THE ROLE OF HPV-16 E6/E7 ALTERNATIVELY SPLICED TRANSCRIPTS IN UTERINE CERVICAL DYSPLASIA

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by

Rachmat Hidajat

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BY

RACHMAT HIDAJAT

A Thesis/Practicum 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

Dedication

This thesis is dedicated to my beloved wife, son, and parents, for their unlimited love, encouragement, understanding, and unselfish sacrifice.

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Abstract

The role of human papillomavirus (HPV) as the causative agent of cervical cancer, one of the most common malignancies in women worldwide, has been established. HPV type 16 accounts for more than 50% of these cases. Expression of E6/E7 open reading frame (ORF) of HPV-16 is essential for transformation of the host cell. The precursor mRNA is processed into two mature mRNA, E6*I and E6*II, by alternative splicing. The primary function of the splicing within E6 ORF is believed to be to facilitate the efficient translation of E7 protein, the predominant factor in oncogenesis. The E7 protein is mainly translated from E6*I mRNA, due to the close proximity of the E6 protein stop codon to the start codon for E7 in the full-length and E6*II transcripts. The splicing pattern for the transcripts is conserved in all grades of cervical intraepithelial neoplasia (CIN) and cervical cancer derived cell lines. In this present work, a modified quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) assay was used to assess quantitative changes in E6*I and E6*II expression in exfoliated cell and biopsy specimens in relation to the grade of CIN. The findings demonstrated that E6*II expression was significantly diminished or absent in biopsy tissue obtained from CIN II/CIN III, which suggested that the activation of 3' splice sites within E6 ORF is associated with cellular differentiation. The relationship between expression of SF2 and hnRNP A1 splicing factors and 3' splice site selection within the HPV-16 E6/E7 ORF was also evaluated by using the modified QRT-PCR. These splicing factors are known to modulate alternative 5' splice site selection of polycistronic pre-mRNA including those of SV40 and adenovirus, but their role in 3' splice site selection is unknown. To test these functions *in vitro*, a plasmid construct expressing HPV-16 E6/E7 was designed. The expression of SF2 positively correlated with E6*II expression. By combining site-directed mutagenesis and inverse PCR, two mutant plasmids with abrogated splice acceptor sites were generated in order to continue to define the roles of E6*I and E6*II in cervical cancer in future studies.

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Introduction

Cervical cancer is one of the most common malignancies and the second worldwide leading cause of death from cancer among women. Most of them live in developing countries where cervical cancer is 6-fold more common than in Western societies (Fisher, 1994). Of the estimated half million new cases per year (Parkin *et al.*, 1993), 50% will ultimately die of this disease.

Enormous studies have been performed to determine the etiologic agents of cervical cancer and their roles in cervical carcinogenesis. Many factors have been proposed to be associated with cervical cancer, in particular sexually transmitted agents. The attempts to demonstrate a link between spermatozoa, smegma, *Neisseria gonorrhea*, *Trichomonas vaginalis*, cytomegalovirus, *Chlamydia trachomatis*, and Herpes Simplex Virus 2 (HSV-2) and cervical cancer have failed to show a strong relationship (Okagaki, 1992). However, in the last two decades a strong correlation between Human Papillomavirus (HPV) infection and cervical cancer has been established and the role of HPV as the etiologic agent of cervical cancer has been accepted widely. Despite numerous investigations, the pathogenesis of HPV infection in causing cervical cancer is still not clearly understood, since few of cancer-related HPV infections will develop into cancer.

Literature Review

Human Papillomavirus and The Cancer of The Uterine Cervix

Epidemiology

HPV DNA is detected in a high proportion of invasive cervical cancers and cell lines derived from cervical cancers. The relative rarity of HPV-negative cervical cancers (de Villiers, 1989; Muñoz & Bosch, 1992) indicates a strong association with HPV infection. HPV DNA and viral transcripts are detected not only in the primary uterine cervical cancer, but also in lymph nodes with and without histological evidence of metastasis (Rose *et al.*, 1991; Rose *et al.*, 1992).

Epidemiological studies of different HPV-types infecting the cervix have defined the "low risk" types which are associated with milder diseases and the "high risk" HPV types which commonly correlate with malignancies. The latter includes at least types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 68 (Schiffman, 1994).

Similar to other human cancer, cervical cancer develops through a multistep process (Morris *et al.*, 1995). It begins as a preinvasive neoplastic change in cells, which may regress, remain unchanged, or progress to invasive cancer. In pre-invasive lesions, namely cervical intraepithelial neoplasia (CIN), the prevalence of HPV infection is very high. In one study, it approached 100% with high risk types of HPV accounting for at least half of the infections (Schiffman, 1994). Nevertheless, the prevalence of HPV DNA in cytologically normal women varies from 100% to less than 5%, depending on the demographic and behavioral profiles of the study group (Schiffman, 1994). Some factors associated with increasing HPV DNA detection correlate with sexual behavior, such as younger age at first intercourse, history of prior sexually transmitted disease, higher lifetime number of sexual partners; lower educational achievement, lower socioeconimic

status, multiparity, hormonal contraceptive use, pregnancy, and current smoking (Bauer *et al.*, 1993; Wheeler *et al.*, 1993; Parazzini *et al.*, 1992; McNicol *et al.*, 1990), yet the associations are inconsistent between investigators.

Some studies also indicate coincident, multiple HPV infections. The prevalence of multiple infections approaches 20-30% of all infected women. However, the prevalence of multiple infections tends to decrease in high-grade lesion (Schiffman, 1994). Infections with other infectious agents such as HIV, herpes simplex virus 2, human herpesvirus 6, or chlamydia may be cofactorial for the development of cervical neoplasia (Chen *et al.*, 1994). Abnormal vaginal microfloras were also reported to be associated with CIN (Guijon *et al.*, 1992).

HPV infections appear to be the most common in young sexually active women, whereas it is uncommon in virgins (Schiffman, 1994). The peak prevalence of HPV infections is between 15-25 years of age (Morrison *et al.*, 1991; Ley *et al.*, 1991; van den Brule *et al.*, 1991). Older women are less likely to be HPV positive (Hildesheim *et al.*, 1993). The lower prevalence of HPV infections in women over 30 might be due to immunologic clearance or regression of existing lesions, combined with less exposure to new HPV types because of fewer new sexual partners (Schiffman, 1994).

It is obvious that cervical HPV infections are sexually transmitted. Risk of obtaining HPV infections increases with increasing numbers of sexual partners. It seems also that the infection occurs soon after the sexual intercourse debut (Schiffman, 1992). Sexual activity with a few new sexual partners leads to high prevalence of HPV DNA in cervical specimens within months. The relationship between HPV-related penile lesion in male partners and women with CIN has also been shown (Barrasso, 1992). The detection of oncogenic-HPV types in prostatic tissue implies a possible reservoir for infection (McNicol & Dodd, 1989; McNicol & Dodd, 1990; McNicol & Dodd, 1991).

Transmission of cervical HPV infections through non-sexual routes seems to be rare. Nevertheless, limited data, such as the discovery of HPVs in uterine cervix among

virgins and celibate females, and in women who have only one lifetime sexual partner (Ley *et al.*, 1991) suggest its possibility. Vertical transmission from women infected with genital HPV to their babies has been reported (Kaye *et al.*, 1994). Hand-genital contact may also be a potential mode of transmission, particularly in the very young (Fairley *et al.*, 1995).

Many studies using various methods for HPV DNA detection in cytologically normal cervical cells found that the detection and typing of the virus is inconsistent between sequential specimens from the same individual (McNicol *et al.*, 1992; Rosenfeld *et al.*, 1992; Schneider & Koutsky, 1992; Evander *et al.*, 1995). Possibly, HPV infections in cytologically normal cervical cells are chronic and persistent, but only intermittently detectable. Alternatively, HPV infections are also hypothesized to be transient (Schiffman, 1992; Evander *et al.*, 1995). A cohort study showed that the number of women with "new" infection equalled the number "losing" infection, and the longer the interval between examinations, the smaller the probability of the same type HPV being detected. However, for CIN, it is much more likely for persistent rather than transient infections to occur. HPV persistence is probably an important risk factor for the development of cervical neoplasia (Schiffman, 1994).

Taxonomy of Human Papillomavirus

Together with polyomaviruses, papillomaviruses are grouped in the *Papovaviridae* family. The members of this family are non-enveloped, have a double-stranded close circular DNA genome, an icosahedral capsid composed of 72 subunits (capsomere), and similar biochemical composition. Papillomaviruses and polyomaviruses have different genomic organizations, in that there is only one coding DNA strand in papillomaviruses whereas separate strands code for vegetative functions and structural proteins of polyomaviruses. Hence, papillomaviruses are subclassified in the subfamily *Papillomavirinae* (reviewed in Pfister & Fuchs, 1994).

Historically, classification of the papillomaviruses was based on host species and then on DNA homology, but not on serologic analysis since many types replicate so poorly that there is not enough antigen for immunization or detection. Viruses isolated from a particular species were named after the natural host, such as cottontail rabbit papillomavirus (CRPV), bovine papillomavirus (BPV), deer papillomavirus (DPV), and human papillomavirus (HPV). Because of host specificity, organotropism, and growth difficulty in a tissue culture environment, the biology and genetic structure of papillomaviruses were not elucidated until molecular biology techniques had been developed.

The development of Southern blot hybridization and DNA cloning in the 1970s revealed that more than one genotype of HPV exist. The papillomaviruses were subsequently subclassified according to DNA homology. If the DNA of a papillomavirus was less than 50% homologous to the DNA of other known types, it was described as a new genotype. If the homology was higher than 50%, it was considered as a subtype and if the homology was close to 100%, it was regarded as a variant of the same viral type. Since sequencing of the DNA genomes became more feasible, the initial definition of an HPV type has recently been modified. At present a new HPV type is defined by a comparison of the DNA sequences of the E6, E7, and L1 open reading frames (ORFs) to the same ORFs of all the known HPV types. If this homology proves to be less than 90%, the full-length genome constitutes a new type. Homology higher than 90% renders it as another subtype (reviewed in de Villiers, 1994).

Currently almost 100 genotypes of HPV have been isolated, and over 70 genotypes have been identified and characterized. Sequence variants of individual HPV types appear clustered in different geographic regions of the world (Chan *et al.*, 1992; Eschle *et al.*, 1992). The existence of European, Asian, and American Indian branches indicates that the viruses spread and co-evolve with humankind over the globe (Ho *et al.*, 1993).

HPV is organotropic. Some genotypes are known to associate with skin warts, while others are associated with benign and malignant mucosal lesions (reviewed in de Villiers, 1989; Moscicki, 1992). The cross infection between organs is very rare, nevertheless the presence of mucosal HPV (HPV-16) in squamous cell carcinoma of the finger has been reported (Sánchez-Lanier *et al.*, 1994).

Papillomavirus genomes contain approximately 7,800 to 7,900 base pairs and have similar genomic organizations (Figure 1). There are nine open reading frames (ORFs) which encode six to seven early (E1 to E7) proteins and two late (L1 and L2) proteins. There is also a long control region (LCR) which does not encode protein, but contains transcriptional enhancers and promoters. All ORFs and LCR region occur on one strand. The early proteins play a role in viral replication while the late proteins are structural proteins required for viral assembly; structural proteins are found only in well-differentiated squamous cells and rarely in cancer cells. The biological function of most ORFs has been identified, mostly from BPV 1. This virus has been used as a reference for other papillomaviruses.

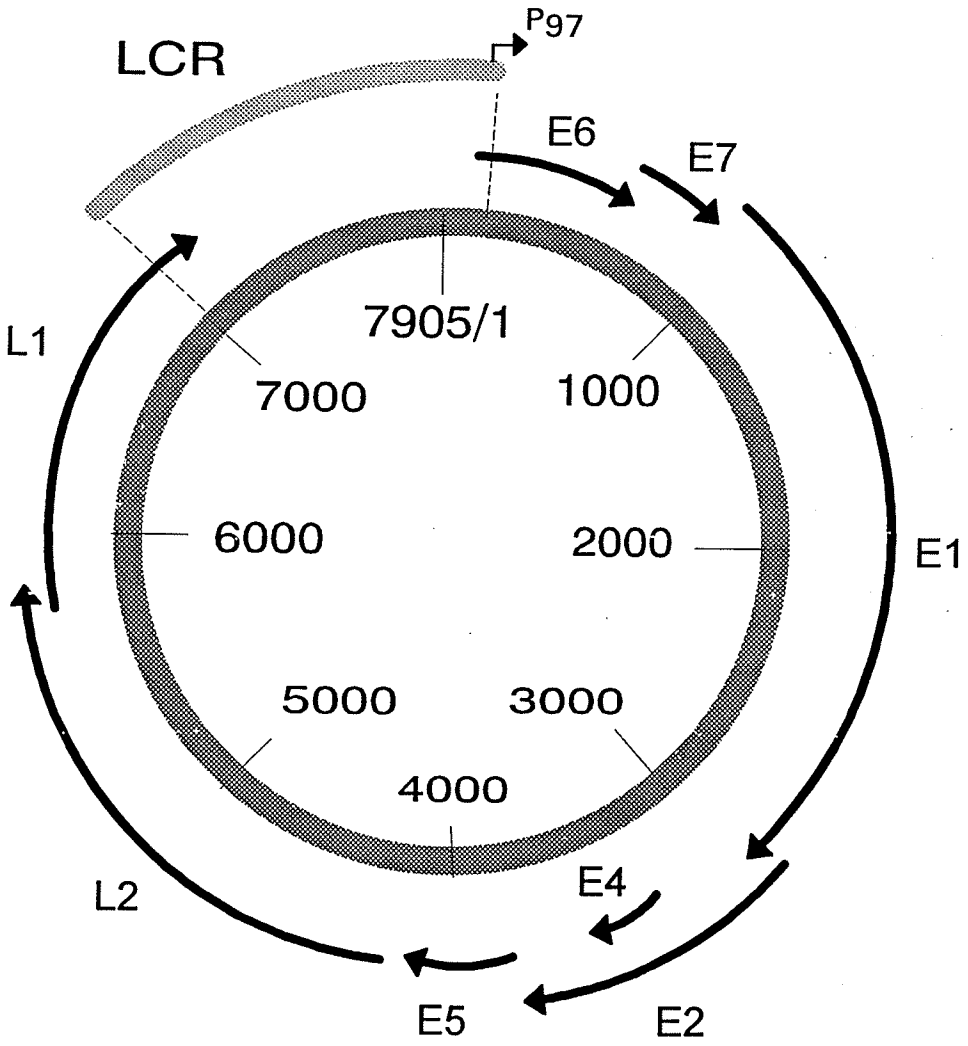


Figure 1. Genomic Map of HPV-16

This schematic diagram of HPV-16 genome shows the early-region (E) and late-region (L) open reading frames. The P_{97} promoter is located within the LCR upstream of the E6 and E7 genes.

The Biology of HPV Genes

E1

Little is known about the role of HPV E1 protein, however, it seems to have essential roles in viral replication for a number of HPV types. E1 is one of the most highly conserved of the papillomaviral genes. *In vitro* biochemical studies of HPV E1 have been hampered by difficulty in producing a purified protein. Significant sequence similarities are noted between 200 amino acids in the C-terminal half of papillomavirus E1 polypeptides and the large-T proteins of polyoma viruses. The strongest similarities were found in two blocks in the C-terminal portion of E1, which correspond in SV40 and polyoma virus to sites involved in ATPase and nucleotide-binding activities. In combination with E2, E1 can support the replication of homologous and heterologous papillomavirus DNA replication origins (Chiang *et al.*, 1992; Del Vecchio *et al.*, 1992).

BPV E1 gene product is a 68-kDa nuclear phosphoprotein which forms a complex with the 48-kDa E2 transcription factor. This complex binds specifically to the viral origin of replication which contains multiple binding sites for E2 located within the viral LCR (Wilson & Ludes-Meyers, 1991). Without the association with E2, E1 binds the origin weakly, whereas the additional binding of E2 increases this affinity (Mohr *et al.*, 1990; Ustav *et al.*, 1991). The cellular proteins that interact with E1 and E2 when bound to the origin of replication are not known yet (Scheffner *et al.*, 1994). Mutation of the E1 gene result in increased levels of viral transcription and a corresponding increase in viral transformation activity (Lambert & Howley, 1988; Schiller *et al.*, 1989). Since E1 has the ability to complex with E2 and repress E2-transactivated transcription from the major early promoter P_{89} of BPV-1 (Sandler *et al.*, 1993), the mutations of E1 might cause decrease or cause a loss of its binding capacity for E2, resulting in a reduction or loss of the repression of E2 activity.

Mutations or deletions of E1 gene of HPV-16 resulted in the increased immortalization capacity of the viral genome (Romanczuk & Howley, 1992), which may depend on efficient E6 and E7 expression (Münger *et al* 1989; Hawley-Nelson *et al.*, 1989). These results suggest that HPV-16 E1 may be able to repress the P_{97} promoter, although the mechanism of such a repression is not known (Scheffner *et al.*, 1994).

E2

As for E1, papillomavirus E2 gene products are relatively well conserved and are important regulators of viral transcription and replication. The E2 proteins regulate transcription from promoters containing E2 binding sites (E2BS) (McBride *et al.*, 1991). The E2 ORF of HPV is thought to encode a papillomavirus-specific transcription factor which also has a role in viral replication. The E2 proteins of all papillomaviruses studied to date have been shown to bind specifically to the common conserved sequence $ACC(N)_6GGT$ found at multiple locations in their genomes. The full-length E2 protein can function as a transactivator or a repressor depending on the context of the E2BS within the enhancer/promoter region (Bouvard *et al.*, 1994). Binding of E2 to its binding site, which overlaps the transcription enhancer factor Sp1 binding site, displaces the Sp1 factor. This displacement may or may not result in repression of E6 promoter in the absence of the proximal E2BS (Demeret *et al.*, 1994; Tan *et al.*, 1994).

Two shorter forms of BPV E2 can inhibit the transcriptional transactivation function of the full-length polypeptide by competing for DNA-binding sites and by forming inactive heterodimers with the full-length transactivator protein (McBride *et al.*, 1991). These repressor proteins fail to complex with E1 suggesting that the 162-amino acid region of BPV E2 that participates in transactivation contains critical determinants for interaction with E1 (Mohr *et al.*, 1990).

The high-risk HPVs' E2 proteins can repress expression of the E6 and E7 genes by binding to E2-binding sites located in close proximity to the E6/E7 promoter and most

probably interfere with the assembly of the pre-initiation complex (Thierry & Yaniv, 1987; Bernard *et al.*, 1989; Romanczuk *et al.*, 1990). Kinetic studies show that the E2-DNA complexes are very stable, with half-lives ranging from 2.15 to greater than 240 minutes (Sanders & Maitland, 1994).

There are four E2BS within the LCRs of all genital HPVs genomes. Two E2BS are located immediately adjacent to the TATA box of the P_{97} promoter of HPV 16 which possesses a basal activity which can be repressed by full-length E2 gene products (Thierry & Yaniv, 1987; Bernard *et al.*, 1989). The basal activity of this promoter is dependent on the keratinocyte-dependent enhancer contained within the LCR.

Wild-type HPV 16 DNA immortalizes primary human keratinocytes with low efficiency, whereas a genome with a mutation in the E2 ORF is perhaps ten fold more efficient in inducing cellular immortalization (Romanczuk & Howley, 1992). The E2 gene is usually disrupted in cervical cancer, which may account for high expression of E6 and E7 ORFs. Absence of E2 gene was observed in biopsy samples of cervical lesions with different histopathological diagnoses despite episomal or integrated viral forms (Krajinovic *et al.*, 1993). Introduction of BPV E2 to cell lines derived from cervical carcinoma results in specific inhibition of the expression of the resident HPV E6 and E7 genes and in inhibition of cell growth (Hwang *et al.*, 1993). However, mutation of the E2-binding sites adjacent to the P_{97} promoter only partially diminish E2-mediated repression, suggesting that an additional mechanism must also be involved in regulation of this promoter (Romanczuk & Howley, 1992).

E5

Much of our understanding of the action of E5 protein has come from studies of BPV E5. BPV E5 is responsible for the major transforming activity of the virus (Schiller *et al.*, 1986). Recent studies showed that HPV E5 is a potential mediator of immortalization or transformation of human cells. The role of HPV-6 E5, and HPV-16 E5

in transformation of rodent cell lines has been shown by several groups (Chen & Mounts, 1990; Leptak *et al.*, 1991). HPV-16 E5 contains 83 amino acids and is strongly hydrophobic. It can transform murine fibroblasts and keratinocytes by cooperation with the epidermal growth factor receptor (EGFR), it disturbs the processing of stimulated receptor, resulting in slower turnover. BPV E5 has been shown to act through its association with ductin, a 16 kDa gap junction protein and a component of the vacuolar H^+-ATPase (V-ATPase). Using ^{35}S-methionine metabolically labeled cell lysate of human keratinocyte cell line, HPV-16 E5 was shown to associate with a cellular protein with a similar molecular weight (Kell *et al.*, 1994). The immortalization or transformation of human cells by HPV E5 has yet to be proven, moreover, the E5 gene is likely to be a part of HPV genome fragment deleted during integration into the host cell genomes (Schwarz *et al.*, 1985; Kaur *et al.*, 1989).

E6

Like the SV40 large T-antigen, E6 proteins of high-risk HPVs are able to complex with and degrade p53 (Werness *et al.*, 1990; Mietz *et al.*, 1992) (Figure 2.). Whereas some studies suggest that the E6 proteins encoded by HPV-6 and 11, viral types which are normally limited to benign lesions, may also interact with p53, the association is weaker (Werness *et al.*, 1990). The specific domain of E6 involved in complex formation with p53 is distinct from the degradation domain (Mansur *et al.*, 1995). It has been shown that the high-risk HPV E6 but not mutant E6 can efficiently abrogate the transcriptional regulatory activity of p53 following genotoxic stress (Gu *et al.*, 1994; Sverdrup & Khan, 1994). Crook *et al.* (1994) indicated that association between E6 and p53 is necessary for this interruption. Huibregtse *et al.* (1991) found that complex formation between E6 and p53 required an additional cellular factor which was designated as E6-associated protein (E6-AP) and had a molecular weight of 100 kDa. Later, they also reported the purification and cloning of a cDNA for E6-AP (Huibregtse *et al.*, 1993) which contains a

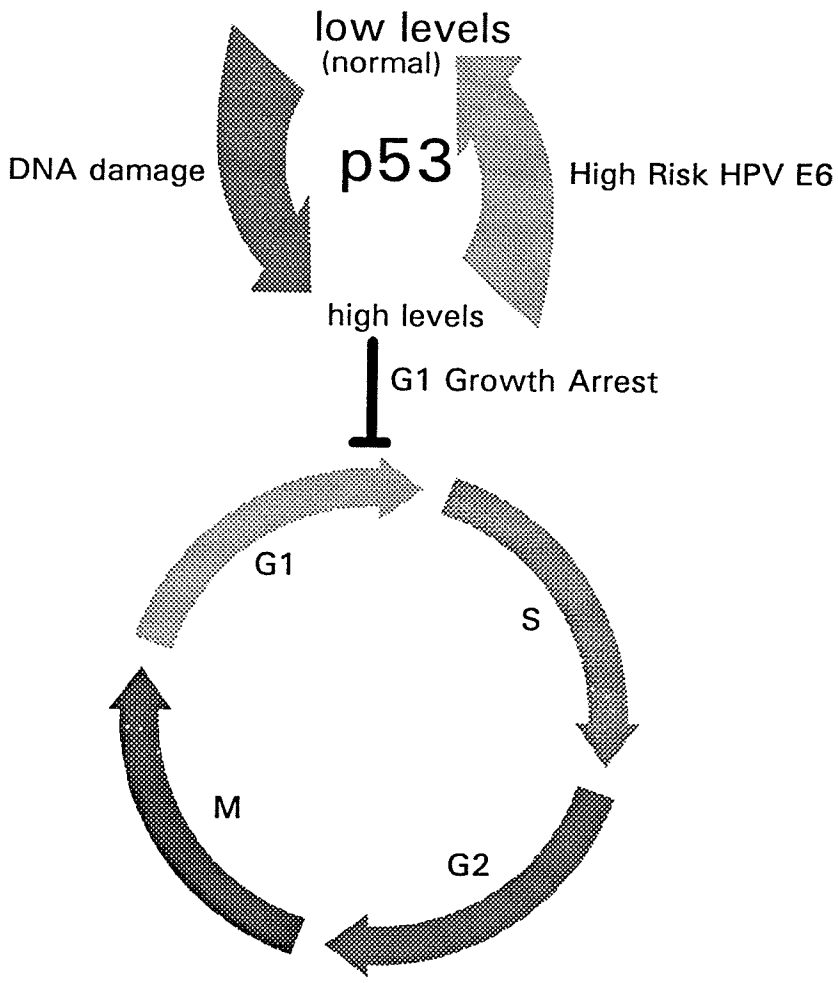


Figure 2. Interaction between high risk HPV E6 and p53

The level of p53 in primary cells is usually low. Damaging agents increase the level of cellular p53. The elevated levels of p53 can lead to a cell cycle checkpoint arrest in G1 possibly to allow the damaged DNA to be repaired. High risk HPV E6 protein interferes with this negative growth regulatory function of p53 by forming a complex leading to inactivation and degradation of p53.

novel ORF encoding 865 amino acids.

Wild-type p53 behaves as a negative growth regulator or tumor suppressor gene (reviewed in Donehower & Bradley, 1993). The ability of the p53-deficient mice to develop normally demonstrates that p53 is not essential for normal cell division, cell differentiation, or embryonic development, however the mice are susceptible to tumors at a young age. In normal cells, if DNA is damaged, p53 is induced, and arrests the cell in G_1 of the cell cycle until the damage is repaired (Reich & Levine, 1984; Kastan *et al.*, 1991). If the damage cannot be repaired, the p53 might initiate programmed cell death (apoptosis). Apparently, mutant forms of p53 are commonly found in human malignancies but not in HPV-induced cancers. The level of p53 in HPV-infected cells is low compared to uninfected primary host cells (Scheffner *et al.*, 1991). The HPV E6 protein inactivates p53 through degradation which involves the ubiquitin-dependent protease system (Scheffner *et al.*, 1990) and this process is induced by E6-AP (Huibregtse *et al.*, 1993). Levels of p53 in E6 immortalized cells or in HPV-positive cervical carcinoma cells are, on average, two or three fold decreased compared to primary cells. In the *in vitro* system, E6 leads to an almost complete removal of p53 (Scheffner *et al.*, 1990; Scheffner *et al.*, 1991). The half life of p53 is reduced from 4 hours to 15-30 minutes in human keratinocytes and fibroblasts expressing E6 (Hubbert *et al.*, 1992). While in normal cells, intracellular p53 level increases in response to DNA damaging agents (Kastan *et al.*, 1992), in E6-expressing cells, increase in p53 level is not observed (Kessis *et al.*, 1993; Gu *et al.*, 1994). However, p53 degradation is not the only mechanism for p53 inactivation since HPV E6 mutants with no detectable degradative activity, were able to some extend to abrogate p53-mediated transcriptional *trans*-regulation (Crook *et al.*, 1994; Thomas *et al.*, 1995).

A recent study showed that HPV-16 E6 associated specifically with several other proteins designated pp212, pp182, p81, p75, and p33 (Keen *et al.*, 1994). By using the yeast two-hybrid system another E6-binding protein was found (Chen *et al.*, 1995). The latter protein is identical to a putative calcium-binding protein, ERC-55, localized in the endoplasmic reticulum. The possible roles of these cellular proteins in tumor progression remain to be elucidated.

Independent of p53, the HPV E6 protein exhibits transcriptional effects, including *trans*-activation of the HPV non-coding region (Gius *et al.*, 1988; Desaintes *et al.*, 1992) and adenovirus E2 promoter (Shirasawa *et al.*, 1994), and repression of the moloney murine leukemia virus long terminal repeat and the cytomegalovirus immediate early promoter, although the degree of the repression was low (Etscheid *et al.*, 1994). Interestingly, it was also found to repress its autologous promoter (Shirasawa *et al.*, 1994), suggesting a negative feedback control mechanism of the oncogene expression.

Despite the detrimental effect of E6 protein on the host cell, several lines of evidence suggest that p53 inactivation may not an obligatory factor in cervical carcinogenesis. Many studies showed that the potential coding transcript of E6 protein was often undetectable in many cervical lesions and cell lines (Böhm *et al.*, 1993). There was no significant association between the presence or absence of HPV DNA and p53 positivity in CIN (Pöllänen *et al.*, 1993). An increase of functional nuclear p53 protein levels upon treatment with genotoxic agents was still observed in HPV-positive cell lines, indicating that the tumorigenic phenotype of HPV-positive cancer cell lines does not necessarily correlate with a lack of DNA damage-induced p53 activity (Butz *et al.*, 1995).

E7

HPV E7 protein of high risk HPV has some functional similarities and also shares regions of significant amino acid sequence similarity with the adenovirus (Ad) 12S E1A product (Phelps *et al.*, 1988; Dyson *et al.*, 1992). It can *trans*-activate the Ad E2

promoter (Phelps *et al.*, 1988), stimulate DNA synthesis in cells (Sato *et al.*, 1989; Watanabe *et al.*, 1992), cooperate with *ras* in rodent cell transformation assays (Heck *et al.*, 1992; Phelps *et al.*, 1988; Matlashewski *et al.*, 1987) and complex to some cellular proteins. One of the cellular proteins known to bind to HPV E7 is pRB (Figure 3).

pRB is the product of the retinoblastoma tumor suppressor gene which acts as a negative regulator of cell growth at the G_1/S border. The transition from active to inactive forms of pRB is regulated by phosphorylation-dephosphorylation mechanisms. The hypo- or under-phosphorylated form of pRB is believed to be the active form with respect to cell growth suppression, while the hyperphosphorylated form is the inactive form. HPV-16 E7 binds preferentially to the hypophosphorylated form of pRB which may result in the functional inactivation of pRB and permits progression to the next steps in cell cycle. Like adenovirus E1A protein and the simian virus 40 large tumor antigen, E7 protein can disrupt the transcription factor E2F-pRB complex (Chellapan *et al.*, 1992; Pagano *et al.*, 1992). E7 binding affinity to pRB correlates with its transformation capacity (Heck *et al.*, 1992). Several observations suggest that the interaction of pRB with E2F is functionally significant with respect to the action of pRB as a tumor suppressor (reviewed in Nevins, 1992). Rabbit polyclonal antiserum against HPV-16 E7 (Sato *et al.*, 1989) detected both nuclear and cytoplasmic E7 proteins transiently expressed in the COS-1 cells, but the monoclonal antibodies only detected the cytoplasmic E7 (Kanda *et al.*, 1991), supporting the hypothesis that the intranuclear E7 protein formed a complex with cellular proteins. Demers *et al.*, (1994) reported that even though p53 protein levels increased in response to DNA damage in cells expressing HPV-16 E7, G_1 growth arrest was bypassed, suggesting a connection between the p53 and pRB pathways in controlling the growth arrest signal after DNA damage. *Trans*-activation by HPV-16 E7 in pRB-deficient breast tumor cell lines (Carlotti & Crawford, 1993), and the fact that mutations in E7 which inhibit binding of pRB did not abrogate the ability of the HPV-16

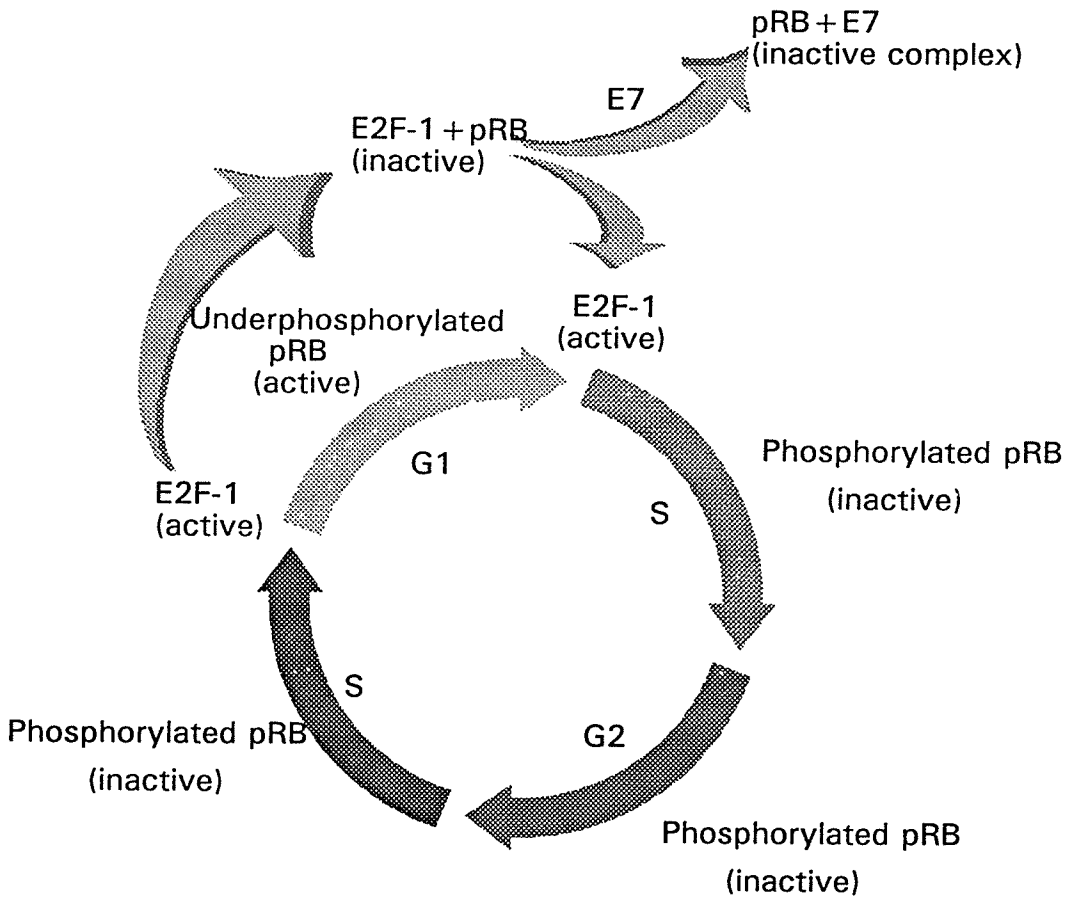


Figure 3. Interaction between HPV-16 E7 and pRB

The active form of pRB is detected only in the G_0/G_1 phase of the cell cycle, and acts as a negative regulator of cell cycle progression. The cellular transcription factor E2F-1 is preferentially bound to the active form of the RB protein which presumably results in the functional inactivation of E2F-1. Interaction with a viral oncoprotein such as HPV-16 E7 may result in the release of E2F-1 allowing it to function as a transcriptional activator on target promoters, activating expression of specific genes at the beginning of S phase.

DNA to immortalize primary human keratinocytes (Jewers *et al.*, 1992) indicate the existence of a pRB-independent pathway for immortalization.

L1 and L2 Late Proteins

L1 and L2 proteins are responsible for the composition of the viral capsid. The L1 proteins have an average molecular mass of 55,000 and their amino acid sequences are highly conserved among all papillomaviruses (Doorbar & Gallimore, 1987). The second, minor component of the PV particles is the viral L2 protein. The L2 polypeptides with an apparent M_r of about 76 kDa are less well conserved (Komly *et al.*, 1986; Doorbar & Gallimore, 1987). Expression of L1 and L2 genes is limited to the very last stages of the viral replication in more differentiated epithelial layers. L1 and L2 proteins which compose the viral capsid are not detected in the basal and spinous layers of epithelia infected with HPVs. Since high-grade cervical dysplasia and cervical carcinoma lesions are undifferentiated, it is unlikely that L1 and L2 ORFs are expressed in these cells (Böhm *et al.*, 1993). Moreover the segment from E2 to L2 is likely to be missing in the integrated form found in high-grade dysplasia and cervical cancer cells (Schwarz *et al.*, 1985).

Long Control Region

The long control region (LCR) spans between the end of the gene L1 and the start of gene E6. It is a non-coding region of about 1,000 bp DNA, containing binding sites for SP1 factor, E2 protein, and clusters of transcription factor binding sites termed enhancers. It plays an important role in regulating viral transcription. Numerous transcription activators that bind HPV enhancers have been identified, in particular Nuclear Factor I (NFI), AP1, progesterone/glucocorticoid receptors, *oct*-1, TEF-1, TEF-2, and cEBP (reviewed in Bernard & Apt, 1994). Transgenic mice harboring the HPV-18 LCR, E6 and E7 genes, revealed that the lesions were restricted to genital organs and E6 and E7

transcripts were limited to the affected organs and kidney, suggesting that the LCR may direct expression specifically to the urogenital tract (Comerford *et al.*, 1995).

Detection of HPV Infection in Cervical Lesions

Methods for diagnosing HPV infections of the cervix include cytology, colposcopy, histopathology, electron microscopy, immunohistochemistry, and nucleic acid techniques. Serologic tests have not been proven to be useful for detecting HPV infection due to high prevalence of HPV antibody positivity in the population, and specific antibodies against certain proteins of HPV are limited to invasive carcinoma (Gosh *et al.*, 1993). The narrow host range of HPV and tissue tropism have made it impossible to detect HPV by using tissue culture or animal inoculation methods. Cytology, colposcopy, and histopathoiogy are the most common clinical methods used, although they are rather insensitive and nonspecific because they are unable to verify the presence of HPV DNA and its specific type.

Cytologic evaluation of the cervix was introduced for the first time by Papaniculaou in 1941. Exfoliative cervical cytology evaluation or Pap smear screening is still regarded as the most effective tool currently available for early detection of cervical cancer because of its simplicity and low false-positivity especially in detecting invasive cervical cancer and high-grade cervical intraepithelial neoplasia, although it is less specific for low-grade cervical intraepithelial neoplasia. A Pap smear is easily obtained by scraping the metaplastic epithelium of cervix. It has led to a more than 70% reduction in cervical cancer mortality since the test was introduced more than 50 years ago (Larsen, 1994). About a third of women diagnosed with cervical cancer still die because the cancer was not detected at an early enough stage for successful treatment. Some of this residual mortality may result from problems with the Pap smear, including a high false-negative rate of between 20% and 40% depending on the rates of error in sampling and laboratory

detection or interpretation (Larsen, 1994). A single follow-up Pap smear failed to detect one half of the cases of biopsy-proven CIN (Slawson *et al.*, 1993).

