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Suppression of human papillomavirus gene expression in vitro and in vivo by herpes simplex virus type 2 infection

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Abstract

Recent epidemiological studies have found that women infected with both herpes simplex virus type 2 (HSV-2) and human papillomavirus (HPV) type 16 or HPV-18 are at greater risk of developing cervical carcinoma compared to women infected with only one virus. However, it remains unclear if HSV-2 is a cofactor for cervical cancer or if HPV and HSV-2 interact in any way. We have studied the effect of HSV-2 infection on HPV-11 gene expression in an in vitro double-infection assay. HPV transcripts were down-regulated in response to HSV-2 infection. Two HSV-2 vhs mutants failed to reduce HPV-16 E1^E4 transcripts. We also studied the effect of HSV-2 infection on preexisting experimental papillomas in a vaginal epithelial xenograft model. Doubly infected grafts demonstrated papillomatous transformation and the classical cytopathic effect from HSV-2 infection. HPV and HSV DNA signals were mutually exclusive. These studies may have therapeutic applications for HPV infections and related neoplasms.

Keywords: Human papillomavirus; Herpes simplex virus type 2; Sexually transmitted diseases; Virus host shutoff; Human xenografts; Cervical neoplasias; Cervical cancer

Introduction

Infection with a high-risk type human papillomavirus (HPV) constitutes the major risk factor for developing cervical cancer (zur Hausen, 1994). HPV DNA has been found in more than 90% of cervical cancers, and HPV-16 represents about 50% of the viral types identified (Bosch et al., 1995). In addition to the presence of HPV DNA in cervical neoplasias, active transcription of HPV DNA within dysplastic cervical lesions further establishes a strong molecular association of HPV with cervical neoplasia (for review see Stoler, 2000). The expression of early genes E6 and E7 predominates in high-grade CIN and invasive cancers, and E6 and E7 are also actively transcribed in cervical carcinoma cell lines containing HPV-16 or HPV-18 (Stoler, 2000). Experimental studies on the viral oncoproteins E6 and E7 and their interactions with host cellular proteins provide a mechanistic basis for the link between HPV infection and cervical cancer. Several in vitro transformation assays have demonstrated that high-risk types of HPV E6 and E7 can immortalize primary human foreskin or cervical keratinocytes (McDougall, 1994). However, the E6 and E7 can transform primary cells only in the presence of an active oncogene (Storey et al., 1995), and continued expression of E6 and E7 is required to maintain the transformed phenotype (DiPaolo et al., 1993). The E6 proteins of the high-risk types inactivate the tumor suppressor protein p53 by inducing its degradation through the ubiquitin-mediated proteolysis pathway (Scheffner, 1998). The E7 proteins of high-

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risk HPV bind to the tumor suppressor retinoblastoma protein (pRb). The binding of HPV E7 to pRb destabilizes pRb and results in the release of E2F (Berezutskaya and Bagchi, 1997), which is capable of activating transcription of a variety of host genes, many of which are involved in DNA synthesis and cell-cycle progression (zur Hausen, 2000). The activity of these oncoproteins results in genomic instability, and this may further contribute to the deregulation of HPV gene expression during carcinogenic progression. Cells with a growth advantage may be selected and give rise to clonal expansion and tumorigenic progression, which will ultimately result in full malignant transformation.

Although the role of high-risk HPV in cervical cancer has been well established, it is clear that HPV is not sufficient for full malignant transformation. Patients infected with high-risk HPV do not necessarily develop cervical cancer, indicating that other risk factors are involved. Investigations in the 1970s and 1980s tried to establish herpes simplex virus type 2 (HSV-2) as a sole etiologic agent of cervical cancer but resulted in failure to identify a persistent viral oncogene. Two distinct and separate transforming regions (mtrII and mtrIII) have been identified within the HSV-2 genome (Jones, 1995). Using a focus formation assay and colony formation in a soft agar assay, mtrII was shown to transform established rodent cells (Galloway and McDougall, 1981). The minimal transforming fragment is within a 737-bp fragment on the left end of mtrII (Galloway et al., 1984). Primary hamster cells transformed by mtrIII formed foci in monolayer cultures and transformed lines were tumorigenic in newborn hamsters (Jariwalla et al., 1980). HSV-2-transforming DNA sequences were not consistently retained in these transformed cells, indicating that HSV-2 may be necessary for the initiation of transformation but not required for the maintenance of the transformed phenotype (Galloway and McDougall, 1983). Gene products encoded by minimal transforming fragments were not present or have not been identified. The existing data favor a “hit-and-run” hypothesis for HSV-2 transformation (Galloway and McDougall, 1983).

HSV-2 infection has also been proposed as a cofactor in the development of cervical cancer (zur Hausen et al., 1984). HSV-2 DNA has been found in 10–30% of genital tumors (Di Luca et al., 1987). An epidemiological study found that women infected with both HSV-2 and HPV-16 or -18 are at greater risk of developing cervical carcinoma compared to women infected with only one of the viruses (Hildesheim et al., 1991). A more recent, pooled analysis of seven case-control studies that were conducted in different countries indicated that HSV-2 seropositivity was associated with increased risks of invasive cervical cancer among the HPV DNA positive women, after adjustment for potential confounders, suggesting that HSV-2 infection may act in conjunction with HPV infection to increase the risk of invasive cervical cancer (Smith et al., 2002). However, a perspective study conducted by Vonka and colleagues with a cohort of 10,000 women revealed no correlation of HSV-2 infection and increased risk of cervical cancer (Vonka et al., 1984a, 1984b). A recent longitudinal nested control study with a Nordic cohort of 550,000 women and the meta-analysis of six longitudinal seroepidemiologic studies also showed that HSV-2 did not play a role in the development of cervical cancer (Lehtinen et al., 2002). Transfection of nontumorigenic HPV-16-immortalized human keratinocytes with an HSV-2 subfragment containing mtrII converted these cells to tumorigenic squamous carcinoma cells (DiPaolo et al., 1990). HPV-16-immortalized human fibroblasts transfected with mtrIII formed colonies in soft agar and benign squamous epithelial lesions in athymic mice (Dhanwada et al., 1992, 1993) However, a recent study showed that the right end of the subfragment of HSV-2 *Bgl*II N, *Xho*2, can induce the tumorigenic conversion of HPV-immortalized human genital epithelial cells (DiPaolo et al., 1998). Retention and expression of this subfragment correlated with malignancy, suggesting that either the sequences or the products encoded by this *Xho*2 subfragment are required for the initiation and maintenance of the transformed phenotype. Thus, mechanisms other than hit-and-run may be possible in regard to HSV-2 and HPV interactions.

Several additional studies have demonstrated close interactions of HPV and HSV. HSV-1-encoded transactivators activated HPV-16 and HPV-18 long control region (LCR) reporter gene constructs (Gius and Laimins, 1989; McCusker and Bacchetti, 1988). HPV-16 E2 can also activate the promoter for the HSV-2 ribonucleotide reductase large subunit (Wymer and Aurelian, 1990). HSV-1 infection down-regulated HPV-18 E6 transcripts in HeLa cells (Karlen et al., 1993). HSV infection resulted in amplification of integrated HPV-18 DNA in HeLa cells, which contain 10–50 copies of integrated HPV-18 DNA (Schwarz et al., 1985), and in replication of transfected HPV-18 plasmid DNA in A431 cells (Hara et al., 1997). HSV-2 infection of HeLa 229 cells was reported to increase the transcription of E1 and E6 genes after 20 h (Pisani et al., 2002). We studied the effect of HSV-2 infection on HPV gene expression in vitro in a double-infection assay and the effect of HSV-2 infection on preexisting papillomas in vivo. In vitro double-infection in A431 cells and RT-PCR analyses demonstrated that HSV-2 infection down-regulates HPV transcripts as a result of the HSV-2 virion associated host shutoff protein (vhs) function. The incoming vhs associated with HSV-2 virions was sufficient for this effect. In vivo studies, using a vaginal epithelial xenograft model in which preexisting papillomas were superinfected by HSV-2, demonstrated that HPV-11 and HSV-2 signals are mutually exclusive, suggesting that HSV-2 infection may inhibit HPV transcription and replication in papillomatous cells.

