analyzed with multiple-cut restriction enzymes. As shown in Fig. 1C, the appearance of forms I, II, and III in undigested DNA and the appropriate sized bands in the digested lanes indicates that the viral genome is episomal and that no large deletions or rearrangements were present.

Early and late HPV-16 gene expression in 3A trophoblasts

Given that HPV-16 was replicating its DNA in the 3A trophoblasts, next, mRNA expression was analyzed by reverse transcription-PCR (RT-PCR) using DNase I-treated, poly(A) selected RNA as a template. E6, E2, and L1 targeting primer sets were used to observe the po-

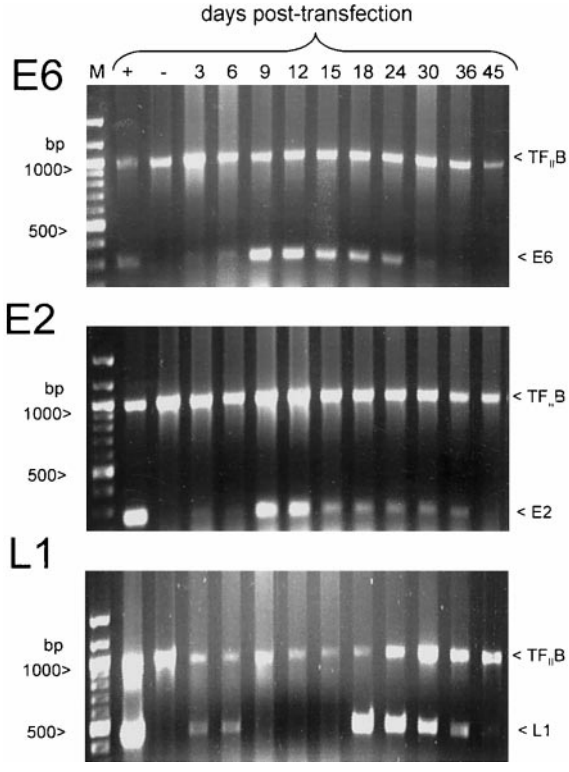

FIG. 2. HPV-16 RNA expression in 3A trophoblasts. (E6) E6 mRNA expression. E6 mRNA [poly(A)selected] was analyzed by RT-PCR as described under Materials and Methods. Note that the expression of TFIIB is relatively stable over the time course while significant E6 RNA expression starts at day 9 and then declines thereafter. "+" refers to the PCR products generated from both the $TF_{II}B$ and E6 primer sets using the $TF_{II}B$ and pAT-HPV-16 cloned DNAs as templates. "−" refers to the RT-PCR products generated from both the $TF_{II}B$ and E6 primer sets using mRNA from untransfected 3A cells as a template. (E2) E2 mRNA expression. E2 mRNA was analyzed by RT-PCR as described under Materials and Methods. Note that E2 expression is like that of E6, starting at day 9 and then declining. (L1) L1 mRNA expression. L1 mRNA was analyzed by RT-PCR as described under Materials and Methods. Note that L1 mRNA expression is different than that of E6 or E2, starting at day 18 and then declining. The small blip of early L1 expression was reproducible but much lower than expression at later times. The mRNA samples were also analyzed by direct PCR and failed to generate products (data not shown).

tential of "early" and "late" gene expression. Also included was a primer set for $TF_{II}B$ as a constitutive cellular control. After RT-PCR amplification, the products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The results for E6 oncoprotein mRNA expression, shown in Fig. 2, E6, demonstrate that significant E6 expression does not take place until day 9 and then slowly declines. The expression of E2 transcription factor mRNA was similar (Fig. 2, E2) and follows that of the HPV-16 DNA replication. Next, the expression of L1 capsid mRNA was analyzed, as shown in Fig. 2, L1. As can be seen, the expression of significant L1 mRNA levels does not take place until day 18, 9 days later than E2 and E6. Thus, this late gene of HPV in skin is expressed late relative to the early genes in trophoblasts. The early low-level expression of L1 on days 3 and 6 was a reproducible event. As L1 mRNA was expressed at day 18, we further analyzed for L1 protein expression by Western blot analysis. Again, 3A cells were transfected with HPV-16 DNA and harvested at days 9 and 18. Total proteins were isolated, Western blotted, and probed with anti-HPV-16 L1 monoclonal antibody. The results are shown in Fig. 3 and demonstrate that L1 protein is expressed at day 18, but not at day 9, consistent with the mRNA findings.