Pap smear results showing atypical squamous cells without evidence of dysplasia or carcinoma may be secondary to inflammatory atypia, human papillomavirus infection, reparative changes, endocervical atypia, or cervical neoplasia (Fowler, 1993). Nuclear atypia is characterized by enlargement of the nucleus relative to the cytoplasmic volume (increased nuclear:cytoplasmic ratio), coarse chromatin that may marginate to the nuclear membrane, increased chromocenters, and hyperchromasia (Koss & Durfee, 1956). A study of atypical cytology demonstrated that 18% of atypical Pap smears had dysplasia, and 25% had HPV infection, both documented by histologic evaluation of directed cervical biopsies (Pearlstone *et al.*, 1992). Endocervical atypia is associated with clinically significant cervical lesions in as high as 50% of cases (Goff *et al.*, 1992).

Koilocytes that have an enlarged nucleus, have been associated with HPV infection (Meisels & Fortin, 1976). Koilocytes and giant cells are more frequent in low-grade lesions and are not usually observed in invasive carcinomas (Okagaki, 1992). However, the presence of koilocytes does not alter the grading of a squamous intraepithelial lesion.

Any result of Pap smears suggestive of squamous neoplasia, including dysplasia, cervical intraepithelial neoplasia, carcinoma *in situ*, microinvasive carcinoma, or invasive carcinoma, requires further evaluation. Unless there is an obvious invasive lesion, colposcopy has become the standard procedure for evaluating abnormalities in appearance or stain result of Pap smears. Characteristic color changes, vascular patterns, and margins of the cervix can be assessed by colposcopy. The results may be clinically correlated with the results of the referral Pap smear, and the lesion can be localized and a biopsy site can be selected. Colposcopy with directed biopsy provides clinical evidence for an accurate diagnosis of cervical neoplasia. Widespread use of colposcopy has decreased the incidence of unnecessary cervical conization and hysterectomy (Fowler, 1993).

Grading of Cervical Intraepithelial Neoplasia

Neoplasia is the growth of cells that exceeds and is uncoordinated with the growth of normal tissue. Neoplastic epithelium in the cervix ranges from minimally abnormal growth (low grade) to growth identical to carcinoma except confined to the epithelium (high grade and *in situ*). In all grades of intraepithelial neoplasia, the thickness of the epithelium is morphologically abnormal, reflecting aberrant cellular proliferation. Atypical nuclei are present throughout the epithelium.

Grading squamous cervical intraepithelial neoplasia (CIN) is based on the amount of proliferation of basaloid cells. The presence of a basaloid cell population of more than one cell layer indicates an increase in the immature cell population in the epithelium. Mitotic activity in low-grade squamous intraepithelial neoplasia is confined to the basal layer and the few cell layers just above it. Mitoses become both more frequent and occur more superficially in high-grade lesions. Greater degrees of mitotic abnormality have been associated with lesions that are higher grade and more likely to progress to invasive carcinoma.

The lowest histological grade of squamous intraepithelial neoplasia is cervical intraepithelial neoplasia grade 1 (CIN I) or mild dysplasia. Abnormal cells with altered nuclear/cytoplasmic ratio are present throughout the epithelium, and basaloid cells occupy as much as the lower third of the epithelium. Bizarre, enlarged, and multinucleated cells may be present at the surface.

Moderate dysplasia or CIN II is identified by an abnormal cellular proliferation throughout the epithelium with the basaloid population extending into the middle third of the epithelium. Mitotic activity is likely to be greater, with more abnormal forms, in this lesion than in CIN I. Severe dysplasia and carcinoma *in situ* are considered together as CIN III because the separation has no present therapeutic or prognostic implication. Severe dysplasia has some surface maturation, whereas carcinoma *in situ* has a basaloid population throughout the epithelium.

Many studies have shown there can be a difficulty with consistency of grading CIN on both the inter-observer and intra-observer levels. The Bethesda System combines condylomas and mild dysplasia (CIN I) into low-grade squamous intraepithelial lesions (LSIL) and moderate dysplasia to carcinoma *in situ* (CIN II and III) into high-grade squamous intraepithelial lesions (HSIL) (Lundberg, 1989).

New Arsenal in Early Detection of HPV Infection

To improve early detection of HPV infection, researchers need to optimize the Pap smears and also to develop new screening modalities. Auxiliary screening modalities to complement the Pap smear might overcome some of the inherent problems.

Molecular biologic hybridization techniques have improved the ability to identify human papillomavirus infection in the lower genital tract. These techniques can identify human papillomavirus DNA or RNA, but they differ in their sensitivity, specificity, and predictive values. A method of detection of HPV DNA was described by Southern in 1975 and the further development of DNA technology, based on the Southern blot, has resulted in the characterization of different HPV types. Several genotypes have been identified in the human genital tract. Simpler methods such as dot-blot and *in situ* filter hybridization techniques have also been employed. Application of a recently developed PCR amplification technology provided a higher sensitivity of HPV detection. While these techniques may identify a certain viral type considered high-risk, the meaning of the presence of these types in conjunction with a normal-appearing cervix is not understood.

Use of HPV testing as a screening tool is limited by its high prevalence. HPV DNA can be detected in cervical smears of 5% to 40% of women with normal cytology (Bauer *et al.*, 1991). When cytologically normal women attending a STD clinic were tested with a sensitive PCR method, 46% of women tested positive (Bauer *et al.*, 1991). A similar study of women who were members of a health maintenance organization undergoing annual cytological screening showed an incidence of 17% (Schiffman, 1992).

It is clear that HPV DNA testing alone lacks the specificity to be used instead of the Pap smear as a screening tool. However, HPV gene expression may be a good indicator of prognostic risk for the general population.

Detection of HPV mRNA has been proven to have more significance than DNA detection *per se*, since it reflects the viral activity. The copy number of mRNA in a cell will usually exceed that of DNA, making mRNA an ideal target. Several techniques are currently available to measure changes in gene expression. These include the Northern blot, the RNase protection assay, *in situ* hybridization, and the reverse transcriptase polymerase chain reaction (RT-PCR). For many purposes, the Northern blot or the more sensitive RNase protection assay, is sufficient for detecting quantitative differences between samples. However, if the sample quantity is low or the target message is rare, these techniques are no longer practical, and the more sensitive quantitative RT-PCR should be used.

In the RT-PCR method, RNA is initially reverse-transcribed to cDNA and then the desired target cDNA species are amplified using specific primers. Fewer than 10 copies of target RNA is required for this procedure, and it has even been successful when the RNA was isolated from a single cell (Razin *et al.*, 1991).

Because of this high sensitivity, RT-PCR is being used increasingly to quantify small but physiologically relevant changes in gene expression that would otherwise be undetectable. The sensitivity of RT-PCR is a result of a chain reaction in which the products from one cycle of amplification serve as substrates for the next cycle, resulting in an exponential increase in product. Theoretically, the amount of product doubles during each cycle of the PCR, or if described in the formula:

$$cDNAn = cDNA_0 \times (1 + R)^n$$

the ideal *R* value is 1. In actuality, the efficiency of amplification, or *R* value, is less than that.

A number of factors may cause the decrease in the efficiency, including the re-association of the denatured PCR target before primers can anneal or be extended, the degradation of nucleotides or primers, the inactivation of the DNA polymerase enzyme, substrate excess, where there is more DNA than the amount of enzyme available to replicate it after a high number of cycles, competition by nonspecific amplification products, and the accumulation of other inhibitors of polymerase activity.

In order to quantify absolute values of target cDNA by using this amplification technique, a method called competitive RT-PCR was developed. This method relies on the use of an external standard that mimics or closely imitates the target cDNA with respect to primer binding variables which may affect PCR amplification. Ideally, the standard and the target sequences amplify with the same efficiency, but can be distinguished from each other by gel electrophoresis. For accurate quantification by PCR, the amplifications must be within the exponential range (Murphy *et al.*, 1990).

There are two categories of standard templates: homologous and heterologous. Homologous standards differ only slightly from the target sequence with the addition or mutation of a restriction site, or the presence or deletion of an intron which increases the molecular weight of the standard (Piatak *et al.*, 1993; Gilliland *et al.*, 1990). However, the homologous standards bear a potential problem, that the standard may form heteroduplexes with the target sequences during the amplification process. The formation of heteroduplexes could interfere the quantification (Murphy *et al.*, 1990).

Heterologous standards differ from the target except for the flanking primer-template regions, which are identical (Cottrez *et al.*, 1994). Heteroduplexes are unlikely to form between the heterologous standard and the target sequences, however the efficiency of amplification of those two templates may also be different.

RNA purity and RNA integrity are also major sources of variability of RT-PCR amplification. In competitive RT-PCR, since the RNA preparations of the standard and target sequences are through different processes, this variability may affect the end results. In assays where DNA standards instead of RNA standards are used, there is no control for reverse transcription yield and uniformity. A mathematical model demonstrated that absolute quantification by competitive quantitative PCR requires a highly stringent fulfillment of its basic assumption that the efficiency of amplification should be the same for the target and sample (Raeymaekers, 1993).

Another method of quantitative RT-PCR is called non-competitive RT-PCR. This method relies on the observation that there is a linear relationship between the quantity of input RNA and final product during PCR amplification. A standard curve relating the amount of input RNA and the amount of PCR product is generated, so that the initial amount of a quantified amplification product can be estimated relative to the standard sample (Gendelman *et al.*, 1990; Singer-Sam *et al.*, 1990; Svetic *et al.*, 1991; Wynn *et al.*, 1993; Graziosi *et al.*, 1994). The sample used for generating the standard curve should have high expression of endogenous target gene.

Hsu and McNicol (1992) introduced a modification of non-competitive RT-PCR based on the assumption that the amplification efficiency is constant between cycles of amplification and between samples. A standard curve of the amount of the amplification products as the function of number of cycles was generated for every specific product amplified, so that the efficiency of amplification of each product from a specific pair of primers could be determined. By knowing the amount of PCR products of a specific target after a certain number cycles of amplification and the efficiency of the amplification reaction, the initial amount of cDNA in the specimen could be determined. However, since there are differences in the reverse-transcription efficiency between reaction tubes within an assay and between assays, the method did not facilitate comparison between samples.

In order to allow the detection of differences in the efficiency between reaction tubes within an assay or between assays, it is important to include an internal standard in the quantitative RT-PCR assay. DNA standards have been used for the quantification of RNA targets, however variation in the RT efficiency is not taken into account. There are two types of internal standards commonly used: an exogenous fragment added to the amplification reaction or an endogenous sequence or gene transcript that is normally present in the sample. Wang *et al.* (1989) demonstrated that the exogenous standard could also be used to quantify the absolute level of target or cDNA in the original sample. However, this method relies on the assumption that the efficiency of the PCR product is the same for both the target and standard mRNA.

A "housekeeping" gene is commonly used as the endogenous standard, typically β-actin, hypoxanthine phosphoribosyl transferase (HPRT), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Gendelman *et al.*, 1990; Svetic *et al.*, 1991; Svetic *et al.*, 1993; Peterson *et al.*, 1994). The endogenous standard permits the detection of relative differences in the integrity of individual RNA samples. Even if the same quantity of total mRNA is used for each RT PCR, the final quantity of amplification product may be greatly affected by differences in RNA integrity and the presence of inhibitors of reverse transcriptase. This is a particular problem, because the degree of RNA degradation can vary significantly between samples within a given experiment, and RNA degradation is not detectable spectrophotometrically.

The involvement of "housekeeping" mRNA from the same mRNA preparation in the reaction could detect the variation in RNA degradation between samples. However, co-amplification of the target and the standard in the same tube is not practical when the housekeeping gene is expressed in high level, because it reaches plateau prematurely and inhibits the amplification of the target (Murphy *et al.*, 1990; Braga & Gendler, 1994).

Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA is a good choice for internal standard because its level in tissue culture cells is independent of the

degree of confluence of the culture and its expression is not influenced by exogenous factors. Edwards and Denhardt (1985) reported an mRNA species transcribed from the mitochondrial genome whose abundance did not change in the rate of transcription following serum stimulation. This mitochondrial gene is the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. GAPDH has been proven to be a better internal control than actin and is widely accepted for this purpose on Northern blots, RNase protection assays, and in nuclear run-on experiments (Dieffenbach *et al.*, 1989; Inghirami *et al.*, 1990; Dukas *et al.*, 1993).

GAPDH is an enzyme involved in the cellular glycolysis pathway. It is composed of four subunits and is not tissue specific. It has been shown that GAPDH is encoded by only one mRNA. It is highly conserved, and the level of expression is constant during different phases of the cell cycle. Therefore, GAPDH is widely used as an internal control for RT-PCR.

HPV 16 Oncogenes

HPV-16 is the most common type of HPV associated with genital infections and is present in at least 50% of the cervical lesions. HPV-16 was the predominant type in all countries except Indonesia, where HPV-18 was more common (Bosch *et al.*, 1995). Hence, future discussion is confined to the HPV-16, and particularly the most prominent early ORFs responsible of neoplastic transformation.

The HPV 16 genome was initially isolated from a German patient (Dürst *et al.*, 1983). Subsequent isolates of HPV 16 genomes from patients throughout the world showed mutational differences from that initial type. These latter isolates were designated as "variants" (Ho *et al.*, 1991; Icenogle *et al.*, 1991; Chan *et al.*, 1992; Eschle *et al.*, 1992; Ho *et al.*, 1993).

HPV DNA detection may be a good marker of HPV infection occurring before cancer development (Muñoz & Bosch, 1992). However, the finding of HPV-16 DNA or

other high risk HPV DNA in cervical cells is not a guarantee that cervical cancer will develop. Prospective studies showed that only a few of HPV infected cervical cells would progress into higher stages (Schiffman, 1994).

The presence of this oncogenic virus in infected cells has little significance, unless its oncogenes are expressed. It becomes obvious that the expression of the genes is more important in carcinogenesis. Falcinelli *et al.* (1993) reported that no mRNA of HPV oncogenes was detectable, using the most sensitive method, in cytological normal cervical scrapings positive for the presence of HPV-16 DNA.

Several lines of evidence have supported the idea or the finding that expression of E6 and E7 ORFs of high-risk HPVs is associated with malignant progression. HPV-16 DNA is commonly found integrated within the cellular genome of cervical cancer cells. This integration seems to happen randomly, as viral antisense signals were observed on rare occasion (Vormwald-Dogan *et al.*, 1992), however, all of HPV DNA containing cervical carcinoma cells retain and express E6 and E7 genes. The integrated sequences usually extend from the mid-portion of the L1 ORF through the E7 ORF and end within the E1 ORF (Kaur *et al.*, 1989). The integration of HPV-16 DNA leads to increased steady-state levels of mRNA encoding the viral oncoproteins E6 and E7 (Jeon & Lambert, 1995). In most cases, despite the lower HPV-16 DNA copy number, the cells with integrated viral DNA have higher levels of E7 protein and grow faster than those cell populations harboring extrachromosomal viral DNA (Jeon *et al.*, 1995).

Transfection of E6/E7 genes derived from HPV 16 leads to extension of the life span and immortalization of primary human keratinocytes and embryonic fibroblasts (Hawley-Nelson *et al.*, 1989; Münger *et al.*, 1989; Watanabe *et al.*, 1989; Hudson *et al.*, 1990), human mammary epithelial cells (Band *et al.*, 1990), bronchial cells, oral and nasal keratinocytes (Park *et al.*, 1991), and human corneal endothelial cells (Wilson *et al.*, 1995). After several extensive passages, the immortalized cell lines become tumorigenic when inoculated into immunocompetent animals (Pecoraro *et al.*, 1991).

Using transgenic mice, Auewarakul *et al.*, (1994) demonstrated the effects of HPV-16 E6 and E7 expression in the epidermis, i.e., an increase in the number of growing cells in the undifferentiated basal layer and abnormal proliferation of differentiated cells in the suprabasal strata, higher expression of c-*myc* expression in the skin, and overexpression of transforming growth factor β1 in the supra basal layers.

On the other hand, by using antisense construct approaches, the role of E6/E7 gene products of high-risk HPVs in growth regulation and maintenance of the malignant phenotype in cervical carcinoma derived cell lines and HPV-transformed mouse cell lines was shown (von Knebel Doeberitz *et al.*, 1988; von Knebel Doeberitz *et al.*, 1992; Watanabe *et al.*, 1993). Upon introduction of complementary RNA to the HPV E6 and E7 ORFs, the expression of the constitutive E6 and E7 was inhibited, and decreased cell growth was observed.

The expression of HPV-16 E7 ORF alone under the control of a strong promoter could immortalize primary human keratinocytes (Halbert *et al.*, 1991). In baby rat kidney (BRK) cells transformed by HPV-16 E7 in cooperation with the activated *ras*, continued expression of HPV-16 E7 is required for maintenance of the transformed phenotype, *i.e.*, grows easily in soft agar and induces large tumors in athymic nude mice (Crook *et al.*, 1989; Liu *et al.*, 1995). Similar transforming potential of the HPV-16 E6 ORF cotransfected with an activated *ras* gene was reported in BRK cells (Liu *et al.*, 1994), but it has never been observed in human cell lines. In an immortalized rat fibroblast line transfected with HPV-16 E6, or E7, or both E6/E7 ORFs, DNA stimulation was observed in the cells containing E7 or E6/E7 ORFs, but not in those containing E6 ORF (Sato *et al.*, 1989). When transfected alone, HPV-16 E7 could induce hyperproliferation in human foreskin keratinocytes, whereas E6 exhibited no activity (Hawley-Nelson *et al.*, 1989), indicating that expression of the HPV-16 E7 gene is mitogenic. The transforming potential of E7 is directly correlated to the expression levels of the oncoprotein and a threshold level of E7 may be required before the cells can be fully transformed (Liu *et al.*,

1995). In HPV-associated CIN, invasive cancer, and cervical cancer-derived CaSki cell line, most transcripts observed coded for E7 protein (Böhm *et al.*, 1993) and only a minor mRNA could code for E6 (Smotkin & Wettstein, 1986). However, E6 protein is able to contribute to the efficiency of the E7 immortalizing function (Hawley-Nelson *et al.*, 1989; Halbert *et al.*, 1991).

HPV-16 E6/E7 ORF Transcripts

From the P_{97} promoter of HPV-16 E6/E7 ORFs, three transcripts are generated; a full-length unspliced transcript and two transcripts generated by alternative splicing, arbitrary named E6*I and E6*II (Smotkin *et al.*, 1989; Cornelissen *et al.*, 1990). This alternative splicing phenomenon does not occur in low-risk HPVs. The unspliced transcript with a coding potential for a full-length E6 protein is rarely detected in cervical lesion and cell lines, whereas the E6*s are observed more frequently. These two spliced transcripts of HPV-16 E6/E7 ORFs have the same splice donor site at nucleotide (nt) 226, but have different acceptor sites, i.e., at nt 409 and nt 526, respectively (Cornelissen *et al.*, 1990) (Figure 4). The E6*I transcript potentially encodes a truncated E6 protein and a full-length E7 protein, whereas the E6*II transcript is more likely to encode merely another truncated E6 protein due to the close proximity of the truncated E6 protein translation termination codon to the initiation codon for E7 protein (Yamada *et al.*, 1995). Unlike the benign HPVs, an equivalent promoter located within E6 designated for transcribing mRNA encoding the E7 protein has not been found in HPV-16 containing cancers and cancer-derived cell lines (Smotkin *et al.*, 1989), suggesting that E7 protein is encoded solely through the splicing mechanism. A mutation that prevented splicing in E6-E7 mRNA severely reduced the level of E7 protein (Sedman *et al.*, 1991). Little is known about the biological activity of the truncated E6 proteins. Shirasawa *et al.* (1994) demonstrated that E6*I protein *trans*-activated the autologous P_{97} promoter and the heterologous adenovirus E2 promoter. The biological activity of the other truncated E6

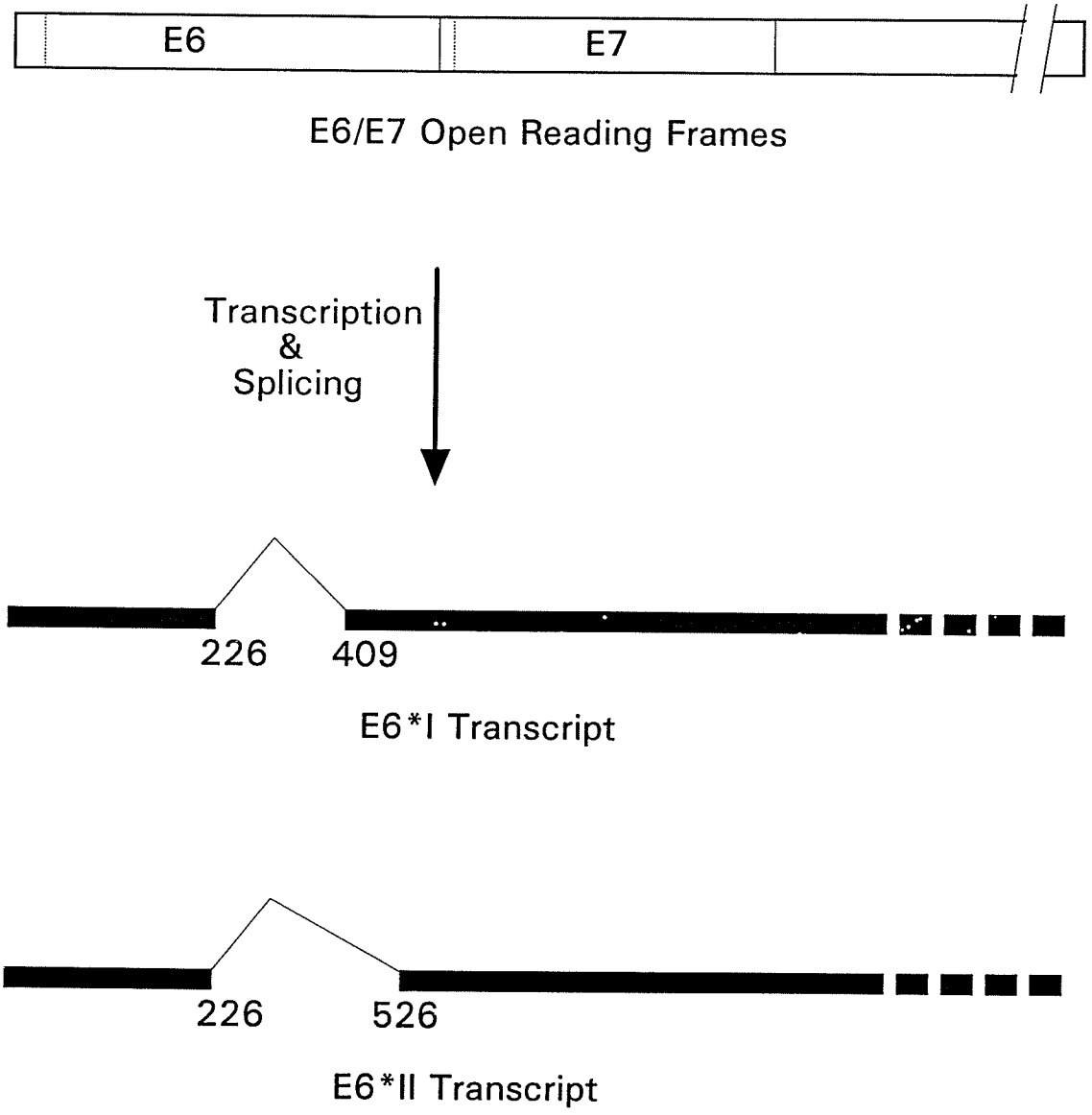


Figure 4. HPV-16 E6*I and E6*II transcripts are produced by use of alternative 3' splice sites

The slanted lines represent intervening sequences removed by splicing.

protein, E6*II, has not been reported. The splicing mechanism of this polycistronic transcript is poorly understood, but it seems necessary for malignant occurrence.

The Expression of HPV-16 E6/E7 ORFs Changes Quantitatively with Disease Progression

The attempts to demonstrate variations of the splicing pattern in cervical intraepithelial neoplasia (CIN), invasive carcinoma and cytologically normal cervical tissue failed to show qualitative changes in E6/E7 ORFs transcripts but rather substantial quantitative differences between normal and cytologically abnormal cervical cells (Johnson *et al.*, 1990). The splicing pattern is conserved in premalignant cervical lesion, carcinoma, and HPV-16-transformed human fibroblasts regardless of whether it was controlled by a homologous or a heterologous promoter (Cornelissen *et al.*, 1990). Shirasawa *et al.* (1991) noted that the expression levels of spliced and unspliced E6-E7 transcripts were variable in CIN and carcinoma. Between the spliced E6-E7 transcripts, E6*II was much less abundant than E6*I (Cornelissen *et al.*, 1990; Shirasawa *et al.*, 1991; Sánchez-Lanier *et al.*, 1994). However, in established cervical tumors, no relationship was found between the E6/E7 transcription patterns and the histological type and differentiation of the tumors (Rose *et al.*, 1995). In CaSki cells, E6/E7 are constitutively produced in a differentiation-independent manner (Choo *et al.*, 1994).The link between relative proportion of E6*I/E6*II mRNA and CIN status was demonstrated by Hsu *et al.* (1993).

Alternative Splicing

As mentioned previously, two transcripts, namely E6*I and E6*II, are generated from HPV-16 E6/E7 ORFs by an alternative splicing mechanism. In general, the process of alternative splicing, where transcripts from a polycistronic gene are cut and joined to yield multiple forms of mature mRNA and thus encode multiple protein products, is still poorly defined. Since intron removal occurs in constitutive splicing and alternative

splicing, most constitutive splicing factors are expected to be utilized in alternative splicing as well. Furthermore, it now appears that several of the *trans*-acting molecules involved in alternative splicing, are constitutive splicing factors which regulate splice site choice by their concentration and/or activity.

The *cis*- and *trans*-acting elements that permit constitutive splicing, which are most likely involved in splicing of alternative exons as well, have been characterized through biochemical and genetic manipulations in yeast and mammalian systems. Each single intron in a nuclear pre-mRNA is removed in a two-step, ATP-dependent process (reviewed in Green, 1986; Padgett *et al.*, 1986; Bindereif & Green, 1987; Sharp, 1987; Guthrie, 1991; Wassarman & Steitz, 1991; Norton, 1994). The first step involves cleavage at the boundary between 3' end of the upstream exon and 5' end of the intron (the 5' splice site or splice donor) and concomitant formation of a 2'-5' phosphodiester bond between the 5' terminal guanine of the intron and the 2' hydroxyl of a conserved adenosine located in the intron, forming a branched nucleotide (Figure 5). Intermediates generated during the first step of splicing are: free exon 1 and the intron with its 5' end joined to the branchpoint, known as a lariat structure due to its configuration, while its 3' end is still joined to exon 2. In the second step of splicing, the lariat intermediate undergoes cleavage at the junction between 3' end of the intron and the downstream exon (the 3' splice site or splice acceptor) and simultaneous ligation of the two exons occurs. The spliced exons and the intron lariat are the end products of the splicing episode (Figure 5).

Splicing reactions are catalyzed in spliceosomes, complexes containing small nuclear ribonucleoprotein particles (snRNPs), heterogeneous ribonucleoprotein particles (hnRNPs), and various splicing factors (Guthrie & Patterson, 1988; Gall, 1991; Guthrie, 1991; Wassarman & Steitz, 1991). Spliceosome assembly is initiated by formation of the H complex, which contains pre-mRNAs and hnRNPs. This is followed by production of the E complex, which contains U1 snRNP and U2AF in addition to hnRNPs, and commits the pre-mRNA to being spliced (Michaud & Reed, 1993). Then in a series of ATP-

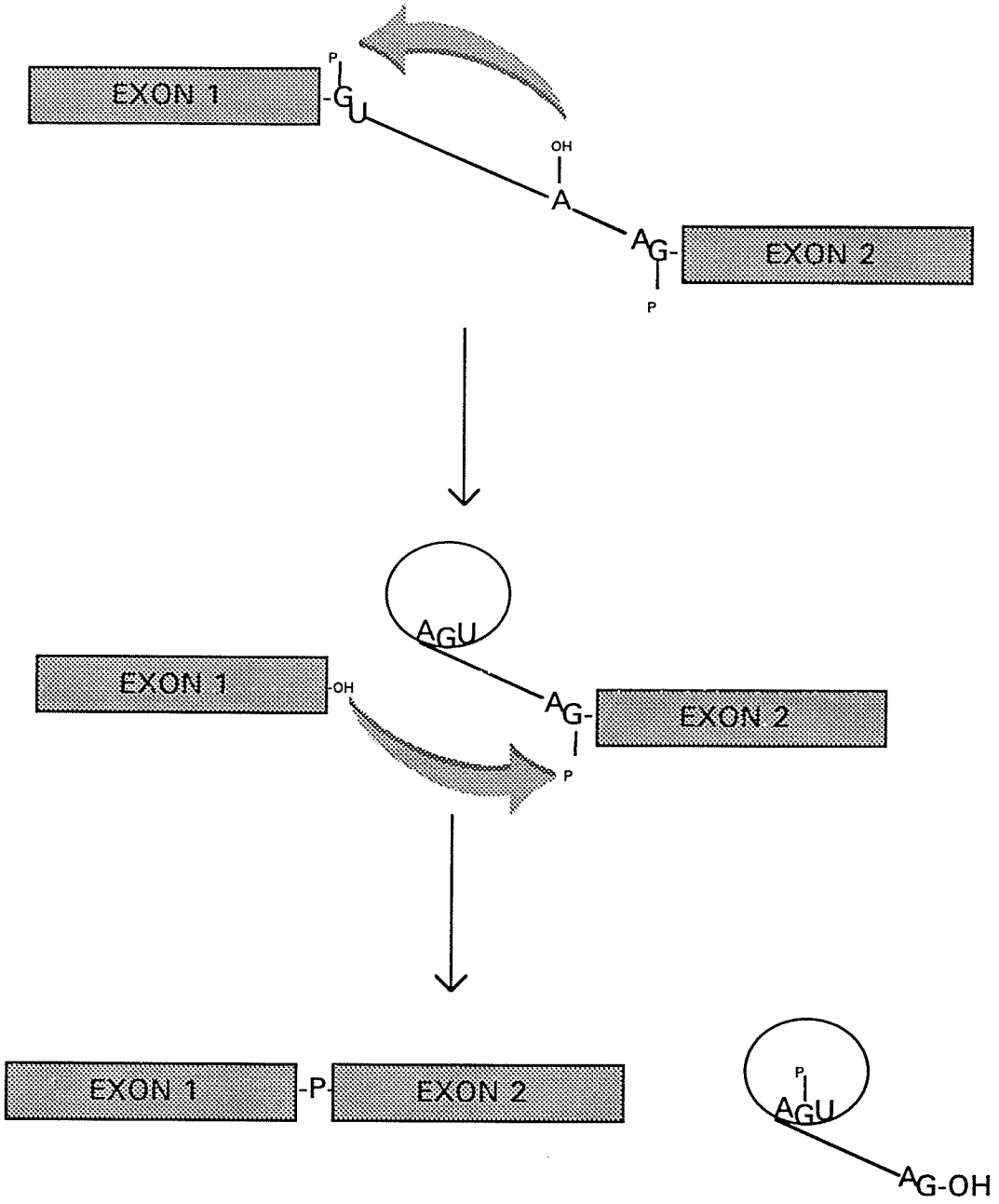


Figure 5. Two Step Splicing Pathway for Pre-mRNA

Step 1 involves joining of the branchpoint adenosine to the 5' end of the intron via a 5'-2' phosphodiester bond. This results in free exon 1 and a lariat structure consisting of the intron attached to exon 2. Step 2 involves cleavage at the 3' end of the intron and ligation of exon 1 to the 5' end of exon 2. This results in exon 1 and 2 being joined by a phosphodiester bond and release of the intron lariat.

dependent steps, the A complex containing U1 and U2 snRNPs; the B complex containing U4, U5, and U6 snRNPs in addition to U1 and U2, and the C complex, which has the same snRNP components are produced. Both biochemical and genetic studies show that phosphorylation and dephosphorylation of spliceosome protein components are important in pre-mRNA splicing (Mermound *et al.*, 1992; Tazi *et al.*, 1992; Alahari *et al.*, 1993; Tazi *et al.*, 1993). Following splicing, the spliced product dissociates from the complex.

Sequence comparisons and mutagenesis experiments have identified RNA elements necessary for splice site definition. The conserved sequences $^{C}/_{A}$AG↓GURAGU or G↓GUAUGU, in mammals or yeast, respectively, are present at the 5' splice site, and the sequence Py_{11}NYAG↓ is generally found at the 3' splice site in mammals (where ↓ indicates the splice junction) (Breathnach *et al.*, 1978; Mount, 1982; Ruskin & Green, 1985). Choice of a 5' splice site depends upon its match with the consensus sequence, its surrounding sequences, and its distance from the 3' splice site (Reed & Maniatis, 1986; Lear *et al.*, 1990; Nelson & Green, 1990). The terminal Gs of the intron may interact via non-Watson-Crick base pairing to bring the 5' and 3' exons together (Parker & Siliciano, 1993).

The branchpoint sequences UACUAAC in yeast and YNCUGAC in mammals (where the underlined adenosine is the residue that forms the 2' to 5' link) are usually found 17 to 40 nucleotides (nt) upstream of the 3' splice site (Keller & Noon, 1984; Langford *et al.*, 1984; Ruskin *et al.*, 1984; Zeitlin & Efstratiadis, 1984; Ruskin *et al.*, 1985; Konarska *et al.*, 1985; Nelson & Green, 1989). This conforms with the observation that cryptic branchpoints 22 to 37 nucleotides upstream of the 3' splice site are activated when the natural branchpoints located as far as 177 nts upstream from the 3' splice junction are used efficiently in several alternatively spliced pre-mRNAs (Helfman & Ricci, 1989; Smith & Nadal-Ginard, 1989).

Correct choice of 5' and 3' splice site pairs in pre-mRNAs is not just dependent on recognition of the conserved sequence elements since there are many sequences which

match the consensus but are not used (Ohshima & Gotoh, 1987). Communication among splicing factors has been shown to be responsible for correct 5' and 3' splice site pairs in constitutively spliced transcripts. The splicing factors bound at the 5' and 3' ends of introns interact directly or indirectly to promote recognition and removal of introns (Lamond *et al.*, 1987).

Identification and purification of proteins that influence the use of 5' and 3' splice sites, and cloning of their cDNA sequences, augment the understanding of splicing mechanism. ASF/SF2 (Ge & Manley, 1990; Krainer *et al.*, 1990; Ge *et al.*, 1991; Krainer *et al.*, 1991) is an essential 33-kDa factor that is required for the first cleavage and lariat formation during pre-mRNA splicing. ASF/SF2 contains an amino terminal RNA binding domain consisting of an RNP consensus sequence (an 80-90 amino acid sequence present in a number of RNA binding proteins) and a carboxy terminal serine-arginine (SR)-rich domain (reviewed in Bandziulis *et al.*, 1989). Although ASF/SF2 binds RNA nonspecifically, it has been shown to promote annealing of complementary RNAs. The finding that it strongly stimulates use of the 5' splice site (splice donor) closest (most proximal) to the 3' splice site (slice acceptor) in a dose-dependent manner suggests that it may mediate alternative splicing choices as well. Recent structure and function studies on ASF/SF2 show that the SR domain is essential for the general splicing process, but is not required for activating proximal 5' splice site use (Cáceres & Krainer, 1993; Zuo & Manley, 1993).

Mayeda and Krainer (1992) purified a protein termed SF5 from HeLa cells. In contrast to ASF/SF2, this protein has a similar activity with distal splicing factor (DSF), which promotes use of distal 5' splice sites (Harper & Manley, 1991). The 34-kDa SF5 protein was determined to be hnRNP A1. The hnRNP A1 N-terminus contains two copies of the RNP consensus sequence and the C-terminal has 12 repeats of a glycine-rich sequence believed to contribute to cooperative binding of the protein (Buvoli *et al.*, 1988; Biamonti *et al.*, 1989; Mayeda & Krainer, 1992). hnRNP protein A1 is abundant and

believed to act stoichiometrically. Its activity counteracts the action of ASF/SF2 and the relative amounts of ASF/SF2 versus hnRNP A1 determine whether a proximal or distal 5' splice site is selected. These activities are thus likely to play an important role in alternative RNA splicing (Mayeda *et al.*, 1993).

Polypyrimidine tract binding protein (PTB) is a splicing factor of 57-62 kDa that mediates 3' splice site selection by directly binding to the upstream polypyrimidine tract and is required for efficient U2 snRNP interaction with the pre-mRNA (Garcia-Blanco *et al.*, 1989; Wang & Pederson, 1990; Gil *et al.*, 1991; Patton *et al.*, 1991). Patton *et al.* (1991) demonstrated proteins of 33 kDa and 100 kDa copurify with PTB. The 33-kDa protein has been identified as hnRNP A1 (Bothwell *et al.*, 1991). PTB was recently shown to be hnRNP I (Bennett *et al.*, 1992).

SV40 early pre-mRNA is alternatively spliced to produce two mRNAs encoding large T or small t antigen by use of two different 5' splice sites (Figure 6 A). Use of the more distal 5' splice site (relative to the common 3' splice site) generates large T mRNA whereas use of the more proximal 5' splice site (only 66 nt upstream of the 3' splice site) produces small t mRNA. The ratio of these two products varies among different cell lines; in HeLa cells five times more large T than small t mRNA is produced but the reverse ratio is observed in embryonic kidney 293 cells. Ge and Manley (1990) purified a splicing factor, ASF/SF2, from 293 cells and demonstrated that it promotes use of the proximal small t 5' splice site and decreases use of the large T 5' splice site.