Results

Infectivity of HPV-11 viral stock

RT-PCR of RNA extracted from A431 cells infected with HPV-11 at various dilutions, using nested primers for spliced HPV-11 E1^E4 transcripts, showed that E1^E4 transcripts were detectable from serial dilutions of 10^{-2} to 10^{-6}. This indicates that the viral stock was infectious (Fig. 1), and the infected culture dose $_{50}$(ICD$_{50}$) was 10^6/ml.

Down-regulation of HPV-11 E1^E4 transcripts by HSV-2 infection

The effect of HSV-2 infection on HPV-11 gene expression was studied using an in vitro double-infection assay. A431 cells were infected either with HPV-11 alone (Fig. 2A) or with HPV-11 and HSV-2 (Fig. 2B). Repeated RT-PCR analyses with nested primer sets for HPV-11 E1^E4 showed that HSV-2 infection down-regulates HPV-11 E1^E4 transcripts at various time points as early as 8 h post-HSV-2 infection (44 h post-HPV-11 infection) as indicated (Fig. 2). To test if the down-regulation of HPV-11 E1^E4 transcripts by HSV-2 infection is cell-type-specific, we then examined the effect of HSV-2 infection on HPV-11 gene transcripts using the same in vitro double-infection assay in human keratinocytes immortalized by the catalytic subunit of human telomerase (N-Tert) (Dickson et al., 2000). The down-regulation of HPV-11 E1^E4 was also observed in N-Tert cells (Fig. 3). In this experiment, down-regulation of E1^E4 was observed after 12 h of HSV-2 infection (48 h post-HPV-11 infection). Nested primers for β-actin were included. Production of β-actin cDNA was measured as a quality control for RNA samples. The level of β-actin transcript appeared unchanged but decreases in this transcript might not be detected here because this is an abundant message and any remaining transcripts would still score positive in this nonquantitative assay.

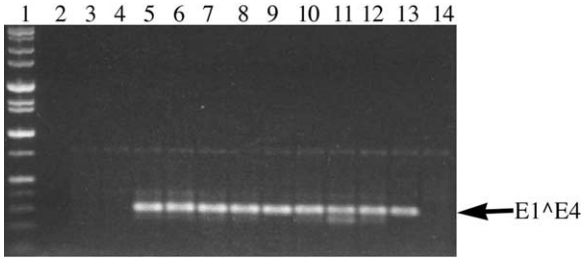

Fig. 1. Detection of HPV-11 E1^E4 transcription in infected A431 cells. RT-PCR analysis using nested primers for HPV-11 E1^E4. Lane 1: high–low molecular marker. Lane 2: H_2O control. Lanes 3 and 4: uninfected controls. Lanes 5 and 6: 10^{-2} dilution of viral stock. Lanes 7 and 8: 10^{-3} dilution of viral stock. Lanes 9 and 10: 10^{-4} dilution of viral stock. Lanes 11 and 12: 10^{-5} dilution of viral stock. Lanes 13 and 14: 10^{-6} dilution of viral stock. The ICD$_{50}$ was 10^6/ml.

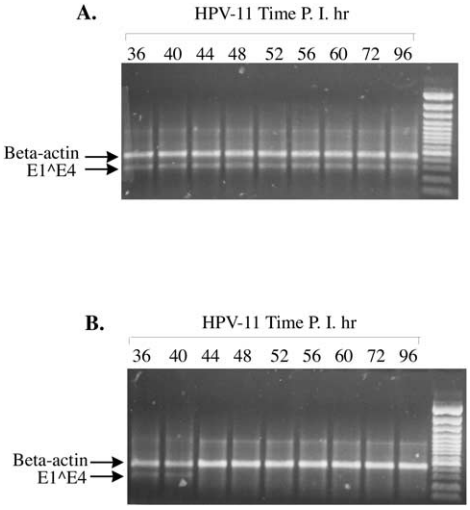

Fig. 2. Down-regulation of HPV-11 E1^E4 transcripts by HSV-2 infection in A431 cells. RT-PCR analysis using nested primers for HPV-11 E1^E4 and β-actin. HPV-infected cells were infected with wild-type 333 HSV-2 at an m.o.i. of 10 PFU/cell in 0.5 ml medium 36 h post-HPV infection. Expected size for the E1^E4 amplicon was 294 bp and for β-actin was 429 bp as indicated by arrows. (A) A431 cells infected with HPV-11 alone. (B) A431 cells infected with HPV-11 and HSV-2 at 36 h post-HPV-11 infection. RNA samples were harvested at various time points as indicated.

Down-regulation of HPV-11 E7 transcripts by HSV-2

To test if the HPV-11 E1^E4 transcripts were exclusively targeted by HSV-2 infections and to further quantitate the shutdown of HPV transcripts by HSV-2 infection, HPV-11 E7 transcripts were examined in the in vitro double-infection assay. Analysis by real-time RT-PCR using primers and probe specific for HPV-11 E7 showed that E7 transcripts were also reduced following HSV-2 infection as early as 4 h postinfection (Fig. 4).

Down-regulation of HPV-16 E6 transcripts by the HSV-2 vhs function

To examine if viral oncogene transcripts from high-risk types of HPV were also down-regulated by HSV-2 infection, HPV-16 E6 transcripts in HSV-2-infected CaSki cells were examined. Because the down-regulation of HPV-11 transcripts was neither cell-type-specific nor specific for a particular transcript, and because HSV-2 vhs is known to mediate mRNA degradation in a sequence nonspecific manner, we suspected that HSV-2 vhs may have played a role in the down-regulation of HPV transcripts. CaSki cells infected with wild-type HSV-2 were therefore also compared with cells infected with the HSV-2 vhs mutant, 333-vhsB (a generous gift from Dr. J. Smiley at University of Alberta, Edmonton, Alberta, Canada). Total RNA samples extracted from CaSki cells infected with either the wild-type HSV-2 or the vhs mutant were subjected to RT-PCR analysis using nested primers specific to transcripts containing HPV-16 E6 (Sotlar et al., 1998). Results showed that different spliced

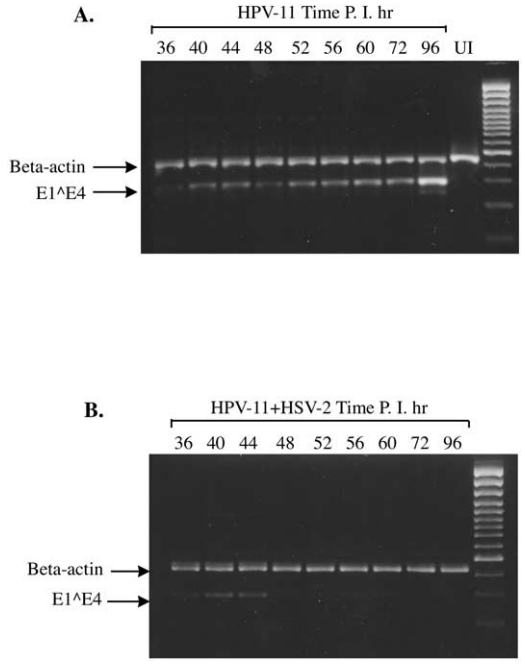

Fig. 3. Down-regulation of HPV-11 E1ˆE4 transcripts by HSV-2 infection in N-Tert cells. In vitro double-infection assay using human keratinocytes immortalized by the catalytic subunit of human telomerase (N-Tert). RT-PCR analysis using nested primers for HPV-11 E1ˆE4 and β-actin. (A) N-Tert cells infected with HPV-11 alone. RT-PCR products of HPV-11 E1ˆE4 transcripts are detected from 36 h post-HPV infection. (B) N-Tert cells infected with HPV-11 and with HSV-2 at 36 h post-HPV-11 infection. RNA samples were harvested at various time points as indicated.

forms of HPV-16 E6 transcripts, E6* I and E6 *II, are dramatically down-regulated in wild-type HSV-2-infected cells as early as 4 h postinfection and remained reduced up to 48 h post-HSV-2 infection (Fig. 5A). In contrast, CaSki cells infected with the HSV-2 vhs mutant failed to shut down these transcripts under the same conditions, suggesting that the observed shutdown of HPV transcripts was a result of HSV-2 vhs function (Fig. 5B).

