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Cloning and analysis of human papillomavirus type 18 codon optimized Flag-tagged E2 protein

Bachelor thesis

12 EAP

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Cloning and analysis of human papillomavirus type 18 codon optimized Flag-tagged E2 protein

Human papillomaviruses (HPVs) are highly prevalent pathogens, which are associated with wide variety of disorders. HPVs encode nine proteins. E2 protein is a key regulator of HPV life cycle. The E2 proteins function in initiation of DNA replication, transcriptional regulation and partitioning of viral genome. However, E2 proteins are expressed at very low level, which significantly complicates studies of these proteins. In order to increase expression of HPV18 E2 protein, codon optimized sequence of E2 was inserted to the pCMV-Flag-4 vector, which contains Flag epitope. Flag-tag potentially may influence E2 protein properties and expression level. Results have shown that codon optimization of E2 protein encoding sequence increased the expression level of HPV18 E2 proteins in 293FT cells; codon optimized E2-Flag protein supported replication of HPV18 $E2^-$ deficient genome in U2OS cells. New HPV18 E2c.o.pCMV-Flag construct is functional.

KEYWORDS: HPV, papillomavirus, E2, replication, expression

CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

Inimese papilloomiviiruse tüüp 18 koodon optimiseeritud Flag-märgisega E2 valgu kloneerimine ja analüüs

Inimese papilloomiviirused on väga levinud patogeenid mis on seotud paljude erinevate haigustega. HPVd kodeerivad 9 valku. E2 valk on HPV elutsükli võtmeregulaator. E2 valgud reguleerivad viiruse DNA replikatsiooni, transkriptsiooni ja viiruse genoomi jaotust tütarrakkude vahel. Kuid E2 valke ekspresseeritakse väga madalal tasemel, mis oluliselt raskendab nende valkude uurimist. E2 valgu ekspressiooni suurendamiseks sisestati koodon-optimeeritud järjestus pCMV-Flag-4 vektorisse, mis sisaldab Flag epitoopi. Flag- märgis võib potentsiaalselt mõjutada valgu omadusi ja ekspressioonitaset. Tulemused näitasid, et E2 valgu koodoni optimeerimine suurendas HPV18 E2 valkude ekspressioonitaset 293FT rakkudes; koodon-optimeeritud E2-Flag valk toetas replikatsiooni HPV18 E2- puudulikus genoomis U2OS rakkudes. Uus HPV18 E2c.o.pCMV-Flag konstrukt on funktsionaalne.

Märksõnad: HPV, papilloomiviirus, E2, replikatsioon, ekspressioon

CERCS: B230 Microbiology, bacteriology, virology, mycology

CONTENTS

LIST OF ABBREVIATIONS

BPV – bovine papillomavirus

BS – binding site

C-terminal – carboxyl-terminal

DBD – DNA binding domain

dNTP – deoxynucleotide triphosphate

dsDNA – double-stranded DNA

GFP – green fluorescent protein

HPV – human papillomavirus

LCR – long control region

MHC – major histocompatibility complex

N-terminal – amino-terminal

ORF – open reading frame

TBP – TATA binding protein

URR – upstream regulatory region

INTRODUCTION

Human papillomaviruses (HPVs) are highly prevalent pathogens with great medical importance due to their association with several types of human cancer. HPVs are the most common sexually transmitted pathogens. They can be spread between partners from the skin and mucous membranes. Sexually transmitted HPV types are divided into two categories: “high-risk”, which can cause cancer, and “low-risk”, which can cause skin warts. HPV causes majority of cervical cancer among women, but also can be a reason of other anogenital and head and neck cancers (zur Hausen., 2009). Some specific types have been linked to cutaneous cancers. In fact, all cases of cervical cancer are caused by HPV. Constant research has increased the understanding of biology and host responses to HPV in molecular details. More precise understanding of HPV life cycle enables creating of virus-specific treatment. Currently the only way to prevent HPV infection is vaccination.

Papillomaviruses infect epithelial cells of skin or mucosa in wide variety of vertebrates and induce cell proliferation. The viral genome is maintained as a circular dsDNA extrachromosomal replicating element within these cells (McBride., 2008). There are three modes of replication of papillomaviruses. The first one is establishment phase, which occurs upon the entry, when the viral genome is amplified to a low copy number. The second, maintenance phase, takes place when the viral genome replicates at a constant copy number synchronously with the cellular DNA in dividing cells, partitioning its genome between daughter cells. In the third, vegetative replication phase, the viral DNA is amplified to a high copy number in differentiated cells. This is followed by the packaging of genomes into the capsids (McBride., 2008).

Papillomaviruses produce approximately nine proteins. Seven of them are expressed in the early stages of infection and two of them in the late stages. One of the best studied proteins is E2 early protein, which is the regulator of papillomavirus life cycle. E2 has also shorter forms, which are the products of alternative splicing

Together with second early protein E1, E2 supports papillomavirus DNA replication, all other essential proteins and enzymes, which are needed for replication are provided by the host cell. The viral E1 protein acts together with E2 initiating viral replication. Moreover, E2 is needed for proper partitioning of viral genome in dividing cells in order to establish persistent infection. In addition, E2 can regulate the transcription of the viral genes. Thus, E2 serves as a

very important protein in PVs life cycle. Generally, research of E2 is challenging issue because of it very low expression level in host cells.

The first part of the thesis gives a theoretical overview on papillomavirus life cycle and E2 protein structure and function. The practical part aims at cloning of codon optimized sequence coding for E2 protein of HPV18 and subsequent testing of its expression level and biological activities.

Present thesis was made in the Tartu University Institute of Technology in the Papillomavirus research group.

1. LITERATURE REVIEW

1.1 Papillomaviruses - an overview

Papillomaviruses are small, non-enveloped, double-stranded DNA viruses that belong to the *Papillomaviridae* family and infect the basal cells in epithelial tissue of a wide variety of animals. There are five major known HPV genera: α-papillomavirus, β- papillomavirus, γ-papillomavirus, mu-papillomavirus and nu-papillomavirus, 49 species and over 200 types. The taxonomic classification of HPVs is based on the nucleotide sequence of the L1 gene, which encodes capsid protein (Bzhalava *et al.,* 2015).

Papillomaviruses infect keratinocytes in the basal layers of a stratified squamous epithelium (Zheng and Baker., 2006.) and cause wide range of diseases in their host: from benign tumors (warts, papillomas) to malignancies (de Villiers *et al.*,2004). HPV types of α-papillomavirus genera are known as a major cause of cervical cancer (Walboomers *et al.*, 1999). These viruses are classified into high-risk or "oncogenic" (HPV16, HPV18), which immortalize human keratinocytes, causing cervical and anal cancer (de Villiers *et al.*,2004; Munoz *et al.*, 2003) and low-risk or "non-oncogenic" (HPV6, HPV11), which can may cause warts (Lacey, 2005) and are very rarely found in genital malignancies (Zheng and Baker., 2006.).