A number of characteristics of the SV40 precursor RNA make the small t 5' splice site an unfavorable choice (Fu & Manley, 1987; Noble *et al.*, 1987; Zhuang *et al.*, 1987; Fu *et al.*, 1988; Noble *et al.*, 1988). The size of the small t intron sterically hinders the simultaneous interaction of splicing factors with the 5' and 3' splice sites. The small intron size also constrains the small t splice usage since the branchpoints are located at positions -18 and -19 relative to the 3' splice site. The polypyrimidine tract just downstream of the branchpoints is interrupted by several purines, which may not allow efficient binding of

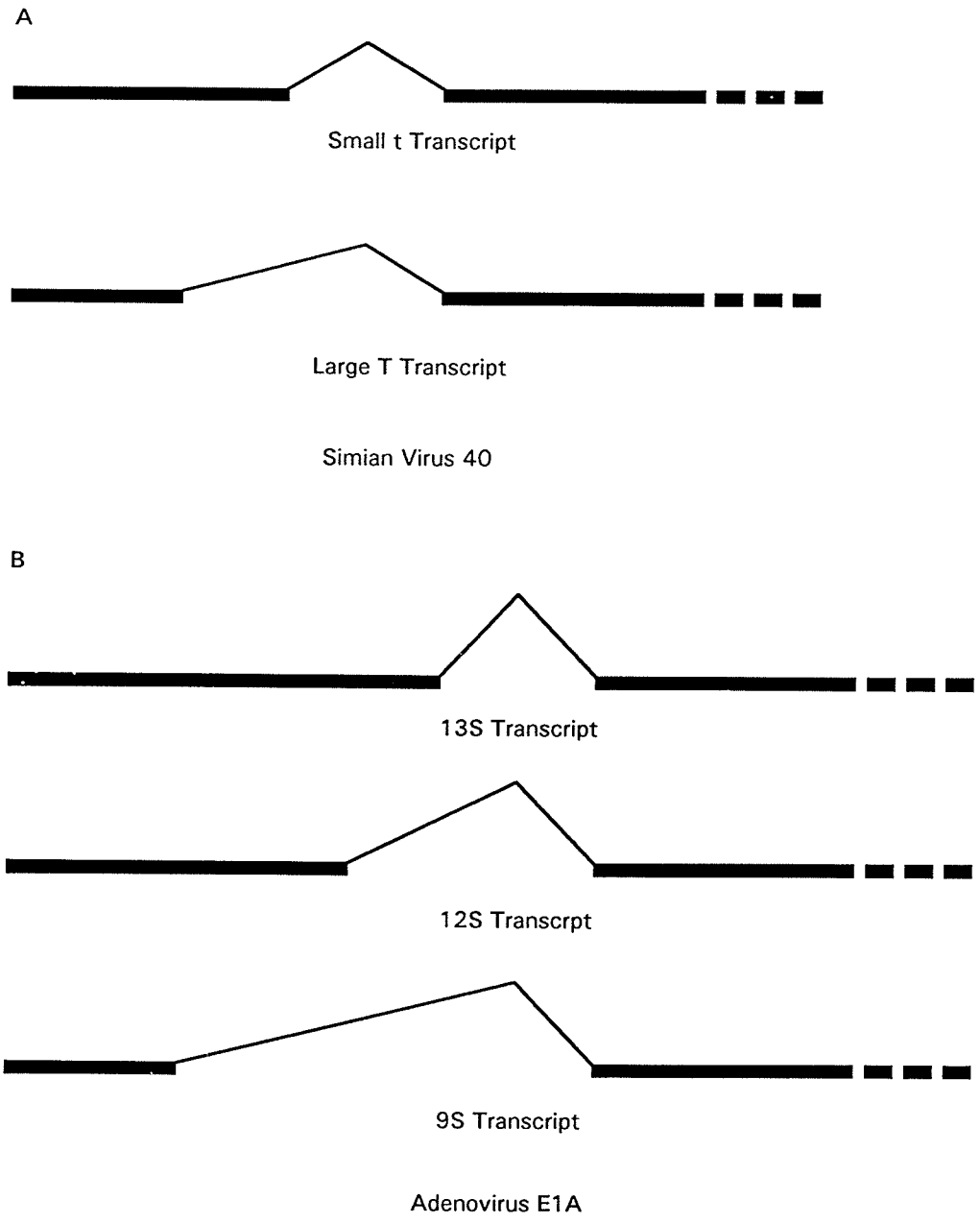


Figure 6. Alternative Splicing in SV40 T/t and Adenovirus E1A Transcripts

SV40 large T and small t antigens and adenovirus E1A transcripts (9S, 12S, and 13S) are produced by use of alternative 5' splice sites. The slanted lines represent intervening sequences removed by splicing.

splicing factors to the 3' splice site. Mutations that increase the strength of the polypyrimidine tract increase the T/t mRNA ratio, which suggests that increased interaction of splicing factors at the 3' end of the intron inhibits the binding of splicing factors to the small t 5' splice site. Both small t and large T 5' splice sites are suboptimal, and improvement of the large T 5' splice site permits it to out-compete the small t 5' splice site *in vivo*.

Adenovirus E1A RNA is spliced to generate 9S, 12S, and 13S mRNA by ligation of any of three alternative 5' splice sites to a common 3' splice site (Figure 6B). Use of the more proximal 12S and 13S 5' splice sites predominates early in infection but utilization of the distal 9S site increases late in infection. Replacement of the common E1A branchpoint-3' splice site region with a heterologous branchpoint and 3' splice site increased use of the 12S and 9S 5' splice sites, with concomitant decreased use of the 13S site (Ulfendahl *et al.*, 1989). Thus there is a cooperative interaction of particular 5'-3' splice site pairs. It was proposed that late in infection the virus modifies splicing factors that bind to the E1A 3' splice site, resulting in selection of different 5' splice sites. Schmitt *et al.* (1987) also induced a switch from predominant use of the proximal 5' splice sites to use of the distal 5' splice sites by altering ion concentrations *in vitro* (Schmitt *et al.*, 1987). This *in vitro* effect is most likely due to pre-mRNA structural changes. Whether such changes occur during the course of infection is unknown.

Hypotheses and Objectives

It is unequivocal that the expression of E6 and E7 ORFs of HPV-16 is essential for the progression of HPV infected cervical uteri toward malignant lesion. The alternative splicing mechanism processes the pre-mRNA transcript from these ORFs into E6*I and E6*II mRNA species. The pattern of splicing sites has been shown to be conserved between different grades of CIN as well as in malignant diseases. It was hypothesized in this laboratory that the expression of the HPV-16 E6/E7 ORFs changes quantitatively rather than qualitatively. Previous study has shown that there is a direct correlation between the increase of the relative proportion of E6*I/E6*II mRNAs and increased disease severity in exfoliated cervical cell specimens collected from premalignant lesions (Hsu *et al.*, 1993). However, how the expression of E6*I and E6*II change quantitatively in biopsy tissue specimens was not known. It was of great interest to determine whether the same phenomenon would be observed in biopsy tissue specimens. It was also of interest to know whether a similar pattern of E6*I and E6*II expression would be observed in exfoliated cell and biopsy tissue specimens collected from the same woman.

High risk HPVs share a great number of similarities with SV40 and adenovirus. The proteins encoded by these DNA tumor viruses bind with and inactivate p53 and pRB tumor suppressor proteins. SV40 large T antigen, adenovirus E1B and HPV E6 bind with p53, whereas SV40 large T antigen, adenovirus E1A and HPV E7 bind with pRB. In addition, all of these viral oncoproteins are translated from mRNAs generated via alternative splicing from their mRNA precursors. In contrast to SV40 and adenovirus, where they have two or more alternative 5' splice sites and a common 3' splice site, the high risk HPVs have two 3' alternative splice sites and a common 5' splice site. The relative amounts of ASF/SF2 versus hnRNP A1 have been shown to determine the alternative splice site that is selected in SV40 large T/small t pre-mRNA. Similar phenomenon has not been observed in HPV. Thus, it is of a great interest to know

whether the levels of ASF/SF2 and hnRNP A1 also determine the levels of splicing product of HPV-16 E6/E7 ORFs, i.e., E6*I and E6*II in cells.

Based on the above evidence, I proposed that the pattern of quantitative change in E6*I and E6*II expression level, where the level of E6*I expression increases relative to the level of E6*II expression with the progression of the disease, would also be observed in biopsy tissue specimens. As the histopathologic examination shows more definitive result than cytologic examination and thus it is regarded as the golden standard for determining the grade of pre-malignant cervical lesion, the biopsy tissue specimens were expected to yield better and more consistent result than the exfoliated cervical cell specimens.

In order to be able to compare the level of expression of E6*I and E6*II between specimens collected from different patients, and between different specimens collected from the same patient, the quantitative RT-PCR assay (Hsu & McNicol, 1992) was modified; GAPDH primers were included in amplification reactions. The level of expression of E6*I and E6*II in different stages of cervical dysplasia was then standardized relative to the expression level of GAPDH gene. This standardization compensated for differences in the integrity of mRNA and reverse-transcription reaction efficiency, and eliminated the necessity to determine the quantity of cells from which the mRNA was isolated.

I also proposed that the selection of the splicing sites is regulated post-transcriptionally by cellular splicing factors, namely SF2 and hnRNP A1. The expression of the splicing factors varies with cellular differentiation and with different cell types. This hypothesis was tested by determining the level E6*I and E6*II expression in transiently transfected cell lines, in association with different levels of endogenous expression of ASF/SF2 and hnRNP A1 of the cell lines. Adaptation of quantitative RT-PCR facilitated the quantitative determination of the levels of ASF/SF2 and hnRNP A1 expressions in cell lines, and the determination of the level of E6*I and E6*II expression. A plasmid

construct expressing the HPV-16 E6/E7 ORFs was prepared for this purpose, by cloning the PCR amplified fragment of HPV-16 from nt 71 to nt 873 into a mammalian expression plasmid pCDNA3.

The results of this study will provide more insight into the pathogenesis of HPV in developing uterine cervical malignancy, and yield a better understanding of the role of HPV-16 E6/E7 ORFs transcripts in the progression of the pre-malignant lesions. The cellular splicing factor study would also bring more understanding of the mechanism of E6*I and E6*II transcript processing in conjunction with cellular differentiation.

Materials and Methods

Specimen Collection

Paired specimens, biopsy tissues and exfoliated cervical cells, were collected from women referred to a colposcopy clinic at the Health Sciences Centre, University of Manitoba, Winnipeg, Manitoba, Canada because of a previous abnormal Papanicolaou smear result. Exfoliated cervical cells were taken by scraping the uterine cervix with an Ayres spatula and sampling the endocervical canal with a cytobrush. A colposcopically-directed biopsy was taken at the same time as exfoliated cervical cell specimen was collected. All specimens were divided for molecular and histopathological studies. Specimens for molecular studies were suspended in 5 ml of phosphate-buffered saline solution (pH 8.0) (Appendix) and held at -20°C until molecular analysis (Figure 7). Histopathologic samples were evaluated at the Department of Pathology, University of Manitoba, for determination of the grade of cervical intraepithelial neoplasia (CIN). All specimens were taken by Dr. Fernando Guijon (Department of Obstetrics, Gynecology and Reproductive Sciences, University of Manitoba).

Specimen Selection

The process of specimen selection is summarized in Figure 8. DNA from approximately one fifth of a suspension of the exfoliated cervical cell specimen was isolated by digesting the cells with 2 units of proteinase-K (Boehringer Mannheim) in 200 µl of tissue suspension buffer (TSB) (Appendix). The digestion was performed in a 37°C waterbath (Precision) for 2 hours, followed by phenol:chloroform extraction and sodium acetate-ethanol precipitation (Appendix). The remainder of the specimen was maintained at -20°C. To test the capacity of isolated cellular DNA to be amplified by the polymerase chain reaction (PCR), we used HLA DQ-α primers (Table 1). A DNA sample that showed the specific HLA DQ-α amplification product was subsequently screened for the

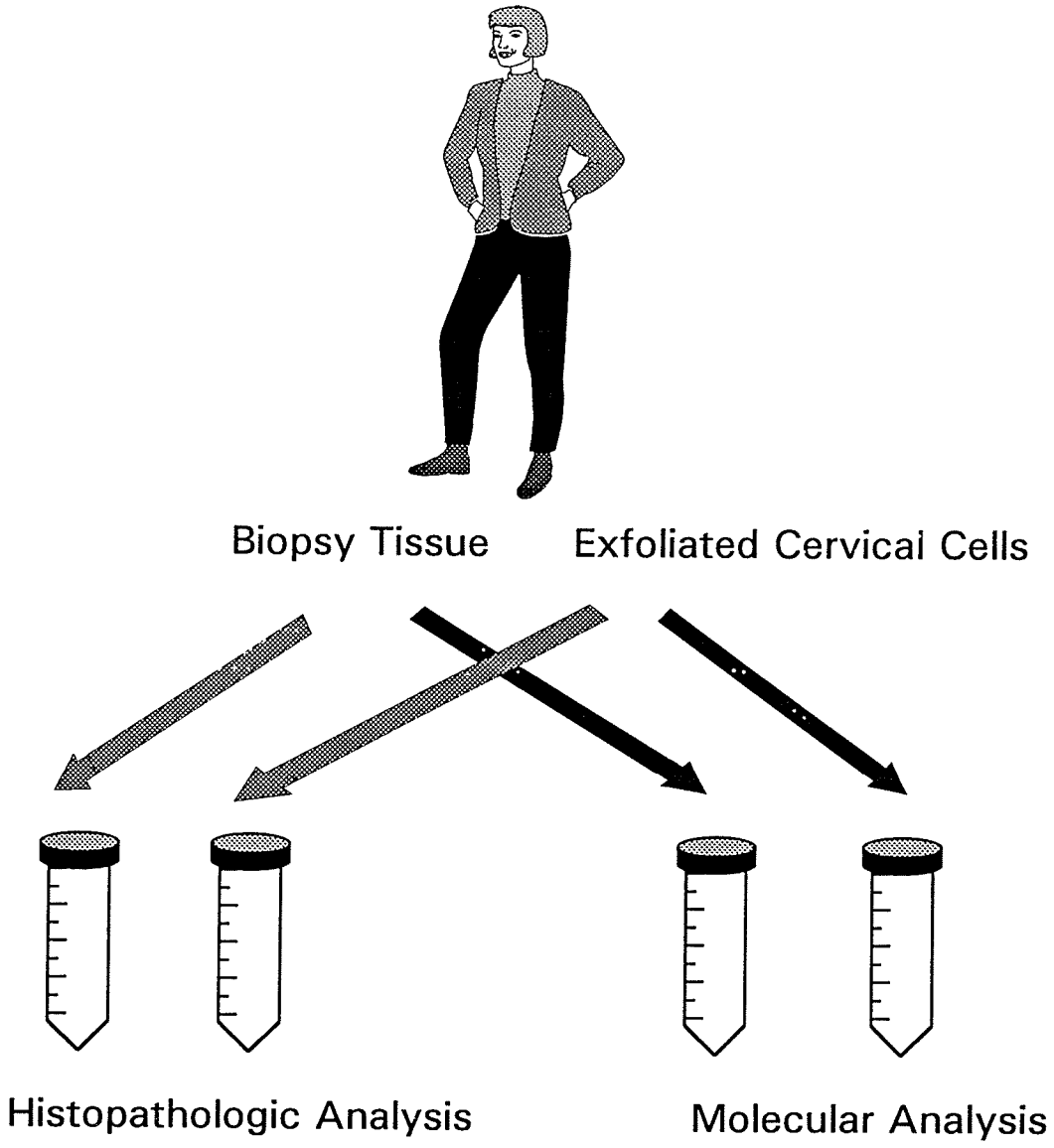


Figure 7. Specimen collection

A pair of specimens, biopsy tissue and exfoliated cervical cells are collected from every woman referred to the colposcopy clinic because of a previous abnormal cytological result. Each specimen is divided for histopathologic and molecular study.

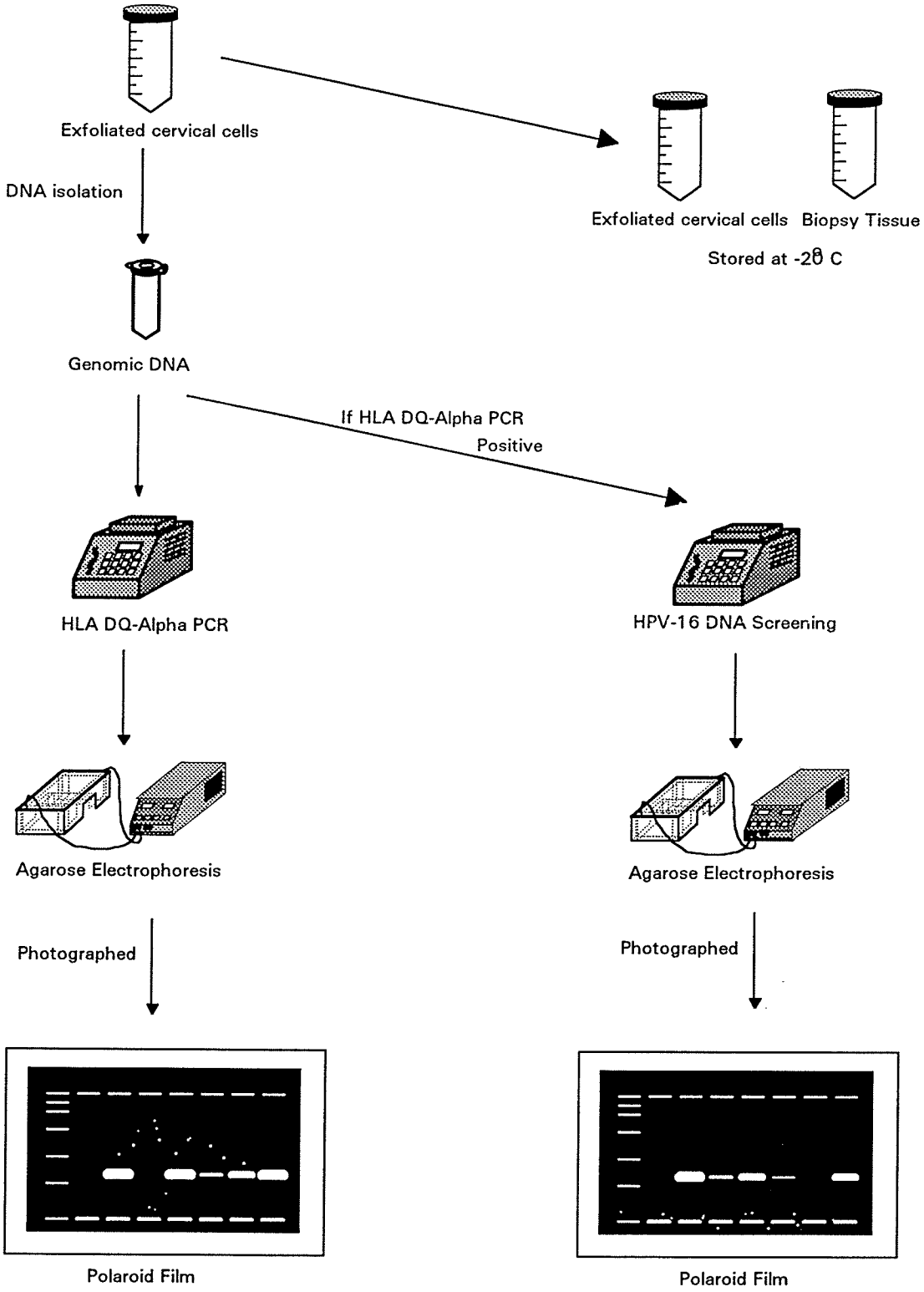


Figure 8. Specimen selection

Table 1. Primer sequences

Primer Name	Sequence (5'-3')	Genome Location (nt)	Fragment size (bp)
HLA-DQα			
Primer 1	GTGCTGCAGGTGTAAACTTGTACCAG		242
Primer 2	CACGGARCCGGTAGCAGCGGTAGATTG		
HPV-16 DNA Detection			
Primer 1	GATGGGAATCCATATGCTGTA	E6 269-289	240
Primer 2	TCGACCGGTCCACCGACCCCT	E6 508-488	
Glyceraldehyde-3-phosphate dehydrogenase			
Primer 1	CATCTCTGCCCCCTCTGCTGA		305
Primer 2	GGATGACCTTGCCCACAGCCT		
HPV-16 E6/E7 spliced transcripts			
Primer 1	CAAGCAACAGTTACTGCGACGTG	E6 202-224	503 (DNA)
Primer 2	TCCGGTTCTGCTTGTCCAGCTGG	E6 704-682	321 (E6*I) 204 (E6*II)
SF2 splicing factor			
Primer 1	ATGTTTACCGAGATGGCACTG		279 (cDNA)
Primer 2	ATCTGCTATGACGGGGAGAAT		
hnRNP A1 splicing factor			
Primer 1	AGGTGGATGCAGCTATGAATG	exon 3 1856-1876	372 (DNA)
Primer 2	TGCCTCGGTCAGTCATGATTT	exon 4 2228-2208	223 (cDNA)
HPV-16 E6/E7 ORF cloning primers			
Primer 1	CACGT↓GGATCC|AGCAGACATTTTATGCACCA (*Bam*HI)	E6 71-90	803
Primer 2	ATGACC↓TCGAG|ATCAGCCATGGTAGATTATG (*Xho*I)	E7 854-873	791 (cloned)
Abrogation of nt 409 splice acceptor in HPV-16 E6			
Primer 1	TTAATTA*CT*TGTATTAACTGTCAA (GG in wild type)	E6 401-424	amplify the entire plasmid
Primer 2	CAAATCACACAACGGTTTGT	E6 400-381	
Abrogation of nt 526 splice acceptor in HPV-16 E6			
Primer 1	TTGTTGCA*CC*TCATCAAGAA (GA in wild type)	E6 517-536	amplify the entire plasmid
Primer 2	GACATACATCGACCGGTC	E6 516-499	

presence of HPV-16 genomic DNA by using the same method but with HPV-16 primers. Specimens positive for HPV-16 DNA were selected for further analysis.

Determination of DNA and mRNA Concentration

The concentration of DNA or mRNA was determined spectrophotometrically following the method of Sambrook *et al.* (1989). Absorbance readings were taken at wavelengths 260 nm and 280 nm in a DU-20 ultraviolet light spectrophotometer (Beckman Instruments). An absorbance of 1 at 260 nm corresponds to approximately 50 μg/ml for double stranded DNA and 40 μg/ml for single stranded nucleic acid, and ~20 μ g/ml for single-stranded oligonucleotides. The ratio of absorbances at 260 nm and 280 nm estimates the purity of the nucleic acid isolates. An absorbance ratio A260/A280 less than 1.8 for DNA or 2.0 for mRNA indicates a potential contamination with protein or phenol.

PCR Amplification

Because of the problem of potential contamination by PCR products, nucleic acid isolation and PCR set-up was performed in a room physically separated from where PCR amplification and analysis of the products were done. Additionally, positive displacement pipettes with disposable plastic tips and pistons (Gilson, Microman, Mandel Scientific) were used for setting up PCR. By using specific oligonucleotide primers (table 1) in stringent amplification conditions, only the specific DNA or cDNA sequences perfectly complementary to the oligonucleotide primers were targeted for amplification. The cap of the tube used for the PCR amplification has a hollow plastic plunger to minimize the chances of aerosolized PCR products condensing underneath the cap (Bio/Can Scientific) and eliminates the use of an oil overlay.

The PCR cocktail contains a final concentration of 50 mM KCl, 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 2 units *Taq* DNA polymerase (Bio/Can Scientific), 1 μM of appropriate primers and 500 ng genomic DNA in a total volume of

100 µl. Controls were included in every batch of amplification. A characterized template was used as the positive control, while the negative control was a reaction containing all reagents except DNA template. The amplification was performed on a PTC-100 programmable thermal cycler (MJ Research).

The thermal cycler program for HLA DQ-α amplification was: initial denaturation at 94°C for 2 minutes, followed by 30 cycles of 94°C for 1 minute, 50°C for 1.5 minutes and 72°C for 1 minute, and ended by final elongation at 72°C for 9 minutes. Genomic DNA isolated from a DU-145 (ATCC HTB 81) cell lysate was used as the positive control for HLA DQ-α amplification.. For HPV-16 DNA screening, the thermal cycler program was: initial denaturation at 95°C for 3 minutes, 5 cycles of 1 minute for each temperature at 95°C, 58°C, and 72°C, followed by 25 cycles of 1 minute for each temperature at 95°C, 55°C, and 72°C, and ended with final elongation at 72°C for 9 minutes. The positive control was plasmid carrying HPV-16 DNA sequence.

Agarose Gel Electrophoresis

The products of the amplification were precipitated in sodium acetate (Appendix) and ethanol, resuspended in 15 µl of tracking dye (Appendix), and separated on the basis of molecular size. This was done on 4% agarose gels (Appendix) using a horizontal gel electrophoresis system, Bio-Rad Wide Mini sub-cell (Bio-Rad). The gel was prepared using Tris-Acetate EDTA (TAE) buffer (Appendix) and Ultrapure DNA grade agarose (Bio-Rad). Ethidium bromide (Sigma) was added to the gel to visualize the position of bands under ultraviolet light (Appendix). The gel was run for approximately 2 hours at 70 volts, using a Bio-Rad Model 1000/500 power supply (Bio-Rad), then was visualized on an ultraviolet transilluminator (Fotodyne Incorporated) and photographed using a Polaroid Type 57 (ASA 3000) high speed 4×5 instant sheet film with a Polaroid MP4 camera through a 25 Red 1 filter (Tiffen) and a Wratten Gelatin filter (Kodak).

Cell Culture

CaSki (ATCC CRL 1550), HeLa 229 (ATCC CCL 2.1), G-401 Wilms' tumor (ATCC CRL-1441), and C-33A (ATCC HTB-31) cell lines were purchased from the American Type Culture Collection (ATCC). A human Foreskin Fibroblast (HuFF) cell line was obtained from the virology section of Cadham Provincial Laboratory. All cell lines were grown in a T150 flask in modified Leibovitz-15 (L-15) media with glutamine (Flow Laboratories) supplemented with 10% fetal bovine serum (Appendix) at 35°C. The cells were harvested by trypsinization (Appendix), washed in phosphate-buffered saline (pH 8.0) and held at -70°C for subsequent mRNA isolation.

Cell lines that are recipients of transfection were also grown to confluence in modified L-15 media with glutamine (Flow Laboratories) supplemented with 10% fetal bovine serum at 35°C. One day prior to transfection, the cells were trypsinized, counted, seeded in 60 mm cell culture dishes with 1-5 X 10^5 cells per dish, fed with 5 ml of L-15 media containing 10% fetal calf serum, and incubated at 35°C overnight.

Isolation of mRNA

The exfoliated cells and biopsy specimens known to harbor HPV-16 DNA were subjected to mRNA isolation by using a Micro Fast-track mRNA isolation kit (Invitrogen) as per the manufacturer's instructions. Briefly, this kit utilizes a detergent lysis (Appendix) of cells with simultaneous inactivation of nucleases and degradation of proteins by an enzymatic reaction held at 45°C, followed by batch binding with NaCl (Appendix) and oligo d(T) cellulose. Degraded proteins and DNA were washed off in high salt wash buffer (Appendix) several times, then the non-polyadenylated RNA was removed with low salt wash buffer (Appendix). After the washes, mRNA was eluted from a microcentrifuge spin column and collected by precipitation with glycogen carrier (Appendix), sodium acetate (Appendix), and ethanol (Corby Distilleries Limited).

mRNA from cell lines (less than 5 X 10^6 cells) was also isolated using a Micro Fast-track mRNA isolation kit. For more cells, the Fast-track mRNA isolation kit was used. This kit uses the same principle as the Micro Fast-track kit, except on a larger scale.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

The level of E6*I and E6*II transcripts in mRNA isolated from exfoliated cervical cells and biopsy tissues were analyzed by using Quantitative Reverse Transcriptase-Polymerase Chain Reaction (QRT-PCR) assay. Figure 9 briefly depicts the consecution of QRT-PCR.

1. Reverse Transcription

In order to amplify mRNA isolated from cell lines or specimens using PCR, the mRNA has to be converted into double stranded cDNA by reverse transcriptase (RT). The reverse transcriptase reaction was performed in a reaction consisting of 400 ng mRNA, 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 units RNA guard RNase inhibitor (Pharmacia), 2.5 U avian myeloblastosis virus (AMV) reverse transcriptase (Pharmacia), and 2.5 mM each of dGTP, dCTP, dATP, and dTTP (Pharmacia) in a total volume of 20 µl. The reaction was incubated at 23°C for 10 minutes, followed by primer extension at 42°C for 45 minutes and enzyme inactivation at 95°C for 10 minutes. Samples were immediately refrigerated at 5°C until PCR amplification. The positive control was mRNA isolated from CaSki cells. CaSki cells are known to harbor and express the HPV-16 genes (Yee *et al.*, 1985). The negative control was a reaction containing all reagents except RNA template. For every sample a reaction containing all reagents and RNA template, but lacking reverse transcriptase was included. The latter reaction functioned as the background control for the quantification studies.

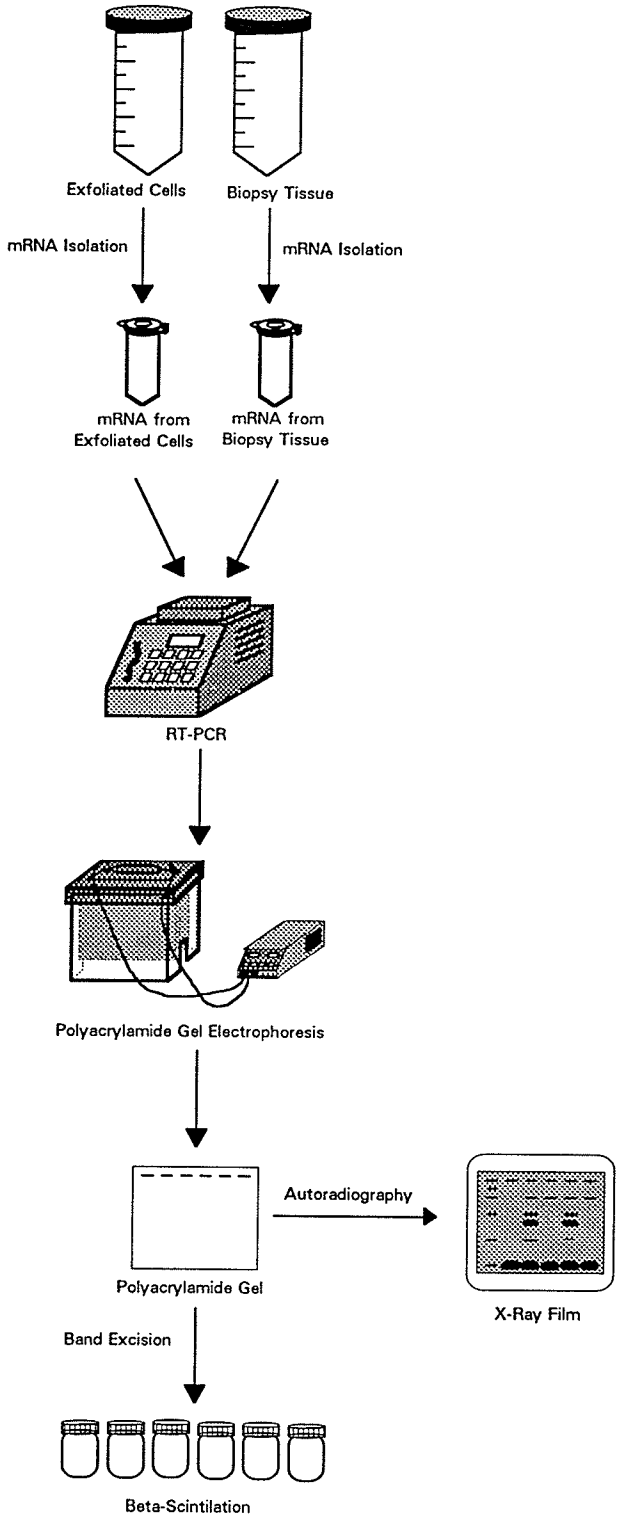


Figure 9. Quantitative Reverse Transcriptase-Polymerase Chain Reaction (QRT-PCR)

2. 5' End Labeling of Primers

Oligonucleotide primers (Table 1) utilized in the PCR amplification of cDNAs were 5' end-labeled with [γ-^{32}P]-ATP (ICN Biomedicals Inc.) so that the PCR products could be quantified. For this purpose, the 5' DNA end-labeling kit (Boehringer Mannheim) was used as per the manufacturer's instructions. A total of 100 pmol of primers was utilized in each PCR reaction, however, only half of the total required primer was labeled, and the remainder in each reaction volume was left unlabeled. Similarly labeled was 1 μg of a 100 base pair molecular weight ladder (Pharmacia) to facilitate the determination of molecular weight of the PCR products. The labeling reaction was carried out in a 37°C waterbath (Haake) for 1 hour and the products were precipitated with sodium acetate (Appendix) and ethanol. The molar ratio of the primers for HPV-16 E6/E7 and GAPDH in QRT-PCR was 1:9, respectively.

3. PCR Amplification of cDNA Products

The specific cDNA sequences reverse-transcribed in the reverse transcription reaction as described above, were co-amplified in PCR. Amplification was done using a programmable thermal cycler (MJ Research). The total volume of the reverse transcription reaction was added to a PCR cocktail of 80 μl containing a final concentration 50 mM KCl, 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 2 U *Taq* DNA polymerase (Bio/Can Scientific), and radiolabeled primers at a final concentration of 1 μM. Initial DNA denaturation at 94°C for 2 minutes was followed by 20 to 30 cycles of amplification. Each cycle consisted of primer annealing (60°C for 1 minute), extension (72°C for 1 minute), and denaturation (94°C for 1 minute). Final annealing (60°C for 1 minute) and final elongation (72°C for 7 minutes) steps were done after the final cycle. The amplification products were kept at 4°C until precipitation with sodium acetate (Appendix) and ethanol. The precipitates were resuspended in 15 μl

tracking dye (Appendix) and subsequently separated using polyacrylamide gel electrophoresis.

4. Quality Control of mRNA Isolation

Since the cDNA reverse-transcribed from mRNA encoding human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was coamplified in the assay, the quality of isolated mRNA could be directly determined. Specimen which had amplifiable mRNA showed a specific band of amplified GAPDH cDNA in the polyacrylamide gel.

5. Polyacrylamide Gel Electrophoresis

The products of RT-PCR with radiolabeled primers were separated on 8% polyacrylamide gels using the Bio-Rad Protean II xi cell (Bio-Rad) 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 (TBE) buffer (Appendix). The paired PCR products, one with and the other without reverse transcriptase, were loaded side by side. The gel was run at 150 volts for approximately 5 hours, after which it was wrapped in polyvinyl chloride laboratory wrap (Fisher Scientific), sealed in a plastic bag, and autoradiographed with an x-ray film.

6. Autoradiography

The products of RT-PCR separated by polyacrylamide gel electrophoresis were radioactive due to the incorporation of radioactively labeled primers. Therefore direct autoradiography of the polyacrylamide gels was done to visualize the position of the bands of interest. A sheet of Kodak X-omat AR film was placed over on the sealed 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.).

7. Scintillation Counting

Autoradiography of the 8% polyacrylamide gel revealed the positions of the specific RT-PCR amplified products. The site in the gel containing the band of interest was carefully excised and transferred to a disposable scintillation vial (Fisher Scientific) filled with 5 ml EcoLite scintillation fluid (ICN Biomedicals Inc.), and the beta particle decay was counted in LKB Wallac 1217 Rackbeta Liquid scintillation counter. The site in the lane of reaction without reverse transcriptase was also scintillation counted; this value was subtracted from the corresponding value for the reaction containing reverse-transcriptase. The result obtained was the net amount of the amplification.

8. Quantification of Expression Levels of Transcripts

The value obtained from scintillation counting could be used to reveal the relative amount of a target DNA after a specific number of cycles of amplification. By assuming that the efficiency of PCR amplification was constant for each successive cycle, the relative quantity of the target cDNA entering first cycle of PCR could be extrapolated using the method developed by Golde *et al.* (1990).

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

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 radiolabeled primer during each cycle, the amount of cDNA at the beginning of the PCR amplification, $cDNA_0$, could be determined. This quantity represents the amount of target mRNA in the sample.

9. Determination of Amplification Efficiency of Each Pair of Primers

The calculation of the relative amount of each transcript required a determination of the amplification efficiency of each pair of primers used in RT-PCR. Since the length or primary sequence differences between templates could have an effect on relative amplification efficiency, an assumption that the efficiency, R, of all pairs of primers was equal would have resulted in an erroneous calculation of the amount of $cDNA_0$, and subsequently of the amount of mRNA.

To determine the efficiency of amplification of each pair of primers, the CaSki cell line was used as a model system. CaSki cells are known to harbor and express the HPV-16 E6/E7 ORFs (Yee *et al.*, 1985). At time intervals of 20, 22, 24, 26, 28, and 30 cycles of PCR, a pair of samples (with and without reverse transcriptase) were removed from the thermal cycler and held on ice. After all the samples had been amplified, the amplification products were precipitated with sodium acetate (appendix) and ethanol. The precipitated products were re-suspended in 15 µl of tracking dye (Appendix), separated on an 8% polyacrylamide gel, autoradiographed, excised, and scintillation counted as described above. This experiment was repeated three times for each time point, and the log of the mean disintegration per minute (dpm) obtained was plotted as a function of cycle number, n. The regression line was generated by using SigmaPlot for Windows version 2.01 (Jandel Scientific).

The equation described above can be rewritten in the log form as followed:

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

Since $\log(1+R)$ is equal to the slope of the regression line, the R value or efficiency of amplification for each pair of primers was determined.

10. Standardization Relative to GAPDH Expression

The values obtained for $cDNA_0$s of E6*I and E6*II or SF2 and hnRNP A1 were adjusted relative to the value obtained from GAPDH $cDNA_0$ in each mRNA specimen. This adjustment compensated for differences in efficiency of the reverse-transcription reaction and for differences in the number of cells per specimen. The standardization is calculated as followed:

$$cDNA_{std} = \frac{C}{GAPDH} \times cDNA_0$$

where $cDNA_{std}$ is the standardized value of the transcript, C is the quantity of GAPDH expression of a specimen arbitrarily chosen as a standard, $cDNA_0$ is the initial quantity of a specific transcript calculated using the formula described previously, and $GAPDH$ is the initial quantity of the corresponding GAPDH expression.