Down-regulation of HPV-16 E1ˆE4 transcripts by HSV-2 vhs function

To further quantitate the down-regulation of HPV-16 transcripts in HSV-2 infected CaSki cells and to confirm the vhs-mediated shutdown, two different vhs mutants were used followed by quantitative RT-PCR analyses, with primers and probes for HPV-16 E1ˆE4. The results indicated that HPV-16 E1ˆE4 transcripts were also reduced as a result of HSV-2 infection as early as 4 h postinfection. On the other hand, cells infected with the two different HSV-2 vhs mutants, 333-vhsB and 333-41d (a generous gift from Dr. D. Leib, Washington University School of Medicine, St. Louis, MO), failed to shut down HPV-16 E1ˆE4 transcripts (Fig. 6).

De novo protein synthesis is not required for the HSV-2 vhs mediated shutdown of HPV transcripts

To investigate whether de novo protein synthesis is required for the HSV-2 vhs mediated shutdown of HPV transcripts, CaSki cells were infected with HSV-2 at a multiplicity of infection (m.o.i.) of 10 plaque forming units (PFU)/cell in the presence or absence of 50 μg/ml cycloheximide and compared with uninfected cells in the presence or absence of cycloheximide. Labeling experiments to measure incorporation of ^{3}H-labeled amino acids indicated that at 50 μg/ml of cycloheximide the inhibition of protein synthesis was highly effective (data not shown). Using the same HPV-16 E1ˆE4 primers as in the real-time PCR, the 80-bp amplicon expected was shown as a single band. Contamination of the RNA preparation with HPV genomic DNA might have led to an amplified product of 2558 bp. This was not observed. The RT-PCR results showed that the presence of cycloheximide did not change the level of HPV E1ˆE4 transcripts in uninfected cells over a 12-h time course (Fig. 7A), and the down-regulation of HPV E1ˆE4 transcripts consequent to HSV-2 infection was still observed when de novo protein synthesis was inhibited by cycloheximide (Fig. 7B). This implies that the shutdown of HPV transcripts under these conditions was mainly due to the early shutdown mediated by HSV-2 virion associated vhs protein.

Optimized transformation of human vaginal xenografts by HPV infection

Previously, we published HPV-11-specific papillomatous transformation of human vaginal epithelial xenografts by HPV-11 (Howett et al., 2000). To optimize the transformation rate of human vaginal xenografts by HPV-11 infection, three different conditions were tested. Human vaginal xenografts were infected with HPV-11 by preincubation of the tissue with the virus before grafting, or grafted directly and then infected at 1 or 2 weeks postgrafting by injecting the HPV-11 virus stock into the lumen of the grafted vaginal tubes. The results showed that preincubation of the vaginal epithelial tissue with HPV-11 before grafting or infection of the vaginal grafts at 1 week postgrafting resulted in a higher papillomatous transformation rate than was observed in xenografts infected at 2 weeks postgrafting (Table 1).

Inhibition of HPV-11 activity by HSV-2 infection in the vaginal xenograft model

The human vaginal xenograft model was adapted for the study of HPV and HSV-2 interaction in vivo. Human vaginal grafts infected with HPV-11 at 1–2 weeks after xenotransplantation were known to be transformed by 10–13 weeks; such grafts were then infected with HSV-2. Harvested grafts infected with HPV-11, alone, demonstrated areas of papillomatous transformation with koilocytes char-

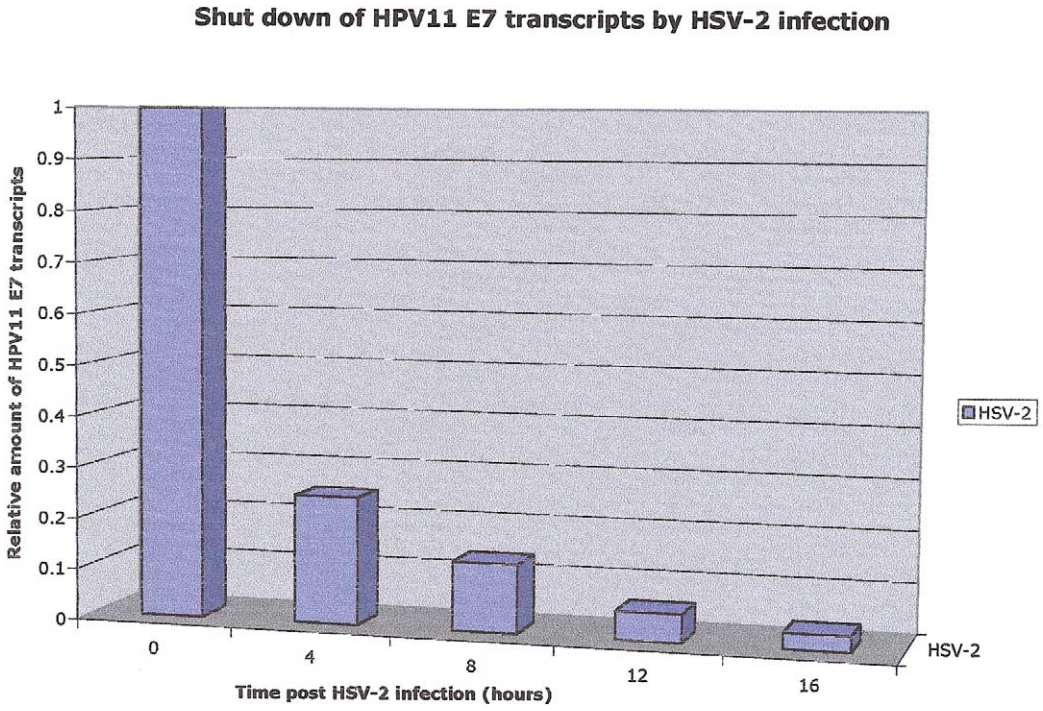

Fig. 4. Down-regulation of HPV-11 E7 transcripts by HSV-2 infection in A431 cells. In vitro double-infection assay using A431 cells established from human epidermoid carcinoma. Total RNA was subjected to the real-time PCR analysis for HPV-11 E7. The amount of HPV-11 E7 in cells infected with HPV-11 and HSV-2 relative to the amount of HPV-11 E7 in control cells infected with HPV-11 alone was plotted against time postinfection. HPV-11 E7 transcripts were dramatically down-regulated by wild-type HSV-2 infection.

acteristic of HPV infection as previously reported (Howett et al., 2000). Double-infected grafts demonstrated areas of the HSV-2 cytopathic effect (◀) by histology in addition to areas of papillomatous transformation (→) (Figs. 8A1 and B1). In situ hybridization using biotin-labeled HPV-11 and HSV-2 DNA probes on adjacently cut tissue sections demonstrated that papillomatous transformed cells are HPV-11-positive, while HSV-2-positive cells were located at the surface and edges of the papillomatous-transformed epithelial areas. The HPV-11 (Fig. 8A2 and B2) and HSV-2 (Figs. 8A3 and B3) -positive signals were mutually exclusive in grafts infected with HPV-11 and wild-type HSV-2. We have observed this mutually exclusive pattern of HPV and HSV DNA signals in 10 different doubly infected vaginal xenografts, suggesting that HSV-2 infection may also inhibit HPV activity in HPV-11 transformed tissues.

Discussion

The direct role of HSV-2 in the development of cervical cancer was extensively studied in the 1980s. However, the link between cervical cancer and HSV is not conclusive from early virological and epidemiological studies, suggesting that HSV-2 may not be directly associated with cervical cancer causality. The possibility remains that HSV-2 could function as a possible cofactor in the development of cervical cancer. Several studies have demonstrated close interactions of HPV and HSV (Dhanwada et al., 1992, 1993; DiPaolo et al., 1990, 1998; Gius and Laimins, 1989; Hara et al., 1997; Karlen et al., 1993; McCusker and Bacchetti, 1988; Pisani et al., 2002; Wymer and Aurelian, 1990). However, it remains unclear whether and how HSV-2 would function as a cofactor in the development of cervical cancer and whether HPV and HSV-2 interact in any way during the carcinogenic process.