Human papillomavirus (HPV) is among the most common sexually transmitted infections worldwide and is associated with 95% of cervical cancer. (Health Canada. Human papillomavirus (HPV) (http://www.hc-sc.gc.ca/ hl-vs/alt_formats/pdf/iyh-vsv/diseases-maladies/hpv-vph-eng.pdf; Munoz *et al*., 2003). This knowledge has led to development of vaccines, screening strategies and HPV testing (Cuschieri and Cubie, 2005). At the present time, there is no cure for HPV infection and prevention through vaccination is essential step for reducing of HPV-related disease. Available preventative vaccines are based on virus-like-particles (VLPs) containing only of L1 major capsid protein which has an ability to assemble into VLPs. Gardasil, the quadrivalent HPV 6, 11, 16 and 18 recombinant VLP vaccine (4vHPV) is designed by Merck. The vaccine protects against HPV 16 and HPV18 that cause approximately 70% of cervical cancers and HPV 6 and 11 that cause 90% of genital warts. Another licensed vaccine is bivalent HPV 16 and 18 VLP, Cervarix (2vHPV) developed by GlaxoSmithKline. The nine-valent HPV VLP vaccine (9vHPV), Gardasil 9, which is the second generation HPV vaccine, contains HPV31, 33, 45, 52 and 58 VLPs connected with the quadrivalent VLP vaccine HPV types. Those vaccines were expanded to include males and were licensed in 2014 (Bryan *et al.*, 2016).

1.2 Life cycle of papillomaviruses

Human papillomaviruses usually invade through the skin damages and infect basal epithelial cells, which is the only epithelium cell layer that divides constantly. Gene expression and replication is closely associated with keratinocyte differentiation of the host (Figure 1.). To start infection, virus must reach basal cells and bind to cellular receptors. Virions initially attach to the heparan sulfate proteoglycan (HSPG) on the basal membrane, and transfer to the receptor expressed on the keratinocytes. (Kines *et al.*, 2009). After the entry and uncoating, viral genomic DNA is transported to the nucleus, where it is maintained at a low-copy number as extrachromosomal element or episome (Moody and Laimins, 2010). HPV genome is small and does not encode polymerase, which is necessary for virus replication. Therefore, HPV requires DNA replication machinery of the host (Münger *et al.*, 2004). The cell division of the host is necessary for viral DNA replication. E6 and E7 are expressed in the lower epithelial layers and drive cells to S-phase, which conduce to viral genome replication and cell proliferation (Doorbar, 2005). E6 and E7 inactivate p53 and retinoblastoma (pRB) tumor suppressors, maintaining the cell DNA replication potential (Münger *et al.*, 2004).

Papillomavirus replication and assembly takes place exclusively in the nucleus (Zheng and Baker, 2006). HPV amplification occurs in differentiating keratinocytes. With division of infected basal cell, viral DNA is distributed between both daughter cells: one of daughter cells migrates from basal layer to granular layer and initiates a program of differentiation. The other daughter cell keeps dividing in the basal layer, providing a storage of infected cells (Münger *et al.*, 2004). Once genome amplification has been completed, the synthesis of the capsid proteins in upper layer of infected tissue is started (Doorbar, 2005). The capsid proteins L1 and L2 are required for viral packaging. This is followed by virion assembly and external release with peeled keratinocytes (Kajitani *et al.*, 2012).

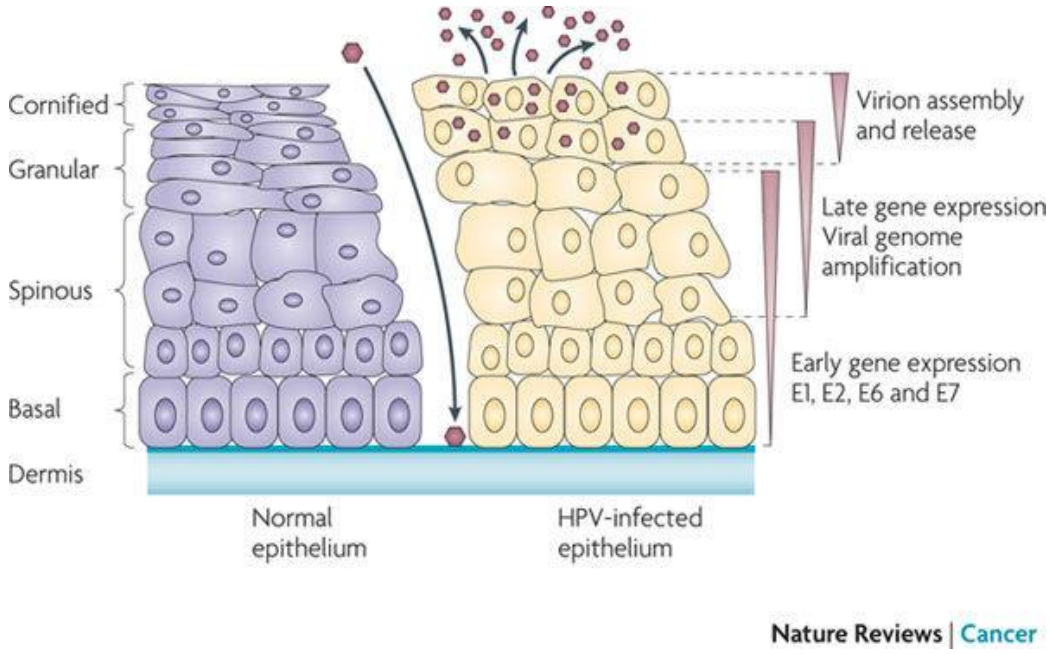

Figure 1. Stratified squamous epithelium and viral gene expression during the life cycle of the papillomavirus. (Moody and Laimins, 2010).

1.3 Genome organization and proteins of papillomaviruses

All papillomaviruses have circular double-stranded DNA that is approximately 8000 bp in size. The genome is divided into three major regions: early, late and long control region (LCR or Upstream Regulatory Region (URR). Six open reading frames (ORFs) in the early genome region encode the E1, E2, E4, E5, E6, E7 and E8 proteins, which are expressed in the lower layers at different stages during epithelium cells differentiation. The late region encodes L1 and L2 capsid proteins, which are expressed in the upper epithelial layers (Zheng and Baker., 2006.). LCR is does not encode any proteins but contains important elements such as replication origin, promoters and binding sites for viral E1 and E2 proteins and cellular transcription factors (McBride, 2008)

E1 is the primary replication protein that binds specifically to the origin and initiates replication. E1 has ATPase and helicase activity, although the interaction with E2 is required for binding to ori site and initiation of viral DNA replication. After loading, E2 dissociates from the origin, E1 converts into double-helix hexameric ring and unwinds the viral replication origin to allow access of the cellular replication proteins. (McBride, 2008)

E2 protein plays a crucial role in viral genome transcription, replication and maintenance during cell division. The structure and role of the E2 protein is described in more details in the next chapter.