Production of HPV-16 infectious units in 3A trophoblasts

The strong mRNA and protein expression of L1 on day 18 suggested the possibility that HPV-16 progeny virions were being produced at this time. Production of the L1 protein leads to self-assembly of pseudo-particles even in the absence of DNA (Kirnbauer *et al.,* 1992; Muller *et al.,* 1997). To analyze whether HPV-16 virions were being produced, we generated putative virus stocks from HPV-16-transfected trophoblast cultures at day 18 (L1 expression) and day 9 (no L1). The trophoblasts were lysed to generate a putative virus stock, which was then used to infect normal primary keratinocytes. The keratinocytes were then seeded onto collagen rafts containing J2 fibroblasts and then raised to the air to stimulate the formation of a stratified squamous epithelium that would be favorable for HPV replication (the organotypic epithelial raft culture system) (Meyers, 1996). The experiment is schematically described in Fig. 4A. In this experiment any HPV-16 virions produced in the trophoblasts would infect the keratinocytes and be amplified in the raft "skin" by day 12. At day 12 total raft DNA was isolated and analyzed for HPV-16 sequences by Southern blot. The results, shown in Fig. 4B, give no evidence of HPV-16 infection and replication resulting from the day 9 lysate (RILT, rafts infected with lysed trophoblasts), while the rafts infected with the day 18 lysates show a strong 8-kb

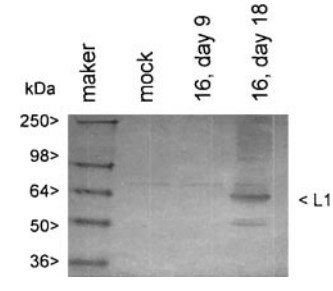

FIG. 3. Production of L1 capsid protein by HPV-16 in trophoblasts. Cells were treated as in Fig. 2 except that total proteins were isolated at days 9 and 18 and analyzed for L1 protein. Western blot, using anti-HPV-16 L1 monoclonal antibody and peroxidase-labeled secondary antibody. Note that no L1 protein was expressed in untransfected cells or transfected cells harvested at day 9. In contrast, the cells transfected and harvested at day 18 show a strong band at the size consistent with L1 protein.

