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MART USTAV Jr

Molecular Studies of HPV-18 Genome Segregation and Stable Replication

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Molecular Studies of HPV-18 Genome Segregation and Stable Replication

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Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia

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"Don't go with the flow, be the flow"
Shawn Corey Carter

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LIST OF ORIGINAL PUBLICATIONS AND INVENTIONS

(I) Geimanen, J.; Isok-Paas, H.; Pipitch, R.; Salk, K.; Laos, T.; Orav, M.; Reinson, T.; Ustav, M. Jr.; Ustav, M.; Ustav, E. (2011). Development of a cellular assay system to study the genome replication of high- and low-risk mucosal and cutaneous human papillomaviruses. Journal of Virology, 85(7), 3315–3329.

(II) Toots, Mart; Männik, Andres; Kivi, Gaili; Ustav, Mart JR.; Ustav, Ene; Ustav, Mart (2014). The Transcription Map of Human Papillomavirus Type 18 during Genome Replication in U2OS Cells. PLoS ONE, 9(12): e116151. doi:10.1371/journal.pone.0116151

(III) Ustav M Jr., Rodriguez Castaneda F, Reinson T, Männik A, Ustav M. 2015. Human papillomavirus type 18 *cis*-elements crucial for segregation and latency PLoS ONE 10(8): e0135770. doi:10.1371/journal.pone.0135770

My contributions to these publications are as follows:
I – I cloned the E1 and E2 frameshift mutants and performed the assay demonstrating E1/E2 dependent replication, analyzed keratin expression in U2OS cells, and participated in writing of the manuscript.
II – I analyzed and demonstrated the replication of HPV-18 genomes used in the transcription mapping, performed and analyzed the primer extension assay, developed the E2C antibody and performed the WB analysis of novel E2C proteins, performed the 5' RACE analysis of the E2C mutant genomes, and participated in writing of the manuscript.
III – I designed the experiments used in this manuscript, designed and performed the segregation assay and cloned the assay plasmids for HPV-18, developed the E2BS linkermutants and analyzed the transient and stable replication of these mutants, performed 5' RACE analysis of linkermutant HPV-18 genomes, and wrote the manuscript.

INVENTIONS
Method and a kit for identifying compounds capable of inhibiting human papilloma virus replication; Omanik: **Icosagen Cell Factory OÜ**; Authors: Mart Ustav, Ene Ustav, Jelizaveta Geimanen, Regina Pipitš, Helen Isok-Paas, Tormi Reinson, Mart Ustav, Triin Laos, Marit Orav, Anu Remm, Kristiina Salk, Andres Männik; Prioriteedinumber: PCT/EE2010/000010; Prioriteedikuupäev: 19.05.2010

Development of an assay system to identify novel HPV replication inhibitors by high-throughput screening; Authors: Andres Männik, Mart Toots, Mart Ustav Jr., Andres Tover, Meelis Kadaja, Mart Ustav; Applicant – Icosagen Cell Factory OÜ. **Submitted 05/2015.**

LIST OF ABBREVIATIONS

ATM – ataxia telangiectasia mutated
ATP – adenosine triphosphate
ATR – ataxia telangiectasia and Rad3-related protein
BM – basement membrane
bp – base pair
BPV – bovine papillomavirus
Brd4 – bromodomain-containing protein 4
BS – binding site
CD21 – complement receptor type 2
CDC25 – cell division cycle 25 homolog A protein
CHK1 – checkpoint kinase-1
C-terminal – carboxyl-terminal
DBD – DNA binding domain
DDR – DNA damage response
DH – double hexamer
DS – dyad symmetry element
DSB – DNA double-stranded break
dsDNA – double-stranded DNA
DT – double trimer
E1BS – the E1 protein binding site
E2BS – the E2 protein binding site
E6-AP – E6 associated protein
EBNA-1 – Epstein Barr Virus Nuclear Antigen 1
EBP2 – EBNA1 binding protein 2
EBV – Epstein Barr Virus
ECM – extracellular matrix
EGF – epidermal growth factor
EMSA – electrophoretic mobility shift assay
EP400 – E1A-binding protein p400
FasL – Fas ligand
FR – family of repeats
GFP – green fluorescent protein
H2A – histone H2A
H2B – histone H2B
HDAC3 – histone deacetylase 3
HLA - human leukocyte antigen
hnRNP – heterogeneous nuclear ribonucleoproteins
HPV – human papillomavirus
IRF-3 – interferon regulatory factor 3
KSHV – Kaposi Sarcoma Associated Herpesvirus
LANA 1 – latency associated nuclear antigen 1
LBS – LANA-1 binding site
LCR – long control region

MHC – major histocompatibility complex
Mklp2 – mitosin like kinase
MME – minichromosome maintenance element
mRNA – messenger RNA
NBS1 – nibrin 1 protein
NcoR1 – nuclear receptor co-repressor 1
N-terminal – amino-terminal
Oligomeric (HPV) genome – viral DNA containing several copies of the HPV genome
ORF – open reading frame
ori – replication origin
pAE – early polyadenylation site
pAL – late polyadenylation site
PaVE – papillomaviruse episteme
PDGF-beta – platelet-derived growth factor beta
pRB – retinoblastoma family protein
pre-RC – pre-replication complex
PV – papillomavirus
RALYL – RNA-binding Raly-like protein
RPA - replication protein A
SETDB1 – histone-lysine N-methyltransferase
SMC1 – structural maintenance of chromosomes 1 protein
SMCX – lysine-specific demethylase 5C
Sp1 – specificity protein 1
ssDNA – single-stranded DNA
TAD – transactivation domain
TBP – TATA box-binding protein-like protein
TLR-9 – Toll-like receptor 9
TopBP1 – topoisomerase II β-binding protein 1
Topo I – type I topoisomerase
TRAIL – TNF-related apoptosis-inducing ligand
TRIM28 – tripartite motif-containing 28

1. INTRODUCTION

An association between papillomaviruses and epithelial malignancies had already been proposed by the 1970s; Dr. Harald zur Hausen was one of the first to hypothesize that human papillomaviruses (HPV) might be the cause of certain cancers. In 1983 and 1984 he was able to isolate two types of HPV genomes from cervical cancer samples – HPV-16 and HPV-18. For his ground breaking discovery he was awarded the Nobel Prize in Physiology and Medicine in 2008. After discovering the link between infection with certain subtypes of HPV and cancer, papillomaviruses have been intensively studied, resulting in new information about the viral DNA replication, transcription, maintenance and oncogenicity of papillomaviruses and their genes. In 2006, the first vaccine against cancer was introduced – a preventative vaccine against HPV. Nevertheless, scientists have continued to study the molecular aspects of papillomavirus functions as millions of people are and likely will be infected and are therefore at risk of acquiring cancer. The pursuit of identifying novel antivirals continues with the objective of inhibiting viral gene expression, certain steps of DNA replication or segregation, thereby eliminating the infection. Elucidating the mechanisms of cellular transformation and oncogenesis induced by the E5, E6 and E7 viral oncoproteins may reveal novel antiviral targets for treating cervical cancer.

A suitable model system is required to analyze the different aspects of a studied phenomenon. For a long period of time, the model system for papillomavirus molecular biology was based on bovine papillomavirus type 1 (BPV-1). For BPV-1, the suitable cellular system is based on the mouse tumor cell line C127, where establishment of viral infection, initiation of DNA replication, persistence of the episomal genome, transcription, segregation and cellular transformation have been studied. This model system has provided most of the current information on viral genome features, viral genes, mechanisms of transcription, gene expression, DNA replication and segregation as well as cellular transformation by BPV-1.

There are also model systems described for HPV; however, until recently, a similar robust cellular system was lacking for HPV (Chow 2015). Thus, there was no good platform on which to investigate molecular mechanisms of the virus and that could also become the basis for screening antivirals. In this thesis, I refer to data that use the human osteosarcoma-derived cell line U2OS, as this cellular platform supports the replication different subtypes and genera of HPV genomes. Our studies have shown that viral DNA replication, early gene transcription, splicing and other functions reflect the natural molecular mechanisms, as these exist during natural viral infection of primary keratinocytes. The only exceptions that we currently cannot reconstruct in U2OS cells are expression of late capsid proteins and assembly and maturation of infectious viral particles. After discovering the potential of the cellular system for HPV genome-level studies, our workgroup has extensively used this to characterize the molecular mechanisms of high and low risk mucosal HPVs and cutaneous HPVs. Some of

these studies are included in my thesis. In particular, I focus here on describing the molecular mechanisms and factors of clinically important oncogenic human papillomavirus HPV-18. In these studies, we described the HPV-18 transcription map in U2OS cells. In addition, the thesis includes the characterization of specific elements and factors required for the maintenance and latency of the viral genome in infected cells.

2. LITERATURE OVERVIEW

2.1. General introduction to papillomaviruses

The Papillomaviridae (PV) family consists of large group of DNA viruses infecting a wide variety of vertebrates. PVs that are able to infect humans are defined as human papillomaviruses (HPVs). Over 200 HPV subtypes have been identified from genetic information obtained from patient epithelial tissue samples, and nearly 140 animal Papillomaviruses are listed in the Papillomaviruse Episteme (PaVE) database. Furthermore, the recent implications of next generation sequencing have enabled the discovery of several new Papillomavirus genotypes from asymptomatic tissue samples that would otherwise have been undetected or could not be cloned due to their low copy number (Eklund, Forslund et al. 2014) (Arroyo, Smelov et al. 2013). Newly discovered genotypes are constantly uploaded onto the PaVE database and verified by the International Papillomavirus Reference Center (Bzhalava, Eklund et al. 2015).

Papillomaviruses infect cutaneous and mucosal epithelia and are associated with several malicious diseases – cervical cancer, oropharyngeal cancers, penile cancer, anal cancer in mucosal epithelia and squamous cell carcinoma (SCC), actinic keratosis (AK) and keratoacanthoma (KA) in cutaneous epithelia (Walboomers, Jacobs et al. 1999) (Asgari, Kiviat et al. 2008) (Harwood, Surentheran et al. 2004) (Mackintosh, de Koning et al. 2009) (Forslund, DeAngelis et al. 2003) (IARC 2007). Papillomaviruses are usually classified as high-risk and low-risk PVs, depending on their association with malignancies. Papillomaviruses are also responsible for benign tumors, such as genital warts or condylomas that are mainly due to infection with low-risk HPV 6 and 11. HPV DNA has been found in approximately 99% of cervical cancer lesions; however, in condylomatas, the number of HPV-positive samples had been approximately 96%, providing evidence that HPV-independent condylomas exist (Arroyo Muhr, Bzhalava et al. 2015). However, new metagenomic sequencing technologies have challenged the hypothesis of the existence of papillomavirus-negative condylomata, as deep sequencing has detected HPV in almost all condylomata biopsies (Johansson, Bzhalava et al. 2013) (Arroyo Muhr, Bzhalava et al. 2015). As HPV-16 and HPV-18 are known to be the cause of the absolute majority of cervical cancer cases, these viruses can be considered some of the strongest carcinogens (Burd 2003).

The current therapies for treating HPV infections primarily involve surgery or cryotherapy for the excision or destruction of the infected tissue, respectively. Although effective in many cases, these techniques are invasive and complicated, especially in facial and genital regions. Non-surgical treatments involve the topical administration of immunomodulators, such as imiquimod or podophyllin. However, the efficacy of non-specific immunotherapy is limited, and the rate of recurrence is relatively high, especially in immunocompromised patients (Scheinfeld and Lehman 2006). A more effective approach has been to prevent viral infection by the development of vaccines. In 2006, Cervarix and

Gardasil were approved as preventive HPV vaccines, initially covering 4 HPV subtypes (6, 11, 16, 18). In 2014, the FDA approved the 9-valent HPV vaccine Gardasil-9 that protects against up to 90% of all cervical cancer-causing HPV types (Siddiqui and Perry 2006) (Schmiedeskamp and Kockler 2006) (Gizzo, Noventa et al. 2013). Nevertheless, there is an unmet medical need for HPV antivirals to combat the virus in already infected people. Thus, as not all vaccination programs include the HPV vaccine, millions of people are diagnosed with HPV infections every year.

Very limited numbers of anti-HPV drugs have been found. These few examples include inhibitors for ATPase activity of the viral replication protein E1, inhibitors for the interaction of viral replication proteins E1-E2, and compounds targeting the oncoproteins E6 and E7, which are reviewed in (D'Abramo and Archambault 2011). For these compounds, which have been identified mainly by biochemical assays *in vitro* and/or by surrogate cell-based assays, the antiviral effects are generally weak and are usually specific to particular types of HPVs. The development of relevant cellular model systems and the description of viral mechanisms of action are key for setting up efficient screening for antivirals that can be used to cure existing viral infections.

2.2. The papillomavirus life cycle

The papillomavirus life cycle is a complex process; it takes advantage of physical injury to the epithelial tissue, interacting intensively with the host cell to ensure its productive life cycle. Simultaneously, it activates the appropriate countermeasures to avoid an antiviral response from the host. In this chapter, I have dissected the viral life cycle into three distinct sections: (i) Mechanism of viral entry to the host cell; (ii) Establishment of infection; (iii) Host differentiation and productive phase of the PV.

2.2.1. Viral entry

Papillomaviruses infect epithelial tissues, and their life cycle is linked to the differentiation of epithelial cells. For entering the epithelium, microinjury of the epithelial tissue is required for viral particles to reach and attach to the basement membrane (BM). The receptors responsible for viral particle binding are comprised of heparan sulfate proteglycan (HSPG) and laminin 5, which are components of the extracellular matrix (ECM). Virion affinity for these receptors enables the guidance of virions to the basal proliferating epithelial layer of keratinocytes, of which infection is targeted (Culp, Budgeon et al. 2006) (Culp, Budgeon et al. 2006). After binding of the virions to the basal membrane, a series of conformational changes is triggered within the viral particles that induces the cleavage of L2 by furin-PC5/6, after which the virions attach to a receptor on proliferating basal epithelial cells, which are permissive for infec-

tion (schematically represented in Fig. 1) (Kines, Thompson et al. 2009). The wound generated in the epidermis induces the proliferation and migration of basal keratinocytes with mitotic capability to heal the wound. The presence of mature HPV virions capable of infecting the proliferating cells would grant the right cellular target for HPV. Studies have shown that only the epithelial cells proximal to the BM are mitotic (Blanpain and Fuchs 2006), and papillomaviruses are only able to infect cells that are in M phase (Pyeon, Pearce et al. 2009). Thus, the targeting of a specific period of the cell cycle assures that viral genome replication will be initiated and that infection may be established.