Since infectious HPV-11 virus, which can be generated using the athymic mouse model, produces low-grade dysplasias of cervix, vagina, and foreskin epithelium, we took advantage of this model to study the interaction of HPV-11 and HSV-2. The effect of HSV-2 infection on HPV-11 gene expression was examined in an in vitro double-infection assay. Unlike several other in vitro studies, where the effect of a particular HSV gene product on HPV gene expression was studied using an expression plasmid and HPV LCR-driven reporter gene constructs in the absence of the whole viral genomes, our double-infection assays allow the study of HPV and HSV interaction in cell lines and tissues which can be infected by both viruses. The presence of the whole genomes of both viruses may more closely mimic a natural infection. Infection of 5×10^5 cells with a 10^{-2} dilution of HPV stock in 2 ml of medium corresponds to an m.o.i. of at least 0.04 to 0.4 ICD_{50}/cell. Under these conditions, HPV-11 E1^E4 transcripts can be detected by nested RT-PCR beginning at 36 h postinfection. Total cellular RNAs were then harvested at different time points within 48 h post

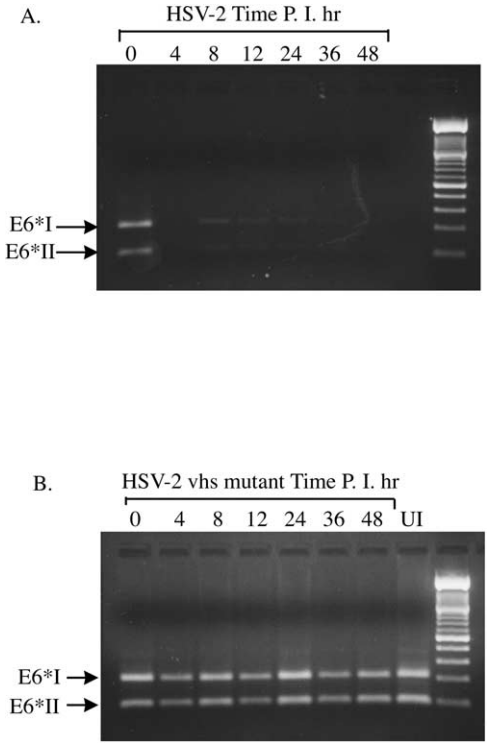

Fig. 5. Down-regulation of HPV-16 E6 transcripts by HSV-2 vhs function. RT-PCR analysis for HPV-16 E6. The expected size for various forms of HPV-16 E6 transcripts (Table 2) are 395 bp (E6/E7 unspliced), 213 bp (E6*I), and 95 bp (E6*II). (A) CaSki cells infected with wild-type HSV-2. (B) CaSki cells infected with HSV-2 vhs mutant. RNA was harvested at various times postinfection as indicated.

HSV-2 infection. These time points are early enough to observe the effect of the HSV-2 infection on HPV transcripts before the cells undergo complete lysis as a result of HSV-2-induced cytopathic effects. The time for HSV-2 inoculation is also late enough to allow HPV transcripts to accumulate to a detectable level so that any effect of HSV-2 on HPV transcripts will be easily detected.

Repeated RT-PCR analyses in A431 cells with nested primer sets for HPV-11 E1^E4 showed that HSV-2 infection down-regulates HPV11 E1^E4 transcripts as early as 8 h postinfection. This down-regulation was not cell-type-specific or specific for a particular transcript. We subsequently examined HPV-11 E7 transcripts in response to HSV-2 infection by real-time RT-PCR analysis. The amount of HPV transcripts in total RNA isolated from HPV-11 and HSV-2 wild-type virus-infected cells, relative to that from cells infected with HPV-11 alone, was plotted.

We suspected that HSV-2 vhs protein may be involved, since HSV-2 vhs is known to shut off host protein synthesis by disruption of preexisting polyribosomes and accelerated degradation of host mRNA in a sequence-nonspecific manner and that binding of vhs by VP16, another HSV-2 protein, is required to allow accumulation of viral transcripts at a later stage of infection (Smiley et al., 2001). We found that HSV-2 vhs mutants failed to down-regulate HPV-16 E1^E4 and E6 transcripts in CaSki cells. Similar results were found by real-time RT-PCR for HPV-16 E1^E4 transcripts in CaSki cells using two independently derived HSV-2 vhs mutants. However, this conclusion does not eliminate the involvement of other HSV-2 proteins in vivo. The level of HPV transcripts was not restored to the level at zero time point of infection, especially at later time points, suggesting that other HSV-2 proteins may also be involved. It has been suggested that the delayed secondary shutoff requires de novo protein synthesis and is mediated by the immediate-early HSV-2 protein, ICP27. ICP27 causes host shutoff through regulation of small nuclear ribonucleoprotein distribution and inhibition of mRNA splicing (Phelan and Clements, 1998). The immediate early protein ICP22, which acts through an alteration in the phosphorylation state of RNA polymerase II to specifically repress cellular gene transcription, also aids in host shutoff (Phelan and Clements, 1998). The results of our studies are not in agreement with observations from several other studies where several HSV transactivators were found to enhance HPV gene expression in in vitro systems when studied in the absence of the whole viral genomes (Gius and Laimins, 1989; McCusker and Bacchetti, 1988). This suggests that HPV and HSV interact differently in the context of intact viral genomes during a natural infection compared to genes in isolation. Our results are similar to the observation made in HSV-1-infected HeLa cells where hypersensitivity of HPV-18 E6 transcripts to the HSV-1 vhs function was observed (Karlen et al., 1993). Apparently, the vhs function of the different types of HSV is conserved and the effect of HSV on HPV of both high-risk types and low-risk types is similar.

The presence of cycloheximide did not affect the shutdown of HPV transcripts, suggesting that our observations are mainly due to the early shutdown mediated by the HSV-2 virion-derived vhs. Using this semiquantitative assay, HPV transcript levels appeared to be slightly higher in HSV-2-infected cells in the presence of cycloheximide compared to those cells in the absence of cycloheximide; this may be due to the stabilization effect on polyribosomes by cycloheximide (Arbuzov, 1978). This result also implies that in the situation of an abortive HSV infection or in the presence of defective particles, the vhs-mediated shutdown of HPV is equally likely. If the infected cells are not killed by HSV infection, the effect of vhs-mediated shutdown on cells and the repertoire of HPV genes expressed may affect the course of HPV infection and contribute to the carcinogenic process.

The shutdown of HPV viral oncogene transcripts by HSV-2 vhs function raises the possibility of using regulated expression of HSV vhs as a therapeutic application for cervical neoplasms. Previous reports have shown that expression of vhs reduced gene expression from a cotransfected reporter plasmid (Pak et al., 1995). Vhs expression has also been shown to inhibit human immunodeficiency virus replication in transfected cells (Hamouda et al., 1997). Therefore, it may be possible to use regulated expression of vhs targeted to cervical cancer cells as a therapeutic agent

