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Regulation of Human Papillomavirus Type 16 Late L1 mRNA Splicing

Xiaoze Li



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DOCTORAL DISSERTATION

By due permission of the Faculty Medicine, Lund University, Sweden.

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Abstract <p>Human papillomaviruses (HPVs) cause almost half of the human cancers that are attributable to viruses. HPV type 16 is the most carcinogenic type among the HPVs and is detected in 50% of all cervical cancers. HPV-16 infects epithelial cells and HPV-16 gene expression is tightly linked to the differentiation stage of the infected cells. Early HPV-16 genes are expressed in basal layers of the epithelium whereas the late genes which encode highly immunogenic viral structural proteins are only expressed in the suprabasal layers. HPV-16 infections are normally cleared within 18-24 months, but HPV-16 can establish persistent infections that progress to cancer. Such HPV-infected cancer cells express early HPV-16 genes but never expressed the late genes. We speculate that inhibition of HPV-16 late gene expression is a prerequisite for viral persistence and progression to cervical cancer.</p> <p>HPV-16 uses alternative splicing to regulate expression of early and late genes. HPV RNA elements and cellular factors control the expression level of viral proteins by regulating alternative splicing. This project was carried out to enhance our understanding of the regulation of HPV-16 gene expression, in particular at the RNA splicing level. The goal of this thesis was to identify viral RNA elements and cellular factors that regulate the processing of HPV-16 early and late mRNA splicing. These studies may also contribute to the identification of diagnostic biomarkers for premalignant infections at risk of progressing to cervical cancer.</p> <p>We identified a splicing silencer that interacts with hnRNP D proteins and hnRNP A2/B1 to suppress HPV-16 late gene expression in mitotic cells, including cervical cancer cells. We also characterized a splicing enhancer that promotes HPV-16 early gene expression, thereby indirectly inhibiting late gene expression. Mutation in this enhancer reduced its binding to the ASF/SF2 splicing factor. This resulted in decreased expression of the viral oncogenes E6 and E7 and a reduced ability of HPV-16 to immortalize human epithelial cells, thereby, linking HPV-16 mRNA splicing regulation to its pathogenic prospects. We also identified the hnRNP G protein binding to this enhancer and has opposite effects to ASF/SF2 on splicing matched by antagonism in RNA binding.</p>			
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List of Papers

This thesis is based on the following manuscripts, which are referred to in the text by their roman numerals:

- I. **Xiaoze Li**, Cecilia Johansson, Jacob Glahder, Ann-Kristin Mossberg and Stefan Schwartz. Suppression of HPV-16 late L1 5'-splice site SD3632 by binding of hnRNP D proteins and hnRNP A2/B1 to upstream AUAGUA RNA motifs. Nucleic Acids Research. 2013; 1-21
- II. **Xiaoze Li**, Cecilia Johansson, Carlos Cardoso, Anki Mossberg, Soniya Dhanjal, Monika Somberg, Stefan Schwartz. Eight nucleotide substitutions inhibit splicing to HPV-16 3'-splice site SA3358 and reduce the efficiency by which HPV-16 increases the life span of primary human keratinocytes. PloS One. 2013; 8 (9): e72776.
- III. **Xiaoze Li**, Jacob Glahder and Stefan Schwartz. (2013). Characterize the role of hnRNP G/RBMX protein in RNA binding and regulating the splicing of HPV-16 pre-mRNA.(submitted)

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Contents

Abbreviations.....	15
Introduction	1
Human papillomaviruses and cancer.....	2
Classification of HPVs	2
The HPV virion	4
HPV genetic organization and gene products	4
LCR	6
E5, E6 and E7 oncoproteins	6
E6.....	7
E7	7
E5.....	8
E1, E2, E8^E2 and E4 proteins.....	8
E1	8
E2	9
E8^E2.....	10
E4.....	10
L1 and L2 capsid proteins.....	10
The HPV infectious cycle.....	11
Regulation of gene expression.....	12
Transcriptional modulation of gene expression	12
Post-transcriptional regulation of gene expression	13
Capping.....	13
Polyadenylation.....	13
Splicing.....	13
Spliceosome assembly	17
Alternative splicing	17
Trans/cis-acting splicing factors.....	17
SR proteins regulate pre-mRNA splicing.....	18
hnRNP proteins regulate pre-mRNA splicing.....	19
Regulation of HPV-16 gene expression.....	19
Polyadenylation and splicing in HPV-16 gene expression.....	20
Cellular splicing factors in this thesis	22
hnRNP D family and hnRNP A2/B1	22
hnRNP G.....	22
ASF/SF2 (SRSF1)	23
Present investigation	24
Aims of the present study.....	24

Introduction to papers	25
paper I.....	25
paper II	26
paper III.....	26
Overall conclusions	28
Concluding Remarks and Future perspectives.....	29
论文摘要 (In Chinese)	31
Acknowledgement.....	32
References.....	34

Abbreviations

3'ss	3' splice site, splice acceptor
3'UTR	3'untranslated region
5'ss	5' splice site, splice acceptor
AdoMet	Adenosylmethionine
AP1	Activator protein-1
AP2	Activator protein-2
ASF/SF2	Alternative splicing factor/splicing factor2
Bak	Bcl-2 homologous antagonist/killer
BCC	Basal cell carcinoma
BPS	Branch point sequence
C/EBPs	CCAAT-enhancer-binding proteins
CDP/Cut	CCAAT displacement protein/human Cut protein
CFI/CFII	Cleavage factorsI/ cleavage factorsII
CIN	Cervical intraepithelial neoplasia
CPSF	Cleavage and polyadenylation specificity factor
CR	Conserved regions
CstF	Cleavage stimulation factor
CTLs	CD8+ cytotoxic T cells
DBD	DNA-binding domain
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
ESE	Exonic splicing enhancer
ESS	Exon splicing silencer
E6AP	E6-associating protein
E6C	E6- carboxyl-terminus
E6N	E6- amino-terminus
eUTR	Early untranslated region-Papillomavirus
GTP	Guanosine-5'-triphosphate
hDlg	Human disc large
hnRNP	Heterogeneous ribonucleoprotein
HPV	Human papillomavirus
HR	High-risk
ICTV	International Committee on Taxonomy of Viruses
IFN	Interferon
ISE	Intron splicing enhancer
ISS	Intron splicing silencer
KRF-1	Keratinocyte-specific transcription factor
IUTR	Late untranslated region
LR	Low-risk

LCR	Long control region
mRNA	Messenger ribonucleic acid
MAG1	Membrane-associated guanylate kinase, WW and PDZ domain-containing protein
MAPK	Mitogen-activated protein kinases
MHC	Major histocompatibility complex
NMSCs	Non-melanoma skin cancers
NRF	Nuclear factor kappa B-repressing factor
Oct-1	Octamer-motif-binding factor I
ORF	Open reading frame
Ori	Replication origin site
PAP	Polyadenylate Polymerase
PV	Papillomaviruses
pAE	Early polyadenylation signal- papillomavirus
pEarly	Early promoter- papillomavirus
pLate	Late promoter- papillomavirus
pAL	Late polyadenylation signal- papillomavirus
p53	Protein 53
PPT	Polypyrimidine tract
pRb	Retinoblastoma protein
pre-mRNA	Precursor messenger RNA
RB	Retinoblastoma
RBDs	RNA-binding domains
RCA	Rolling circle amplification
RNA	Ribonucleic acid
RS domain	Serine/arginine-rich domain
RRM	RNA recognition motif
SCC	Squamous cell carcinoma
SLIM	Site-directed, ligase-independent mutagenesis
Sp1	Specificity protein 1
snRNA	Small nuclear RNAs
snRNPs	Small nuclear ribonucleic particles
SR protein	Serine-arginine rich protein
TEF-1	Transcriptional enhancer factor
USP15	Ubiquitin carboxyl-terminal hydrolase 15
VLPs	Virus-like particles
YY1	Yin Yang 1

Introduction

Each year an estimated 530 000 new cases of cervical cancer are diagnosed and more than 275 000 women die from cervical cancer worldwide (2). Cervical cancer is caused by sexually-acquired infections with a subset of human papillomavirus (HPV) (359, 360). HPV is the most common viral infection of the anogenital tract (2). Almost all sexually active individuals will be infected by HPV at least once but probably multiple times during their lives. Most HPV infections do not cause any symptoms or disease as they spontaneously resolve, often within one to two years after infection. However, infections with specific types of HPVs (most frequently type 16 and 18) may persist and lead to precancerous lesions or cancer if they are not appropriately treated.

More than 40 types of HPVs are sexually transmitted and infect the anogenital region. HPV types that infect the genital mucosa are divided into two groups: High-risk (HR) types like HPV-16, 18, 31 and 33 that can cause genital, mouth, or oro-pharynx cancer, and low-risk (LR) types like HPV-6 and 11 that may cause warts, but are not found in malignancies. HR HPV-16 and 18 are the most frequently detected HPV types in cervical cancer (244).

Although vaccines against HR HPV-16 and 18, and LR HPV-6 and 11, are available nowadays, there is a large number of new cases of cervical cancer and deaths each year, especially in low and middle income countries (2). Vaccination is only freely available for girls aged 9-13 years in a few developed countries. Papanicolaou test and liquid-based cytology are two available methods in cervical cancer screening (44, 247). However, there are no efficient biomarkers or medicines available for diagnostic or treatment of HPV infections at risk of progressing to cancer. Therefore, it is important to fully understand the mechanism of HPV persistence in order to uncover novel biomarkers for disease, or targets for antiviral treatment. In this study, we have investigated how HPV-16 late gene expression is regulated at the level of RNA processing. These results will enhance our understanding of the ability of HPV-16 to hide from the immune system by using a highly regulated gene expression program. This is highly significant since establishment of HPV-16 persistence is one of the most important risk-factors for development of cervical cancer.

Human papillomaviruses and cancer

Virus-related cancer accounts for about 15% of all human cancers (244), half of which is attributable to human papillomaviruses (HPVs) (25, 244). Genital tract infection by HPV is the most common sexually transmitted virus infection (192). Decades ago, researchers postulated and identified the role of HPV in cervical cancer (198, 338, 357, 358). The first experiments to search for papillomavirus DNA in cervical cancers were performed in 1974 (361) and papillomavirus was identified in cervical smears in 1976 (208). The human papillomavirus family were shown to be heterogeneous (102, 103, 241), and various HPV types were isolated from various genital cancers, genital warts and laryngeal papillomas (101, 104). 60-65% of vaginal cancers, 20-50% of vulvar cancers and about 40-50% of all penile cancers were found to contain HPV-DNA (1, 244). High prevalence of HPV-DNA are also found in oro-pharynx cancers, head and neck cancers, and anal cancers but the strongest correlation between HPV and cancer was observed for cervical cancer as it has been shown that 99.7% of all cervical cancers contain HPV (2, 244).