The entry of the virus by endocytosis of clathrin-coated vesicles is itself a slow process, with viral disassembly detected 6-8 h post-entry (Day, Lowy et al. 2003) (Day, Baker et al. 2004). Approximately 24 h post-infection, the viral genome can be found in the nucleus. During transport of the viral genome, the minor viral capsid protein L2 plays an additional significant role in granting efficient infection. After furine cleavage, the C-terminal fragment of the L2 protein acts as a vehicle for efficient release from the endosome and also transport to the nucleus. This has been confirmed by the fact that both the C-terminus of L2 and the genome can be detected in the nucleus. At the same time, the N-terminus of L2 is captured in the endosomes (Richards, Lowy et al. 2006).

2.2.2. Establishment of infection

After entry of the viral genome into the nucleus of the cell, the early promoters of HPV are activated, triggering viral early gene expression and leading to the initial replication of viral genomes in the S- and G2 phases of the cell cycle. Viral E1 and E2 proteins are necessary and sufficient to replicate and maintain viral genome as an episome in dividing cells (Ustav and Stenlund 1991) (Ustav, Ustav et al. 1991) (Ustav, Ustav et al. 1993) (Chiang, Ustav et al. 1992) (Wilson, West et al. 2002). The viral transforming proteins E6 and E7 are expressed from the viral genome, and there is an increase in the number of cells harboring viral genomes shortly after infection. This is triggered by E6- and E7-induced cellular proliferation of the infected cells in the early phase of the infection (Doorbar 2005). Due to the initial transient amplificational DNA replication in basal cells, viral infection can be established, after which HPV maintains itself at a relatively low copy number of 20–200 copies per cell, and all viral early genes are expressed at a low level (Doorbar, Quint et al. 2012). The initial amplificational replication and stable maintenance is further discussed in the respective chapters of this thesis.

2.2.3. Productive phase

Keratinocytes normally initiate and undergo terminal differentiation after migrating from the basal to the suprabasal cell layers, after which they normally

no longer enter S-phase. However, in virus-infected cells in the differentiating epidermis, the expression of oncoproteins still induces activation of the host replication machinery and thereby viral DNA replication (Banerjee, Genovese et al. 2006; Banerjee, Wang et al. 2011). Viral oncoproteins act via the association with cell cycle regulators and are positive stimulators of the cell cycle (Munger, Basile et al. 2001).

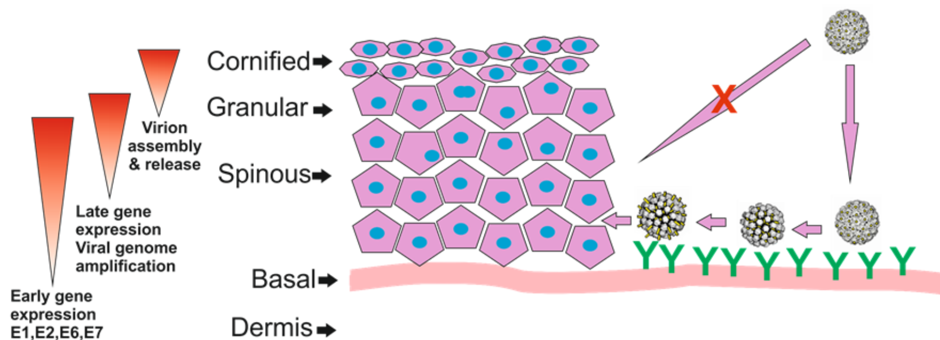


Figure 1. Simplified view of stratified mucosal and cutaneous epithelium and viral gene expression dependent on the state of the virus-infected cell. PVs cannot infect keratinocytes directly; rather, viral capsids have to undergo conformational changes at the basal membrane (BM) before particles become infectious.

Virus-infected cells migrate to the suprabasal level and enter the differentiation phase. During that process, the change in the cellular microenvironment yields change of the splicing pattern and expression of viral proteins, amplification of viral genome and eventually results in the production of the viral capsid proteins, packaging of the viral particles as the virus-infected cell reaches the final layers of terminally differentiated epithelial cells (Cheng, Schmidt-Grimminger et al. 1995) (Johansson and Schwartz 2013). Before the production of late proteins and assembly of viral particles, the viral copy number is increased during the second amplificational replication stage. In uninfected cells, the cell cycle is retarded. In HPV-infected cells, the viral oncogenes E6 and E7 are capable of overcoming this block; they induce S-phase to replicate DNA through inducing endoreduplication without cellular division (Cheng, Schmidt-Grimminger et al. 1995).

Papillomaviruses activate the ATM DDR pathway that acts through three different branches - p53/p21, CHK2/CDC25 and NBS1/SMC1 (Yazdi, Wang et al. 2002) (McKinney, Hussmann et al. 2015). PV amplification has been shown to be mediated through the interaction of these DNA damage response pathways, and this is discussed further in the replication overview chapter of this thesis (Moody and Laimins 2009) (Gillespie, Mehta et al. 2012) (Sakakibara, Mitra et al. 2011) (Kadaja, Sumerina et al. 2007) (McKinney, Hussmann et al. 2015). To amplify the viral DNA in the second amplification phase, all viral

early genes are expressed, including E4 and E5 (Ozbun and Meyers 1998) (Longworth and Laimins 2004).

The viral genome is amplified by the cellular replication machinery, which is activated by E7 and directed at the viral origin by the E1 and E2 proteins. Throughout the viral life cycle, promoter usage and splice site variation regulate the expression of early proteins. To amplify the viral DNA in the second amplificational phase, an increase in the level of E1 and E2 proteins is required. The exact mechanism for the upregulation of E1 and E2 protein expression is not well understood, but a possibility is that a slight increase of promoter activity might trigger the increase of early gene expression, thereby also increasing replication by positive feed-back – the newly synthesized viral genomes would act as templates for the expression of E1 and E2 that would facilitate the additional amplification of viral DNA (Doorbar 2005).

As the cell migrates to the upper level of epithelial tissue, the early polyadenylation site of HPV is repressed, viral mRNA is transcribed through the L2 and L1 ORF, and the late polyadenylation site is used to generate mRNA to express structural proteins L1 and L2 to encapsulate the amplified viral genomes (Doorbar 2005) (Doorbar 2006). During the initial and stable infection phase, the late polyadenylation site is repressed by cellular factors as they are expressed in the basal layer cells (Somberg, Zhao et al. 2008) (Johansson and Schwartz 2013). hnRNP family proteins have been shown to be responsible for the inhibition of pAL, and repression of hnRNP has been shown to activate the expression of L1 and L2 (Zhao, Rush et al. 2004) (Oberg, Fay et al. 2005). More recently, RALYL, a protein that belongs to the hnRNP C family, has been shown to activate late gene expression (Dhanjal, Kajitani et al. 2015). E2 recruits L2 protein to the viral genome, after which L1 and L2 form the viral capsid in the nucleus. The papillomavirus genome is encapsulated within a capsid that contains 360 copies of L1 protein and 72 copies of L2 protein. Viral maturation occurs in the upper most layer of the epithelium within terminally differentiated keratinocytes. The change from the reducing to oxidative environment enables accumulation of disulfide bonds between L1 proteins, thereby resulting in extremely stable viral particles (Finnen, Erickson et al. 2003) (Buck, Thompson et al. 2005). E4 protein seems to play a role in viral exit from cells as E4 disrupts the keratin structure by assembling into amyloid fibers, thereby enabling infectious viral particles to exit the cells (McIntosh, Martin et al. 2008) (Wang, Griffin et al. 2004) (Brown, Kitchin et al. 2006).

2.3. Genomic organization of papillomaviruses and viral proteins

Papillomaviruses have a circular double-stranded DNA genome that is approximately 8 kb in size. The genome can be divided into three regions – early (E), late (L) and noncoding long control regions (LCR). The early region of papillomaviruses encodes seven common open reading frames (E1, E2, E4, E5,

E6, E7 and E8). The late region encodes two structural proteins L1 and L2 (Fig. 2). The LCR does not encode any protein coding sequences; however, it contains crucial *cis*-elements required for viral DNA replication, transcription and stable maintenance. All ORFs are encoded from one strand and are structurally and functionally conserved between different HPV subtypes. As papillomavirus mRNAs are polycistronic, it has been difficult to identify all minor transcripts that might have a role in the viral life cycle.

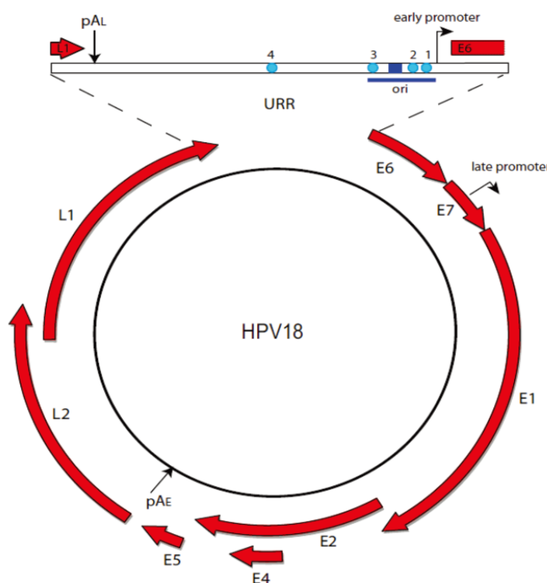


Figure 2. Schematic representation of the HPV-18 genome. The LCR with replication origin and E2BS is represented at the top. The early (E) and late (L) gene ORFs are marked. The positions of early and late promoter and polyadenylation sites are marked with arrows. Adopted from (McBride and Jang 2013).

Two viral proteins, E1 and E2, are the cornerstones of viral DNA replication and play a crucial role throughout the viral lifecycle. E1 is expressed from a polycistronic mRNA and is conserved among all Papillomaviruses with main functions of replication origin recognition and viral ATP-dependent helicase activity (Remm, Remm et al. 1999) (Ustav and Stenlund 1991) (Ustav, Ustav et al. 1991; Chiang, Ustav et al. 1992) (Sun, Thorner et al. 1990) (Bergvall, Melendy et al. 2013). Recent studies have shown novel functions for E1. The overexpression of E1 protein can cause double-strand DNA breaks (DSB), genomic instability and activate the DNA damage response (DDR) pathways (Sakakibara, Mitra et al. 2011) (Reinson, Toots et al. 2013) (Fradet-Turcotte, Bergeron-Labrecque et al. 2011) (Kadaja, Sumerina et al. 2007).

The role of E2 protein in the papillomavirus life cycle can be considered as very universal; E2 acts as a major viral transcriptional regulator, viral DNA

replication initiation protein, factor responsible for segregation and for viral particle assembly (McBride 2013). BPV-1 E2 is a strong transcriptional activator, while the E2 proteins of the *alpha*-genus subtypes, such as HPV-16 and HPV-18, are considered transcriptional repressors of the promoter for expressing the viral oncogenes E6 and E7 (Knipe 2007). E2 protein also binds with E1, stabilizes the protein and increases the specificity of E1 for recognizing the replication origin (Stenlund 2003). The third major function of the E2 protein is its ability to tether the viral genome to the mitotic chromatin, thereby participating in the relatively equal distribution of viral genomes to daughter cells (McBride 2013). A number of potential cellular partners have been described for E2, but certain differences have been identified between different subtypes. The bromodomain family protein Brd4 is probably the most intensively studied cellular partner, playing a role as a transcriptional modulator as well as part of the cellular tethering complex (You, Croyle et al. 2004) (Ilves, Maemets et al. 2006) (McPhillips, Ozato et al. 2005) (Jang, Kwon et al. 2009) (Wang, Helfer et al. 2013). Another cellular protein ChlR1 has been shown to play an important part in the stable maintenance of viral genomes upon binding with E2 (Parish, Bean et al. 2006). Mitosin like kinase Mklp2 and TopBP1 have also been suggested as cellular partners for stable maintenance of the viral genome (Yu, Peng et al. 2007) (Donaldson, Boner et al. 2007). The role of E2, the truncated forms of E2 and its interaction partners in the viral life cycle are discussed in more detail in further chapters of this thesis.

E4 protein (E1ˇE4) expression is dependent on KC differentiation, and it is mainly expressed in the upper layers of the epithelium. E4 protein has been described to play important roles in preparing viral particle exit. For example, a link has been reported between blocking the cell cycle and disrupting cytokeratins by forming amyloid filaments (Doorbar, Ely et al. 1991) (Doorbar 2013). E4 interacts with keratin, and their structures are modified by different kinases as the cell differentiation, thereby enhancing E4's association with cellular keratin (McIntosh, Laskey et al. 2010) (Doorbar 2013). In the final differentiation stages, E4 is cleaved by Calpain and forms the amyloid fibers for disruption of the cellular cytokeratins that aid viral exit from the cells (Khan, Davy et al. 2011). As E4 is easily detected by immunostaining biopsy materials, it could serve as a possible biomarker for viral infection.

E5 proteins are short hydrophobic transmembrane proteins and are considered to hold transforming activities, although their role in productive infection is poorly defined. Most information about E5 has been derived from BPV-1 and HPV-16 studies (DiMaio and Petti 2013). The E5 protein of BPV-1 has been shown to induce transformation by binding directly and activating the PDGF-beta receptor (Petti, Nilson et al. 1991) (Petti and Ray 2000; Talbert-Slagle and DiMaio 2009) (Edwards, Xie et al. 2013). HPV E5 proteins have been shown to possess a low transforming ability. HPV-16 E5 does not have sequence similarity with BPV-1 E5; instead of binding the PDGF-beta receptor, it binds epidermal growth factor (EGF) to induce cellular growth and proliferation (Straight, Hinkle et al. 1993; Auvinen, Crusius et al. 1997) (Genther Williams, Disbrow et

al. 2005). Although the transforming ability of E5 *in vitro* is low, it still seems to play a role in carcinogenesis as mouse studies have shown E5 to contribute to the generation of neoplastic cervical cancer (Maufort, Shai et al. 2010). HPV-16 E5 also plays a role in avoiding the activation of apoptotic pathways by impairing TRAIL- and FasL-mediated apoptotic pathways (Kabsch, Mossadegh et al. 2004) (Kabsch and Alonso 2002). In the basal layer, E5 is considered to play a role in the immune evasion of HPV-infected cells. E5 helps to downregulate MHC/HLA class I by the alkalinization of endomembrane compartments (Schapiro, Sparkowski et al. 2000) (Stanley, Pett et al. 2007) (Ashrafi, Haghshenas et al. 2006).

