QUANTITATION OF HPV-16 E6/E7 TRANSCRIPTION IN CERVICAL INTRAEPITHELIAL NEOPLASIA BY RT-PCR.

A Thesis Presented to the
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by
Euginette M. Hsu
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QUANTITATION OF HPV-16 E6/E7 TRANSCRIPTION IN CERVICAL INTRAEPITHELIAL HEOPLASIA BY RT-PCR

BY

EUGINETTE M. HSU

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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ABBREVIATIONS

nt	nucleotide
bp	base pair
DNA	deoxyribonucleic acid
mRNA	messenger ribonucleic acid
HPV	human papillomavirus
HSV	herpes simplex virus
ORF	open reading frame
LCR	long control region
GRE	glucocorticoid responsive element
CIN	cervical intraepithelial neoplasia
OC	oral contraceptive
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase-PCR
HET	human papillomavirus type 16 E6/E7 transcription
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
dpm	disintegrations per minute

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1. Abstract

Human papillomavirus type 16 (HPV-16) is associated with neoplastic lesions of the uterine cervix. Viral transforming functions have been localized to the E6/E7 open reading frame (ORF) and this ORF is conserved consistently in cervical intraepithelial neoplasia (CIN). Two mRNAs, generated by alternative splicing, are expressed from the E6/E7 ORF. These are known as E6*I and E6*II, and potentially encode the viral E7 and E6 proteins, respectively. It is believed that the HPV-16 transforming capability is mediated by the E6 and E7 proteins. The viral E6 protein forms an inactivating complex with the cellular tumor suppressor protein known as p53, while E7 binds to the retinoblastoma cellular protein. Both p53 and retinoblastoma are important cell cycle regulatory proteins. A quantitative RT-PCR assay was developed using the CaSki cervical carcinoma-derived cell line, in order to characterize the relative expression of E6/E7 spliced transcripts. The ratio of 97.1% to 2.9% for E6*I to E6*II is thought to be representative of the transformed phenotype of CaSki cells. This assay was applied to exfoliated cervical cells obtained from patients in varying stages of clinically defined CIN, and the relationship between viral expression, disease stage, oral contraceptive use, and age was studied. There was a direct correlation between relative proportions of E6*I/E6*II mRNAs greater than 95%/5%

and increased disease severity. As disease severity increased, there was a trend toward a greater proportion of E6/E7 mRNA comprised of the E6*I species. Characteristic trends in E6/E7 transcription profile were observed in association with normal histology and CIN. In CIN, E6*I expression tended to be higher while E6*II was lower; however, in normal cells, this trend was reversed. Additionally, there was a significant association ($p = 0.05$) between increased age and greater expression of E6*I relative to E6*II for oral contraceptive users. This study underscores the importance of the relationship between viral transforming gene transcript quantities and the course of cervical disease. It also suggests that quantitation of HPV-16 E6/E7 genes may be useful as a prognostic tool to indicate women at increased risk for developing cervical cancer.

2. Introduction

Uterine cervical carcinoma is an important health concern; worldwide, it kills millions of women each year [114]. Epidemiological observations suggest that since the disease behaves like a sexually transmitted infection [1,43,80,87,124,130,148,160], the etiology may lie in a transmissable agent. In 1974, zur Hausen [179] proposed that the human wart virus, human papillomavirus (HPV) was responsible for malignant cervical neoplasia. The association between the two has been intensely studied, and is based along several lines of evidence. It has been shown that HPV is found in almost all of cervical dysplasias and carcinomas [180], and the viral DNA is often integrated into the human genome [32]. Cell lines derived from cervical carcinomas frequently contain HPV DNA [117,144], and transfection by HPV DNA can cause transformation and immortalization of cultured cells [75,159].

Of particular interest is HPV type 16 (HPV-16), which is the genotype of HPV most frequently isolated from carcinomas and precancerous dysplasias [180]. The transforming potential of HPV-16 has been mapped to the E6/E7 open reading frame (ORF), since it is invariably retained during viral genomic integration into the cellular genome [3,16]. The mRNAs expressed from this ORF are found in transformed cells [75,169], and transfection by the E6/E7

ORF is sufficient to cause transformation and immortalization of cultured cells [54,159,169].

Two mRNAs of interest are expressed from the E6/E7 ORF, and are generated by alternative splicing. They are known as E6*I and E6*II, and potentially encode the E7 and E6 proteins, respectively [149,150]. It is believed that HPV-16 effects transformation via these proteins. The E7 gene product has transactivating properties as well as transformation functions in a fashion similar to adenovirus type 5 E1A proteins [123]. The protein induces aneuploidy in epidermal keratinocytes [53], and is able to bind to the cellular tumor suppressor protein retinoblastoma [34,105]. The viral E6 protein has also been shown to have transactivating properties in vitro [27], and forms an inactivating complex with the cellular tumor suppressor protein known as p53 [142,171]. Thus, there appears to be a role for both E6 and E7 proteins in transformation.

Cervical neoplasia is a progressive disease that can be clinically defined into different stages of cervical intraepithelial neoplasia (CIN). Mild dysplasia is known as CIN I, while the most severe form of disease, which is the stage immediately preceding invasive carcinoma, is known as CIN III [52]. It has been shown that both E6 as well as E7 are required to transform and immortalize human keratinocytes [54,104], and in fact both E6*I and E6*II mRNAs can be found in all stages of CIN [21,65,146] as well as a cell line derived from a cervical carcinoma [150].

Early data suggested that the degree of expression of E6/E7 genes correlated with the proliferation of cervical carcinoma cells [165], and no change in splicing was observed in relation to advancement of disease [21,65,146]. Thus, the hypothesis of this investigation states that quantitative variation in the transcription of E6*I and E6*II is associated with disease progression. To study this correlation, a quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay was developed using a cervical carcinoma-derived cell line known as CaSki [59]. This technology was then applied to exfoliated cervical cells obtained from patients in varying stages of disease. Additionally, it was of interest to determine if there was any association between transcriptional patterns and demographic factors, such as age and the use of oral contraceptives (OC), since epidemiological data suggests that younger women may be at increased risk for cervical disease [12,29], and oral contraceptive data remains controversial [61,119,173].

It was found that for CaSki cells, the proportion of mRNA expressed from the HPV-16 E6/E7 ORF was comprised of 97.1% E6*I, and 2.9% E6*II [59]. This substantiated Northern blot data which showed that the major E6/E7 mRNA species was E6*I [150]. It is speculated that these relative proportions are reflective of the fully transformed status of CaSki cells.

When the assay was applied to patients' cells, it was found that some patients had HPV-16 E6/E7 expression detectable (HET+) while others did not (HET-). HET+ patients tended to have higher grade disease than HET- patients. Among the HET+ women, three transcription profiles were observed: expression of E6*I alone or E6*II alone, or co-expression of E6*I and E6*II. No association was seen between transcription profile type and stage of CIN. Of the patients who expressed both E6*I and E6*II, it was found that there was a correlation ($p = 0.05$) between high-grade CIN and E6*I/E6*II $\geq$ 95%/5%. When compared to CaSki cells, it was shown that with an increase in the degree of transformation, there was greater expression of E6*I and less expression of E6*II.

The findings of this study show that the expression of viral transforming genes may quantitatively vary in association with progression of disease as well as other demographic factors. It has been demonstrated that studies of viral expression dynamics will be invaluable to the understanding of tumor virology.

3. Literature review

Cervical cancer

Carcinoma of the uterine cervix is the third most frequently occurring human gynecological cancer, with 13,500 new cases in the United States in 1992. However, this statistic does not include about 55,000 new cases of carcinoma in-situ of the uterine cervix annually, and large numbers of precancerous dysplasias [7]. The incidence of uterine cervical cancer is declining in Canada [107], and in the USA, survival rates have shown a gradual increase [7]. This may be attributable to the introduction of the Papanicolaou smear, a now routine screening procedure that detects precancerous epithelial cell abnormalities which are treatable before the progression to invasive carcinoma. However, there is a higher prevalence of cervical cancer among North American aboriginal populations [74,107,177] compared to overall Canadian and American incidence, and it is in fact the most frequently diagnosed type of cancer among Canadian Inuit women [57]. In developing nations, cervical cancer kills more women than any other cancer, and on a worldwide scale, it ranks second to breast cancer as a neoplastic cause of death [114]. Clearly, an understanding of the etiology of uterine cervical carcinoma is of great importance.

The mechanism for the development of cervical carcinoma is beginning to be understood; it is a small question within the overall scheme that this thesis attempts to answer.

Etiology of cervical cancer

The notion that the etiology of cervical cancer lies within a sexually transmitted infectious agent has its roots in numerous epidemiological observations. Firstly, cancer of the uterine cervix is almost unknown in Catholic nuns [43,160]. Secondly, extramarital sexual activity [87,124], early age of first sexual intercourse [1], history of prostitution [130], history of syphilis [80,130] and marriage to men with penile cancer [148] are all associated with an increased risk of this malignancy. Consequently, a search for a horizontally transmitted oncogenic agent began, with viruses emerging as the most promising candidates.

Initially, it was thought that Herpesvirus type 2 (HSV-2) was responsible for cervical carcinoma. This was based on seroepidemiological observations showing a high correlation between HSV-2 infection and the incidence of cervical carcinoma. Moreover, a higher incidence of antibodies to HSV-2 was found in women with carcinoma than in matched control women [66,106,127,128]. Additionally, <u>in vitro</u> studies have shown that malignant transformation of hamster cells occurs after infection by partially inactivated HSV. Further studies have revealed HSV-specific RNA in premalignant and malignant cervical tissue

[35,85,95]. However, the hypothetical role of HSV in human genital cancer has been weakened by the inability to detect HSV-specific DNA in cervical cancer biopsy samples [178], except in one study by Frenkel et al. [41]. Furthermore, careful analysis of epidemiological data shows no support for HSV-2 involvement in cervical neoplasia [118,166]. Thus evidence, although suggestive of a possible link between HSV-2 infection and cervical carcinoma, is circumstantial. A direct cause-and-effect relationship has yet to be shown.

Condylomas and human papillomavirus: a brief history

Great interest has developed in the relationship between papillomaviruses and cervical cancer in the past twenty or so years. In 1956, Koss and Durfee [69] were the first to describe what they called "koilocytotic changes" in the uterine cervical squamous epithelium. These cells were characterized as being large cells with small, irregular and hyperchromatic nuclei, surrounded by a transparent cytoplasm which resembles an empty space. These cells were then designated "koilocytes", "koilos" being Greek for "hollow" or "cavity". However, it was not until 1976 that Meisels and Fortin [98] again reviewed epithelial atypia; they described the presence of koilocytes in condylomatous warts. Condyloma acuminata, or warty lesions, were described by the ancient Greeks. It is now known that they are sexually transmitted, and that the agent responsible for condyloma acuminata is the human papillomavirus (HPV) [30,110].

Koilocytes, as seen in condylomas, are now considered hallmarks of HPV infection [99], and it has been shown that HPVs of different types can be isolated from these warts [71].

It was Shope who first proposed the papillomavirus as the etiological agent responsible for causing infectious warts, or papillomas in rabbits [147]. The association between papillomavirus and cancer was first noted in 1935, by Rous and Beard [134] who described how the viral agent causing benign papillomas (also known as Shope rabbit papillomas) on wild cottontail rabbits caused malignant tumors to arise when used to induce papillomas on rabbits of the domestic species.

HPVs and human cancers: early studies

With respect to human cancers and HPVs, one of the earliest observations showing a relationship between the two was seen in patients suffering from epidermodysplasia verruciformis, a rare autosomal recessive disease characterized by persisting disseminated flat warts. It has been shown that approximately 30% of these patients develop skin cancer [84] which tends to arise during the third or fourth decade, and that wart biopsies display malignant changes typical of invasive squamous cell carcinomas [135]. Moreover, papillomavirus particles have been detected in benign warts, in contrast to malignant tumors [174] wherein no complete viral particles are synthesized.

The carcinomas are primarily found at sites exposed to sunlight, such as the forehead [62]. This suggests that papillomaviruses act synergistically with some extrinsic environmental factor (most likely ultraviolet light) to effect malignant conversion. Therefore, epidermodysplasia verruciformis serves as a useful model in the study of papillomavirus infection and the steps involved in neoplastic transformation.

The recognition that HPVs were present in the warts that could potentially undergo malignant conversion led to subsequent molecular analyses of virus DNA in genital wart lesions and cervical carcinomas. The first of such studies began in 1974, when zur Hausen et al. [179] attempted to hybridize virus-specific DNA sequences of tumors, using complementary RNA transcribed from benign wart virus DNA. It was found that although benign plantar warts hybridized well, condylomata acuminata, laryngeal papillomas, and all malignant tumors did not anneal, suggesting that different types of HPVs were responsible for variant lesions.

Papillomaviruses: biology and taxonomy

Until recent advances in recombinant DNA technology, papillomavirus research was hampered by the inability to propagate the virus in vitro in cell culture. Furthermore, they could not be analyzed easily from wart material because intact virus particles are produced in low quantities. However, current techniques, particularly the advent of

molecular cloning, have led to a tremendous surge in knowledge of the biochemistry and biology of papillomaviruses (PVs).

PVs are one of two genera in the viral family Papovaviridae, group A being the PVs and group B including the polyoma and vacuolating viruses. Within the PVs, taxonomy is based first on host range. Further classification is based not on serological similarity, but by DNA homology as determined by reassociation kinetics in liquid phase [20,90]. By definition, less than 50% cross-hybridization of heterologous viral DNAs indicates an individual type. Isolates showing greater than 50% homology but differing in restriction endonuclease cleavage patterns are designated as subtypes. So far, about a dozen animal PVs and more than 70 human PVs have been described, with numerous subtypes.

The PV virion is composed of a double-stranded DNA genome enclosed by a protein capsid made up of viral encoded proteins. The outer capsid size is 55 nm as determined by electron microscopy, is non-enveloped, and has icosahedral symmetry. The PV DNA is a double-stranded circular molecule with type-specific size differences; it has a molecular weight of about 5 megadaltons, corresponding to a length of approximately 8,000 base pairs (bp) [47,68]. The G+C content also shows type specificity, ranging from 41% to 50% [23,103].

PVs are widespread in nature; they have been identified in numerous animal species such as cattle, sheep, dogs, deer, rabbits, mice, monkeys, and finches [73,103,112,120,126]. Amongst these, it is the bovine PVs which have been most extensively studied, and in fact bovine papilloma virus type 1 (BPV-1) is used as a model for studying other PVs.

Infection

PVs cause proliferations of the surface or mucosal epithelium, and as with most virus types, the target cell is confined to these types of cells. PV-induced tumors are usually benign, have limited growth, and often spontaneously regress [88,136]. Malignant conversion can occur, but usually after long persistence of lesions.

PV DNA can exist as two forms: either as stable episomes or integrated into the cellular genome. It has been found that production of viral particles is seen only in highly differentiated keratinocytes. In other words, epidermal cells are non-permissive at the beginning of their differentiation process, yet become increasingly permissive with increased differentiation. In latently infected transformed cells where the virus exists exclusively as a genome, genomic replication is possible but no viral progeny is produced. The cellular environment does not allow for expression of late viral genes and thus mature virions are not assembled [121,153], although the mechanism is unclear.

Papillomavirus genetic organization

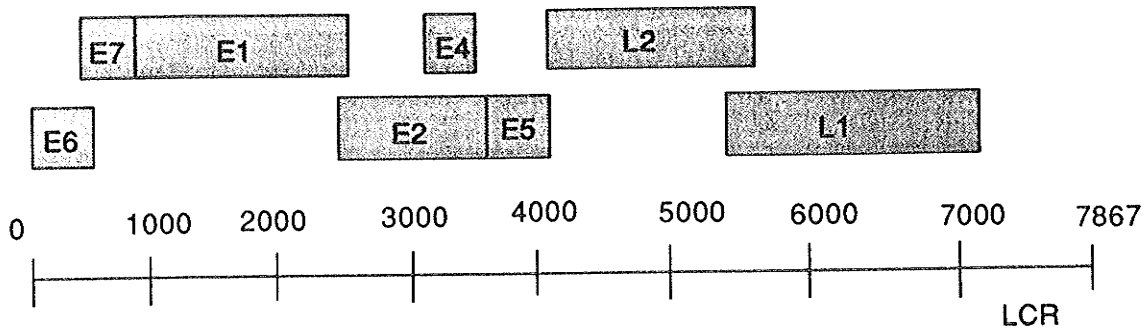
Generally speaking, all PVs have a similar genetic organization, (see Fig. 1) with approximately ten open reading frames (ORFs) encoding protein located on the same strand of DNA [153]. Transcripts derived from this strand fall into two categories; the first is the E (early) coding region, which contains the early genes E1-E8, although the E3, E5 and E8 ORFs are not conserved in all PVs [70]. The second region encodes the late (L) genes, L1 and L2. The early genes encode proteins responsible for viral replication and transformation, and the late genes contain information for structural viral proteins such as the capsid proteins, and are expressed only in differentiated keratinocytes. The long control region (LCR) or upstream regulatory region (URR) is a noncoding stretch of DNA of about 1,000 bp upstream of the E6 ORF. It has been found to contain the origin of replication, AT-rich tracts, transcriptional promoter sequences, transcription enhancer sequences, and several copies of the $ACCN_6GGT$ palindrome, which is the binding site for viral E2 regulatory protein [14,82,154,168].