Cervical cancer is the second most common cancer in women worldwide and causes about 15% of all female cancers (86). The first HPV type isolated from cervix cancer was HPV-16 (79, 359). A role for HPVs in cervical cancer was strengthened when it was shown that specific viral genes, the E6 and E7 genes, were always detected in cervical cancer cell lines and cancer biopsies (218, 273). Furthermore, the E6 and E7 oncogenes could immortalize human primary cells (78, 254). It was later shown that E6 and E7 inhibit tumor suppressor proteins p53 and pRb, respectively (221, 359), thereby preventing apoptosis and inducing cell proliferation. Some HPV types establish persistent infections that may progress to cervical cancer as well as other anogenital cancers and a subset of head and neck cancers (359). It is therefore important to investigate how HPV regulates gene expression to establish persistence.

Classification of HPVs

HPVs are commonly classified as cutaneous or mucosal based on the location of the lesion in which the HPV virus is consistently identified. Cutaneous HPV infections are ubiquitous (9), but most infections have no clinical symptoms and are spontaneously cleared by the immune system within months. Some cutaneous

HPV types may cause benign lesions, such as HPV types 2 and 27 that are frequently found in common warts; or HPV types 1, 2, 3 and 4 that cause plantar warts. Some cutaneous HPV types contribute to premalignant or malignant cutaneous lesions such as melanoma and non-melanoma skin cancers (NMSCs), including squamous cell carcinoma (SCC) (321) and basal cell carcinoma (BCC) (5). For example, HPV-17 and 38 are found in malignant melanoma (268) and HPV-96, 94 and 92 are found in SCC or BCC (68, 89, 268, 321). Mucosal HPV types infect epithelial cells in genital or oral mucosa. Forty seven mucosal HPV

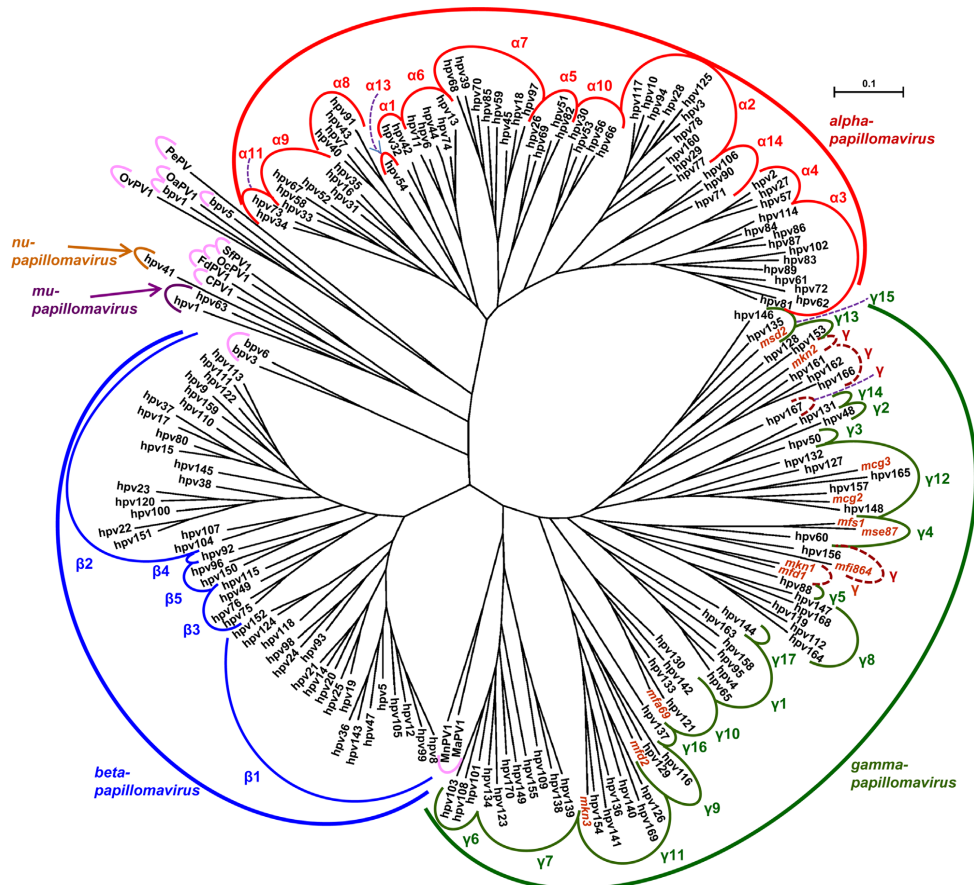


Figure 1. Phylogenetic analysis based on the L1 ORF sequences of 170 HPV types. Adapted from (25, 67).

types have been identified so far (41), and of those, about 12 are carcinogenic to humans, including HPV-16, 18 and others (136). These HPVs are termed HR HPV types (29). Other mucosal HPV types such as HPV-6, 11 and 61 may cause benign tumors, mostly genital warts (41). These HPVs are termed LR HPV types (29). Persistence of HR HPV infections is the most significant risk factor for

cervical cancer (29, 30, 222, 359) and may also cause cancer of the uterine cervix, vulva, vagina, penis, oropharynx or anus (244).

The L1 ORF has been used to identify new types of papillomaviruses (PV) since the L1 ORF is the most conserved gene within the genome (68). A new PV type must be completely cloned and the DNA sequences of the L1 ORF must have less than 90% similarity to the closest known HPV type (68). The Greek alphabet is officially used for the systematic naming of PVs by the International Committee on Taxonomy of Viruses (ICTV) (68) (Fig. 1). About 174 HPV types have been identified to date and classified into genus, species, type, subtype, and variant (68). Variants have less than 2% sequence difference in the L1 ORF (68). Based on the comparison of L1 genes, the HPVs are classified into alpha, beta, gamma, nu and mu genera (41, 67, 68, 90) (Fig. 1). Only HPV types in the alpha and beta genera can cause malignant lesions in mucosal and cutaneous epithelium, while HPVs in the other genera are benign (68). The alpha genus contains all high-risk mucosal HPV types, for instance HPV-16 and 18 that are the most frequently detected HPV types in cancer (187). HPV-16 was investigated in this thesis.

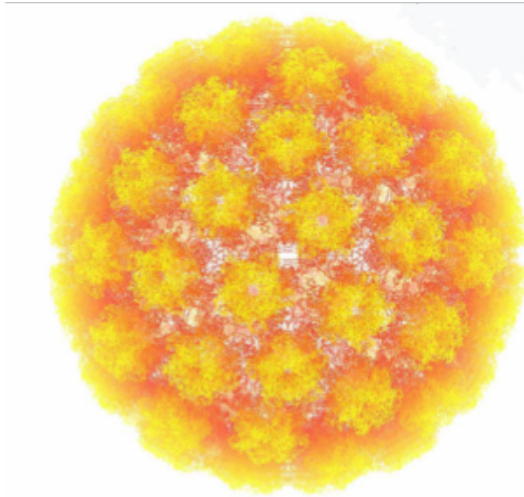
The HPV virion

HPVs are non-enveloped viruses. The capsid has icosahedral symmetry and is approximately 55-60 nm in diameter (Fig. 2A). The viral genome is about 7.9 kb in length and consists of circular double-stranded DNA. It encodes the early genes E6 and E7 that maintain viral DNA replication competence of the cells, early and late genes E1, E2, E4, E5, and E8 that modulate viral DNA transcription, replication and late functions, and the two late genes encoding the L1 and L2 capsid proteins. The genome also contains a long control region (LCR) that contains the viral replication origin (ori) and a promoter with multiple binding sites for viral and host cell proteins (29, 127, 144, 267) (Fig. 2B).

HPV genetic organization and gene products

All HPVs have a common genetic organization (Fig 2B). The viral genome is divided into two parts by the virus early promoter, differentiation-dependent late promoter and two polyadenylation signals that are coordinately regulated during cell differentiation (Fig. 3). The Early promoter (pEarly) controls E6 and E7 oncogene expression assisted by the early polyadenylation signal (pAE) that is

A.



B.

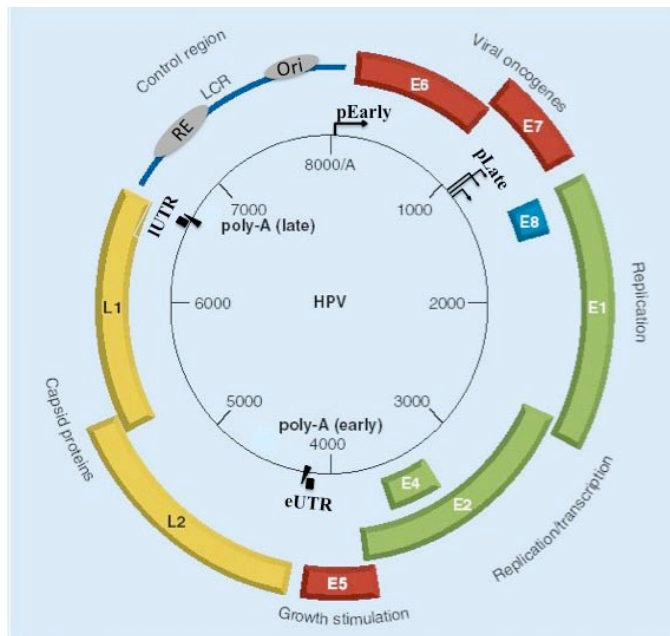


Fig 2. Human papillomavirus (A). assembled virion (Adapted from virology.wisc.edu/virusworld). (B). Schematic presentation of the HPV genome. Ori: The replication origin, RE: transcriptional regulatory elements, LCR: Long control region. pEarly: virus early promoter, pLate: differentiation-dependent late promoter, eUTR: early 3' untranslated region, IUTR: late 3' untranslated region. Open reading frames (ORFs) are colored to indicate at which phase of the virus life cycle each is most highly expressed (See Figure 3).

preceded by the 3'UTR (3'untranslated region, eUTR). As infected cells proliferate and differentiate, E1, E2, E4, E5 and E8 genes are expressed for both early and late promoters (pEarly and pLate) (Fig. 3 and 8). The late L1 and L2 genes are expressed from the late promoter (pLate) that produces late mRNAs that are terminated at the late polyadenylation signal (pAL), which is preceded by the late 3'UTR (lUTR) (29, 144) (Fig. 2B).