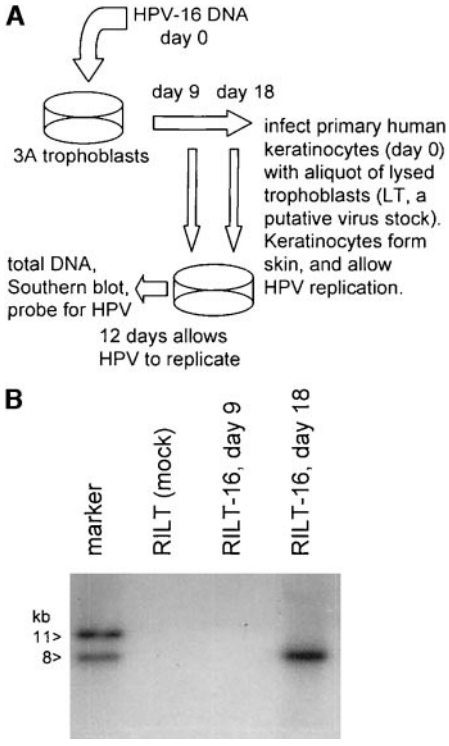

FIG. 4. Production of HPV-16 virions in the trophoblasts. The generation of HPV-16 virions was measured by the ability of trophoblast lysates to infect normal keratinocytes, which were then placed into an organotypic raft culture system to generate a stratified squamous epithelium product for HPV. (A) The experimental scheme for showing infectious virus production is diagramatically presented. (B) As in Figs. 1 and 2, 3A trophoblasts were mock or HPV-16 DNA lipofected, and at the indicated times a putative virus stock was generated by freeze–thawing the cells. The virus stocks were used to infect normal primary keratinocytes, which were then introduced in the organotypic epithelial raft culture system. At 12 days postinfection, total DNA was isolated from the rafts and analyzed by Southern blot for HPV-16 sequences. Note that the mock and day 9 lysates did not produce any evidence of HPV-16 infection, while the day 18 lysate showed a significant 8-kb HPV-16-specific band.

band upon *Bam*HI digestion. These data are consistent with HPV-16 virion production on day 18.

To further support the notion that HPV-16 generated infectious virions in trophoblasts, an antibody neutralization experiment was undertaken using a series of well-characterized antibodies. The HPV-16 virus stocks/lysed trophoblasts were mixed with H16.V5 (HPV-16 neutralizing antibody), H16.J4 (HPV-16 nonneutralizing antibody), HPV-18.J4 (HPV-18 neutralizing antibody), and H11.H3 (HPV-11 neutralizing antibody) (Roden *et al.,* 1997; Christensen *et al.,* 1996). Finally, H16.V5 was also treated with 0.2 M β-mercaptoethanol (β-ME) before it was also mixed with the HPV-16 virus stock. The infections were 4-h incubations, after which the keratinocytes were transferred on top of the collagen matrixes and the experiment was allowed to proceed as in Fig. 4. The results (Fig. 5) show that only the antibody H16.V5, known to neutralize HPV-16, had a neutralizing effect on the HPV-16 virus stock/trophoblast lysate, while all other

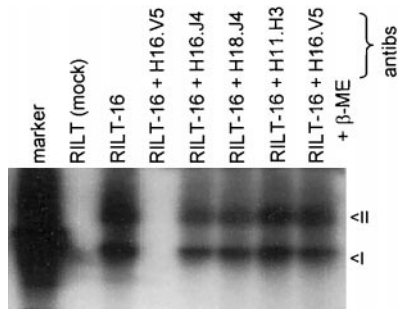

FIG. 5. Specific antibody neutralization of HPV-16 virions produced in trophoblasts. This experiment was similar to Fig. 4, except that the HPV-16 virus stocks were pretreated with the indicated antibodies before the keratinocytes were infected. In addition, H16.V5 was also treated with β-ME. The marker lane was 0.5 ng of pHPV-16 plasmid cut with *Bam*HI. Note that only untreated H16.V5, known to neutralize HPV-16, was able to prevent the infection of the raft tissue.

antibodies did not. Furthermore, treatment of H16.V5 with β-ME inhibited this neutralization.

To further indicate that HPV-16 generated infectious virions in trophoblasts, an analysis of E6^E7 and E1^E4 spliced transcripts in the initial HPV-16 lipofected 3A cells and in secondary RILT was undertaken. The results are shown in Fig. 6. Both transcripts were present in both the initial 3A culture and the secondary RILT cells. However, observation of the E6^E7 transcript in RILT required