The oncogenic potential of papillomaviruses

Amongst the strongest data suggesting an oncogenic potential of PVs is the fact that certain types of PVs are found in association with malignant conditions. For

Figure 1. HPV-16 genome

linear representation



base pairs

example, as previously described, cottontail rabbit virus (CRPV) causes malignant conversion in infected domestic rabbits [134]. It was found that few virus particles are produced in the lesions of these animals, yet CRPV genome can be isolated from all of the malignant tumors [157,170].

In contrast, viral genome could not be detected in malignant tumors of cattle with alimentary tract cancer, which results after infection with BPV-4 and subsequent ingestion of bracken fern [64,140]. This may indicate that malignant conversion in the presence of certain co-factors no longer requires the PV genome.

As stated previously, BPV has served as a model for understanding the molecular genetics of PVs. It has been found that a fragment encompassing 69% of the BPV-1 genome between unique HindIII and Bam HI restriction endonuclease sites, of length 5.6 kb, is sufficient for transformation of mouse cells in vitro [81]. Furthermore, all the polyadenylated RNAs expressed in transformed cells are transcribed from this region and range in size from 1,000-4,000 bases [55]. Sequencing cDNA copies of these transcripts has shown that transformed cells appear to contain RNAs derived from differential splicing. All the RNA transcripts detected in transformed cells are polyadenylated at the same site (base 4203), suggesting that RNAs present during transformation are a subset of the transcripts produced during a productive infection [37,175]. The E2 ORF appears to encode two proteins regulating an

enhancer element in the LCR [155] and thus indirectly controls transcription of the E5 and E6 ORFs. The dissection of the BPV-1 genome has also revealed the importance of other early ORFs: the E1 ORF appears necessary to maintain the BPV genome as an episome, and E7 is thought to maintain a high copy number of episomal viral DNA [83,139]. The E5 region, which is predicted to encode a hydrophobic low molecular weight peptide, has also been found to have transforming ability [143]. Deletion mutants lacking the E6 and E7 ORFs are still capable of effecting transformation, but at much lower efficiency [139].

HPV-associated human cancers show similar patterns: HPVs are often found in association with warts of patients suffering from epidermodysplasia verruciformis as described above. In cases of malignant conversion, it is the DNA of HPV-5 or HPV-8 which are found most often [111]. Additionally, the HPV-5 genome has been isolated in metastatic lesions associated with the disease, which indicates the continued presence of the viral genome in the maintenance of the malignant state. Moreover, the observation eliminates the possibility that HPV-5 DNA was present due to contamination from adjacent benign lesions [113].

HPV infection: the spectrum of clinical manifestation

HPV and its association with warts has long been known, however it is also correlated with numerous other conditions, encompassing a spectrum ranging from clinically inapparent infections to benign or malignant lesions. Because HPV-associated lesions often recur, it is speculated that the virus is able to persist in an asymptomatic infection. This has been shown to be true in numerous cases wherein HPV DNA was demonstrated in normal cervices [22,26], the respiratory tracts of children with recurrent respiratory papillomatosis [156] and in the histologically normal skin of the genital area of patients with genital warts [39]. In condylomas, possibly the most frequent hallmark of HPV infection, lesions may show varying degrees of histological alterations. Mucosal epithelium (such as vagina, cervix, rectal mucosa) that has been infected shows little or no keratinization, while lesions on cutaneous epithelium (such as vulva, penis, perianal area) are keratinized. Viral DNA is often present in all layers of the infected epithelium, including the basal layer, yet expression of genes is closely tied to the degree of cellular differentiation [131]. Thus, cellular factors likely play a prominent role in the transforming capability of the virus.

Dysplasia represents another category of HPV infection and decribes the precancerous conditions that can progress

to invasive carcinoma of the cervix [108]. Mild dysplasias begin in the cervical epithelium as flat condylomas. These condylomas, which resemble warts, show characteristic koilocytosis--cytoplasmic vacuolization and nuclear pleomorphism. The dysplasia eventually may become increasingly less differentiated, with accompanying modifications in cell and nuclear size. These stages are clinically defined as cervical intraepithelial neoplasia (CIN) grades I-II, and can progress to CIN III, or severe dysplasia. At this stage, greater variation of cell and nuclear size occurs, cellular orientation becomes disorderly and differentiated surface cells and koilocytotic changes are nearly absent. At this point, also known as carcinoma in-situ, epithelial alterations are confined to the epithelium; the next stage is invasive carcinoma. It is at this time that cancerous epithelial changes begin to invade underlying tissue, and is designated "invasive carcinoma" [129].

Malignant transformation is the final clinical manifestation in the continuum of HPV infection. As in the case of epidermodysplasia verruciformis, HPV DNA has been reported in several types of cancers. Vulvar and penile carcinomas have been found to contain HPV DNAs [92,125,158,164], as have laryngeal, tonsillar, and prostate carcinomas [67,94,152,165], supporting the hypothesis of HPV's involvement in neoplastic disease. HPV DNA in cervical carcinomas is of particular interest; using HPV-6

DNA cloned from a condyloma acuminatum and HPV-11 DNA, it was demonstrated that these types of HPV are associated with benign (condylomata acuminata) and precancerous (mild dysplasia) lesions [reviewed in 46]. Using nonstringent hybridization conditions, zur Hausen et al. identified, then cloned HPV-16 and HPV-18 from cervical carcinomas [9,31]. Several other types are implicated in genital cancers, although HPV-16 is present in approximately 50% of cervical carcinomas. HPV-18 is found in about 20% of cervical cancers, and HPV-33 is detected in an additional 10% [reviewed in 44,45]. Other types such as HPV-31 and HPV-35 account for up to 10% [49].

Another line of evidence suggestive of the role of HPV in transformation is seen in studies of cell lines that have been derived from cervical cancers. For example, the commonly used HeLa cell line has been shown to contain HPV DNA integrated into the cellular genome. [144,145].

It has been shown that the physical state of the HPV genome generally correlates with tissue pathology; nonmalignant tissues frequently contain viral DNA that exists as an episome, while anogenital cancer biopsies generally carry the genome integrated into the cellular genome [17,32]. Although there appear to be no insertional "hotspots", that is, locations on the host genome that are consistently disrupted during integration of the viral genome, it appears that integration of the circular viral genome commonly occurs within the 3′ end of the E1 ORF or

the 5′ end of the E2 ORF, which potentially disrupts viral autoregulation [144,155].

Mounting evidence strongly supports the hypothesis that HPV is the etiologic agent responsible for cervical carcinoma. A closer investigation of the molecular mechanisms behind this phenomenon follows below.

Transcription of HPV-16

As stated previously, HPV-16 DNA is often integrated into the cellular genome in single or multiple copies in malignant tumors, while remaining in episomal form in benign lesions [32]. In transformed cells, it has been shown that it is consistently the E1/E2 ORFs that are disrupted when the circular viral genome linearizes, and that the E4 and E5 ORFs have been found to be deleted [15,75,79,117]. It is therefore the viral long control region (LCR) and the ORFs at the 5′ end of the early region, E6 and E7, which are immediately downstream of host cell sequences.

In contrast to gene expression during a productive infection, transforming infections involve transcription of early genes only. The HPV late genes, which encode virus structural proteins, are neither consistently conserved during viral integration into the cellular genome nor cause transformation of cells. Therefore the following discussion will be confined to the HPV-16 early ORFs most salient to the process of neoplastic transformation.

The E2 ORF

The HPV E2 ORF and that of the more extensively studied BPV-1 E2 ORF encodes a regulatory protein that increases the enhancer activity of the LCR of the viral genome [58,86,155]. For HPV-16, the E2 gene product binds to $ACCN_6GGT$ motifs in the LCR, upstream of the P_{97} promoter that controls expression of the E6 and E7 ORFs. In CAT assays, the E2 gene product has been found to increase levels of transcription of the E7 ORFs within the cell, resulting in increased ability to transform primary epithelial cells in co-operation with an activated ras oncogene [77]. Recently, it has been reported that a repressor function has also been found with the E2 protein of some virus types, and thus the E2 protein probably has either enhancing or repressing effects on transcription, mediated by the E2-binding sites in the LCR [78,132,161]. Thus, disruptions of the viral E2 ORF during integration must have important effects on the control of viral transcription. In particular, this perturbation may have important ramifications in controlling transforming gene expression.

The LCR

The long control region of papillomaviruses plays a significant role in the process of viral transformation. In BPV-1, a strong enhancer element in the LCR is regulated by

a cellular transcription factor and the E2 repressor, and is essential for viral DNA replication, transformation, and the maintenance of the BPV-1 genome in latently infected cells [162]. In a human keratinocyte cell line immortalized by HPV-16, it has been shown that the LCR contains an epidermal growth factor (EGF)-responsive element. The addition of EGF to the media resulted in down-regulation of HPV-16 E6/E7 at the mRNA level [176]. This suggests that growth-related specific cellular factors may mediate transcriptional regulation of the HPV-16 E6/E7 transforming genes by interacting with LCR elements. The LCR of HPVs may also be in itself an important determinant of the viral transforming ability. The LCR/E6/E7 region of HPV-18 is more efficient at immortalization than the corresponding region in HPV-16. The E6/E7 genes of both types of HPVs show similar immortalization efficiencies, suggesting that the difference may lie in varying transforming capabilities in the transcriptional regulatory regions upstream of these genes. Chimeric HPV-16 and HPV-18 LCR/E6/E7 constructs confirm this observation, indicating that the LCR upstream of the E6 and E7 genes is a major determinant of the differential immortalization potentials of human papillomavirus types 16 and 18 [133].

The E6/E7 ORF

Disruption of the viral early ORFs during integration may result in altered expression from the E6/E7 ORF

immediately downstream of the integration site. Several studies have strongly implicated the involvement of this ORF in malignant transformation. It is this ORF which is conserved when the HPV-16 genome integrates into the human chromosome in cervical carcinomas [16,167] and cervical carcinoma cell lines [117], and abundant E6/E7 transcripts are found in transformed cultured cells [3,75,169]. Products of the E6/E7 region have been observed to cooperate with activated Ha-ras oncogene in transforming primary rodent fibroblasts [89]. Additionally, it has been shown that HPV-16 E6 and E7 proteins together immortalize human fibroblasts and keratinocytes in vitro [54,169]. The E6 and E7 genes of the oncogenic HPVs appear to have intrinsically different biological activity and regulation of expression compared to those of the HPVs considered to be low-risk for causing malignant transformation [5], and transcription of these genes occurs via different mechanisms [149]. The mechanisms of these properties are under intense scrutiny, and thus far it has been found that the E6 protein of HPV-16 and HPV-18 is capable of binding to the cellular protein p53, which has transformation suppressing properties [40,171]. Moreover, this association appears to stimulate the degradation of p53, which may inactivate cellular control mechanisms and thus allow abnormal proliferation [24,142]. Preliminary studies have also shown that the E6 protein of HPV-16 behaves as a transcriptional activator of heterologous promoters [27], however whether the binding of

p53, transactivation, and transformation functions of E6 are related remains to be clarified.

The E7 gene product has been found to encode transactivating properties as well as cellular transformation functions similar to those of the adenovirus type 5 E1A proteins [123], and induces aneuploidy in epidermal keratinocytes [53]. Furthermore, the HPV E7 protein is able to bind to the cellular retinoblastoma tumor suppressor gene product (Rb), and E7 proteins derived from the "low-risk" HPV-6 and HPV-11 show lower affinities for the retinoblastoma gene product than does E7 protein encoded by the oncogenic HPVs [34,105]. Deletion mutagenesis studies have suggested that transformation and transactivation require overlapping domains of the E7 gene product [122]. The region of overlap is analagous to a conserved domain in adenovirus E1A, which suggests that binding with the Rb gene product may be necessary for both activities.

The importance of the role played by the p53 and Rb tumor suppressors in cervical neoplasms remains unresolved. The HPV-18 cervical cancer-derived HeLa cell line has recently been shown to have an extremely unstable p53 protein [91], in keeping with the in vitro observation that the E6 protein of HPV-16 or 18 degrades p53. Two HPV-negative cell lines, C-33A and HT-3 have mutations in the p53 and Rb genes, respectively [141]. However, in the case of p53, similar findings have not extended to patient

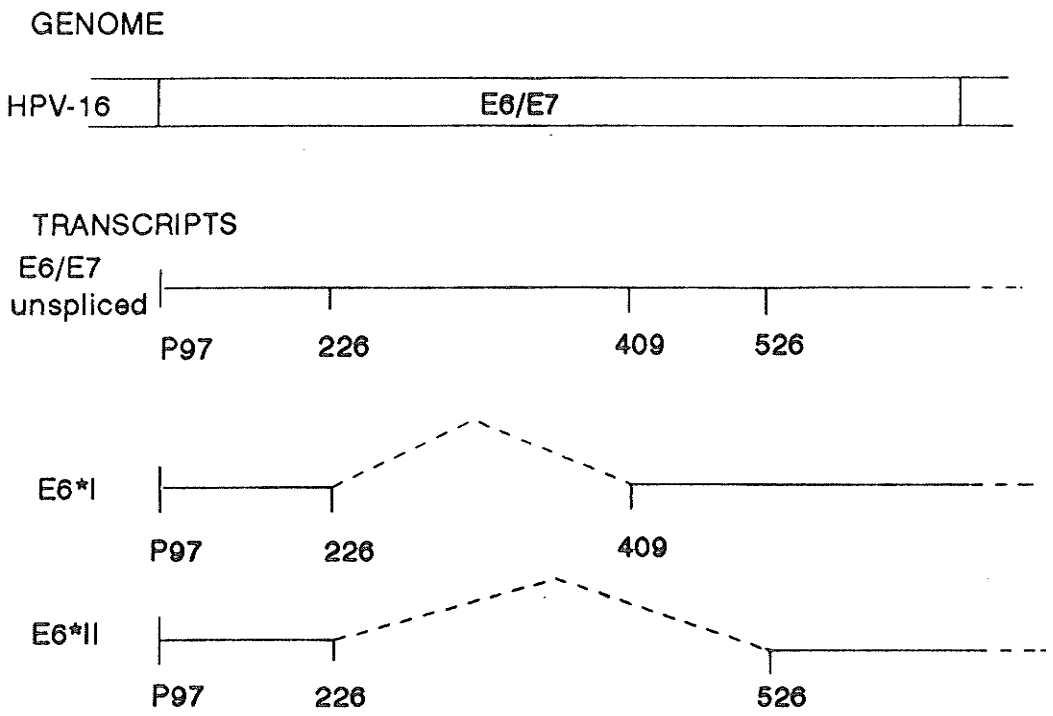
isolates [8,10,42,96], and the implication that somatic mutation in the absence of HPV causes transformation remains controversial.

Depending on the system studied, there appears to be varying requirement for E6/E7 gene products to effect transformation. In vitro, E6 and E7 are both required to transform human foreskin keratinocytes; E7 transfected alone induces hyperproliferation, but E6 is necessary to maintain the immortalization phenotype [54]. Both the E6 and E7 genes of HPV-16 were found to be required for the transformation of primary human fibroblasts [169], and transcripts from this region have been detected in primary human epidermal cells as well as carcinomas [21,65,93,150]. However, in rodent cells, transforming functions have been mapped to the E7 gene alone [123,159]. This implies that amongst varying species there exists host-specific elements that may influence the transformation abilities of proteins produced from this ORF. The E6 and E7 proteins have been found to contain the Cys-X-X-Cys motifs characteristic of zinc finger proteins; this may be indicative of DNA binding activity [4,50]. The E7 protein is found mainly in the cytoplasm, is phosphorylated at unspecified serine residues, and is thought to interact with itself or with cellular proteins [151].