LCR

The HPV LCR is approximately 850 bp in size (Fig. 2B). It controls viral gene expression and viral replication through its multiple transcription factor binding sites and ori (6, 151, 186, 253, 285, 325). LCR variants within a HPV type may differ by up to 5 % (68). Transcription of E6 and E7 oncogenes is regulated by cellular and viral factors binding to pEarly in the LCR. Variations in HPV-16 and HPV-18 LCR could also affect ori function (6, 131). Nucleotide variation in LCR therefore affects viral oncogenic potential and biological properties (85).

Many cellular transcription factors that bind to the LCR of HPV and regulate the activity of the E6/E7 promoter have been identified, for example, KRF-1 (192), AP-1 (279), AP-2 (277), YY1 (18), Oct-1 (297), Sp1 (125), TEF-1 (139), CDP/Cut (235), and C/EBP (19). Mutations in the LCR that increase expression of the E6/E7, could potentially affect the carcinogenic process (253). Naturally occurring mutations in the LCR also affects the binding of the E2 protein that regulates viral transcription in trans (212). European LCR variants of both HPV type 16 and 18 have lower transcriptional activities than Asian-American variants (151, 324, 325). Both Asian-American and European LCR variants of HPV-18 have higher transcriptional activity than African variants (6).

E5, E6 and E7 oncoproteins

HR HPV E6 and E7 oncoproteins are well known to interfere with the cellular tumor suppressor protein p53 and retinoblastoma protein (Rb) to induce cell transformation and resistance to terminal cell differentiation (78, 234, 263, 294, 320). These proteins drive cell immortalization and the carcinogenesis process (29, 221, 226) and are consistently expressed in malignant cells. In addition to promoting cell growth and inhibit apoptosis, E6 and E7 may also interact with host immune functions (31, 220). Several beta genus HPV E6 and E7 also have transforming and immortalization activities in several experimental models (3, 42, 94, 126, 223, 326, 331). Inhibition or depletion of E6 and E7 halted cell division of differentiation-resistant E6 and E7 expressing transformants (69, 221), whereas overexpression of these proteins dramatically increases transforming activity (14,

118). HPV E5 may also be an oncoprotein since E5 alone can induce cancer in transgenic mice (197). The E5 protein may therefore be involved in cervical carcinogenesis by cooperation with E6 and E7 (34, 73, 299, 318, 323). Another mechanism that may contribute to HPV induced tumorigenesis is alleviation of the repressive effects of E2 on E6 and E7 via viral integration into host chromosomes that deletes E2 (216).

E6

HPV E6 is an approximately 150 amino acid-protein containing two zinc-finger domains (N-terminal and C-terminal) (180, 230, 231). HPV E6 is well-known for its ability to degrade the tumor suppressor protein p53 (340) by binding to p53 and the E3 ubiquitin ligase E6-associating protein (E6AP) (48, 133, 134, 266). Degradation of p53 is necessary for malignant progression (216) and is activated by E6 only when E6AP and p53 bind to E6 (154, 341). Both E6 N-terminal and C-terminal domains are needed for p53 degradation (340). The HPV E6 N-terminal self-association is also required to promote degradation of p53 (96, 233, 339-341). LR HPV E6 also self-associates via its N-terminal domain and binds to p53, even though it cannot degrade p53 (36, 314). HR HPV E6 is involved in several E6AP-mediated pathways that block apoptosis, alter the transcription machinery, interfere with cell-cell interactions (an important step towards malignancy), increase life span of cells, and promote cell immortalization (46, 161, 173). E6 may also degrade p53 independently of E6AP ligase activity (43, 195, 232, 278). E6 proteins are ubiquitinated and degraded by the proteasome (298), but are stabilized by E6AP (316). HPV-16 E6 may also contribute to carcinogenesis by interacting with cellular proteins containing PDZ domains (115, 313), for example, human discs large (hDlg) (160), the MAG1 family of proteins (105, 312), the proapoptotic protein Bak (311), c-Myc (116) and the deubiquitinating enzyme USP15 (327).

E7

The E7 protein is primarily located in cell nucleus and associates with the hypophosphorylated pRb to facilitate cell cycle transition into S phase by preventing pRb binding to E2F (359). E7 also mediates ubiquitinated-degradation of pRb (143). In addition to binding to several pocket proteins (pRb, p107 and p130), HPV E7 also directly interacts with many additional cellular targets (219) to maintain the episomal HPV DNA and to promote cells amplification (55, 88, 206). E7 has three conserved regions (CR): CR1, CR2, and CR3 (252). The CR1 and CR2, which are located in the N-terminal domain of E7 protein (95), have pRb binding affinity and are associated with pRb destabilization. These regions are indispensable for early viral activities (122, 147). For example, CR1 competes with E2F transcription factors and is involved in pRb destabilization. CR2 is required for cell transformation (81). The CR3 region which is located in the C-terminal zinc finger domain and has high binding affinity for pRb (246), inhibits

pRb function by cooperating with the CR1 and CR2 regions (228). HR HPV E7 is expressed in infected epithelium and is associated with cell replication, immortalization, and carcinogenesis (219, 228). HPV-16 E7 also inhibits IFN- γ -mediated enhancement of major histocompatibility complex (MHC) class I antigen processing (58, 171) and T-cell-induced target cell lysis (175, 319, 350, 351), thereby allowing HPV to persist in the presence of a functional immune system. Vaccines containing HPV-16 E6 and E7 epitopes for MHC class I human alleles could potentially prevent HPV-induced tumor formation (215).

E5

The HR E5 proteins are small, hydrophobic peptides, approximately 83 amino acids in length that primarily exist at the endoplasmic reticulum (ER), Golgi apparatus and nuclear membrane (57, 74, 75, 99, 304). Tumors with episomal HPV-16 may have more aggressive properties (323), since E5 may only be produced from episomal HPV DNA. HPV E5 could potentially promote cell proliferation and induce viral replication (216, 260). The N-terminus of E5 is bound to the ER lumen, whereas the C-terminus is exposed in the cytoplasm (162) where it interacts with cytoplasmic- and ER- proteins (11, 49, 57, 117, 170, 240, 262). HPV E5 is involved in various cellular pathways, primarily at the early stage of cervical carcinogenesis (156). HPV-16/18 E5 can enhance the percentage of cells in the S phase (178). HPV-16 E5 also inhibits TRAIL- and FasL-mediated apoptosis (149) and downregulates CD1d-mediated innate and adaptive immunity (149, 209, 323). Overexpression of HPV-16 E5 significantly suppresses cancer-related proteins (269, 309) and down-regulates tumor suppressor proteins (248, 317, 323). Moreover, HPV-16 E5 reduces the cell surface levels of MHC class I (11). The same effects were observed in CIN lesions (59). The HPV-16 E5 protein may induce viral integration and accelerate aggressive cervical carcinogenesis via stimulation of the type I IFN response and activation of antiviral genes (224, 250). Some animal studies have shown that HPV-16 E5-adenovirus vectors as tumor vaccines inhibit tumor development in mice by reducing the growth of tumors through induction of CD8⁺ cytotoxic T cells (CTLs) (181). The HPV DNA genome usually integrates and the E5 gene is lost during tumor progression (29, 323). Therefore, the role of HPV E5 in the viral life cycle is still poorly understood (216, 360).

E1, E2, E8^{E2} and E4 proteins

E1

E1 and E2 play an important role in initiating replication of episomal HPV DNA (60). E1 protein is expressed at the early stage of the viral life cycle. E1 initiates viral DNA replication via binding to the viral origin of replication. The HPV E1

consists of three functional domains: an N-terminal regulatory domain, a DNA-binding domain (DBD), and a C-terminal ATPase/helicase domain (97, 132, 275, 315). E1 binds to the HPV DNA origin with very low specificity. In contrast, E2 binds HPV DNA with high specificity. Binding of E1 to E2 therefore increases the specificity and efficiency of recognition of the HPV DNA (47). In addition, E1 can recruit cellular replication factors to the viral origin by interacting with DNA polymerase alpha-primase, topoisomerase I and the single-stranded DNA binding protein RPA to form a replication complex (56, 185, 196, 243, 300). Integration to human genome usually disrupts/deletes the E1 and E2 ORFs (10, 60) and results in dysregulation of viral gene expression and replication. This dysregulation might contribute to the progression of cervical neoplasia to invasive cancer (10, 128).

E2

The full-length E2 gene produces a 45-48 kDa protein which is consistently expressed at early and intermediate stages of the viral life cycle. The E2 protein is mainly located in nucleus (130), with the exception of one report that shows nuclear-cytoplasmic shuttling of HR E2 proteins (27). E2 protein contains a conserved N-terminal “transactivation” domain and a C-terminal DNA binding/dimerization domain connected by a flexible linker called the “hinge” (100, 203). The hinge region of the E2 protein is not well conserved either in sequence composition nor in length (100, 201). E2 is a multifunctional protein and is involved in several viral processes by associating with viral or cellular proteins (22, 144, 200, 202). E2 protein is the major transcriptional regulator in PVs (51, 61, 167, 291) and acts by specifically binding to PV genomes and recruiting cellular factors to the viral genome (35, 93, 229, 274, 286, 336). E2 protein regulates viral transcription mostly by silencing the promoter that controls expression of viral E6 and E7 oncogenes (286). Restoration of E2 expression leads cells to senescence in HPV-associated cancer cells that depend on E6/E7 expression (71, 77, 109). E2 protein also supports and loads E1 onto the replication origin to initiate viral DNA replication (91, 211, 265). E2 is involved in the RNA post-transcriptional regulation by promoting HPV late gene expression (145). In transgenic mice, expression of the HPV-8 E2 protein in skin results in the development of skin tumors, demonstrating that HPV-8 E2 may play an important role in cell transformation (174, 251). Moreover, E2 links viral genomes to cellular chromosomes to ensure that viral genomes are segregated in approximately equal numbers in daughter cell nuclei (138, 163, 164, 172). In a few cases, E2 protein can regulate cellular gene expression (146, 328) and enhance genome-packaging into virions (344). The hinge region of E2 proteins of different HPVs can promote nuclear localization, link E2 to nuclear matrix, and interact with cellular proteins (166, 276, 355). The stability of E2 protein can be modulated by phosphorylation (276), binding to E4 (66), and interaction with E1 (158).