E4 protein is an E1^E4 splicing product and is synthesized at very upper levels in the differentiated layers of the papillomavirus infection site, when the vegetative viral genome amplification begins (Davy *et al.*, 2004). The E4 ORF region is located in E2 gene. Due to its abundant expression in late stages, it may serve as a biomarker of active virus infection (Middleton *et al*., 2003). E4 associates with keratin filaments that disrupt the stability of keratin networks and leads to particle release from the cell (Davy *et al.*, 2004).

E5 is short, membrane-associated, hydrophobic protein. (Schlegel *et al*., 1986). BPV1 E5 is located in the membranes of the endoplasmic reticulum and Golgi apparatus of transformed cells as a dimer (Horwitz *et al.,* 1988). E5 proteins affect activity of growth factor receptors by acidification of Golgi apparatus, which contributes to cell transformation, stimulate the proliferation of infected basal cells (DiMaio and Mattoon, 2001) and protect host cells from apoptosis (Hu *et al.*, 2010). Also, E5 downregulates surface expression of major histocompatibility complex (MHC) class I molecules, helping the virus to evade the host immune system (Ashrafi *et al.,* 2006; Zhang *et al.*, 2003).

E6 and E7 proteins are powerful oncoproteins. They both are sufficient for the efficient immortalization of primary human squamous epithelial cells (Münger *et al.*, 1989). E6 and E7 both interact with tumor suppressor gene products. The E6 protein associates with p53, which leads to degradation of p53 tumor suppressor through the ubiquitin-dependent proteolysis system. This prevents p53 from inducing apoptosis of abnormal cells, which contributes to tumorigenesis (Huibregtse *et al.*, 1991). The E6 proteins from high-risk HPV types have higher affinity for p53, than low-risk HPV types (Li and Coffino, 1996).

E7 protein is a major HPV oncoprotein, and E7 expression in high-risk HPVs is essential for both cell transformation and viral pathogenesis. E7s' main target is retinoblastoma (Rb) tumor suppressor and its related proteins including p107 and p130 (Garnett and Duerksen-Hughes, 2006). E7 proteins destabilize pRB through ubiquitin-proteasome-mediated proteolysis (Boyer *et al.*, 1996). In addition, interaction between pRB and E7 proteins results in the release of E2F family of transcription factors. This leads to activation of genes necessary for cell S-phase entry and progression (McLaughlin-Drubin and Münger, 2009). Same as E6 proteins, the E7 proteins of high-risk HPV types bind pRb with higher efficiency compared to low-risk E7 (Münger *et al.*, 1989).

All papillomaviruses encode an E8^E2 repressor proteins, which consist of a short E8 peptide fused to the entire hinge and DNA binding domains of E2. These proteins are powerful repressors of viral transcription and replication (Zobel *et al.*, 2003; Stubenrauch *et al.*, 2001)

L1 and L2 are papillomavirus structural proteins and are essential for DNA packaging. The expression and assembly of infectious virions occurs in terminally differentiated layers of epithelial tissues. L1 is major protein and can efficiently self-assemble into virus-like particles (Kirnbauer *et al.*, 1992; Kirnbauer *et al.*, 1993). L2 is a minor protein and is required for efficient encapsidation of HPV DNA during the late phase of infection (Zhao *et al.*, 1998).

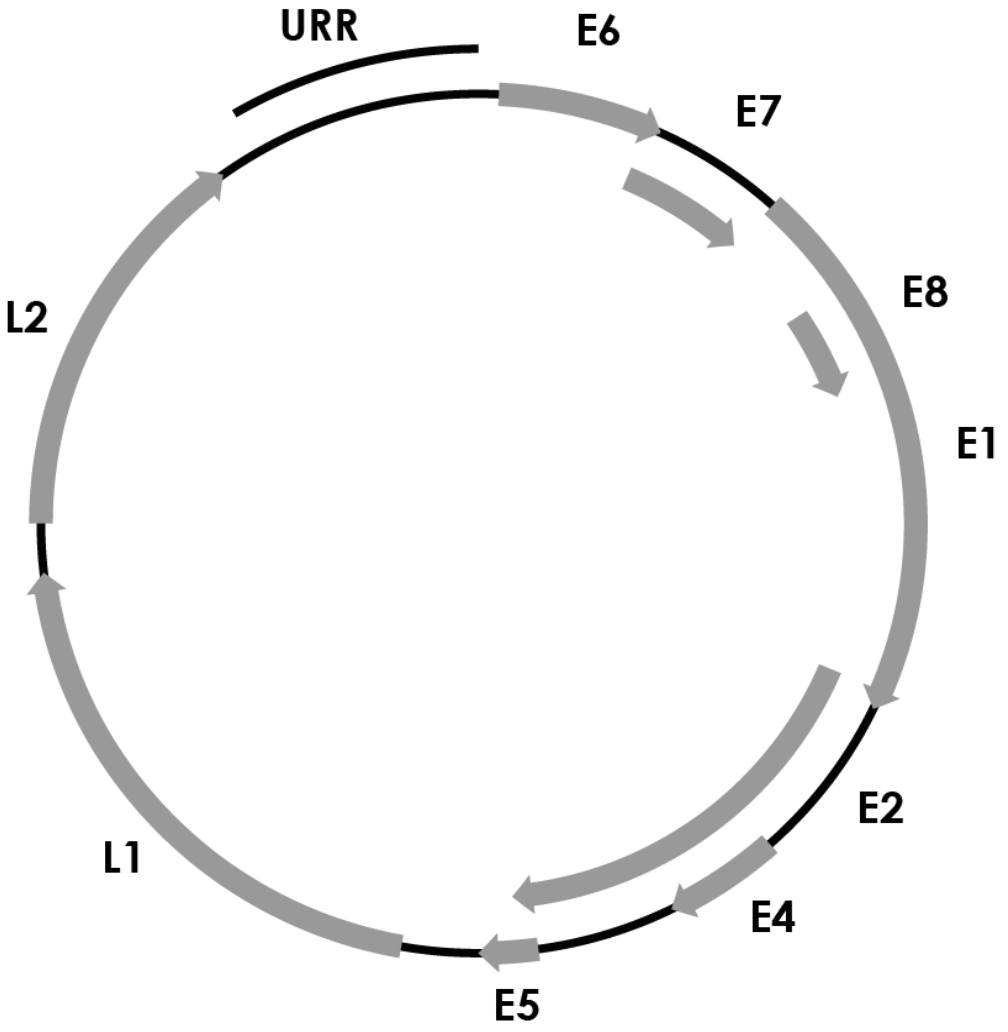

Figure 2. Schematic representation of papillomavirus genome. Open reading frames (ORFs) are shown as grey arrows. The upstream regulatory region (URR) is marked as a black line.

