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LIISI HENNO

Study of the human papillomavirus genome replication and oligomer generation

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

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LIST OF ORIGINAL PUBLICATIONS

I. Marit Orav, **Liisi Henno**, Helen Isok-Paas, Jelizaveta Geimanen, Mart Ustav, Ene Ustav (2013) Recombination-dependent oligomerization of human papillomavirus genomes upon transient DNA replication. *Journal of Virology*, **87** (22), 12051–12068

II. Marit Orav, Jelizaveta Geimanen, Eva-Maria Sepp, **Liisi Henno**, Ene Ustav, Mart Ustav (2015) Initial amplification of the HPV18 genome proceeds via two distinct replication mechanisms. *Scientific Reports* **5**, 15952

III. **Liisi Henno**, Eva-Maria Sepp, Marit Orav, Jelizaveta Geimanen, Ene Ustav, Mart Ustav (2017) Analysis of human papillomavirus genome replication using two- and three-dimensional agarose gel electrophoresis. *Current Protocols in Microbiology*, **45**, 14B.10.1–14B.10.37

My contributions to the listed articles are as follows:

Ref. **I** I performed the analysis of transient assays of HPV18 E8 mutant and HPV11 genome replication and oligomer formation over time; co-transfection analysis of the HPV18 and HPV18E genomes; and 1D and 2D N/N analysis of DNA replication of HPV18 URR minicircle in the presence of E1 and E2 expression vectors or the HPV18 E8 mutant. I participated in interpretation of the data.

Ref. **II** I participated in performance of the experiments in U2OS cells related to the analysis of linearized HPV18 genomes and sub-genomic fragments. I participated in interpretation of the data.

Ref. **III** I analyzed the data and wrote the manuscript. I participated in optimization of the 2D methods and in performance of the experiments.

Articles not included in this dissertation are as follows:

I. Tormi Reinson, **Liisi Henno**, Mart Toots, Mart Ustav Jr, Mart Ustav (2015). Engagement of the ATR-dependent DNA damage response at the human papillomavirus 18 replication centers during the initial amplification. *Journal of virology,* **87**, 951–964

LIST OF ABBREVIATIONS

1D	one-dimensional
2D	two-dimensional
3D	three-dimensional
AG	agarose gel
AGE	agarose gel electrophoresis
ATM	ataxia-telangiectasia mutated kinase
ATP	adenosine triphosphate
ATR	ATM- and Rad3-related kinase
BPV1	bovine papillomavirus type 1
ccc	covalently closed circle
D-loop	displacement loop
DDR	DNA damage response
DSB	double-stranded DNA break
dsDNA	double-stranded DNA
E	early region
E1BS	E1 protein binding site
E2BS	E2 protein binding site
EBV	Epstein-Barr virus
EtBr	ethidium bromide
HIV	human immunodeficiency virus
HJ	Holliday junction
HPV	human papillomavirus
HR	homologous recombination
HSV-1	herpes simplex virus type 1
L	late region
LMW	low molecular weight
N/A	neutral/alkaline
N/N	neutral/neutral
N/N/A	neutral/neutral/alkaline
NHEJ	non-homologous end-joining
oc	open circular
ORF	open reading frame
ori	origin of replication
pRB	retinoblastoma protein
PV	papillomavirus
RCR	rolling circle replication
RDR	recombination-dependent replication
RI	replication intermediate
ssDNA	single-stranded DNA
SV40	Simian Virus 40
URR	upstream regulatory region
UV	ultraviolet
wt	wild type

INTRODUCTION

Human papillomaviruses (HPVs) are prevalent pathogens that infect either mucosal or cutaneous epithelium. Most infections are asymptomatic; however, a small fraction lead to hyperproliferative lesions, such as warts and condyloma, or in the case of high-risk human papillomaviruses, to malignant tumors. As a result, HPVs are the major cause of cervical cancer (zur Hausen 2002).

Cervical cancer is the third most common cancer worldwide (Jemal et al. 2011), and HPVs are the etiologic agent of 5% of all lethal cases of cancer worldwide. In addition, a major proportion of anal, perianal, vulvar, and penile cancers appear to be primarily linked to HPVs. Moreover, papillomavirus infection has also been identified in non-genital cancers, including lung, colon, esophageal, laryngeal, oropharyngeal and urothelial cancers (Mammas et al. 2011). Thus, great effort needs to be put into patient screening, vaccine development, and therapeutic strategies.