E8^{E2}

All HPVs have the potential to encode shorter E2 forms which may play a role in the control of viral DNA copy number and in the stable maintenance of HPV episomes, as well as transcription and replication (7, 165, 302, 354). For example, alpha genus HPVs produce E8^{E2} proteins that contain a short E8 peptide fused to the entire hinge and DNA binding domain of E2 (301) (Fig. 8) that are strong repressors of viral transcription and replication (87, 302, 354).

E4

The PV E4 ORF overlaps the E2 ORF. The most abundant products of E4 transcripts are E1^{E4} proteins which contain five amino acid residues of E1 at N-terminus of E4 (76) (Fig. 8). The E1^{E4} protein is hereafter called E4, E4 proteins are intensely expressed at differentiated layers of the infected lesions suggesting that E4 protein can serve as a useful biomarker for HPV-specific infection (114, 120, 296). E4 is likely to have many functions in the viral life cycle since it is the most abundant protein in the mid to upper layers of the epithelium. Suggested functions of E4 are induction of G2/M cell cycle arrest, RNA processing (21, 272), and association of mitochondrial functions (256). E4 also appears to be involved in genome amplification and virus assembly (76). E4 is required for viral late gene expression, but it is not known how (225, 249, 334). In HR HPV-16 and HPV-31, but not in HPV-18, E4 is involved in maintenance of viral genome (225, 335). Phosphorylation of HPV-16 E4 promotes the interaction with cytokeratins (332).

L1 and L2 capsid proteins

Late L1 and L2 genes which encode the major L1 and minor L2 viral capsid proteins, are only expressed in the upper most layers of the epithelium (13, 144). Expression of L1 and L2 is controlled at the level of transcription by the late differentiation-dependent promoter p670, and at the level of RNA processing by viral and cellular factors, including HPV E2 (144, 145) and cellular SR proteins (287) and hnRNPs (50, 177).

The viral capsid is a T=7 icosahedral lattice containing 360 copies of the L1 protein and associated with 37-72 copies of the L2 protein (37, 205). L1 can spontaneously self-assemble into capsid structures that lack L2 and the viral genome. The viral particles are released from the top of the epithelium. Expression of L1 alone in eukaryotic cells results in virus-like particles (VLPs) with similar morphology and immunogenicity as native virions (159). VLPs of L1 is the component in prophylactic vaccines against HPV infection (45, 205).

The HPV infectious cycle

The HPV life cycle is unique and differs from most other virus families in that HPV only infects proliferating epithelial cells and that the virus life cycle is highly linked to the differentiation of the host cells (157, 359). HPV infection preferentially initiates at basal epithelial cells through microwounds. Infection often occurs in the transformation zone. In basal layer cells, only early genes (such as E6, E7) are expressed to enhance cell proliferation and lateral expansion (Fig. 3). The early to late genes (such as E2 and E4), which control replication and transcription of viral DNA, are expressed in proliferating cells. The late genes are only expressed in superficial layers and is followed by production, assembly, and release of viral particles in the upper layers of the mucosa (29, 359) (Fig. 3).

Viral capsids initially bind to the basement membrane of infected cells which is followed by entry into basal cells and uncoating of the viral genome. Expression of E6 and E7 promote cell division to maintain an intracellular environment that is permissive for HPV DNA replication. E7 binds to tumor suppressor protein pRb and induces the expression of DNA replication factors (for example DNA polymerase, thymidine kinase, and cdc6) by liberating E2F transcription factors

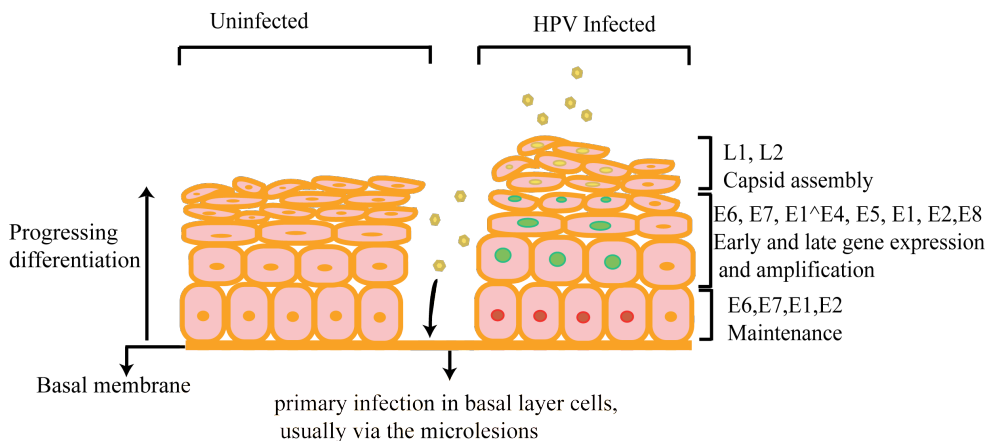


Figure 3. Structure of the cervical epithelium and human papillomavirus life cycle. Nuclei are colored to indicate the different viral proteins expressed during the life cycle (See Figure 2).

(220). E7 also induces the tumor suppressor protein p53 to activate DNA repair proteins, arrest cell growth and initiate apoptosis (220), whereas E6 inhibits p53 activity by binding to p53 and induces E6AP-mediated p53 degradation (98). Expression of E1 and E2 link the episomal viral genome to the cellular DNA polymerase and result in viral DNA replication (144, 219, 295). E1 initiates viral DNA replication by binding to early promoter p67 associated with E2. The E2

protein controls the early viral promoter to regulate expression of E6, E7, E1, as well as E2 itself (91, 100, 325). The HPV genome is present at about 50-200 episomal copies per cell in undifferentiated cells (29, 216). Viral protein production is low in undifferentiated cells for immune evasion purpose and viral persistence (359). As cells differentiate, the late viral promoter is activated to produce high levels of viral proteins, in particular E1, E2, and E4, and viral copies increase to thousands per cell (293). Both E4 and E5 play essential roles at the late stage of viral infection (334). The capsid proteins L1 and L2 are highly immunogenic proteins and are expressed in the superficial level of the epithelium (144, 270, 271). Then virions assemble. Virus delays the L1 and L2 late gene expression to avoid the host defense systems. For instance by using differentiation dependent gene expression program and by switching from early to late polyadenylation signals (270).

In HR HPV infected cancer cells, the viral genome may integrate into the host genome with disruption/deletion of the E1, E2 and E5 genes as a consequence (199). Therefore, high levels of E6, E7 genes are expressed which facilitates progression to cervical neoplasia and invasive cancer (29, 144). The immunogenic L1 and L2 proteins are never expressed in cancer cells.

Regulation of gene expression

Regulation of gene expression is important for a cell to produce the exact amount of functional gene products when they are needed. It is for example vital for cells to respond to external and internal signals when the environment changes, or for example for cellular differentiation. Gene regulation includes transcriptional modulation, post-transcriptional regulation and translational modification. Post-transcriptional regulation is executed on processes that convert precursor RNA (pre-mRNA) into mature mRNA and is essential to control the levels of mRNAs that are made available for translation. Post-transcriptional regulation may occur in the cell nucleus and mainly target three pre-mRNA processing events: 5'-capping, 3'-polyadenylation, and RNA splicing.

Transcriptional modulation of gene expression

Transcription is the first step of gene expression which occurs in the nucleus and begins with the binding of RNA polymerase to the promoter in DNA (40). The TATA box is a common core promoter in eukaryotes.

Transcription factors play important roles in modulation of transcription. For example, TATA-binding protein and Transcription Factor II D bound TATA box to form a preinitiation complex. Transcriptional activator factors can specifically bind to DNA sequence and stimulate transcription(92, 238).

Post-transcriptional regulation of gene expression

Capping

5' cap is a specially altered nucleotide on the 5' end of pre-mRNA. The process of 5' capping is important as the cap structure stimulates mRNA splicing and polyadenylation. It is also recognized by the protein synthesis machinery (52). Capping occurs soon after transcription initiation when the nascent RNA is 25-30 nucleotides in length (148, 258). The capping reaction undergoes three steps (210): first, the 5' γ -phosphate end of the nascent RNA molecule is removed by RNA triphosphatase. Then, mRNA guanylyltransferase form a covalent enzyme-GMP intermediate with GTP to transfer GMP onto the 5'-diphosphate end. Finally, S-adenosylmethionine synthetase methylates the cap at position 7 of the terminal guanosine (4).

Polyadenylation

In eukaryotes, polyadenylation is a part of the process that produces mature mRNA. It begins as the transcription passes the polyadenylation signal. First, cleavage and polyadenylation specificity factor (CPSF) binds to a polyadenylation signal sequence 5'- AAUAAA-3' located near the 3' end of the pre-mRNA molecule. Cleavage stimulation factor (CstF) binds to a GU-rich sequence presented downstream of the polyadenylation signal sequence (Fig. 4A). This protein complex recruits additional cleavage factors (CFI/CFII) and the enzyme Polyadenylate Polymerase (PAP) to cut the RNA at the cleavage site (5'-CA-3') located between the polyadenylation sequence and the GU-rich sequence (Fig. 4A). PAP then adds 200-300 adenosine units to the 3' end of the new RNA molecule, followed by binding of multiple copies of poly(A) binding protein (PABII) to protect the 3'-end from ribonuclease digestion (Fig. 4B).