The other two viral oncoproteins are E6 and E7, which are expressed from a polycistronic mRNA directed from the early viral promoter. The E6 proteins are comprised of approximately 150 amino acids and contain two zinc-fingers that are flanked by the N- and C-terminal domains (Howie, Katzenellenbogen et al. 2009). E6 is one of the tumor-inducing oncoproteins of HPV. The HPV E6 oncoprotein binds to a cellular protein of 100 kD, termed E6-associated protein (E6-AP). The E6/E6-AP complex specifically binds to p53, resulting in the rapid ubiquitin-dependent degradation of p53 (Huibregtse, Scheffner et al. 1991). E6AP is unable to bind p53 without the interaction of E6 with its N-terminal domain. (Huibregtse, Scheffner et al. 1993) (Huibregtse, Scheffner et al. 1993). This process helps the virus to prevent cell cycle block and induction of apoptotic pathways. E6 has a number of other interaction domains and partners (White, Kramer et al. 2012). In addition to inactivating p53 and blocking apoptosis, it has been shown to play a role in the evasion of the immune response by interacting with IRF-3 and TLR-9 (Ronco, Karpova et al. 1998) (Hasan, Bates et al. 2007). Furthermore, an interaction with proteins responsible for G protein modulation, and therefore cell growth, has been demonstrated (Howie, Katzenellenbogen et al. 2009).

The E7 protein is the third oncoprotein of HPV; similarly to E6, E7 also contains a zinc-finger. It is hypothesized that E6 and E7 may have derived from a duplication event of one of the core motifs (Cole and Danos 1987). E7 is encoded by all HPVs, but their differences in oncogenicity are due to the binding affinity of E7 with the pRb family proteins. E7 inhibits the retinoblastoma (pRB) family proteins, thereby releasing and activating the transcription factor E2F that drives the expression of S-phase genes (Moody and Laimins 2010). This enables papillomavirus DNA replication in differentiating suprabasal cells.

L1 and L2 are the two structural proteins expressed by papillomaviruses and are conserved throughout the different subtypes. Together they form the viral capsid that is composed of 360 copies of L1 protein and 72 copies of L2 protein (Finnen, Erickson et al. 2003). The late proteins encapsulate the viral genomes and also play a key role in viral entry; they are important targets of inducing sterilizing immunity against the virus (Richards, Lowy et al. 2006) (Conway and Meyers 2009; Kines, Thompson et al. 2009) (Fahey, Raff et al. 2009).

2.4. E2 proteins – characteristics and functions

The E2 protein is an essential regulatory protein that is expressed by all papillomaviruses. It plays a role in viral transcriptional regulation, DNA replication and the segregation of viral genomes to daughter cells as well in late phases of the virus infection cycle. In addition, several truncated versions of E2 protein are expressed that have been shown to participate in the regulation of different aspects of the papillomavirus life cycle. All E2 proteins carry the C-terminal DNA binding dimerization domain and bind to the specific 12bp palindromic motif, -5'-ACCGNNNNCGGT-3', that is found several times in the LCR. The full-length E2 protein consists of three structural domains. The N-terminal transactivation (TA) domain has been shown to contain many of the interaction domains with cellular interaction partners and is connected with the C-terminal DNA-binding domain (DBD), responsible for binding to the viral binding motif. The two domains are connected by a flexible hinge region, as schematically represented in Fig. 3 (Giri and Yaniv 1988) (McBride, Byrne et al. 1989) (McBride, Schlegel et al. 1988).

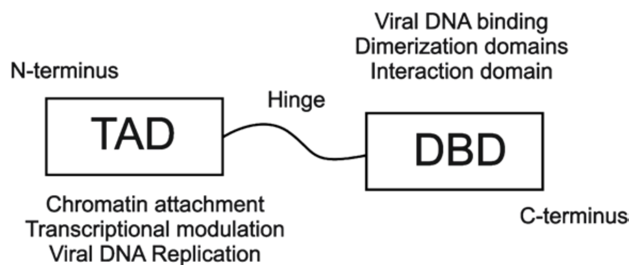


Figure 3. Schematic representation of E2 protein with its functional domains.

Many papillomaviruses have the ability to encode truncated versions of E2 protein that mostly contain the DBD, hinge and sometimes residues from the TA domain (Chiang, Broker et al. 1991) (Lace, Anson et al. 2008) (Lace, Ushikai et al. 2012) (Lambert, Hubbert et al. 1989) (Toots, Mannik et al. 2014). In general, these truncated versions have been characterized as transcriptional and replicational repressors; however, a certain function has not been allocated to all isoforms. As E2 is usually present in the cell as a dimer, it has been shown that E2 heterodimers with the truncated E2 versions are mostly prevalent. Studies in our group have shown that such heterodimers are active as transcriptional regulators and are effective in the initiation of DNA replication but not for stable maintenance and segregation of viral DNA (Kurg, Tekkel et al. 2006) (Kurg, Uusen et al. 2009) (Kurg, Uusen et al. 2010). One of the most frequently described variants of E2 protein is E8^E2C (Stubenrauch, Hummel et al. 2000). The E8^E2C protein is generated by splicing of a transcript that contains a short exon located in the E1 ORF that encodes E8 peptide fused with the E2 ORF at

an acceptor site to encode E8^E2C. All papillomaviruses have been shown to potentially encode truncated E2 transcripts that contain the E2C domain, the hinge region and a part of a sequence of an upstream ORF (McBride 2013). E8^E2C has been described as an inhibitor of viral genome replication for a number of high-risk PVs in undifferentiated cells. There are several ways that this protein can repress viral functions. First, this protein carries no transcription activation domain, and it can compete with full-size E2 for binding at E2 binding sites. Second, this protein can form heterodimers with all E2 proteins and can thus eliminate or acquire certain specific functions. Third, E8^E2C uses the E8 peptide to recruit repressor complexes to the viral DNA (Ammermann, Bruckner et al. 2008) and thereby acts as a repressor of the viral early promoter and an inhibitor of E1/E2-dependent viral origin replication (Lace, Anson et al. 2008) (Zobel, Iftner et al. 2003). The E8^E2C contains an 11 amino acid peptide that is the shortest known protein sequence to target the cellular nuclear matrix (NM) (Sankovski, Karro et al. 2015). Mutational analysis has shown that elimination of expression of the E8^E2C results in overreplication of PV genomes throughout the viral life cycle (Ammermann, Bruckner et al. 2008) (Lace, Anson et al. 2008) (Kurg, Uusen et al. 2010) (Straub, Dreer et al. 2014).

2.5. HPV transcription and the role of E2

As the virus enters the epithelial cells of the basal layer, the viral genome migrates to the cell nucleus, after which the virus genome has to link and adapt itself to the cellular regulatory circuit to become biologically active. To enable this, the early viral genes have to be expressed using the main early promoter P102 for HPV-18 (Fig. 4) and p97 for HPV-16. All species of mRNAs initiated from this promoter are polycistronic, use the early polyadenylation site (pAE) and have the potency to translate different early viral proteins (Graham 2010) (Johansson and Schwartz 2013). The pAE in papillomaviruses is located before the late coding region, and the pAL is located in the upstream region of the LCR after the late coding region. During the early stages of differentiation, the mRNAs are still polyadenylated via the pAE; as the cell differentiates to the granular layer of the epithelia, pAE activity is suppressed, and the mRNA readthrough reaches the L2 and L1 region, where the mRNA is polyadenylated at the late polyadenylation site (pAL) (Doorbar, Parton et al. 1990) (Grassmann, Rapp et al. 1996) (Milligan, Veerapraditsin et al. 2007).

The regulation of promoter and polyadenylation activity is heavily dependent on the cellular transcription factors, and as the cell enters differentiation, the composition of cellular regulators is also alternated. For example, it was recently shown that the heterogeneous nuclear ribonucleoprotein C interacts with HPV-16 3’UTR and thereby activates late L1/L2 gene expression. The levels of hnRNP are very low in basal keratinocytes but are increased in upper differentiated layers of the skin (Dhanjal, Kajitani et al. 2015). Although viral transcription is heavily dependent on cellular regulators, a major role is played by viral

regulators that either directly manipulate transcription or act as interaction partners that recruit transcriptional modulators to the viral genome.

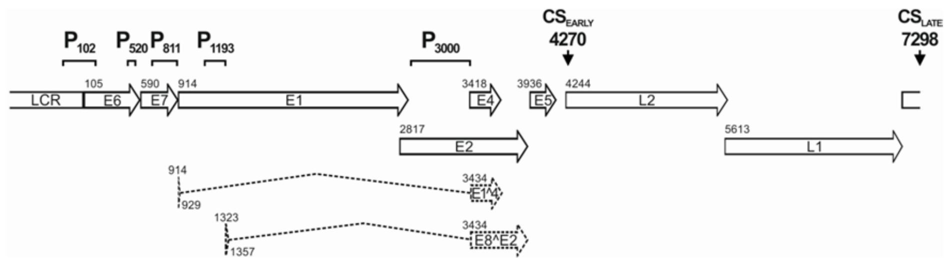


Figure 4. The HPV-18 genome schemata with marked promoter regions and positions of the early and late polyadenylation cleavage site.

One of the major viral transcriptional regulators of papillomaviruses is the E2 protein. A screen mapping E2 and host interaction partners of 12 HPV genotypes revealed that E2 interacts with highly interconnected cellular proteins. The proteins and interactions regulate a number of cellular processes – transcriptional regulation, apoptosis, RNA metabolism, ubiquitination and intracellular transport (Muller, Jacob et al. 2012). Interestingly, the interactome of different cellular transcription factors with E2 is quite heterogeneous among the different genotypes. Ten transcription factors, including Brd4, were found to be targeted by all E2 proteins, and most probably play an important role in transcriptional regulation for all 12 studied genotypes (Muller, Jacob et al. 2012). E2 has been shown to recruit cellular factors to the viral genomes, which then act as either activators or repressors of viral transcription and can also participate in replication or viral genome tethering processes (Ilves, Maemets et al. 2006) (McPhillips, Oliveira et al. 2006) (Parish, Bean et al. 2006) (Wang, Helfer et al. 2013). E2 proteins bind to their specific E2BSs located in the viral genome and act either as activators or repressors depending on the related location of the BS and the interacting cellular proteins. Mucosal HPV types have E2BSs located proximal to the early promoter, and E2 binding to these sites represses transcription. In cutaneous HPV genomes, E2BSs are located distal from the early promoter, and E2 binding to its BSs have been shown to act as transcriptional activators (Guido, Zamorano et al. 1992). In the case of mucosal HPV types, E2-mediated repression of the early promoter has been demonstrated, resulting from the fact that the cellular transcription factor binding sites are partially overlapping or are within near proximity of the specific E2BS and interfere with each target protein binding. For example, when E2 is bound to the viral genome, the TBP and Sp1 are sterically blocked from binding to the viral genome (Thierry 2009) (Dong, Broker et al. 1994). However, E2-mediated repression of the early promoter is due to steric interference as well as E2 protein recruitment of a number of cellular transcriptional repressors to the viral

genome. E2 interacts with histone modulators, such as demethylase SMCX and the EP400 component of histone acetyltransferase complex (Smith, White et al. 2010).

A large number of cellular factors has been shown to interact with E2 proteins that have pronounced roles in regulating different aspects of the viral life cycle, of which a large number are described as transcriptional repressors. (Muller and Demeret 2012) (Muller, Jacob et al. 2012). One of the major partners of E2 is the cellular bromodomain protein Brd4 that has been shown to play a central role in both transactivation and repression (Ilves, Maemets et al. 2006) (McBride and Jang 2013) (You, Croyle et al. 2004) (Wu and Chiang 2007). Brd4 directs E2 by interacting with the TA-domain of the protein to transcriptionally active cellular chromatin (McPhillips, Ozato et al. 2005). Brd4 is a component of the HPV-11 E2 transcriptional silencing complex that is responsible for repression of the E6/E7 oncoproteins promoter repression (Wu, Lee et al. 2006). E2 can also bind to the oncoproteins E6 and E7, resulting in modulation of their functions. E2 and E7 bind to each other through the hinge and zinc-binding domains, respectively, resulting in inhibition of E7-induced transformation and recruiting E7 to the mitotic chromosomes in later stages of mitosis (Gammoh, Grm et al. 2006) (Gammoh, Isaacson et al. 2009). E2 and E6 have also been shown to interact directly through sequences located in the C-terminal domain of E2 and residues between aa28-31 of E6. E6 binding to E2 limits viral replication but simultaneously elevates the transcriptional activity of E2 (Grm, Massimi et al. 2005). Studies have also shown that E2 can induce apoptosis through different pathways. E2 can induce apoptosis via binding to p53 and induce apoptosis in both HPV-transformed and non-transformed cells, and secondly, E2 can bind to the viral genome of HPV-transformed cells (Webster, Parish et al. 2000) (Parish, Kowalczyk et al. 2006).

In addition to full length E2, the truncated forms of E2 have been shown to compete with full length E2 protein for binding to their respective binding sites, thereby repressing the functions of full length E2 protein. The most pronounced of these versions is E8^E2C, which has been shown to recruit cellular repressor complexes to the viral genome (Ammermann, Bruckner et al. 2008). The E8^E2C of HPV-31 has been described to interact with NcoR1/HDAC3, a histone methyltransferase SETDB1 and TRIM28 protein to function as an independent repressor domain (Powell, Smith et al. 2010) (Ammermann, Bruckner et al. 2008).

2.6. HPV DNA replication

Papillomaviruses exclusively infect cutaneous or mucosal keratinocytes, and they have adapted their life cycle to the stages of differentiation of epithelial cells. During normal epithelial homeostasis, keratinocytes exit the cell cycle and undergo terminal differentiation that ultimately leads to cell death. In contrast, papillomaviruses have evolved extensive mechanisms to manipulate the

infected cells and integrate their productive life cycle with cells going through differentiation. The papillomavirus DNA replication cycle in infected cells can be dissected into three modes – (i) the initial amplificational replication for propagation of the genome copy number during the establishment of infection in the host cell; (ii) stable maintenance replication to keep the genome copy number relatively constant during latency period, when viral DNA is spread into the progeny of the infected cell and; (iii) vegetative replication in the upper layer of the epithelium for large amplification of new viral genomes for package and release into virions (schematically represented in Fig. 5).