Our research group has focused on understanding what happens to HPV genomes after transfecting them into the nucleus of U2OS cells, including revealing the mechanism(s) behind the formation of genomic oligomers and analyzing an unidentified replication mechanism that functions in addition to the well-documented theta type of bidirectional replication.

One of the best methods for analyzing replication intermediates (RIs) of the HPV genome is two-dimensional agarose gel electrophoresis (2D AGE). This method provides clear visualization of molecules of different structures and sizes. However, it is also difficult and time-consuming to perform and provides results that are even more difficult to interpret. Therefore, in the interest of reproducibility, I have included an overview of various applications of these methods, together with an analysis of the results.

Based on results obtained from 2D assays, we have identified the oligomeric state of HPV DNA and intricate mechanisms of viral DNA replication.

LITERATURE REVIEW

Papillomaviruses

Human papillomaviruses are a large family of small double-stranded DNA viruses that infect basal keratinocytes in stratified epithelia. Papillomaviruses (PVs) are extremely species and tissue specific; they only infect the keratinocytes of amniotes (mammals, birds, and reptiles) (Bernard et al. 2010; Rector and Van Ranst 2013).

Human papillomaviruses commonly cause cutaneous and genital warts, less commonly anogenital cancers, oropharyngeal cancer, and recurrent respiratory papillomatosis. Evidence suggests that different types of HPVs have existed since the evolutionary origin of humans (Bernard et al. 2006; Chen et al. 2009); therefore, it is believed that humans have always suffered from warts and anogenital cancers.

Genomic organization

PVs have maintained their basic genomic organization for more than 100 million years. The PV genome is composed of genetically stable double-stranded DNA that is replicated by the host cell's DNA replication machinery. Diversification of PV genomes has occurred at a low evolutionary rate due to slow DNA replication, the proofreading abilities of DNA polymerases and the absence of intra-type recombination (Bernard et al. 2006).

To date, more than 300 PVs have been identified and completely sequenced (PaVE: Papillomavirus Episteme *https://pave.niaid.nih.gov/*).

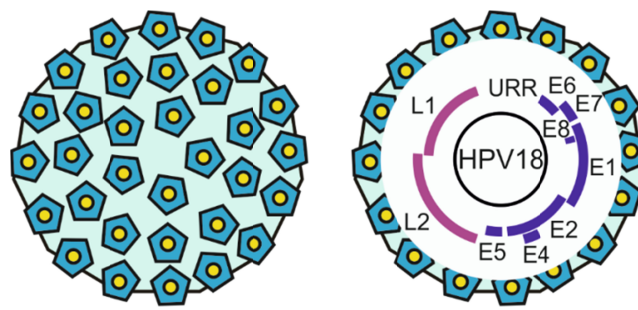

Figure 1. Schematic representation of HPV18 virion. Blue pentagons represent major capsid protein L1 and yellow circles represent minor capsid protein L2. The HPV18 genome is indicated inside the virion. Adapted from (Tristram and Fiander 2007).

The HPV genome consists of an approximately 6–8 kbp long circular double-stranded DNA (dsDNA). All HPVs have the same general organization of their genome, which can be functionally divided into three general components – a noncoding upstream regulatory region (URR), an early (E) region, and a late (L) region (Fig. 1, HPV18 genome inside the virion).

The 400–1000 bp long URR region contains the viral origin of replication, binding sites for viral and host regulatory proteins, early promoter, enhancer, and silencer sequences that work together to control transcription from the early open reading frames (ORFs), viral DNA amplification, and cellular tropism (reviewed in (Longworth and Laimins 2004)). The early region carries ORFs for non-structural regulatory proteins E1, E2, E4, E5, E6, and E7 (E5, E6 and E7 are not universally present in all PV types), which function to adapt viral activities to changes in the cellular milieu, regulate viral genome replication, cell cycle control, viral transcription, cellular transformation and proliferation, elude the host immune system, and modify the host cell to facilitate the release of progeny virions (reviewed in (Longworth and Laimins 2004; Egawa et al. 2015)). Additional early proteins have also been identified (E3, E8); however, their expression is not uniform throughout the *Papillomaviridae* family (Harari et al. 2014).