Three transcripts expressed from the E6/E7 ORF in cervical cancer-derived cell lines (CaSki, SiHa) have been identified. All initiate from the p97 promoter and two are

generated by alternative splicing of one intron [149,150]

Figure 2. Spliced transcripts of the HPV-16 E6/E7 ORF.



p97 is the promoter-binding site

hatched lines indicate splice site

(Figure 2). The unspliced mRNA encodes the full-length E6 protein, which has been found to have trans-activating as well as transforming properties in cultured keratinocytes [145]. There are two transcripts generated by processing at a splice donor site at nucleotide (nt) position 226, E6*I having a splice acceptor site at nt 408, potentially encoding the E7 protein, and E6*II with a splice acceptor site at nt 526, putatively encoding the E6 protein.

Recently it was shown using the polymerase chain reaction (PCR) that both spliced E6 transcripts were detected in HPV-16 transformed human fibroblasts, premalignant lesions and carcinomas, indicating no qualitative variation in splice patterns in association with disease progression [21]. This was corroborated by Johnson et al. [65] who found the same complement of spliced mRNAs in cervical intraepithelial neoplasia (CIN), invasive cervical carcinoma, and in lower amounts, in cytologically normal cervical tissue. These findings were verified and extended by Sherman et al. [146] who found no relationship between splicing pattern and tumor progression of E6/E7 mRNAs. The results suggest that quantitative rather than qualitative differences in E6/E7 gene products may correlate with the progression of the disease and may be a better predictor of disease risk since the intracellular concentration of HPV-16 E6 and E7 proteins are directly linked to the proliferative capacity of cervical cancer cells [165].

This current work attempts to quantify HPV-16 E6/E7 gene expression in association with stages of CIN and other demographic factors. Development of a modified polymerase chain reaction, employing a reverse transcription step prior to amplification (RT-PCR) facilitated the relative quantitation of HPV-16 E6/E7 gene expression.

4. Patient demographics

Cervical intraepithelial neoplasia (CIN)

The work of Johnson, Cornelissen, and Sherman has shown that there exists no difference in the types of transcripts expressed from the HPV-16 E6/E7 transforming gene in cells at different stages of CIN [21,65,146]. Thus, it is of great interest to determine if the relative amounts, or quantitative differences of these spliced products show any correlation with disease progression.

Age

It was recently found that the prognosis of cervical carcinoma patients may be distinguishable on the basis of age and detectable HPV RNA. A younger group with detectable HPV RNA had a better disease outcome while increased age and mortality were associated with carcinomas negative for HPV RNA [56]. HPV infection is common among sexually active young women [6], and CIN may now be more prevalent among women in their second and third decade [12,29] than older women. That is, epidemiological studies indicate that

younger age may be a risk factor for developing CIN, which is reflected by HPV infection rates. Therefore, age was included as a variable for study.

Oral contraceptive use

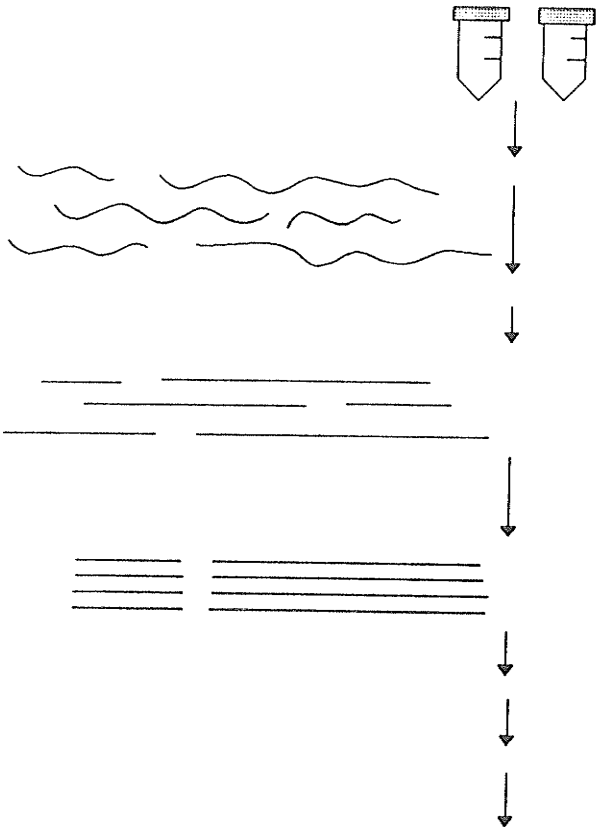
The use of oral contraceptives and its possible correlation to relative quantities of HPV-16 E6/E7 spliced transcript was investigated because the HPV-16 LCR has been shown to contain a glucocorticoid-responsive element [116]. This element augments the ability of HPV-16 to cause transformation, anchorage-independent growth, and tumor induction capability of primary cells [116]. Furthermore, progestins and progesterone found in oral contraceptives may induce the same effect [115]. Additionally, it has been proposed that the use of oral contraceptives may predispose cervical epithelial cells to HPV-associated neoplastic transformation [163]. Thus, it was of interest to determine whether oral contraceptive users showed significant differences in HPV-16 E6/E7 transcription in association with CIN compared to non-users.

5. **Materials and methods** (Figure 3)

Patient population

One hundred and ten patients were consecutively enrolled between January, 1990 to February, 1992 from women referred to a colposcopy clinic at the Health Sciences Centre, University of Manitoba, Winnipeg, Manitoba, Canada.

Figure 3. Methodology.



CELLS COLLECTED
CIN STATUS DETERMINED

PCR (HPV-16)

mRNA ISOLATION

REVERSE TRANSCRIPTION

cDNA

PCR: END LABELLED PRIMERS

SPECIFIC PCR PRODUCTS

ELECTROPHORESIS

AUTORADIOGRAPHY

BETA-SCINTILLATION COUNTING
ANALYSIS

Approval was obtained from the Human Ethics Committee of the University of Manitoba and participants gave their informed written consent. Women attending the clinic were referred because of an abnormal Pap smear, although later evidence after biopsy indicated that some were found to have normal histopathology. Laboratory personnel were not informed of the CIN status of the patients under study. Exfoliated cervical cells were taken by scraping the cervix with an Ayres spatula, sampling the endocervical canal with a cotton swab, then suspending the cells in 5 mL phosphate-buffered saline solution (pH 8.0) (Appendix). Specimens were frozen at -20°C until DNA isolation. Biopsies taken at the same time as swab specimens were evaluated by the Department of Pathology, University of Manitoba, for determination of the grade of cervical intraepithelial neoplasia. All specimens were taken by Dr. Fernando Guijon.

Specimen selection

The DNA from approximately 10^5 cells was isolated as previously described [97]. The remainder of the specimen was maintained at -70°C. The cellular DNA was then screened for the presence of HPV-16 genomic DNA. Specimens positive for HPV-16 infection and amplifiable mRNA were selected for further analysis, as described in this thesis.

Cell culture

CaSki cells (ATCC CRL 1550) were grown to confluence in modified Leibovitz-15 media with glutamine (Flow Laboratories) supplemented with 10% fetal calf serum (Appendix) at 37°C and 1% CO_2. After seven days they were trypsinized with 1 mL of a 0.25% trypsin in Modified Hank's Balanced Salts Solution (Appendix), washed in phosphate-buffered saline (pH 8.0) and held at -70°C until mRNA isolation.

Isolation of mRNA

The specimens to be analyzed were exfoliated cervical cells. Relative to biopsy tissue, there was a paucity of cells on which to perform the analysis. Thus, the conventional methods of mRNA isolation, such as guanidinium isothiocyanate/$CsCl_2$ gradient [138], were not applicable. Therefore various methods of mRNA isolation were assessed [138], including methods tailored to polymerase chain reaction (PCR) [60]. Commercially available kits (Pharmacia QuickPrep mRNA Purification kit, Invitrogen Micro FastTrack mRNA Isolation kit) were evaluated as well. Ultimately, the kit marketed by Invitrogen Co. (Micro Fast-Track mRNA Isolation kit) was chosen and used as per manufacturer's instructions. Briefly, this kit utilizes a quick lysis of cells with simultaneous inactivation of nucleases, followed by binding of mRNA to oligo d(T) cellulose. After numerous

washes, mRNA is eluted from a microcentrifuge spin column and collected by precipitation with sodium acetate and ethanol (Appendix).

Determination of mRNA concentration

The concentration of mRNA was determined spectrophotometrically after the method of Sambrook et al. [138]. Absorbance readings were taken at 260 nm and 280 nm in a DU-20 ultraviolet light spectrophotometer (Beckman Instruments, Canada, Inc.). An absorbance of 1.0 at 260 nm corresponds to a concentration of 4.0 ug mRNA per mL for single-stranded nucleic acid. The ratio of absorbances at 260 nm and 280 nm estimated the purity of the mRNA isolate. An absorbance ratio A_{260}/A_{280} of 2.0 indicates a preparation of mRNA essentially free of protein [138].

Reverse transcription

Reverse transcription of mRNA isolated from CaSki cells and exfoliated cervical cells was performed in order to produce a double-stranded product that was amplifiable by PCR [21,172]. The final concentrations for the reverse transcriptase reaction consisted of 4 mM $MgCl_2$, 0.015% gelatin, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 100 ng random hexamer primer (Pharmacia), 20 U RNAguard RNAse inhibitor (Pharmacia), 2.5 U avian myeloblastosis virus reverse transcriptase (Pharmacia), and 200 mM each of dGTP, dCTP, dATP, and TTP (Pharmacia) in a total volume of

20 uL. The reaction was incubated at 23°C for 10 minutes, followed by primer extension at 42°C for 45 minutes and denaturation at 95°C for ten minutes. Samples were then refrigerated at 5°C. Negative control reactions included a reaction containing all reagents except RNA template, and reactions containing all reagents and RNA template but lacking reverse transcriptase.

PCR amplification

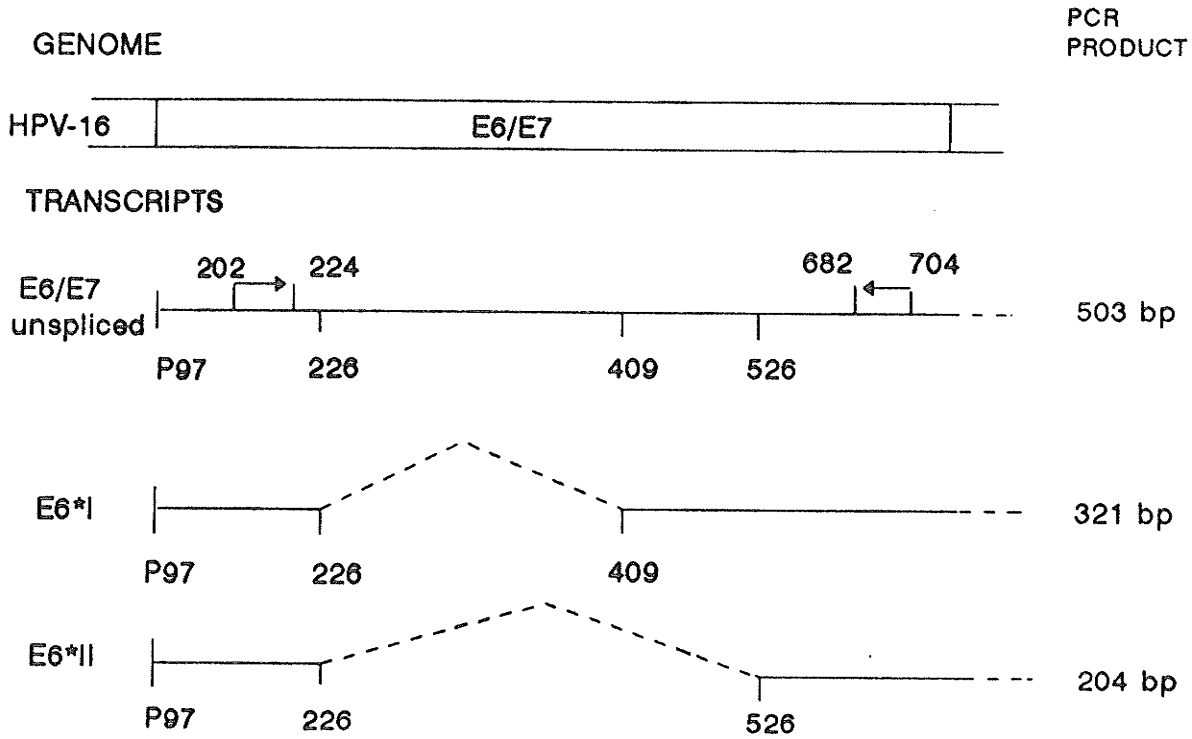
The polymerase chain reaction [137] procedure was used to amplify specific cDNA sequences transcribed in the reverse transcription reaction. Because of the problem of potential contamination by PCR products, physically separate laboratories were used: one for mRNA isolation and reverse transcriptase-PCR (RT-PCR) set-up, and one for amplification and analysis of PCR products. Additionally, positive displacement pipettes with disposable plastic tips and pistons were used (Gilson, Microman, Mandel Scientific). By using specific oligonucleotide primers of our design (Figure 4A), only the cDNAs transcribed from the E6/E7 region of HPV-16 were targeted for amplification. The positions of the primers relative to the HPV genome is shown in Figure 4B. The total volume of the reverse transcription reaction was added to a PCR cocktail of 80 uL, which contained a final concentration of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 2 U Taq DNA polymerase (Bio/Can Scientific), and primers at a final

Figure 4. Primer sequences and PCR products.

A.

Primer	Sequence (5'-3')	Genome location (bp)
P1ST	CAAGCAACAGTTACTGCGACGTG	E6 202-224
P2ST	TCCGGTTCTGCTTGTCCAGCTGG	E6 682-704

B.



arrows indicate primer annealing sites

concentration of 1 uM. The tubes used for the PCR amplification had plastic plungers to minimize the chances of aerosolized PCR products escaping out of the tube (Bio/Can) and eliminated the use of an oil overlay. Between 20 to 30 cycles of amplification were done using a programmable thermocycler (MJ Research, Watertown, NY), after initial DNA denaturation at 94°C for 2 minutes. Each cycle consisted of primer annealing (60°C for 45 seconds), extension (72°C for 45 seconds), and denaturation (94°C for 1 minute). A final annealing step (60°C for 1 minute) and elongation step (72°C for 7 minutes) was done after the final cycle, after which specimens were kept at 4°C. Amplified products were precipitated overnight with sodium acetate to a final concentration of 0.3 M and two volumes of ethanol (Appendix).

Quality control of mRNA isolation

In order to ensure that specimens with no detectable E6/E7 mRNA were negative due to non-expressing HPV, a control mRNA was also amplified. For this purpose the mRNA of a "human housekeeping" gene was RT-PCR amplified, thus indicating that the mRNA isolated in the specimen was of sufficient integrity to be amplified. Initially the mRNA of human beta-actin was selected; however it was found that a genomic DNA pseudogene [100,101,109] of the same molecular weight as the mRNA was co-amplified, resulting in false-positive signals in the control reaction lacking reverse

transcriptase. Subsequently, the mRNA encoding human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [2] was used as a target for RT-PCR. The reagents and reaction conditions of RT-PCR using the GAPDH primers (Appendix) were the same as previously described, except that 200 ng of input mRNA was used, 30 cycles of PCR were done, and the primers were not end labelled. Specimens which had mRNA amplifiable with GAPDH primers as detected by agarose gel electrophoresis were then selected and stored at -70°C until quantitative RT-PCR with HPV-16 E6/E7 primers.

Agarose gel electrophoresis

The products of the GAPDH RT-PCR were run on 4% agarose gels using a horizontal gel electrophoresis system (Bio-Rad Wide Mini sub-cell, Bio-Rad Laboratories, Richmond, CA). PCR-amplified products were resuspended in 15 ul of 33% glycerol/TE/tracking dye (Appendix). The gels, of total volume 100 mL, were prepared using Tris-Acetate buffer (Appendix), 4 g agarose (Bio-Rad Ultrapure DNA grade agarose) and 3 ul of 10 mg/ml ethidium bromide (Appendix) (Sigma Chemicals), to visualize the migration of bands. Gels were run for 2.5 hours at 55 mA, using a Bio-Rad Model 1000/500 model power supply. They were then visualized on a transilluminator (Johns Scientific, Ultraviolet products line) and photographed with a Polaroid MP4 camera through a filter (55 mm Tiffen 25 Red 1, USA). Type 57 Polaroid 4 x 5 land film was used.