Cloning of The HPV-16 E6/E7 ORFs

In order to investigate the role of the cellular splicing factors in the HPV-16 E6/E7 splice acceptor site selection, a plasmid construct was prepared by cloning the HPV-16 E6/E7 DNA fragment into the mammalian expression vector pcDNA3 (Invitrogen).

1. HPV-16 E6/E7 Cloning Fragment Amplification

The PCR technique was used to amplify HPV-16 E6/E7 ORF from nt 71 to nt 873. The upstream and downstream primers (Table 1) contained restriction sites for *Bam*HI and *Xho*I respectively, to facilitate a directional cloning of the product. The reaction consisted of 100 pmol template DNA, 50 mM KCl, 10 mM Tris-HCl (pH 9.0),

0.1% Triton X-100, 1.5 mM $MgCl_2$, 2 units *Taq* DNA polymerase (Bio/CAN Scientific), 200 μM each of dGTP, dCTP, dATP, and dTTP (Pharmacia), and 1 μM primers in a total volume of 100 μl. Initial denaturation at 94°C for 4 minutes was followed by 30 cycles of denaturation (94°C, 1 minute), primer annealing (45°C, 2 minutes 30 seconds), and primer extension (72°C, 4 minutes). The PCR product, subsequently called "insert", was then processed and cloned into the mammalian expression vector pcDNA3 (Invitrogen).

2. Insert and Vector Preparation

The insert was digested in a reaction containing 5 μg insert, 20 mM Tris-acetate (pH 7.5), 20 mM magnesium acetate, 100 mM potassium acetate, 40 units *Bam*HI (Pharmacia) and 45 units *Xho*I (Pharmacia), in a total volume of 50 μl. The mammalian expression vector pcDNA3 (vector) was also digested in a separate reaction containing 2 μg vector, 20 mM Tris-acetate (pH 7.5), 20 mM magnesium acetate, 100 mM potassium acetate, 20 units *Bam*HI, and 22.5 units *Xho*I, in a total volume of 30 μl. Both reactions were incubated in 37°C waterbath (Precision) for 1 hour. The enzymes were heat inactivated by incubating at 85°C for 30 minutes, then the reaction was cooled down for 20 minutes at room temperature. The insert was purified by passing through a Chromaspin -100 column (Clontech). Only DNA with size greater than 100 bp is able to pass through the column. The purified insert was collected in a sterile microcentrifuge tube by spinning the column at 700 × g for 5 minutes, precipitated with sodium-acetate (Appendix) and ethanol, and resuspended in TE buffer (Appendix). Meanwhile, vector DNA was dephosphorylated in a reaction containing 1 μg digested vector, 10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate, and 0.1 unit Calf Alkaline Phosphatase (Pharmacia), in a total volume of 50 μl. The reaction was incubated for 30 minutes at 37°C, then an additional 0.1 unit of Calf Alkaline Phosphatase was added. The reaction was re-incubated at 37°C for another 30 minutes. The enzyme was inactivated by heating at 85°C for 15 minutes, then cooled down to room temperature.

The dephosphorylated vector was extracted by mixing with an equal volume of buffer-saturated phenol (Gibco BRL), then with equal volume of phenol:chloroform, followed by sodium acetate (pH 7.0) (Appendix) and ethanol precipitation. The precipitate was rinsed with 70% ethanol, then was re-suspended in TE buffer.

3. Ligation of Insert to Vector

The purified insert (150 ng) and the dephosphorylated vector (300 ng) were warmed to 45°C for 5 minutes, chilled immediately on ice, and then ligated in a 10 µl reaction containing 10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate, 10 nmol ATP (Pharmacia), and 3-4 Weiss units of T4 DNA ligase (Pharmacia). The reaction was carried out at 10°C for 4 hours, and stopped by heating to 65°C for 10 minutes.

4. Bacterial Transformation

To propagate the plasmid, the ligated DNA was transformed into the susceptible bacterial host by using the method of Cohen *et al.* (1972). Briefly, 1 ml overnight culture of *Escherichia coli* strain TOP10F' (Invitrogen) is grown in 100 ml Luria broth at 37°C on a shaker for 1-2 hours, chilled on ice for 15 minutes, and washed in 50 ml of ice cold 0.1 M $MgCl_2$. The cells were resuspended in 25 ml of ice cold 0.1 M $CaCl_2$ for 30 minutes, and then concentrated to 1 ml of bacterial suspension in 0.1 M $CaCl_2$. Plasmid DNA in the amount of 50-200 ng is added to every 0.1 ml bacterial suspension. The mixture is chilled on ice for 30 minutes, heat shocked at 42°C for 2 minutes, chilled on ice again for 10 minutes. Luria broth is then added to bring the final volume to 1 ml. The suspension is incubated at 37°C for 1 hour. After the incubation, approximately 0.1 ml bacterial culture is inoculated to each trypticase soy agar plate containing 20 mg ampicillin/litre. Since the plasmid vector carries an ampicillin resistance gene, only bacteria taking up the plasmid are able to grow and form colonies. Controls included in the transformation were:

untransformed bacteria as the negative control; bacteria transformed with a characterized plasmid as the positive control; bacteria transformed with digested and religated vector, and bacteria transformed with digested, dephosphorylated, and religated vector to determine the efficiency of dephosphorization; and bacteria transformed with insert DNA only to exclude the possibility of contamination with other plasmids. The ampicillin resistance of the growing colonies was confirmed by β-lactamase enzyme detection.

5. Detection of β-lactamase enzyme

The vector plasmid carries a gene encoding an enzyme which inactivates ampicillin by hydrolyzing its β-lactam ring. In order to confirm that the growing colonies were taking up the plasmid, all the selected colonies were tested for β-lactamase enzyme production using Cefinase® paper discs (Becton-Dickinson). The disc is impregnated with the chromogenic cephalosporin, nitrocefin. This compound exhibits a very rapid color change from yellow to red as the amide bond in the β-lactam ring is hydrolyzed by a β-lactamase. When a bacterium produces this enzyme in significant quantities, the yellow-colored disc turns red in the area where the isolate is smeared. In the assay, a Cefinase® disc was placed in a sterile Petri dish and moistened with a loop full of sterile water. With a sterile needle, a portion of a colony to be tested was removed and smeared onto the disc surface. Color change was observed in seconds. Colonies confirmed for β-lactamase enzyme production were subsequently subcultured and further analyzed.

6. Plasmid Mini-Preps

Recombinant plasmids were isolated from the host cells by using a mini-prep procedure. Briefly, a loop full of bacterial colonies from overnight culture on a trypticase-soy agar plate containing 20 mg ampicillin/litre were gently lysed in 80 µl lysis buffer (Appendix) containing 0.2% (w/v) of lysozyme (Sigma). The reaction is held on ice for 10 minutes. Two volumes of solution containing 0.2 M NaOH, 1% SDS (Appendix) is

added, then chilled on ice for 5 minutes. One hundred µl of 3 M sodium acetate (pH 4.6) (Appendix) is added, and chilled on ice again for 5 minutes. Cell debris is removed by centrifugation. Cell proteins are removed from the supernatant by mixing with an equal volume of phenol:chloroform solution. Possible RNA contaminants are then digested by adding 10 µg RNase A (Sigma) and 2 units RNase T1 (Boehringer Mannheim) and incubating at 37°C for 15 minutes. The plasmid DNA is then precipitated with an equal volume of iso-propanol.

7. DNA Sequencing

In order to verify the correct DNA sequences had been cloned, the sequence of the insert was analyzed by DNA sequencing. The commercially available ^{T7}Sequencing Kit marketed by Pharmacia was chosen for DNA sequencing analysis. This kit adapted the Sanger method (Sanger *et al.*, 1977). Briefly, four separate reactions are performed, all containing primer (Table 2), template, and the four deoxynucleotides including radioactive α-^{32}P dATP (Dupont NEN), but each reaction includes a different chain-terminating dideoxynucleotide. The products of the four reactions are electrophoresed in a 6% polyacrylamide gel containing 7 M urea (Bio-Rad) using a Sequi-Gen II Sequencing Cell (Bio-Rad) and powered by Bio-Rad PowerPac 3000 (kindly provided by Dr. Janice Dodd), at 1,500 volts for approximately 2 hours. When the samples reached the bottom of the gel, the electrophoresis was stopped, and the sequencing apparatus was disassembled. The gel was carefully transferred to a sheet of Whatman 3MM paper, covered with polyvinyl-chloride laboratory wrap (Fisher Scientific) and dried using a vacuum gel dryer model 583 (Bio-Rad) (kindly provided by Dr. Grant McClarty) for 1 hour at 80°C. The sequence bands are visualized by autoradiography at room temperature without intensifying screens. Sequence data were compared to HPV-16 Genebank database. Clones carrying the correct DNA sequence were selected and stored as stock cultures and subsequently called pHPV800 (Figure 10).

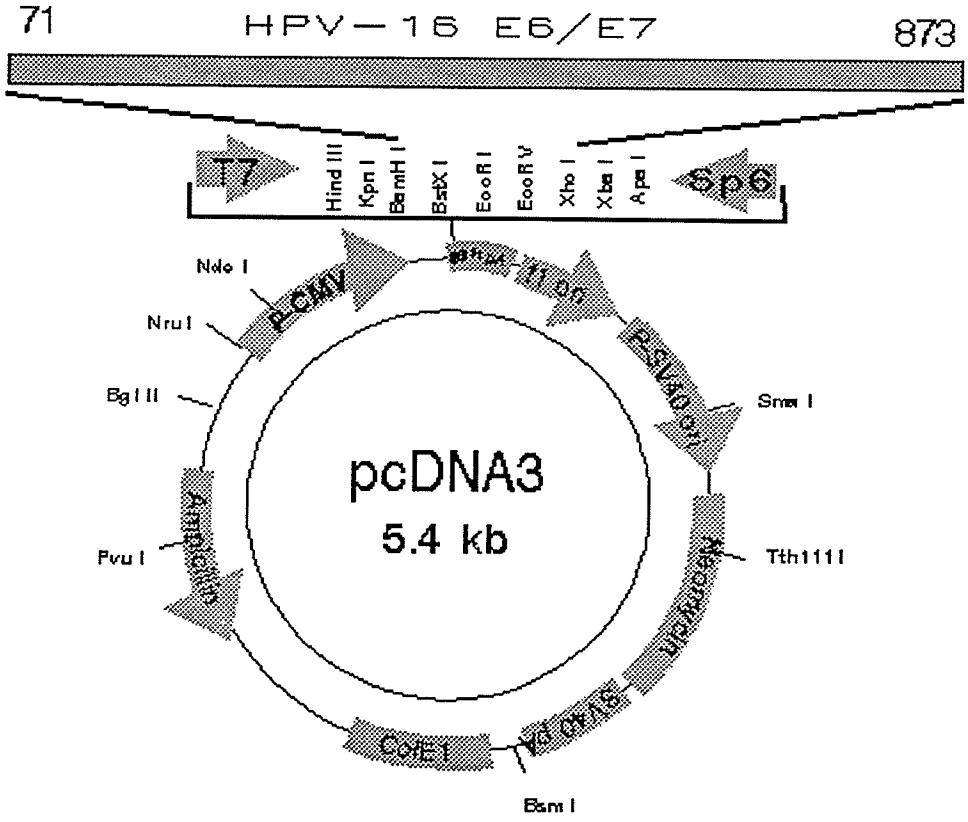


Figure 10. pHPV800

HPV-16 E6/E7 ORFs from nt 17 to nt 873 is inserted at *Bam*HI and *Xho*I insertion sites of pcDNA3 mammalian expression plasmid.

Table 2. Probe and Sequencing primer sequences of HPV-16 E6

Probe	Sequence (5'-3')	Genome Location (nt)	Hybridization Temperature (°C)
nt 409 Mutant	TTAATTA*CT*TGTATTAACTGTCAA	E6 401-424	58°C
nt 526 Mutant	TTGTTGCA*CC*TCATCAAGAA	E6 517-536	56°C

Sequencing Primer	Sequence (5'-3')	Genome Location (nt)
Primer 1	TCGACCGGTCCACCGACCCCT	E6 508-488
Primer 2	TCCGGTTCTGCTTGTCCAGCTGG	E6 704-682

Introduction of Plasmid DNA into Mammalian cells

1. large-Scale Plasmid Preparation and Purification

Transfection of DNA into eukaryotic cells requires a relatively large amount of exceptionally pure plasmid DNA. Impurities in the DNA preparation can be detrimental to transfection efficiency. The standard method for plasmid DNA purification is by cesium chloride gradient centrifugation. However, this method can be both laborious and time consuming, and often requiring an overnight centrifugation. The Wizard Maxipreps DNA Purification System marketed by Promega, offers a simpler and more rapid procedure, yet yields large quantities of purified plasmid DNA, therefore it was chosen and used for large-scale plasmid preparation. Briefly, overnight bacterial culture grown in trypticase-soy broth containing 20 mg ampicillin/l media is lysed in alkaline solution (Appendix) in about 20 minutes and neutralized with Neutralization Solution (Appendix). DNA is separated from cell debris by centrifugation and filtering it through Whatman #1 filter paper. DNA is precipitated in isopropanol and resuspended in TE buffer (Appendix). Plasmid DNA is then purified by binding to silica-based resin, washed several times with buffer containing ethanol (Appendix), eluted from a column and collected by centrifugation in a centrifuge tube.

2. Calcium Phosphate Transfection

The pHPV800 and pCMVβ-Gal (Stratagene) plasmids were co-introduced and expressed transiently in mammalian cell lines using a modified $Ca_3(PO_4)_2$ technique. A commercially available transfection kit optimized for transient transfection from Stratagene was chosen. The transfection MBS Mammalian Transfection Kit is based on a modified $Ca_3(PO_4)_2$ technique introduced by Chen and Okayama (1987). Briefly, the circular plasmids are mixed with $CaCl_2$ and BES-buffered saline (pH 6.95) (Appendix), then the mixture is added to exponentially grown mammalian cells which are freshly fed

with MEM containing 6% modified bovine serum (Stratagene). The cells are incubated at 35°C under 3% CO_2 for 3 hours. In the low pH of the buffer, calcium phosphate-DNA precipitates develop gradually in the medium during incubation. The precipitates are then removed by washing with PBS (Appendix). The cells are refed with fresh media containing 10% fetal calf serum, and harvested after 72 hours with a Teflon scraper (Nunc). Cells are divided for β-Galactosidase activity assay to determine the efficiency of transfection and QRT-PCR assay to determine E6*I and E6*II expression levels in the transfected cells. The controls were untransfected cells and cells co-transfected with pcDNA3 and pCMVβ-Gal plasmid.

3. β-Galactosidase Activity Assay

In order to determine the efficiency of transfection, plasmid carrying HPV-16 E6/E7 ORF was co-transfected with β-galactosidase expressing plasmid (pCMVβ-Gal) (Stratagene). β-galactosidase, the protein product of the plasmid, is an enzyme that catalyzes the hydrolysis of β-galactosides including lactose. The protein is extremely stable and resistant to proteolytic degradation in cellular lysates and is easily assayed. The β-galactosidase assay kit from Stratagene was used for measuring β-galactosidase activity in transfected cells lysate. In brief, the cells are lysed in a detergent lysis buffer (Appendix), and then incubated in a reaction buffer (Appendix) containing 0-phenyl-galactopyranoside (ONPG) substrate at 37°C. β-galactosidase hydrolyzes this colorless substance into a yellow solution. The reaction is stopped by adding 1 M Na_2CO_3. The amount of the hydrolyzed ONPG is quantified by measuring the absorbance of the reaction at wavelength 420 nm using a spectrophotometer. One OD unit is equal to 222.22 nmoles of ONPG hydrolyzed per ml reaction during the timed incubation period. By adjusting to the volume of the lysate used in the reaction, the total volume of the assay, duration of incubation, and protein concentration of the lysate, the β-galactosidase activity can be calculated as Units per mg of protein in the lysate using the following formula:

$$\beta - Gal = \frac{OD420}{0.0045} \times Volume \times \frac{1}{time} \times \frac{1}{lysate} \times \frac{1}{protein}$$

where β-*Gal* is β-galactosidase activity in Units per mg of lysate, *OD 420* is OD reading at λ420 nm, *Volume* is the total assay volume in ml, *time* is the total incubation time at 37° C in minutes, *lysate* is the volume of lysate used in the assay in μl, and *protein* is the protein concentration of 1 μl lysate in milligrams

4. Protein Assay

The amount of total protein in the cell lysate was determined using *DC* Protein Assay kit (Bio-Rad). This kit is based on Lowry assay (Lowry *et al.*, 1951). In this assay there are two steps which lead to color development: the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. The blue color developing after 15 minutes reaction is measured by using a spectrophotometer at wavelength 750 nm, and the protein concentration of the cell lysate is determined by comparing to a standard curve. A standard curve is prepared each time the assay is performed by plotting absorbance at wavelength 750 nm of various concentrations of standard bovine serum albumin.

5. Determination of E6*I and E6*II Expression in Transfected Cells

The expression levels of E6*I and E6*II of the transfected cells were determined by using QRT-PCR assay as described previously. The results were normalized to β-galactosidase activity to compensate for differences in efficiency of the transfection by using the following formula:

$$cDNA_{adj} = cDNA_0 \times \frac{S}{\beta - Gal}$$

where $cDNA_{adj}$ is the adjusted value of the transcript, $cDNA_0$ is the initial amount of E6*I or E6*II of the transfected cells, β-*Gal* is β-Galactosidase activity of the transfected cells, and S is the β-Galactosidase activity of a sample chosen as the standard.

6. Characterization of SF2 and hnRNP A1 Expression in Cell Lines

The expression of SF2 and hnRNP A1 of C-33 A, G-401, HeLa, and HuFF cells were determined by using QRT-PCR assay as described above, except in the RT reaction 100 ng of mRNA is used and in the PCR amplification the ratio of SF2, hnRNP A1, and GAPDH primers was 4.5:4.5:1. A standard curve for determining the efficiency of each pair of primers was also generated for SF2, hnRNP A1, and GAPDH primers as previously described for E6*I, E6*II, and GAPDH.

Site-Directed Mutagenesis

Oligonucleotide-directed mutagenesis has become an important method for modifying certain bases of a DNA sequence and a powerful tool for studying the expression and function of a specific gene (Picard *et al.*, 1994). Initially, the procedure required cloning of an appropriate fragment of DNA into a bacteriophage M13 vector in order to generate a single-stranded DNA template (Kunkel, 1985) which was used as a template. The template was annealed with a synthetic oligonucleotide which contains one or several bases differing from the wild-type sequence functioning as the primer for *in vitro* DNA polymerization. When the double-stranded molecule was subsequently transfected into a suitable host, the mutation was sustained by replication of the hybrid molecule (reviewed in Smith, 1985). Many modifications of the method have been introduced (Vandeyar *et al.*, 1988; Sugimoto *et al.*, 1988), however, the desired mutants can be obtained only inefficiently.

The development of the polymerase chain reaction (PCR) led to new approaches to oligonucleotide-directed mutagenesis (Barettino *et al.*, 1993; Chen & Przybyla, 1994; Juncosa-Ginestà *et al.*, 1994; Owen *et al.*, 1994). Construction of single-stranded circular DNA yielded recombinant bacteriophage is no longer essential since double-stranded DNA can be used as the template. In these methods, the oligonucleotides with the base changes function as primers, which after PCR, are incorporated into the PCR products. Generally the procedures require three oligonucleotide primers: two flanking primers, which are upstream and downstream of the mutation site, and one mutagenic primer. After amplification, the products were cloned into a suitable vector.

The ability to amplify PCR products of several kilobases long makes it possible to amplify an entire recombinant plasmid by using an inverse PCR method. Combination of inverse-PCR and PCR-based mutagenesis eliminates the cloning step. The inverse-PCR-based mutagenesis has been reported (Hemsley *et al.*, 1989; Weiner *et al.*, 1994). However, the procedures use *Taq* DNA polymerase, an enzyme with the highest error rate of the known thermostable DNA polymerases.

The error rate of *Taq* DNA polymerase is in the range of 0.1×10^{-4} to 2×10^{-4} per nucleotide per pass of the polymerase, depending on the reaction conditions (Keohavong & Thilly, 1989). Over the course of PCR, the cumulative error rate is roughly 10^{-3} per nucleotide. For amplification over a longer segment, this high error rate becomes unfavorable since undesired mutations are likely to occur. By increasing template concentration and reducing cycle number the high error rate can be diminished (Weiner *et al.*, 1994; Owen *et al.*, 1994), but these modifications inevitably diminish the amounts of mutagenized DNA.

An alternative method for PCR-based oligonucleotide-directed mutagenesis by utilizing high-fidelity *Pwo* DNA polymerase is developed in this current work. *Pwo* DNA polymerase is the recombinant form of an enzyme originally isolated from the hyperthermophilic archaebacterium *Pyrococcus woesei*. It possesses a highly processive

5'-3' DNA polymerase activity, and unlike *Taq* DNA Polymerase, it also possesses a 3'-5' exonuclease "proofreading" activity. Because of its proofreading activity, *Pwo* has ten times higher fidelity rate than *Taq* DNA Polymerase, thus the number of the PCR cycles can be maintained at 30-40 cycles while the potential second-site mutation during the PCR remains low. However, the 3'-5' exonuclease activity of *Pwo* DNA polymerase acts also on single stranded DNA in the absence and presence of dNTPs, hence the bases mismatched in the primers should be centrally located.

Taq DNA polymerase inherently has a terminal-transferase like activity, which usually adds an additional dA to the ends of PCR products (Hemsley *et al.*, 1989). Thus, the PCR products have to be polished either by T4 or *Pfu* polymerase (Costa & Weiner, 1994) to yield blunt-ended fragments prior to recircularization. *Pwo* DNA Polymerase provides blunt-ended fragments as it does not add an additional adenosine to the ends of PCR products the way *Taq* DNA Polymerase does, hence the PCR product can be directly phosphorylated and ligated.

Amplification of long template requires an increase in the primer extension time (Kainz *et al.*, 1992; Ponce & Micol, 1992; Yen *et al.*, 1992; Sitaraman & Rashtchian, 1994) and consequently increases the entire PCR time. This may result in decreased polymerase activity. The *Pwo* DNA Polymerase has a longer half-life at high temperature compared with *Taq* DNA polymerase, so it is not necessary to add enzyme stabilizing agents, such as glycerol.

Efficient primer annealing is important in double stranded site-directed mutagenesis. The primers must anneal before reassociation of the long, complementary template strands can occur. To promote the strand separation, and facilitate the kinetics of primer annealing, the strand destabilizing agent DMSO was added to the reaction. Addition of certain denaturants including DMSO to PCR mixtures has been demonstrated to improve the specificity of the amplified product (Qian & Kibenge, 1994; Rasmussen *et al.*, 1994; Varadaraj & Skinner, 1994). Incomplete strand separation of long DNA

template was overcome by the use of denaturants such as formamide and DMSO. (Ivey *et al.*, 1994). DMSO was also reported to reduce the melting temperature of the primers (Chester & Warshak, 1993) and revert the inhibitory effect of certain polysaccharides on PCR amplification (Demeke & Adams, 1992).

1. Inverse PCR for Site-Directed Mutagenesis

Inverse PCR was performed in order to produce mutation in splice acceptor sites at position nt 409 or nt 526 of HPV-16 E6 ORF. A pair of primers located 'back to back' was designed for each mutation. The primers spanning the splice acceptor sites contained two mismatches at nt 408 and 409, and nt 525 and 526, respectively (Appendix). The PCR reaction consisted of 100 pmol pHPV800 as the template, 25 mM KCl, 10 mM Tris-Cl (pH 8.85), 5 mM $(NH_4)_2SO_4$, 5% DMSO, 200 μM each of dGTP, dCTP, dATP, and dTTP (Pharmacia), and 1 μM primers in a total volume of 100 μl. For optimal enzyme activity, *Pwo* DNA Polymerase requires slightly alkaline PCR buffer. The high pH buffer may cause decomposition of dNTPs, thus dNTPs should be added immediately before starting the reaction. Two drops of mineral oil were added to prevent evaporation of the buffer during high temperature incubation. Five units of *Pwo* DNA polymerase (Boehringer-Mannheim) was added through a layer of mineral oil when the temperature of the mixtures reached 80°C. Initial DNA denaturation at 94°C for 4 minutes was followed by 40 cycles of amplification consisting of denaturation at 94°C (1 minute), primer annealing at 46°C for nt 409 mutation and 42°C for nt 526 mutation (1 minute), and extension at 72°C (6 minutes). Mineral oil was extracted with 200 μl of chloroform, and the PCR product was precipitated with sodium acetate (Appendix) and ethanol. The amplification reaction produced a linear version of the entire plasmid but with mutated splice acceptor sites.

2. Phosphorylation of PCR Product

In order to be able to recircularize the linear plasmid in a ligation reaction, the PCR product should carry a phosphate at 5' end. Since the 5' end of the primers do not carry a phosphate, then the PCR product should be phosphorylated prior to recircularization. The 5' end phosphorylation was carried out in a reaction containing 4 pmol of 5' ends, 10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate, 10 pmol of ATP (Pharmacia), and 4 units of T4 polynucleotide kinase (Pharmacia) in a total volume of 10 µl. The reaction was incubated in 37°C waterbath (Haake) for 1 hour, precipitated with sodium acetate (Appendix) and ethanol.

3. Recircularization

The phosphorylated PCR product was recircularized to form a circular double stranded plasmid (Figure 11). The reaction contained 1 pmol of 5' ends, 10 nmol ATP, 10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate, 15 Weiss units of T4 DNA ligase (Pharmacia) in a total volume of 20 µl. The reaction was incubated at 16°C overnight, followed by transformation into a competent bacterial host as described previously (Cohen *et al.*, 1972).

4. Differential Screening to Identify Mutated Clones

In order to differentiate clones containing mutated plasmids from those containing wild-type plasmid, clones were screened by colony hybridization. The primers containing the base changes were used as probes (Table 2). The probes were 5' end-labeled with [γ-^{32}P]-ATP using a 5' DNA end-labeling kit (Boehringer Mannheim) as per manufacturer's instructions. The hybridization was performed under highly stringent conditions equal to the temperature of dissociation of the probes (Table 2). Briefly, the growing colonies were transferred onto a Hybond-N Hybridization transfer membrane (Amersham) laid on a trypticase soy agar plate containing 20 mg Ampicillin/l media. A duplicate was also

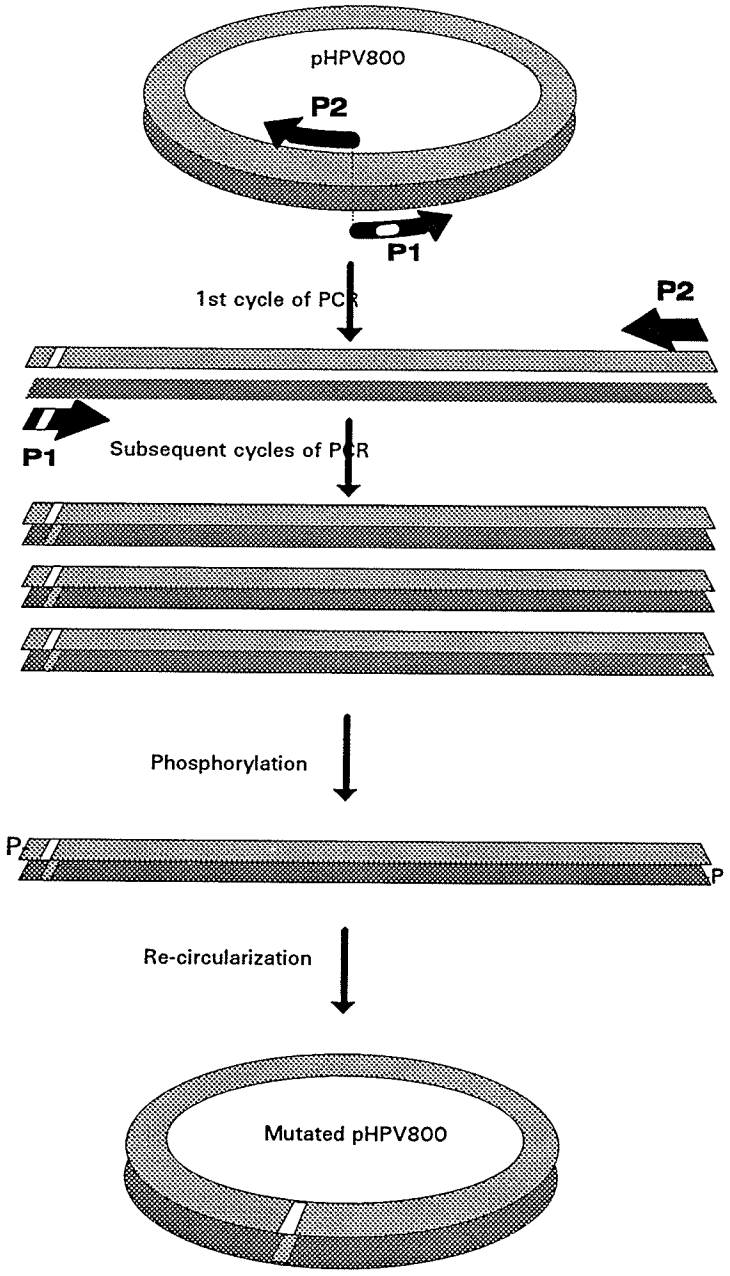


Figure 11. Site-Directed Mutagenesis Inverse PCR

The first cycle of inverse PCR generates a linear double stranded DNA, which serves as template in subsequent cycles. The base changes are incorporated in the PCR products. PCR products are 5' end phosphorylated to facilitate recircularization.

prepared on a similar agar plate but without a nylon membrane. The plates were incubated at 37°C overnight. The nylon membrane, with growing colonies on it, was lifted up. The colonies were denatured by laying the membrane with colony-side up on several layers of 3MM Whatman paper presoaked with denaturation solution (Appendix) for 10 minutes, transferred to dry 3MM Whatman paper to remove excess liquid, then placed on several layers of 3MM Whatman paper presoaked with neutralization solution (Appendix) for 10 minutes. The membrane was blotted and air dried on 3MM Whatman paper, baked in an oven at 80°C for 2 hours, cooled down and stored at room temperature until hybridization. The hybridization was performed by wetting the membrane with 6× SSC (Appendix), placing the membrane in a sealed plastic bag containing 20-30 ml preheated prehybridization solution (Appendix) and incubating in a shaking waterbath (Haake) at hybridization temperature for 1.5 hour. Twelve and a half pmol of 5' end-labeled probe suspended in 50 µl TE buffer (Appendix) was added into the plastic bag. The plastic bag was resealed and returned to the shaking waterbath (Haake) for 1 hour. The hybridized membrane was taken out from the bag, washed in prewarmed 6× SSC, 0.1% SDS (Appendix) in the shaking waterbath (Haake) for approximately 15 minutes, then in prewarmed 2× SSC, 0.1% SDS (Appendix) for another 15 minutes. The membrane was placed on a dry 3MM Whatman paper to absorb excess liquid, sealed in a plastic bag and autoradiographed with intensifying screen overnight at -70°C. Since the sequence of the probes is complementary to that of mutated plasmids, the probes bind more strongly to mutated plasmids, thus clones containing mutated plasmids showed stronger signals than those containing wild-type plasmid. Clones with stronger signals were selected and subsequently plasmid DNA was isolated from the clones for DNA sequencing analysis (Figure 12).

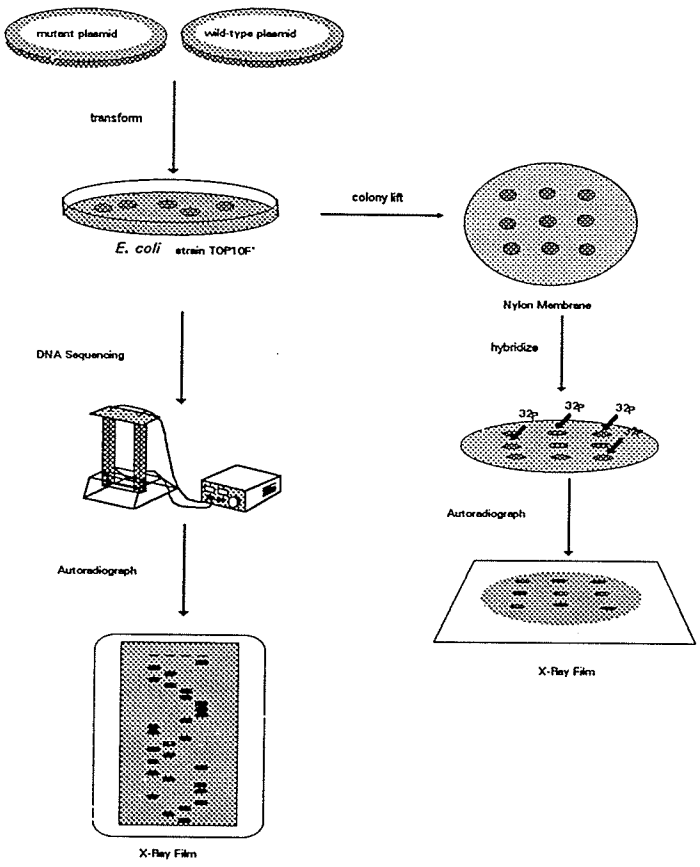


Figure 12. Differential Screening to Identify Mutated Clones

Recircularized inverse PCR products containing both mutant and wild-type plasmids are transformed into *Escherichia coli* strain TOP10F' with ampicillin selection. The growing colonies are transferred to a nylon membrane and hybridized with radiolabeled primers carrying the base changes acting as the probes. Colonies possessing mutant plasmids show stronger signals than those harbor wild-type plasmids. Desired mutation is confirmed by DNA sequencing.

Statistical Analysis

All statistical analyses were performed at the Biostatistical Consulting Unit, University of Manitoba. Tests performed were the Spearman Correlation Coefficient analysis for determining correlation between HPV 16 E6*I and E6*II expression and the grade of CIN. The data for CIN grade 0 and CIN grade I lesion were grouped as low-grade lesions, while data for CIN grade II and CIN grade III lesions were grouped as high-grade lesions for purposes of statistical analysis.

Comparisons in the level of expressions of E6*I, E6*II, SF2, and hnRNP A1 between cell lines were tested by using Analysis of Variance (ANOVA) and Tukey's Studentized Range Test, and correlation between SF2 and E6*I, SF2 and E6*II, hnRNP A1 and E6*I, hnRNP A1 and E6*II, ratio SF2/hnRNP A1 and E6*I, and SF2/hnRNP A1 and E6*II were analyzed by using Pearson Correlation coefficients.

Results

Isolation of Genomic DNA

The isolation of genomic DNA from roughly one fifth of the suspension of an exfoliated cervical cell specimen, yielded approximately 2.5 μg of genomic DNA as determined spectrophotometrically. The isolated genomic DNA was used as a screening specimen harboring HPV-16 genome once its quality was proven to be sufficient for PCR amplification.

Quality Control of Genomic DNA Isolation

Amplification of a conserved human gene, HLA DQ-α, was performed to examine the quality of isolated genomic DNA. Lack of a PCR product indicated that the genomic DNA was of poor quality. This eliminated the need to process inadequate specimens. The amplification rendered a specific product of 242 bp, shown by agarose gel electrophoresis (Figure 13). Genomic DNA not yielding a specific product was subjected to repurification or reisolation. Those specimens yielding the HLA DQ-α PCR product were assayed for HPV-16 DNA detection.

HPV-16 DNA Detection

The PCR for HPV-16 amplified the E6 gene fragment between nt 269- nt 508, generating a specific 240 bp product. The product is also shown in an agarose gel electrophoresis (Figure 14). Specimens positive for HPV-16 DNA were selected for analysis by quantitative RT-PCR.

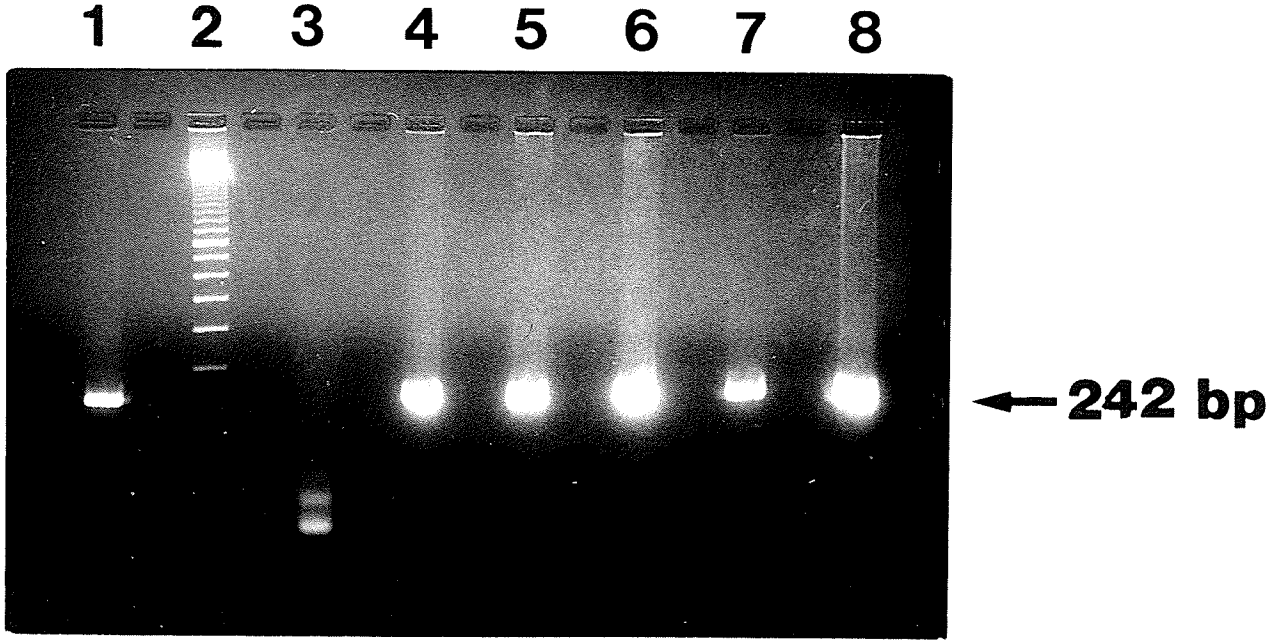


Figure 13. HLA DQ-α PCR amplification of genomic DNA

HLA DQ-α PCR amplification generates a specific product of 242 bp long. Lane 1, positive control; lane 2, 100 bp molecular weight marker; lane 3, negative control; lane 4-8, amplification products from specimens.