1.5 Papillomavirus E2 protein

1.5.1 E2 protein - an overview

All papillomaviruses encode E2 protein, which is an essential regulatory protein. E2 protein is multifunctional and involved in many processes in the viral life cycle, such as replication, transcription and maintenance of the viral genome. E2 is expressed at establishment and maintenance phases of the viral life cycle and known as E2-TA or E2 transactivator (McBride, 2013).

The full-length E2 protein is expressed from the entire open reading frame (ORF). E2 protein consists of a conserved N-terminal transactivation domain of about 200 amino acids long and C-terminal binding domain of about 100 amino acids long. (McBride *et al.*, 1989b). N-terminus is necessary for replication, transcriptional activation and repression, interaction with the E1 protein, as well as many cellular factors. C-terminal domain has sequence specific DNA and dimerization properties (McBride and Myers, 1997). The linker sequence provides a flexible connection between two domains, which varies among the papillomavirus genera by length and sequence (McBride *et al*., 1989b).

Besides full-length E2 protein, all papillomaviruses have a potential to encode shorter forms of E2 that contain C-terminal domain. These proteins have been alternatively named E8^E2, E8^E2C, E1^E2, E1M^E2 and E9^E2. The shorter forms of E2 serve as repressors of viral replication and transcription. Elimination of E8^E2 expression leads to over-replication of HPV genomes (McBride, 2013).

1.6 E2 functions

1.6.1 Transcription

The E2 proteins are the main transcriptional regulators of papillomaviruses (Lambert *et al.*, 1987). E2 regulates the viral transcription through binding as a dimer to its specific recognition sites. The binding sites for E2 protein are located within URR region and are highly conserved. The URR in HPVs most often contains 4 binding sites (Spalholz *et al.*, 1985) and the sites with the highest affinity have the sequence $ACC(N)_4GGT$ (Androphy *et al.*, 1987; Li *et al.*, 1989). E2 also uses non-consensus binding sites with lower affinity (Newhouse and Silverstein, 2001), such as $AAC(N)_6GGT$ or $AAC(N)_6GTT$ in BPV1 (Li *et*

al., 1989). The E2 protein activates or represses the viral transcription, depending on the context of binding sites and nature of the associated cellular factors, which virus recruits. E2 binding sterically hinders the binding of cellular factors such as Sp1 and TBP (TATA binding protein) to proximal promoter elements in the viral genome (Dostatni *et al.*, 1991; Romanczuk *et al.*, 1990; Tan *et al.*, 1994) and prevents the formation of the transcriptional initiation complex, which leads to transcriptional repression of the early promoter (Thierry, 2009).

In many viruses, the full-length E2 protein can either activate or repress viral transcription in dose-dependent manner (Bouvard *et al.*, 1994; Fujii *et al.*, 2001; Steger and Corbach, 1997; Thierry and Yaniv, 1987). Studies have shown, that the HPV18 early promoter P105 is activated in the presence of low E2 amounts and repressed, when the E2 concentration is increased. E2 binds with highest affinity to the most distal E2 binding site 4 (BS-4) and can upregulate viral early gene expression. The early promoter is activated in the presence of low concentrations of E2, when it binds BS-4. As E2 concentration rise, E2 occupies BS-2 and BS-1 with reduced affinity that are involved in transcriptional repression. (Steger and Corbach, 1997, Demeret *et al.*, 1997). This dose-dependent sequence of DNA binding events correlates with an increasing the expression of E1, E2, E6 and E7 (Demeret *et al.*, 1997)

The shorter forms of E2 act as transcriptional repressor. Repression can be mediated by competition for binding to E2 binding sites (Lambert *et al.*, 1987) or by binding the repressor complexes to viral DNA with the help of the E8-derived peptide. (Ammermann *et al.*, 2008; Fertey *et al.*, 2010; Powell *et al.*, 2010). Dimerization of shorter forms of E2 with full-length E2 cam also repress transcriptional activation (Kurg *et al.*, 2006).

In HPV-associated cancers E2 is often disrupted, which negatively affects the E2-mediated repression and leads to increased expression of the E6 and E7 oncogenes. Also, high-levels of E2 can induce the expression of the late HPV genes L1 and L2, causing read-through at the early polyadenylation signal into the late region of HPV genome (Johansson *et al.*, 2012).

1.6.2 Initiation of viral DNA replication

To initiate viral DNA replication, E2 must load the E1 protein, which serves as helicase, onto the replication ori, which contains an E1 binding site, an A/T regions and at least one E2 binding site (Ustav *et al.*, 1993; Ustav *et al.*, 1991). The initiation of replication occurs in several steps: specific recognition of the origin precedes origin unwinding, helicase delivery

and possessive DNA synthesis. E2 is required to enhance and support the function if E1, the primary replication protein (Frattini and Laimins, 1994; Mohr *et al.*, 1990). After loading, E1 converts to a double-hexameric helicase to unwind the DNA, which is followed by E2 dissociation from the complex (Sanders and Stenlund, 1998). Replication activity correlates very well with the formation of E1-E2-ori complex. Also, E2 protein determines the binding specificity of E1 helicase at the HPV origins. (Sedman and Stenlund. 1995).

1.6.3 Genome maintenance

In order to maintain the viral genome as an independent, episomal element, it has to be preserved and divided uniformly among daughter cells during mitosis. Virus association to cellular chromatin provides distribution of viral genomes to daughter cells in approximately equal numbers and ensures their retention in the nucleus after cell division. The partitioning and episomal maintenance of the viral genomes in the maintenance phase depends on the viral E2 protein (Ilves *et al.*, 1999; Skiadopoulos and McBride, 1998). Long-term and stable maintenance of replicons requires, in addition, the minichromosome maintenance element, a cis element containing multiple E2 binding sites (Piirsoo *et al.*, 1996). The E2 protein tethers the viral genome to the host mitotic chromosomes to ensure retention, maintenance and partitioning of the viral genome (Bastien and McBride, 2000; Ilves *et al.*, 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). The DBD of E2 protein binds to binding sites in the viral genome and other regions of E2 (such as TA domain) tether the genome to the mitotic chromosome of the host through protein-protein interactions (McBride, 2013). However, studies have shown that E2 represses transcription by recruiting factors that manipulate cellular chromatin processes (Nishimura *et al.*, 2000; Schweiger *et al.*, 2007; Smith *et al.*, 2010; Wu *et al.*, 2006). Heterodimers, formed by long and short forms of E2, support the initiation of replication but are defective for partitioning (Kurg *et al.*, 2006, 2009).