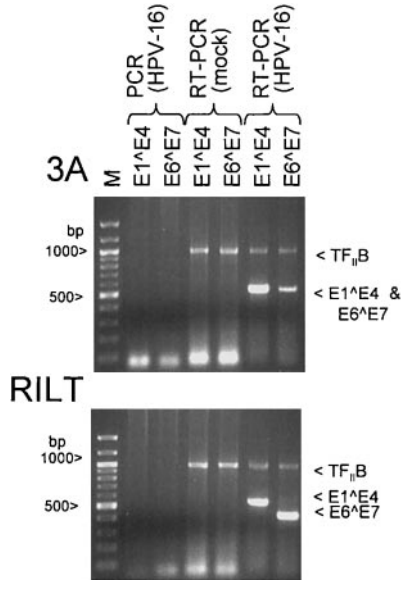

FIG. 6. HPV-16 E6^E7 and E1^E4 spliced transcripts are present in RILT-16 as analyzed by RT-PCR. Details are presented under Materials and Methods. Briefly, 3A trophoblasts were transfected with HPV-16 DNA as in Fig. 1. At day 21 some initial 3A cells were used for poly(A) RNA isolation, while others were lysed and used to generate a virus stock as in Fig. 4. Poly(A) RNA was also isolated from day 12 keratinocyte rafts infected with trophoblast lysates (RILT) and analyzed by RT-PCR. Mock-lipofected 3A cells and mock-infected RILT were included as controls. Note that the E6^E7 and E1^E4 transcripts were present in both the original 3A and secondary RILT. The E6^E7 transcripts in the RILT required nested PCR to be observed, resulting in the smaller size.

a second, nested PCR amplification. These data provide a third set of evidence, in addition to the temporal appearance of infectious units (Fig. 4) and the neutralization of infectious units with specific antibodies (Fig. 5), all consistent with the production of infectious HPV-16 virions in trophoblasts.

DISCUSSION

This study demonstrates that HPV-16 is fully active in trophoblasts. HPVs are already well known to be pathogenic viruses and are the largest risk factor in the development of cervical cancer (Melbye and Frisch, 1998). Thus, 3A trophoblasts may represent a new host cell type, in addition to the well-studied replication of HPVs in differentiating keratinocytes. These data also support the hypothesis that HPV infection of trophoblasts may be linked to some spontaneous abortions. The search for factors that may be implicated in spontaneous abortion has led to the examination of numerous factors, including maternal age, environmental exposures, tobacco use, and nutritional factors (Barrington *et al.,* 1996; Cordier *et al.,* 1991; Coste *et al.,* 1991; Dominguez *et al.,* 1991; Kline *et al.,* 1995; Neuqebauer *et al.,* 1996; Parazzini *et al.,* 1991; Risch *et al.,* 1988; Stucker *et al.,* 1990; Tan *et al.,* 1995). However, the studies exploring these relationships have been inconsistent in their findings. For every study supporting an association with spontaneous abortions, there is another study that does not support a relationship. The only uncontested factor, thus far, is maternal age. However, two studies place HPVs in 60–70% of spontaneous abortions (Hermonat *et al.,* 1997; Malhomme *et al.,* 1997). Furthermore, preliminary data suggest that HPV-11 and HPV-18 are also able to replicate in 3A trophoblasts (You, Liu, and Hermonat, unpublished results). If these miscarriages are, in fact, due to HPV infection, then the number of resulting human gestational fatalities may be large, possibly numbering in the millions in the United States per year. If the link between HPV infection and gestational loss can be further substantiated, then specific treatment protocols might be developed.

Replication levels of HPV-16 peak at days 15–27 and then start to decline. What is responsible for this decline is unclear. Possibly this is due to the death of the cells that are productive for HPV, but further experiments are needed to determine whether this is the correct interpretation. Finally, these data represent a technological advance in the ease of studying HPV over the tedious and expensive organotypic raft culture system.

MATERIALS AND METHODS

Infectious center and Southern blot analyses of replication

The infectious center technique was carried out in a manner similar to that described previously (Schlehofer *et al.,* 1983). Briefly, 3A trophoblasts (ATCC CRL1583) were grown at 39.5–40°C in Dulbecco's modified Eagle's medium with 7% fetal bovine serum. Reconstituted HPV-16 genomic DNA was generated by cutting the HPV-16 sequences from the cloned HPV-16 plasmid (pAT-HPV-16) (Bubb *et al.,* 1988) with *Bam*HI, followed by ligation and recircularization. For infectious center analysis, 1×10^6 3A cells were transfected with 1 μg of HPV-16 genomic DNA using FuGENE 6 (Roche Diagnostics Corp.). At the indicated times postlipofection the 3A cells were trypsinized and 1×10^4 cells were applied to and lysed on nylon membranes under continuous suction. The membranes were then probed with ^{32}P-labeled HPV-16 DNA. For Southern blot analysis of HPV-16 infection, 1×10^6 3A cells were transfected with 1 μg HPV-16 genomic DNA and harvested at the indicated times (days 3–45). Twenty micrograms of the total cellular DNA from each time was digested by both *Dpn*I and *Bam*HI, agarose gel electrophoresed, Southern blotted, and probed with ^{32}P-labeled HPV-16 DNA.