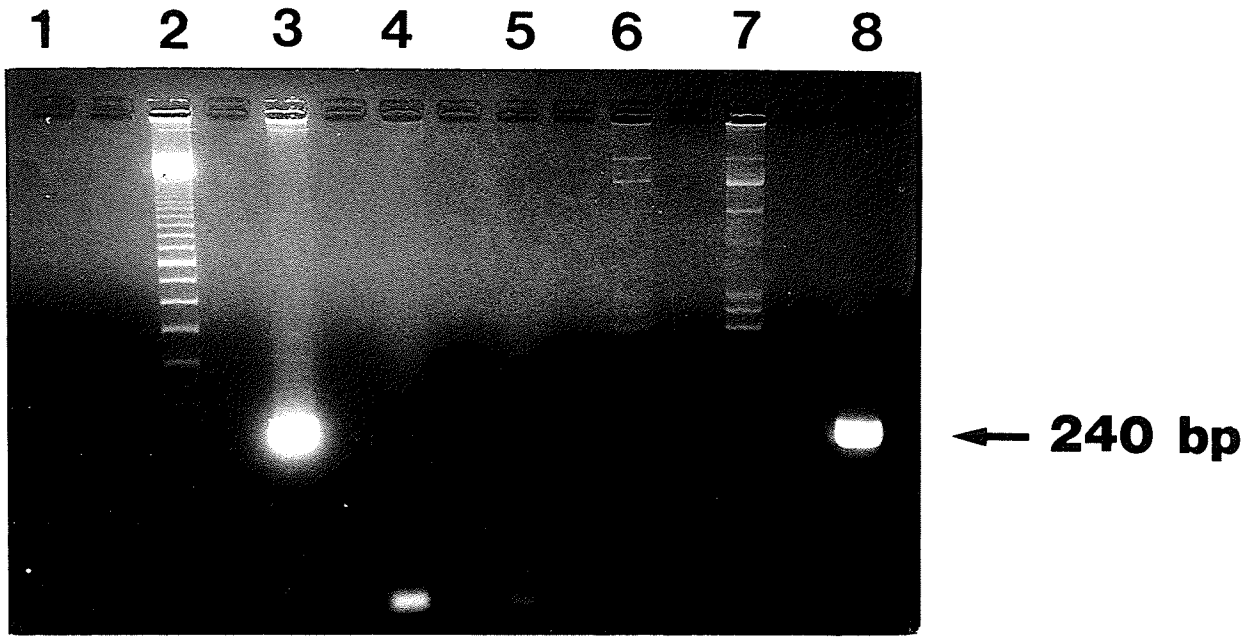


Figure 14. HPV-16 E6 PCR amplification of genomic DNA

HPV-16 DNA PCR amplifies E6 gene fragment between nt 269 - nt 508, rendering a specific product of 240 bp long. Lane 1, negative control; lane 2, 100 bp molecular weight marker; lane 3, positive control, lane 4-7, specimens with no ampifiable HPV-16 DNA; lane 8, specimen with ampifiable HPV-16 DNA.

Isolation of mRNA by Micro-FastTrack Kit

The remaining exfoliated cervical cell specimens which had been proven to harbor HPV-16 DNA was subjected to mRNA isolation. The isolation typically yielded between 0.5-2.0 μg of mRNA. The mRNA from the corresponding biopsy specimens was also isolated. The biopsy specimen yielded between 1-5 μg of mRNA.

The mRNA from transfected C-33A, G-401, HeLa, and HuFF cells were isolated from approximately one half of a cell suspension harvested from a 60 mm dish grown almost to confluence. The yields were between 0.5-1.0 μg of mRNA.

Isolation of mRNA by FastTrack Kit

CaSki, HeLa, C-33a, G-401, or HuFF cells yielded between 7-20 μg of mRNA from a confluently grown cell culture in a T150 flask containing approximately 3×10^7 cells.

Quantitative Reverse Transcriptase-PCR

The mRNA was reverse-transcribed to cDNA with random hexamer primers, which were subsequently amplified by PCR using specific primers. For determining HPV-16 E6/E7 expression level, HPV-16 E6/E7 primers were used, while for determining splicing factors expression level, both pairs of SF2 and hnRNP A1 primers were utilized. When the HPV-16 E6/E7 primers were used, there were two specific products amplified, with sizes of 321 bp and 204 bp, representing E6*I and E6*II transcripts, respectively. The SF2 primers yield a 279 bp product, while the hnRNP A1 primers yield a 223 bp product. Amplification of a "human housekeeping" gene, glyceraldehyde-6-phosphate dehydrogenase (GAPDH), was also performed concurrently is the same tube, functioning both as a control and a standard. This gene is constitutively expressed in the cell, therefore, lack of an RT-PCR product of this gene indicates that the total mRNA isolated is of poor quality. Since this gene is expressed at a constant level independent, of cell

stage, the quantity of the product is also indicative of the efficiency of the RT reaction and the number of cells in the specimen. GAPDH amplification yields a 305 bp product, which can be clearly separated from other specific RT-PCR products by polyacrylamide gel electrophoresis. Only the specimens that had detectable GAPDH products were included in the analysis.

1. Determination of RT-PCR Efficiency of HPV-16 E6/E7 and GAPDH Primers

The autoradiograph of PCR coamplification of CaSki mRNA using HPV-16 E6/E7 and GAPDH primers demonstrates the expected bands of 321 bp, 305 bp, and 204 bp, corresponding to E6*I, GAPDH, and E6*II, respectively (Figure 15). With an increase in the number of cycles of PCR amplification, there is a concurrent increase in the amount of specifically amplified products. This is confirmed by the scintillation counting (Table 3). The disintegration per minute (dpm) obtained for each specific PCR cycle were plotted as the function of the number of PCR cycles, and a regression line was drawn for each specific PCR product (Figure 16). By applying the equation shown below,

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

the amplification efficiency R, can be determined for each transcript by using the value determined for the slope of each line, which is equal to log $(1 + R)$.

From the E6*I/E6*II standard curve, the value of R for E6*I, E6*II, and GAPDH was calculated to be 0.244, 0.621, and 0.288, respectively.

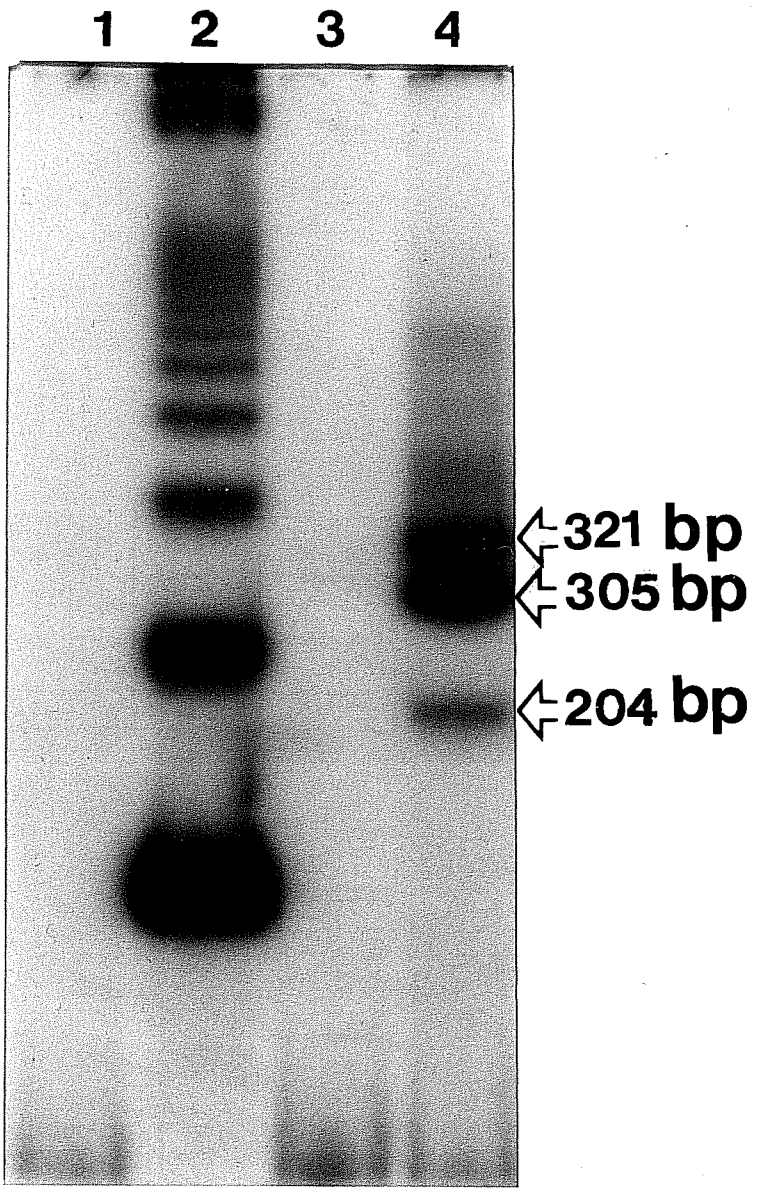


Figure 15. Autoradiograph of GAPDH and HPV-16 E6/E7 Expression Assayed by QRT-PCR. Amplification products were separated on 8% polyacrylamide gel electrophoresis. Lane 1, negative control includes all reagent except mRNA; lane 2, 123 base-pair ladder; lane 3, reaction lacking reverse transcriptase acts as a background control; lane 4, amplification products generated from E6*I, GAPDH, and E6*II transcripts, with sizes of 321 bp, 305 bp, and 204 bp, respectively.

Table 3. The determination of RT-PCR efficiency of HPV-16 E6/E7 and GAPDH primers

Cycle no.	E6*I [log dpm (SD)]	E6*II [log dpm (SD)]	GAPDH [log dpm (SD)]
20	4.86 (0.60)	3.65 (0.78)	4.98 (0.83)
22	5.01 (0.71)	4.26 (0.39)	5.19 (0.70)
24	5.41 (0.24)	4.77 (0.19)	5.47 (0.35)
26	5.45 (0.43)	4.93 (0.26)	5.55 (0.43)
28	5.52 (0.09)	5.27 (0.18)	5.56 (0.16)
30	5.63 (0.09)	5.30 (0.07)	5.87 (0.01)

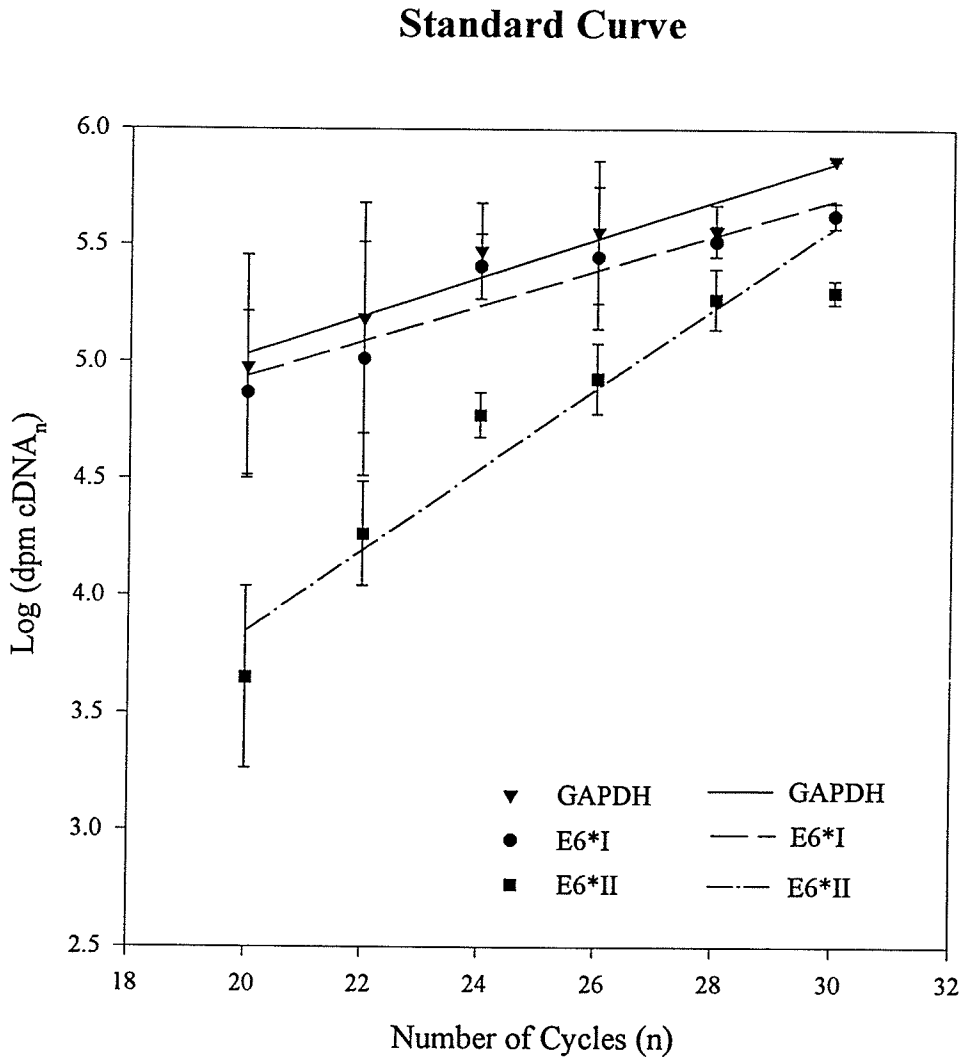


Figure 16. Log-linear relationship between cycles of amplification and PCR products The regression lines for amplification of E6*I, E6*II, and GAPDH cDNAs were generated from the mean values obtained from three experiments.

2. Determination of The Relative Quantities of HPV-16 E6*I AND E6*II Transcripts From Clinical Specimens

The amplification products from clinical specimens were not as easily visualized by autoradiography as the CaSki mRNA control, indicating fewer cells infected with HPV-16, less abundant viral mRNA per cell, or lower expression of HPV-16 genes *in vivo*. However, scintillation counting revealed a significantly higher counts for samples with reverse transcriptase and for those without reverse transcriptase. The $cDNA_n$ was determined by calculating the difference in dpms between the sample and its corresponding control lacking reverse transcriptase. In this way, the background counts were eliminated. The $cDNA_0$ was calculated for each specimen by using the following formula:

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

where n is equal to 30, $cDNA_n$ values were obtained from scintillation counting, and R values were determined using the standard curve described previously. Thus the quantity of cDNA before PCR ($cDNA_0$) of each mRNA species could be determined. The values obtained for each mRNA species were adjusted relative to an equivalent content of GAPDH $cDNA_0$ by using the following formula:

$$cDNA_{std} = \frac{C}{GAPDH} \times cDNA_0$$

The C value was chosen from GAPDH $cDNA_0$ value of a biopsy tissue which was 6.14 dpm. The standardized expression of E6*I and E6*II from exfoliated cervical cells and biopsy specimens collected from 22 women is shown in Table 4.

Table 4. Expression of E6*I and E6*II in biopsy tissue and exfoliated cervical cells

Patient No.	CIN Grade	Biopsy Tissue		Cervical Cells	
		E6*I	E6*II	E6*I	E6*II
		(log dpm × 10^4)		(log dpm × 10^4)	
1	0	0	2.10	0	0
2	0	4.98	1.62	ND	ND
3	0	5.02	1.75	0.55	1.53
4	0	5.09	1.47	5.10	1.80
5	0	5.32	2.46	5.66	1.80
6	I	5.04	2.26	5.20	2.10
7	I	5.12	0.79	4.83	0
8	I	5.13	0	5.34	1.62
9	I	5.20	2.10	5.31	2.12
10	I	5.26	2.24	4.44	0
11	I	5.29	2.00	5.17	1.90
12	I	5.59	2.48	5.38	1.97
13	II	4.22	0	5.55	0
14	II	4.90	1.49	5.24	2.51
15	II	5.26	1.68	ND	ND
16	II	7.05	0	7.31	2.25
17	III	4.42	1.81	4.91	1.71
18	III	4.49	0	6.27	2.28
19	III	4.80	0.62	4.83	1.63
20	III	5.62	0.94	6.98	2.14
21	III	5.89	0	ND	ND
22	III	6.26	1.07	6.70	1.33

ND, specimens not included in statistical analysis due to lack of GAPDH cDNA amplification.

3. Histopathologic Results

The histopathologic evaluation of biopsy specimens collected from the 22 HPV-16 positive women, received from the Department of Pathology, University of Manitoba, revealed 7 women with CIN I, 4 women with CIN II, and 6 women with CIN III. The remaining 5 women were histopathologically normal, which were classified as CIN 0. The histopathologic assessment was compared to the expression of E6*I and E6*II (Table 4 and Figure 17).

4. Comparison of E6*I and E6*II Expression in Paired Specimens

In low grade of dysplasia (CIN 0 and CIN I), the levels of E6*I and E6*II expression in most of the paired biopsy and cell specimens were comparable. However, in paired specimens taken from women with high grade of dysplasia (CIN II or III), the expression of E6*I was similar in biopsy tissue and exfoliated cells, while E6*II expression was notably less in the biopsy tissue compared to exfoliated cells collected from the same woman (Figure 17).

5. Expression of E6*I and E6*II in Biopsy Specimens

Except for one specimen collected from normal cervix (CIN 0), the expression of E6*I was detected in all biopsy specimens. In general, the expression of E6*II was lower than of E6*I. There was a tendency that E6*I expression to increase while E6*II expression decreased in higher grades of dysplasia (Figure 18 A). In higher grades of dysplasias (CIN II and III), loss of E6*II expression became more frequent. The decreased or lack of expression of E6*II relative to E6*I was significantly associated (P=0.014) with high grade CIN.

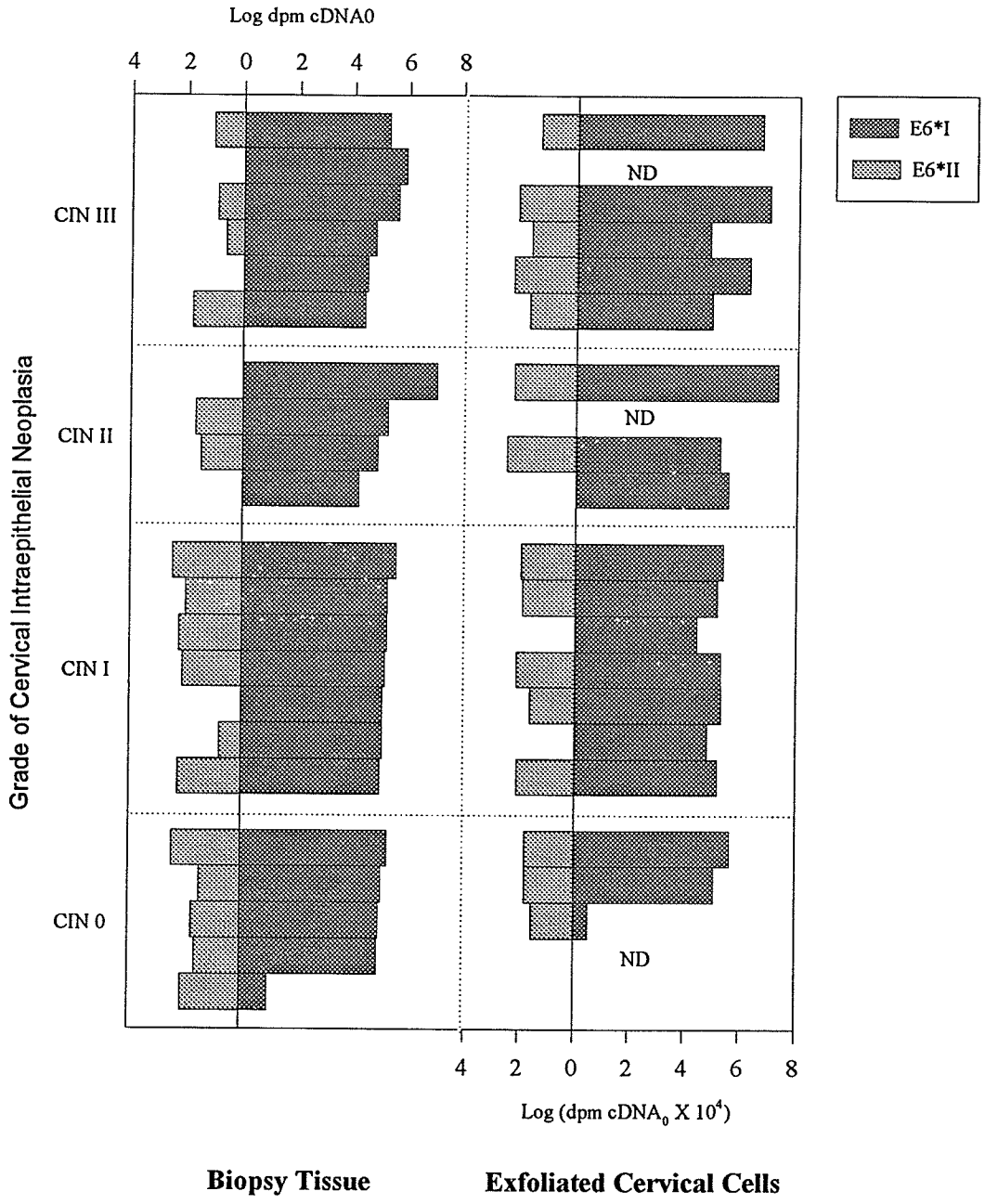


Figure 17. Expression of HPV-16 E6/E7 ORFs in biopsy (left) and exfoliated cervical cells (right). ND, GAPDH expression is not detected due to insufficient quality of mRNA.

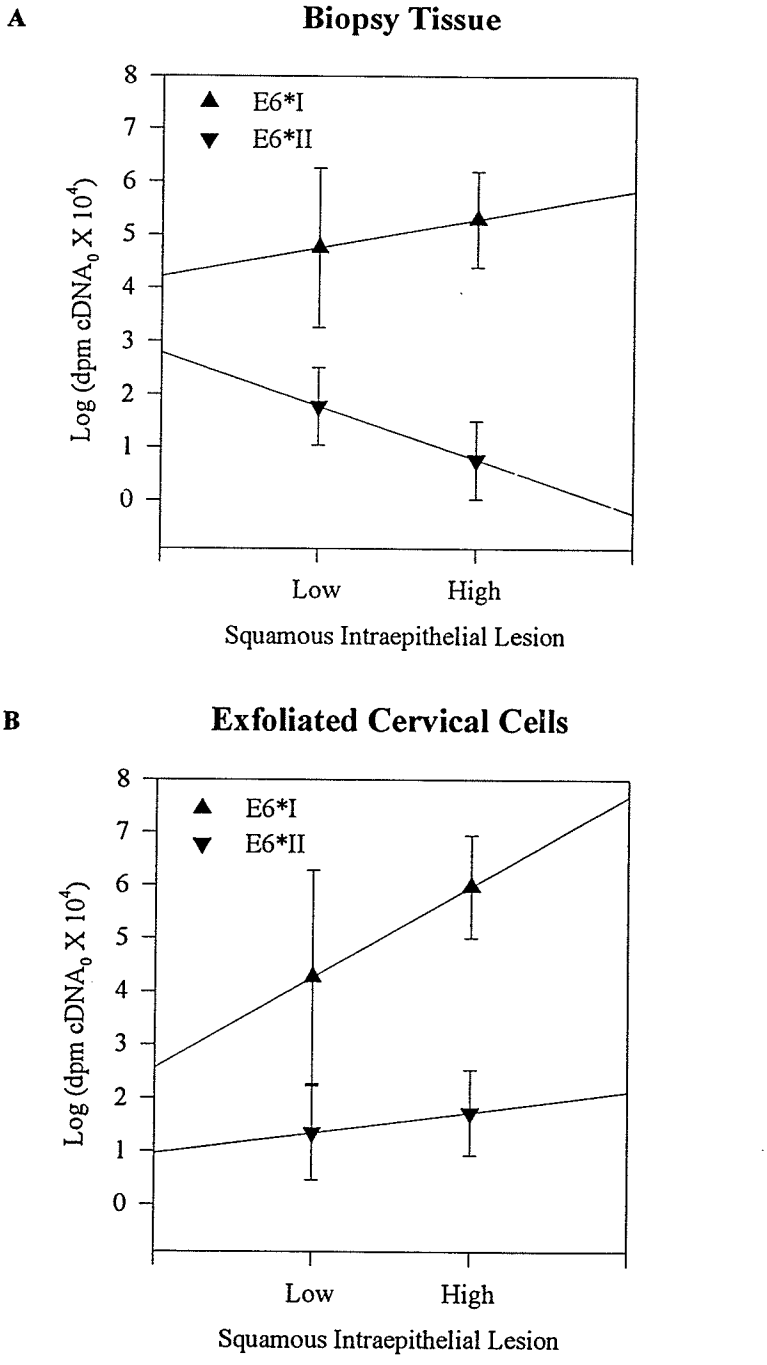


Figure 18. Correlation between HPV-16 E6/E7 ORF transcripts and degrees of lesions.

6. Expression of E6*I and E6*II in Exfoliated Cervical Cell Specimens

Three specimens had no detectable GAPDH RT-PCR product indicating poor quality mRNA had been isolated. Hence, those were eliminated from statistical analysis. Although all of the remaining 19 specimens were proven to harbor HPV-16 DNA, one specimen collected from a normal cervix (CIN 0) had no detectable E6*I and E6*II expressions. This suggested that the virus might be in a latent stage or the E6/E7 ORFs were expressed in a very low level, below the limit of the assay. E6*I expression was observed to increase in higher grade of lesion (Figure 18 B). In contrast to biopsy tissues, E6*II expressions in exfoliated cervical cells were slightly increased in higher degree of dysplasia (Figure 18 B). Hence, the increase in E6*I expression relative to E6*II for CIN II and III specimens was not statistically significant ($p=0.137$).

Cloning of The HPV-16 E6/E7 ORFs

The PCR amplification of HPV-16 plasmid with the HPV-16 E6/E7 cloning primers yielded approximately 20 μg of PCR product (insert) as determined by spectrophotometry. From the 5 μg of insert digested with *Bam*HI and *Xho*I, purified by Chromaspin-100 column (Clontech) chromatography, and precipitated with sodium acetate (Appendix) and ethanol, about 2 μg of purified insert was recovered. Figure 19 shows a picture of an agarose gel electrophoresis of the PCR product and purified digested insert, with the expected size of 791 bp. Figure 20 shows a picture of an agarose gel electrophoresis of linearized pcDNA3 vector, with the size of 5.4 kbp. The purified insert and linearized vector were ligated and directly transformed into *E. coli* strain TOP10F' with ampicillin selection.

There was no colony formation for all plates inoculated with untransformed bacteria (negative control), indicating the host bacterium was susceptible to ampicillin, and no contamination with other ampicillin-resistant bacteria had occurred. There was also no colony formation on plates inoculated with bacteria transformed with insert only, which excluded the possibility of contamination with other plasmids carrying an ampicillin resistance gene. About 5-30 colonies per plate were growing on plates inoculated with bacteria transformed with a characterized plasmid (positive control), on plates inoculated with bacteria transformed with digested and re-ligated vector, and plates inoculated with insert ligated to dephosphorylated vector. These results showed a successful bacterial transformation. No colonies were observed on plates inoculated with bacteria transformed with digested, dephosphorylated, and religated vector, suggesting a successful dephosphorylation reaction. Colonies confirmed for β-lactamase production were selected and a portion of the insert was sequenced. The sequence of the DNA was compared to HPV-16 genome database, and the correctness of the cloned HPV-16 E6/E7 ORF was confirmed. The plasmid was named pHPV800.

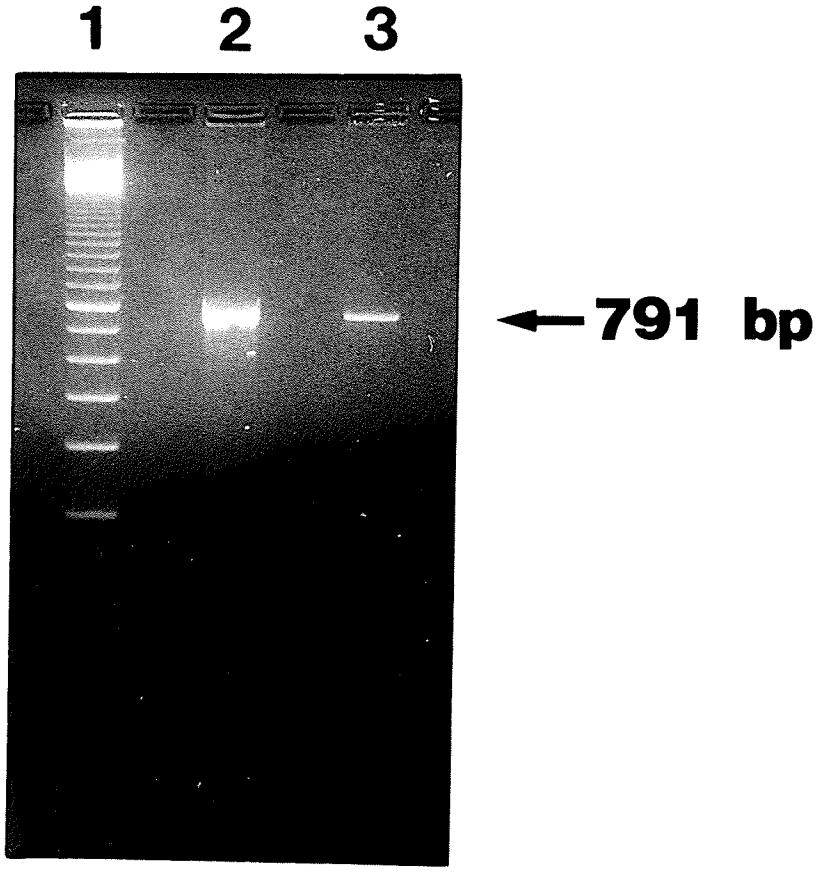


Figure 19. Purified digested insert with size of 791 bp

Lane 1, 100 bp molecular weight marker ladder; lane 2, purified undigested insert; lane 3, purified insert digested with *Bam*HI and *Xho*I.

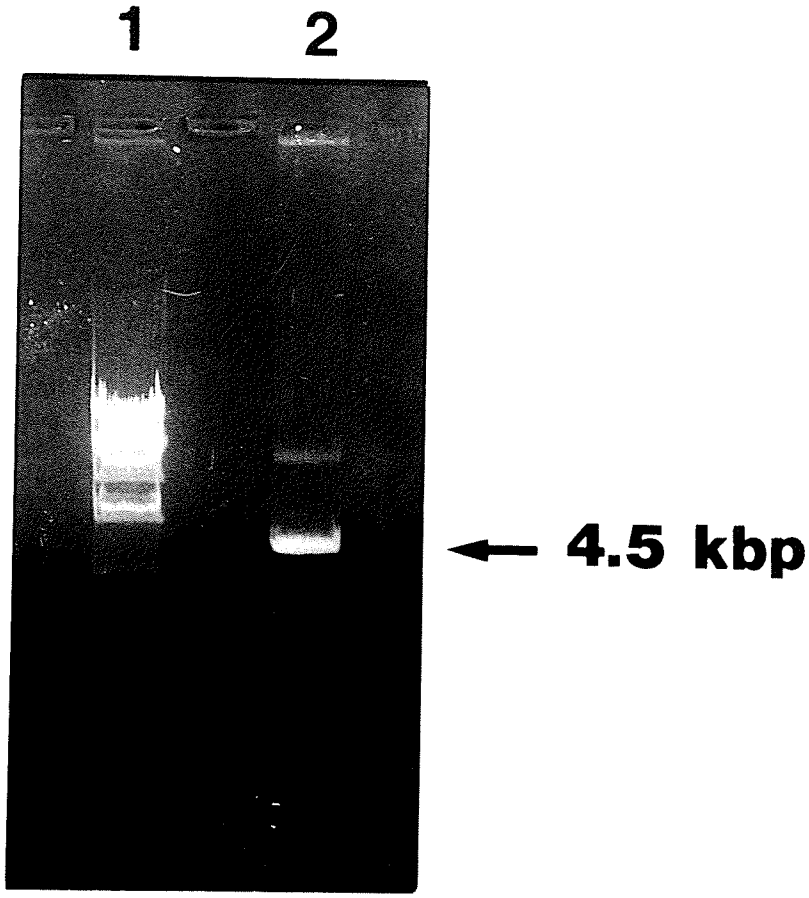


Figure 20. Linearized pCDNA 3 vector

Lane 1, λ bacteriophage DNA digested with *Hind*III as the molecular weight marker; lane 2, pcDNA 3 vector digested with *Bam*HI and *Xho*I.

Transfection of C-33A, G-401, HeLa, and HuFF cell lines

From 500 ml overnight culture grown in trypticase-soy broth containing 20 mg ampicillin/l media, between 0.5-1 mg of purified plasmid DNA as determined by spectrophotometry. Each cell line was co-transfected with 5 μg pHPV800 and 2.5 μg pCMVβ-Gal. The β-galactosidase activity was calculated in unit/mg of protein of lysate by using formula:

$$\beta - Gal = \frac{OD420}{0.0045} \times Volume \times \frac{1}{time} \times \frac{1}{lysate} \times \frac{1}{protein}$$

Where *Volume* is equal to 0.85 ml, *time* is equal to 300 minutes, and *lysate* is equal to 50 μl. The value of *protein* was obtained from the protein assay, by plotting the OD 750 nm readings and the standard curve generated using bovine serum albumin (Figure 21).

The baseline β-galactosidase activity obtained from untransfected cells was subtracted from the β-galactosidase activity of transfected cells, to get the corrected β-galactosidase activity of the transfected DNA. The β-galactosidase activity is indicative of the efficiency of transfection (Table 5). C-33 A cell line shows highest β-galactosidase activity from transfected plasmid, while HeLa cell line shows the lowest β-galactosidase activity.

Determination of E6*I and E6*II Expression Level in Transfected Cell Lines

Quantitative RT-PCR was performed to measure expression of E6*I and E6*II in transfected cell lines. The $cDNA_0$ of the transcripts was calculated using formula:

$$cDNA_0 = \frac{cDNA_n}{(1+R)^n}$$

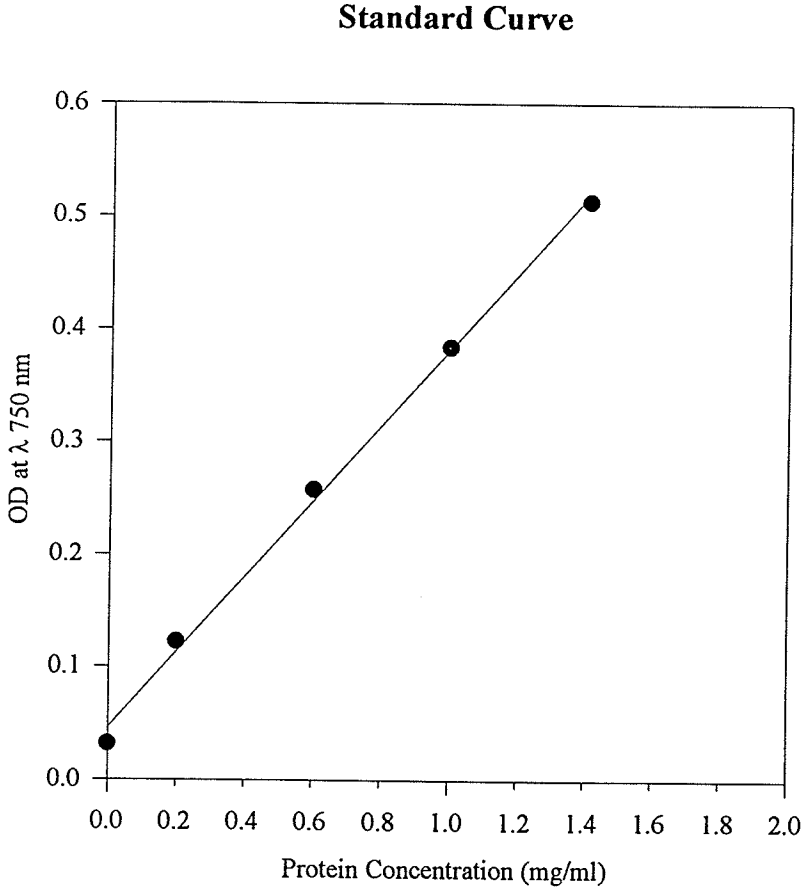


Figure 21. Linear correlation between protein concentration and OD 750 nm readings Standard curve of OD at λ 750 nm as a function of protein concentration of bovine serum albumin was prepared in every protein assay.

Table 5. β-Galactosidase activity of transfected Cell lines

Cell line	β-Galactosidase activity [Units/mg protein of lysate (SD)]
C-33A	3.1258 (0.7537)
G-401	0.4493 (0.3138)
Hela	0.3255 (0.2904)
HuFF	1.8402 (0.4627)

where n is equal to 30, R is equal to 0.244 for E6*I and 0.621 for E6*II, and $cDNA_n$ was determined by scintillation counting.

The values were then adjusted relative to β-Galactosidase activity, to compensate for differences in transfection efficiencies by using the formula:

$$cDNA_{adj} = cDNA_0 \times \frac{S}{\beta - Gal}$$

The standard value S, was chosen from β-Gal activity of transfected HuFF cells, which was the closest to the average of β-Gal activity of all transfected cells. The value is 1.3762 units/mg of protein of the lysate (Table 6).

2. Determination of RT-PCR Efficiency of SF2, hnRNP A1, and GAPDH Primers

Amplification of CaSki mRNA using GAPDH, SF2, and hnRNP A1 primers resulted in corresponding products of 305 bp, 279 bp, and 223 bp, respectively (Figure 22). By using the same method as for determining RT-PCR efficiency of primers for E6*I, E6*II, and GAPDH amplification, a standard curve of SF2, hnRNP A1 and GAPDH was generated (Figure 23 and Table 7). By applying the formula:

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

where the slopes of the curves were equal to log (1+R), the calculated R values for SF2, hnRNP A1, and GAPDH were determined to be 0.336, 0.374, and 0.307, respectively.