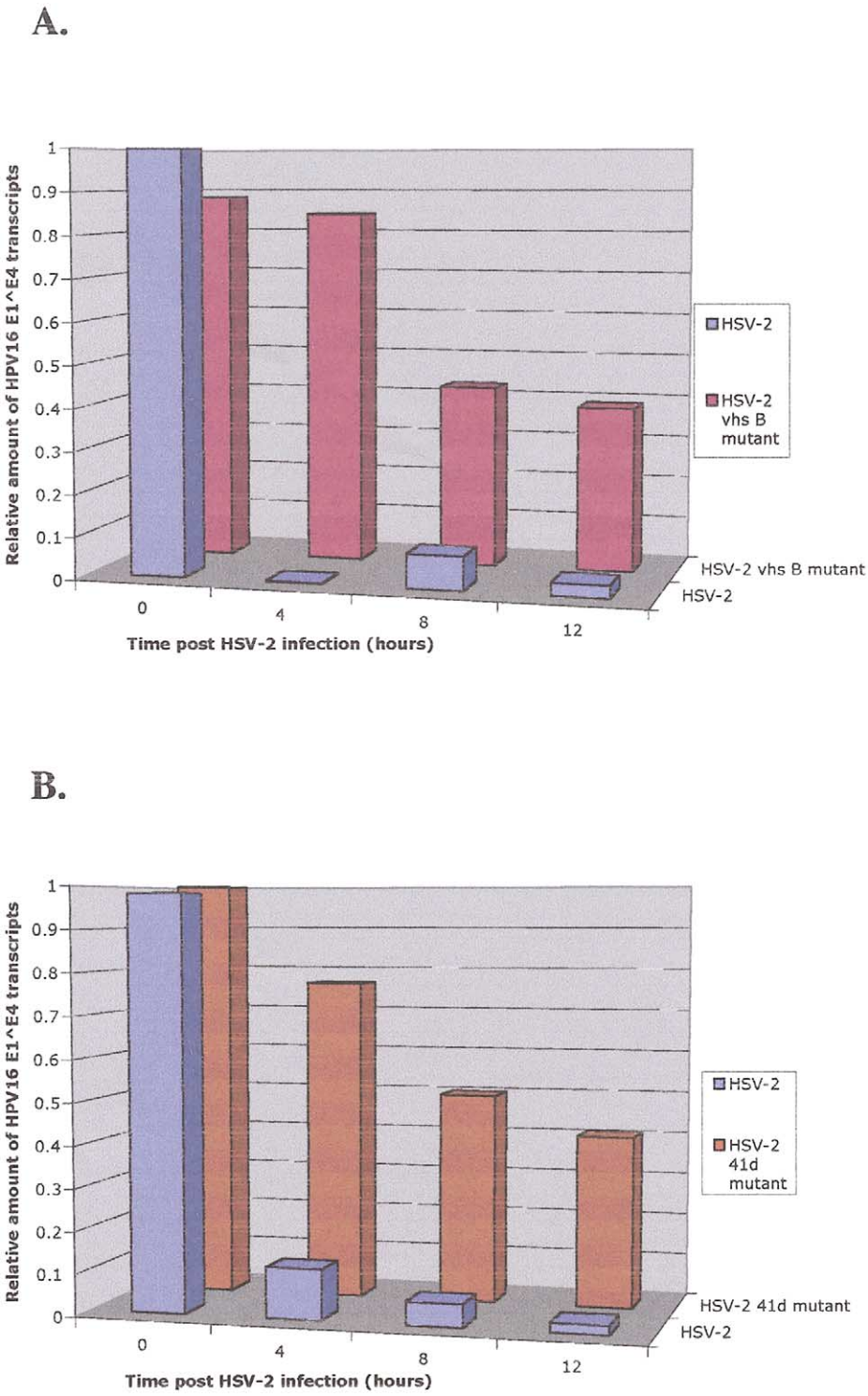

Fig. 6. Down-regulation of HPV-16 E1ˆE4 transcripts by HSV-2 vhs function. Real-time PCR analysis for HPV-16 E1ˆE4. The amount of HPV-16 E1ˆE4 in infected cells relative to the amount of HPV-16 E1ˆE4 in mock-infected cells was plotted against time postinfection. Bars represent the relative amount of HPV-16 E1ˆE4 transcripts from cells infected with wild-type HSV-2 or two different HSV-2 vhs mutants at various times postinfection, as indicated. (A) Relative amount of HPV-16 E1ˆE4 transcripts at various time points in cells infected with wild type HSV-2 or HSV-2 333-vhsB mutant. (B) Relative amount of HPV-16 E1ˆE4 transcripts at various time points in cells infected with wild-type HSV-2 or 333-41d mutant.

for cervical papillomas or cervical cancer. However, the effects of vhs on cellular functions need to be systematically studied before it can be developed into a therapeutic agent.

No study has ever examined the interaction of HPV and HSV in vivo in the context of natural human vaginal tissue. Previous experiments in our laboratory have demonstrated

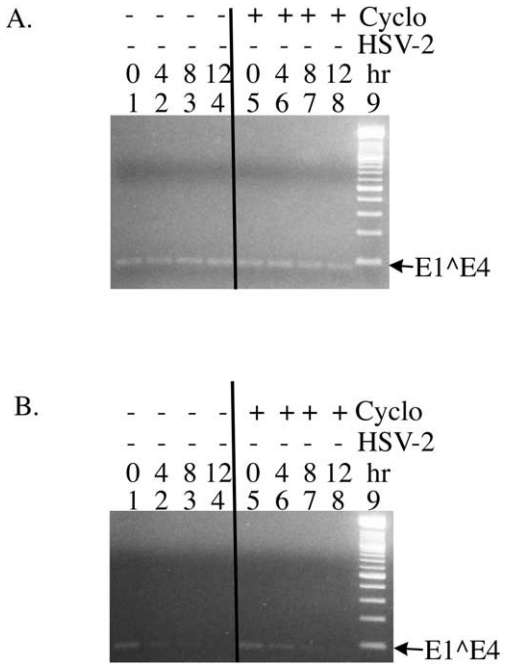

Fig. 7. De novo protein synthesis is not required for the HSV-2 vhs mediated shutdown of HPV transcripts. RT-PCR analysis for HPV-16 E1ˆE4Ê4 using the same primers as in the real-time PCR. (A) Uninfected CaSki cells. Lanes 1–4: uninfected CaSki cells without cycloheximide harvested at 0, 4, 8, and 12 h. Lanes 5–8: uninfected CaSki cells with cycloheximide harvested at 0, 4, 8, and 12 h. Lane 9: 100-bp molecular marker. (B) HSV-2-infected CaSki cells. Lanes 1–4: HSV-2-infected CaSki cells without cycloheximide harvested at 0, 4, 8, and 12 h postinfection. Lanes 5–8: HSV-2-infected CaSki cells with cycloheximide harvested at 0, 4, 8, and 12 h. Lane 9: 100-bp molecular marker.

that the vaginal epithelium can serve as a target for productive infection and neoplastic transformation by HPV-11 (Howett et al., 2000). Results showed that double-infected grafts, by histology, demonstrated areas of the HSV-2 cytopathic effect in addition to areas of papillomatous transformation. In situ hybridization using HPV-11 and HSV-2 DNA probes on adjacently cut tissue sections demonstrated that papillomatous transformed cells are HPV-11-positive, while HSV-2-positive cells were located at the surface and edges of the papillomatous transformed epithelial areas or at basal layers of the epithelia. HSV-2 and HPV-positive signals were mutually exclusive, indicating that HSV-2 infection, acute, recurrent, or persistent, may inhibit HPV macromolecule synthesis in preexisting papillomas. Although it is difficult to show that HSV-2 virions are exposed to the same cells that are infected by HPV, we have observed this mutually exclusive pattern of HPV and HSV DNA signals in 10 different doubly infected vaginal xenografts. The inhibition of HPV activity was also observed in layers above HPV-11-positive cells in terminally differentiated layers of the epithelia, which would normally stain positive for HPV-11 all the way to the cornified layers of the epithelia (Fig. 8B2). Areas which are negative for HPV-11 stained positive for HSV-2 (Fig. 8B3). These results suggest that HSV-2 infection may inhibit HPV activities. The data are in agreement with our in vitro data for the down-regulation of HPV transcripts by HSV-2 infection, suggesting that the effect of HSV on HPV gene expression observed in vitro could happen in vivo as well. We do not know, however, that the effects observed in vivo are solely mediated by vhs.

The most direct interpretation of our in vitro and in vivo data suggests that HSV-2 infection inhibits HPV activities and therefore may result in ablation of papillomas. Epidemiological studies found conflicting results for the association of HSV-2 and increased risk of cervical cancer. The recent longitudinal nested control study with a Nordic cohort of 550,000 women, and the meta-analysis of six longitudinal seroepidemiologic studies on the association of HSV-2 with cervical neoplasia showed that HSV-2 did not play a role in the development of cervical cancer (Lehtinen et al., 2002). In fact, these studies revealed that among HPV-seropositive women, HSV-2 seropositivity was associated with a slightly decreased risk of developing cervical cancer. The association of HSV-2 infection with cervical cancer found in several cross-sectional case control studies may be biased, since the cross-sectional design itself cannot exclude the possibility that cervical neoplasia predisposes to HSV-2 infection.