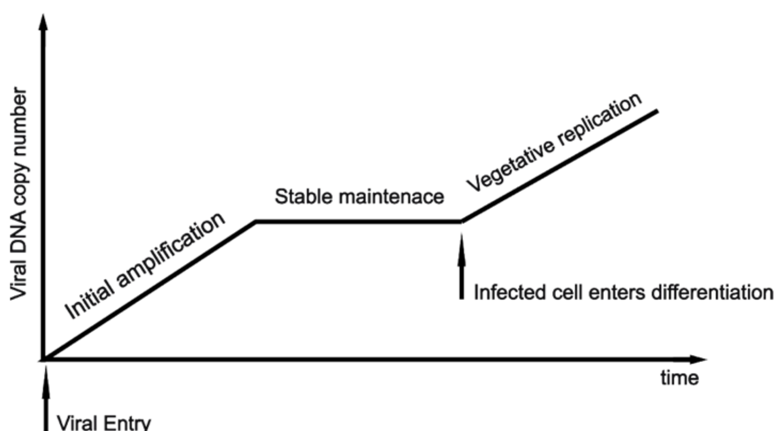


Figure 5. Representation of the three replicational phases of the papillomavirus genome. After viral genome entry into the nucleus, initial amplification is achieved by multiple rounds of viral DNA replication per cell-cycle, followed by stable maintenance replication that takes place in synchrony with the cellular DNA replication, resulting in doubling of DNA per cell cycle. When the host cell enters differentiation, then amplificational replication is initiated again to produce viral genomes for efficient packaging into virions.

2.6.1. Initial amplificational replication and mechanism of viral DNA replication

It is very difficult to study molecular mechanisms of viral genome replication *in vivo* using differentiating epidermis. For such studies, three-dimensional models of differentiating epidermis have been developed and used with several limitations (Chow 2015). Most of the basic mechanistic studies about the function of papillomaviruses have been executed in model systems based on transformed cell lines. In these studies, papillomavirus DNA replication in the initiation phase has been considered to consist of both bi-directional theta-type and recombination-dependent replication modes (Orav, Geimanen et al. 2015).

The E1 and E2 proteins have been shown to be the major viral trans factors for PV replication, as several studies have demonstrated that they are the only

viral proteins required for initiation and elongation of replication *per se* (Kuo, Liu et al. 1994; Sverdrup and Khan 1994) (Del Vecchio, Romanczuk et al. 1992) (Chiang, Ustav et al. 1992). The viral replication origin is located in the LCR, and the minimal replication origin of HPV has been described to contain the E1BS flanked by two E2BSs (Lu, Sun et al. 1993) (Sverdrup and Khan 1995) (Remm, Brain et al. 1992). The replication initiation process begins with the formation of the pre-replication complex (pre-RC) by E1/E2 complex formation and traveling of the complex to the viral origin, where E1 initially binds the replication origin as a dimer (Mohr, Clark et al. 1990) (Frattini and Laimins 1994) (Sedman and Stenlund 1995) (Masterson, Stanley et al. 1998). However, the replication initiation process itself is E2-independent, and cell-free replication studies have shown that the E2 is displaced from the complex after E1 has bound to the viral origin (Kuo, Liu et al. 1994). As the E1 specificity to its binding site is low, the role of E2 is crucial for recruiting E1 to the replication origin, and this is mainly due to the ability of E2 to simultaneously bind E1 and viral DNA, as seen from the first step in Fig. 6 (Seo, Muller et al. 1993) (Frattini and Laimins 1994). After the E2 is displaced from the complex, E1 forms a double trimer (DT) to bind viral DNA and melt the DNA strands in origin (Fig. 6. Step 2). Then, the formation of a double hexameric (DH) helicase is possible with the presence of ATP hydrolysis (Fig. 6. Step3) (Sedman and Stenlund 1996) (Schuck and Stenlund 2005) (Sedman and Stenlund 1998). Only ssDNA can be bound within the hexameric ring of the E1 helicase, and the DNA binding hairpins of the hexamer form a spiral staircase, enabling the DNA to pass through the helicase as it rotates (Enemark and Joshua-Tor 2006). ATP hydrolysis occurs at the top of the staircase, and ADP is released in the bottom of the staircase. As the hairpin reaches the bottom of the staircase, it releases the bound ssDNA and is ready to bind new ATP as it moves to the top of the staircase and binds the new nucleotide.

As papillomaviruses express only a limited number of proteins, they rely heavily on recruiting cellular factors for executing different mechanisms required for the viral life cycle. Viral DNA replication is not an exception, and efficient viral DNA replication relies on the appropriate partners, including the cellular DNA polymerases α and δ (Bergvall, Melendy et al. 2013) (Conger, Liu et al. 1999). Topo I is an ATP-independent protein that enables the modification of supercoiled DNA topology by reducing the torsional stress of the DNA. Papillomavirus E1 interacts directly with Topo 1 at DBD, and this interaction increases Topo1 activity (Clower, Fisk et al. 2006). At the same time, Topo 1 enhances E1 binding to the origin and E1 oligomeric formation to functioning as a helicase. E1 DBD involves the interaction with the replication protein A (RPA) and the loading of RPA molecules to ssDNA during lagging strand synthesis (Loo and Melendy 2004) (Hu, Clower et al. 2006). As the viral helicase rotates and progresses along the replicating DNA, E1-bound RPA molecules are directed proximally to the naked ssDNA molecules, enabling binding of RPA from E1 to the ssDNA. Only free RPA molecules are able to re-bind E1, as already ssDNA-bound RPA molecules have very low affinity for E1, probably

due to steric alterations (Loo and Melendy 2004). The α-primase DNA polymerase interacts using its p70 or p180 subunits with the helicase domain of E1. It plays a role in re-initiation of replication during synthesis of the lagging strand of viral DNA (Park, Copeland et al. 1994) (Bonne-Andrea, Santucci et al. 1995) (Amin, Titolo et al. 2000) (Conger, Liu et al. 1999).

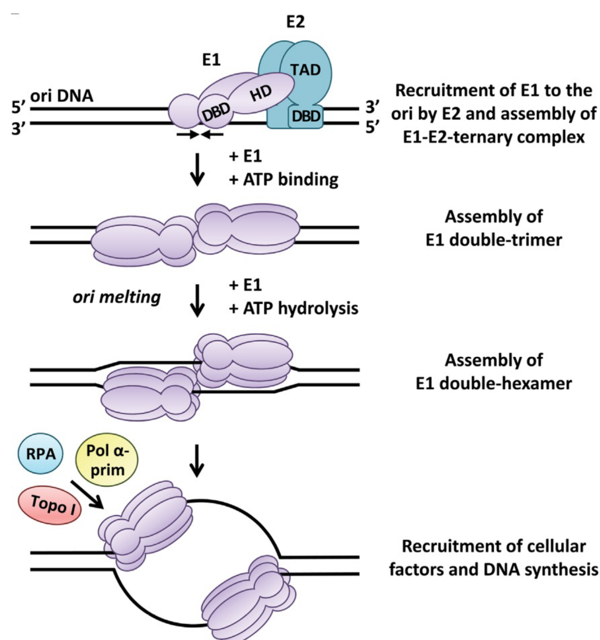


Figure 6. Cartoon describing the initiation of viral DNA replication: unwinding of dsDNA and formation of the pre-RC. First, E2 recruits E1 to the viral origin (ori), after which the E1 double trimer (DT) is formed. The ori melting enables assembly of the E1 double hexameric helicase (DH) and further recruitment of cellular DNA replication factors. Adopted from (Bergvall, Melendy et al. 2013).

2.6.2. Stable maintenance replication

After the initial rapid amplification of viral genomes, the virus is maintained in dividing basal and suprabasal cells at a relatively constant copy number. This is believed to be achieved by two functions of viral genome persistence: first, by slowing down the initiation of replication, resulting in approximately once per cell cycle replication, granting doubling of virus genome copies every cell cycle; and second, by efficient segregation of the its extrachromosomal genome into daughter cells during the division of latently infected cells.

Most information about the stable maintenance replication phase has been generated from BPV-1 studies. For BPV-1 in C127 cells, it has been demonstrated that the viral genome replicates following a random mechanism of

initiation of replication during the stable replication phase (Gilbert and Cohen 1987). For HPV, no definitive results have been published. Studies of the HPV-16 and HPV-31 mode of replication in W12 and CIN612 cells, respectively, have indicated that HPV DNA replicates either by random-choice (CIN612) or by once-per-cell cycle (W12) mechanisms. However, when HPV-16 and HPV-31 DNAs were separately introduced into the immortalized keratinocyte cell line NIKS, they both replicated randomly (Hoffmann, Hirt et al. 2006). These controversial results were interpreted such that papillomaviruses can infect two types of epithelial cells – stem-like cells and transiently amplifying cells and that differences observed in the replication modes between W12 and CIN612 are caused by the different nature of the host cells (Kim, Garner-Hamrick et al. 2003; Hoffmann, Hirt et al. 2006). This explanation is highly speculative, and authors have not demonstrated that both cell lines, W12 and CIN612, actually carry the episomal copies of the virus. An alternative explanation is that the virus genome is integrated in the W12 cells and therefore is independent of E1 and follows the once-per-cell cycle cellular DNA replication mode, while the HPV-31 positive cell line CIN612 carries the episomal viral genomes in the cells. HPV-16 genome integration in W12 cells has been demonstrated convincingly (Pett, Herdman et al. 2006).

The BPV-1 E1 and E2 proteins are necessary for efficient maintenance replication of the viral genome (Ilves, Kadaja et al. 2003). It has also been suggested that in stable maintenance replication, papillomavirus genomes can replicate episomally and be maintained without any viral protein expression (Kim and Lambert 2002). One hypothesis is that E1 might be required only initially for establishing viral DNA in the nucleus and for vegetative replication but is not required for the maintenance phase (Egawa, Nakahara et al. 2012). This statement contradicts the findings that the origin of replication for BPV-1, HPV-11 and HPV-16 during stable episomal replication has been mapped into the LCR where the E1/E2 dependent replication origin is located, and 2D-gel electrophoresis indicates that the same replication intermediates exist during the stable replication phase as during the E1/E2 dependent amplificational replication phase.

The stable maintenance replication of the viral genome in proliferating cells is ultimately linked with effective segregation of the viral episomes into daughter cells. Several cellular partners in the segregation process have been identified, and the mechanisms of stable maintenance of viral genomes will be discussed further below.

2.6.3. Vegetative viral DNA replication

Vegetative HPV DNA replication, or the second amplificational replication, occurs as virus-infected keratinocyte migrate into the upper layers of the epidermis and enter the differentiation path. During this phase, the cellular micro-environment changes, including the expression of cellular miRNAs, inducing a

switch of viral gene expression, which is needed for viral exit from the cells (Gunasekharan, Hache et al. 2012) (Zheng and Wang 2011). It is crucial for the virus to amplify the genome copy number for efficient packaging of the genome into virions, which is possible after the production of viral late proteins. The exact mechanism of initiation and mode of HPV genome replication is still under study; however, a number of cellular cofactors have been described to play a role in this process.

The fact that such a replicative phase exists is derived from the information of the change of viral DNA copy numbers (20–200) during replication of basal cells from the viral DNA copy number (100–1000) obtained from episome-containing cervical cell lines. In the case of vegetative amplificational replication, there are studies showing that rolling circle replication might be used instead of bidirectional theta type replication (Dasgupta, Zabielski et al. 1992) (Flores and Lambert 1997). Rolling circle replication (RCR) would allow more rapid synthesis of viral DNA molecules for encapsulating into virions without the need for initiation of replication for duplication of viral genomes, a prerequisite for recombination-related replication (RDR) (Orav, Henno et al. 2013) (Orav, Geimanen et al. 2015). Nevertheless more data are required to clarify switching between replication modes as viral-infected cell enters differentiation. Our group has recently demonstrated that stable HPV DNA replication occurs in the S-phase, but in the case of increased E1 levels, viral DNA replication is extended to the G2 phase (Reinson, Henno et al. 2015). The ability to replicate also in G2 phase might be important from the virus perspective, as differentiated cells are usually blocked in G2 (Wang, Duffy et al. 2009).

How amplificational replication is triggered is still unclear, but several studies have proposed an increase in E1 and E2 expression, which is regulated at the transcriptional level and by activation of the ATM-dependent pathway (Bedell, Hudson et al. 1991) (Fradet-Turcotte, Bergeron-Labrecque et al. 2011) (Moody, Fradet-Turcotte et al. 2007) (Mehta, Gunasekharan et al. 2015) (Sakakibara, Mitra et al. 2011). An increase of E2 expression has been described in the case of BPV-1, and an increase of mRNAs encoding E1 and E2 has been shown for HPV-31 (Klumpp and Laimins 1999) (Burnett, Strom et al. 1990) (Penrose and McBride 2000).

The DNA damage response (DDR) pathways have been described to play an important role. Papillomaviruses have been shown to regulate the expression of certain cellular proteins as cells differentiate. In HPV-positive keratinocytes, the activation of caspase-3, -7 and -9 was detected upon differentiation, but activation of these in HPV-negative cells was not monitored (Moody, Fradet-Turcotte et al. 2007). Caspase inhibitors hampered viral amplificational replication, indicating that HPV-activated caspases play an important role in the vegetative replication phase. Caspase cleavage has been shown to be mediated by CHK2, which was previously activated by E7 oncoprotein binding to the ATM kinase (Moody and Laimins 2009). Another independent pathway of the ATM DNA damage response includes the cohesin protein SMC1. HPV-infected cells have been shown to phosphorylate SMC1 upon differentiation. This activation of

SMC1 and related factors results in the recruitment of this complex to the viral genome through binding of the insulator transcription factor CTCF to its respective binding sites on the viral genome, thereby facilitating viral genome amplification (Mehta, Gunasekharan et al. 2015).

2.7. Stable maintenance of PV DNA

Viruses have evolved different approaches and mechanisms to support their life cycle and confirm their transmission into the progeny of infected cells. Some viruses, like retroviruses, convert their genomic information from RNA into DNA and integrate it into the host cell genome, becoming an integral part of the infected cells DNA. Therefore, they express, replicate and maintain their DNA as part of the chromosomal DNA. In contrast, all viruses that maintain their genomes as an extrachromosomal genetic element must have developed specific mechanism(s) to persist in the cell as it divides – first, the viral DNA has to replicate and second, it has to segregate nearly equally into daughter cells. Currently, most of the information we know about papillomavirus maintenance and segregation has been derived from studies of BPV-1. A *cis*-element, named the minichromosome maintenance element (MME), and E2 protein have been shown to be necessary for these functions. The MME is defined as a minimum of 6 E2BS out of 12 located in the LCR that tethers the viral genome to chromatin and ensures efficient segregation and maintenance of the corresponding plasmid in the dividing cell population (Piirsoo, Ustav et al. 1996). The studies of segregation mechanisms have revealed that E2 binds its C-terminal DBD to the viral genome, and the N-terminal TAD forms complexes with cellular tethering proteins that load and keep the E2/DNA complex at the mitotic chromatin. As the cell divides, relatively equal copies of the viral genome are distributed to the daughter cell as seen from the scheme in Fig. 7.