Table 6. The determination of E6*I and E6*II expression levels in transfected C-33 A, G-401, HeLa, and HuFF cell lines

Plasmid	pHPV800		pcDNA3	
Cell line	E6*I	E6*II	E6*I	E6*II
	[log (dpm $cDNA_0 \times 10^4$)(SD)]		[log dpm $cDNA_0 \times 10^4$]	
C-33A	5.4658 (0.0504)	1.8890 (0.0812)	0	0
G-401	5.0690 (0.3262)	1.0607 (0.4278)	0	0
HeLa	6.2485 (0.1357)	1.4180 (0.2602)	0	0
HuFF	4.6502 (0.1424)	0.3510 (0.1540)	0	0

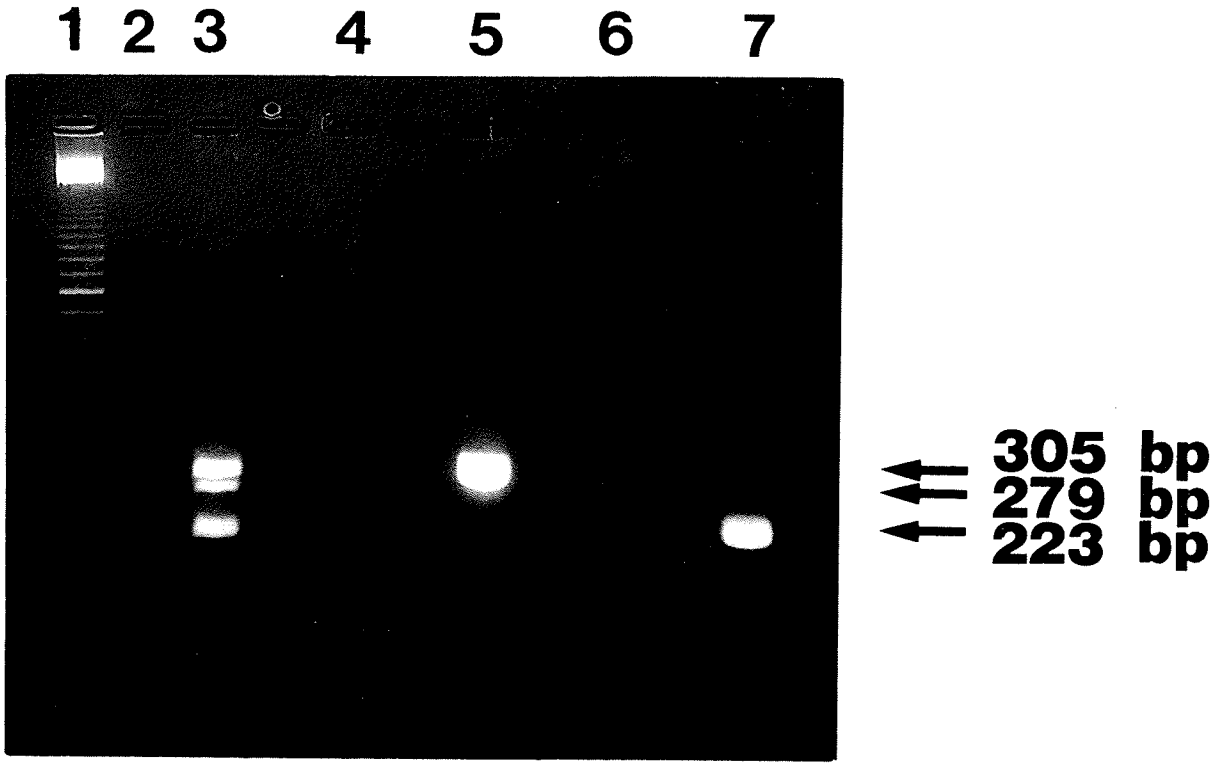


Figure 22 . Photograph of SF2, GAPDH, hnRNP A1 PCR RT-PCR Products on Gel Electrophoresis

Lane 1, 100 bp molecular weight marker ladder; lane 2, a reaction containing all reagents except mRNA; lane 3, a reaction containing GAPDH, SF2, and hnRNP A1 primers; lane 4, a reaction containing all reagents except reverse-transcriptase; lane 5, RT-PCR product of GAPDH mRNA; lane 6, RT PCR product of SF2 mRNA; lane 7, RT-PCR product of hnRNP A1 mRNA.

Table 7. The determination of RT-PCR efficiency of SF2, hnRNP A1, and GAPDH primers.

Cycle no.	SF2 [log dpm (SD)]	hnRNP [log dpm (SD)]	GAPDH [log dpm (SD)]
20	3.66 (0.21)	4.44 (0.50)	4.44 (0.31)
22	4.44 (0.07)	5.35 (0.19)	5.04 (0.19)
24	4.65 (0.28)	5.70 (0.07)	5.42 (0.09)
26	5.05 (0.05)	5.90 (0.06)	5.54 (0.09)
28	4.81 (0.53)	5.88 (0.17)	5.65 (0.05)
30	5.12 (0.08)	6.02 (0.08)	5.68 (0.15)

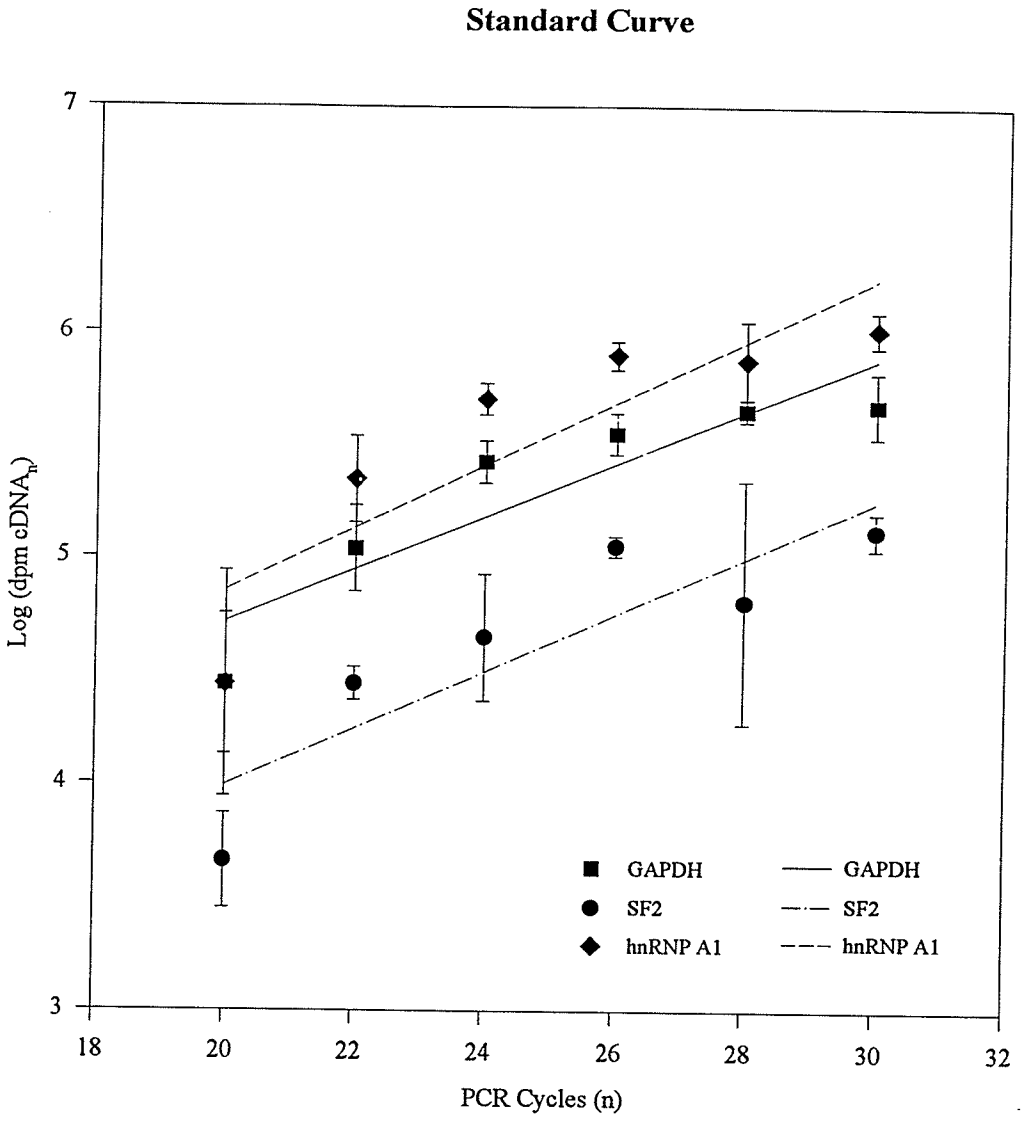


Figure 23. Log-linear relationship between number of cycles of amplification and the formation of the PCR-amplified product

The regression lines for amplification of GAPDH, SF2, and hnRNP A1 cDNAs were generated from the mean values obtained from three experiments.

3. **Determination of The Relative Quantities of SF2 and hnRNP A1 Transcripts**

By knowing the R values for SF2, hnRNP A1, and GAPDH, the value of $cDNA_0$ for SF2, hnRNP A1, and GAPDH of each sample could be determined by using formula:

$$cDNA_0 = \frac{cDNA_n}{(1+R)^n}$$

where n is equal to 30, R is equal to 0.336, 0.374, and 0.307 for SF2, GAPDH and hnRNP A1 respectively, and $cDNA_n$ was determined by scintillation counting. The values obtained for each mRNA species were adjusted relative to an equivalent content of GAPDH $cDNA_0$ by using formula:

$$cDNA_{adj} = \frac{C}{GAPDH} \times cDNA_0$$

The C value was chosen from the GAPDH $cDNA_0$ value of G-401 cells, which was the closest to the average of GAPDH $cDNA_0$ of all cell lines. The value is 109.6101 dpm. The values of standardized expression of SF2 and hnRNP A1 are shown in Table 8. The expression levels of SF2, hnRNP A1, E6*I, and E6*II of the transfected cell lines are summarized in Figure 24.

Table 8. The determination of SF2 and hnRNP expression levels in C-33 A, G-401, HeLa, and HuFF cell lines

Cell line	SF2 [log dpm × 10^4(SD)]	hnRNP A1 [log dpm × 10^4(SD)]
C-33A	5.6948 (0.0141)	5.8104 (0.0168)
G-401	5.9338 (0.0287)	5.9619 (0.0287)
HeLa	5.3923 (0.0411)	5.9142 (0.0737)
HuFF	4.9249 (0.0124)	5.5270 (0.0669)

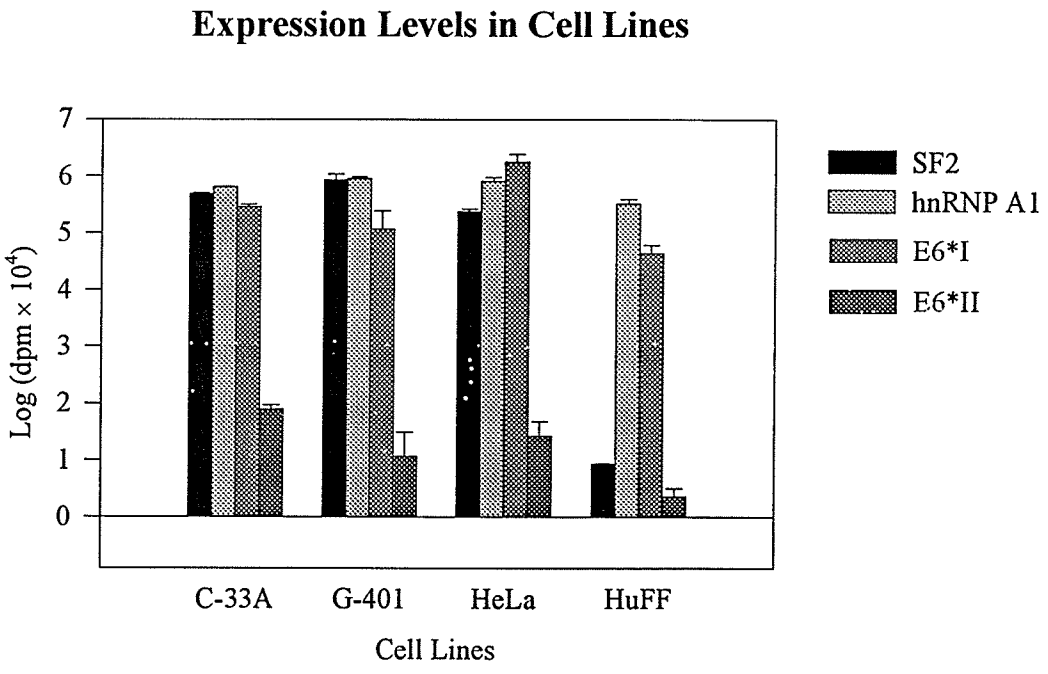


Figure 24. Histogram of SF2, hnRNP A1, E6*I, and E6*II expression in tranfected C-33 A, G-401, HeLa and HuFF cell lines

4. Comparison of The Level of E6*I and E6*II Expressions in Transfected Cell Lines

In order to verify differences among transfected cell lines, the level of E6*I and E6*II expression was analyzed by using ANOVA. It was found that there is a significant difference in the level of E6*I expression among transfected cell lines ($p=0.0001$). The order of the level of E6*I expression from high to low is HeLa, C-33 A, G-401, and HuFF cells. The E6*I expression is significantly higher in HeLa cells than in other cell lines ($p<0.05$). There is no significant difference in the level of E6*I expression between C-33 A and G-401 cells. The lowest level of E6*I expression was observed in HuFF cells. Although the level of E6*I expression in G-401 cells was higher than in HuFF cells, there was no statistically significant difference.

The level of E6*II expression is also different among transfected cell lines ($p=0.0011$). The order of the level of E6*II expression from high to low is C-33 A, HeLa, G-401 and HuFF. However, by using Tukey's Studentized Range Test, it was found that there is no significant difference in the level of E6*II expression in C-33 A and HeLa cells, in HeLa and G-401 cells, and in G-401 and HuFF cells ($p>0.05$).

E6*II/E6*I ratio is significantly different among cell lines ($p=0.014$). The order of the E6*II/E6*I ratio is C-33A, HeLa, G-401, and HuFF cells. There is no significant difference in the E6*II/E6*I ratio between C-33 A and HeLa cells, and between HeLa and G-401 cells. Unexpectedly, HuFF cells showed a significantly lower E6*II/E6*I ratio than other cell lines.

5. Comparison of SF2 and hnRNP A1 Expressions in Cell Lines

Analysis of the level of SF2 expression among different cell lines showed a significant difference ($p=0.0001$). The cell lines, ranked order of SF2 expression from high to low, are G-401, C-33 A, HeLa, and HuFF. Tukey's Studentized Range Test determined that the level of SF2 expression varies significantly between different cell lines ($p<0.05$).

The hnRNP A1 expression is also significantly different among transfected cell lines ($p=0.0001$). While there is no significant difference among continuous cell lines (C-33 A, G-401, and HeLa), the level of hnRNP A1 expression in HuFF cells is significantly lower ($p<0.05$).

6. Correlation Between SF2 Expression and E6*I or E6*II Expression in Transfected Cell Lines

Figure 25 A shows that there is a slight increase in the level of E6*I expression with the increase in the level of SF2 expression. However, this correlation is weak (correlation coefficients (r) = 0.184, 0.222, and 0.229; p values = 0.568, 0.488, and 0.472, respectively). Stronger correlation was observed between SF2 and E6*II expression (Figure 25 B) ($r = 0.541$, 0.579, and 0.618; $p = 0.069$, 0.048 and 0.032, respectively). These results suggest that the increase of SF2 expression correlates with the increase of E6*II expression rather than of E6*I expression. This correlation is also observed between SF2 expression and ratio of E6*II/E6*I (Figure 25 C) ($r = 0.595$, 0.642, and 0.677; $p = 0.041$, 0.024, and 0.016, respectively).

7. Correlation Between hnRNP A1 Expression and E6*I or E6*II Expression in Transfected Cell Lines

Figure 26 A and Figure 26 B show the correlation between E6*I and hnRNP A1 expression and between E6*II and hnRNP A1 expression, respectively. Although E6*I expression is slightly increased with the increase of hnRNP A1 expression, it is not statistically significant ($r = 0.537$, 0.574, and 0.585; $p = 0.072$, 0.051, and 0.046, respectively). However, this is also the case with E6*II expression ($r = 0.526$, 0.542, and 0.617; $p = 0.079$, 0.068, and 0.033, respectively). Correlation analysis between the ratio of E6*II/E6*I and hnRNP A1 expression revealed no statistically significant association (Figure 26 C) ($r = 0.509$, 0.514, and 0.591; $p = 0.091$, 0.088, and 0.043, respectively).

These results demonstrate that the selection of splice acceptor sites is independent of hnRNP A1 expression alone.

8. Correlation Between E6*I or E6*II Expression and SF2/hnRNP A1 Ratio in Transfected Cells

Since SF2 and hnRNP A1 are thought to act antagonistically in the selection of 3'-end splicing sites, the E6*I and E6*II expression levels were compared to the ratio of SF2/hnRNP A1. The level of expression of E6*I was not affected by the ratio of SF2/hnRNP A1 (Figure 27 A). The r values are -0.072 -0.014; and 0.004, and p values are 0.825, 0.966, and 0.989, respectively. The E6*II expression level was also not correlated with ratio of SF2/hnRNP A1 (Figure 27 B), with r = 0.475, 0.521, and 0.527; and p values = 0.119, 0.082, and 0.078, respectively. However, significant correlation was found between ratio of E6*II/E6*I and ratio of SF2/hnRNP A1 (Figure 27 C), (r values = 0.560, 0.628, and 0.631; p = 0.058, 0.029, and 0.028, respectively).

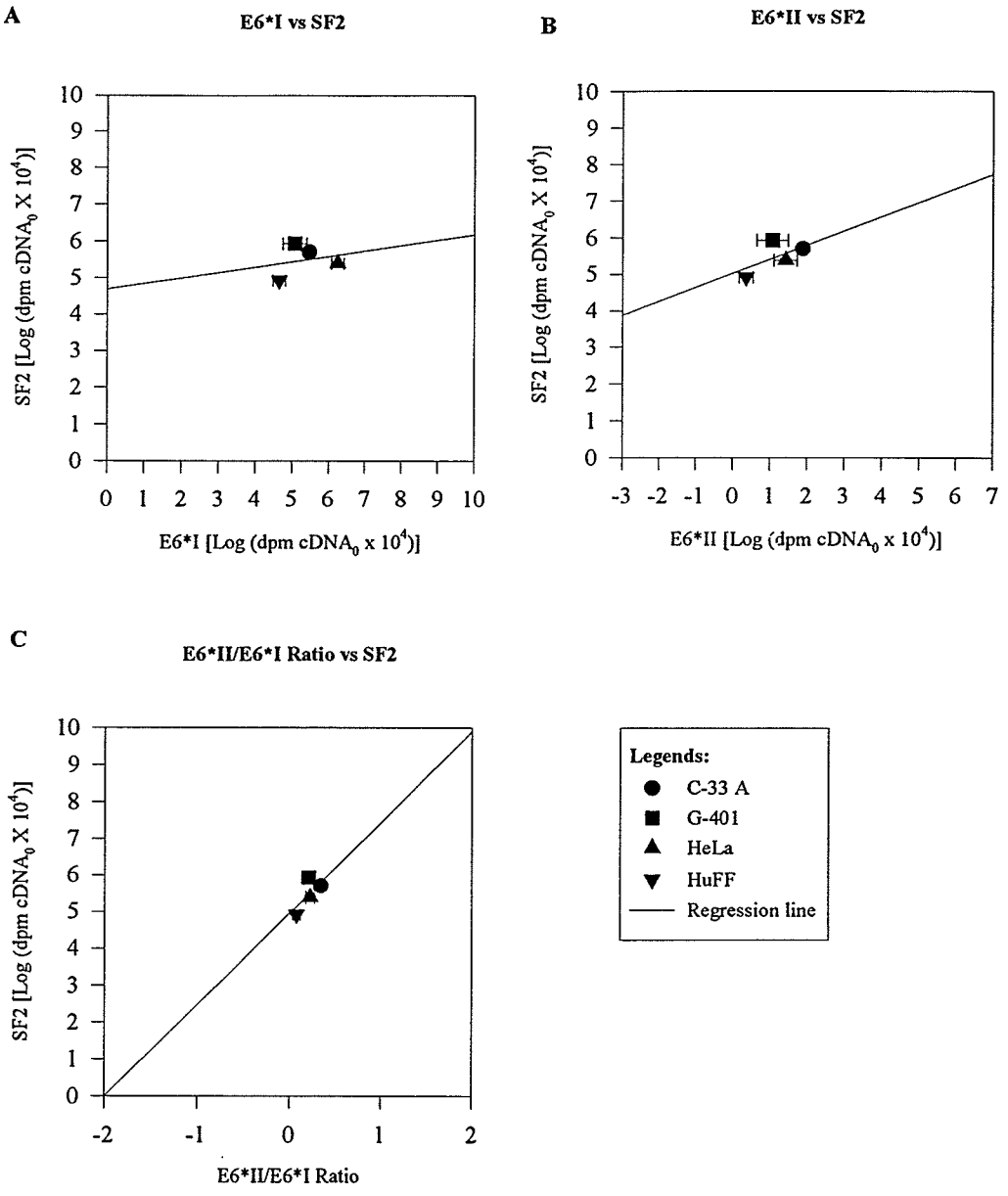


Figure 25. Correlation between The Levels of HPV-16 E6/E7 Transcripts and SF2 Expression

A. Correlation between E6*I and SF2 expression.

B. Correlation between E6*II and SF2 expression.

C. Correlation between E6*II/E6*I ratio and SF2 expression.

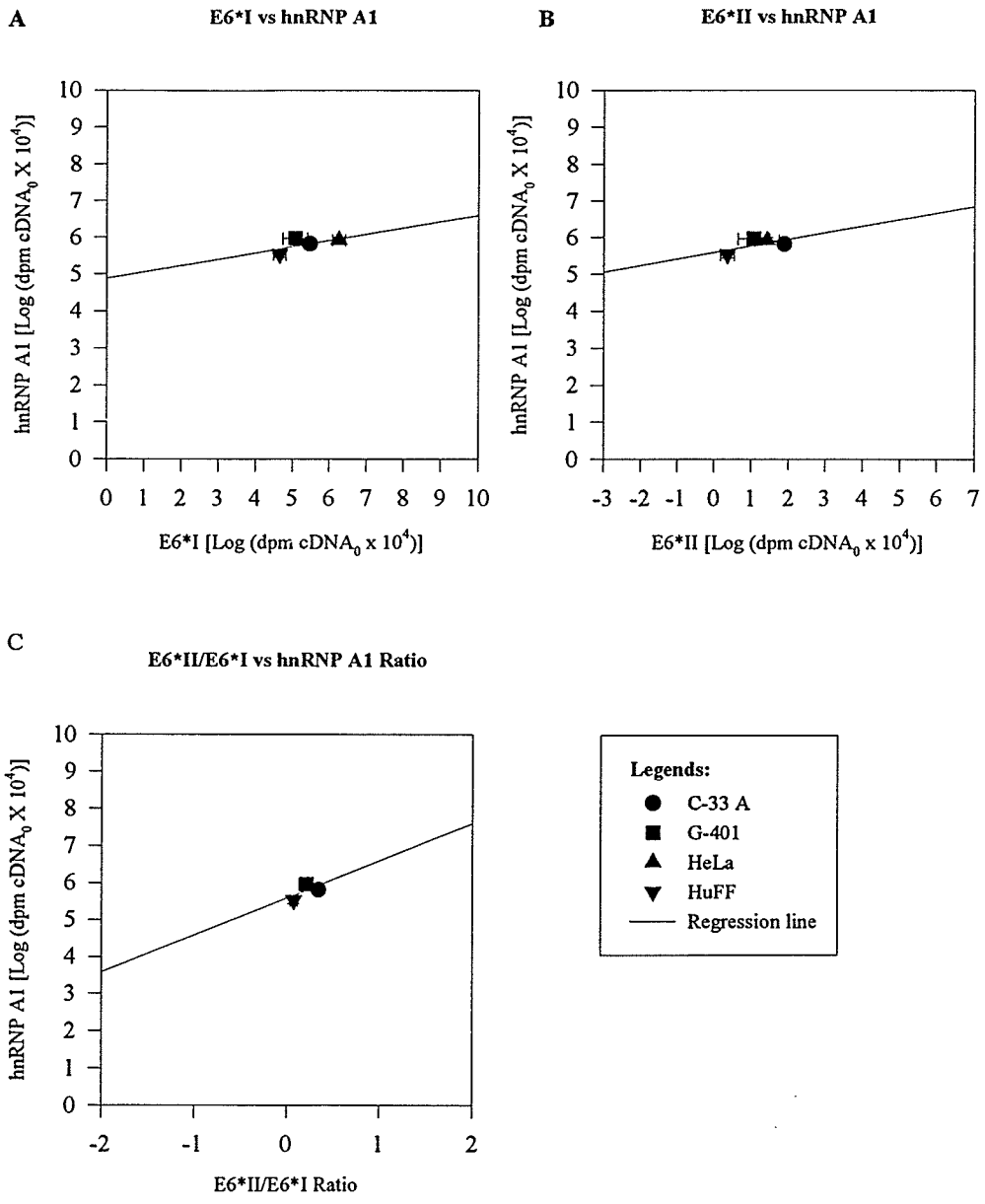


Figure 26. Correlation between The Levels of HPV-16 E6/E7 Transcripts and hnRNP A1 Expression

A. Correlation between E6*I and hnRNP A1 expression.

B. Correlation between E6*II and hnRNP A1 expression.

C. Correlation between Ratio E6*II/E6*I and hnRNP A1 expression.

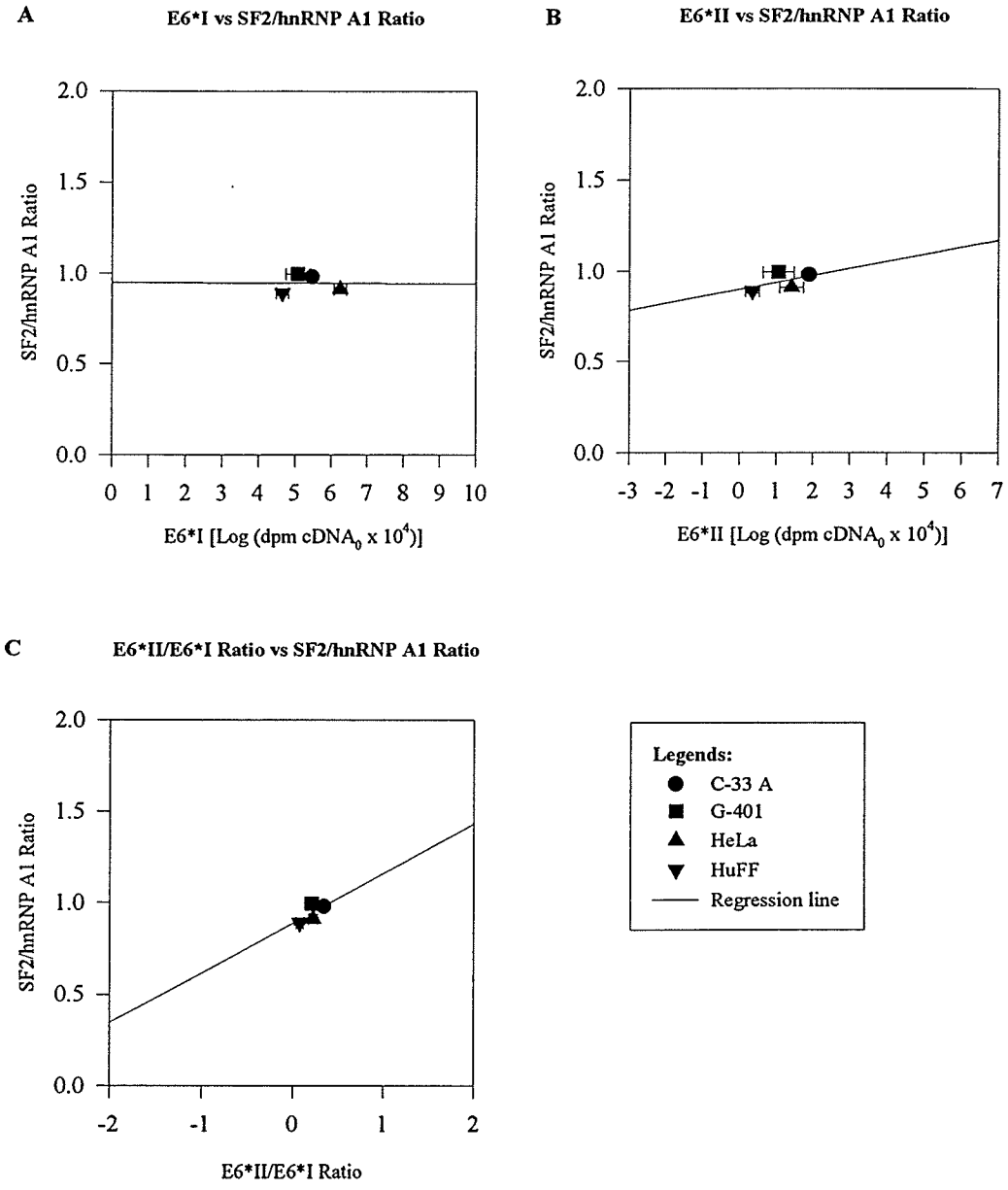


Figure 27. Correlation between The Levels of HPV-16 E6/E7 Transcripts and SF2/hnRNP A1 Ratio

A. Correlation between E6*I and SF2/hnRNP A1 Ratio.

B. Correlation between E6*II and SF2/hnRNP A1 Ratio.

C. Correlation between E6*II/E6*I Ratio and SF2/hnRNP A1 Ratio .

Site-Directed Mutagenesis by Using Inverse PCR

Due to the long DNA template (6.2 kbp), the inverse PCR reaction was not as efficient as the regular PCR. The yield of 1 PCR reaction tube was about 10 μg DNA, which was more than sufficient for bacterial transformation. Figure 28 shows a successful amplification of the entire plasmid. However, some bands with sizes smaller than 6.2 kbp were also observed. They might be non-specific PCR products or PCR products with incomplete elongation. However, they were unlikely to be viable plasmids when recircularized.

1. Bacterial Transformation of Mutated Plasmids

Besides the bacteria transformed with the recircularized PCR products, some controls were also performed, *i.e.*, bacteria transformed with a characterized plasmid as the positive control, bacteria transformed with unprocessed PCR product as the background control, and untransformed bacteria as the negative control. No colonies were observed in the negative control plates, indicating the bacteria were susceptible to ampicillin, and no contamination with other ampicillin-resistant bacteria had occurred. Some colonies grew on the positive control plates, indicating the bacteria were competent for transformation. Between 0-3 colonies were observed in the background control plates, suggesting the template plasmid which carried the wild-type sequence of HPV-16 E6/E7 ORF might be still viable. About 150 colonies were growing in plates inoculated with bacteria transformed with recircularized PCR products, and all colonies were transferred onto hybridization membranes. A colony of bacteria with wild-type HPV-16 E6/E7 was also inoculated onto each hybridization membrane, functioning as the point of orientation, and point of reference for the hybridization.

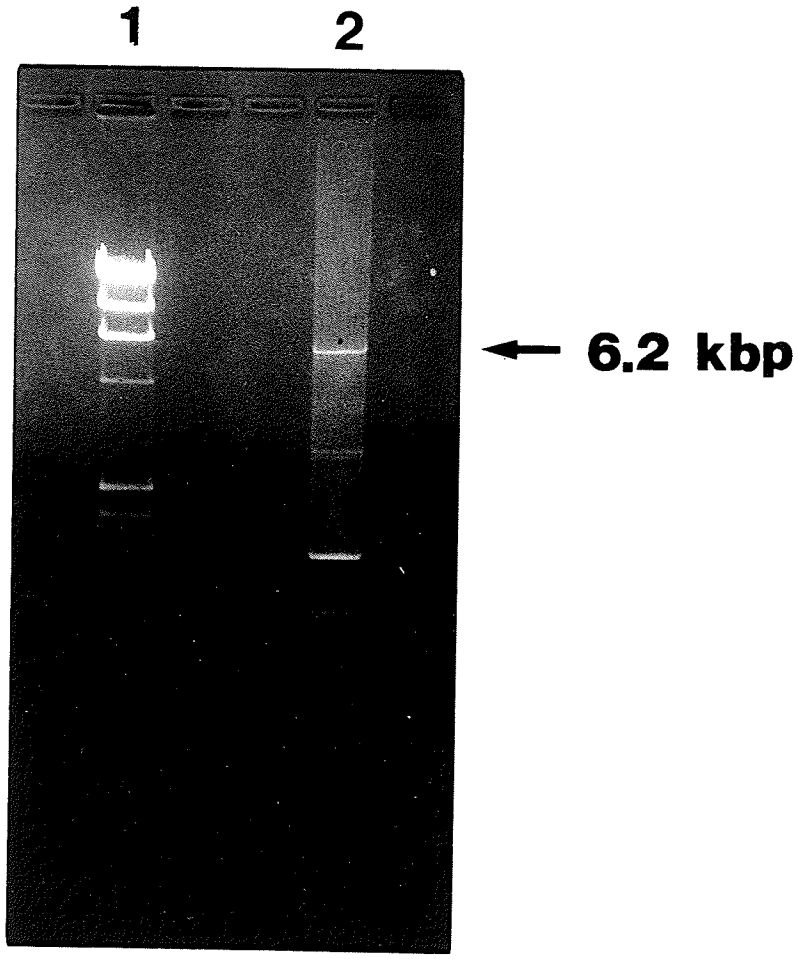


Figure 28. Photograph of Amplification Products of Inverse-PCR with Size of 6.2 kbp Lane 1, λ bacteriophage DNA digested with *Hind*III as the molecular weight marker; lane 2, amplification product of the inverse-PCR with size of 6.2 kbp.

2. Differential Screening to Identify Mutated Clones

All colonies grew well on the hybridization membranes and on the master plates. After the hybridization with radiolabeled probe (Table 2), most of the colonies showed stronger binding to the probe than the known wild type colony, and a few colonies did not hybridize with the probe. Four colonies from each hybridization membrane which showed the strongest binding were selected (Figure 29).

3. DNA Sequence Analysis of The Mutated Plasmids

Figure 30 and Figure 31 show the autoradiographs of DNA sequence of the mutated plasmids. All have the desired base changes at the correct location. No additional base at the point of ligation and no other mutations was observed. The plasmid carrying nt. 409 and nt 526 mutations were subsequently called p409Mu and p526Mu, respectively.

4. Transfection of C-33A with Mutated Plasmids

C-33A cells transiently transfected p409Mu plasmid revealed no expression of E6*I, and C-33A cells transiently transfected p526Mu plasmid revealed no expression of E6*II, suggesting that the mutation knocked out the splicing acceptor sites (Table 9).