1.7 Replication

E2 is a multifunctional protein, which is involved in the viral genome replication. The first replication phase is initial amplification, during which the viral copy number is increased to a few hundred copies per cell to establish infection in dividing cells and occurs, when the viral particle enters cell. E1 and E2 are early viral proteins, which are essential for replication

initiation and elongation. All other proteins and enzymes, needed for replication, are provided by the host cell replication machinery. The second phase of PVs is stable maintenance replication, where the viral copy number is kept constant during several rounds of cell division (McBride, 2008). E2 distributes genomes between daughter cells by binding to the viral genome via its DBD domain and to the chromosomes via its TAD domain (Ilves *et al.*, 1999). The first and second phase take place in the basal cells in the bottom of the layer of epithelium. (McBride, 2008)

The last stage of viral replication is vegetative replication, where viral genomes are amplified to a high number of copies. It occurs in the differentiated keratinocytes, when they move upward in the epidermal layer. The second round of amplification requires E1 and E2 proteins. The abundant increase of E1 and E2 proteins is followed by the upregulation of the differentiation-dependent late promoter (Hummel *et al.*, 1992; Ozbun and Meyers, 1998b). The E6 and E7 proteins are absolutely required for S-phase re-entry. Moreover, E7 protein is needed for G2-phase prolonging in differentiated cells (Banerjee *et al.*, 2011), when vegetative amplification of HPV DNA takes place (Wang *et al.*, 2009). There are two mechanisms suggested for vegetative replication. During differentiation, the cellular environment changes and cells no longer produce factors, which are necessary for DNA replication. That means that environment becomes unsuitable for DNA replication and the virus must overcome these restrictions. Rolling-circle replication is unidirectional and one initiation event generates multiple copies of the genome thereby enabling rapid synthesis of viral DNA. As theta mode of replication requires initiation with every round of replication, the virus can switch to rolling-circle replication mode, facilitating the generation of large amounts of DNA and escaping the unfavorable conditions of the differentiated cell (Flores and Lambert, 1997). After amplification phase, the viral genome is packaged into the capsids. (McBride., 2008).

2. EXPERIMENTAL PART

2. Objective of the study

E2 is a very important HPV protein. It plays a crucial role in the viral life cycle regulation. HPV E2 proteins are expressed at very low levels and generally it is very difficult to detect them using Western blot analysis (Kurg *et al*., 2010). This fact significantly complicates studies of these proteins. In order to increase their expression level, nucleotide sequence of the 5' end of HPV18 E2 ORF was changed to have the most frequent codons in mammalian cells (Oliveria *et al.*, 2006). To facilitate detection of the expression level of E2 protein, codon optimized E2 protein encoding sequence was cloned into pCMV-Flag-4 plasmid, which contains polypeptide protein tag DYKDDDDK, where D is aspartic acid, Y is tyrosine, and K is lysine.

The objective of this study is:

- cloning of codon optimized sequence coding for E2 protein of HPV18 into pCMV-Flag-4 plasmid;
- control of the expression level of the Flag-tagged E2 protein using Western blot analysis;
- testing biological activity of the modified E2 protein using HPV18 genome deficient for E2 expression (HPV18E2$^-$) and U2OS cells;

2.2 Materials and methods

2.2.1 Plasmids

- HPV18 E2 expression vector (pQM18E2)

pQM18E2 vector comes from laboratory collection. This vector encodes codon optimized E2 which biological activity has been tested previously (Kurg *et al.*, 2010).

- pJET1.2/blunt cloning vector

2974 bp length cloning vector. It is suitable for cloning of PCR products and sequencing of cloned fragments (Thermo Fisher Scientific).

- pCMV-Flag-4 vector

6271 bp length vector (Sigma Aldrich). It contains HindIII and BamH restriction sites and Flag tag encoding sequence before the HindIII site.

- E2-Flag HPV18 plasmid

This plasmid comes from laboratory collection. It encodes Flag-tagged wild type E2 of HPV18 cloned in pCMV-Flag-4 vector in HindIII and BamHI restriction sites.

- Wild type HPV18 minicircle

This plasmid comes from laboratory collection and encodes wild type HPV18 genome.

- HPV18E2- minicircle

This plasmid comes from laboratory collection. It encodes HPV18 genome containing point mutation in E2 first AUG and therefore deficient for expression of E2 protein and replication.

2.2.2 PCR and agarose gel electrophoresis

Codon optimized E2 was amplified using PCR, pQM18E2 plasmid (Kurg et al., 2010) as a template and Eppendorf 5331 Mastercycler Gradient PCR Thermal Cycler. PCR mix included 1µl of template DNA (20 ng/µl), 4µl of 5xbuffer, 0,5 µl of oligo mix (10 µM), 0,4 µl of dNTP (10mM), 0,2 µl of Phusion Polymerase (Thermo Fisher Scientific) and 14,9 µl of H_20. The following primers were used:

HPV18 E2 RV BamHI	GGATCCTTACATTGTCATGTATCC
HPV18 E2 FW HindIII	AAGCTTCAGACCCCCAAAGAGACC

PCR program consisted of 3 min 96 ºC of initial denaturation of the template and 20 cycles of denaturation at 96 ºC for 30 sec, primer annealing at 59 ºC for 20 sec and synthesis of the product at 72 ºC for 1 min. The samples were analyzed on 0,8% agarose gel in TAE buffer.

2.2.3 Purification of PCR products from the gel

Amplified codon optimized E2 DNA fragment was excised and purified from the agarose gel using Zymoclean™ Gel DNA Recovery kit (Zymo Research) according to manufacturer's instructions. A piece of agarose gel was melt at 55 ºC for 10 min, the suspension was transferred to column containing DNA binding silica. After the series of washes, DNA was eluted in 20 µl of nuclease-free water.

2.2.4 Ligation of purified PCR products into pJET1.2/blunt cloning vector

Ligation reaction contained 0,5 µl of pJET1.2/blunt cloning vector, 7,5 µl of the gel purified PCR product, 1 µl of 10x buffer, and 1 µl T4 ligase. Ligation mix was incubated at 16 ˚C overnight.

2.2.5 Transformation of DH5a

Eschericia coli DH5a competent cells were transformed with the ligation mix (pJET1.2/blunt + purified codon optimized E2 PCR product) and plated on LB agar supplemented with 50 µg/ml of ampicillin. Cells were incubated overnight at 37˚C. Only bacterial cells containing recombinant plasmids, were able to form colonies. Next, five colonies were selected and inoculated into LB supplemented with 200 µg/ml of ampicillin. Cells were grown at 37˚C 220 rpm overnight.