Several alternative possibilities for the outcome of HSV-2 infection-mediated down-regulation of HPV can be proposed. HSV-2 infection may initially cause the cytopathic effect and tissue destruction, which may result in partial ablation of the preexisting papillomas. Tissue destruction followed by immune clearance of HSV-2, however, may lead to stimulation of papilloma growth during repair of injury and therefore promote carcinogenic progression. In addition, defective particles may not kill infected cells, and the effect of vhs mediated shutdown on these surviving cells and the repertoire of HPV genes expressed may alter HPV infection and promote tumor growth through changes in HPV integration, amplification, or mutagenic events. It is unknown at this point if alterations in HPV gene expression by HSV-2 contribute to the carcinogenic process or if the down-regulation of HPV macromolecule synthesis results in in vivo ablation of the papilloma. Further studies using various HSV-2 mutants in this xenograft model, such as HSV-2 vhs or HSV-2 replication defective mutants, may

Table 1
Optimization of human vaginal xenograft athymic mouse model for the rate of HPV-11 induced transformation

Method	Papillomas/total xenografts
A	13/15
B	9/10
C	8/46

A: Preincubation of human vaginal epithelia tissue with virus before grafting; B: virus inoculation at one week postgrafting; C: virus inoculation at 2 weeks postgrafting.

allow longer incubation times for HSV-2-mediated carcinogenic conversion of HPV-induced papillomas. In addition, the effect of the vhs on preexisting papillomas may be studied by transduction of the vhs gene to preexisting papillomas. These studies are currently in progress. The potential limitation of this animal model is that HPV-11-induced transformations are not uniform and HSV-2 infections on preexisting papillomas are opportunistic as well. Therefore, large numbers of human xenografts in animals may be needed to make definitive conclusions.

The in vivo and in vitro results also suggest the possibility of using HSV-2 for the treatment of cervical carcinoma. The idea of using viruses as oncolytic agents has been suggested in the past (Kenney and Pagano, 1994). The fact that cervical epithelial cells are the natural targets of HSV-2, and that HSV-2 is highly cytolytic, makes it a good candidate as an oncolytic agent for cervical cancer. The toxicity associated with HSV-2 infection would clearly prevent the use of wild-type HSV-2 for treatment of human cancer. However, with advanced genetic manipulation, it may be possible to develop HSV-2 mutants which will maximize the killing of tumor cells and minimize the toxicity to normal cells. Replication-competent HSV has been demonstrated to be an effective oncolytic agent for several malignant tumors. These HSV mutants have been engineered to reduce virulence in normal tissues while maintaining oncolytic ability. Deletion mutants of HSV-1, such as thymidine kinase (TK) deletion and ribonucleotide reductase (RR) deletion, have been made for the treatment of brain tumors (Andreansky et al., 1997; Kramm et al., 1997). Inhibition of oral cancer tumor growth by HSV-1 in a mouse model has been shown, and the inhibition was dependent on the ability of the virus to replicate in the tumor (Lou et al., 2002). The HSV-1 ICP34.5 null mutant has been shown to effectively treat experimental human melanoma in mice, and virus replication was severely attenuated in normal tissue through unclear mechanisms (Randazzo et al., 1997). HSV-1 G207, which was constructed with deletions in both the ICP34.5 gene and a *lacZ* gene inserted into the ICP6 gene, demonstrated efficient and selective cytotoxicity and tumor growth inhibition of human head and neck squamous cell carcinoma (Carew et al., 1999). However, mutants that work for brain tumors or oral cancer may not work for cervical cancer. Other mutants may have to be developed to target cervical cancer cells specifically. One way to regulate the replication of HSV-2 would be to replace the promoter of an essential gene with a promoter that is selectively active in cervical cancer cells.

Materials and methods

Generation of experimental HPV-11-infected papillomas

Experimental papillomas and HPV-11 virus stocks were generated as previously described (Kreider et al., 1987). Briefly, human foreskins were obtained from neonatal circumcisions and placed into minimum essential medium (MEM) containing gentamycin (0.4 mg/ml), penicillin and streptomycin (0.08 mg/ml each), fungizone (2.5 μg/ml), sodium bicarbonate (0.05%), and HEPES (10 mM). Human foreskin tissue was then cut split-thickness into pieces measuring approximately $2 \times 2 \times 0.5$ mm. Tissues were incubated with HPV-11 virus inoculum for 1 h at 37°C. The original cell-free extract of HPV-11 used in the xenograft system was obtained from pooled patient *condylomata acuminata* (Kreider et al., 1985). Infected tissue chips were then transplanted under the mouse renal capsule. The grafts were allowed to grow for 3–5 months. Infected foreskin grafts transformed by HPV-11 often produce *condylomatous* cysts of 1–2 cm in diameter. Keratin cores were removed from experimentally developed *condylomatous* cysts and kept at −70°C; these cores were used for preparation of additional virus stocks.

Preparation of HPV-11 viral stock

HPV-11 viral stock was prepared as previously described (Kreider et al., 1987). Briefly, keratin cores removed from experimentally developed *condylomatous* cysts and kept at −70°C were thawed and weighed. Keratin was then ground with sterile sea sand and 2 volumes (w/v) of PBS with a mortar and pestle. The resultant paste was centrifuged at 11,000 *g* at 4°C for 5 min. The supernatant was removed and stored at −70°C.

Preparation of HSV-2 virus stock

HSV-2 viral stocks were prepared as described previously (Adelman et al., 1980). Briefly, confluent African green monkey kidney cells were infected with the HSV-2 strain 333 virus stock at a low m.o.i. (0.1 PFU/cell) in a small volume of sterile Tris-buffered saline (137 mM NaCl, 2.68 mM KCl, 25 mM Tris, pH 7.4). The virus inoculum was replaced by fresh Dulbecco's MEM (supplemented with 10% fetal calf serum, 1.5 g/L $NaHCO_3$, 100 U/ml penicillin G, 100 mg/ml streptomycin) after 1 h of incubation at 37°C. Incubation was continued for 24–36 h until the cells showed complete cytopathic effects. Flasks were frozen and thawed and media were pooled. Cell debris was pelleted by centrifuging for 5 min (200 *g*) at 4°C. The supernatant was aliquoted and stored at −70°C.

Assay for infectivity of HPV-11 viral stock

The infectivity of HPV-11 viral stock was determined as described previously (Ludmerer et al., 2000; Smith et al., 1995). Briefly, the HPV-11 viral stock was serially 10-fold diluted in Dulbecco's medium (supplemented with 10% FCS, 100 U/ml penicillin G, 100 mg/ml streptomycin, 1.5 g/L $NaHCO_3$, 2.8 g/L HEPES). A431 cells established from human epidermoid carcinoma (Giard et al., 1973) were