Quantitation of transcription products

Oligonucleotide primers (Appendix) used in the PCR amplification of HPV-16 E6/E7 cDNAs were 5′ end-labelled with [γ-^{32}P]-ATP using a 5′DNA end-labelling kit (Boehringer Mannheim) as per manufacturer's instructions. Only 50 pmol of the total required complement of each primer was labelled, and the remainder in each reaction volume was left unlabelled. The labelling reaction was carried out at 37°C for one hour and products were precipitated with sodium acetate and ethanol as previously described. Similarly labelled was 2 ug of a 123 base pair molecular weight ladder (BRL) to facilitate the determination of mass of the PCR products. Following PCR amplification, ethanol precipitated products were electrophoresed through 8% polyacrylamide gels.

Polyacrylamide gel electrophoresis

The products of RT-PCR with the HPV-16 E6/E7 primers were run on 8% polyacrylamide gels using the Bio-Rad Protean II xi cell (Bio-Rad Laboratories, Richmond, CA) powered by a Bio-Rad model 1000/500 power supply. The gel was prepared as described in the Appendix, cast to 0.75 mm thickness, and run in Tris-Borate-EDTA buffer (Appendix). Gels were run at 150 volts for 5 hours, after which they were wrapped in polyvinylchloride laboratory wrap (Fisher Scientific) and used to expose x-ray film (X-Omat AR film, Rochester, NY.).

Autoradiography

The polyacrylamide gels used to separate the products of HPV-16 E6-primed RT-PCR were radioactive due to the incorporation of radioactively labelled primers. Therefore direct autoradiography of the polyacrylamide gels was done to visualize the position of the bands of interest. X-ray film (Kodak X-Omat AR film, Rochester, NY) was overlaid on the wrapped polyacrylamide gel and was exposed between 16 and 48 hours at room temperature without intensifying screens. The film was subsequently developed using a Pako 14X x-ray film processor (PAKO Corp., Minneapolis, USA)

Scintillation counting

Autoradiography of the 8% polyacrylamide gels revealed the positions of the specific RT-PCR amplified products. The bands of interest were excised, 5 mL EcoLite liquid scintillation solution was added (ICN Biomedicals Inc., Irvine, CA) and beta decay was counted (LKB Wallac 1217 Rackbeta Liquid scintillation counter).

Relative quantitation of E6/E7 spliced transcript

By adapting the method developed by Golde et al., [48], based on a quantitative paradigm proposed by Chelly et al., [13], the relative amounts of spliced mRNAs transcribed from the E6/E7 region of the HPV-16 genome in CaSki cells were determined. Since the efficiency of PCR amplification is

assumed to be constant for each successive cycle, an equation relating the formation of product as a function of efficiency, initial amount of HPV E6/E7 mRNA, and the number of cycles can be derived:

$$cDNA_n = (cDNA_0)(1 + R)^n$$

where $cDNA_0$ is the initial quantity of cDNA after reverse transcription, $cDNA_n$ is the amount of cDNA produced after n cycles of PCR amplification, and R is the efficiency of the reaction. Since the amplified products, ($cDNA_n$) are quantifiable due to the incorporation of radiolabelled primer during each cycle, the amount of cDNA at the beginning of the PCR amplification, $cDNA_0$ can be calculated. This quantity represents the amount of mRNA present in the sample.

Standardization reactions

As stated previously, the CaSki cell line was used as a model system on which to develop the assay that was subsequently applied to patients' specimens. Therefore, it was necessary to perform standardization reactions using CaSki cells to determine the parameters of amplification efficiency, relative abundance of different HPV-16 E6/E7 mRNA species, and limits of sensitivity of the reactions.

i/ Determination of amplification efficiency

The calculation of the relative amount of each spliced transcript necessitated a determination of the amplification efficiency of the RT-PCR. An assumption that the efficiency, or R, of both species of mRNA was equal would have resulted in an erroneous extrapolation of the amount of $cDNA_o$, or mRNA. Thus the R values for each of the two spliced transcripts designated as E6*I and E6*II had to be determined. This was done by performing RT-PCR on numerous 1 ug samples of CaSki mRNA, each containing the reagents described previously. At time intervals of 20, 22, 24, 26, 28, and 30 cycles of PCR, a sample was removed from the thermocycler and refrigerated at 4°C. After all the samples had been run, the amplification products were precipitated with sodium acetate and ethanol, separated on 8% polyacrylamide gel electrophoresis, and scintillation counted. This experiment was repeated three times, and the log of the mean disintegrations per minute (dpms) obtained was plotted as a function of cycle number, n. The regression line generated (Sigmaplot, Jandel Scientific Version 3.00, USA) was used to calculate R. The efficiency of the amplification was determined by calculating the slope of the regression line. By rearranging the equation previously described to the form below,

$$\log cDNA_n = n\log(1 + R) + \log cDNA_0$$

the amplification efficiency, R, was determined for each PCR product by using the value determined for the slope of each line.

ii/ Quantitative accuracy

To evaluate the accuracy of the assay with respect to input quantity of mRNA, serial dilutions of CaSki mRNA (between 10 pg and 2 ug) were used in the RT-PCR amplifications. Samples were reverse transcribed and amplified as described above, and the products of the reaction were quantified. By graphing the log dpm $cDNA_0$ of detectable specific transcript as a function of total quantity of input mRNA, the lower limits of sensitivity of the assay were established.

iii/ Standardization for <u>in vivo</u> conditions

CaSki cells represent a model system for studying the molecular events of transformation by HPV-16. However, these cells were treated under ideal conditions and were maintained under controlled conditions in the laboratory. The reality of <u>in vivo</u> conditions included various cervicovaginal microflora, the presence of nucleases, fluctuations in pH, etc. all of which potentially affected the quality of mRNA in the exfoliated cervical cell specimens. Moreover, specimens taken from the patient often remained at room temperature for up to a day before being

frozen. To study the influence of specimen handling on the quantitative RT-PCR assay, 5 x 10^5 CaSki cells were incubated (in duplicate) in 5 mL pooled supernatant taken from 8 patient specimens for 0, 8, and 24 hours at room temperature. As a control, the same number of cells were incubated with sterile phosphate-buffered saline. At each time interval, 0.1 mL of supernatant from test and control tubes was streaked on blood agar (Appendix) and incubated at 37°C overnight under aerobic conditions to verify the presence of viable microflora. The mRNA from all samples was isolated and each E6/E7 spliced transcript was quantified as described above.

Study population demographics

A detailed history was taken on the patients at the time of enrollment into the study in order to analyze the correlation, if any, between host factors and the relative quantity of HPV-16 E6/E7 spliced transcripts. Such information included the degree of cervical intraepithelial neoplasia (CIN) as evaluated by the Department of Pathology, University of Manitoba, the patient's age, use of oral contraceptives, smoking history, and the stage of the menstrual cycle at the time of sample collection. The specimens were then analyzed as described under blinded conditions.

Statistical analysis

All statistical analyses were performed at the Biostatistical Consulting Unit, University of Manitoba. Tests performed were the Spearman Correlation Analysis for determining correlations between age, oral contraceptive use, and E6/E7 expression; Mantel-Haenszel Chi-square analysis for evaluating E6/E7 transcription in association with CIN grade; and McNemar's Test for evaluating the relationship between relative proportions of E6/E7 mRNAs and grade of CIN.

6. Results

Isolation of mRNA

The isolation of mRNA from CaSki cells yielded between 7-20 ug of mRNA from 3 x 10^7 cells. From patient specimens, wherein it was estimated that a maximum of 1 x 10^6 cells were obtained, the yield of mRNA was typically between 1-5 ug, as determined by spectrophotometry.

Quality control of mRNA isolation

Amplification of a "human housekeeping gene" was performed because such a gene is constitutively expressed by the cell. Therefore, lack of an RT-PCR product indicates that the total mRNA isolated was of poor quality. This obviated the need to process inadequate specimens. The gene initially chosen for this purpose was the beta-actin gene,

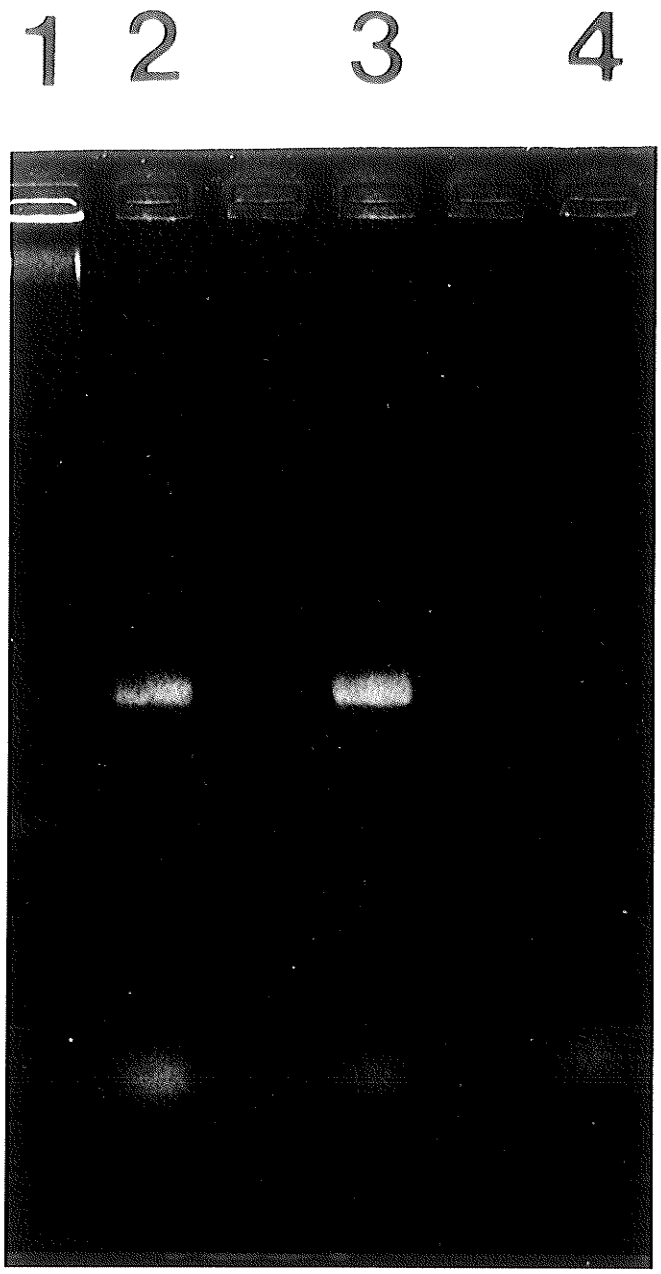


Figure 5. RT-PCR amplification of the beta-actin pseudogene.

Lane 1, 123 bp molecular weight marker.

Lane 2, PCR amplification without prior reverse transcription.

Lane 3, RT-PCR amplification.

Lane 4, RT-PCR amplification lacking RNA template.

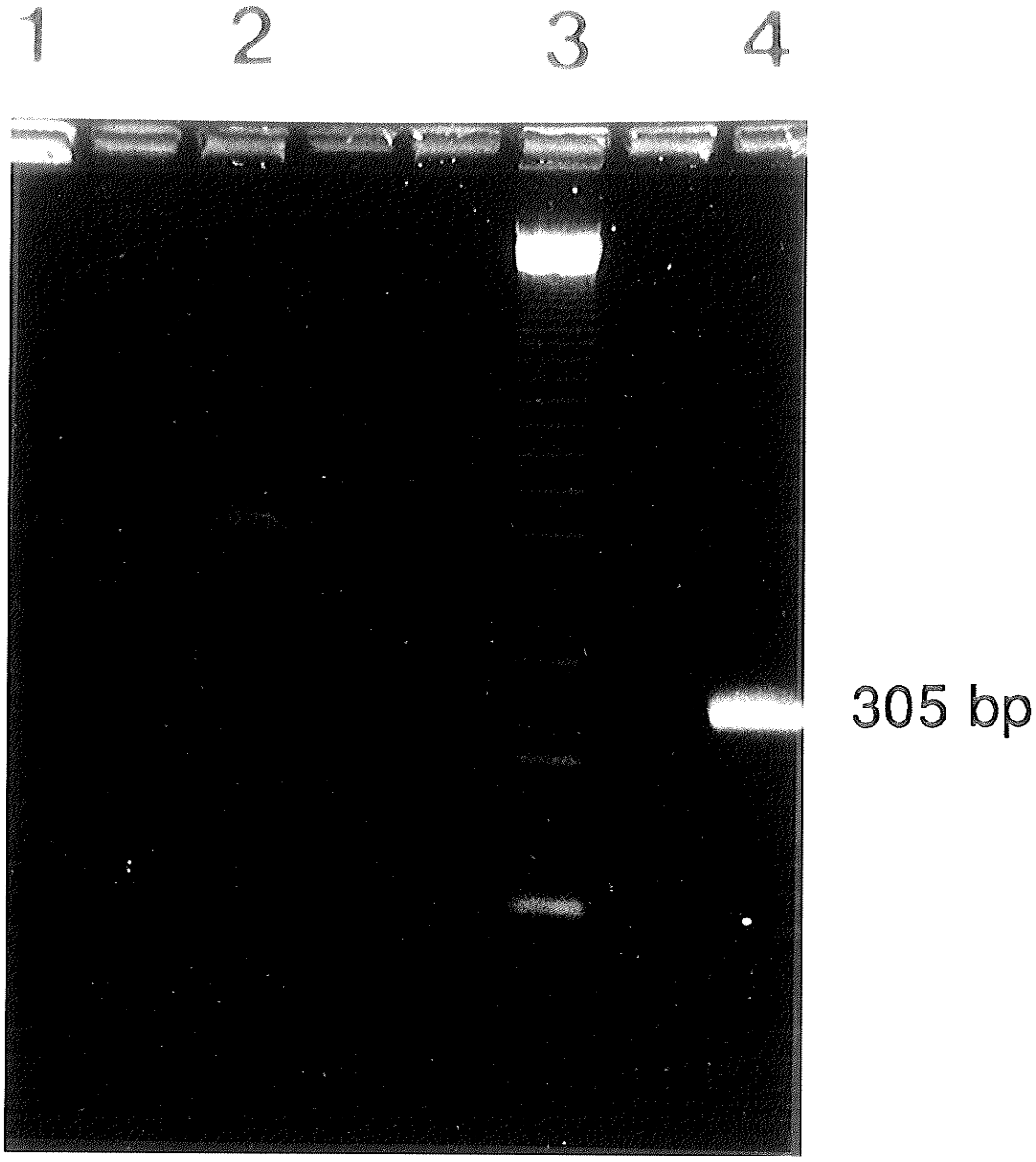


Figure 6. RT-PCR amplification of GAPDH mRNA in CaSki cells.

Lane 1, RT-PCR amplification lacking RNA template.

Lane 2, PCR amplification lacking prior reverse transcription.

Lane 3, 123 bp molecular weight marker.

Lane 4, RT-PCR amplification.

however, it was found that a genomic DNA pseudogene [100,101,109] was co-amplified with the mRNA (Figure 5). Because the product of RT-PCR amplification of this pseudogene was similar to that of the mRNA, thus yielding false-positive signals, the mRNA of the glyceraldehyde-6-phosphate dehydrogenase gene (GAPDH) [2] was alternatively selected as a control. Sporadically, there was detection of what appeared to be a transcript even in the absence of reverse transcriptase, due to the amplification of a pseudogene located on genomic DNA [33]. However, because the 305 bp product, when present, was less abundant than that seen in the reaction containing reverse transcriptase, it did not interfere with interpretation of the results (Figure 6). The absence of the band on the gel in negative control reactions indicated the presence of genomic human DNA in the mRNA preparations rather than contamination of reagents. The specimens that had detectable GAPDH mRNAs were then selected for further RT-PCR amplification with the HPV-16 E6/E7 primers.