Splicing

Pre-mRNA splicing was first described in adenovirus late mRNAs (23, 24, 53). Later, it was found that splicing is a common event and that the majority of all cellular mRNA are spliced. Splicing is required to remove non-coding regions called introns and join protein coding sequences called exons to form a single continuous mRNA molecule (Fig. 5). Introns typically have a GU at their 5' ends

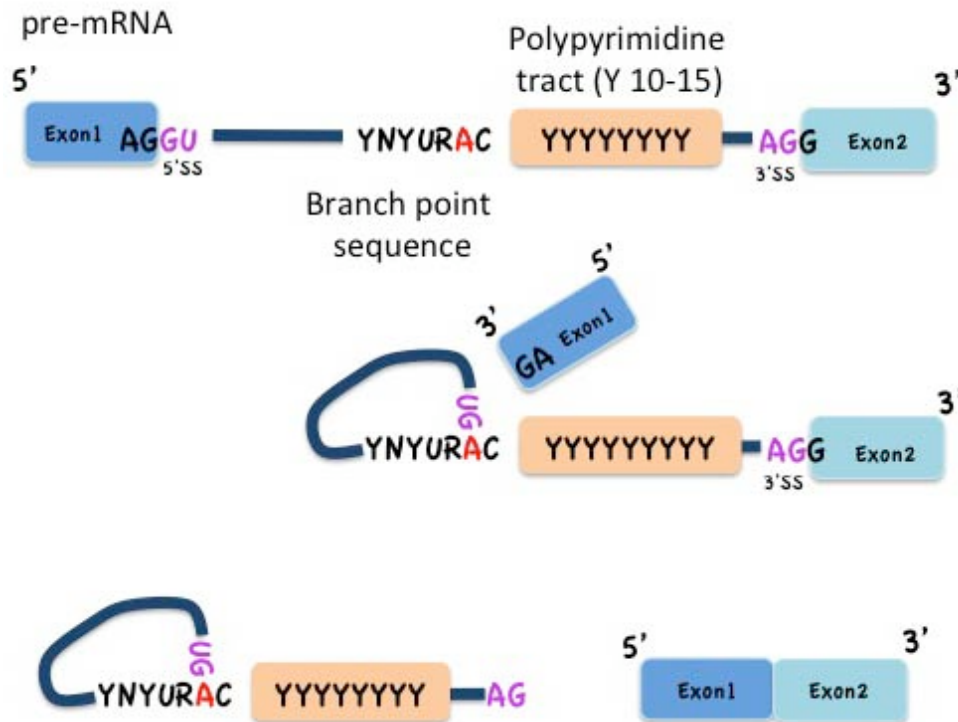


Figure 5. Schematic diagram showing the structure of pre-mRNA(top), and the mechanism of pre-mRNA splicing reaction (middle to bottom).

eukaryotes, pre-mRNAs undergo splicing to produce mature mRNAs before they are transported to the cytoplasm for translation. In prokaryotes, splicing is a rare event and occurs in non-coding RNA. In yeast, approximately 5% of genes contain one or two intron(s) which undergo splicing (80). Splicing is catalyzed by the spliceosome, which is a complex of small nuclear RNAs (snRNA) and a range of associated protein factors that recognize splice sites, PPT and BPS. The 5'ss is cut first and the GU end of the 5'ss binds to the BPS. Then, the 3'ss is cut, followed by ligation of the two exons (Fig. 5 middle and bottom) (182). There are two types of splicing: constitutive and alternative splicing. Constitutive splicing removes introns and joins exons together to form mature mRNAs and alternative splicing alternatively joins exons from multi-exon pre-mRNAs to produce different mRNA isoforms that may encode different proteins. Many diseases have been found that are caused by splicing deficiencies in humans (15, 28, 189). Recent experiments suggest that correction of defective splicing could become new way to treat Genetic diseases (15, 28, 189).

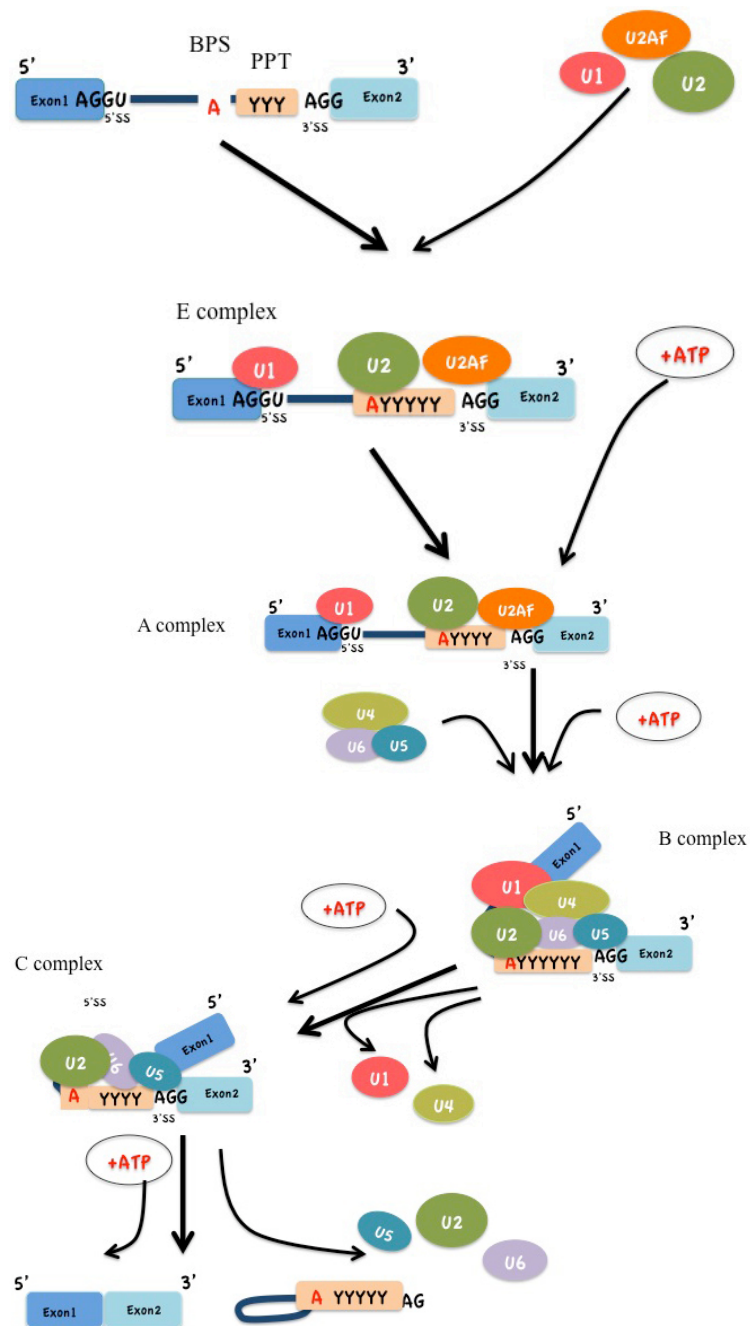


Figure 6. Schematic representation of the splicing process and spliceosome assembly.

Spliceosome assembly

A transcribed pre-mRNA needs a spliceosome to catalyze the removal of introns and to ligate the flanking exons. There are five snRNAs (U1, U2, U4, U5, and U6) that make up the major spliceosome. Combined with protein factors, they make RNA-protein complexes, called small nuclear ribonucleic particles (snRNPs) (8). Spliceosomes assemble snRNPs onto the pre-mRNA (330). First, U1 snRNP binds to the 5'ss with other non-snRNP associated factors to form the early (E) complex (64, 141) (Fig. 6). Then, U2 snRNP binds to BPS with the E complex component U2AF (U2 snRNP auxiliary factor) and possibly U1 snRNP in an ATP-dependent manner forming the A complex (Fig. 6). Next, the U4/U5/U6 tri-snRNP is recruited to the pre-spliceosome to form the B complex with U6 and U2 forming the U2/U6 helix II (Fig. 6). Then, with several rearrangements, the B complex transfers the 5'ss from U1 to U6 and releases both U1 and U4 snRNPs, leading to complex C. The U5 snRNP is thought to align the two exons via the invariant loop of U5 snRNA (8, 204) (Fig. 6).

Alternative splicing

Alternative splicing is a common phenomenon occurring in eukaryotes. It splices a single pre-mRNA into a range of unique mRNAs, which encode different protein sequences that might have different biological functions. This is achieved by varying the exon composition of the mRNA. About 95% of multiexonic genes are alternatively spliced in humans (242). Alternative splicing of some genes occurs under specific cellular conditions and/or in a tissue-specific manner (242). Five types of alternative splicing have been detected so far, including intron retention, exon skipping (or cassette exon), mutually exclusive exons, alternative 5' splicing (alternative donor site), and alternative 3' splicing (alternative acceptor site) (Fig.7).

Trans/cis-acting splicing factors

Competition between the splice sites occurs and different splice sites are used with different efficiencies (193). Alternative splicing is regulated by cis-acting RNA splicing elements (enhancers and silencers) on the pre-mRNA transcript that are bound by trans-acting splicing factors (activators and repressors) (352). These trans/cis-acting splicing factors induce or reduce the usage of a particular splice site (32, 33). Regulation of alternative splicing plays an important role in gene expression (32, 33, 39). Various human diseases, including cancer, have been linked to perturbed alternative splicing (65, 150, 352). There are exonic splicing enhancers (ESE), exonic splicing silencers (ESS), intronic splicing enhancers (ISE), and intronic splicing silencers (ISS). The major cellular trans-acting splicing factors are proteins from the serine/arginine rich protein family (SR proteins) (112, 123) and the heterogeneous nuclear ribonucleoproteins (hnRNPs) (39, 119, 271). Although many SR proteins act as splicing activators (15), and

hnRNPs as repressors (179) by binding to pre-mRNAs, it has been shown that trans/cis-acting factors regulate splicing in both time and position-dependent manners (179). For example, exon sequences that act as enhancers may also inhibit splicing if they are moved into an intron (111). Furthermore, a splicing activator protein induces splicing when it is bound to an intronic enhancer, served as a repressor when it was bound to an exon sequence (137, 176, 179, 207, 287, 288).