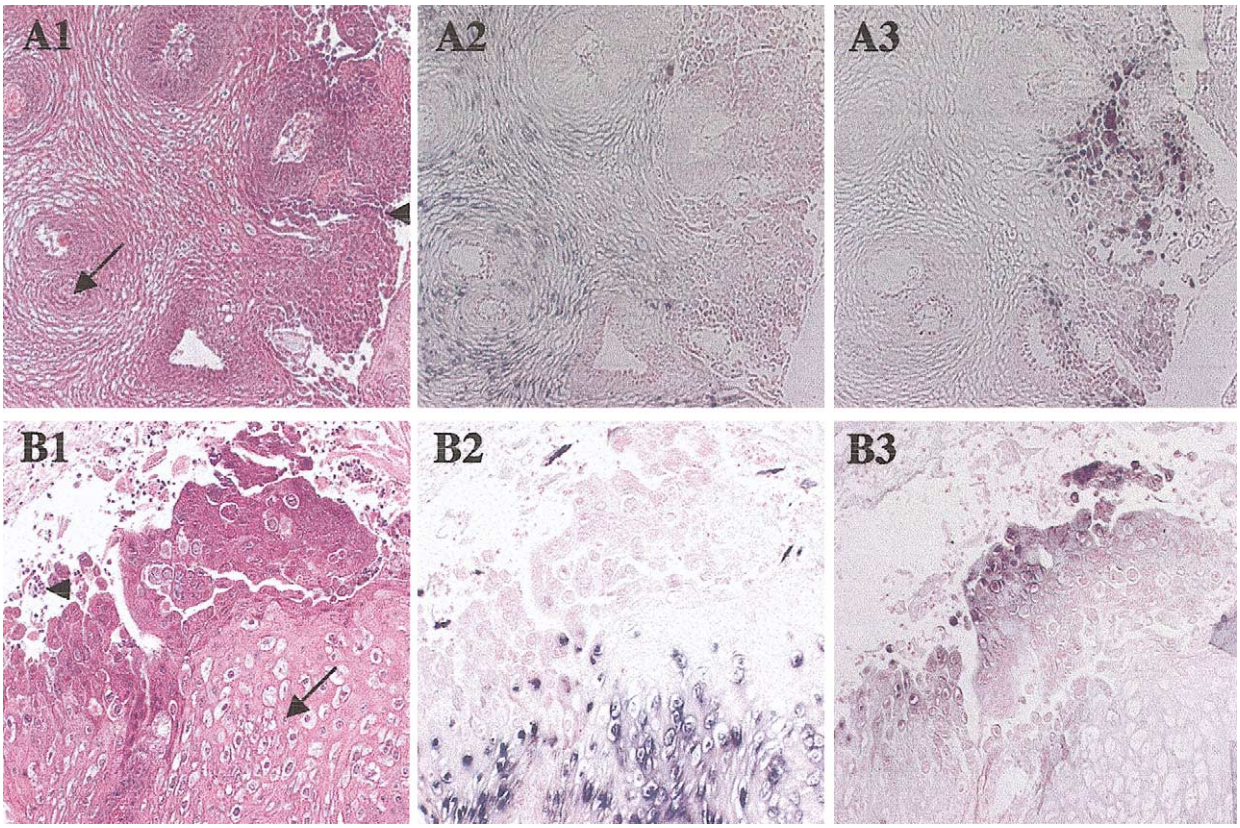

Fig. 8. Inhibition of HPV-11 activity by HSV-2 infection in the vaginal xenograft model. Human vaginal grafts infected with HPV-11 at 1–2 weeks after xenotransplantation were then infected with HSV-2 and harvested 1 week post-HSV-2 infection. (A1 and B1) Hematoxylin and eosin staining of two different formalin-fixed and paraffin-embedded doubly infected xenograft longitudinal sections. Grafts demonstrated areas of papillomatous transformation with koilocytes characteristic of HPV infection (◀) and areas of HSV-2 cytopathic effect located at the edge and surface of the graft (→). (A2 and B2) In situ hybridization of adjacently cut sections of the same area as in A1 and B1, respectively, probed with biotin-labeled HPV-11 DNA. Areas of papillomatous transformed cells stained dark purple are HPV-11 positive. (A3 and B3) Adjacently cut section of the same areas as in A2 and B2, respectively, probed with biotin-labeled HSV-2 DNA probe. HSV-positive cells stained dark purple are located at the surface and edges of the papillomatous-transformed epithelial cells and the HPV-11- and HSV-2-positive signals are mutually exclusive in the doubly infected grafts.

seeded in six-well plates (5×10^5 cells/well) 1 day prior to infection with serially diluted HPV-11 stock in 1 ml of culture medium. An additional 2 ml of culture medium was added after overnight incubation at 37°C, and cells were incubated for an additional 2 days. Total cellular RNA was extracted 72 h postinfection using a QIAshredder and RNeasy mini kit (Qiagen, Valencia, CA). RT-PCR analysis of the HPV-11 E1ˆE4 transcript was used to determine the end-point dilution of virus that still initiated infectivity.

Titration of HSV-2 by plaque assay

HSV-2 viral titers were determined as described previously (Adelman et al., 1980). Briefly, the virus samples to be titrated were serially 10-fold diluted in Dulbecco's MEM as described above. Confluent African green monkey kidney cells (CV-1 or Vero) seeded in 60-mm tissue culture plates were inoculated with diluted viruses and incubated at 37°C for 1 h. At the end of incubation, virus inoculum was removed and 5 ml of methylcellulose medium was added to each plate. The plates were incubated for 1 to 4 days and fixed and stained with 5% formaldehyde and 0.5% crystal violet. Plaques were counted and the virus titer was expressed in plaque-forming units.

In vitro double-infection assay

A431 cells were seeded in six-well plates (5×10^5/well). On the next day, cells were infected with 10^{-2} diluted HPV-11 viral stock in 2 ml of medium. HPV-infected cells were then infected 36 h post-HPV infection with wild-type 333 HSV-2 or HSV-2 mutants (333-vhsB and 333-41d) at an m.o.i. of 10 PFU/cell in 0.5 ml medium. The HSV-2 333-vhsB mutant was constructed by disruption of the UL41 ORF in HSV-2 strain 333 DNA by insertion of an expression cassette consisting of the *Escherichia coli lac*Z gene driven from the HSV-1 ICP6 promoter into a *Bst*X1 restriction site at codon 30. The mutation was transferred to HSV-2 by marker rescue. The 333-d41 mutant HSV-2 has a 939-bp deletion in vhs. The HSV-2 inoculum was replaced by medium after a 1-h incubation. Total cellular RNA was extracted every 4 h from the point of HSV infection for 24 h

Table 2
Reverse transcriptase and polymerase chain reaction primers for HPV-11 E1^E4 and β-actin

Primer description	Nucleotide sequence (5′ → 3′)
HPV-11	
11-UO	^{778}GCTGGGCACACTAAATATTGT^{798}
11-UI	^{808}CTGCGCACCAAAACCATAACA^{828}
11-DO	^{3684}GCCCAATGCCACGTTGAAGA^{3665}
11-DI	^{3578}TAGGCGTAGCTGCACTGTGA^{3559}
β-Actin	
A-UO	^{395}GATGACCCAGATCATGTTTG^{414}
A-DO	^{1036}GGAGCAATGATCTTGATCTTC^{1016}
A-UI	^{423}AACACCCCAGCCATGTACGTTG^{444}
A-DI	^{852}ACTCCATGCCCAGGAAGGAAGG^{831}

Note. Primer pairs, specific for HPV-11 E1^E4 and β-actin and their corresponding nucleotide sequences are shown as published in Ludmerer et al. (2000) and Smith et al. (1995).

using the RNeasy mini kit (Qiagen). RNA samples were stored at −70°C.

RT-PCR analysis of HPV-11 E1^E4 transcripts

Total cellular RNA (200 ng) extracted from infected A431 cells was reverse transcribed in the presence of RT buffer, 5 mM $MgCl_2$, 1 mM of dNTPs, 5 μM 11-DO, and A-DO primers (Table 2) and 2.5 U of reverse transcriptase in a total volume of 20 μl at 42°C for 1 h. The product was amplified using nested primer sets (Table 2) and two 30-cycle PCR rounds to detect the HPV-11 E1^E4 spliced transcript (Ludmerer et al., 2000; Smith et al., 1995). β-actin primer sets (Table 2) were included in PCR reactions to serve as an internal control for RNA integrity. Following cDNA synthesis primers 11-UO and A-UO were used for 30 cycles of PCR (temperature file: 94°C/30 s, 60°C/30 s, 72°C/55 s with a final extension of 72°C/10 min). A portion (5 μl of 100 μl) of the PCR reaction mixture was then utilized for a second 30 cycles of PCR primed with primers 11-UI, 11-DI, A-DI, and A-UI using the same temperature profile. PCRs were conducted according to the manufacturer's instruction (GeneAmp RNA PCR kit, Perkin–Elmer, Branchburg, NJ). The final concentration of deoxynucleotide triphosphates during PCR was 0.2 mM and final concentrations of primers were 0.2 μM in all cases. The final expected size for the E1^E4 amplified product was 294 bp, and the amplified product was for β-actin was 429 bp.

RT-PCR analysis of HPV-16 E6 transcripts

Total cellular RNA (200 ng) extracted from CaSki cells was reverse transcribed in the presence of RT buffer, 5 mM $MgCl_2$, 1 mM dNTPs, 0.2 μM S4 primer as previously described (Sotlar et al., 1998), and 2.5 U of reverse transcriptase in a total volume of 20 μl at 42°C for 1 h. The primers are shown in Table 3. Following cDNA synthesis, primers S4 and S3 specific for HPV-16 E6 (Table 3) were used at 0.04 μM for 30 cycles of PCR (temperature file: 94°C/30 s, 60°C/30 s, 72°C/55 s). The first cycle is preceded by a 2-min denaturation at 94°C and the final cycle is followed by a final extension of 72°C for 10 min. A portion (5 μl of 100 μl) of the PCR reaction mixture was then utilized for a second 15 cycles of PCR primed with nested primers S1 and S2 (Table 2) at 0.4 μM using the same temperature profile. PCRs were conducted according to the manufacturer's instruction (GeneAmp RNA PCR kit from Perkin–Elmer). The final concentration of deoxynucleotide triphosphates during PCR was 0.2 mM. The final expected size for various forms of HPV-16 E6 transcripts are 395 bp (E6/E7 unspliced), 213 bp (E6*I), and 95 bp (E6*II). PCR products (10 μl) were analyzed by electrophoresis on agarose gels and stained with ethidium bromide.