Although BPV-1 and HPVs are structurally very similar, and many studies can be extrapolated into the context of HPVs, there are significant differences between the segregation elements of BPV-1 and their counterparts in HPVs. The major difference comes from the number of E2BS that is required for viral genome segregation. First, the alpha-genus papillomaviruses have only 4 of E2BSs located in the LCR. This is significantly less than the 12 BS of BPV-1 or the 6 E2BS defined as MME for BPV-1. Second, several mutational analyses of BPV-1 have shown that the N-terminal transcription activation domain of E2, which is bound to many components of cellular transcription machinery, is required for guiding viral DNA to the transcriptionally active chromatin and for attachment to the mitotic chromatin (Silla, Mannik et al. 2010) (Ilves, Kivi et al. 1999) (Abroi, Ilves et al. 2004). In accordance with this, the N-terminally truncated forms of E2 – E2C and E8/E2 repressor proteins – do not interact with the mitotic chromatin for foci formation (Skiadopoulos and McBride 1998). However, the alpha genus HPV E2 has been shown to act as a transcriptional repressor rather than having activator properties; thus, its cellular interaction part-

ners may be different from partners of BPV-1 E2. Thus, the segregation competent complex, formed in the case of HPVs, may also be different and composed of different host factors.

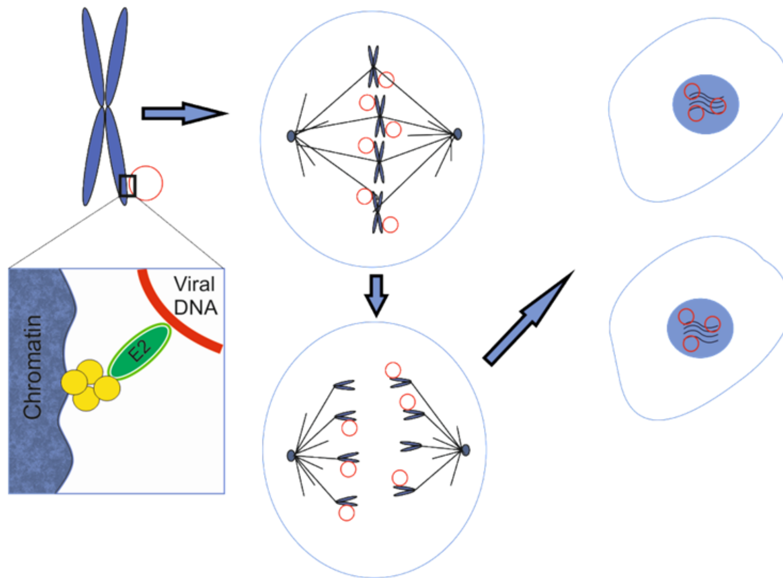


Figure 7. Schematic representation of the current model of PV segregation. E2 binds with the C-terminal DBD to the viral genome, and the N-terminal TAD forms complexes with cellular tethering proteins that load and keep the E2 / viral DNA complex at the mitotic chromatin. As the cell divides, relatively equal copies of the viral genome are divided to the daughter cell.

E2 does not bind directly to the DNA of the mitotic chromatin, but tethering is mediated through interacting with cellular proteins of the chromatin. One of the best described cellular interaction partners in the viral genome tethering process is the bromodomain family protein Brd4 (You, Croyle et al. 2004), which is also the first and most pronounced protein to have been described for the E2 interaction with mitotic chromatin. Studies have shown that blocking the interaction of Brd4 with E2 by overexpression of the C-terminal domain of Brd4 or by expression of inhibiting peptides results in the loss of E2 attachment to the mitotic chromatin. The E2 interaction with Brd4 has been shown to be crucial for the attachment to chromatin for BPV-1, HPV-16 and HPV-31 (McBride and Jang 2013) (Wang, Helfer et al. 2013). From the other side, the universal requirement of Brd4 for chromatin attachment is not completely clear, as not all PV subtype E2 proteins interact with Brd4. In addition, the data obtained for Brd4 binding with E2 in the case of HPV-16 are derived from cell lines that do not support the transient or the stable replication of the viral genome. Additionally, downregulating Brd4 with siRNA does not affect the binding of BPV-1 E2

and HPV-11 E2 to mitotic chromatin (Parish, Bean et al. 2006). To conclude, Brd4 definitely plays a role in the viral life cycle and also in the segregation of some papillomavirus genomes, but it does not seem to be the only receptor for E2 attachment to the mitotic chromatin in the process of viral genome segregation.

Another cellular protein that interacts with E2 and seems to play a role in viral DNA maintenance in dividing cells is the cellular helicase ChlR1. The point mutation W130R, described for BPV-1 E2, failed to associate with ChlR1. Although transient viral DNA replication has not been hampered by this mutation, the viral genomes are not maintained stably in the host cells. At the same time, the W130R mutation does not affect the binding of Brd4 nor the transcription modulated by it. Downregulation of ChlRI with siRNA disrupted E2 binding to the mitotic chromosomes. At the same time, the downregulation of Brd4 did not affect E2 binding with the mitotic chromosomes. Interestingly, ChlR1 is co-localized with E2 and the viral genomes in the S-phase but not in mitosis (Feeney, Saade et al. 2011). These facts suggest that ChlR1 may be required for loading of E2 and the viral genome to the chromatin, but it is dispensable during the segregation process to daughter cells where other cellular proteins interact with the E2.

A third cellular interaction partner that seems to be important for the stable maintenance of papillomaviral genomes is the topoisomerase II-binding protein (TopBP1). It was initially shown to act as a transcriptional and replicational activator of HPV genomes (Boner, Taylor et al. 2002). Then, it was shown that E2 and TopBP1 colocalize during late mitosis and play a role in regulating the association of E2 and viral genomes in the attachment to mitotic chromatin (Donaldson, Boner et al. 2007). It was also demonstrated that TopBP1 is required for the efficient establishment of viral replication; viral transcription of the E2 mutant that does not bind to TopBP1 is not affected, but it is unable to establish DNA replication (Donaldson, Mackintosh et al. 2012).

In addition, the mitotic kinesin-like protein 2 (MKlp2) that is expressed only during mitosis (Yu, Peng et al. 2007) has been shown to interact with BPV-1 E2 protein. MKlp2 colocalizes with E2 in mitosis, but it is very difficult is to analyze its exact role as downregulation of MKlp2 results in growth arrest in telophase and subsequent cell death (Neef, Preisinger et al. 2003) (Zhu, Zhao et al. 2005).

In conclusion, the factors and mechanisms that ensure HPV genome segregation and maintenance remain poorly understood.

2.8. Stable maintenance and tethering of the extrachromosomal genomes for other DNA viruses

In addition to papillomaviruses, there are examples of other DNA viruses that maintain their genomes as episomal nuclear DNA elements. In principle, they use similar mitotic chromatin tethering-based mechanisms to maintain their genomes in dividing cells. They also recruit cellular partners to participate in

DNA replication and in tethering the viral genome to the chromosome upon cell division. Most information has been collected for Epstein-Barr virus (EBV), a human gamma-herpesvirus that infects a large population of humans but remains largely asymptomatic. This virus infects B-lymphocytes, and the 165 kb circular genome is generated upon entering the cell nucleus to complete the viral life cycle (Deng, Wang et al. 2012). EBV has been described to possess four different latency types (Rowe, Rowe et al. 1987) (Babcock, Hochberg et al. 2000), with stable maintenance and tethering of the extrachromosomal genome without production of viral progeny (Thorley-Lawson 2005).

EBV nuclear antigen 1 (EBNA1) is the major viral protein responsible for viral transcription, replication and maintenance by binding to the viral latent replication origin OriP (Wu, Kapoor et al. 2002). As with E2, the EBNA1 has N-terminal TA and C-terminal DBD domains. The N-terminal domain contains sequences responsible for replication, transcription and partitioning. Interestingly, the EBNA1 DBD has remarkable structural similarities with papillomavirus E2 DBD (Bochkarev, Barwell et al. 1995). The OriP that the EBNA1 binds is composed of two components – the dyad symmetry element (DS) and the family of repeats (FR) (Reisman, Yates et al. 1985). The FR element consists of 20 copies of a 30 bp sequence that also contains the 18 bp palindromic EBNA1 binding site (Rawlins, Milman et al. 1985). The FR region has been shown as the only necessary *cis*-element responsible for viral segregation to daughter cells, as the DS element contains the origin of replication activity (Gahn and Schildkraut 1989). On the other hand, EBNA1 has been demonstrated as the only viral protein needed for genome segregation function, mediated only through EBNA1 and FR (Sears, Kolman et al. 2003) (Nanbo, Sugden et al. 2007). This is in accordance with findings that, during type I latency in proliferating B-cells, only nuclear antigen (EBNA1) and non-coding RNA expression from the EBV genome can be observed (Thorley-Lawson 2005).

EBNA1 and viral DNA is not restricted to certain regions on the host mitotic chromosomes but rather is distributed randomly, and the FR element has been found to direct the viral genome to chromatin regions that are modulated by histone modifications and therefore also transcriptionally active (Harris, Young et al. 1985) (Deutsch, Ott et al. 2010). Recently, the AT-hook DNA binding ability of EBNA-1 has also been shown to be required for the maintenance of viral genomes (Chakravorty and Sugden 2015). Additionally, a cellular partner EBP2 has been described to be important for the attachment of EBNA1 to mitotic chromatin and efficient segregation (Shire, Ceccarelli et al. 1999) (Wu, Ceccarelli et al. 2000). The depletion of EBP2 resulted in loss of the segregation function (Kapoor, Lavoie et al. 2005), but EBP2 is probably not the only partner in the segregation, as it is only associated with telophase during mitosis (Nayyar, Shire et al. 2009). This indicates that the initial contact with chromosomes is probably mediated by an alternative partner and mechanism.

Another well-known example is Kaposi's sarcoma associated human herpesvirus type 8, which is a gamma-herpesvirus that was first identified from an AIDS patient with Kaposi's sarcoma (KS) (Chang, Cesarman et al. 1994).

Following primary infection of the host cell, latency is established. Viral latent infection involves silencing of viral lytic gene expression and the stable maintenance and segregation of viral episomes to daughter cells.

The KSHV genome is composed of a 140 kb unique region that is flanked by an 803 bp long region containing the terminal repeat (TR) regions (Russo, Bohenzky et al. 1996). The circularization process of the KSHV DNA in the host cell nucleus is necessary to form an intact episomal maintenance element, which consists of tandem copies of the TR sequence (Ballestas, Chatis et al. 1999). The KSHV encodes six viral genes by using only 11 kb of its whole genome for transcription (Laurent, Meggetto et al. 2008). The main viral protein for the segregation and stable maintenance is LANA1 – an orthologue of EBNA1 of EBV. Binding to the viral genome and having chromatin binding activity, LANA1 mediates segregation of the KSHV genome during latency. LANA1 is a large 222–234 kDa nuclear protein that interacts with various viral and cellular proteins and also dysregulates cellular pathways (Verma, Lan et al. 2007). LANA1 asymmetrically binds to its binding sites in the TR DNA in contrast with EBNA-1. Recent crystal structure studies of the C-terminal domain of LANA1 have shown that there are at least three LANA1 binding sites (LBS) in the TR (Hellert, Weidner-Glunde et al. 2015). The maintenance of Kaposi's sarcoma-associated herpesvirus (KSHV) episomal genome relies on LANA and the TR located on the viral genome (Ballestas, Chatis et al. 1999) (Ballestas and Kaye 2001) (Cotter, Subramanian et al. 2001). It has been shown that approximately 16 copies of the TR sequence are required for efficient partitioning of the viral genome, and two 71 bp minimal replication elements separated by 801 bp center-to-center spacing are required for efficient stable replicative maintenance (Shrestha and Sugden 2014). For the efficient establishment and maintenance of viral genomes, LANA1 is responsible for viral DNA replication interacting by the C-terminal DBD with two sites in the TR (Garber, Hu et al. 2002). A number of cellular proteins has been described to act as potential partners for KSHV genome segregation (reviewed in (Feeney and Parish 2009), but perhaps the most interesting target would be LANA docking with the nucleosomes. LANA uses its N-terminal domain to dock with nucleosomes through binding with histones H2A-H2B and might use this attachment to nucleosomes to persist during cell division (Barbera, Chodaparambil et al. 2006). Additionally, similarly to papillomavirus E2 proteins, LANA1 interacts with the bromodomain family proteins Brd2 and Brd4 to colocalize on mitotic chromatin (You, Srinivasan et al. 2006) (Viejo-Borbolla, Ottinger et al. 2005). As with the studies of papillomaviruses, the exact mechanisms of the tethering process are still unknown, and current data indicate that the process could involve several cellular partners that are participating and active in different stages of the cell cycle, thereby working in tandem.

3. MATERIALS AND METHODS

One of the pillars of this thesis is the development of a new cellular system that would allow efficient study of various characteristics of the viral life cycle using molecular biology techniques. The U2OS cells described herein are a cell line capable of replicating various HPV genotypes, and we demonstrate the relevancy of the cell system in depth regarding DNA replication and the transcriptional map of HPV-18 replicating in U2OS cells (Ref I & II). For propagation of HPV genomes, these were cloned into regular cloning vectors suitable for *E. coli* strains. However, for studies of HPV genomic DNA, the bacterial backbone must be removed. Instead of restricting out the backbone and transfecting cells with re-ligated viral genomes, we implemented the minicircle attB/attP-Bxb1 recombination system to generate viral (ccc) genome molecules that could be used directly to transfect cells (Kay, He et al. 2010) (Wang, Meyers et al. 2011).

The first transient amplification of HPV genomes was achieved by transfecting U2OS cells with minicircle plasmids containing HPV genomes; the stable maintenance replication of HPV was obtained by generating stable HPV-positive subclones from U2OS cell lines after selection, and the vegetative amplificational replication of HPV genomes was achieved by using dense cell culture conditions cultivating stable HPV-positive cell lines.

In Fig. 8 I represent the general workflow approach that was used in these studies. Several cloning techniques have been used to generate DNA plasmids expressing either viral, cellular or marker proteins, including gene synthesis, PCR, and single nucleotide mutagenesis. Considering the design of the experiments, plasmid DNA was transfected via electroporation into the cells, after which downstream analytical methods were performed. A detailed overview of the materials and methods used in this study are located in the appropriate section of each publication.