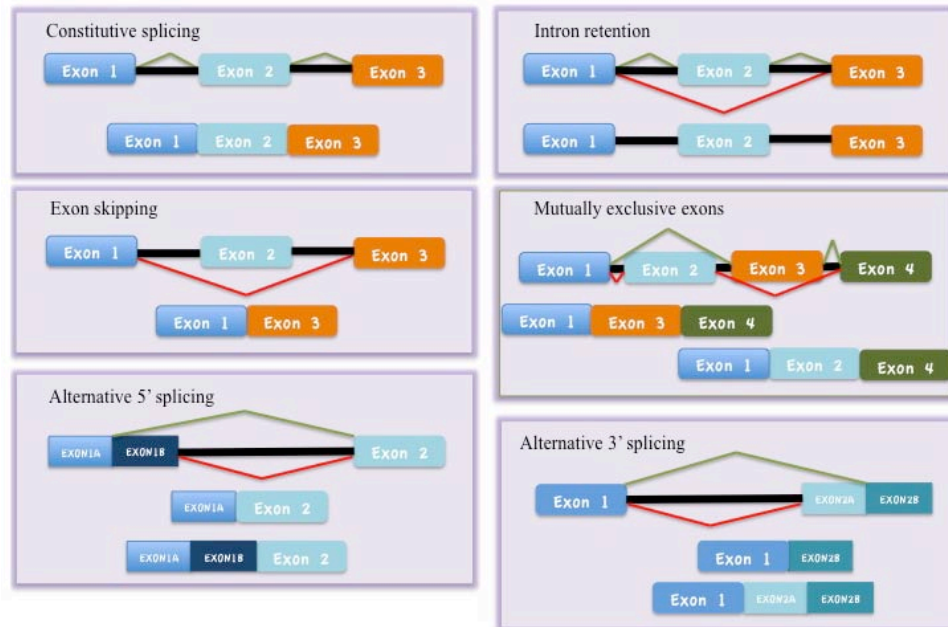


Figure 7 Schematic representation of constitutive splicing and five types of alternative splicing patterns..

SR proteins regulate pre-mRNA splicing

SR proteins are splicing control factors involved in both constitutive and alternative splicing (292), and may play a role in the evolution of splicing (38, 257). SR proteins also interact with the cap-binding complex and the polyadenylation machinery to regulate splicing (63, 292). SR proteins are highly conserved splicing regulators which contain an RNA recognition motif (RRM) and a serine/arginine-rich domain (RS domain) (184, 338). SR proteins are RNA binding proteins containing RRM as certain hnRNP proteins (39, 308). But the RS domain is unique for SR proteins and separates SR proteins from other RNA binding proteins. Although SR proteins have different RNA binding specificities and many different functional properties, they also have common properties in the

splicing process (352). SR proteins generally bind ESE sequences to enhance exon inclusion during splicing, either by antagonizing the effect of negative regulators such as hnRNP proteins, by promoting splicing factors (such as U1/U2snRNPs) to splice sites, or by regulating spliceosome assembly. SR proteins also have the ability to induce exon skipping by binding to ESE sequences (176). Recently studies showed that some SR proteins suppress splicing (280, 281). For example, SRp38 acts as a splicing repressor in mitotic cells and in response to heat shock (280, 281).

hnRNP proteins regulate pre-mRNA splicing

The hnRNPs are primarily nuclear proteins that contain RNA-binding domains (RBDs), except for hnRNP U. Many of them also contain RGG boxes (repeats of Arg-Gly-Gly tripeptides) or glycine-rich, acidic or proline-rich domains. Like SR proteins, hnRNP proteins regulate pre-mRNA splicing by binding directly to the target RNA through the RBD domains. After binding to the pre-mRNA, hnRNPs promote protein-protein interactions via the RGG domains, or glycine-rich, acidic or, proline-rich domains (39). Unlike SR proteins, hnRNPs are only known to interfere with some steps of the splicing process. For example, they repress spliceosomal assembly (353), block the recruitment of snRNPs, or interfere with splicing by looping out exons (194). It has been reported that the function of hnRNPs in splicing depends on their location on pre-mRNA and that they can both repress and activate splicing (39, 119).

Regulation of HPV-16 gene expression

HPV-16 initiates the infection in the basal layer of epithelium. The viral genome migrates to the cell nucleus after uptake of the virion by endocytosis (144, 305). In the basal epithelium, HPV-16 uses the cellular transcription machinery to activate viral transcription from the early promotor p97 (143, 144). All mRNAs generated from this stage undergo different patterns of alternative splicing and are polyadenylated at pAE (Fig. 8) (144, 270, 271). Many viral and cellular factors are involved in these processes (270, 271). Cellular factors KRF-1 (192), AP-1 (279), AP-2 (277) and YY1 (18) regulate viral transcription activity. E1 and E2 bind to the viral LCR sequence and initiate replication (47, 60). As cells differentiate and leave the basal layer, the differentiation-dependent promotor p670 is activated and will be used to produce high levels of E4, E2, and probably E7, and subsequently promotes expression of the L1 and L2 genes. All mRNAs encoding the early genes, such as E4, E1, and E2 are polyadenylated at pAE (144, 270, 271). Whereas mRNAs encoding the late genes L1 and L2 are polyadenylated at pAL. There is an abundance of regulatory factors linked to these processes (12, 17, 270, 271). For

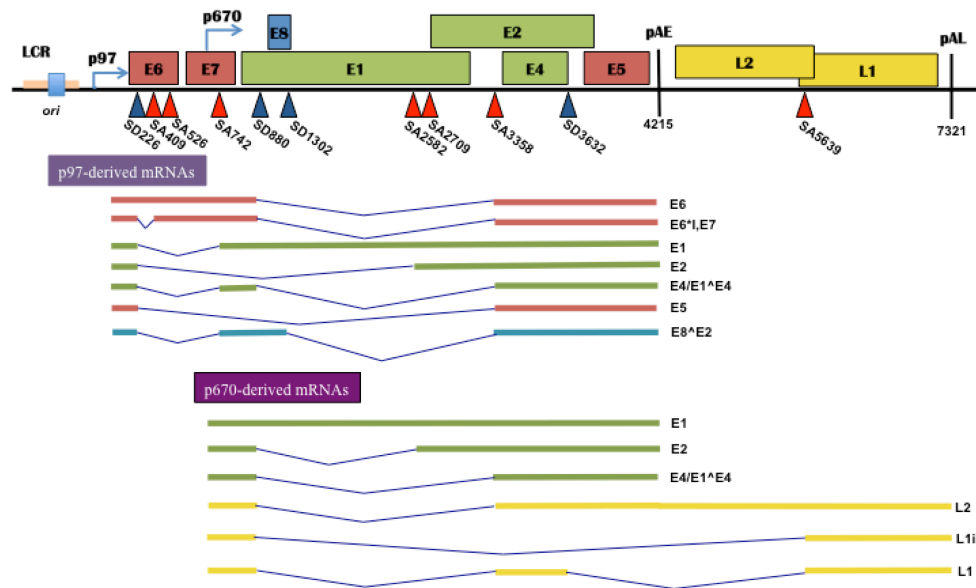


Figure 8. The HPV-16 genome shown linerised above, encoding early (E) genes from E1-E8, and late (L) genes L1 and L2. The LCR is located before p97 and contains the DNA replication origin (ori). The Early promoter p97 and the late promoter p670 are shown. Two polyadenylation signals are present, pAE and pAL. All known splice sites are indicated (blue triangle for splicing donor, red triangle for splicing acceptor). A subset of the alternatively spliced mRNAs are indicated separated as p97- and p670- derived. All the genes and mRNAs are colored to indicate the different viral genes expressed during the life cycle (also see Figure 2 and 3).

instance, E2 promotes transcription by binding to different LCR sites and also regulates early polyadenylation to induce late gene expression (145). SR and hnRNP families of RNA binding proteins control late gene expression by regulating pre-mRNA splicing (287-289).

Polyadenylation and splicing in HPV-16 gene expression

HPV-16 mRNA splicing and polyadenylation are tightly connected to each other (144, 349) (Fig. 8). HPV-16 genome is divided into an early and a late region by the early pAE signal. The HPV-16 pAE signal not only controls early gene expression, but also prevents premature expression of late genes L1 and L2 during the early stages of infection (144, 271, 349). All mRNAs encoding the early genes are polyadenylated at pAE (Fig. 8). A cryptic polyadenylation site upstream of pAE is efficiently used if HPV-16 pAE is mutationally inactivated, demonstrating that pAE is strongly regulated by surrounding sequences (144, 347).

There are multiple polyadenylation enhancer elements located in sequences downstream of pAE and in the HPV-16 early 3'UTR (eUTR) (347) (Fig. 2B).

Cellular factors regulating polyadenylation bind directly to these enhancer elements (144, 145). For example, Fip1 (155), CstF64, and hnRNP H (236). Reduced usage of pAE results in the expression of the late genes L1 and L2 (144, 145). The L1 and L2 mRNAs undergo alternative splicing and polyadenylation at the late pAL. HPV-16 E2 protein induces HPV-16 late gene expression by interfering with cleavage and polyadenylation specificity factor CPSF-30 (also known as CPSF4) of the early polyadenylation complex (145).

HPV-16 3'-splice site SA3358 is predicted to be weak, yet it is the most commonly used 3' splice site of HPV-16 (270, 271) (Fig. 8). mRNAs encoding to oncogenes E6 and E7 are dependent on splicing to SA3358 (239, 288). Inhibition of SA3358 indirectly induces premature L1i gene expression (288) (Fig. 8). There is a strong splicing enhancer downstream of HPV-16 3'-splice site SA3358 that enhances the splicing to SA3358 (264, 288). Mutational inactivation of these sites reduces splicing to SA3358 and enhances splicing to late splice site SA5639 (288). A number of cellular proteins are involved in the regulation of SA3358. This enhancer contains 10 clusters of serine-and arginine-rich splicing factor 1 (SRSF1) also called ASF/SF2 binding sites (288). ASF/SF2 binds to this enhancer and enhances mRNA splicing to SA3358 (288). High levels of ASF/SF2 at the early stage of the viral life cycle induce expression of E6 and E7 mRNAs, whereas moderate levels of ASF/SF2 in terminally differentiated cells induce expression of L1 and L2 mRNAs (144). SRp30c also binds to this enhancer and causes skipping of SA3358, thereby promoting splicing to SA5639 (287). Another sequence downstream of this enhancer element interacts with SRp20 that inhibits splicing to SA3358 (142).

HPV-16 splice sites SD3632 and SA5639 are used exclusively for production of L1 mRNAs and are regulated in a cell differentiation-dependent manner during the HPV-16 life cycle (144) (Fig. 8). The splice sites SD3632 and SA5639 are activated in terminally differentiated cells, and are completely suppressed in mitotic cells and cervical cancer cells (270, 271). hnRNP A1 inhibits late gene expression by binding to exonic splicing silencers downstream of SA5639 (346, 348). HPV-16 5' splice site SD3632 is suppressed by a splicing inhibitor upstream of SD3632 (264).

Late 3' UTR (IUTR) controls late mRNA levels by either reducing late mRNA stability or by inhibiting polyadenylation at pAL or translation (111, 144, 349). HPV-16 IUTR contains two GUUUG motifs, which are CUGBP1 binding sites, and four 5' splice site-like sequences that interact with U1snRNP (144). Interaction of these factors with the IUTR results in inhibition of polyadenylation, mRNA half-life and translation (111, 144, 349).