mRNA isolation and RT-PCR analysis of unspliced and spliced transcripts

HPV-16 mRNA expression was measured by RT-PCR along with a cellular mRNA control. 3A cells were lipofected at day 0 and total RNA was isolated at the indicated days using Trizol reagent (Gibco BRL Life Technologies Inc.), according to the manufacturer's protocol, and treated with 5 U/μg of RNase-free DNase I (Promega Co.) at 37°C for 2 h. Messenger RNA was then separated using the Oligotex mRNA Mini Kit (Qiagen Inc.) according to the supplier's instructions. The first-strand cDNA synthesis was performed at 37°C for 1 h in a final volume of 25 μl reaction buffer [1 μg mRNA; 50 mM Tris–HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 10 mM DTT; 0.5 μg oligo$(dT)_{15}$ (Promega Co.); 0.5 mM each of the four dNTPs; 30 U of RNasin (Promega Co.); and 200 U of M-MLV Reverse Transcriptase RNase H Minus (Promega Co.)]. PCR amplification of the cDNA was performed in a 100-μl reaction volume that contained 2.5 U *Taq* DNA polymerase (Fisher Scientific Co.); 10 mM Tris–HCl, pH 8.3; 50 mM KCl; 2 mM $MgCl_2$; 0.2 mM each of the four dNTPs; a 1 μM concentration of each upstream and downstream primer specific for the cDNA template; and 10 μl cDNA template. The E6 primer set used was 5′-ACCACAGTTATGCACAGAGC-3′ and 5-AGGACACAGTGGCTTTTGAC-3′, which targeted amplification of the HPV-16 sequences from nt 139 to 420. The E2 primer set used was 5′-AACACAGACGACTATCCAGC-3′ and 5′-ATGCCATGTAGACGACACTG-3′, which targeted amplification from nt 3454 to 3696. The L1 primer set used was 5-GCACAGGGCCACAATAATGG-3′ and 5′-CGTCCTAAAGGAAACTGATC-3′, which targeted amplified from nt 6583 to 7033. The expected product sizes for E6, E2, and L1 RT-PCR were 282, 243, and 451 bp, respectively. A

control RT-PCR analysis of expression of the housekeeping gene TFIIB (Ha *et al.,* 1991) was also undertaken with the primer set 5′-GTGAAGATGGCGTCTACCAG-3′ and 5′-GCCTCAATTTATAGCTGTGG-3′, which amplified nt 356–1314 of that mRNA. Several controls were also undertaken. First, a direct PCR was undertaken (data not shown) to observe any contaminating DNA. Second, a negative-control RT-PCR was undertaken using mRNA isolated from untransfected 3A cells. Third, a positive-control PCR was undertaken to observe correct product sizes using DNA as a template. The products were then analyzed on an agarose gel, stained with ethidium bromide, and visualized by ultraviolet light.