The late region carries ORFs for the structural major and minor capsid proteins, L1 and L2, respectively, which form the icosahedral viral capsid ((Doorbar and Gallimore 1987), reviewed in (Buck et al. 2013; Wang and Roden 2013)). Late transcripts, including E1^E4, are initiated by a late promoter located in the E7 ORF (del Mar Pena and Laimins 2001).

Viral early proteins

HPV proteins E1 and E2, together with the host replication machinery, are necessary and sufficient to replicate the papillomavirus genome (Ustav and Stenlund 1991; Chiang et al. 1992; Sverdrup and Khan 1994). E1 harbors ATPase activity while it melts dsDNA for strand separation. It thus works as a helicase and participates in recruiting host DNA replication factors to the viral origin of replication (Seo et al. 1993b; Park et al. 1994; Han et al. 1999; Bergvall et al. 2013). E2 functions in viral transcription and viral DNA replication and plays a role in viral genome partitioning (reviewed in (McBride 2013)). Viral DNA replication is initiated when a dimeric E1 protein attaches to its binding site with the help of a dimeric E2 protein. E2 protein increases the affinity and site-specificity of E1 protein (Seo et al. 1993a). As a result, more E1 proteins are recruited, followed by the release of E2 proteins from the accumulated complex, thereby resulting in the formation of a double hexamer E1 protein complex, which starts to unwind the dsDNA (Sedman and Stenlund 1998; Auster and Joshua-Tor 2004).

E2 protein is responsible for recruiting cellular factors to the viral genome, which in turn regulate transcriptional processes. E2 is also responsible for the

down-regulation of early promoter activity to maintain a low profile during the early stages of the viral life cycle. This role is supported by the fact that HPV integration into the host genome often results in disruption of the E2 ORF, which in turn dismisses early promoter regulation and thereby avoids over-expression of E6 and E7 (Hegde 2002; Longworth and Laimins 2004; Doorbar 2006). In HPV associated cancers, continued expression of E6 and E7 sustains the continued cancer phenotype, whereas the late genes are not expressed and progeny viruses are not produced (Stoler et al. 1992; Chow and Broker 2013).

E1 has also been shown to function in early induction of the DNA damage response (DDR) pathway, which in turn facilitates a permissive environment for viral genome replication. E1 is capable of inducing breaks in the host dsDNA that activate the ATM (ataxia-telangiectasia mutated) DDR pathway, which signals cell cycle arrest (Fradet-Turcotte et al. 2011; Sakakibara et al. 2011; Reinson et al. 2013).

E5, E6, and E7 stimulate cell proliferation and cell survival and modulate keratinocyte differentiation. The E5 ORF encodes a transmembrane protein that most likely contributes to cell signaling (Chen and Mounts 1990). Because HPV heavily relies on cellular proteins for its genome replication and transcription, the essential role of E6 and E7 in the viral life cycle is primarily to modify the cellular environment to permit viral genome amplification, which mainly occurs by driving S-phase re-entry in the upper epithelial layers.

E6 and E7 play different roles in high- and low-risk HPVs. In the case of high-risk types, E6 and E7 function as oncogenes by disrupting pathways controlled by the two major tumor suppressors, p53 and the pRB family of proteins, and additional host proteins, as well as driving cell proliferation in the basal layers ((Doorbar 2006; McLaughlin-Drubin and Munger 2009; Chow and Broker 2013), reviewed in (Moody and Laimins 2010)). However, in low-risk types, the role that these proteins play is unclear (reviewed in (Roman and Munger 2013; Vande Pol and Klingelhutz 2013)). Differences in the function of E6 and E7 represent a major determinant of HPV disease pathogenicity between HPV types (White and Howley 2013).

During the HPV life cycle, E4 is first synthesized as part of the E1^E4 fusion protein from spliced E1^E4 transcripts, where the first few amino acids contain the N terminus of E1 (Nasseri et al. 1987). E4 protein accumulates massively in the upper epithelial layers, where viral genomes are packaged into virus particles (Doorbar 2013). E4 protein optimizes viral genome amplification and participates in viral release (Egawa et al. 2017). In high-risk HPV types, E4 protein assembles into amyloid fibrils that can disrupt the structure of keratin and compromise the normal assembly of the cornified envelope. It has been suggested that E4 amyloid fibers may contribute to virion release from the epithelial surface, and as a result, they may influence both infectivity and transmission (McIntosh et al. 2008; McIntosh et al. 2010). However, E4 probably does not have precisely the same function in all papillomaviruses (Egawa et al. 2017).