Cellular splicing factors in this thesis

hnRNP D family and hnRNP A2/B1

The hnRNP D family contains hnRNP D, hnRNP DL and hnRNP AB proteins (62). The hnRNP D gene produces four alternatively spliced mRNAs giving rise to four isoforms of hnRNP D: hnRNP D37, hnRNP D40, hnRNP D42, and hnRNP D45 (329). hnRNP D37 lacks exons 2 and 7, hnRNP D40 contains only exon 2, hnRNP D42 contains only exon 7 and hnRNP D45 contains both. It has been shown that hnRNP D37 and hnRNP D42 isoforms destabilize cellular IL-6 mRNAs (245), while hnRNP D42 and hnRNP D45 activate transcription (255). hnRNP D may stabilize cytoplasmic mRNAs (337) and play a role in mRNA translation (188) and splicing (140). It has been shown that hnRNP D can bind RNA in a cooperative manner (356). The hnRNP D proteins can be posttranslationally modified by methylation (237), phosphorylation (342), and ubiquitination (169) in an isoform specific manner. Overexpression of the hnRNP D37 and D40 isoforms suppresses the expression of the HIV-1 gag capsid protein, while overexpression of hnRNP D42 and D45 has the opposite effect (191). hnRNP D can specifically bind to the pre-mRNA 3' splice site sequence r(UUAG/G) and the human telomeric DNA sequence d(TTAGGG)_n (140). Overexpression of hnRNP D has also been shown to prevent cellular senescence (255), thereby, leading to tumorigenesis (110).

hnRNP DL, known as hnRNP D-like protein or JKT41-binding protein, has several isoforms and has affinity for polyG (70). It binds to AU-rich RNA instability elements in TNF- α and COX-2 mRNAs (70), and may regulate translation of human nuclear factor kappa B-repressing factor (NRF) (259). hnRNP AB regulates splicing and is present in prespliceosomal complexes (20).

hnRNP A2/B1 is a splicing factor that binds to splicing silencers and inhibits splicing to both 5'- and 3'-splice sites (26, 106, 135). It also binds to RNA trafficking elements (168) and RNA stability elements (108), contributing to many steps in the cellular RNA processing pathway. hnRNP A2/B1 protein is overexpressed in many different cancer forms and can drive tumorigenesis (107). It also controls invasive cell migration (217) and epithelial-mesenchymal transition (310).

hnRNP G

hnRNP G, which belongs to hnRNP family, is a well conserved nuclear protein with a RNA binding domain at its amino terminus (124, 290). It contains one RRM and one RBD which have been shown to be important for RNA binding and for performing the function of hnRNP G (121, 152). hnRNP G binds directly to CC(A/C)-rich single-stranded RNA sequences and influences alternative splicing (6). It can also change splice site selection independent on its RRM motif (6).

hnRNP G has been shown to be involved in the tissue-specific regulation of transcription of some genes (345) and is essential for neural and muscle development in zebrafish and *Xenopus laevis* embryos (72). In mammals, hnRNP G is important for normal sperm development (82, 153, 322) and regulates alternative splicing of several pre-mRNAs by specifically binding to certain RNA sequences (153, 227, 322). hnRNP G has a negative effect on RNA alternative splicing in skeletal muscle (227, 306). whereas hnRNP G can efficiently activate the testis-specific exon splicing by specific binding to the pre-mRNA (183). hnRNP G has been shown to compete with Tra2 β and ASF/SF2 for the same target RNA with opposite effects on RNA splicing (83, 353). hnRNP G has tumor-suppressive activity by binding to the promoter of the tumor suppressor gene *Txinp* in mice and mutations of hnRNP G RNA-binding domain were found in a tumor-derived cell line (282, 283). Expression of hnRNP G is dramatically decreased in premalignant and malignant epithelial tissues, while levels are relatively high in normal or hyperplastic non-dysplastic epithelium (284). Knockdown of hnRNP G protein inhibits neurogenesis, causing brain defects and abnormal muscle development in *Xenopus* (72).

ASF/SF2 (SRSF1)

ASF/SF2 is a key regulator of RNA processing (32, 113). It also plays an important role in nonsense-mediated mRNA decay (343), mRNA export (129), and translation (303). Expression of ASF/SF2 increases with the grade of severity of the HPV positive cervical lesion (84). High level of ASF/SF2 were detected in the entire epithelium of high-grade, CIN-III cervical intraepithelial lesions, and all cervical cancers (84). Relatively little ASF/SF2 is expressed in low-grade lesions and normal HPV-negative cervical epithelium (84, 214). ASF/SF2 is required for production of HPV-16 mRNAs that are spliced to HPV-16 SA3358 (288). It has also been reported that HPV-16 E2 enhances ASF/SF2 expression by transcriptional activation of the ASF/SF2 promoter (214).

Present investigation

Aims of the present study

A strict regulation of HPV-16 late gene expression may be necessary to avoid premature production of L1 and L2, and detection by the immune system. This paves the way for viral persistence which is the most important risk factor for HPV-16-induced cervical cancer. Exploring of HPV-16 late gene expression will therefore further increase our understanding of the progression of HPV-16 infection to cervical cancer.

The goal of this thesis project was:

- 1) to determine how the HPV-16 late 5'-splice site SD3632 is regulated by cis-acting RNA elements and cellular trans-acting factors
- 2) to determine how HPV-16 3'-splice site SA3358, which is used by the majority of early and late HPV-16 mRNAs, is regulated, and how it contributes to the ability of HPV-16 to immortalize human cells.

Introduction to papers

paper I

Suppression of HPV-16 late L1 5'-splice site SD3632 by binding of hnRNP D proteins and hnRNP A2/B1 to upstream AUAGUA RNA motifs.

HPV-16 5'-splice site SD3632 is the only 5'-splice site used to produce late L1 mRNAs. This splice site is suppressed in proliferating cells, including cervical cancer cells, thereby, preventing L1 production and contributing the ability of HPV-16 to hide from the immune system and establish persistence. Studies on the regulation of this splice site would further our understanding of HPV-16 gene expression and the establishment of persistence and progression to cancer.

In order to identify cis-acting RNA elements that regulate SD3632, subgenomic HPV-16 plasmids that contain the strong viral CMV promoter and the CAT/sLuc reporter gene as a surrogate marker for L1 expression were constructed. We found that the 232 nucleotides immediately upstream of SD3632 inhibited production of spliced L1 mRNA. Next, we inactivated SD3632 by using a site-directed, ligase-independent mutagenesis (SLIM) method, and found that SD3632 is suboptimal and subject to negative regulation. Deletions revealed that the splicing inhibitory elements were located in the 34 nucleotides immediately upstream of HPV-16 late 5'-splice site SD3632. We found two HPV-16 specific AUAGUA motifs that are located in these 34 nucleotides inhibited SD3632 and two nucleotide substitutions in each of the AUAGUA motifs alleviated splicing inhibition and induced late L1 mRNA production from episomal forms of the HPV-16 genome in primary human keratinocytes.

We identified cellular proteins that bind specifically to the AUAGUA motifs in the inhibitory RNA element by RNA affinity purification assay. These cellular proteins are hnRNP D proteins (p37, p40, p42, and p45), hnRNP DL and hnRNP AB, as well as hnRNP A2/B1. We found that siRNA knock-down of these proteins induced HPV-16 late L1 mRNA expression, and overexpression of hnRNP A2/B1, hnRNP AB, hnRNP DL and the two hnRNP D isoforms hnRNP D37 and hnRNP D40 further suppressed L1 mRNA expression. In conclusion, we have identified a splicing inhibitory RNA element that suppresses HPV-16 late 5'-splice site SD3632 and identified the cellular factors that interact with this inhibitor.

paper II

Eight nucleotide substitutions inhibit splicing to HPV-16 3'-splice site SA3358 and reduce the efficiency by which HPV-16 increases the life span of primary human keratinocytes.

HPV-16 3'-splice site SA3358 is the most commonly used 3'-splice site producing the mRNAs encoding early genes E4, E5, E6 and E7, and late genes L1 and L2. Previously we have showed that SA3358 is suboptimal and is strictly controlled by a downstream splicing enhancer containing 15 clusters of potential ASF/SF2 binding sites.

In this paper we used subgenomic HPV-16 plasmids that contain the CAT/Luc/sLuc reporter genes as markers for L1 expression to better map and characterize this splicing enhancer. We found that only one of the predicted ASF/SF2 sites accounts for the majority of the enhancer activity. This ASF/SF2 binding site consists of 8 nucleotides. Mutations inactivated the splicing enhancer at SA3358, thereby redirecting to an induction of L1 mRNA expression from these plasmids.

Further mutagenesis revealed that single nucleotide substitutions in this predicted ASF/SF2 site impaired the enhancer function. We provide evidence that HPV-16 mRNAs that are spliced to SA3358 interact with ASF/SF2 *in vitro* and in living cells by *in vitro* RNA pull-downs, and *in vivo* UV cross-linking, followed by immunoprecipitation and RT-PCR. In addition, mutational inactivation of the enhancer weakened splicing to SA3358 in episomal forms of the HPV-16 genome leading to induction of HPV-16 late gene expression.

Furthermore, inactivation of the splicing enhancer at SA3358 reduced the ability of E6- and E7-encoding HPV-16 plasmids to increase the life span of primary keratinocytes *in vitro*. We concluded that efficient usage of HPV-16 3'-splice site SA3358 was dependent on one ASF/SF2 binding site and that an intact splicing enhancer of SA3358 is required for efficient production of the E6 and E7 mRNAs.

paper III

hnRNP G/RBMX inhibits splicing to the HPV-16 3'-splice site SA3358

This is a continuation of the project on the enhancer element at HPV-16 3'-splice site SA3358. Splice site SA3358 is totally dependent on a splicing enhancer downstream of SA3358. Here we have investigated if there are other cellular factors binding to the enhancer, in addition to ASF/SF2. We first incubated biotin-labelled enhancer RNA sequences with HeLa nuclear extract, followed by a pull down of the RNA-protein complexes with streptavidin beads. After SDS-PAGE,

silver staining, and mass spectrometry analysis, we found that one of the hnRNP proteins hnRNP G (also known as RBMX) binds to this enhancer. These results were confirmed by Western blot assay.

hnRNP G is a RNA-binding protein and a tumor suppressor in human oral squamous cell carcinomas (HOSCC) (282, 284). To determine if hnRNP G affects HPV-16 late gene expression, we cotransfected the cDNA plasmid encoding hnRNP G protein with HPV-16 subgenomic plasmids containing sLuc/Luc reporter genes in place of HPV-16 L1. We found that overexpression of hnRNP G inhibited HPV-16 SA3358, presumably by interfering with the downstream enhancer, thereby redirecting splicing to late L1 3'-splice site SA5639. In contrast, hnRNP G had little effect on SA3358 when the downstream enhancer had been mutated. RT-PCR and q-PCR of the RNAs extracted from the transfected cells suggested that hnRNP G induced late HPV-16 L1i mRNAs by negatively interfering with the eight-nucleotide splicing enhancer element downstream of HPV-16 SA3358.