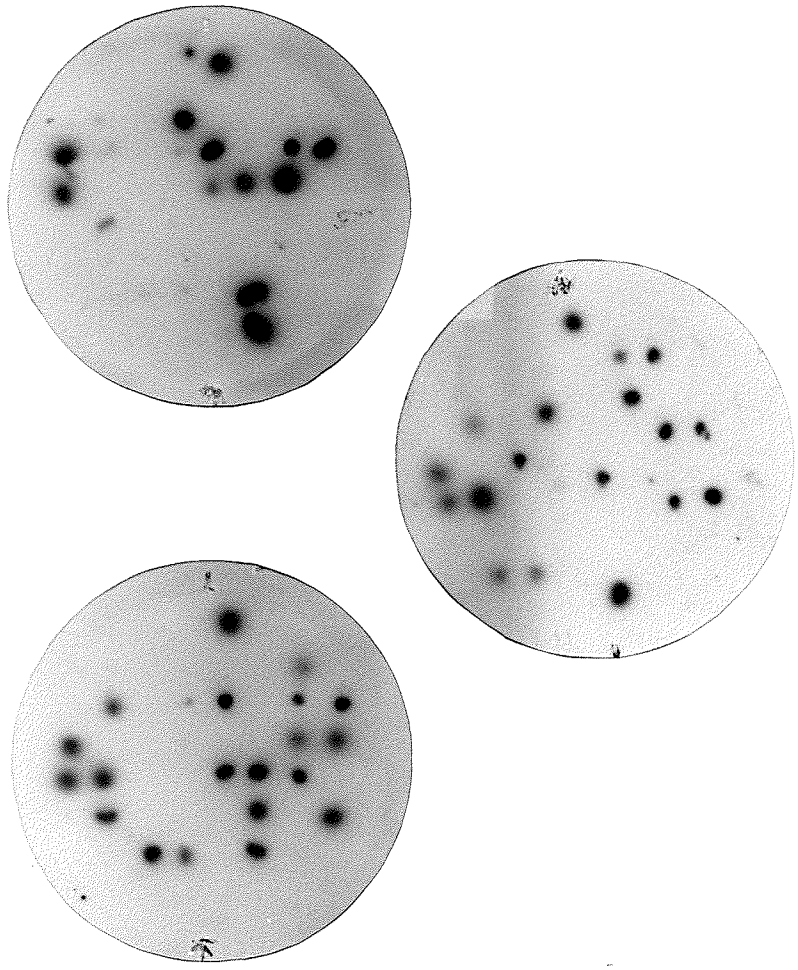


Figure 29. Autoradiograph of Colony Lift Hybridization

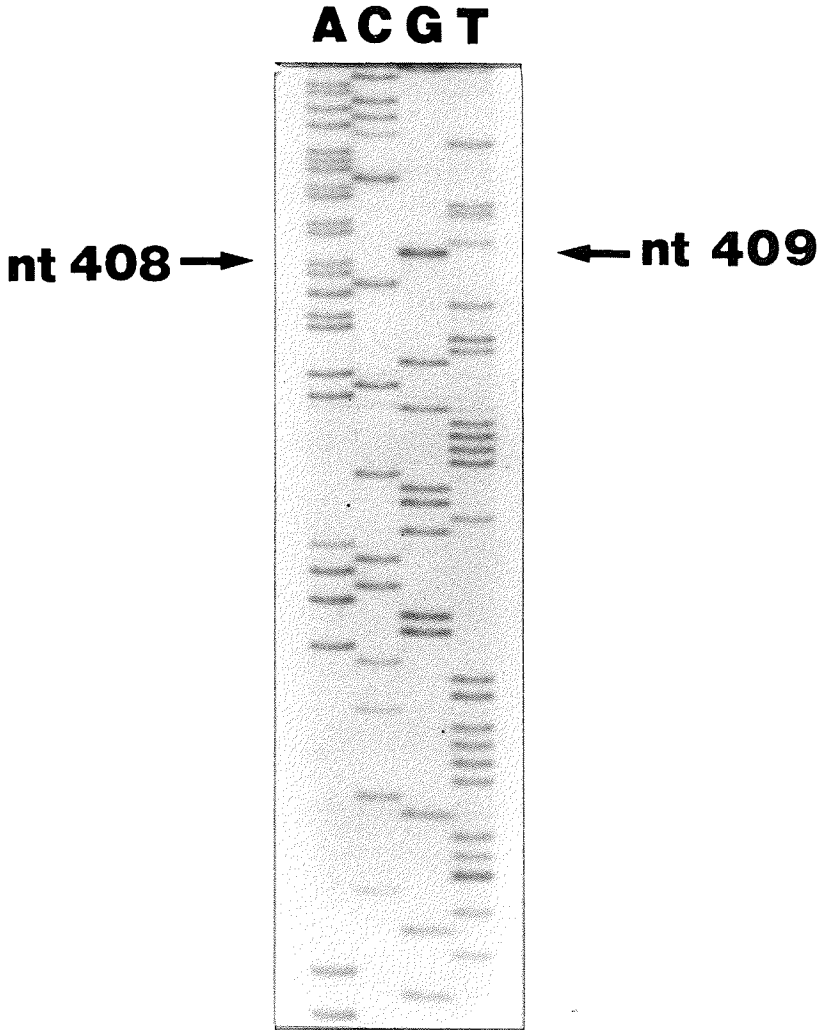


Figure 30. Autoradiograph p409Mu DNA Sequence

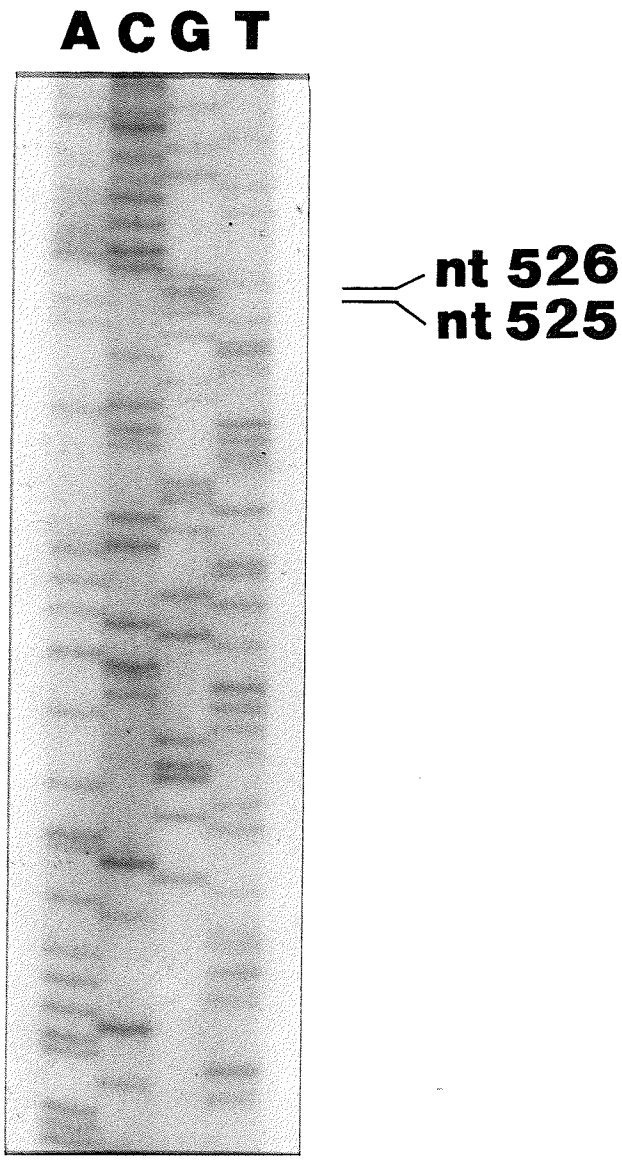


Figure 31. Autoradiograph p526Mu DNA Sequence

Table 9. The Determination of E6*I and E6*II Expression Levels in Transfected C-33A Cell Lines

Plasmid	E6*I (log dpm×10^5)	E6*II (log dpm×10^5)
pcDNA3	0	0
p409Mu	0	0.6559
p526Mu	4.7601	0

Discussion

Detection of HPV Infection in Cervical Lesions

The observation that the incidence of cancer of the cervix was higher in married women made by Rigoni-Stern in 1842 (Rigoni-Stern, 1987, Translation), eventually led to the hypothesis of causality by a sexually transmitted infectious agent. The hypothesis that the infectious agent causing cancer of the cervix is HPV was raised for the first time by zur Hausen (zur Hausen, 1976). The identification of specific HPV types in cervical cancer in 1983, 1984 and in subsequent years substantially boosted activities in papillomavirus research (Dürst *et al.*, 1983). Large scale epidemiological studies during the past few years have identified HPV infection as being the major risk factor for cervical cancer. The overwhelming amount of evidence linking specific HPV infections to cancer of the cervix is gradually leading to a shift of interest from questions related to causality towards the understanding of the mechanism by which HPV, the infected host cells, their cellular environment, and other factors contribute to cancer development (zur Hausen, 1994).

In this study, the quantitative RT-PCR method introduced by Hsu and McNicol (1992) was refined by co-amplifying GAPDH mRNA as the internal standard together with mRNA derived HPV-16 E6/E7 ORF, in the same tube. As other "housekeeping" genes, GAPDH is inherently expressed in all living cells. GAPDH mRNA is always recovered during mRNA isolation from living cells. Since GAPDH is known to be expressed at a constant level per cell, the amount of GAPDH mRNA is equivalent to the cell number. The number of cells used in each analysis can vary, hence it is necessary to normalize the results. (Kellogg *et al.*, 1990). In this investigation, GAPDH mRNA underwent the identical mRNA isolation, reverse transcription and PCR amplification processes as the HPV-16 E6/E7 transcripts did. The standardization of the level of expression of E6*I and E6*II mRNA to the level of GAPDH expression compensates for

the differences in the integrity of mRNA and reverse-transcription reaction and eliminates the necessity to determine the quantity of cells from which the mRNA is isolated. It also facilitates comparison between tubes within and between assays.

Determination of Amplification Efficiency of HPV-16 E6/E7 and GAPDH Primers

A small variation in efficiency of PCR amplification results in drastic changes in product yields due to the exponential nature of the amplification (Kellogg *et al.*, 1990). In order to determine the amplification efficiency of each pair of primers, it is necessary to generate a standard curve for each transcript. All initial reactions and standardization were developed by using CaSki cells. The standard curve was generated by measuring the amount of amplification product made between 20 to 30 cycles, as a linear relationship was observed after 20 cycles and reached a plateau after 30 cycles (Kellogg *et al.*, 1990). In this current study, the efficiency of amplification, or *R*, values for E6*I, E6*II, and GAPDH were determined to be 0.244, 0.621, and 0.288, respectively. These values also suggest that the efficiency of amplification is in inverse proportion to the length of the products. The shorter the amplicon, the more efficient is the reaction. This is probably due the ease by which the template strands dissociate, facilitating primer annealing.

Determination of the Level of E6*I and E6*II Expression in Clinical Specimens

In this study, exfoliated cervical cells and biopsy tissue specimens collected from 22 women were evaluated for the level of E6*I and E6*II expression by using the quantitative RT-PCR technique. The normalization of the results to the internal standard facilitated comparison between specimens collected from different patients and between specimens taken from the same individual. It was found that the level of E6*I expression was higher than of E6*II expression in most cases. The level of E6*I expression in both types of specimens was higher in high grade lesions. Conversely, in biopsy tissue specimens the level of E6*II expression in high grade lesions decreased. Nevertheless, in

exfoliated cell specimens, the E6*II expression level remained constant. The diminished or absence of E6*II expression relative to E6*I expression in biopsy specimens was statistically significant (p=0.014). These results confirm the hypothesis that there is a decrease in the level of E6*II expression relative to E6*I expression with the progression of the disease in biopsy tissue specimens. Although in exfoliated cell specimens the difference in the level of E6*I and E6*II expression was greater for high grade lesions than in low grade lesions, the change in the level of E6*I expression relative to the level of E6*II expression with the progression of the disease was not proven to be statistically significant (p=0.137).

Despite a great variation in inter-observer and intra-observer findings, histopathologic examination is still regarded as the gold standard for determining the grade of CIN. Histopathologic assessment is more related to clinical condition than cytologic assessment. Histopathologic examination and molecular analysis of biopsy tissue share the trait of assessing the same type of sample, hence the results of molecular analysis of a biopsy specimen would be expected to be more in accord with the histopathological finding. The results of this study proved that molecular analysis of biopsy tissue specimens was more consistent with the histopathologic assessment than analysis of the exfoliated cervical cell specimens.

Some exfoliated cervical cell specimens did not yield amplifiable GAPDH mRNA (patient number 2, 15, and 21), making E6*I and E6*II expression analysis impossible. This was likely caused by poor quality of the isolated mRNA. The exfoliated cervical cell specimen consists mainly of sloughed off cells from the epithelial surface, thus many of these cells may be senescent. Since there is no metabolic activity in these cells, GAPDH mRNA may not be present although the DNA might be intact. Moreover, the exterior location of the cells brings them in direct contact with vaginal microflora. Abnormal microflora is observed more frequently in CIN patients than in normal patients (Guijon *et*

al., 1992). The metabolites of the organisms and concomitant alteration in vaginal pH may affect the viability of the cells and the integrity of their nucleic acid.

Overall, a biopsy specimen is preferred to an exfoliated cervical cell specimen for molecular analysis since it provides better and more consistent results.

1. Differences in Cell Composition of Biopsy Tissue and Exfoliated Cervical Cell Specimens Influence the QRT-PCR Results

The quantitative RT-PCR measures the average of the expression level of all cells in the specimen. In biopsy tissue specimen, it measures the average of the level of E6*I and E6*Il expression of cells from the basal membrane to the superficial layer of the worst lesion in the epithelium of the cervix, whereas in exfoliated cervical cell specimen, it measures the average of the level of E6*I and E6*II expression in cells collected from the surface of the uterine cervix. The difference in the cell composition between the types of specimens accounts for the different results in the level of E6*I and E6*II expression, especially in high grade lesions.

2. Analysis of the Level of E6*I and E6*II Expression in Low Grade Lesions

In low grade CIN, only a third or fewer of cells located close to the basal membrane undergo prolonged proliferation, whereas the rest of the cells undergo normal differentiation. Low grade CIN is also considered as the early stage of the disease, thus the adjacent tissue is unlikely to be infected with HPV and contain normally differentiating cells. It was reported that by using the *in situ* hybridization assay, the abundance of expression of HPV-16 E6/E7 ORFs in low-grade lesions was mapped in the upper layer of the epithelium (Crum *et al.*, 1988; Dürst *et al.*, 1991; Böhm *et al.*, 1993). These observations were confirmed by the more sensitive method, the RT *in situ* PCR (Nuovo, 1994). This evidence suggests that in low grade lesions the transcription of these ORFs is increased in the differentiating cells.

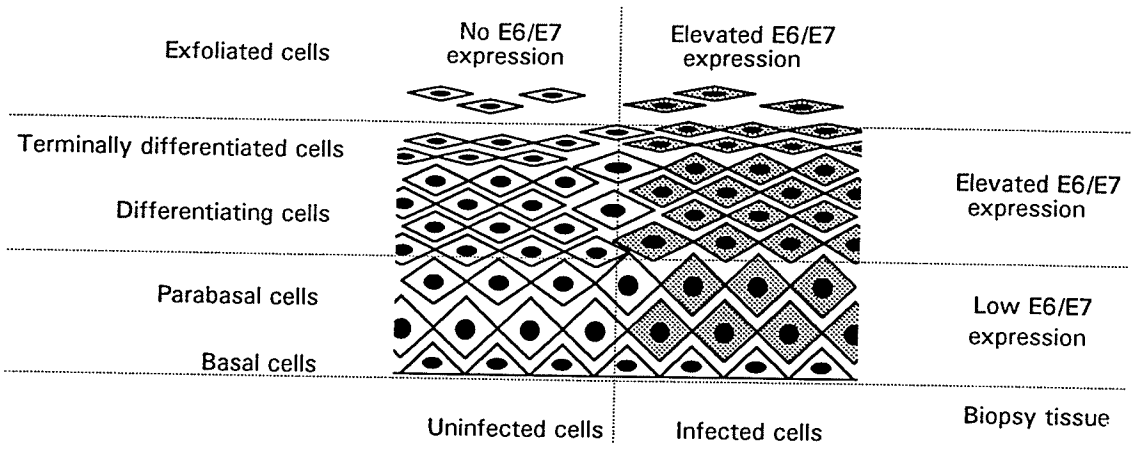
The biopsy tissue specimen is a combination of cells from the lower aspect of cervical epithelium, in which the expression of E6/E7 ORFs is low (Dürst *et al.*, 1991), and cells from the upper layer of the epithelium with an elevated expression of E6/E7 ORFs (Figure 32 A). The exfoliated cervical cell specimen is a combination of cells from the upper layer of the epithelium with a high expression of E6/E7 ORFs and normal cells from the adjacent tissue which are not infected with HPV and thus do not express HPV's oncogenes. Hence, in terms of HPV-16 E6/E7 ORFs expression, there is no significant different in the cell composition between exfoliated cervical cell and biopsy specimens of low grade dysplasia. Comparison of specimens collected from the same woman with low grade disease showed no difference in the level of E6*I and E6*II expression.

3. Analysis of the Level of E6*I and E6*II Expression in High Grade Lesions

The architecture of the epithelium of high grade lesions is totally defective. Two thirds to the entire thickness of the epithelium is mainly composed of undifferentiated cells. Undifferentiated cells are more abundant in the epithelial layers of high grade lesions than of low grade lesions. It was demonstrated that in high grade lesions and tumor tissues, the cells in both the basal and superficial aspects of the lesion express very high level of E6/E7 mRNA (Böhm *et al.*, 1993; Dürst *et al.*, 1991; Nuovo, 1994). Thus, a biopsy tissue specimen taken from a high grade lesion contains invariably undifferentiated cells which express very high level of E6/E7 mRNA.

Since the biopsy tissue specimen was collected from the worst area seen during the colposcopy examination, the degree of dysplasia of the surrounding tissue might be lower than of the area where the biopsy tissue was collected. The exfoliated cell specimen sampled the cells from the worst area, as well as from the surrounding tissue, therefore it contains an admixture of undifferentiated cells and normally differentiated cells. Obviously, the cell composition of the biopsy tissue specimen is considerably different

A



B

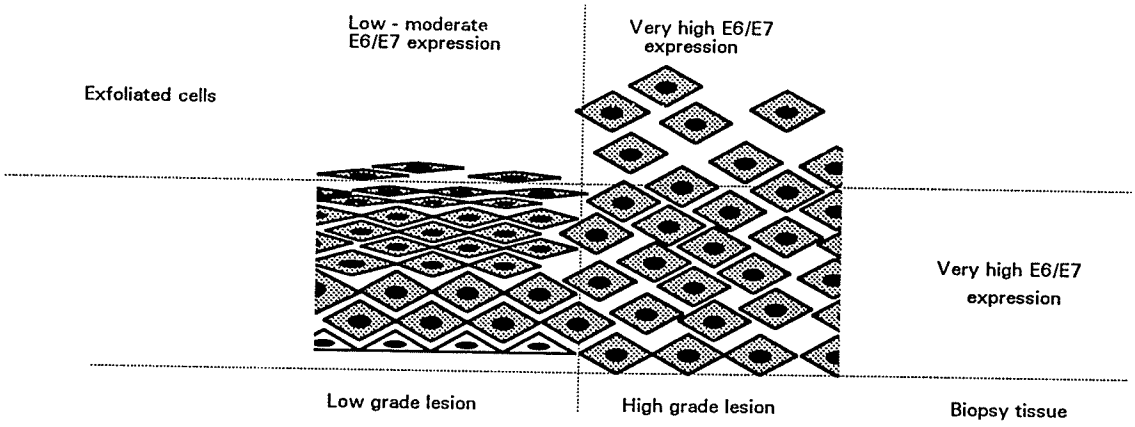


Figure 32 . Exfoliated Cell and Biopsy Tissue Specimens in Low and High Grade Lesions Shaded cells are cells infected with HPV-16. In low grade lesion, exfoliated cell specimen consists of infected as well as non-infected sloughed off cells from the surrounding tissue, while biopsy specimen consists of cells expressing low HPV-16 E6/E7 from lower aspect of the epithelium and cells with elevated E6/E7 expression from upper aspect of the epithelium (A). In high grade lesion, exfoliated cell specimen consists of sloughed off undifferentiated cells as well as cells from the surrounding tissue with lower grade of dysplasia, while biopsy specimen consists of uniformly undifferentiated cells expressing high level of HPV-16 E6/E7 (B).

than that of the exfoliated cell specimens and consequently the level of E6/E7 expression in both types of specimen was also different (Figure 32 B).

4. The Expression of Alternate Transcripts Correlates with Degree of Cellular Differentiation

The biopsy tissue specimen collected from a high grade dysplasia or from a cervical tumor is a good model for HPV-16 associated malignancy *in vivo*, since it contains uniformly undifferentiated cells with high level of E6/E7 expression. In this study, the level of E6*I expression was high in high grade dysplasias, while of E6*II expression was very low to absent. The exfoliated cervical cell specimens collected from high grade lesions showed also high level of E6*I expression while the level of E6*II expression did not change with the increase in the degree of dysplasia. The fact that the exfoliated cervical cell specimen consists of many more differentiated cells than the biopsy specimens may explain the difference in the expression of E6*II between the two types of specimens. It can be inferred from this result that the E6*II expression is higher in more differentiated cells.

Higher level of E6*I expression in high grade lesions in biopsy tissue specimens compared to the E6*I expression level in low grade lesions can be explained by the lack of the differentiated cells. This would also account for lower or loss of E6*II expression in high grade lesions compared to low grade lesions. Constant level of E6*II expression in low grade lesions as well as in high grade lesions of exfoliated cervical cell specimens is explained by the fact that specimens from both low and high grade lesions contain the differentiated cells collected from "normal" regions of the cervix.

Taken together, expression of E6/E7 in undifferentiated cells is likely to be in the form of E6*I mRNA species, while the differentiated cells favor the E6*II expression. Thus, there is a strong correlation between cell differentiation and E6*I/E6*II expression. The fact that E6*I and E6*II mRNAs are generated from the same pre-mRNA by

alternative splicing, suggests that the selection of the splicing sites is modulated by cellular splicing factors selectively expressed in relation to cellular differentiation.

5. Putative Role of E6*I Transcripts in Malignant Progression

HPV-16 E6*I mRNA transcribed from the polycistronic E6/E7 ORFs, initiated from the P_{97} early promoter (Smotkin & Wettstein, 1986) is the major transcript in cervical carcinoma tissues, premalignant lesions, metastatic tissue, and cell lines derived from cervical cancer (Smotkin & Wettstein, 1986; Rose *et al.*, 1991; Shirasawa *et al.*, 1991; Rose *et al.*, 1994; Rose *et al.*, 1995). The primary function of the splicing within E6 ORFs is to facilitate the efficient translation of E7 protein (Sedman *et al.*, 1991). The E6*I encoding mRNA has a much larger spacing between the stop codon of the E6*I ORF and the start codon of the E7 ORF (Smotkin *et al.*, 1989). The E7 protein of HPV-16 has been shown to be the predominant factor in oncogenesis (Crook *et al.*, 1989; Hawley-Nelson *et al.*, 1989; Sato *et al.*, 1989; Liu *et al.*, 1995). The transforming potential of E7 is directly correlated to the expression levels of the oncoprotein and a threshold level of the E7 may be required before the cells can be fully transformed (Liu *et al.*, 1995).

The role of the other protein encoded by this mRNA, *i.e.*, the putative truncated E6 protein, E6*I, is not known. By developing a monoclonal antibody against E6*I fusion protein of HPV-18, the existence of this protein was demonstrated in an HPV-18 DNA containing human cervical carcinoma established in nude mice (Schneider-Gädicke *et al.*, 1988). Recently, it was shown that the E6*I protein of HPV-16 was able to *trans*-activate the autologous P_{97} promoter (Shirasawa *et al.*, 1994).

The results presented here showed that the level of the E6*I mRNA is increased in high grade dysplasia. This supports the hypothetical role of E7 in malignant progression, and that the transforming capacity of E7 protein is directly correlated with its level of expression. The basis of why the expression of E7 is increased in high grade lesion is not

well understood. The preferential splicing of E6/E7 pre-mRNA to produce E6*I mRNA in undifferentiated cells as shown in this study, may explain this phenomenon. Besides the E7 protein, E6*I protein is also translated from this mRNA. The capability of E6*I protein to activate its autologous promoter results in higher transcription of E6/E7 ORFs. In undifferentiated cells, this pre-mRNA is then spliced to E6*I mRNA, completing a loop that promotes cell proliferation (Figure 33).

An alternative explanation can be based on the integration of HPV genome into the host chromosome, as is commonly observed in high grade dysplasias and cervical cancers. The integration appears to occur at random in the human genome, but selectively in the region of E1 and E2 ORFs of the viral genome. This integration disrupts the E2 ORF resulting in the loss of control of E6/E7 expression. Despite lower viral copy number, the integration of HPV-16 DNA has been demonstrated to lead to increased steady-state levels of mRNAs of E6/E7 ORFs (Jeon & Lambert, 1995). The undifferentiated state of the cell favors processing of the pre-mRNA to the E6*I transcript and the loop is again completed.

The low grade dysplasia also expresses E6*I mRNA. However, the cells only undergo slightly prolonged mitotic activity before entering the differentiation phase. The results of this study revealed that the level of E6*I expression was lower in low grade dysplasia than in high grade dysplasia, supporting the hypothesis that a certain threshold has to be met before the cell enter infinite proliferation.

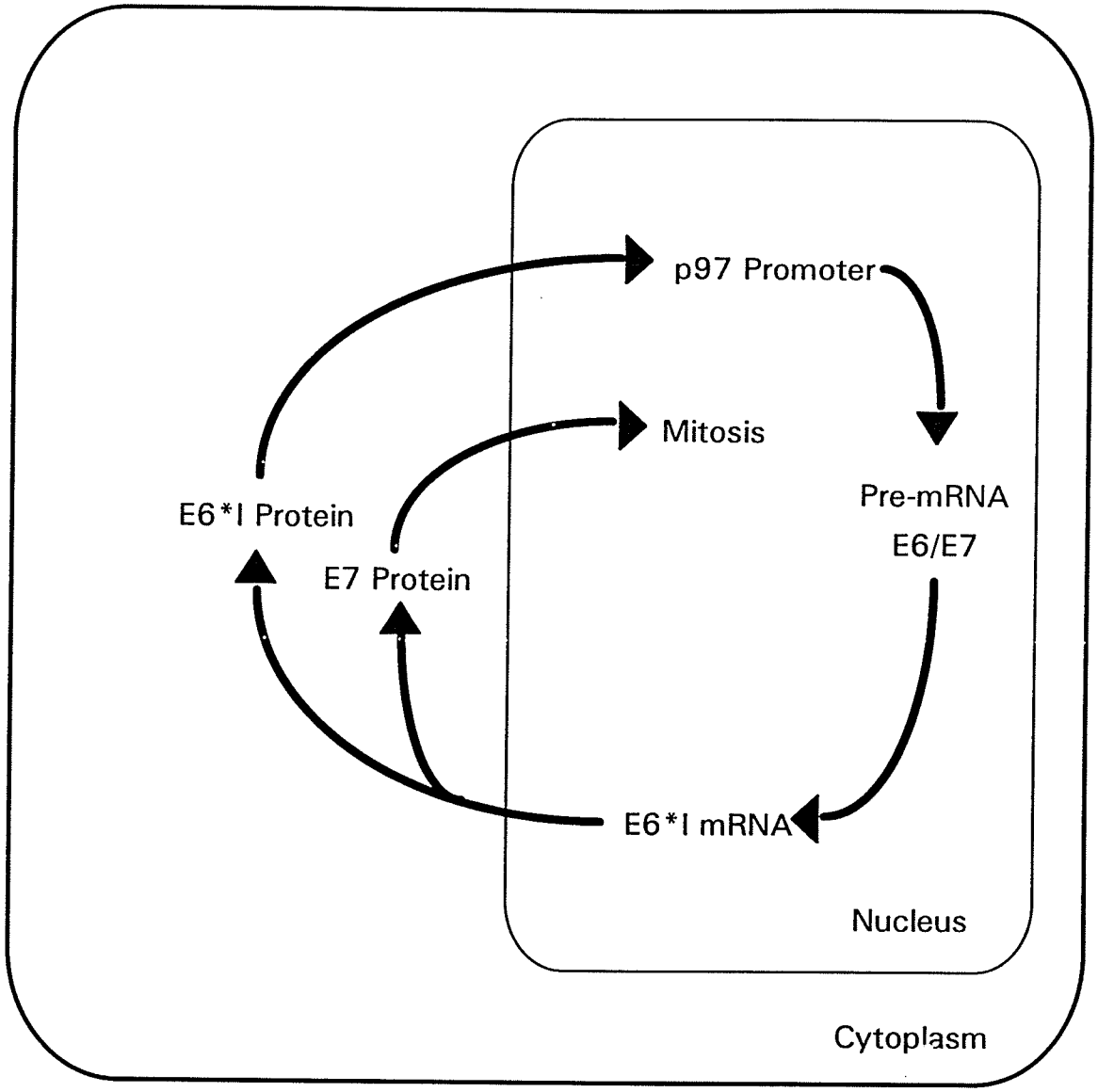


Figure 33. Putative role of E6*I transcript in malignant progression

In an undifferentiated cell E6/E7 pre mRNA is preferentially spliced into E6*I mRNA. The E6*I mRNA is then transported across the nuclear membrane and translated into E6*I and E7 proteins. The E6*I protein activates its autologous P_{97} promoter, resulting in the increase of E6/E7 transcription. The E7 protein induces the DNA replication of the host cell, restraining it from differentiation.

6. Putative Role of E6*II Transcripts in Malignant Progression

The E6*II mRNA is known to be the second spliced transcript of E6/E7 ORFs (Smotkin & Wettstein, 1986). It was detected less abundantly than the E6*I mRNA in clinical specimens and in HPV-16 containing cell lines (Shirasawa *et al.*, 1991; Hsu & McNicol, 1992). The function of this second spliced transcript is not yet understood. E6*II potentially encodes a second truncated E6 protein, E6*II, however the presence and the role of this protein in clinical specimens as well as in cell lines have not been reported. Although the E6*II transcript also possesses E7 ORF, it is unlikely that the E7 protein is translated from this mRNA due to the close proximity of the termination codon of E6*II protein to the initiation codon for E7. In this study, E6*II mRNA was mainly observed in low grade dysplasias. *In situ* hybridization of biopsy tissues revealed that transcripts carrying E7 ORF were confined to isolated nuclei of differentiated cells (Böhm *et al.*, 1993). This suggests that E6*II mRNA remains intranuclear and is not transported into the cytoplasm. The fact that the differentiated cells still express E6*I mRNA, suggests that E6*II mRNA may also block the transportation of E6*I mRNA into the cytoplasm (Figure 34). Lower E6*II expression in high grade lesions may be an indicator of the progression of the disease, and thus the determination of E6*II expression may be used as a prognostic tool.

The role of the E6*II protein has not been explored. In order to identify E6*II protein in cells, one needs to generate monoclonal antibodies in order to specifically immunoprecipitate the putative E6*II protein. This monoclonal antibody would enable the detection of the putative E6*II protein in human cervical carcinoma cell lines and in a HPV-16-positive human cervical carcinoma, aiding in elucidation of its biological role.

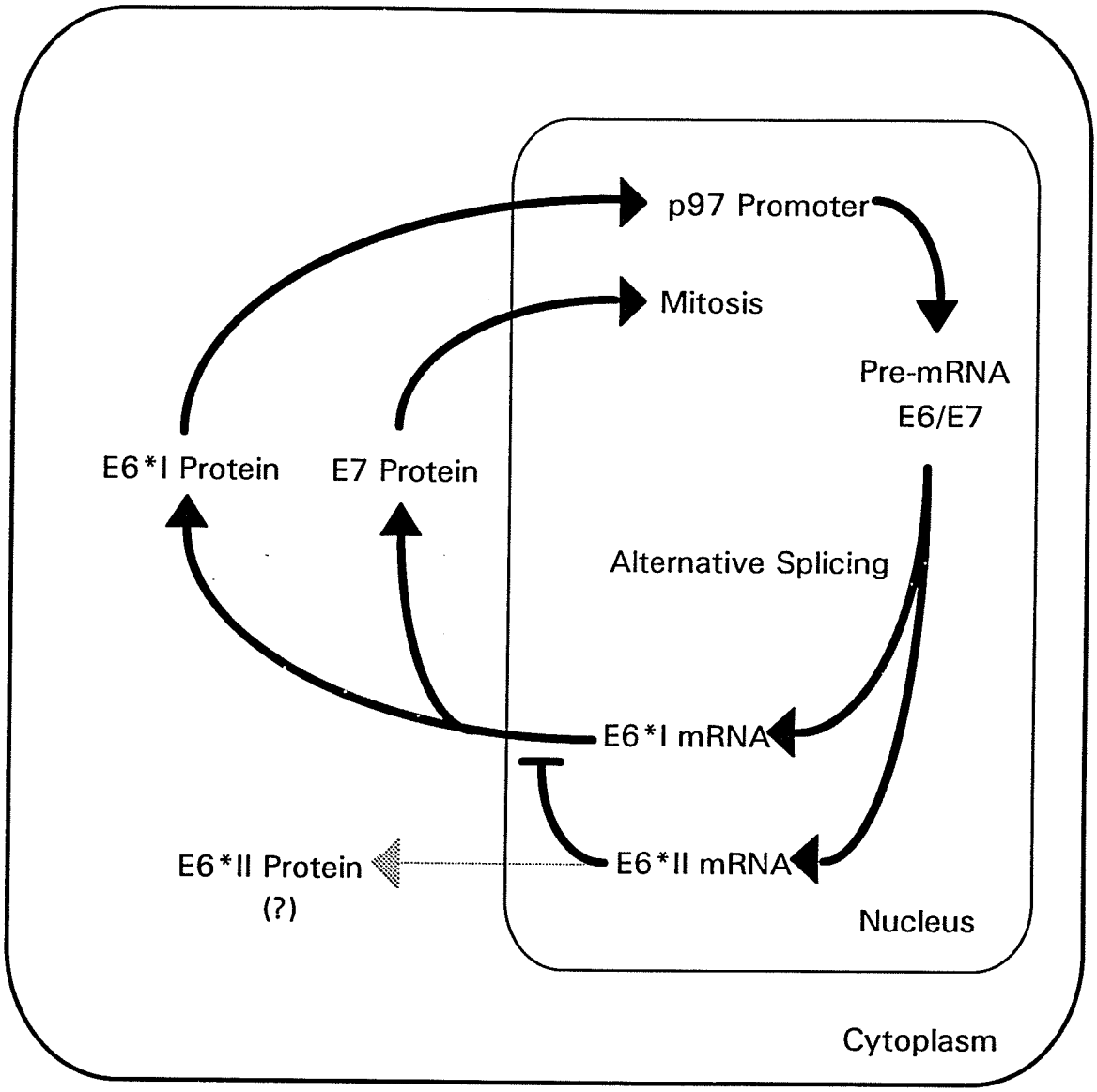


Figure 34. Putative Role of E6*II Transcripts in Differentiated Cell

In differentiated cell, E6/E7 pre-mRNA is preferentially spliced into E6*II mRNA. The E6*II mRNA inhibits or competes with E6*I mRNA in the transportation into the cytoplasm, causing both transcripts to be retained in the nucleus.

Determination of the Effect of Cellular Splicing Factors on HPV-16 E6/E7 ORFs Transcripts

The work presented here has shown that there is a direct correlation between splicing site selection of HPV-16 E6/E7 ORF pre-mRNA and cellular differentiation. In low grade lesions where the cells are more differentiated, higher E6*II level is observed, which means increased selection of nt 526 or distal splice acceptor site, whereas in high grade lesions which contain mainly less differentiated cells, higher E6*I expression is observed. This means the nt 409 or proximal splice acceptor site is activated. The process of cellular differentiation was shown to repress T-antigen expression of SV40 (Estervig *et al.*, 1989). A more recent study demonstrated that alternative splicing of SV40 T/t antigen is regulated by cellular splicing factors, SF2 and hnRNP A1 (Ge & Manley, 1990; Harper & Manley, 1991). High ratio of SF2/hnRNP A1 activates proximal splice donor site, resulting in higher large T antigen being produced. Transient expression of hnRNP A1 shifted 5' splice site selection toward the more distal donor site on the adenovirus E1A pre-mRNA (Yang *et al.*, 1994). The effect of SF2 and hnRNP A1 on the HPV-16 E6/E7 ORF expression in several different cell lines were evaluated in this study.

1. Degree of Differentiation of Cell Lines

When cells are cultured and propagated as a cell line, the resultant cell phenotype is often different from the characteristics predominating in the tissue from which it was derived. This is probably due to the highly proliferating cells that overgrow cells with reduced proliferative capacity. Cells with reduced proliferative capacity are usually in a more differentiated phenotype. Differentiation is the process of cell progression from committed precursor cells such as blast cells and epidermal basal cells, leading to the expression of phenotypic properties characteristic of the functionally mature cell *in vivo*. Cells in terminal differentiation fully express the mature phenotype and cannot progress beyond this point. The process of differentiation is usually irreversible such as in neurons,

skeletal muscle, and keratinized squamous cells, however, some type of cells can revert to a less differentiated phenotype and resume proliferation, such as a fibrocyte.

As differentiation progresses, cell division is reduced and eventually lost. However, in most continuous cell lines derived from tumors, the proliferation is incompatible with the expression of differentiated properties, although the differentiated properties are not always fully expressed. The expression of specific properties can be used as markers of differentiation. Apparently tumor cells may often retain the ability to respond to induction of differentiation.

The HeLa cell line was derived from a biopsy taken from a carcinoma of the cervix of a 31 year old woman. It was the first established aneuploid epithelial-like cell line originating from human tissue. Since its origin, it has been one of the most widely studied cell lines. Initially the tumor was diagnosed as epidermoid carcinoma. Re-examination of the original biopsy taken in February 1951 showed cells with large dark nuclei with a revealing acinous formation of gland cells growing directly from the ordered glandular cords (Jones *et al.*, 1971). This characteristic is consistent with adenocarcinoma. It was also reported to be a very aggressive adenocarcinoma of the cervix. On the basis of the aggressiveness of the disease, histomorphology, growth in soft agar, and high tumorigenicity in laboratory animals, one may consider HeLa cell as a highly undifferentiated cell.

The G-401 cell line was derived from a tumor of the kidney of 3-month old male baby. It was believed to be derived from a Wilms' tumor . However, current classification of Wilms' tumor does not consider other renal neoplasms of childhood, such as the clear cell sarcoma and rhabdoid tumor of the kidney to be variants of Wilms' tumors (Beckwith & Palmer, 1978). The original tissue blocks utilized in diagnosis of the tumor from which G-401 originated could not be located, hence re-examination of histopathological section of the original tumor can not be done. Histological, ultrastructure, and mRNA expression examinations of heterotransplants derived from the G-401 cell line revealed that the

properties of G-401 heterotransplants were consistent with a rhabdoid phenotype rather than that of a classical Wilms' tumor (Garvin *et al.*, 1993). It was reportedly a diploid cell line which is highly transformed, forms large progressive tumors in inoculated nude mice (Weissman *et al.*, 1987), and grows in soft agar, thus it is likely to be a highly undifferentiated, epithelial-like cell.

The C-33 A cell line was grown from a poorly differentiated, invasive cervical carcinoma. It is established as a hypodiploid human tumor cell line. The original contributor reported that C-33 A cell line was the most anaplastic of all cell lines established at the same time, *i.e.*, C-4, C-27 and C-33 (Auersperg, 1964).

The fibroblast cell is a unique cell since it retains the capability to revert to less differentiated phenotype and resume proliferation. In tissues, the fibroblast is in quiescent state and expresses its differentiation-specific properties. Some histologists prefer to name the quiescent fibroblast as fibrocyte. However, as a cultured cell, when the number of cells is maintained low and supplied with sufficient growth stimulator such as serum, fibroblasts proliferate actively. In many experiments, fibroblast cells have been dealt with like homogeneous non-differentiating cells until recently. Human fibroblasts remain predominantly euploid throughout their culture life-span and never give rise to continuous cell lines. Studies of fibroblast cells from different animals demonstrated that fibroblast cell systems are stem cell systems and eventually undergo differentiation. At high passages, fibroblasts cease proliferation but remain viable for a considerable period of time. This post mitotic phase resembles to terminal differentiation (Bayreuther *et al.*, 1992).

In this study, the human fibroblast (HuFF) was derived from foreskin of a newborn baby and designated to be the most differentiated cell line. In normal conditions, the fibroblast culture can be split up to 24 passages before it halts proliferation. In order to be as close as possible to the terminally differentiated fibroblast, while the cells are still in exponential growth, high passage of the fibroblast line was used.

Depending upon the type of lineage, there are many kinds of markers of differentiation known, *e.g.*, keratin for keratinocytes, collagen for fibrocytes, and hemoglobin for erythrocytes. However, a universal marker for differentiation has not been discovered, hence comparison of degree of the differentiation between cell lines is difficult to carry out.