Determination of RT-PCR efficiency

Figure 7 shows an autoradiograph which demonstrates that PCR amplification using these primers yielded the expected products of 321 base pairs (bp) and 204 bp, corresponding to E6*I and E6*II, respectively. Co-amplification of any viral genomic DNA co-isolated with mRNA generated a fragment of 503 bp. This product is easily

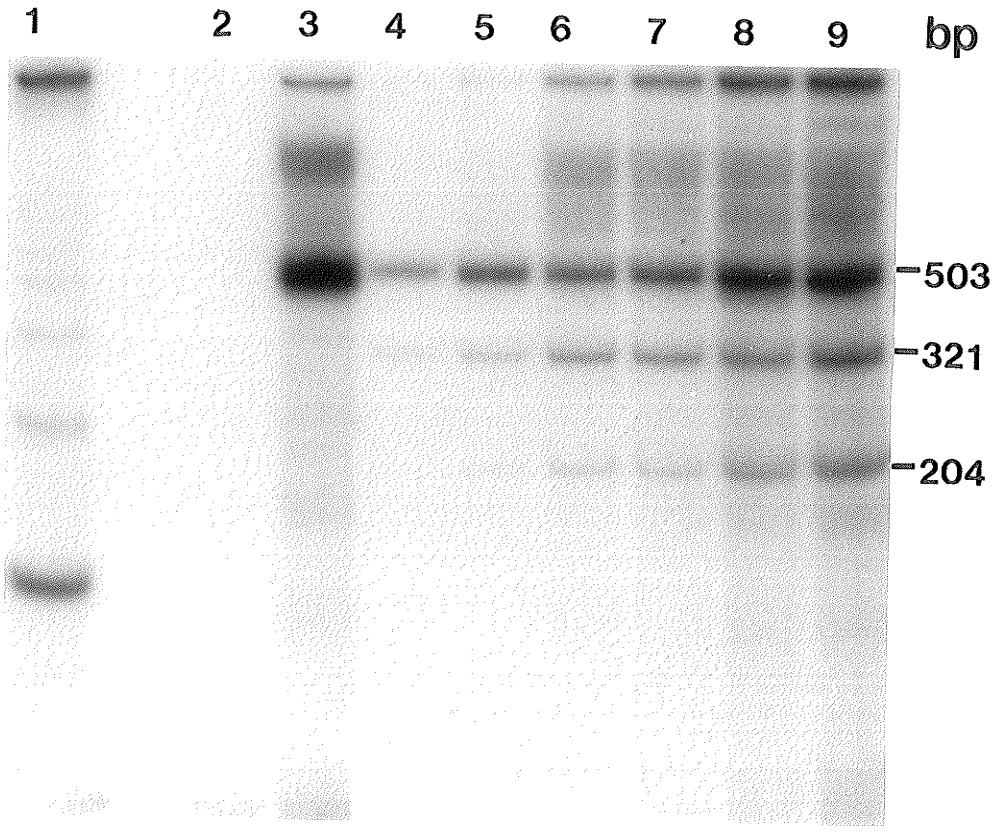


Figure 7. Autoradiographic detection of HPV-16 amplified transcripts.

Lane 1, 123 bp molecular weight marker.
Lane 2, RT-PCR amplification lacking RNA template.
Lane 3, PCR amplification without prior reverse transcription.
Lane 4, 20 cycles of PCR.
Lane 5, 22 cycles of PCR.
Lane 6, 24 cycles of PCR.
Lane 7, 26 cycles of PCR.
Lane 8, 28 cycles of PCR.
Lane 9, 30 cycles of PCR.
The presence of the 503 bp amplified product in Lane 3 indicates amplification of viral genomic DNA.

Table 1. The determination of RT-PCR efficiency.

cycle no.	log dpm ($cDNA_n$) E6*II	log dpm ($cDNA_n$) E6*I
20	2.09	2.65
22	2.37	2.98
24	2.75	3.26
26	3.14	3.49
28	3.57	3.73

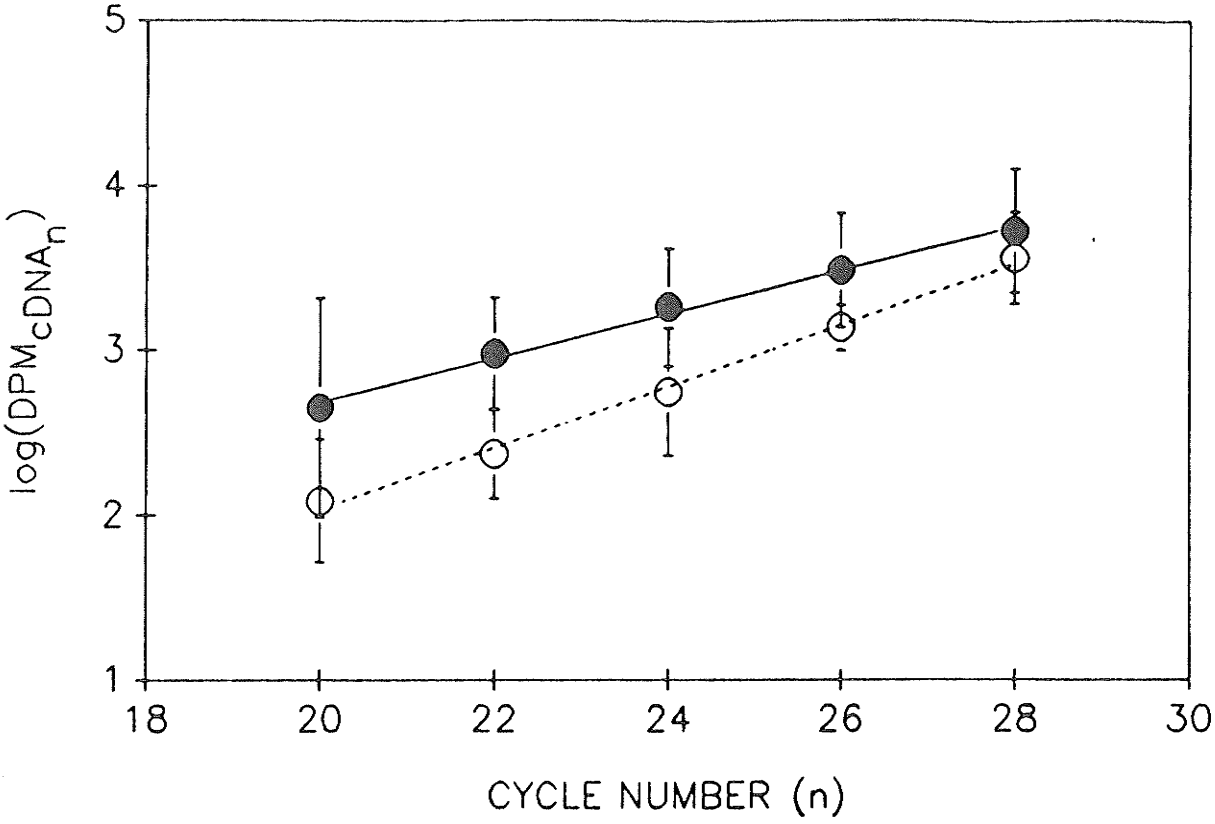


Figure 8. Log-linear relationship between cycles of amplification and PCR product.

The line through the solid circles represents the increase in E6*I product observed with increasing number of PCR cycles.

The line through the open circles indicates the corresponding increase in E6*II. The regression lines were generated from the mean of values obtained in three experiments.

identified as it is observed in the presence or absence of reverse transcriptase.

With an increase in the number of cycles of PCR amplification, there is a concurrent increase in the amount of E6*I and E6*II specific amplified product (Fig. 7). This is confirmed by the scintillation counting (Table 1), as shown in Figure 8. It should be noted that past 28 cycles, a plateau effect was observed, whereby efficiency of amplification declines.

As stated previously (Materials and Methods), by applying the equation shown below,

$$\log cDNA_n = n\log(1 + R) + \log cDNA_0$$

the amplification efficiency R, can be determined for each spliced transcript by using the value determined for the slope of each line in Fig. 8. For E6*II, the slope, or log(1 + R) was 0.185, therefore R equals 0.53. This value indicates that 53% of cDNA amplified in one cycle is copied during the subsequent cycle. The amplification efficiency of E6*I was calculated to be somewhat lower, at 0.38.

Determination of the relative quantities of E6*I and E6*II mRNA

By using the formula described above, disintegrations per minute (dpm) values for $cDNA_0$ were determined, facilitating calculation of the fraction of the total quantity of HPV-16 spliced transcript comprised by each mRNA

Table 2. Relative quantities of HPV-16 E6/E7 transcript in CaSki cells.

transcript	$cDNA_{24}$ (log dpm)	$cDNA_0$ (log dpm)	R (efficiency)	total mRNA (%)
E6*I	3.19	0.676	0.38	97.1
E6*II	2.74	0.020	0.53	2.9

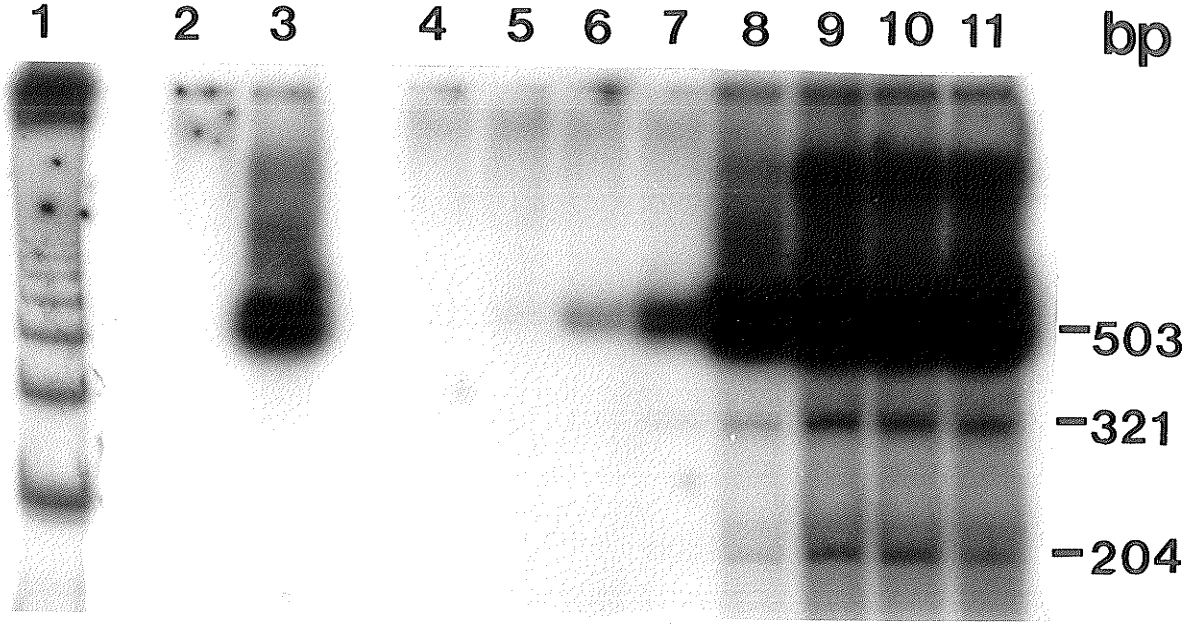


Figure 9. Autoradiographic assessment of quantitative accuracy of amplification of HPV-16 E6/E7 mRNAs.

Lanes 4-11 show increasing amounts of input CaSki mRNA into RT-PCR amplification.

Lane 1, 123 bp molecular weight marker.
Lane 2, RT-PCR without mRNA template.
Lane 3, PCR amplification without prior reverse transcription.
Lane 4-11, 10 pg, 100 pg, 1 ng, 10 ng, 100 ng, 1 ug, 1.5 ug, 2 ug of mRNA, respectively.

species (Table 2). Calculation of $cDNA_0$ after 24 cycles of amplification was chosen because this point falls into the middle of the linear portion of the graph shown in Figure 8, where amplification efficiency is most consistently determined. After 24 cycles of amplification, log dpm $cDNA_{24}$ for E6*I was found by scintillation counting to be 3.22. By using the calculated value of R and the determined value for log dpm $cDNA_{24}$, the log $cDNA_0$ for this transcript can be deduced with the following calculation:

$$\log \text{dpm } cDNA_0 = 3.19 - 24\log (1 + 0.38)$$

$$\text{dpm } cDNA_0 = 0.676$$

On the basis of the calculated values, the E6*I transcript accounts for 97.1%, and the E6*II transcript constitutes 2.9% of spliced mRNAs transcribed from the E6/E7 region of HPV-16 in CaSki cells (Table 2).

Quantitative accuracy

In order to evaluate the accuracy of the assay with respect to input quantity of mRNA, serial dilutions of CaSki mRNA were used in the RT-PCR amplifications (Figure 9). For the E6*I transcript, there was a linear correlation between $cDNA_0$ values and input CaSki mRNA between 10 ng and 1 ug (Figure 10A). Below 10 ng of input mRNA there was no significant amplification of E6*I, thus defining the limit of sensitivity of the assay. For E6*II, the $cDNA_0$ values

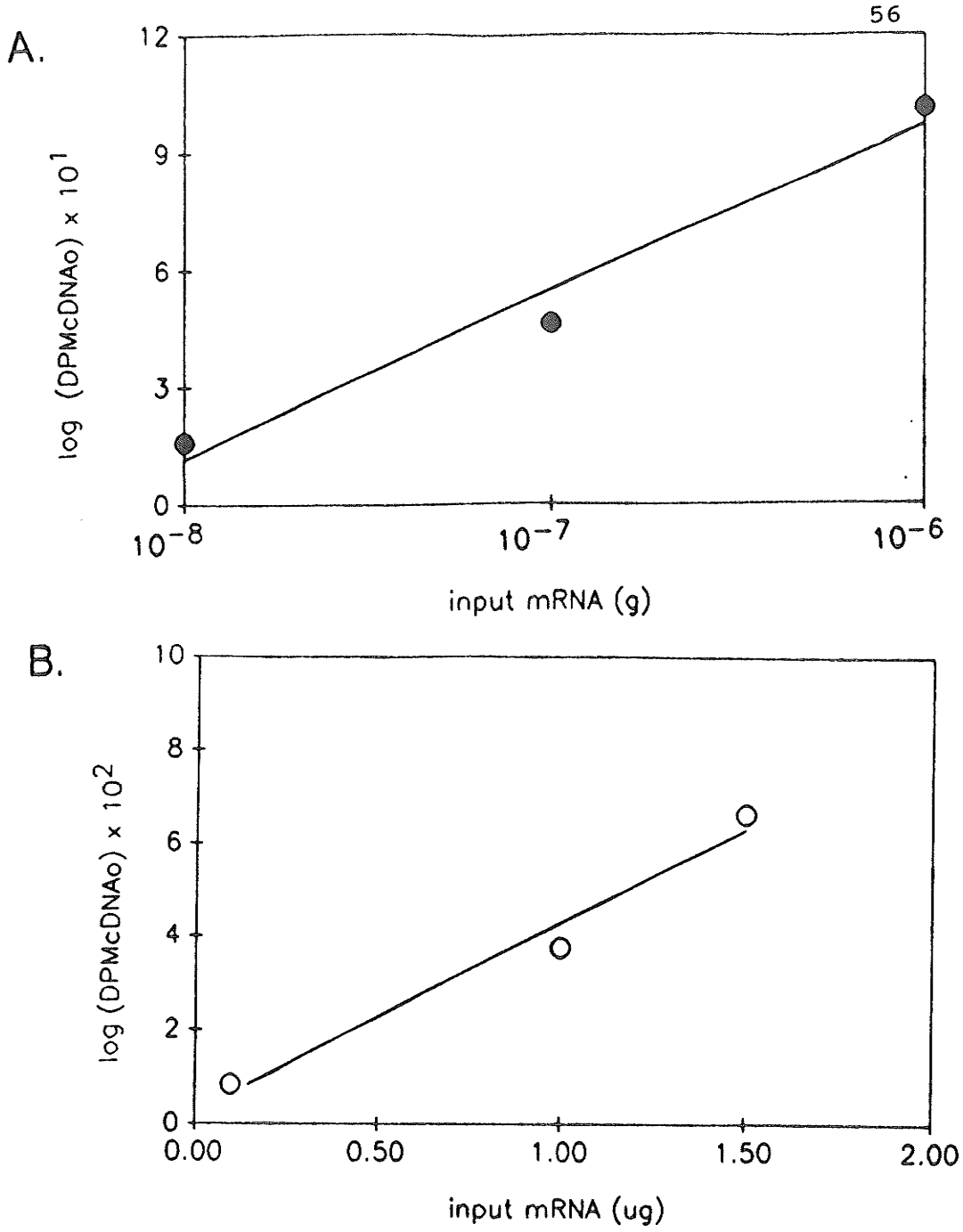


Figure 10. Relationship between input CaSki mRNA and calculated $cDNA_o$ for E6*I and E6*II mRNAs.