We also performed siRNA knock-down of hnRNP G in a cell line that contains HPV-16 subgenomic expression plasmid pBELsluc integrated in the genome (177). The results showed that knock-down of hnRNP G decreased the expression level of sluc. This is the first report on the role of hnRNP G in the regulation of HPV-16 pre-mRNA splicing. Our results suggest that the hnRNP G protein plays an important role in the HPV-16 gene expression program.

Overall conclusions

I. HPV-16 late 5'-splice site SD3632 project.

- 1) A splicing silencer inhibits HPV-16 late 5'-splice site SD3632.
- 2) The splicing silencer located immediately upstream of HPV-16 SD3632 is 34-nucleotide long and contains two AUAGUA motifs.
- 3) Two AUAGUA motifs are required for inhibition of SD3632.
- 4) Cellular proteins hnRNP A2/B1, hnRNP DL, hnRNP AB, and hnRNP D isoform p37 and p40 specifically interact with the AUAGUA motifs and inhibit splicing at SD3632.

II. HPV-16 3'-splice site SA3358 project.

- 1) One ASF/SF2 binding site consisting of eight-nucleotide accounts for the majority of the activity of the splicing enhancer downstream of HPV-16 SA3358.
- 2) Cellular protein ASF/SF2 and hnRNP G specifically bind to eight-nucleotide sequence and have opposite effects on splicing at HPV-16 3'-splice site SA3358: ASF/SF2 enhances splicing to splice site SA3358, whereas hnRNP G inhibits splicing to SA3358.
- 3) Mutational inactivation of the single ASF/SF2 site reduces HPV-16 E6/E7 oncogene expression and the ability of HPV-16 to immortalize human keratinocyte *in vitro*.

Concluding Remarks and Future perspectives

More than 99% of cervical cancers are attributable to HPV and HPV-16 accounts for about half of the cases. Expression of the immunogenic viral proteins L1 and L2 is inhibited to allow the virus to hide from immune system. One of the mechanisms is to strictly regulate HPV-16 gene expression at the level of viral RNA processing during the infection (Fig. 8). Cis-acting RNA elements and Trans-acting splicing factors regulate HPV-16 splice sites SA3358 and SD3632 two vital splice sites controlling HPV-16 late gene expression (Fig. 9).

In this thesis, we identified two exonic splicing elements: 34-nucleotide inhibitor and an eight-nucleotide enhancer that regulate HPV-16 SD3632 and SA3358 (Fig. 9), respectively. The 34-nucleotide exonic splicing inhibitor efficiently blocks the usage of SD3632 to prevent the expression of late gene L1, whereas the eight-nucleotide enhancer stimulates the usage of SA3358, thereby further blocking late gene expression. It has been reported that mutations in exonic splicing elements might cause human genetic diseases that are linked to splicing defects (28). An increasing number of human genetic diseases are linked to point mutations that cause splicing defects, and many of these mutations are located within exons (28). Nucleotide substitutions in splicing inhibitor or enhancer elements induce high levels of HPV-16 L1 expression. These findings gain a deeper understanding of the regulation of HPV-16 late gene expression.

Cellular hnRNP D and hnRNP A2/B1 proteins were found to interact with the exonic inhibitor elements and downregulate HPV-16 L1 expression (Fig. 9). hnRNP D proteins and hnRNP A2/B1 are highly expressed in cervical cancer cells and no or little expression is seen in normal, terminally differentiated cells (www.proteinatlas.org). Although hnRNP D proteins have been reported that have various effects on gene expression (16, 190), there are no investigations on hnRNP D proteins and HPV-16 in cervical cancer. It would be interesting to determine the role of hnRNP D proteins and hnRNP A2/B1 in the HPV-16 life cycle. We also identified cellular protein ASF/SF2 and hnRNP G that interact with eight-nucleotide splicing enhancer. ASF/SF2 is a proto-oncogene that shows increased expression levels in high-grade cervical lesions (213) and hnRNP G is a

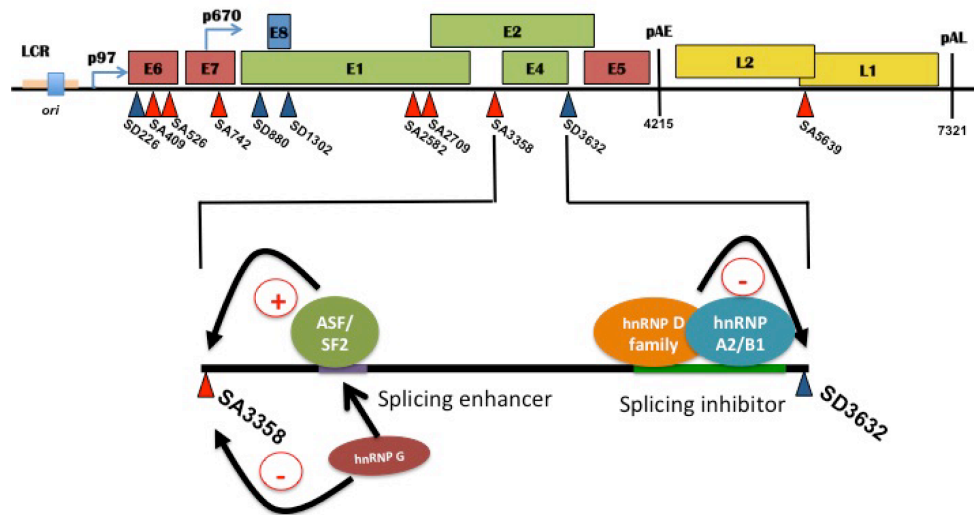


Figure 9. HPV-16 splice sites are controlled by multiple cellular proteins. Position of splicing enhancer element at 3'-splice site SA3358 (purple) and splicing silencer element (green) at 5'-splice site SD3632 are indicated. Arrows show the effect of the viral regulatory RNA elements and cellular trans-acting factors on different HPV-16 splice sites.

tumor suppressor protein, of which expression level was dramatically decreased in premalignant and malignant oral epithelial tissues (282, 284). Ongoing work on this project is to characterize the role of these proteins in the HPV-16 life cycle. It will enhance our understanding of how the functions of these splicing factors are altered in cervical cancer. The results undoubtedly will contribute insights into the mechanisms of HPV-16 gene expression.

Cytology is a screening method that has contributed to a significant reduction of cervical cancer, yet false-positive results are common (54). Biomarker is could enhance sensitivity and specifictiy. The hnRNP D proteins, hnRNP A2/B1, ASF/SF2, and hnRNP G proteins investigated in the thesis could potentially be used as biomarkers for detection of lesions that have a high risk of development to cervical cancer.

论文摘要 (In Chinese)

人类乳突病毒引起的癌症占由病毒引起癌症总数的百分之五十，其中高危型人类乳突病毒十六型病毒是导致癌症的最危险的病毒类型，百分之五十的宫颈癌都可以检测到人类乳突病毒十六型病毒。高危人类乳突病毒十六型病毒是一种嗜上皮性病毒，它的基因表达与被感染细胞的分裂和分化有密切关系。人类乳突病毒十六型病毒早期基因在表皮细胞的基底层表达，而编码具有高度免疫原性的病毒结构蛋白的两个晚期基因只在表皮细胞的上层表达。大多数人类乳突病毒感染可在短期内消失，机体通过自身免疫系统使病毒逐渐清除，感染一般持续十八到二十四个月左右。但如果细胞持续感染高危型人类乳突病毒十六型病毒可引起宫颈癌变。在被人类乳突病毒十六型病毒感染的癌细胞中，只有早期基因表达，晚期基因被完全抑制，不能表达。我们推测病毒潜伏感染和细胞癌变需要抑制人类乳突病毒十六型病毒的晚期基因表达。

人类乳突病毒十六型病毒利用选择性剪接来调控早期和晚期基因的表达。同时，病毒调控因子和细胞调控因子也参与控制病毒蛋白的表达。我们的研究项目是为了增强在核糖核酸调控水平上，对人类乳突病毒十六型病毒高危型病毒的基因表达调控的了解。本论文的目的在于鉴别调控人类乳突病毒十六型病毒早期和晚期信使核糖核酸表达的病毒核糖核酸调控因子，和结合在该核糖核酸调控因子上的，共同调控病毒基因表达的细胞调控因子。这些研究结果可能为宫颈癌早期诊断和预测患病危险度的生物标记物的鉴定提供重要依据。

我们鉴定出一个在有丝分裂细胞和宫颈癌细胞中，抑制人类乳突病毒十六型病毒晚期基因表达的外显子剪切抑制因子。并鉴定和分析了与这个抑制因子结合的四种细胞质蛋白（人异质性胞核核糖核蛋白 D, DL, AB, 和 A2/B1），他们互相结合并共同调控人类乳突病毒十六型病毒晚期基因的表达。我们还鉴定和分析了一个上调人类乳突病毒十六型病毒早期基因表达的外显子剪切增强因子，该因子因此可以间接的抑制人类乳突病毒十六型病毒晚期基因的表达。突变的增强因子与细胞质剪切调控蛋白可变剪接因子一/剪切因子二结合的能力明显减弱，从而抑制了人类乳突病毒十六型病毒 病毒癌基因早期基因六和早期基因七的表达，影响了早期基因六和早期基因七对细胞生长周期的调控，降低了人类乳突病毒十六型病毒使细胞永生化和恶变的能力。因此，确定人类乳突病毒十六型病毒信使核糖核酸的剪接和调控与其致病机制有较高的相关性。

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