2.2.6 Isolation of plasmid DNA (Miniprep)

Plasmid DNA was isolated according to the protocol of FavorPrep™ Plasmid Extraction Mini Kit. Briefly, bacteria were lysed, DNA was bound to the silica matrix, washed and eluted in 50 µl of nuclease-free water. Concentration of DNA was measured with NanoDrop™ spectrophotometer.

2.2.7 Restriction analysis and sequencing

Restriction reaction contained 0,5 µg DNA, 2 µl of 10xFastDigest buffer, 0,5 µl of HindIII enzyme, 0,5 µl of BamH enzyme and H_20 (up to 20 µl). The reaction was incubated at 37˚C for 1 hour. To detect the presence of the correct bands, the samples were run on 0,8% agarose gel, and 1kB O'GeneRuler DNA ladder (Thermo Fisher Sientific) was used as a reference. The size of the expected fragments was 2,7 and 1,1 kb.

In order to check the sequence of the cloned codon optimized E2, samples were sent to the Core Laboratory for DNA Genotyping and Sequencing in the Institute of Molecular and Cell Biology. Sequencing analysis did not reveal any mutation in E2.

Next, coding sequence the codon optimized E2 was excised from pJET1.2/blunt vector, purified from agarose gel and ligated into the pre-cleaved and agarose gel-purified pCMV-Flag-4 vector. Restriction reaction contained 5 µg DNA, 2 µl of 10xFastDigest buffer, 1,5 µl of HindIII enzyme, 1,5 µl of BamHI enzyme and H_20 (up to 20 µl). Ligation reaction was performed as described in 2.2.4. The obtained construct was defined as HPV18 E2c.o.pCMV-Flag.

2.2.8 Cell lines and transfection of U2OS cells and 293T cells

In my work, I used the human osteosarcoma U2OS cells and 293FT cells (a derivative of Human Embryonic Kidney cell line HEK293). U2OS cells were cultured in the IMDM (Iscove's Modified Dulbecco's Medium) + 10% Fetal Bovine Serum + 1% penicillin/ streptomycin mix. 293T cells were grown in DMEM-High Glycose (Dulbecco's Modified Eagle Medium) + 10% Fetal Bovine Serum + 1% penicillin/ streptomycin mix. Cells were

plated on 6-well plates 24 hours before transfection. Cells were incubated at 37˚C and 5% CO_2.

Transfection of U2OS cells

U2OS cell were transfected using Reagent 007 (Icosagen). Different amounts of HPV18 E2c.o.pCMV-Flag plasmid were added for each transfection: 0, 0,5 ng, 1 ng, 10 ng and 100 ng. Also, 0,5 µg of mutant HPV18E2$^-$ genome was added to each sample. GFP-encoding plasmid (100 ng) was used as a control. Also, 200 ng of reference HVP18 wild type genome was transfected separately. The transfection mixes contained 20 µl of reagent 007 and respective DNA in sterile water. DNA/Reagent 007 mixture was incubated at room temperature for 5-10 min. Then, cells were washed with PBS and 1,5 ml of pure IMDM and DNA/007 mixture were added to each well. After 2-3 hours, normal growth medium (IMDM supplemented with FBS and ampicillin/streptomycin) was added to the cells, and cells were incubated for 72 h.

Transfection of 293FT cells

293FT cells were transfected using Polyethylenimine reagent (PEI) (Inbio). First, 100 µl of pure DMEM was added to each microtube. Different amounts of HPV18 E2c.o.pCMV-Flag plasmid were diluted in DMEM: 200 ng of HPV18 E2c.o.pCMV-Flag + 1800 ng empty vector, 1000 ng of HPV18 E2c.o.pCMV-Flag + 1000 ng empty vector and 2000 ng of E2c.o.pCMV-Flag. To compare expression of codon optimized and wild type E2, 2000 ng of plasmid encoding Flag-tagged wild type E2 of HPV18 was added to the separate microtube. Next, 10 µl of PEI stock solution (concentration 80 mg/ml) was diluted in 790 µl of sterile water (resulted concentration 1 mg/ml) and 5 µl of the diluted PEI were added to each microtube. DNA/PEI mixture was left at room temperature for 5-10 min. The cells were washed with PBS and 1,5 ml of pure DMEM and DNA/PEI mixes were added. After 2-3 hours, 2 ml of normal growth medium (DMEM supplemented with FBS and antibiotics) was added to the cells, and cells were incubated for 48 h.

2.2.9 Replication assay using different concentrations of E2c.o.pCMV-Flag construct and HPV18E2- and HPV18wt genomes

Total DNA from transfected U2OS cells was extracted as described in Ref. (Geimanen et al., 2011)**.** Five µg of total DNA was cut with restriction enzymes BglI (2 µl per sample), which linearizes HPV DNA, and DpnI (2 µl per sample), which cuts only bacterially methylated DNA. The products were run on 0,8% gel, 30 V, in TAE buffer overnight and then blotted to nylon membrane in 10x SSC buffer using capillary transfer method, which was followed by pre-hybridization in solution containing 9 ml 20x SSC, 3 ml 50x Denhardt's Reagent (Termo Fisher Scientific), 1.5 ml 10% SDS, 0.6 ml of denaturated salmon sperm DNA and 16 ml of sterile water at 65 oC for 2 h and hybridization with a radioactively labelled HPV18 specific probe at 65 oC under slow rotation overnight. The probe was synthesized using 100 ng of linearized and gel-purified HPV18 template, α-32P-dCTP (PerkinElmer) and DecaLabel DNA Labeling Kit (Thermo Fisher Scientific) according to manufacturer's instructions. After hybridization, the membrane was washed and exposed to X-ray film.

2.2.10 Immunoblotting

For immunoblotting analysis, transfected 293FT cells were lysed in 200 µl of RIPA buffer (50 mM Tris pH 7,5, 150 mM NaCl, 2 mM EDTA, 0,1% SDS and 0,1% TRITON-X100) for 15 min on ice. The lysates were cleared by centrifugation at 4 ºC 13 000 rpm for 10 min. Next, 15 µl of lysate was mixed with 15 µl of 2x Laemmli Sample Buffer (0.125M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 100 µM DDT, 0.004% bromophenol blue). Samples were denaturated at 100 oC for 10 min. Proteins were separated by polyacrylamide gel electrophoresis and transferred to PVDF membrane (Millipore). The membrane was blocked in PBS containing 0,1 % Tween-20 and 5 % of non-fat dry milk and then incubated with anti-Flag-HRP antibody (Sigma Aldrich) diluted 1:5000 in PBS-Tween solution containing 2,5 % of non-fat dry milk at RT and slow end-to-end rotation for 1 hour 20 min. The membrane was washed in PBS-Tween solution 3 times for 15 min. Enhanced chemiluminescence (ECL) HRP substrate (SuperSignal™ West Dura Extended Duration kit, Thermo Fisher Scientific) was added to membrane and incubated for 1 min at RT. The membrane was rinsed in PBS-Tween for 10 sec and exposed to X-ray film.