A linear relationship was observed between input mRNAs and amplified product between

A. 10 ng-1 ug CaSki mRNA for detection of E6*I, and
B. 100 ng-1.5 ug CaSki mRNA for detection of E6*II.

also showed a linear increase with increasing input mRNA within the range of 100 ng to 1.5 ug (Figure 10B).

Standardization for in vivo conditions

To examine the influence of specimen handling on the quantitative RT-PCR, 5 x 10^5 CaSki cells were incubated in pooled supernatant taken from patient specimens as described previously. The mRNA was then isolated and each E6/E7 transcript was quantified as before. It was found that over 0, 8, and 24 hours, there was no significant difference in the amount of detectable transcript of either species (E6*I, $p = 0.566$; E6*II, $p = 0.159$) compared to that isolated from control CaSki cells that were incubated in sterile phosphate-buffered saline (PBS) (Table 3). Furthermore, there was no significant difference in the ratios of transcripts detected in CaSki cells that were incubated in clinical supernatant compared to PBS controls (Table 3). Thus, the quantity of mRNA available for isolation from patient specimens as well as the ratio of transcripts of interest, or quality, is unaffected by an in vivo environment, and the standardization parameters determined on CaSki cells were applicable to patient specimens.

To verify the presence of viable microflora in the patient isolates which could potentially affect the integrity of the isolated mRNA, the pooled cell supernatants were streaked on blood agar and incubated overnight under

Table 3. Relative quantities of HPV-16 E6/E7 transcript in CaSki cells subjected to in vivo conditions.

mean ratio of E6*I/E6*II

time (hours)	PBS control	clinical supernate
0	92.3/7.7	93.5/6.5
8	96.0/4.0	97.5/2.5
24	94.0/6.0	90.3/9.7

aerobic conditions. The organisms which grew were typical aerobic cervicovaginal microflora such as E. coli, S. aureus, Lactobacillus spp., yeast, and Streptococcus spp. Thus, the presence of viable microflora in the patients' specimen supernatants was confirmed.

Analysis of patient specimens

i/ Amplification with HPV-16 E6/E7 primers

Figure 11 shows a typical autoradiograph of amplification of clinical isolates. As expected, the amplification products generated were of 321 bp and 204 bp for E6*I and E6*II, respectively. It was obvious that although CaSki mRNA, as a control, amplified well, the mRNA of clinical isolates were not as easily visualized by autoradiography, indicating less abundant viral mRNA per cell or fewer cells infected with HPV-16. However, upon careful excision of the polyacrylamide gels and subsequent scintillation counting, the $cDNA_n$ was determined and $cDNA_0$ was calculated for each spliced transcript (Table 5). For each specimen, the dpms of each spliced transcript were found by calculating the difference in dpms between the sample and its corresponding control lacking reverse transcriptase. In this way, the background counts were eliminated.

By using quantitative RT-PCR, it was reported that the relative quantities of E6/E7 mRNA in CaSki cells are 97.1% and 2.9% for E6*I and E6*II, respectively (see "Results:

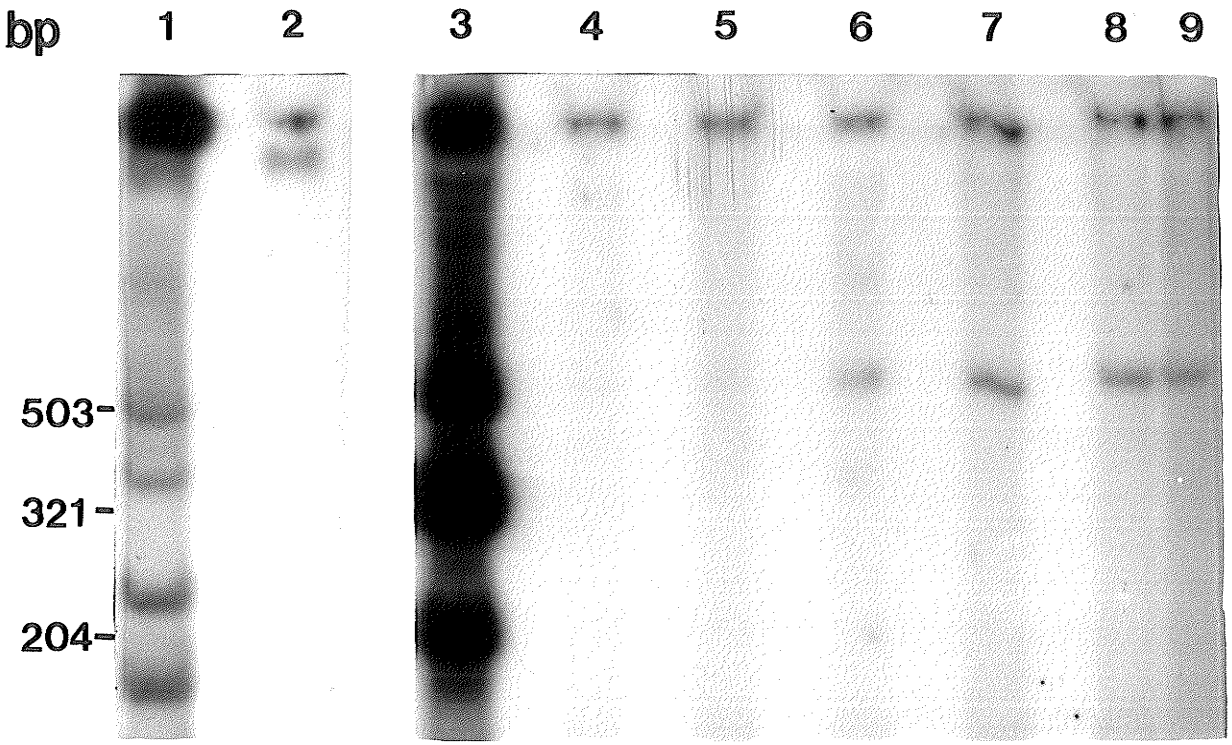


Figure 11. Autoradiographic detection of RT-PCR-amplified E6*I and E6*II.

Lane 1, 123 bp molecular weight marker.
Lane 2, PCR amplification of CaSki mRNA without prior reverse transcription.
Lane 3, RT-PCR amplification of CaSki mRNA.
Lane 5,7,9, RT-PCR amplification of cervical cell mRNA obtained from three patients.
Lane 4,6,8, PCR amplification of mRNA in Lane 5,7,9, respectively, but lacking prior reverse transcription.

Table 4. Cohort characteristics and relationship to HPV-16 E6/E7 mRNA expression.

patients' characteristics	expression + (n = 37)	expression - (n = 17)
mean age (years)	26.2 (14-46)	27.8 (17-39)
OC* (n = 19)	14	5
CIN grade		
CIN 0 (n = 12)	10	2
CIN I (n =16)	8	8
CIN II (n = 9)	6	3
CIN III (n = 17)	13	4

*OC = oral contraceptive use

Table 5. Determination of E6*I and E6*II relative quantities in the study cohort.

CIN grade	OC[1]	AGE (years)	E6*I (dpm $cDNA_n$)	E6*II (dpm $cDNA_n$)	E6*I/E6*II[2] (%/%)
0	+	20	22.4	0	100/0
0	-	26	26.8	0	100/0
0	-	36	3.6	0	100/0
I	-	36	13.6	0	100/0
I	-	40	96.1	0	100/0
II	+	22	60.9	0	100/0
II	-	28	34.0	0	100/0
III	-	14	72.0	0	100/0
III	-	19	123.7	0	100/0
III	+	23	46.4	0	100/0
III	+	34	89.7	0	100/0
0	-	21	0	20.4	0/100
0	-	22	0	33.2	0/100
0	-	39	0	42.8	0/100
I	+	17	0	2.8	0/100
I	+	21	0	39.6	0/100
I	-	22	0	64.0	0/100
II	+	20	0	14.0	0/100
II	-	22	0	19.2	0/100
III	+	20	0	67.6	0/100
III	-	37	0	2.8	0/100
III	-	41	0	10.0	0/100
III	-	46	0	5.6	0/100
0	-	20	157.3	110.9	94.5/5.6
0	+	22	173.0	358.6	85.2/14.8
0	+	26	34.4	70.4	85.3/14.7
0	-	42	28.8	134.4	71.9/28.1
I	+	17	60.4	57.1	92.6/7.4
I	+	19	72.0	5.6	99.3/0.7
I	-	26	48.4	98.1	85.5/14.5
II	+	20	93.3	31.4	97.2/2.8
II	+	20	43.2	33.2	93.9/6.1
III	-	19	36.4	106.4	80.3/19.7
III	N/D	26	166.1	44.2	97.8/2.2
III	-	28	242.9	54.0	98.2/1.8
III	-	28	159.7	69.6	96.5/3.5
III	-	32	93.2	11.2	99.0/1.0

[1]OC denotes oral contraceptive use. N/D= no data available
[2]E6*I/E6*II indicates the relative proportion of HPV-16 E6/E7 mRNA comprised of each species.

Determination of relative quantities of E6*I and E6*II). The procedure was applied to a patient population as detailed in Table 4. The isolation of amplifiable mRNA from patients' specimens is demonstrated by the presence of amplified glyceraldehyde-3-phosphate dehydrogenase mRNA from CaSki cells and two representative clinical isolates shown in Figure 12.

ii/ HPV-16 E6/E7 transcription in the patient population

It was found that 37 (68.5%) of the 54 women in the study population had detectable HPV-16 E6/E7 transcript (HET+), while HPV-16 E6/E7 transcription was not detectable (HET-) (Table 4) within the limits of sensitivity of the assay (see "Results. Quantitative accuracy") in the remaining 17 patients (31.5%) The HET- women had a mean age of 27.8 years (range 17-39), while the HET+ women were between 14 and 46 years old (mean age of 26.2 years). Both HET+ and HET- patients were observed in the study population comprised of 12 women with normal cervical cytology and 42 with CIN (Table 4). Eight (50%) of the 16 women with CIN I were HET+ while 19 (73%) of the 26 women with high grade lesions (CIN II and CIN III) were HET+. Thus, of the patients with CIN, the women with high grade lesions comprised a greater proportion of women demonstrating HPV-16 E6/E7 transcription. Although all the women in the study cohort presented to the colposcopy clinic with abnormal

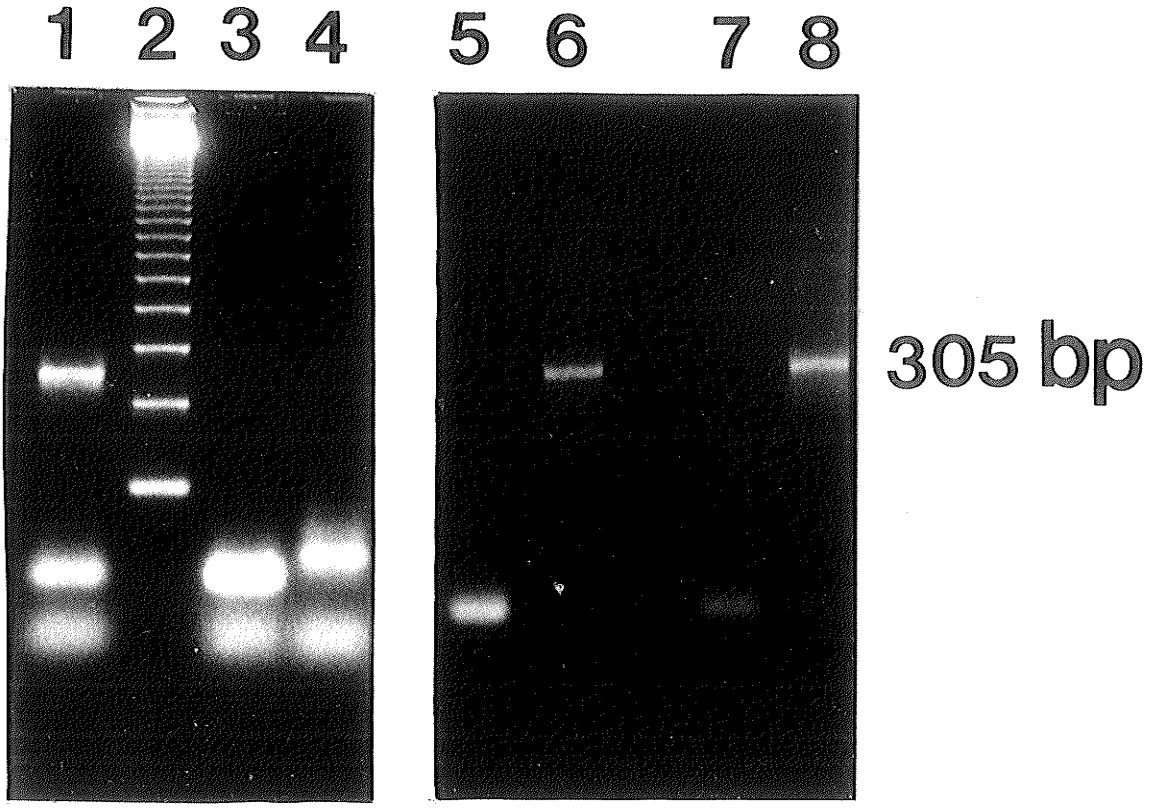


Figure 12. RT-PCR amplification of GAPDH mRNA in patient cells.

Lane 1, RT-PCR amplification of CaSki mRNA.
Lane 2, 123 bp molecular weight marker.
Lane 3, PCR amplification of CaSki mRNA, without prior reverse transcription.
Lane 4, RT-PCR amplification without mRNA template.
Lane 6,8, RT-PCR amplification of cervical cell mRNA obtained from two patients.
Lane 5,7, PCR amplification of mRNA in Lane 6,8, respectively but without prior reverse transcription.

Nonspecific bands below 123 bp are primer dimers.

cervical cytology, some of these patients were diagnosed upon biopsy as having a histopathologically normal cervix, with an inflammatory response or other benign process. Thus, the study population also included women who were infected with HPV-16 who were diagnosed as normal, or CIN 0. Of the 19 women who used oral contraceptives, 14 had detectable HPV-16 transcription (Table 4).

iii/ Quantitation of E6/E7 mRNA in the patient population

The data derived from detection of E6*I and E6*II transcripts from the 37 HET+ women are shown in Table 6. The HET+ patients had one of three possible transcription profiles: expression of E6*I only, expression of E6*II only, or expression of both E6*I and E6*II. The three transcription profiles were equally distributed throughout the HET+ population.

The E6*I transcript by itself was detected in 11 patients, while in 12 patients only the E6*II transcript was detected. Expression of one transcript to the exclusion of the other was not related to CIN grade, age, or OC use.

Among the 14 HET+ women who expressed both E6*I and E6*II, the relationship between the relative quantities of the two transcripts and the grade of CIN was investigated (Table 7) A relative proportion of $\geq$ 95% for E6*I was significantly associated with high grade CIN ($p = 0.05$) (Fisher's exact test). That is, with an increase in disease

Table 6. Expression profiles in HET+ patients for low and high grade CIN.

transcripts	CIN I	CIN II&III
E6*I (n = 8)	2 (25%)	6 (75%)
E6*II (n = 9)	3 (33.3%)	6 (66.7%)
E6*I and E6*II (n = 10)	3 (30%)	7 (70%)

Table 7. Relative quantities of HPV-16 E6/E7 transcript.

CIN grade	% E6*I	% E6*II
CIN 0 (n = 4)	$\bar{X}$ = 84.2 (71.9-94.5)	$\bar{X}$ = 15.8 (5.6-28.1)
CIN I (n = 3)	$\bar{X}$ = 92.5 (88.5-99.3)	$\bar{X}$ = 7.5 (0.7-14.5)
CIN II&III (n = 7)	$\bar{X}$ = 94.6 (80.3-99.0)	$\bar{X}$ = 5.4 (1.0-19.7)
CaSki cells	$\bar{X}$ = 97.1 (96.3-98.9)	$\bar{X}$ = 2.9 (1.1-3.7)

severity, there was a corresponding increase in the expression of E6*I relative to E6*II. For the population as a whole, expression of E6*I and E6*II was inversely correlated ($p = 0.0001$) (Spearman's Correlation Test).

iv/ Trends in transcription: correlation analysis

The study population was stratified on the basis of oral contraceptive (OC) use. Correlation analysis was used to identify significant associations between age, expression of E6*I and expression of E6*II in the presence and absence of OC. This analysis was applied to the patient population as a whole, as well as to HET+ patients only (Table 8, Table 9A and 9B). A description of these results follows.

v/ The effect of CIN and OC use on E6/E7 transcription

When OC use was studied in association with CIN, several trends in viral expression became apparent (Table 8). While not statistically significant ($p > 0.05$), it was observed that patients with a diagnosis of CIN tended to have a higher expression of E6*I and lower expression of E6*II. This trend was seen with low grade (CIN I) as well as high grade disease, in both OC users as well as non-users.