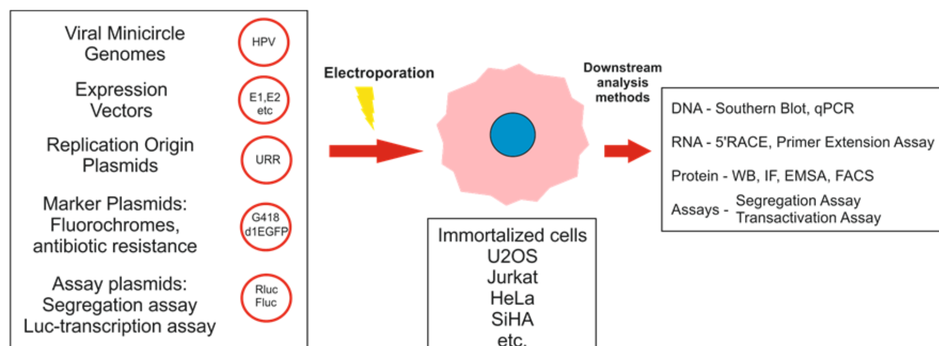


Figure 8. Schematic representation of the general workflow of HPV-related studies used for this thesis. Depending on the experiment design, plasmids were transfected to immortalized cells, after which downstream analytical methods were performed.

The second methodical pillar to be highlighted is the development of the HPV-18 segregation assay (Ref III). The principle of this assay has been published previously in the context of BPV-1 (Abroi, Ilves et al. 2004). The assay consists of a non-replicating plasmid that expresses two factors – the viral E2 protein for the tethering of the plasmid to mitotic chromatin and a short-lived fluorescent protein d1EGFP (Li, Zhao et al. 1998) expression cassette for monitoring the persistence of the plasmid in dividing cells using FACS. The assay plasmid also contains a region that allows the insertion of DNA sequences bearing the E2 binding sites. Due to short half-life of the marker, the fluorescence indicates the presence and expression of the plasmid in the cell. Using FACS for the measurement of GFP-expressing cell numbers over time allows us to calculate the relative segregation efficiency of plasmids with different combinations of *cis*-elements. We have used this approach to create the assay plasmid in the context of HPV-18 and studied the segregation properties of HPV-18 *cis*-elements. As described in Figure 10 below, by monitoring the change of GFP-positive cells over time it is possible to conclude the segregation efficiency of the plasmid, i.e., in comparison of a plasmid lacking any segregation competent elements. In case of a segregation positive plasmid, we see the number of GFP positive cells increasing over time as the plasmids are tethered to the mitotic chromatin and thereby hitchhike to the daughter cell. In case we have a plasmid that is segregation incompetent, we do not see an increase in numbers of the GFP-positive cells, and they instead show a decrease over time as the plasmids either segregate randomly to daughter cells or are lost during the cell cycle. As the E2 protein is a major viral regulator, mutations of E2BS in the context of the viral genome or LCR-replication plasmids result in modulation of transcription and DNA replication. Therefore, it is difficult to make any conclusions on the specific mutation in the context of DNA segregation and partitioning. Our assay is advantageous in the aspect that it allows us to specifically dissect the role of E2 in the segregation/partitioning process. Using this assay system, we proceed to describe the *cis*-elements for segregation of HPV-18 DNA in the following chapter of this thesis (Ref III).

4. RESULTS AND DISCUSSION

4.1. Objectives of the study

Although a preventive HPV vaccine is available for several subtypes, the pressing issue of a large number of cervical cancer cases globally remains. However, people who are already infected or unable to vaccinate against HPV are still at risk. A limitation in the development of therapeutic solutions against HPV infection is caused by the lack of suitable cellular model systems that are capable of hosting HPV and supporting different facets of viral mechanisms – DNA replication, transcription and latency. These systems would allow to study the aspects of HPV molecular biology and use the knowledge to identify novel antiviral strategies. For this thesis, the establishment of such a model system has been described. This creates the opportunity for comprehensive studies of fundamental HPV properties also included herein. The objectives of the thesis are:

a) To characterize the human U2OS cell line as a suitable host capable of initiating and supporting stable genomic replication of several different HPVs (Ref I);
b) To describe the HPV-18 transcription map in U2OS cells during transient replication, stable maintenance and vegetative amplification, including the definition of the promoter regions, polyadenylation sites and mRNA species (Ref II);
c) To characterize the specific elements and factors required for viral genome segregation and stable maintenance (Ref III).

4.2. U2OS as a suitable model system for HPV-related studies (Ref I)

When Harald zur Hausen first proposed the hypothesis that cervical cancer is caused by papillomaviruses in 1976, these became one of the most extensively studied viruses. The ability of papillomaviruses to transform cells gave the field the cell lines HeLa and SiHa harboring integrated sequences of HPV-18 and -16, respectively, that have enabled the gathering of invaluable information in a variety of fields – virology, cancer biology, and immunology (Durst, Gissmann et al. 1983) (Boshart, Gissmann et al. 1984). As science and the available tools for research were evolving, a larger emphasis was directed onto the molecular biology of papillomaviruses. As animal papillomaviruses were considered model systems for HPV, a large portion of scientific research was performed in the context of the Bovine Papillomavirus Type-1 (BPV-1), after which the initial findings were mostly confirmed in the context of HPV. The fact that cellular systems suitable for modeling different mechanisms of BPV-1 were available was the main cause of its development as the main target of papillomavirus research. For example, studies from BPV-1 have led to major findings regarding the replication of papillomavirus DNA and have given many insights into how

cellular DNA replication occurs. These type of studies were possible due to the availability of the suitable cell line C127 that supported the replication and transformation of the BPV-1 genome (Law, Lowy et al. 1981). However, these cells do not support the replication of HPV.

Papillomaviruses are species-specific viruses, and the microenvironment of the cell determines the functionality of the virus within the cell. The virus may not perform the same in different cell lines – for example, papillomaviruses replicate in keratinocytes but do not replicate in other cell types. The possibility of generating E1- and E2-expressing cell lines of BPV-1 gave useful information about the stable maintenance of BPV-1 DNA, determining the requirements for viral DNA segregation and mapping the MME (Piirsoo, Ustav et al. 1996). Transferring this approach into the context of HPV has failed several times for our group. Now we can conclude that the reason for this might be the ability of E1 to create double-strand-breaks (DSB) and thereby induce the DNA damage response pathway (DDR), resulting in either cell-death or stalled cell cycle (Sakakibara, Mitra et al. 2011) (Gillespie, Mehta et al. 2012) (Reinson et al. 2013).

For more authentic studies of HPV molecular biology, we needed to find a robust, easily cultivable cell line that supports the genome replication and gene expression of LCR (ori)-containing plasmids as well as intact viral genomes. In the quest for a suitable cell line, our research group tested a number of cell lines for the ability to support replication of high- and low-risk HPV DNA. We found that human osteosarcoma cell line U2OS (previously named 2T) supported the replication of HPV DNA. The U2OS cell line is derived from a 15-year-old girl who had a moderately differentiated osteogenic sarcoma of the tibia (Ponten and Saksela 1967). U2OS cells have been characterized to have an epithelial-like growth pattern and retain mitotic activity even when cultivated at high-density. In comparison with primary keratinocytes, cultivation of U2OS is much more robust – they are easily transfectable, are cultivated in standard cell culture medium, and undergo unlimited cell divisions with an approximate doubling time of 29 hours. As infection with viral particles is not a possibility, to mimic viral entry we transfected HPV viral genomes into U2OS cells via electroporation. As seen from Ref I Fig. 1, after the transfection of 6 different episomal viral genomes, the DpnI-resistant HPV-specific signals were detected for all HPV subtypes. As the U2OS cells enabled replication of the alpha-genus papillomavirus types 6, 11, 16 and 18 DNA that infect the mucosal epithelium, it was even more surprising that it was able to transiently and stably replicate the beta-genus type papillomaviruses HPV5 and HPV8 that infect cutaneous epithelia. A suitable cellular model system had been completely lacking for cutaneous HPV studies, and the fact that these genomes replicate transiently and are maintained in the cells provides a valuable tool for a deeper understanding of the characteristics of these viruses.

To validate that the initial replication of HPV genomes were dependent on the presence of the papillomavirus replication proteins E1 and E2 and not due to an artifact generated by the cell, we constructed E1 and E2 frameshift mutants in the HPV-18 genome. We clearly demonstrated that mutant viruses not

expressing either E1 or E2 protein were unable to replicate in U2OS cells (Ref I Fig. 1E and scheme Fig. 3H). In contrast, when we co-transfected the mutant genomes with either E1 or E2 expression vectors, replication was rescued. Moreover, when co-transfecting the E1 and E2 mutant genomes together in a complementation assay, we could see the replication activity restored for both mutants, as E1 was provided from the E2 minus genome and E2 from the E1 minus genome. Thus, we concluded that HPV genome replication in U2OS is E1- and E2-dependent.

In addition to the transient replication, we were able to see that the replication signals persisted over time, indicating that the replicating viral genomes were tethered properly to the dividing daughter cells. Long-term cultivation of cells transfected with viral genomes under selective conditions, and even without selection, revealed that the cell pools were efficiently harboring viral genomes as seen in Ref I Fig. 2A. Furthermore, subclones of single cells containing the stably maintained viral genomes were obtained for all six tested subtypes with high efficiency (Ref I Fig. 2B). Interestingly, in addition to the transient HPV DNA replication and stable replication of HPV genomes, we were able to monitor induced amplificational replication. Specifically, when U2OS cells that maintained the HPV genome were cultivated in a confluent state, we were able to see a 2-6-fold increase of the viral DNA copy number (Ref I Fig. 3C). In some aspects, the amplification has remarkable similarity to the productive stage of the HPV life cycle. Studies with keratinocytes have shown that cultivation of cells in confluency may lead to differentiation of the cell, meaning also that the microenvironment–transcriptional profile of the cell also changes (Poumay and Pittelkow 1995). Although the U2OS cells are transformed and do not differentiate, the mechanism of transcriptional modulation of cellular proteins in confluency could be used to explain this phenomena. The relative mRNA levels of viral transcripts in HPV-transfected cells in confluency started to rise approximately 5 days after initiation. Interestingly, the mRNA levels of L1 were also observed, although no late viral proteins or viral particles were detected. Studies of the transcription map of HPV-18 in U2OS (Ref II, Fig. 2B) revealed that in the case of 1.13 subclone harboring HPV-18 viral genomes, the use of late polyadenylation sites was detectable, and it increased as cells were kept in confluent conditions. Similar results were observed in case of the HPV-11 transcriptome map, in which case the late polyadenylation site was more active during cell confluency (Isok-Paas, Mannik et al. 2015).

The surprising finding of the U2OS system was the physical state of the viral genomes. As papillomaviruses are known to be 8 kb dsDNA molecules, they are expected to replicate and remain as such in the cell. However, in addition to the monomeric 8 kb molecules, we were able to detect a large prevalence of oligomeric forms of HPV DNA (Ref I Fig. 5A and 5B). In case of the HPV-18 clone #1.13 that harbors the episomal genome, it was evident that the majority of the viral genome was in an oligomeric state. Such oligomeric forms of HPV have previously been described from tissue samples taken from patients infected with HPV, and they were also detected in patient biopsy samples

obtained in this study (Boshart, Gissmann et al. 1984; Durst, Kleinheinz et al. 1985; Cullen, Reid et al. 1991). The exact mechanism and cause of the formation of such oligomeric HPV DNA molecules has remained unknown, but as viral genomes are capsulated in the viral capsid as monomeric molecules, a mechanism to revert this process has to be included in the process. One hypothesis of why the virus maintains its genome as an oligomeric DNA molecule could be explained by superior stable maintenance of the viral genome and efficient segregation to the daughter cell. As has been described for BPV-1, a minimum of 6 E2 binding sites are required for the genome to segregate efficiently to daughter cells. In the case of alpha-genus papillomaviruses, there are only 4 E2BS located in the viral genome. As the viral genome is in a oligomeric form, the number of E2BS and therefore potential "anchorpoints" per molecule are also increased, resulting in more efficient segregation and partitioning to daughter cells.

In the light of the data obtained from this study, we were able to conclude that HPV genome DNA efficiently replicates in U2OS cells, and replication initiation is E1- and E2-dependent. U2OS cells support the replication of both cutaneous and mucosal skin infecting HPVs; low- and high-risk genotypes and the U2OS system are able to mimic all three phases of viral replication – initial amplificational replication, stable replication and even vegetative-like replication upon confluency of the cells. This enables U2OS cells to become a tool for replication and viral genome stable maintenance-related studies, and we will target these approaches further in our studies.

4.3. Characterization of the transcription map of HPV-18 in U2OS and two E2C mRNA species (Ref II)

To confirm the relevancy of the U2OS system for HPV studies beyond E1- and E2-dependent replication, we needed to describe the characteristics of the virus in the cell in more detail. Thus, we aimed to study and characterize the transcription map of the HPV-18 genome replicating in U2OS cells. At the time, a study had just been published about the trancriptional map of HPV-18 during productive infection in raft cultures that had been established as a renowned system for HPV-related studies and could be used as a reference (Wang, Meyers et al. 2011).

It was evident that the transcription maps in Wang et al. and in U2OS cells were very similar in aspects of promoter regions, early PAS usage as well as splicing patterns and mRNA species (Fig. 9). We defined 5 promoter regions in HPV-18 genomes replicating in U2OS cells: P102, P520, P811, P1193 and a less pronounced P3000 (Fig. 9 below). Similar to the observations from raft cultures, the P102 promoter was most actively used for the expression of early viral transcripts, and it had previously been described in cervical carcinoma cell lines, primary human keratinocytes transfected with HPV genomes and in HPV-18-infected raft cultures (Romanczuk, Thierry et al. 1990) (Schneider-Gadicke and Schwarz 1986; Thierry, Heard et al. 1987; Wang, Meyers et al.

2011). The transcripts initiated from P102 have the potential to encode all viral early proteins, with exception of E8^E2C (Ref II. Fig. 6). P520 is located in the E6 ORF and has not been previously defined as a viral promoter, although spliced transcripts initiated from this region have been previously demonstrated in HPV-18 in raft culture (Wang, Meyers et al. 2011). P811 has been described as the late promoter that is activated as cells enter differentiation. In the case of the U2OS system, activation was detected when cells were kept in confluency. The P1193 promoter is also poorly described but is also detected in the raft culture system that has been used as a reference. The initiation of transcription from the region mapped to the P3000 promoter has been published before in *in vitro* transcription studies and in HeLa cells (Karlen and Beard 1993), but the function of these transcripts has not been analyzed in previous studies.