Life cycle of stratified squamous epithelial cells

Epithelial tissue is a multi-layered structure in which each stratum has a certain gene expression pattern and cellular architecture. Therefore, the epithelium is comprised of keratinocytes at various stages of differentiation. Only basal cells are mitotically active in healthy epithelial tissues (Chow et al. 2010). As basal cells divide, one daughter cell retains its position in the basement layer and the ability to propagate. The other daughter cell is pushed upward from the basement layer, after which its gene expression pattern is altered; the cell starts to differentiate, exits the cell cycle, and loses its ability to propagate (Fig. 2, cells with black lines).

During the differentiation of keratinocytes, keratin is synthesized to strengthen the cytoskeleton. The final stage of this differentiation program is the built-in program of apoptosis of the keratinocytes. The normal life cycle of epithelial cells lasts about three weeks with the final step being the release of necrotic keratinocytes from the epithelial surface (Fuchs 2008; Fuchs 2009).

Papillomavirus life cycle

The life cycle of HPV is closely related to the stages of differentiation within the host epithelium. As mentioned above, HPVs are highly tissue specific and infect only mucosal or cutaneous epithelial cells. For a successful infection, HPVs must enter dividing basal cells of the epithelium and connect their life and replication cycle with the host cell differentiation program (reviewed in (Doorbar et al. 2012)).

The viral life cycle is closely related to the viral replication cycle; both can be separated into three phases. An initial amplification phase (also known as transient viral DNA replication) occurs first, after the HPV has entered the basement membrane of either the mucosal or squamous epithelium via an injury. Replication of viral DNA starts in the host cell nucleus and continues until the copy number of HPV genomes is high. In the second phase, the viral DNA copy number is held at a constant level in proliferative basal epithelial cells and thus is termed the stable maintenance phase (latent infection). The third phase occurs when the infected cells are pushed upwards from the basal layer and start to differentiate. The virus prevents the cells from concluding their life cycle, and infected cells aberrantly re-enter the cell cycle and begin to amplify HPV genomes to an even higher copy number. The final phase is termed the second amplification (also known as late or vegetative amplification). As the infected cells move towards the surface of the stratified epithelium, they express late capsid proteins and viral DNA is packed into progeny virions (Fig. 2, cells with red lines, virions are marked with purple hexagons). These infectious virions are then released from the surface of the epithelium (reviewed in (Kadaja et al. 2009; Bodily and Laimins 2011; Doorbar et al. 2012)).

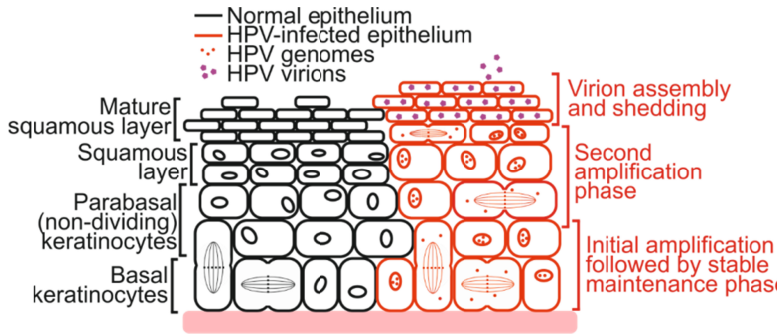

Figure 2. Schematic representation of asymptomatic and infected epithelium. In a normal epithelium, only basal cells are capable of dividing and replicating. After cell division, one cell is pushed upward from the basement layer and begins to differentiate; it loses its ability to replicate and is finally shed form epithelium. Infected basal cells, however, maintain their replicative capability even after being pushed from the basement layer. After infection with HPV, initial amplification occurs, which is followed by a stable maintenance phase. After leaving the basement layer, the second amplification phase begins. HPV produces virions in the uppermost layers of the epithelium, and they are finally shed from the epithelial surface. Figure adapted from (Henno et al. 2017).

It has been proposed that after virions enter the basal cells and amplify their genome, the latent persistence of HPV DNA is established. However, if infection only occurs in cells that have already started their differentiation program, only short-term infection is established because these HPVs are eliminated along with the shedding cells that have finished their normal differentiation program (reviewed in (Bodily and Laimins 2011)).