In non-users of OC with normal cervical histopathology (CIN 0), a reversal of the trend observed for women with CIN was apparent. E6*I expression was higher, while E6*II expression was lower. However, OC users with normal

Table 8. The effect of oral contraceptive use (OC) and CIN on HPV-16 E6/E7 transcription.

CIN grade	E6*I/E6*II no OC	E6*I/E6*II with OC
normal	↓/↑	↑/↓
CIN I	↑/↓	↑/↓
CIN II&III	↑/↓	↑/↓
	(n = 22)	(n = 14)

Table 9. The effect of age and oral contraceptive use on HPV-16 E6/E7 transcription.

A. All patients

	Expression of E6*I/E6*II	
	-OC (n = 33) mean age=29.0 years	+OC (n = 19) mean age=22.8 years
age > mean age	↓/↑	↑/↓

B. Expression+ only

	Expression of E6*I/E6*II	
	-OC (n = 22) mean age=29.3 years	+OC (n = 14) mean age=21.5 years
age > mean age	↓/↑	↑/↓

histopathology had the same transcription profile as the women with CIN.

vi/ The effect of age and OC use on E6/E7 transcription

Two sets of patients within the study population were used to study the effect of age and OC use on HPV-16 E6/E7 transcription. The first, shown in Table 9A, included all patients in the study population for whom information regarding OC use was available (n = 52). That is, data from both HET+ as well as HET- women were included in the analysis. In non-users of OC who were over the mean age of 29 years, the HPV-16 E6/E7 transcription profile increasingly resembled that of the patients with normal cervical cytology. These women showed a decrease or absence of expression of E6*I relative to E6*II. However, when the age of OC users was greater than the mean of 22.8 years, there was a significant association with increasing expression of E6*I and decreasing expression of E6*II ($p = 0.05$); this same transcription pattern was seen in patients with CIN.

The second analysis of age and OC use in relation to HPV-16 E6/E7 transcription specifically tested the HET+ patients only (Table 9B). Similar to the trend observed for all patients, it was seen that when the age of non-users increased (greater than the mean age of 29.3 years), there was a concurrent decrease in expression of E6*I and

increased expression of E6*II. However, in the presence of OC use, there was a reversal of this profile; with increasing age (> 21.5 years) there was a concurrent increase in E6*I expression relative to E6*II.

7. Discussion

The etiology of cervical cancer is not clearly understood; it is known that infection with HPV is one important event that predisposes cervical epithelium to the process of neoplastic transformation. Numerous additional factors, either biological or behavioural, may also be required for the disease to progress. Examples may include genetic susceptibility, cigarette smoking, the use of oral contraceptives, and the age of the patient. In an attempt to clarify the role played by HPV as well as other variables, this study describes the development of an assay to quantify the putative viral transforming gene mRNAs. Furthermore, the technology was applied to cells taken from women with cervical neoplasia in order to assess the association between host-related factors, HPV expression, and cervical disease.

Numerous DNA transfection experiments have shown that the E6/E7 ORF of HPV-16 is integral for transformation and immortalization [54,159,169]. The two spliced mRNAs expressed from this ORF, known as E6*I and E6*II, have been detected in cervical cancer-derived cell lines as well as

cervical cells isolated from patients in various stages of malignancy [21,65,146]. Since both spliced species are observed in various disease stages, it was of interest to determine if quantitative variation in the amounts of E6*I and E6*II was associated with progression of malignancy. To this end, a quantitative RT-PCR assay was developed using the cervical carcinoma-derived CaSki cell line as a model system [59]. This technology was then used to assess whether the relative quantities of viral transforming gene transcripts correlated with the degree of disease severity.

In this study, the relative amounts of spliced E6/E7 transcripts were determined by adapting PCR technology. All initial reactions and standardizations were developed using CaSki cells, a cervical carcinoma-derived cell line, which constitutively expresses the HPV-16 E6/E7 ORF. The first step in the development of the quantitation system was the determination of efficiency, R, of the RT-PCR amplification for each transcript. Assuming equal efficiencies of amplification for each transcript would have resulted in erroneous calculations of the amount of $cDNA_o$s, due to the exponential nature of the amplification. It was found that the R values for E6*I and E6*II were 0.38 and 0.53, respectively. In the example of E6*I, this means that 38% of the amplification product synthesized in one cycle was copied in the subsequent cycle of PCR. The difference in amplification efficiency for the two species was reflective

of several factors; the shorter length of the E6*II PCR product and inherent differences in primer annealing to each transcript may account for the more efficient amplification of this transcript. The Taq DNA polymerase elongation complex dissociates and re-associates from the cDNA template during DNA synthesis, and thus the probability of copying a shorter strand is higher than that of a longer one.

Determination of relative quantities of E6*I and E6*II

It was found that in the CaSki HPV-transformed cell line, 97.1% of spliced transcript is E6*I, which potentially encodes the E7 protein, while E6*II comprises 2.9% of total spliced product. This analysis substantiates Northern blot studies wherein the most common transcript expressed from the E6/E7 ORF in CaSki cells was E6*I [150]. It is possible that the larger proportion of E6*I, and by inference, E7 protein, may reflect the transformed status of CaSki cells. Overexpression of E7 may prove to be pivotal to the process of transformation, thus supporting the hypothesis that quantitative perturbations rather than qualitative differences in HPV-16 gene expression are responsible for initiating or promoting the transformed phenotype.

The E7 protein, putatively encoded by E6*I, is a cytoplasmic phosphoprotein of 20 kDa [151] with retinoblastoma susceptibility protein binding capability [34,105] and trans-activating properties [123]. The E6

protein, encoded by E6*II, has been found to associate with and degrade cellular p53 [142,171]. It has been noted that HPV-negative tumors contain mutant p53 sequences, but in HPV-positive cancers, only wild-type p53 sequences are found [25,141]. Thus, evidence strongly favors a role for HPV-16 E6 and E7 proteins in the development of neoplastic lesions. This is substantiated further by von Knebel Doeberitz et al. [165] who showed that intracellular concentrations of these proteins correlate with the capacity of cervical cancer cells to proliferate.

Quantitative accuracy of RT-PCR

Before applying the quantitative RT-PCR to patient isolates, it was necessary to determine the lowest limits of detection of the procedure, and to verify if specific transcript could be consistently detected over a range of input mRNA into the assay. Thus, serial dilutions of total CaSki mRNA were used in the reaction, and it was found that 10 ng and 100 ng of input mRNA were required to consistently detect E6*I and E6*II, respectively. It was also shown that detection of each transcript showed a linear increase with an increase in total mRNA, over a defined range. The lower threshold amount of input mRNA over which amplification product was detected for E6*I was expected, due to the greater proportion of this spliced transcript compared to E6*II in CaSki cells. Because of the direct correlation of the relationship between predicted $cDNA_o$ and input mRNA for

these transcripts, the quantitative accuracy of the assay was verified.

Standardization of quantitative RT-PCR for in vivo conditions

Specimen handling was a potential source of error in the consistent detection of HPV-16 mRNAs. Specimens were collected from patients, suspended in phosphate-buffered saline, and remained at room temperature, for up to one day. Thus, a standardization experiment was performed wherein CaSki cells were subjected to the same in vivo conditions as clinical specimens. It was found that the PCR-based detection of viral mRNAs was not significantly affected. The relative proportions of E6*I/E6*II remained consistent after 0, 8, and 24 hours of incubation at room temperature in cervical cell collection media taken from patient specimens. Therefore, possible inhibitors of amplification found in clinical isolates had no significant effect, and additional standardization of the quantitative RT-PCR was not required.

Analysis of patient specimens

i/ Cervical intraepithelial neoplasia and HPV-16

Cervical cancer is a disease that exists as a continuum; precancerous dysplasias can be clinically staged on the basis of histological abnormality into cervical intraepithelial neoplasia (CIN) stages I, II, and III. CIN

III represents the most severe stage of dysplasia and immediately precedes invasive carcinoma of the tissue underlying the epithelium. Cross-sectional studies have shown that HPV-16 DNA as well as HPV-16 E6/E7 mRNA can be found in cells at all three stages of CIN. This investigation details a quantitative RT-PCR assay that was developed and standardized using CaSki cells, and the subsequent study of the quantitative relationship between CIN and HPV-16 E6/E7 mRNA. It was hypothesized that a variation in viral transforming gene expression would accompany dysplastic changes. Since fully transformed CaSki cells had an E6*I/E6*II proportion of 97.1%/2.9%, it was thought that the E6*I transcript was quantitatively the more important mRNA in development and/or maintainance of the transformed phenotype. Because of the large excess of E6*I in transformed cells, it was speculated that the proportion of E6*I/E6*II in cells intermediate in the transformation process would have less E6*I and more E6*II. Additionally, it was thought that since both transcripts in a precise quantitative relationship was associated with full transformation, perhaps either of the two, but not both transcripts would be detected in precancerous cells. Both hypotheses proved to be correct.

By using quantitative RT-PCR, the proportion of E6*I and E6*II as a percentage of the total spliced E6/E7 mRNA was determined for each patient in the cohort. The cells

from fifty-four patients were analyzed, and it was observed that the patient population fell into HPV-16 E6/E7 transcript detectable (HET+) and non-detectable (HET-) categories.

ii/ HET- patients and CIN

Seventeen of 54 patients (31%) in the study cohort were found to have no detectable HPV-16 E6/E7 mRNA. This group was comprised of patients with all grades of CIN, as well as two women with normal (CIN 0) histology. Preliminary PCR analysis verified the presence of HPV-16 infection, and GAPDH RT-PCR indicated amplifiable quality of isolated mRNA in this group. These findings are similar to those of Falcinelli et al. [38], and suggest that cells infected by HPV-16 in vivo may enter into a latent, non-expressing stage. These data suggest that continuous expression of the E6/E7 ORF may not be essential for development of cervical neoplasia once the process has begun, as evidenced by the HET- status of a number of CIN specimens. An alternate hypothesis suggests that the development of CIN in these women may occur independently of HPV-16 transforming gene expression. It has been shown using in-situ hybridization that loss of total HPV-16 mRNA in cervical biopsies is associated with older women and a higher mortality rate [56]. The mechanism by which this occurs is not currently known, but it has been proposed that HPV-negative tumours contain non-functional p53 tumor suppressor protein. It

would be of interest to determine the p53 status of this class of patients, since neoplasia may potentially be mediated by means other than HPV-16 transforming gene expression.

iii/ Patients with normal histology

The study cohort consisted of women referred to a colposcopy clinic because of cervical cell abnormalities. Some of these women were diagnosed upon biopsy as having a histopathologically normal cervix, with an inflammatory response or other benign process. A subset of these patients was also found to be HET+. It is possible that HPV-16 E6/E7 expression in these patients is indicative of increased risk for CIN or that expression of the virus is related to underlying cellular perturbations. Possible host-related risk factors in these individuals such as genetic susceptibility [25,141], smoking [163], vaginal microbial flora composition [51], or co-infections with sexually transmitted infections [18,72] may predispose HPV-infected cells to express the viral transforming gene. Thus, the presence of E6/E7 expression in women with normal cervical histology warrants further study. In particular, it would be of interest to follow these women prospectively to see if the development of CIN accompanies the detection of E6/E7 mRNAs.

Latent HPV-16 infection is characterized by viral infection in the absence of detectable pathology.

Therefore, the designation of latent infection may be highly variable, depending upon how virus infection is defined and the sensitivity of the method used to detect disease [102]. Classically, HPV infection has been defined by detection of viral DNA, and varying prevalence of latent HPV-16 infection has been found [6,102]. The detection of HPV-16 transforming gene mRNA may be a more sensitive and informative method of defining clinical latency than DNA detection alone. It would be of interest to follow the disease progress of histologically normal HET- compared with HET+ women to see if detectable E6/E7 mRNA shows an association with the propensity to neoplastic transformation.

iv/ HET+ patients and CIN

For the patients who were HET+, three E6/E7 transcription profiles were identified: expression of either E6*I or E6*II, or expression of both transcripts. The three profiles were equally distributed across all grades of CIN. These data suggest that either transcript is sufficient to initiate pre-malignant change. However, for women who express both transcripts, it was observed that a relative proportion of greater than 95%/5% for E6*I/E6*II was significantly correlated with high grade CIN. When compared to the E6*I/E6*II relative proportion of 97.1%/2.9% for CaSki cells [59], these results suggest that with an increase in the degree of transformation, there is a

corresponding increase in the expression of E6*I relative to E6*II. For women who express both transcripts, with a relative expression of $\geq$ 95% for E6*I, there may be an increased risk for invasive cervical cancer.

The transcripts chosen for study were selected because of their putative roles in causing neoplastic transformation. The E7 protein which is putatively encoded by the E6*I mRNA has the ability to bind to the product of the retinoblastoma (Rb) susceptibility gene [34]. Germline mutations in the retinoblastoma gene of mice results in embryo death [19,63,76], indicating the integral function of Rb in control of cell division and differentiation. Thus, the quantitative characterization of E6*I in cells progressing to neoplastic transformation may underscore the importance of this transcript in disrupting the cell cycle. As such, the observation that E6*I constitutes the major mRNA expressed from the viral transforming gene is suggestive that the viral E7 protein is of significance in the predisposition to the transformed state. Our finding that the E6*I transcript comprises an increased proportion of total transcription of the E6/E7 ORF in conjunction with increasing severity of disease further reinforces this hypothesis.

The E6*II mRNA potentially encodes the HPV-16 E6 protein, which has been shown to have p53 binding and degrading activities [142,171]. It has been proposed that p53 behaves as a tumor suppressor protein, and therefore the

ability of HPV to inactivate p53 may be crucial for transformation. Indeed the loss of functional p53, either through interaction with E6, or by mutation may predispose cervical epithelial cells to transformation in vivo [10,25].

v/ Patients expressing E6*I only

In rodent systems, transfection by E7 alone is sufficient to cause transformation [123,159]. However, in human keratinocytes, in vitro studies have shown that both the E6 and E7 proteins are required for transformation [54,104]. The absolute quantities of these products necessary for this process are presently unknown, yet these results suggest that E6*I mRNA, and by inference, the E7 protein may be quantitatively more important as the degree of cellular abnormality increases. Since the viral E6 protein also behaves as a transactivator of gene expression [27], it is possible that once the process of transformation has begun, E6 protein is no longer necessary, and therefore E6*II expression progressively declines. Interestingly, mouse embryos with deficient mutant p53 genes are able to survive to adulthood, but are more prone to developing cancers [28]. This suggests that p53 may be less critical than Rb in cell cycle control and viability, which perhaps is reflected in the enhanced expression of E6*I relative to E6*II for HPV-16. Clonal selection may confer a survival advantage to HPV-infected cells expressing E6*I as the major

E6/E7 mRNA, which develop into transformed cells and a subsequent neoplastic lesion.

vi/ Patients expressing E6*II only

If E6*I is quantitatively the more important of the spliced species of HPV-16 E6/E7 mRNA, it is not known why there is a subset of patients in the study cohort who solely expressed E6*II in association with clinically defined CIN. Several hypotheses could account for this phenomenon. Firstly, E6*I mRNA could be expressed, but below the limits of detection of the assay. Secondly, host-related co-factors, such as inflammation, could interfere with detection or could be responsible for E6*II-mediated transformation in the absence of E6*I. For example, the role of smoking has not been clearly proved or disproved with respect to the development of cervical neoplasia [163]. Alternatively, since E6*I may effect transformation by inactivating cellular Rb, the absence of viral E6*I suggests that cellular Rb may be absent or mutated in these individuals. Therefore, these cells may have the requirements for transformation: expression of E6*II and non-functional Rb, and thus regulation of the cell cycle is sufficiently disrupted for transformation to occur. A fourth possible explanation for the exclusive detection of E6*II in CIN patients could lie in the progressivity of the disease. It is established that the vast majority of patients with CIN do not progress to invasive carcinoma of

the tissue underlying the cervical epithelium [52]. The results of this study show that for a subset of patients with increasing progression of the disease, there is an increased expression of E6*I and a decrease in E6*II. Thus, lesions expressing only E6*II may have a reduced risk in progressing to more severe invasive cancer. It would be interesting to prospectively follow women in early stages of CIN to see if there is a relationship between progression to high-grade lesions and the transcription of E6/E7 transcripts.

vii/ The role of oral contraceptives

It has been proposed that the use of oral contraceptives predisposes cervical epithelial cells to HPV-associated neoplastic transformation [163]. At the molecular level, in vitro experiments have revealed that binding by glucocorticoid hormones to the LCR of HPV-16 may up-regulate expression of the E6/E7 ORF [116]. To investigate this relationship more closely, the effect of OC on expression of E6/E7 in the study cohort was studied. It was observed that with increasing age of the OC user, there was a corresponding decrease in expression of E6*II. A decrease in the proportion of E6*II relative to E6*I for some women, as previously discussed, may be associated with progression to malignancy. The influence of OC use on E6/E7 expression may already be evident in the epidemiology of CIN. The women who used OC in our study were young, with a

mean age of 22.8 years. This is of interest since CIN has become increasingly prevalent among women in their second and third decade [12,29].