Real-time RT-PCR analysis of HPV-16 E1^E4 and HPV-11 E7 transcripts

Total cellular RNA isolated from CaSki or A431 cells was treated with DNase (DNA-free RNA kit, Zymo Research, Orange, CA) according to the manufacturer's instructions. Quality control (Agilent Technologies 2100 Bioanalyzer, Palo Alto, CA) was done on an aliquot of each DNase-treated RNA to ensure purity and integrity of the RNA. The RNA was then reverse transcribed using the Thermoscript RT-PCR system with random hexamer primers according to the manufacturer's instruction (Invitrogen Life Technologies, Carlsbad, CA). Amplification of the resulting cDNA was carried out in duplicate or triplicate, using TaqMan PCR Core Reagents (Applied Biosystems, Foster City, CA). All primers (Table 4) were synthesized at the Macromolecular Core Facility, Penn State College of Medicine and used at a final concentration of 900 nM. The fluorogenic probes (Table 4) were ordered from MWG Biotech, High Point, NC and used at a final concentration of 200 nM. Standard curves were also run in parallel for each analysis using CaSki or HPV-11-infected A431 cell cDNA with twofold serial dilution down to 1:256. Amplifications were carried out in a 96-well plate in the Applied Biosystems Prism 7700 Sequence Detection System (Applied Biosystems). For HPV-16 E1^E4 amplification, 1 μl (50 ng) of the CaSki cell cDNA was used in a total volume of 50

Table 3
Reverse transcriptase and polymerase chain reaction primers for HPV-16 E6

Primer description	Nucleotide sequence (5′ → 3′)
HPV16	
S1	^{192}-GTGTGTACTGCAAGCAACAG-^{211}
S2	^{586}-GCAATGTAGGTGTATCTCCA-^{567}
S3	^{142}-ACAGTTATGCACAGAGCTGC-^{161}
S4	^{666}-CTCCTCCTCTGAGCTGTCAT-^{647}

Note. Primer pairs specific for HPV-16 E6 and their corresponding nucleotide sequences are shown as published in Sotlar et al. (1998).

Table 4
Reverse transcriptase and real-time polymerase chain reaction primers and probes

Primer description	Nucleotide sequence (5′ → 3′)
HPV11	
E7 Forward	^{647}GTGGACAAACAAGACGCACAAC^{668}
E7 Reverse	^{756}ATGTCTCCGTCTGT-GCACTCC^{736}
E7 Probe	(FAM)-^{370}CAGTCGGACGTTGCTGTCACATCC-A^{706}-(TAMRA)
HPV16	
E1^E4 Forward	^{870}TGATCCTGCAG^{880_3358}AGCAACGAA^{3366}
E1^E4 Reverse	^{3427}TGGTATGGGTCGCGGC^{3412}
E1^E4 Probe	(FAM)-^{3368}TATCCTCTCTCCTGAAATTATTAG-GCAGCACTTGGC^{3401}-(TAMRA)

Note. Names of primer pairs specific for HPV-11 E7 and HPV-16 E1^E4 and their corresponding nucleotide sequences are shown. The numbers represent the nucleotide numbers in the HPV-11 and HPV-16 complete genomes. Note that the HPV-16 E1^E4 forward primer was designed to overlap with the splice donor/acceptor sites.

μl/well. Parallel detection of 18S cDNA for each sample utilized reagents from a commercially available kit (TaqMan Ribosomal RNA Control Reagents, Applied Biosystems) with a fluorescent group, VIC, as the reporter. Alternatively, mock-infected CaSki cDNA of various time points was used as control. The annealing temperature was 55°C. The expected amplicon for HPV-16 E1^E4 from spliced cDNA is 80 bp and the predicted size for the amplification product from the HPV-16 genome is 2558 bp; genomic product is never observed on gels. For HPV-11 E7, 3 μl (67.5 ng) of A431 cell cDNA was amplified in a total volume of 50 μl/well. A431 cDNA from samples infected with HPV-11 alone was used as control. The annealing temperature was 60°C. The expected amplicon for HPV-11 E7 is 110 bp.

In vivo double-infection assay

Human vaginal tissue was obtained from resected vaginal wall removed during reconstructive surgeries. Specimens were held at 4°C until grafting (within 24 h) in MEM supplemented with gentamycin (0.4 mg/ml), penicillin and streptomycin (0.08 mg/ml each), fungizone (2.5 μg/ml), sodium bicarbonate (0.05%), and HEPES (10 mM). Split-thickness human vaginal epithelia (20 × 20 × 1 mm) were rolled into tubes with the vaginal epithelial surface on the inside and grafted subcutaneously to athymic mice receiving estrogen supplementation in silastic tubes (Sigma Chemical Co., St. Louis, MO) to maintain 150–200 pg/ml estrogen in circulation (Robinson et al., 1989). At 1–2 weeks after xenotransplantation, grafted vaginal tubes were infected with 10 μl of infectious HPV-11 virus stock by opening the skin, exposing one end of the graft, and then inserting a micropipette tip into the xenograft lumen. By 10–15 weeks, grafts are known (from previous experiments) to be morphologically transformed and productively infected by HPV-11 (Howett et al., 2000). Grafts were then infected with HSV-2 at 10–15 weeks. One week following HSV-2 infection, infected grafts were harvested by excision and longitudinal sectioning of grafts for standard morphology (standard formalin-fixed, paraffin embedded, hematoxylin and eosin stained histology sections), for virus DNA content (in situ hybridization). (Fig. 8) as previously described (Howett et al., 2000).

^{3}H-labeling assay for assessment of protein synthesis

CaSki cells were seeded at 5 × 10^5 cells/plate in 60-mm tissue culture plates the night before infection. Cells were then infected with HSV-2 at an m.o.i of 10 PFU/cell in the presence of 50 μg/ml cycloheximide (U.S. Biological, Swampscott, MA) or without cycloheximide as a control. After 1-h incubation at 37°C, virus inoculum was replaced by medium containing ^{3}H-labeled amino acids at 10 μCi/ml and incubated at 37°C for 4 h. Cells were then washed twice with PBS and scraped off the plates into 1 ml of PBS. Cells were spun down and the cell pellet was resuspended in 200 μl RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS). Ten microliters of the above cell lysate was spotted onto a filter paper and washed twice with 5% TCA, 95% ethanol and acetone, respectively. The filters were air-dried before counting in the scintillation counter in the presence of Scintisafe Econol 1 (Fisher, Pittsburgh, PA).

In situ hybridization

A biotin label was incorporated into DNA using the random priming method (Amersham, Cleveland, OH). Deparaffinized, rehydrated sections of standard paraffin-embedded tissues were digested with pepsin (4 mg/ml in 0.1 N HCl), washed in Tris–HCl, pH 7.5, dehydrated, and covered with a hybridization cocktail containing 0.6 M NaCl, 10 mM Tris pH 7.4, 0.5 mM EDTA, 1 mg/ml bovine serum albumin (BSA), 0.02% polyvinylpyrrolidone (PVP) (w/v), 0.02% Ficoll (w/v), 0.15 mg/ml yeast tRNA, 5 mM DTT, 10% dextran sulfate (w/v), 50% formamide (v/v), and 1 ng/μl labeled DNA. The probe and tissue were denatured together by heating the slides at 100°C for 6 min. After heat denaturation, slides were incubated in a moist chamber at 37°C for 2 h, washed three times for 10 min each in 2 × SSC (0.3 M sodium chloride and 0.03 M sodium citrate, pH 7.0), and incubated with avidin-conjugated alkaline phosphatase for 30 min. After washing in Tris–HCl buffer (pH 9.5) three times for 10 min, the McGady reagent was used to develop signal color and sections were counterstained with Nuclear Fast Red.

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