To measure the HPV-16 spliced mRNA, RT-PCRs were performed on poly(A) RNA isolated from 3A cells and normal keratinocyte rafts infected with trophoblast lysates using three additional HPV-16 primers sets. One, previously described by White *et al.* (1998), was designed to observe E1^E4 transcripts. Two, previously described by Sotlar *et al.* (1998), were nested and designed to observe E6^E7 transcripts. The E1^E4 primer set used was 5′-TGGAGAACCTGTTAATGGGCACAC-3′ (located at bases 797 to 818 in the HPV-16 genomic sequence) and 5′-TATAGACATAAATCCAGTAGACAC-3′ (located at bases 3826 to 3849 in the HPV-16 genomic sequence). Two of the E6^E7 primer sets used were as follows: A, 5′-ACAGTTATGCACAGAGCTGC-3′ (located at bases 142 to 161 in the HPV-16 genomic sequence) and 5′-CTCC TC CTCTGAGCTGTCAT-3′ (located at bases 647 to 666 in the HPV-16 genomic sequence); and B, 5′-GTGTGTACT-GCAAGCAACAG-3′ (located at bases 192 to 211 in the HPV-16 genomic sequence) and 5′-GCAATGTAGGTG-TATCTCC-3′ (located at bases 568 to 586 in the HPV-16 genomic sequence). The expected lengths of the E1^E4, E6^E7A, and E6^E7B RT-PCR products are 510, 525, and 395 bp, respectively. To analyze spliced transcripts in 3A cells RT-PCRs were carried out with the E1^E4 primer set and E6^E7 primer set A. To analyze spliced transcripts in lysate infected rafts, E1^E4 was analyzed as in the 3A cells. However, observation of E6^E7 transcripts required nested PCR using sequentially set A and then set B. To ensure that DNA was not contributing to the results, a direct PCR was also undertaken.

Western blot analysis

Expression of L1 capsid protein by HPV-16 was measured by Western blot. 3A cells were lipofected with HPV-16 as before, except that total proteins were isolated at days 9 and 18 and analyzed for L1 protein. Western blot was done according to routine protocol. After the electrophoresis and transfer, the nitrocellulose membrane (Amersham Life Science Co.) was treated with blocking buffer (The Protein Detector Western Blot Kit, Kirkegaard & Perry Laboratories, Inc.) for 1 h. Purified mouse anti-human HPV-16 L1 monoclonal antibody (mouse IgG2a, PharMingen International) was added to the buffer (1.5 mg/ml). The membrane was incubated at 4°C overnight and then washed with washing buffer (The Protein Detector Western Blot Kit) five times. The membrane was put into 1× blocking buffer containing peroxidase-labeled secondary antibody [goat anti-mouse IgG (H + L), 1:1000, the Protein Detector Western Blot Kit] and incubated at room temperature for 1 h. After the membrane was washed six times, TMB substrate (The Protein Detector Western Blot Kit) was applied. The reaction was stopped when the suitable color intensity was observed.

Infectious virion and antibody neutralization analysis

The generation of infectious HPV-16 virions in trophoblasts was measured by the ability of trophoblast lysates to infect normal keratinocytes (Clonetics Inc.), which were then placed into an organotypic raft culture system to generate a stratified squamous epithelium product for HPV. "RILT-16" is an abbreviation for "rafts infected with lysed trophoblasts that had been transfected with HPV-16." 3A trophoblasts were mock or HPV-16 lipofected (1 μg), and then at the indicated times the cells were freeze–thawed three times to lyse the cells, and the lysate was filtered (0.22-μm filter) and treated with 100 units/ml DNase for 1 h. The generated putative virus stocks were then used to infect 5×10^6 primary keratinocytes (Clonetics, Inc.), for 4 h. These cells were then trypsinized and seeded onto collagen rafts containing J2 fibroblasts as described by Meyers (1996) to generate an organotypic epithelial raft culture system. HPVs are known to productively replicate in this system. Twelve days after the raft was raised to the air interface, sufficient time to allow for HPV replication, the raft tissues were harvested, and total cellular DNA was isolated, agarose gel electrophoresed, Southern blotted, and probed with ^{32}P-labeled HPV-16 DNA. HPV-16 virion production was further verified by specific antibody neutralization. This experiment was similar to that described above, except that the HPV-16 virus stocks were pretreated with the indicated antibodies, diluted 1/1000, before the keratinocytes were infected. In addition, the antibody H16.V5 was pretreated with 200 mM β-ME.

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