Epidemiological studies have not clearly established the role, if any, of oral contraceptives in the development of cervical dysplasia [163,173]. Such studies are often confounded by detection bias, since oral contraceptive users are more likely to participate in cervical screening [61]. It is currently controversial whether OC use, duration of use [173], dosage of OC [119], or the fact that OC users tend to be a younger group than non-users, accounts for a possible association between OC and CIN. It has been noted that HPV-16 transforms cells more efficiently in the presence of dexamethasone, an effect which is mediated by the glucocorticoid responsive element located in the viral LCR [116]. This effect has also been observed in the presence of progestins and progesterones derived from oral contraceptives [115]. Furthermore, it has been observed that red blood cell folate concentrations are depressed in OC users, which may increase the risk for cervical dysplasia, in association with HPV-16 infection [11]. Therefore, further studies are required to fully elucidate the association between OC use and CIN.

8. Summary and conclusions

This study has yielded several insights into the biology of human papillomavirus and its role in the development of cervical intraepithelial neoplasia. Specifically, the dynamics of expression of the putative viral transforming genes and the association with transformation was investigated. The major findings of this study are summarized below.

1. A quantitative RT-PCR assay was developed, and the relative quantities of mRNA encoding viral transforming proteins were determined in a transformed cervical carcinoma-derived cell line. The proportions of E6*I and E6*II in CaSki cells were 97.1% and 2.9%, respectively.

2. Patients who express HPV-16 E6/E7 mRNA (HET+) tended to have high grades of cervical intraepithelial neoplasias. Women in whom HPV-16 E6/E6 mRNA was not expressed (HET-) tended to have lower grades of CIN.

3. Expression of E6*I relative to E6*II greater than 95% to 5% correlated with high grade CIN ($p = 0.05$). As the degree of transformation increased from CIN I to CIN III and then to fully transformed CaSki cells, the proportion of E6/E7 mRNA that was E6*I increased, and the proportion that was E6*II declined.

4. The trend in transcription seen in women with normal histology was different from that seen in patients with CIN. For normal cells, E6*I was less likely to be expressed and

while E6*II was more likely to be expressed. In CIN, E6*I expression increased relative to E6*II.

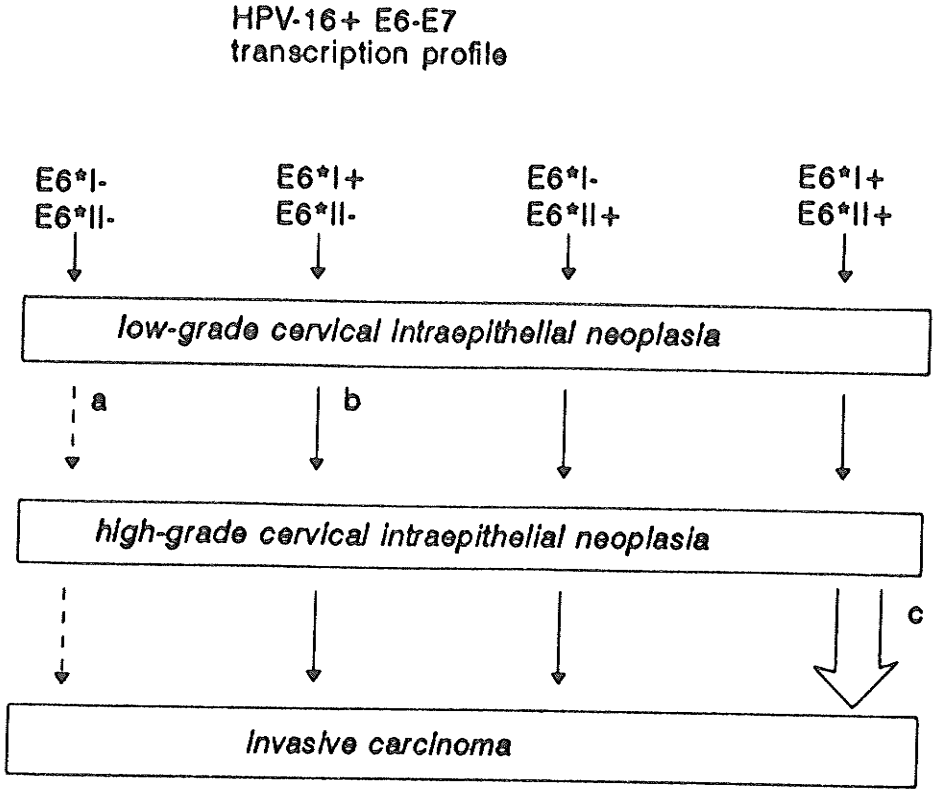
5. For CIN patients who were users of oral contraceptives, the transcription profile was the same as that of the non-users. Specifically, E6*I expression was higher while E6*II lower. However, oral contraceptive users with normal histology showed a trend in transcription that resembled that of CIN, rather than that seen in normal non-users.

6. Increasing age was associated with the transcription profile seen in patients with normal cervical histology. That is, there was a decline in E6*I expression and an increase in E6*II. However, increasing age in the presence of oral contraceptives caused the HPV-16 E6/E7 transcription profile to resemble that of CIN.

HPV-16 and cervical cancer: a complex model (Figure 13)

It is well known that the majority of CIN patients infected with HPV-16 do not progress to more severe disease, and either spontaneously regress or persist at the same stage of dysplasia [52]. The reasons for this are unknown, and it is also unclear whether other mechanisms in conjunction with HPV-16 infection may mediate disease progression. One objective of this study was to evaluate if quantitative expression of the E6/E7 ORF of HPV-16 could be used as a prognostic indicator of the likelihood of increasing disease severity. This model, based on the

Figure 13. A model for HPV-16 and cervical cancer.



	Risk of progression	Involvement of exogenous factors [d] in progression
a ⇠ - - - -	low	high
b ⇠	moderate	moderate
c ⇐	high	low

[d] exogenous factors: host-associated (smoking, other infections), mutant p53 or Rb, exogenous hormones.

findings of this study, proposes that this is possible, and is outlined below.

Cervical cancer in absence of HPV-16 infection

HPV-16 can be detected in about 50% of cervical carcinomas [180]. For patients who are not infected, it is speculated that the molecular events involved in transformation are not related to HPV and are mediated by alternate pathways, such as genetic susceptibility (loss of functional p53 or pRB), hormone status, and smoking. Alternatively, cervical dysplasia and carcinoma may also occur via infection with other oncogenic HPVs, such as HPV-18, 31, 33, and 35 [180].

Cervical cancer with HPV-16 infection

Patients in whom HPV-16 is detected can be analyzed with respect to expression of the E6/E7 ORF, the putative viral transforming genes. Expression patterns in these patients can be seen to fall into four groups: neither transcript is detected, only one of the two transcripts detected, and detection of both together. In this study, it was noted that the patients designated HET- (HPV-16 E6/E7 transcript not detectable) tended to have low-grade neoplastic lesions, while those with detectable transcript of any profile (HET+) tended to have high-grade lesions. Thus, it is speculated that non-expression of HPV-16 E6/E7 genes is associated with the development of low-grade CIN,

and that the progression to high-grade CIN and invasive carcinoma in these women requires interaction with other exogenous factors. In contrast, HET+ patients were found to be commonly associated with high-grade disease. The number of HET+ patients with each type of transcription profile was virtually the same, which suggests that expression of either spliced E6/E7 mRNA is sufficient to drive cervical epithelial cells to high-grade disease.

Although any of the three transcription profiles observed in HET+ patients was associated with high-grade CIN, it was found that for the group expressing both transcripts, a proportion of $\geq$ 95% for E6*I was correlated with high-grade CIN. Furthermore, it was shown that with an increase in the quantity of E6*I relative to E6*II, there was an increase in the degree of transformation in cervical cells. Thus, it is proposed that expression of both transcripts concurrently, and in $\geq$ 95%/5% proportions may cause cervical intraepithelial neoplasia to progress to invasive carcinoma. The HET+ patients expressing E6*I alone, or E6*II alone, may develop to high-grade CIN, but other co-factors must mediate the progression to invasive carcinoma.

The model proposed raises many questions; for example, it would be of interest to know whether or not the aggressiveness of disease or time required for disease progression is correlated with viral E6/E7 transcription. It is known that for invasive carcinoma, the disease may be

more aggressive in younger women than in older women [36]. Since age may be related to viral transcription, further study should reveal if there is a relationship between disease aggressiveness and expression of viral transforming genes.

Another question raised by the results of this study is what are the potential roles of the cellular tumor suppressor proteins p53 and retinoblastoma. Early studies have suggested that in the absence of detectable HPV-16 DNA, transformation may be mediated by loss of functional p53 or Rb [25,141]. However, these studies have not been applied more specifically to HPV-16 E6/E7 transcription-negative specimens, wherein HPV DNA is present but may not be transcriptionally active. As such, the p53 and Rb status of the HET- patients and also the HET+ patients in whom only one transcript was detected would be relevant to development of the model.

This model proposes that patients in whom both E6* mRNAs are expressed are more predisposed to developing high-grade CIN. By extrapolating the observation that an increased proportion of E6/E7 mRNA comprised of E6*I is associated with an increased degree of transformation, it is speculated that these patients would be at particular risk for developing invasive carcinoma. Thus, extending the study to fully transformed cervical cells from patients would be of special significance.

Finally, it was shown that within the subset of HET+ patients, there were patients with normal cervical histology. These women were biopsy-confirmed CIN-negative, but it is possible that benign inflammatory processes had resulted in transcriptionally-active HPV-16. However, it is also speculated that these women may be at increased risk for developing CIN, and it follows that a prospective study including these women may yield further insights into disease risk associated with expression of HPV-16 transforming gene.

From the results of this study, it is evident that quantitation of clinically salient viral mRNAs and subsequent detailing of viral expression dynamics in association with studies of cellular factors may eventually clarify the molecular events of the role of HPV-16 in cellular transformation.

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10. Appendices

Culture Media

Blood Agar

Sheep blood agar base (Oxoid, Hampshire, England)	100 g
Distilled water	2500 mL

Combine and mix to dissolve. Autoclave at 121°C for 40 minutes. Cool to 45 - 50°C. Aseptically add 125 mL 30% citrated sheep blood (Atlas Laboratories, Winnipeg). Mix well and dispense.

L-15 cell culture media (Flow Laboratories, Cat. No. 10-511-22)

Powdered Leibovitz-15 media (modified with glutamine)	2 containers
Distilled water	20 L

Adjust pH of L-15 media to pH 7.6, using approximately 20 mL of 4 N HCl. Filter sterilize.

Fetal Bovine Serum (Flow Laboratories, Cat. No. 29-168-54)
Media supplement

Keep stored at -20°C until needed. When required, incubate at 56°C for 30 minutes to heat inactivate serum. Cool to room temperature. Store at 5°C.

Buffers

Phosphate buffered saline (PBS) pH 8.0

Sodium chloride	24 g
Potassium chloride	0.60 g
Potassium phosphate, monobasic (KH_2PO_4)	0.36 g
Sodium phosphate, dibasic (Na_2HPO_4)	2.63 g
Distilled water	3000 mL

Combine and mix to dissolve completely. Adjust pH to 8.0. Autoclave at 121°C for 15 minutes.

HEPES buffer (N-2-hydroxyethylpipcrazine-N^1-2-ethanesulfonic acid)
(Gibco, Cat. No. 845-1344)

1 M solution

HEPES stock	117.65 g
Distilled water	400 mL

Adjust pH to 7.3 using 5 N NaOH, approximately 20 mL. Filter sterilize and store at 4°C.

Tris-acetate-EDTA buffer 50 X (TAE)

Trizma base (Sigma)	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA	100 mL

Bring to 1000 mL with glass distilled water. Dispense into 500 mL bottles.

0.5 M EDTA

Disodium EDTA	186.1 g
NaOH	20 g
Distilled water	800 mL

Adjust pH to 8.0 with 1 M NaOH and bring volume to 1000 mL with glass distilled water.

Tris-EDTA (TE) buffer

1 M Tris-Cl, pH 8.0	10 mL
0.5 M EDTA	2 mL

Bring volume to 1000 mL with distilled water. Dispense into bottles. Autoclave for 15 minutes at 121°C.

1 M Tris-Cl, pH 8.0

Trizma base (Sigma)	121.1 g
Distilled water	800 mL

Adjust pH to 8.0 with approximately 42 mL of concentrated HCl. Bring volume up to 1000 mL with distilled water.

Tris-borate-EDTA 10X (TBE)

Trizma base (Sigma)	108 g
Boric acid	55 g
0.5 M EDTA (pH 8)	40 mL

Bring volume up to 1000 mL.

Cell culture reagents

Modified Hanks Balanced Salts Solution (BSS) 10X

NaCl	80 g
KCl	4 g
Na_2HPO_4	0.5 g
Dextrose	10 g
KH_2PO_4	0.6 g

Bring volume up to 1000 mL.

Trypsin 1X (0.25%)

10X Trypsin (Flow Laboratories, Cat. No. 16-893-49)	10 mL
HEPES buffer	1 mL
Modified Hanks BSS	90 mL

Adjust pH to 7.4 with 1 N NaOH. Store at 4°C.

Gel electrophoresis

4% agarose gel (total volume 100 mL)

Agarose (Bio-Rad)	4 g
1X TAE buffer	100 mL

Boil while stirring with a magnetic stirrer.
Cool slightly and add 4 uL 10 mg/mL ethidium bromide.

8% polyacrylamide gel (total volume 100 mL)

Acrylamide/bis (29:1) (Bio-Rad)	26.6 mL
10X TBE buffer	10.0 mL
10% Ammonium persulfate	0.7 mL
Distilled water	62.7 mL

Mix gently. Add 35 uL TEMED (Bio-Rad) and cast immediately.

Ammonium Persulfate (10%)

Ammonium persulfate (Bio-Rad)	0.1 g
Distilled water	900 uL

Tracking dye (agarose gel electrophoresis)

Bromophenol blue	0.07 g
Sodium dodecyl sulfate	7 g
glycerol	33 mL
Distilled water	67 mL

Store at room temperature.

Ethidium Bromide (10 mg/mL)

Ethidium bromide	1 g
Distilled water	100 mL

Wrap bottle in aluminum foil and store at 4°C.

Precipitation of mRNA and DNA

3 M Na Acetate

Sodium acetate	204 g
Distilled water	300 mL

Adjust pH to 5.2 with glacial acetic acid. Bring volume to 500 mL and dispense. Autoclave for 15 minutes at 121°C. Precipitate nucleic acid by adding 0.1 volumes 3M sodium acetate and 2 volumes of absolute ethanol.

Primer sequences

Glyceraldehyde-3-phosphate dehydrogenase

Primer 1 CATCTCTGCCCCCTCTGCTGA

Primer 2 GGATGACCTTGCCCACAGCCT

HPV-16 E6/E7 spliced transcript

Primer 1 CAAGCAACAGTTACTGCTACTTG

Primer 2 TCCGGTTCTGCTTGTCCAGCTGG