2.3 Results and discussion

In order to check, if E2-Flag codon optimized protein is functional, its biological activity must be tested. It is known that E2 (together with E1 protein) is absolutely needed for HPV replication. Namely, if virus genome enters the host cell, it can replicate its DNA and partition it to daughter cells only in the presence of E1 and E2 proteins. All other proteins, which are essential for viral genome replication are provided by the host cell replication machinery. Osteosarcoma U2OS cells support viral replication, i.e they have all vital proteins required for HPV replication, except E1 and E2, which are encoded by HPV genome.

If wild type genome of HPV is transfected into U2OS cells, E1 and E2 are expressed and viral genome replication is initiated, and in three days the number of virus genomes in cells will dramatically increase. However, if HPV18E2- is transfected, it will not be able to replicate because of absence of E2, and after three days HPV18E2- DNA will be almost disappeared from the cells. In that case, cells will keep dividing, whereas mutant HPV genome will not partition between daughter cells and number of viral copies will decrease. However, if the plasmid, which encodes the functional protein E2 is added to the cells with mutant HPV genome, then this E2 compensates the absence of endogenous E2 and the mutant genome replicates.

Both E1 and E2 are toxic for cells, i.e. cells can die, if those proteins are in abundance. In case of wild type virus, the amount of E1 and E2 proteins is regulated so, that those proteins are enough for the viral genome replication. If exogenic E2, which is expressed from plasmid is added to the cells, its amount is unclear. It can be very high, much higher than is case of wild type virus and endogenic E2. On the other hand, it can be too low, if less plasmid is added. Besides, introduction of exogenous sequences may alter biological activity of the resulted protein. The modified E2 protein cloned into pCMV-Flag vector contains N-terminal Flag tag that potentially may influence its properties and expression level. Therefore, different amounts of codon optimized E2 encoding plasmid have to be tested together with HPV18E2$^-$ genome. In this way, it is possible to understand, if new codon optimized E2-Flag protein has the same properties as wild type E2 and, which amount of plasmid has to be transfected to manifest these properties.

My work consisted of two parts. First, I generated a new construct by cloning codon optimized HPV18 E2 encoding sequence obtained by PCR into pCMV-Flag-4 expression vector. The obtained construct was defined as E2c.o.pCMV-Flag. Second, I tested the biological activity of the codon optimized Flag-tagged E2 protein using different concentrations of E2c.o.pCMV-Flag construct and HPV18E2- mutant with subsequent detection using Southern blot analysis.

Cloning of E2c.o.pCMV-Flag

In order to obtain the codon optimized HPV18 E2, I amplified the template described in Ref. (Kurg et al., 2010) using primers containing HindIII and BamHI sites on their 5' ends. The obtained PCR product was purified and cloned into vector. Plasmid DNA of five independent clones of HPV18E2co-pJET1.2/blunt was extracted, and the clone lacking mutations was verified by restriction analysis and DNA sequencing using primers specific for pJET1.2/blunt vector. The correct fragment encoding codon optimized E2 was excised from pJET1.2/blunt vector and cloned into pCMV-Flag-4 vector between HindIII and BamHI sites. Plasmid DNA of 5 independent clones of the presumptive E2c.o.pCMV-Flag was purified (their concentrations are shown in Table 1) and controlled by restriction analysis using HindIII and BamHI enzymes (Figure 3). The length of the expected fragments was 6271 nt (corresponding to length of the pCMV-Flag-4 vector) and 1093 nt (corresponding to the length of HPV18 E2 with additional HindIII and BamHI restriction sites on 5' and 3' ends, respectively).

Table 1. The concentration of the obtained E2c.o.pCMV-Flag-4 plasmid DNA

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
215 ng/µl.	127 ng/µl.	185 ng/µl.	189 ng/µl.	229 ng/µl.

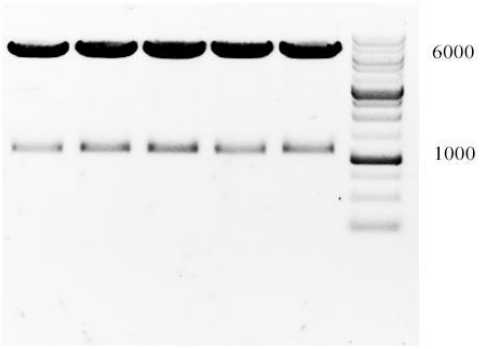

Figure 3. Restriction analysis of the E2c.o. cloned into the pCMV-Flag-4 cloning vector. Clones were cut using HindIII and BamHI restriction enzymes. Expected length of the fragments was 6271 nt (corresponds to pCMV-Flag-4 vector) and 1093 nt (corresponds to E2c.o. PCR product). DNA ladder O'GeneRuler 1kB was used as a reference.

Control of expression level of codon optimized HPV18 E2

In order to check, whether HPV18 codon optimized Flag-tagged E2 is expressed, I used 293FT cells and subsequent Western blot analysis. HPV18 wild type Flag-tagged E2 was used as a control. 293FT cells were transfected with 200, 1000 or 2000 ng of E2c.o.pCMV-Flag encoding plasmid and 2000 ng of HPV18 wild type E2pCMV-Flag construct. The transfected cells were incubated for 48 h and lysed. Whole cell extracts were subjected to Western blot using anti-Flag-HRP antibody (Figure Y). As expected, the analysis revealed that Flag-tagged wt E2 was not detectable, but the Flag-tagged codon optimized E2 was detectable from the sample transfected with 2000 ng of E2c.o.pCMV-Flag encoding plasmid (Figure 4). The band was detected at approximately 42 kDa that corresponds to the expected size of HPV18 E2. Also, non-specific band at approximately 70 kDa was detected in all samples.