Our analysis revealed two transcripts that had the potential to encode truncated forms of E2, containing sequences of the E2C domain. One spliced transcript encoded an E2C-1 protein that contains a peptide encoded by nt 3253–3255 nt in E2 ORF that is then fused to the C-terminal domain of E2 by splicing (Fig. 9, transcript I). The second unspliced mRNA could encode a putative E2C-2 protein that initiated from internal ATG in E2 ORF nt 3426) (Fig. 9, transcript U) .

A similar E2 ORF that initiated expression of truncated E2 variants containing only the C-terminal part of E2 has been described for BPV-1 and HPV-11 (Liu, Kuo et al. 1995) (Lambert, Spalholz et al. 1987; Choe, Vaillancourt et al. 1989) (Lambert, Hubbert et al. 1989; Vaillancourt, Nottoli et al. 1990). These truncated E2 proteins can modulate full-length E2 activities by competing with E2-binding sites in the genome or by forming heterodimers with it. Thus, E2C-1 and E2C-2 are similar to most studied regulator E8‘E2 protein, consisting of a short E8 product (11 amino acids) that is fused to the C-terminal part of the E2 protein. We also analyzed 302 PVs from the PaVE database and found that methionine can be used for translation initiation of E2C-2 and is conserved in all a7 family HPVs (types 18, 39, 45, 59, 68, 70, 85 and 97) as well as in HPV-67 (a9 family).

Due to the lack of available sensitive immunological reagents, we could not analyze the expression of these proteins from the HPV-18 genome. Instead, to analyze the role of E2 DBD-containing proteins in the context of viral genome replication and copy number control, we mutated the ATG codons in E8, E2C-1 and E2C-2 alone and in combinations. We analyzed the transient replication of these mutant genomes (Ref II Fig. 7A) by Southern blot. In accordance with previously published data, mutation of the E8 protein resulted in an approximately 10-fold increase in replication activity (Kurg, Uusen et al. 2010). In the case of the E2C-1 mutation, we did not observe any significant effect on replication, but E2C-2-mutated viral genomes replicated with near 2-fold lower efficiency. The results indicate that E8^E2C represents a major repressor of HPV-18, and copy number control can be achieved mostly by this protein, at least in transient replication. It does not exclude a role for E2C1 and E2C2 in other points or functions of the viral life cycle.

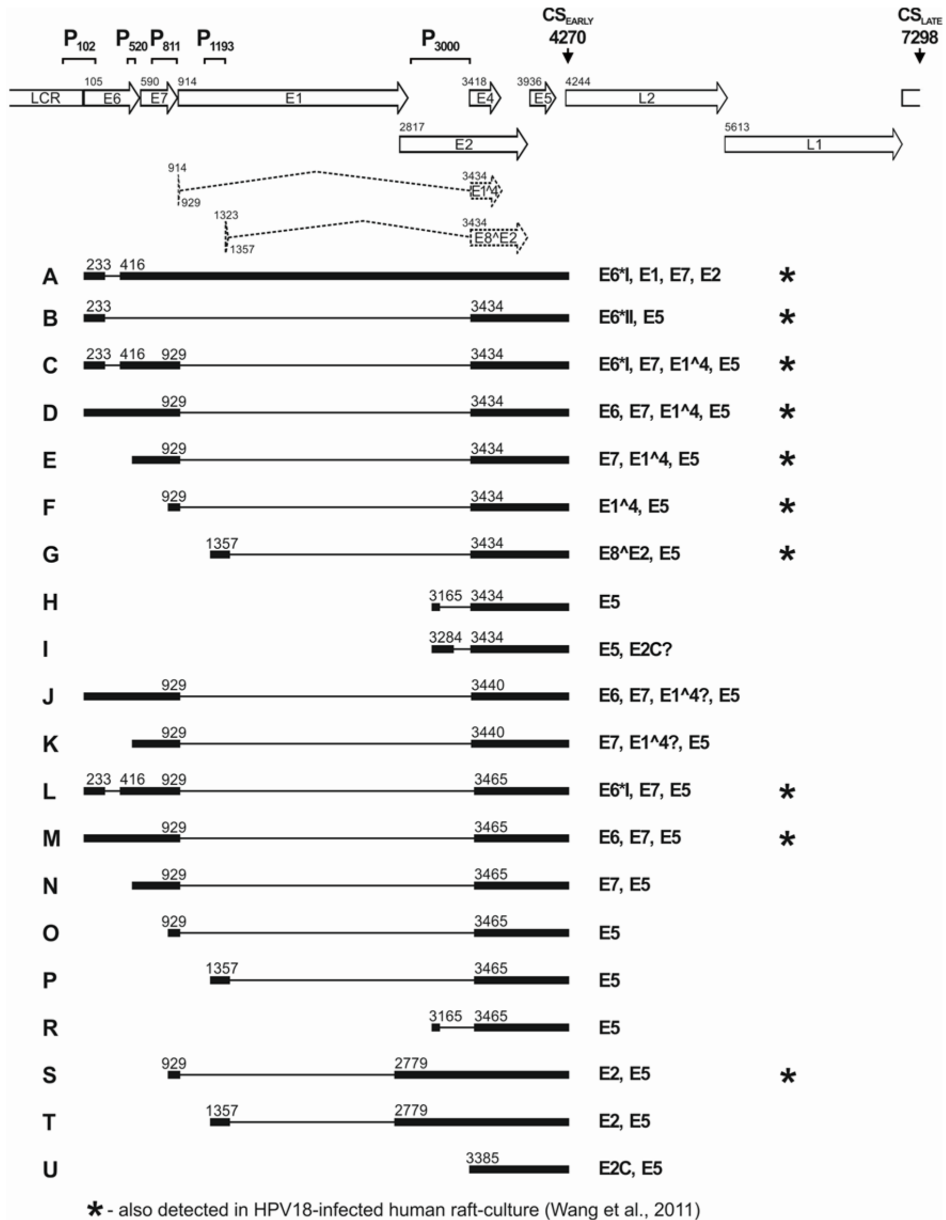


Figure 9. Schematic overview of HPV-18 transcripts mapped in U2OS cells by 5' RACE along with marked promoter regions and cleavage site locations (top).

Thus, we also analyzed the effects of the E2C proteins as transcriptional modulators of the viral promoters. Conducting RACE assays of wildtype vs. mutated genomes revealed that E2C-2 could moderately de-activate the main viral promoter P102, which could explain the weaker replication of this mutant (Ref II

Fig. 8 E–F). Thus, the reduction of transient replication activity by the E2C-2 mutation may be explained by transcriptional repression function of this protein.

Our group has also performed the transcriptome map of cutaneous HPV-5 and low-risk mucosal HPV-11 genomes that actively replicate in U2OS cells (Sankovski, Mannik et al. 2014; Isok-Paas, Mannik et al. 2015). Beta-papillomavirus replication and transcription studies have remained in the background of other cancerogenic forms of HPV, but this has also largely been due to the lack of suitable cellular model systems. It was demonstrated that HPV-5 replicating in U2OS cells is also E1- and E2-dependent, and 4 early promoter regions were described with a total of 14 different viral mRNA species originating from the viral genome. Additionally, the E8^E2C transcript was detected for HPV-5, with similar repressor functions of viral genome replication as other HPV types. The HPV-11 transcriptome map confirmed previous results obtained with HPV-related lesions and additionally confirmed that viral replication and transcription in U2OS truly reflects the effects seen in nature.

In summary, the results of this study strongly suggest that the transcriptome map of HPV-18 (and a separate study published on HPV-11 and HPV-5 replicating in U2OS cells) describe the natural status of the viral genome and thereby further emphasize that the U2OS cells are a relevant system for HPV replication- and transcription-related studies.

4.4. Viral *cis*-elements required for HPV-18 stable maintenance (Ref III)

Papillomaviruses infect basal epithelial cells and, in order for them to be maintained in dividing cells, they need to hitchhike to the daughter cells. For the stable maintenance of latent infection of papillomaviruses and herpesviruses (EBV and KSHV) in dividing host cells, there are two requirements that the virus must fulfill. First, it must replicate its DNA, and secondly, it must segregate its genome equally to daughter cells.

These functions are mediated by E1 and E2 proteins in papillomaviruses (Piirsoo, Ustav et al. 1996), but in the case of herpesviruses, they are mediated by EBNA1 for EBV (Lee, Diamond et al. 1999) and LANA1 for KSHV (Ballestas, Chatis et al. 1999). The binding sites of the chromatin binding viral factors located on the viral genome are crucial anchorpoints for efficient tethering of the viral genomes to the host mitotic chromosome. The latter is used as a vehicle for the safe and loss-less segregation of the viral DNA into the newly forming nuclei of daughter cells (Fig. 7).

As previously described, a large portion of our knowledge of different aspects of the papillomavirus life cycle was initially generated from studies of BPV-1. This is also true for the viral factors and elements for genome segregation and maintenance. Mapping of the minimal *cis*-elements required for BPV-1 stable replication and maintenance was possible because of an E1/E2-expressing cell line. In BPV-1, it has been shown that the E2 protein and 6 E2BS

enable efficient partitioning of viral DNA to daughter cells (Ilves, Kivi et al. 1999) (Abroi, Ilves et al. 2004). The E2 protein is the only viral protein demonstrated to be required for attachment to mitotic chromatin. Attempts to put these approaches in the context of HPV have been problematic due to several aspects that have now become obvious – for example, HPV E1 acting as an activator of the DDR pathway and thereby limiting the possibility of obtaining E1/E2-expressing cell lines and also due to reasons currently unknown (Sakakibara, Mitra et al. 2011).

Due to these difficulties, a different approach had to be developed to study these mechanisms in HPV. Abroi et. al published a study describing a d1-EGFP marker-based segregation assay in a Jurkat suspension cell line that allows monitoring of non-replicating plasmid maintenance in dividing cells (Abroi, Ilves et al. 2004) (see Materials and Methods section). Such an assay system has provided information on the minimal segregation elements of BPV-1, and alanine scanning mutations of E2 have provided information on positions crucial for the efficient segregation complex formation. We used the same approach to construct the segregation assay for HPV-18 (Ref III Fig. 1. A,B).

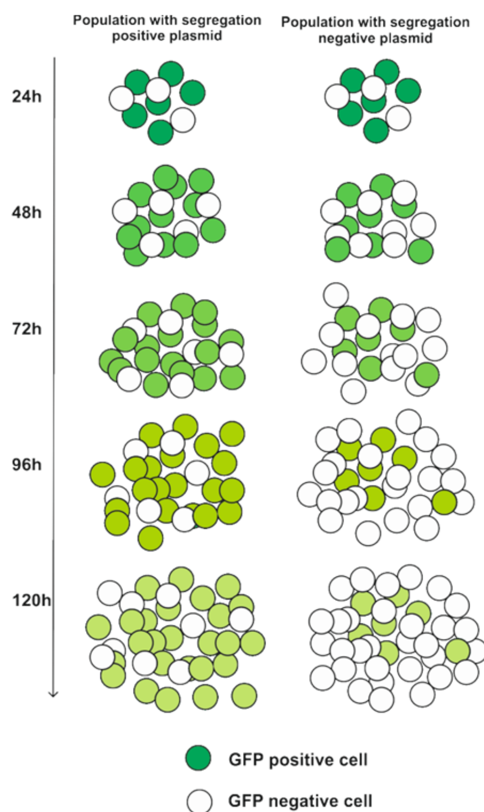


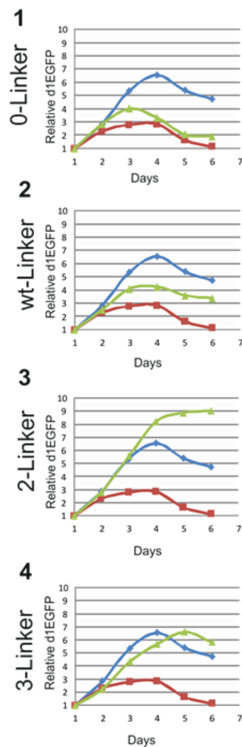
Figure 10. Cartoon representing the division of GFP cells transfected with segregation competent plasmids and segregation incompetent plasmids.

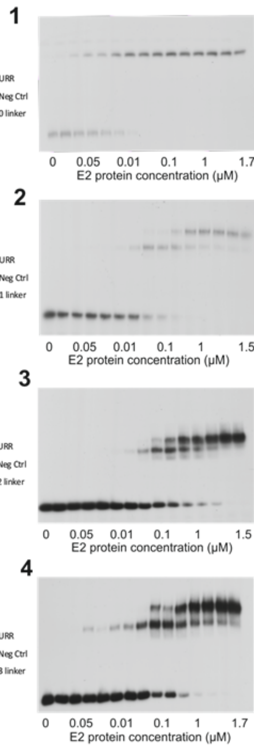
All alpha-genus papillomaviruses have 4 E2BS in their LCR, and we constructed a number of different combinations of HPV-18 E2BS-containing segregation plasmids (Ref III Fig. 2B). As the total number of E2BS in the LCR of HPV-18 (4) is fewer than the minimal number of E2BS needed for the segregation of BPV-1 (6), we hypothesized that several cellular proteins and their respective binding sites in LCR might contribute to the segregation of viral DNA. However, as seen in Ref III Fig. 2C, this hypothesis was not true, as HPV-18 segregation plasmids containing only the plain 4 E2BS and not any other sequence from the LCR were segregating with same efficiency as the full length LCR containing plasmid. In conclusion, we found that 2 E2BS were the minimal *cis*-elements providing plasmid segregation in our assay.

It became evident that the 4bp CGGG linker between the E2BS1 and E2BS2 in LCR seemed to be crucial for the partitioning function. As we created linker modifications by doubling, tripling or deleting the 4 bp linker (Fig. 11.A below), we saw that increasing the spacing between E2BSs enhanced the efficiency of segregation, even exceeding the plasmids that contained the full LCR region (Fig. 11.B below). As we know that the DNA helix requires 10 bp for a full turn (Wang 1979), and E2 bind its respective binding sites cooperatively (Monini, Grossman et al. 1991), we were contemplating that perhaps by changing the spacing between the two E2BS we also sterically enhanced the properties of E2 binding by cooperative binding. Cooperative binding has been shown to occur with many cellular proteins and transcription factors and has been proposed as a reason for synergistic effects during transcription, as the binding of one protein to its ligand improves the affinity of the other protein molecule towards its ligand (Kazemian, Pham et al. 2013). The electrophoretic mobility shift assay (EMSA) confirmed our hypothesis, and we were able to see a 5-fold increase in the cooperative binding of E2 in the case of the doubled linker and a 3-fold increase in the case of the tripled linker (Fig. 11.D below). The plasmid that lacked the linker completely was unable to bind two E2 dimers, and only 1 E2BS could be occupied, likely due to steric interference of the already bound E2 dimer (Fig. 11.C below). These data demonstrated a correlation between the positioning of the E2 binding sites and the cooperative binding of two E2 dimers to the DNA.