2. Analysis of the level of E6*I and E6*II Expression in Transfected Cell Lines

The four cell lines, C-33 A, G-401, HeLa, and Human Foreskin Fibroblast (HuFF), were transiently transfected with a pcDNA3-based (Invitrogen) plasmid construct carrying HPV-16 E6/E7 ORFs. The immediate early cytomegalovirus promoter of pcDNA3 driving transcription of the cloned HPV-16 E6/E7 is independent of species and tissue specificity. Transient transfection results in a higher expression level of the introduced DNA than stable transfection due to the higher copy number of plasmid DNA harbored per cell. Thus it is more sensitive for detecting expression levels. The efficiency of transfection was measured by determining β-galactosidase activity from pCMVβ-Gal plasmid cotransfected with the plasmid construct carrying HPV-16 E6/E7 ORFs (pHPV800). By adjusting the level of E6*I and E6*II expression to β-galactosidase activity, transfection results from different cell lines could be freely compared.

All cell lines transiently transfected with pHPV800 expressed both E6*I and E6*II mRNA. The level of E6*I expression was measurably higher than that of E6*II expression in all cell lines. This is in accordance with the observation in clinical specimens as well as in cell lines expressing HPV-16 E6/E7 ORF, where E6*I is the main transcript.

A number of studies indicated that the small t mRNA of SV40 is an unfavorable choice during the splicing of precursor mRNA (Fu & Manley, 1987; Noble *et al.*, 1987; Zhuang *et al.*, 1987; Noble *et al.*, 1988; Fu *et al.*, 1988). Potentially, E6*II is also an unfavorable choice during the splicing of HPV-16 E6/E7 pre-mRNA. There are some probable mechanisms responsible for this, such as the smaller size of E6*II intron sterically

hindering the simultaneous interaction of splicing factors with the 5' and 3' splice sites, and E6*II utilizing a different branchpoint than E6*I, which may not allow efficient binding of splicing factors.

The level of E6*I and E6*II expression in the normally differentiated HuFF cells is significantly lower than in other tumor cell lines. This is probably due to a lower transcription activity in HuFF cells. Somewhat surprising, the ratio of E6*II/E6*I in HuFF cells is significantly the lowest among cell lines.

3. Analysis of The Level of SF2 and hnRNP A1 Expression in Cell Lines

The expression levels of SF2 and hnRNP A1 of the four cell lines were determined by using quantitative RT-PCR. A standard curve of primers of GAPDH, SF2, and hnRNP A1 was generated, and the efficiencies of amplification of GAPDH, SF2, and hnRNP A1 were determined to be 0.307, 0.336,, and 0.374, respectively. The efficiency of amplification which is in inverse proportion with the length of the products was also observed in this standard curve. The standard curve was used for calculating the expression levels of SF2 and hnRNP A1 relative to GAPDH in the cell lines. The expression levels of SF2 and hnRNP A1 in the cell lines were then adjusted relative to the expression level of GAPDH.

The level of SF2 expression varies significantly between cell lines. G-401 showed the highest SF2 expression, while HuFF cells revealed the lowest. This observation supports the hypothesis that the level of SF2 expression varies significantly between cell lines. The SF2 level in the adenovirus-transformed human embryonic kidney cell line 293 was shown to be significantly higher than in HeLa cells (Ge & Manley, 1990). The evidence that G-401 and 293 cell lines were derived from embryonic kidney suggests that SF2 expression is regulated in a tissue-type specific manner. Low SF2 expression in HuFF cells also indicates that SF2 expression may also be associated with the differentiation stage.

In this study, the level of hnRNP A1 expression in all highly undifferentiated cell lines is significantly higher than in the more differentiated HuFF cells. This result is consistent with the observations from other groups of investigators. Only in a few exceptions, expression of hnRNP A1 was higher in transformed cell lines compared to differentiated cells (Biamonti *et al.*, 1993). Correlation between proliferation rate of the cell and hnRNP A1 level was also demonstrated (LeStourgeon *et al.*, 1978; Celis *et al.*, 1986; Buvoli *et al.*, 1988). Possibly, higher expression of hnRNP A1 in transformed cells is due to higher proliferation rate of those cells. Very recently, it was reported that the amounts of different hnRNP proteins, including hnRNP A1, varied among cell types, perhaps reflecting increased levels of transcription and RNA processing (Kamma *et al.*, 1995).

4. Correlation Between SF2 and E6*I or E6*II

SF2 was reported to promote the use of proximal 5' alternative splice sites (Krainer *et al.*, 1990). This effect is counteracted by hnRNP A1 (Cáceres *et al.*, 1994; Sun *et al.*, 1993; Mayeda & Krainer, 1992; Mayeda *et al.*, 1993). However, the effect of SF2 in 3' alternative splice site selection remains controversial. Krainer *et al.* (1990) reported that SF2 does not strongly influence the selection of competing 3' splice sites. Later, the same group claimed that SF2 favored the selection of the proximal site in a pre-mRNA containing duplicate 3' splice sites (Fu *et al.*, 1992).

The results presented here show otherwise. An increase in SF2 expression significantly correlates with increased E6*II expression, which means SF2 favors the selection of the distal site of 3' alternative splice sites in HPV-16 E6/E7 pre-mRNA. Although there is also an increase in E6*I expression with the increase in SF2 expression to some extent, it is more likely due to general upregulation of transcription in the host cell. This is shown by the significant correlation between SF2 expression and the ratio of

E6*II/E6*I. This result suggests that SF2 regulates the selection of proximal or distal 3' alternative splice sites depending upon the pre-mRNA substrate.

SF2 is an essential factor required for the first cleavage-ligation step of splicing, where recognition of branchpoint and 5' splice site occurs. Branchpoint selection seems to play a crucial role in determining the splicing site. Different spliced mRNA utilizes a different branchpoint (Noble *et al.*, 1988). It was shown that in SV40, splicing of large T mRNA involves the utilization of multiple lariat branchpoints, while small t splicing uses a single, more downstream branchpoint (Noble *et al.*, 1987). As with SV40 where SF2 favors the selection of the downstream small t branchpoint, SF2 also potentially favors the selection of the downstream branchpoint of E6*II transcript in HPV-16.

As described previously, increased E6*II expression correlates with a more differentiated stage of the host cell. Since E6*II expression is correlated with SF2 expression, it was expected that the expression of SF2 and E6*II was elevated in HuFF cells. However, the work in this study fails to show this. All parameters measured in this study were low in HuFF cells, probably due to a general reduction of transcription activity in cells entering terminal differentiation. The correlation between the decrease in abundance of a subset of highly conserved basic nuclear proteins with the loss of proliferative potential in association with the process of terminal differentiation has been reported (Minoo *et al.*, 1989). Although SF2 was shown to act in a dose-dependent manner, it is believed that a certain limit should be met before it can regulate the splicing site selection optimally.

5. Correlation Between hnRNP A1 and E6*I or E6*II

Although hnRNP A1 was demonstrated to have an antagonistic effect with SF2 in 5' alternative splice site selection by favoring the distal splice site, in the study presented here, hnRNP A1 has no effect on splice site selection of 3' alternative splice selection of HPV-16 E6/E7 ORFs. Similar observations on 3' alternative splice sites selection have

also been reported for β-globin pre-mRNA (Krainer *et al.*, 1990). Fu *et al.* (1992) reported that in contrast to the 5' splice site switching activity, the preferential activation of the proximal 3' splice site by SF2 was not counteracted by hnRNP A1. No analogous effect of hnRNP A1 at 3' splice sites has been observed so far (Mayeda & Krainer, 1992). The differences in the effects of hnRNP A1 on 5' and 3' splice site selection may explain the finding that hnRNP A1 has a higher affinity for 5' splice sites than for 3' splice sites.

Since hnRNP A1 has no effect on the selection of 3' alternative splice sites, the SF2/hnRNP A1 ratio does not correlate with E6*I and E6*II expression. Significant correlation between SF2/hnRNP A1 ratio and E6*II/E6*I ratio is mainly due to strong correlation between SF2 and E6*II expression.

6. Putative Roles of SF2 and hnRNP A1 in Alternative Splicing

Figure 35 A shows the putative roles of SF2 and hnRNP A1 in splicing of pre-mRNA with one common acceptor site and two alternative donor sites. SF2 favors the activation of a downstream branchpoint, while hnRNP A1 activates the upstream (distal) donor site. The balance between SF2 and hnRNP A1 determines the outcome of splicing. High SF2 expression relative to hnRNP A1 expression would result in the activation of downstream branchpoint, resulting in the selection of proximal donor site. Conversely, when the hnRNP A1 expression increases relative to SF2 expression, the distal donor site is activated.

On the other hand, the balance between SF2 and hnRNP A1 has no effect on 3' alternative splice site selection (Figure 35 B). Since there is only one splice donor site, changes in hnRNP A1 do not alter the product of splicing. As in pre-mRNA with multiple donor sites, SF2 also favors the activation of distal branchpoint in pre-mRNA with multiple donor sites. When the downstream branchpoint is an unfavored site, low SF2 expression would result in the selection of the upstream branchpoint and the upstream

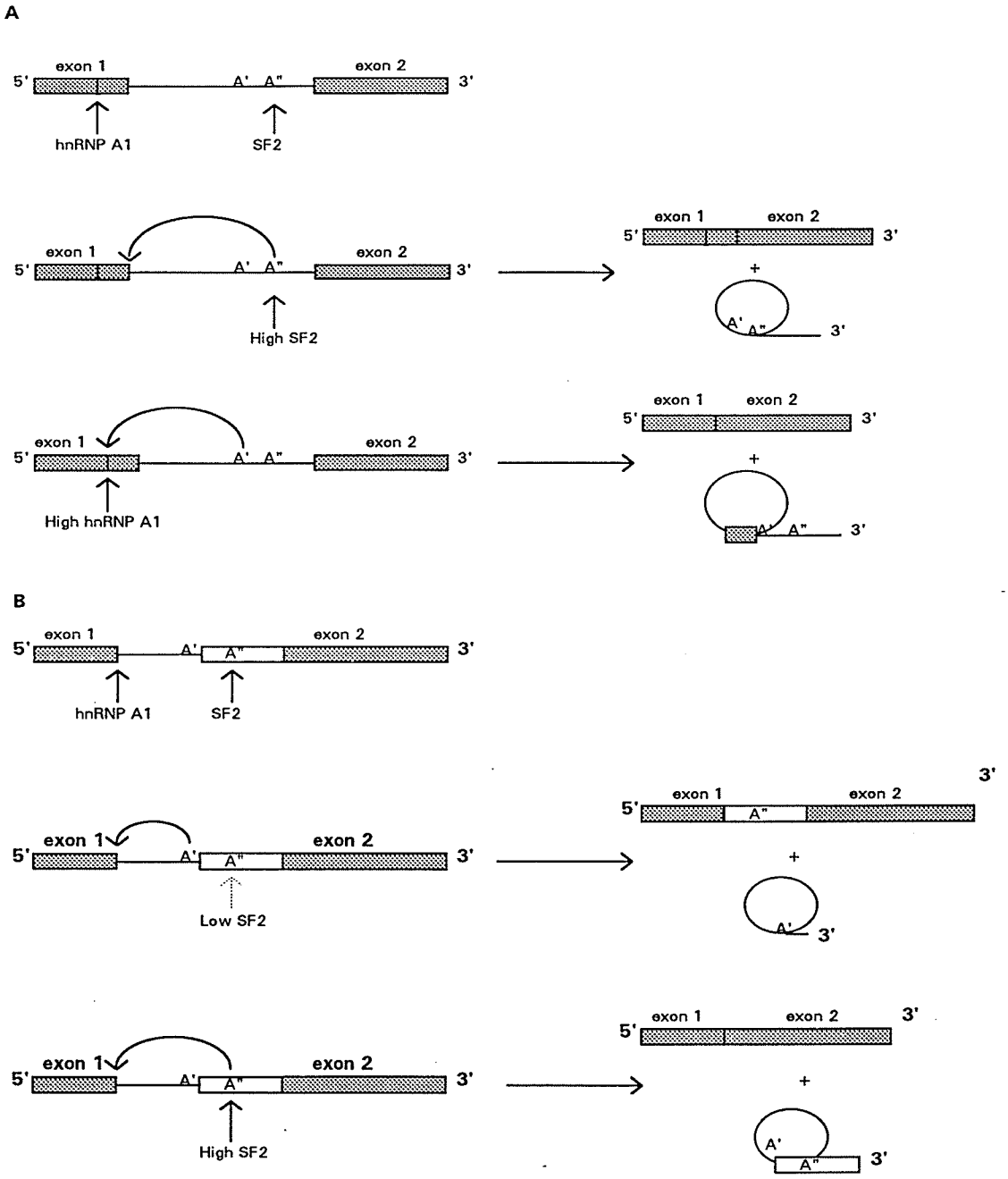


Figure 35. Putative Roles of SF2 and hnRNP A1 in Alternative Splicing

The filled boxes are exons and thin lines are introns removed during splicing. A' is the upstream branchpoint and A" is the downstream branchpoint. (A) Putative roles of SF2 and hnRNP A1 in pre-mRNA with multiple donor sites and single acceptor site. (B) Putative roles of SF2 and hnRNP A1 in pre-mRNA with single donor site and multiple acceptor sites.

acceptor sites is activated. The increase in SF2 expression results in the increase of downstream branchpoint selection, resulting in the selection of the distal acceptor site.

In the case of HPV-16 E6/E7 pre-mRNA, there are two putative branch points, *i.e.*, at nt 404 and nt 482. The activation of the nt 404 branch point results in the production of E6*I mRNA, while the selection of the nt 482 branch point results in E6*II mRNA. Presumably, the nt 404 dominates over the nt 482 during the branch point selection, causing higher expression level of E6*I than of E6*II. SF2 favors the activation of nt 482 branch point. When the expression of SF2 is low, nt 404 branch point is selected, and E6*I mRNA is generated. Higher SF2 expression causes increased selection of nt 482, resulting in increased level of E6*II mRNA.

7. Other Splicing Factors

Additional splicing factors may also be responsible for the selection of 3' alternative splice sites. Recently, a potential pair of proteins with antagonistic activities, namely SF6 and SF7, were suggested (Fu *et al.*, 1992). The SF6 favors proximal 3' splice sites and an excess of SF7 causes the selection of distal 3' splice sites. Once these splicing factors are identified, it should be possible to determine how these competing factors influence the splicing of HPV-16 E6/E7 pre-mRNA.

Polypyrimidine tract binding (PTB) protein may also mediate 3' splice site selection of HPV-16 E6/E7. PTB is an essential recognition element in the determination of branch site location and ultimately in the selection and utilization efficiency of 3' splice sites (Fu *et al.*, 1988; Patton *et al.*, 1991). It promotes the binding of U2 snRNP to the branchpoint region of pre-mRNA (Garcia-Blanco *et al.*, 1989). There is a good correlation between the strength of binding of PTB to a particular polypyrimidine tract and splicing efficiency (Patton *et al.*, 1991). Mutations that increased the number of pyrimidine residues of SV40 pre-mRNA, which presumably increased the binding of PTB to the pre-mRNA, resulted in more efficient utilization of the large T antigen mRNA 5' splice site relative to the small t

5' splice site, while mutations that increased the purine content enhanced small t mRNA splicing (Fu *et al.*, 1988). Determination of PTB level in correlation with 3' splice site selection may disclose its role in the alternative splicing mechanism of HPV-16 E6/E7.

A Model of HPV Infection and Cervical Cancer Development

On the basis of the results presented here and in conjunction with the findings of other investigators, the following model of HPV infection and cervical cancer development is proposed (Figure 36).

1. Mild Dysplasia (CIN I), the Most Common Outcome of HPV Infection

The target of HPV infection is the basal or parabasal cell of cervical epithelium. The site where basal or parabasal cell is relatively exposed to the outer environment of HPV infection is at the junction of the squamous and the columnar epithelium at the transformation zone of the metaplastic epithelium. Alternatively, the virus may enter the target cell through a minor breakage in the squamous epithelium. The HPV receptor is surprisingly ubiquitous. Cells originating from a wide range of tissues and organisms as distantly related as insects and humans bind HPV virus-like particles (VLPs) with similar efficiency and specificity (Müller *et al.*, 1995; Volpers *et al.*, 1995). The binding of HPV to its receptor on the cell surface is followed by an uptake in a small and smooth endocytic vesicle (Volpers *et al.*, 1995). Co-infection with other viruses may increase uptake of HPV since the efficiency of the uptake was greatly enhanced when co-introduced with adenovirus (Müller *et al.*, 1995). After the entry, the virus replicates infrequently and establishes a low copy number of the DNA genome. As the parabasal cells proliferate to replenish the stratified epithelium, the viral transcriptional activity increases and early proteins are expressed . The expression of the E7 protein causes an increase in host cell proliferation, impedes differentiation, and results in an increase in the thickness of the parabasal layer. As the result of self-induction by the E6*I protein, the level of E7

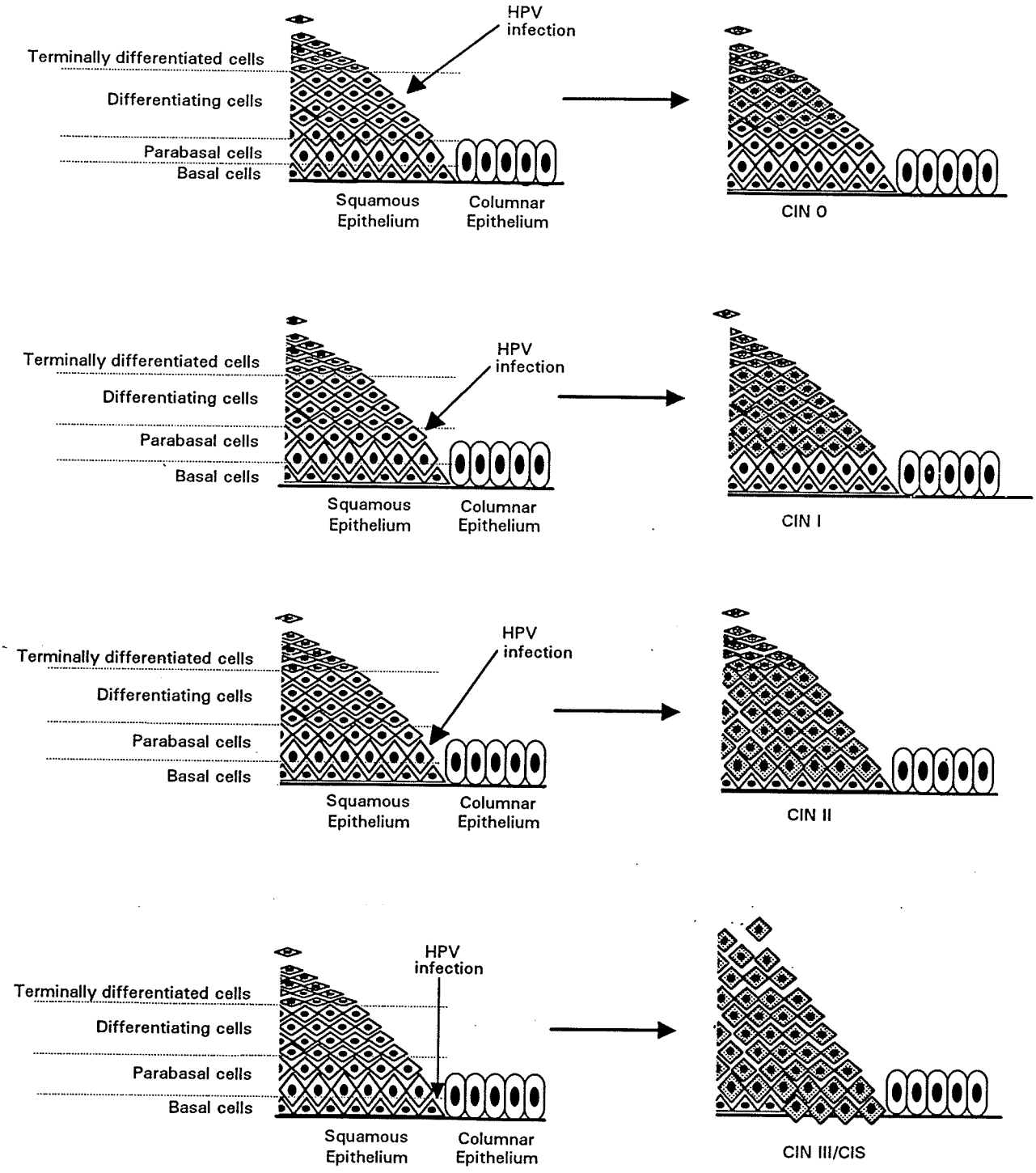


Figure 36. A proposed Model of Pre-Malignant Progression of HPV-Infected Cervical Epithelium Based on the Site of Infection.

Dotted cells are infected cells.

expression increases over time, however, it is not sufficient to abrogate cellular differentiation. As the host cell enters the differentiation phase, it becomes unresponsive to the mitogenic effect of E7 protein. By using an organotypic culture system, it was demonstrated that expression of HPV-16 E7 prevented cell cycle withdrawal in the suprabasal layers of the stratified cultures but had no effect on terminally differentiated layers (Blanton *et al.*, 1992). The host cell differentiation also causes more E6/E7 pre-mRNA to be spliced into E6*II mRNA, putatively due to changes in expression of splicing factors. E6*II mRNA may inhibit the transportation of E6*I mRNA across the nuclear membrane and E7 protein levels fall.

2. HPV Infected Cell with Normal Histopathology (CIN 0)

In some cases, the epithelium is not permissive for viral expression, perhaps due to intervention by the host's immune system. Macrophages have been shown to effectively suppressed HPV transcription in a dose-dependent manner (Rösl *et al.*, 1994). Thus, the viral transcription and replication activity remain low in the cell. Since the E7 is not expressed in a sufficient quantity, the host cell undergoes normal differentiation, and no histopathologic change occurs. Alternatively, the infection may occur in cells above the parabasal layer. These cells have already entered the differentiation phase, and thus are no longer responsive to E7 protein. The E6/E7 pre-mRNA is preferentially spliced into E6*II mRNA in differentiated cells and the host cell is no longer responsive to E7 protein resulting in normal histopathology.

3. High Grade Dysplasia

The host cell permits high level of viral gene expressions, resulting in prolonged proliferation and retardation of differentiation. In CIN II, the increase of E7 protein over time does not exceed the threshold for transformational change, hence the host cell eventually enters differentiation. Whenever the increase of E7 protein exceeds the

threshold, the host cell becomes transformed and remains in a highly proliferating undifferentiated cell form, resulting in CIN III or carcinoma *in situ* (CIS). The vicious circle of E6*I protein promoter enhancement continues, causing a very high level of E6*I mRNA. The integration of the viral genome to the host chromosome, resulting in disruption of the control mechanism of viral oncogene expression is also a possible trigger of the very high expression of E6*I mRNA in high grade dysplasia.

Based on the evidence that HPV nucleic acids are often not detectable in the underlying normal-appearing basal cells of a low grade lesion, even by the sensitive PCR *in situ* hybridization, but are detectable in most of the cells in high grade lesion in both the basal and superficial layers of the lesion (Böhm *et al.*, 1994), the infection in high grade lesion must occur somewhere in the lower layer of parabasal or in basal cells. The longer period of time the infected cell requires to enter the differentiation stage allows time for the E7 protein levels to increase to levels surpassing the threshold and eventually preventing the cell from differentiating. Highly proliferating cells may also facilitate the integration of HPV genome to the chromosome.

4. Are Low Grade Dysplasia and High Grade Dysplasia Distinct Entities ?

Low grade dysplasias (CIN 0 and CIN I) and high grade dysplasias (CIN II, CIN III, and CIS) have been viewed as representing a morphologic and biologic continuum of progressive, consecutive states in the development of invasive cancer. However, many observations show that these are distinct processes. Natural history studies of CIN I have shown that it is a common lesion and the majority regress spontaneously within 2 years of initial detection (Kiviat & Koutsky, 1993). A prospective study found that 61% of 28 women developed high grade dysplasias without a prior history of low grade dysplasia (Koutsky *et al.*, 1992). The topographic distribution of low grade dysplasias and high grade dysplasias is generally different (Burghardt & Ostor, 1983); while low grade dysplasia starts as atypical squamous metaplasia in the transformation zone, the less

common high grade dysplasia occurs as atypical basal hyperplasia of the squamous epithelium.

The organotypic culture system (raft) has proven to most accurately mimic the *in vivo* physiology of the epidermis. Using this system, the effects of HPV-16 and HPV-18 transfected into primary human foreskin keratinocytes have been examined (reviewed in Meyers & Laimins, 1994). However, the conventional methods of introducing DNA into eukaryotic cells such as calcium phosphate coprecipitation, DEAE dextran, electroporation, and liposomes, only allow the DNA introduction to occur prior to the establishment of the raft system. So, the effects of HPV DNA transfected into an established raft system after a certain period of time can not be studied. A new method of delivering DNA directly into cells, called particle bombardment or biolistic, has proven to be highly effective for transferring DNA into cells of living animals as well as into cultured cells (Johnston & Tang, 1994). Using this technology, it is possible to introduce HPV DNA into the established raft system. By introducing the HPV DNA at several different times of the raft system course, the effect of HPV infection in different stage of differentiation can be studied. Such studies will bring to light the consequence of the site of infection to the malignant progression of the infected cell.

Summary

Delineation of the mechanisms contributing to cancer development in human papillomavirus-infected uterine cervix is essential to the understanding of a variety of health-related problems including diagnosis, determination of prognosis, therapy, and ultimately prevention. This study has yielded further insight into the understanding on HPV's role in the development of cervical intraepithelial neoplasia, as related to the dynamics of the viral oncogene expression. The major findings of this study are summarized below.

The expression of E6*I increases with the progression of the disease in pre-malignant lesions, while E6*II decreases. This phenomenon is more discernible in biopsy tissue specimens than in exfoliated cervical cell specimens. Hence, quantification of E6*I and E6*II in biopsy tissue specimens is more relevant to histopathological assessment than in exfoliated cervical cell specimens. This result also suggests that there is a direct correlation between cellular differentiation and the expression of E6*II. The E6*II transcript is less likely to be expressed in undifferentiated cells.

These data provide evidence that high grade dysplasia correlates with high expression of E6*I and diminished or absent of E6*II expression. However, the determination of whether the progression of pre-malignant lesion can be predicted by quantification of HPV-16 E6/E7 transcripts requires a prospective study.

A mammalian expression vector expressing the HPV-16 E6/E7 ORF was constructed in order to study the relationship between the cellular splicing factors, SF2 and hnRNP A1, and expression levels of E6*I and E6*II in transfected cell lines. Although both SF2 and hnRNP A1 expression varied among cell lines, only SF2 was positively correlated with the expression E6*II. In contrast to splicing in multiple 5' splice sites pre-mRNA of SV40 where SF2 favors the selection of the proximal splice donor site, in this study it was found that SF2 favors the selection of distal 3' splice acceptor site.

The specific role of E6*I and E6*II in the development of cervical cancer requires further study. As suggested from the results presented here, over expression of E6*I may lead to the progression of the lesion, while over expression of E6*II may result in the regression of the disease. In order to test this hypothesis, two mutant plasmids, p409Mu and p526Mu which have abrogated splice acceptor sites at nt 409 and nt 526 respectively, were generated. These constructs will be used in future studies based on the findings presented here.

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Appendices

Culture Media

Trypticase Soy Agar with ampicillin

Powdered Trypticase Soy Agar (Becton-Dickinson)	40 g
Distilled water	1 litre

Mix thoroughly. Heat to boil for 1 minute to completely dissolve the powder. Autoclave at 121°C for 15 minutes. Add 20 mg ampicillin when cool.

Tryptic Soy Broth

Powdered Tryptic Soy Broth (Gibco)	30 g
Distilled water	1 litre

Mix and heat to dissolve completely. Dispense 100 ml to bottles. Autoclave at 121°C for 15 minutes.

Tryptic Soy Broth with ampicillin

Powdered Tryptic Soy Broth (Gibco)	30 g
Distilled water	1 litre

Combine and mix to dissolve. Aliquot to 500 ml bottles. Autoclave at 121°C for 15 minutes. Add 20 mg ampicillin when cool.

Luria Broth

Bacto-tryptone	10.0 g
Bacto yeast extract	5.0 g
NaCl	0.5 g
1 M NaOH	2.0 ml

Distilled water to 1000 ml

Combine and mix to dissolve. Dispense 100ml in bottles. Autoclave at 121°C for 15 minutes.

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

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

Adjust pH of L-15 media to pH 7.6, using approximately 20 ml of 4 N HCl. Filter sterilize, dispense 500 ml in Gibco bottles, store at 4°C.

Minimum Essential Medium (MEM)
(Gibco Laboratories, Life Technologies, Inc., Cat. No. 410-1100EB)

Powdered MEM	1 package
Distilled water	1 liter
$NaHCO_3$	2.2 g

Adjust pH of medium to 0.2-0.3 below desired final working pH using 1N NaOH or 1N HCl. Filter sterilize. Keep container closed, store at 4°C.

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

NaCl	24.00 g
KCl	0.60 g
KH_2PO_4	0.36 g
Na_2HPO_4	2.63 g

Distilled water to 3000 ml
Combine and mix to dissolve completely. Adjust pH to 8.0.
Autoclave at 121°C for 15 minutes.

Tissue Suspension Buffer (TSB)

1 M Tris-Cl, pH 7.4	10 ml
5 M NaCl	2 ml
0.5 M EDTA	1 ml
10% SDS	10 ml

Bring volume to 100 ml with glass distilled water.
Dispense into small Gibco bottles.

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.

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.

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.

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.

1 M Tris-Cl

Trizma base (Sigma)	121.1 g
Distilled water	800 ml

Adjust the pH to the desired value by adding concentrated HCl.

pH	**HCl**
7.4	70 ml
7.6	60 ml
8.0	42 ml

Allow the solution to cool to room temperature before making final adjusments to the pH.

Bring volume up to 1000 ml with distilled water. Sterilize by autoclaving.

5 M NaCl

NaCl	292.2 g

Bring volume to 1000 ml with glass distilled water.

10% SDS

Sodium Dodecyl Sulfate	10 g
Glass distilled water	100 ml

It may be necessary to heat this solution to 70°C to get SDS to dissolve. Store at room temperature as solution will precipitate in the cold room.

Cell culture reagents

Trypsin 1X (0.25%)

10X Trypsin (Flow Laboratories, Cat. No. 16-893-49)	10 ml
1M HEPES buffer (Sigma, Cat. No. H 0887)	1 ml
Modified Hanks BSS	90 ml

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

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

Gel electrophoresis

4% agarose gel (total volume 100 ml)

Ultra Pure DNA grade Agarose (Bio-Rad)	4 g
1X TAE buffer	100 ml

Bring to boil in a microwave oven at high power level. Interrupt occasionally to swirl. Cool slightly, add 4µl 10 mg/ml ethidium bromide and pour to gel tray.

Ethidium Bromide (10 mg/ml)

Ethidium bromide	1 g
Distilled water	100 ml

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

8% polyacrylamide gel (total volume 100 ml)

40% Acrylamide/bis (29:1) (Bio-Rad)	20.0ml
10X TBE buffer	10.0 ml
10% Ammonium persulfate	0.7 ml
Distilled water	to 100 ml

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

6% polyacrylamide 7 M Urea sequencing gel

Acrylamide-Urea solution	40 ml
25 % Ammonium persulfate	0.052 ml
TEMED (Bio-Rad)	0.040 ml

Mix gently and cast immediately.

Sequencing gel sealant

Acrylamide-Urea solution	10 ml
25 % Ammonium persulfate	0.070 ml
TEMED (Bio-Rad)	0.050 ml

Mix, pour and seal bottom edge of gel cast immediately.

Acrylamide-urea solution

Urea (Bio-Rad)	21 g
40% Acrylamide/bis (29:1) (Bio-Rad)	7.5 ml
10X TBE	5 ml

Distilled water to 50 ml.

Ammonium Persulfate (10%)

Ammonium persulfate (Bio-Rad)	0.1 g
Distilled water	1 ml

Ammonium Persulfate (25%)

Ammonium persulfate (Bio-Rad)	0.040 g
Distilled water	0.160 ml

Tracking dye

Bromophenol blue	0.25 g
Xylene cyanol FF	0.25 g
Glycerol	30 ml

Distilled water to 100 ml.
Store at 4°C.

Precipitation of DNA and mRNA

3 M Na Acetate

$CH_3COONa.3H_2O$ (Fisher Scientific)	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.

mRNA isolation solutions

Lysis Buffer
200 mM NaCl
200 mM Tris (pH 7.5)
1.5 mM $MgCl_2$
2% SDS
proteases (proprietary mixture of Invitrogen)

High salt wash buffer
500 mM NaCl
10 mM Tris-Cl (pH 7.5)
DEPC-treated water

Low Salt wash buffer
250 mM NaCl
10 mM Tris-Cl (pH 7.5)
DEPC-treated water

Elution buffer
10 mM Tris-Cl (pH 7.5)
DEPC-treated water

2 M Sodium Acetate
2 M Sodium Acetate
DEPC-treated water

5 M Sodium Chloride
- 5 M NaCl
- DEPC-treated water

Glycogen Carrier
- 0.2% (w/v) Glycogen
- DEPC-treated water

Hybridization Solutions

Denaturation Solution

5 M NaOH	5 ml
5 M NaCl	15 ml
Distilled water	30 ml

Neutralization Solution

1 M Tris-Cl (pH 7.4)	25 ml
5 M NaCl	15 ml
Distilled water	10 ml

Prehybridization and Hybridization Buffer

20× SSC	30 ml
10% SDS	5 ml
50× Denhart's Solution	10 ml
10 mg/ml denatured Herring Sperm DNA	1 ml
Distilled water to 100 ml	

Denhart's Solution

Ficoll 400	5 g
Polyvinylpyrolidone mol. wt. 360,000	5 g
BSA Fraction V	5 g
Distilled water to 500 ml.	

20× SSC

NaCl	175.3 g
Sodium Citrate	88.2 g
Distilled water	800 ml

Adjust the pH to 7.2 by adding few drops of concentrated HCl.
Adjust the volume to 1 litre with distilled water.
Sterilize by autoclaving.

10% Sodium Dodecyl Sulfate (SDS) (also called Sodium Lauryl Sulfate)

SDS, Electrophoresis grade (Bio-Rad)	100 g
Distilled water	900 ml

Heat to 68°C to assist dissolution.
Adjust the pH to 7.2 by adding few drops for concentrated HCl.
Adjust the volume to 1 litre with distilled water.

6× SSC, 0.1% SDS

20× SSC	300 ml
10% SDS	10 ml

Bring volume to 1 litre with distilled water

2× SSC, 0.1% SDS

20× SSC	100 ml
10% SDS	10 ml

Bring volume to 1 litre with distilled water

Transfection Solutions

Solution 1 : 2.5 M $CaCl_2$
Filter sterilized and stored at -20°C

Solution 2 : 2X BES-Buffered Saline (2X BBS) pH 6.95 containing:
50 mM N,N,-bis (2-hydroxyethyl) - 2 - aminoethane sulfonic acid (BES) (pH 9.95)
280 mM NaCl
1.5 mM Na_2HPO_4
Adjust pH with HCl at room temperature, filter sterilize and store at -20°C.

Solution 3 : Modified Bovine Serum (MBS) (Stratagene)
Store at -20°C.

β-Galactosidase Assay

Lysis Buffer
0.25 M Tris-HCl (pH 7.8)
0.5% NP40

Reaction Buffer
100 mM NaH_2PO_4 (pH 7.5)
10 mM KCl
1 mM $MgSO_4$
50 mM β-mercaptoethanol (add fresh before use)

ONPG

O-nitrophenyl β-D Galactopyranoside 4 mg/ml in 100 mM NaH_2PO_4 buffer (pH 7.5).

Stop Solution

1 M Na_2CO_3

Double stranded DNA denaturation solution for DNA Sequencing

2 M NaOH

NaOH (Fisher Scientific)	8 g
distilled water	100 ml

3 M Sodium Acetate (pH 4.8)

$CH_3COONa.3H_2O$ (Fisher Scientific)	40.8 g
Distilled water	80 ml

Adjust pH to 4.8 with glacial acetic acid.
Bring volume to 100 ml and dispense.
Autoclave for 15 minutes at 121°C.

Bacterial Transformation

0.1 M Magnesium chloride

$MgCl_2$ (Sigma)	0.95 g
Distilled water	100 ml

Autoclave for 15 minutes at 121°C and store at 4°C.

0.1 M Calcium chloride

$CaCl_2.2H_2O$ (Sigma)	1.47 g
Distilled water	100 ml

Autoclave for 15 minutes at 121°C and store at 4°C.

Plasmids Mini-prep. Solutions

Lysis Buffer

1 M Tris-Cl, pH 8.0	2.5 ml
0.5 M EDTA	2 ml
Sucrose	15 g

Bring the volume to 100 ml with glass distilled water and dispense into small Gibco bottles.

0.5 M EDTA

Disodium Ethylendiamine Tetraacetate (Fisher Scientific)	186.1 g
NaOH (Fisher Scientific)	20 g
Glass distilled water	800 ml

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

0.2 M NaOH, 1% SDS

NaOH (Fisher Scientific)	0.8 g
Sodium Dodecyl Sulfate	1.0 g

Bring volume to 100 ml with glass distilled water.

3 M Sodium Acetate (pH 4.6)

$CH_3COONa.3H_2O$ (Fisher Scientific)	204 g
Distilled water	200 ml

Adjust pH to 4.6 with glacial acetic acid. Bring volume to 500 ml and dispense. Autoclave for 15 minutes at 121°C, and dispense 100 ml into small Gibco bottles.

Maxipreps DNA Purification

Cell Resuspension Solution

50 mM Tris-HCl, pH 7.5
10 mM EDTA
100 mg/ml RNase A

Cell Lysis Solution

0.2 M NaOH
1% SDS

Neutralization Solution

1.32 M Potassium acetate, pH 4.8

Column Wash Solution (concentrations prior to ethanol addition)

200 mM NaCl
20 mM Tris-HCl, pH 7.5
5 mM EDTA

Dilute with 95% ethanol. Final ethanol concentration will be approcimately 55%.