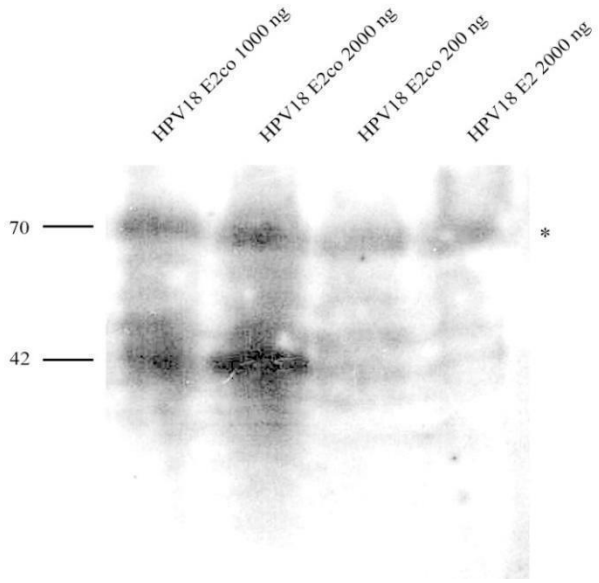

Figure 4. Detection of HPV18 codon optimized Flag-tagged E2 protein by Western blot. Different amounts of the constructs encoding HPV18 E2c.o.-Flag were transfected into 293FT cells. Samples 1 and 3 also contained 1000 ng and 1800 ng of empty vector, respectively. The cells were lysed 48 h after transfection and subjected to WB analysis using anti-Flag-HRP antibody. Asterisk indicates non-specific band.

Control of biological activity of codon optimized Flag-tagged E2 in U2OS cells

To check the biological activity of HPV18 codon optimized Flag-tagged E2, I used U2OS cells and analyzed results using Southern blot. Wild type HPV18 minicircle was used as a control. U2OS were transfected with HPV18 E2$^-$genome and either with 0,5. 1, 10, 100 ng of E2c.o.pCMV-Flag encoding plasmid or 150 ng of GFP. Last transfection contained 200 ng of HPV18 wild type genome used as a control. Transfected cells were incubated for 72 hours and lysed. Results show that new E2c.o.pCMV-Flag plasmid supports viral DNA replication. Eight kb bands correspond to the size of replicated HPV18 DNA (Figure 5, lanes 2 – 5). Highest amount of replicated viral DNA is detected in the sample with 100 ng of E2c.o.pCMV-Flag plasmid (Figure 5, lane 5).

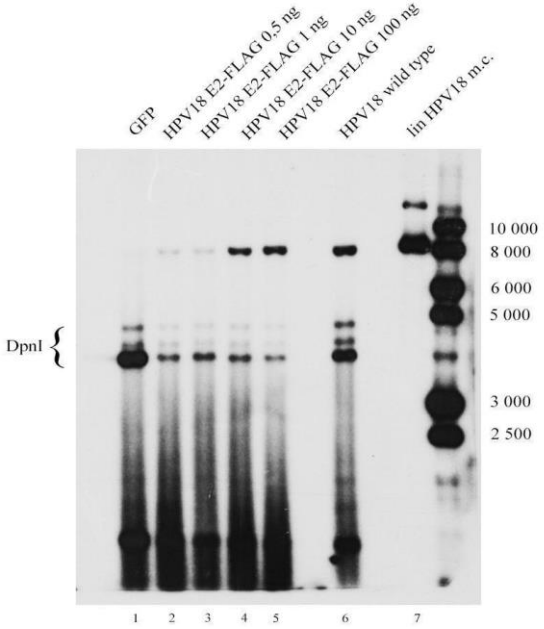

Figure 5. Analysis of E2 biological activity using Southern blot. HPV18E2$^-$ genome was transfected together with either different amounts of the construct encoding HPV18 E2c.o.-Flag (lanes 2 – 5) or GPF (lane 1). Wild type HPV18 genome was transfected as a control (lane 6). in U2OS cells, 72 h. The DpnI-resistant replicated HPV18 DNA was linearized using BglI restriction enzyme. It forms an 8 kb band on the top of the gel. The DpnI-sensitive material (bacterially purified DNA used for transfection) forms a series of lower molecular weight bands. Linearized using BglI HPV18E2$^-$ plasmid (500 pg) used for transfection is shown in lane 7.

CONCLUSION

Present study gives a theoretical overview on the HPV genome organization and life cycle, especially focusing on E2 proteins and their functions.

Papillomavirus E2 protein is a multifunctional protein. E2 protein is a key regulator of HPV life cycle. Studies of E2 protein are complicated due to low protein expression levels. In order to increase expression of E2 protein, codon optimized sequence was inserted to the pCMV-Flag-4 vector, which contains Flag- tag encoding sequence. Flag-tag potentially may influence E2 protein properties and expression level.

The carried out experiments resulted in the following conclusions:

- codon optimization of E2 protein sequence increased the expression level of HPV18 E2 proteins in 293FT cells;
- codon optimized E2-Flag protein supported replication of HPV18 $E2^-$ deficient genome in U2OS cells.

Inimese papilloomiviiruse tüüp 18 koodon optimiseeritud

Flag-märgisega E2 valgu kloneerimine ja analüüs

Kristina Orlova

Resümee

Inimese papilloomiviirused on väga levinud patogeenid, neil on suur meditsiiniline tähtsus, sest juba ammu on leitud nende seos halvaloomuliste kasvajatega. Seksuaalselt levivad tüübid jagunevad kahte kategooriasse: "kõrge riskiga", mis võivad põhjustada vähki ja "madala riskiga", mis võivad põhjustada tüükaid. Põhjalikum HPV uurimine on väga oluline, kuna HPV elutsüklist täpsem arusaamine võimaldab luua viirusvastast ravi. Tänapäeval on ainus võimalus HPV nakkuse ennetamiseks vaktsineerimine.

Papilloomiviirused on väikesed DNA viirused, mis nakatavad epiteelrakke paljudel selgroogsetel ja indutseerivad rakkude proliferatsiooni. Viiruse genoomi säilitatakse sellistes rakkudes tsirkulaarse episomaalse replitseeruva elemendi kujul.

Papilloomiviirused kodeerivad üheksa valku: E1, E2, E4, E5, E6, E7, E8, L1 ja L2. Kõige rohkem on uuritud valku E2. E2 valk on väga oluline viiruse elutsükli reguleerija. Tema funktsiooniks on viiruse replikatsiooni initsieerimine, transkriptsiooni reguleerimine. Samuti on E2 vajalik viiruse genoomi tütarrakkude vahel jagamiseks, püsiva infektsiooni tekkimiseks.'

Esimene töö osa annab teoreetilise ülevaate inimese papilloomiviiruse elutsüklist ja genoomi ülesehitusest, fokusseerudes E2 valgu funktsioonidele.

HPV18 E2 valgud ekspresseeruvad väga madala tasemel ja mõnikord on neid raske detekteerida, kasutades Western blot-i analüüsi. Antud töö eesmärgiks oli E2 koodon optimiseeritud järjestuse pCMV-Flag-4 vektorisse kloneerimine edasise ekspressioni taseme kontrollimisega ja bioloogilise aktiivsusega testimisega.

Antud bakalaureuse töö on valminud Tartu Ülikooli Tehnoloogiainstituudis papilloomiviiruse uurimisrühmas.

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