A

0-Linker ACCGAAAACGGTACCGAAAACGGT
1-Linker ACCGAAAACGGT**CGGG**ACCGAAAACGGT
2-Linker ACCGAAAACGGT**CGGGCGGG**ACCGAAAACGGT
3-Linker ACCGAAAACGGT**CGGGCGGGCGGG**ACCGAAAACGGT





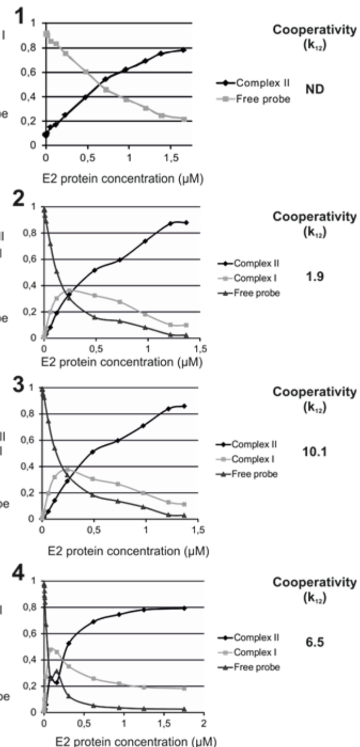


Fig. 11 (A) Schematic representation of the E2BS 1&2 linker mutations **(B)** Segregation assay with the 0-, WT-, 2- and 3-linker segregation plasmids. **(C)** Electrophoretic mobility shift assay (EMSA) performed with purified E2 protein and E2BS (Scheme A) as the probe. 0-linker probe was able to bind only one E2 dimer and formed a single DNA:protein complex. **(D)** Quantitated results of the EMSA and calculated cooperative binding factors.

Next, we used a U2OS-based system to test the effects of implementing these E2BS 1&2 linker mutants in the context of the LCR or intact viral genomic DNA. We tested the ability of these mutant genomes to replicate transiently and even more importantly to maintain the viral genome and see if it confirmed the data obtained from the segregation assay (Ref III Fig. 5). We tested the effect of linkers by transient replication of a HPV-18 LCR-containing plasmid (HPV-18-URR), initiated by co-transfection of E1 and E2 expression vectors. This pure replication assay allowed us to exclude any effects of transcriptional modulation due to the linker mutations laying near the viral main promoter P102. In this

assay, the longer linker did not affect the replication ability, but the 0-linker URR plasmid replicated with a near 3-fold decreased efficiency. Testing the same mutations in the context of the full-length viral genome transient replication, the same 0-linker HPV-18 genome replicated with approximately 5-fold increased efficiency, and increased replication efficiencies were observed with increased E2BS linker mutants (Ref III Fig. 5.B). We were also able to see that, although the 0-linker genome was replicating more efficiently in the transient mode then in stable replication, the mutant genome was not maintained properly, correlating with the results obtained previously in the segregation assay, whereby the 0-linker plasmid was unable to segregate properly to daughter cells.

We attempted a complementation assay by adding extra E2BS combinations into the late region of the 0-linker mutant to test if the rescue of stable replication could be obtained by the addition of a functional E2BS pair (Ref III Fig. 5.D). Unfortunately, the addition of E2 binding sites abolished the viral transient replication for unknown reasons. This might have been due to destabilizing the mRNA of viral transcripts, as deletion of the late region itself did not affect the ability of such "early" genomes to replicate (Geimanen, Isok-Paas et al. 2011). Collectively, the data suggested that the natural positioning of E2BS1 and 2 are crucial for stable replication and segregation of the viral genome.

The possible mechanisms leading to increased transient replication of 0-linker mutation-bearing genomes came from further transcription analyses. Precisely, we performed a 5' RACE assay to detect the early viral mRNAs, similar to Ref II above, and an RT-PCR assay for quantitation of specific mRNAs compared with WT HPV-18. We demonstrated decreased P102 promoter activity together with increased levels of transcripts arising from P1193 for the 0-linker HPV-18 genome. By sequencing the transcripts arising from P1193 transcripts, we were able to detect that the majority of represented mRNAs had the potential to express the E8^E2C repressor protein (Ref III Fig. 6B). There was an increase in E2 transcripts for all mutant genomes, which might also explain the improved transient replication efficiency (Ref III Fig. 6C). Thus, it seems that the effects of the linker mutants result in changes in the optimal balance of the viral transcripts, including those encoding viral replication factors and repressor proteins, and the natural architecture of the E2BSs has been evolutionarily established as optimal for the regulation of the HPV-18 life-cycle.

The fact that E8^E2C mRNA levels are increased but repressor functions are not detectable indicates that either E8^E2C is not able to bind to the single available site or binding to only one E2BS is not sufficient for the repressive function, and E8^E2C repression cannot be mediated by E2BS 3&4. Our results are in agreement with epigenetic studies that have shown that the downstream E2BS 1&2 in the LCR are mediating transcriptional repression, and E2BS 3&4 are required for transcriptional activation (Johannsen and Lambert 2013) (Chaiwongkot, Vinokurova et al. 2013). The E8^E2C inhibited repressor effect also gives ground to the assumption that the 0-linker genome improved

replication ability is due to the activation of E1 and E2 that help to lift the replication level similar to WT-HPV-18 genomes, although the 0-linker is comprised of efficient replication initiation. The role of E8^E2C in stable maintenance of viral genomes is somewhat controversial. Although the repressive function of E8^E2C seems to be conserved throughout different papillomaviruses, a study has shown that E8^E2C is required for efficient maintenance of viral genomes in HPV-31, but in HPV-16, it has been shown to be dispensable for persistence (Stubenrauch, Hummel et al. 2000) (Lace, Anson et al. 2008). Studies in our group with E8^E2C mutants have indicated that a drop of copy number occurs during stable replication, but viral genomes are not completely lost (Kurg, Uusen et al. 2010). Either the loss of viral genomes in the case of the 0-linker genome is due to E8^E2C, or E2's inability to bind to E2BS 1&2 is unclear and requires further investigation. However, it is clear that E2BS 1&2 represent a crucial *cis*-element for segregation, and binding of E2 proteins to these sites is required for segregating efficiently to the daughter cell and for viral genome persistence.

5. CONCLUSIONS

We have identified a human osteosarcoma cell line U2OS that is able to support replication and maintenance of different HPV genomes. We have described this cellular model system in detail and used it to provide new insights into various aspects of HPV-18 molecular biology. The main results of this thesis have been previously published in international peer-reviewed journals and generated two patents. In summary, we have:

- Described the development of a cellular assay system enabling the study of the transient, stable, and amplificational replication of both high- and low-risk HPV genomes;
- Provided a complete overview of the transcriptome map of HPV-18 as it replicates in U2OS cells and showed its similarity to transcription described in productive infection in KCs;
- Characterized viral *cis*-elements that are crucial for HPV-18 segregation and latency and described the importance of E2BS architecture in viral LCR for effective E2 binding and concerted regulation of the viral life cycle.

SUMMARY IN ESTONIAN

HPV-18 genoomi jagunemine ja säilumine rakkudes

Papilloomiviirused on väikesed ligikaudu 8000 aluspaari pikkuse kaheahelalise DNA genoomiga viirused, mis nakatavad spetsiifiliselt keratinotsüüte naha ja limaskesta epiteelkoes. Papilloomiviiruste roll emakakaelavähi tekkes hakkas lahti hargnema 1970ndatel aastatel, kui Prof. Harald zur Hausen esimest korda tuli välja hüpoteesiga, et emakakaelavähi põhjustajaks võivad olla just need viirused. Tõepoolest on praktiliselt kõikides kliinilistes emakakaelavähi proovides võimalik tuvastada papilloomiviiruse DNA ning juba 1980ndatel aastatel eraldati esimesed inimese papilloomiviiruste (HPV-de) genoomid. Sellest alates muutus HPV uurimine ülimalt intensiivseks ning järjest arenevaid molekulaarbioloogilisi meetodeid kasutati ka papilloomiviiruste molekulaarsete mehhanismide – replikatsiooni, geeniekspressiooni ja vähi tekke - uurimiseks.

Kliiniliselt jaotatakse papilloomiviirused kõrge- ja madala riskiga papilloomiviirusteks, sõltuvalt nende võimest tekitada pahaloomulisi kasvajaid. HPV-16 ja HPV-18 on kirjeldatud kui kõige levinumad kõrge riskiga papilloomiviiruse tüübid, mis põhjustavad ligi 90% kogu emakakaelavähi juhtudest. 2006. aastal tulid turule esimesed vähivastased vaktsiinid – preventatiivsed vaksiinid nelja enimlevinud papilloomiviiruse vastu. Paraku pole need vaktsiinid veel jõudnud laialdaselt levida riiklikesse vaktsineerimisprogrammidesse ning miljonid juba nakatunud ja nakatuvad inimesed on siiski ohus. Juba välja kujunenud nakkuse puhuks antud hetkel spetsiifilisi viirusvastaseid ravimeid pole ning nende ravimite leidmisel on olnud oluliseks takistuseks sobiva rakulise uurimisplatvormi olemasolu, mis aitaks efektiivselt kaasa viiruse elutsükli erinevate tahkude uurimisele molekulaarsel tasandil.

Käesolevas töös kirjeldame inimese osteosarkoomi rakuliini U2OS kasutamist inimese papilloomiviiruse molekulaarbioloogia uurimise platvormina. Me avastasime, et sellel rakuliinil on haruldane omadus toetada nii naha, kui ka limaskesta rakke nakatavate HPV subtüüpide genoomi replitsiooni. Lisaks esialgset nakkust mimikeerivale amplifikatsioonilisele replikatsioonile olid viiruse genoomid võimelised jagunevates rakkudes ka säiluma, mis viitas asjaolule, et viiruse genoom jaotatakse ühtlaselt tütarrakkude vahel. Seega on seda süsteemi võimalik kasutada ka latentse viirusnakkusega seotud küsimuste uurimiseks. Kui organismis kulgeval nakkusel viirusega nakatunud rakk lahkub naha basaalmembraanilt, differentseerub ning hakkab epiteelkoe kihis liikuma naha välisosale, toimuvad rakkude sisekeskkonnas muutused ning koostöös viirusvalkudega käivitub viiruse genoomi teistkordne paljunamine ning kapsiidivalkude ekspressioon, et valmistada ette piisavalt viiruse genoomi koopiad, mis omakorda pakkuksid viiruse kapsiidi. Huvitaval kombel on võimalik ka antud replikatsioonifaasi teatud määral mimikeerida U2OS rakkudes, kus sobivate keskkonnatingimuste puhul ilmneb kapsiidivalkude geenide promootori ja polüadenülatsioonisaidi akitveerumine ning viiruse DNA replikatsiooni märkimisväärne võimendamine.

Käesoleva tööga seotud järgnevas publikatsioonis me näitasime täiendavalt U2OS süsteemi autentsust viiruse uurimiseks. Kui senini olime me keskendunud genoomi replikatsioonile, siis nüüd koostasime HPV-18 transkriptoomikaardi U2OS rakkudes. Meie tulemustest ilmnes selgelt, et antud transkriptsioonikaart oli väga sarnane tulemustega, mis samu meetodeid kasutades saadi HPV18 produktiivse nakkuse korral primaarsetes keratinotsüütides. See kinnitab meie poolt avastatud U2OS-põhise süsteemi relevatsust papilloomiviirusega seotud uurimustöödeks. Lisaks kirjeldasime kaht uut repressorvalku, mida HPV-18 ekspresseerib ning millel näib olevat roll viiruse geeniekspressiooni ja replikatsiooni modulleerimisel.

Käesoleva töö kolmandaks tahuks on olnud mehhanismide kirjeldamine, mis on vajalik viiruse genoomi säilumiseks jagunevates rakkudes. Selle valdkonna muudab eriti oluliseks asjaolu, et papilloomiviiruse pikajaline latentne säilimine on üks potentsiaalsetest märklaudadest viirusvastaste ravimite väljatöötamiseks. Valdav enamus teadmistest selle funktsiooni kohta on seni pärinenud veise papilloomiviiruse tüüp-1 (BPV-1) alastest uurimustöödest, mis määratlesid viirusvalgud E1 ja E2 ja piisava arvu E2 valgu seondumiskohti genoomis kui vajalikud latentseks replikatsiooniks jagunevates rakkudes. Samas polnud neid teadmisi võimalik üle kanda inimese papilloomiviirustele, kuna E2 seondumiskohtade arhitektuur viiruse genoomil on HPV'del ja BPVl kardinaalselt erinev. BPV puhul on näidatud et kokku 12st E2 seondumiskohast viiruse genoomi regulatoorses osas on 6 minimaalselt vajalikud selleks, et viiruse DNA saaks päranduda tütarrakku. HPV'del on regulatoorses alas üleüldse kokku 4 E2 seondumiskohta. Meie uurimustööst selgus, et HPV-18 puhul on lisaks E2 valgule vaja minimaalselt 2 E2 seondumiskohta, et tagada DNA pärandumine tütarrakkudesse. Me näitasime, et suurenenud jaotumisfunktsiooni võimekus tuleneb E2 suurenenud kooperatiivse seondumise võimekusest antud järjestustele. Lisaks näitasime me kui oluline on E2 seondumiskohtade täpne paigutus ja kontekst genoomis viiruse elutsükli jaoks. Mutatsioonanalüüs E2 seondumiskohtade vahelises alas osutas, et see element on evolutsiooni käigus optimeeritud viiruse genoomi tasakaalustatud transkriptsiooniks, replikatsiooniks ja segregeerumiseks.

Kokkuvõtvalt me kirjeldame antud töö käigus uudset rakulist mudelsüsteemi, mis on sobilik HPV alasteks uurimustöödeks ning lähme sügavuti jagunevates rakkudes viiruse DNA säilumise mehhanismide uurimisse, kus me defineerime antud töö tulemusena kriitilised elemendid, mis antud protsessis on vajalikud.

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Patents

Method and a kit for identifying compounds capable of inhibiting human papilloma virus replication; Authors: Mart Ustav, Ene Ustav, Jelizaveta Geimanen, Regina Pipitš, Helen Isok-Paas, Tormi Reinson, **Mart Ustav Jr.**, Triin Laos, Marit Orav, Anu Remm, Kristiina Salk, Andres Männik; Priority number: **PCT/EE2010/000010**; Priority date: 19.05.2010

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Patendid

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