Initial amplification of HPV genome

An injury enables HPV virions to attach to the basal keratinocytes within either the mucosal or squamous epithelium. After HPV virions enter the cell, the genome is unpacked and transported to the nucleus. The mechanism of viral attachment and entrance into the host cell is still not precisely known; however, several cellular and viral proteins have been proposed to be involved in the process. Heparin sulfate has been proposed as one possible cellular receptor, and L1 and L2 proteins ensure the infectiveness of HPV virions (Joyce et al. 1999; Horvath et al. 2010). Once in the nucleus, early proteins are expressed from the early promoter and the HPV genome copy number is amplified to ensure a persistent infection. Rapid amplification is achieved by replicating HPV DNA repeatedly during one cell cycle of these host cells ((Demeret et al. 1997; Steger and Corbach 1997), reviewed in (Kadaja et al. 2009; Moody and Laimins 2010)).

It is difficult to study the life cycle of PVs under laboratory conditions. Nevertheless, the initial amplification can be mimicked by transfecting eukaryotic

cells with HPV genomes; however, only a few cell lines are capable of supporting papillomavirus DNA replication. We utilize our U2OS cell line based model system to mimic HPV genome replication cycles (Geimanen et al. 2011). Our experiments on HPV DNA replication are performed during the initial short establishment period by transfecting the U2OS cells with HPV genomes and analyzing the extracted episomal DNA 3 to 5 days after transfection (Orav et al. 2013; Orav et al. 2015; Henno et al. 2017).

Stable maintenance replication of HPV genome

After the initial amplification of viral DNA, the genome copy number is held at a constant level. HPV DNA replication occurs at the same rate as host genome replication. During the latent phase, the necessary proteins for episomal maintenance are expressed at low levels, which presumably occurs to evade detection by the host immune system (reviewed in (Stanley 2012)).

PV replication occurs, on average, once during the cell cycle, concurrently with host replication, and viral genomes are divided almost equally between the daughter nuclei (reviewed in (McBride et al. 2006; Kadaja et al. 2009; Bodily and Laimins 2011)). This observation could mean that either PV replicates in a strictly once-per-S-phase mode (Roberts and Weintraub 1988; Kim and Lambert 2002) or at random modes, where some cells replicate several times during the host cell cycle and others may not replicate at all (Gilbert and Cohen 1987; Ravnan et al. 1992; Piirsoo et al. 1996). It has been shown that PV genomes are capable of both replication modes during the stable maintenance phase, depending on the cell type that carries the viral episomes or on the amount of E1 protein in the host cells ((Hoffmann et al. 2006), reviewed in (Kadaja et al. 2009)).

Cells that support stable replication of HPV can be obtained from cervical lesions (Hoffmann et al. 2006) or they can be created by transfecting cells capable of supporting HPV DNA replication with HPV genomes and cultivating HPV-positive cells under regular culture conditions (Geimanen et al. 2011).

Vegetative amplification of HPV genome

When normal, uninfected stratified squamous epithelial cells are pushed from the basement membrane, they start to differentiate and exit the cell cycle; however, HPV needs the cells to be mitotically active to continue its life cycle with a supply of necessary cellular DNA polymerases and replication factors that are only produced in mitotically active host cells. If the HPV-infected host cell is pushed upwards from the basal cell layer, the HPV replication cycle continues. This continuation is achieved through the action of viral oncoproteins that modify the cellular checkpoint mechanisms so that the cell cycle remains active and an unscheduled re-entry into the S-phase occurs without the induction of

cell apoptosis, which enables the virus to utilize the proteins necessary for host cell replication. However, it has been shown that the virus replicates its DNA also during the G2 phase, after host cell replication (Banerjee et al. 2011), which implies that HPV utilizes other means to obtain the necessary machinery to replicate its DNA. Therefore, it has been proposed that HPV induces and uses DDR pathways for replicating viral genomes in the G2 phase (Moody and Laimins 2009; Fradet-Turcotte et al. 2011; Gillespie et al. 2012; Reinson et al. 2013). DDR is also induced during the replication of other viruses, such as adenovirus, herpes simplex virus type 1 (HSV-1), Epstein-Barr virus (EBV), polyomavirus, SV40, and minute virus of canines, because viral DNA and replication intermediates (RIs) are sensed by the host as damaged DNA (Stracker et al. 2002; Dahl et al. 2005; Lilley et al. 2005; Shi et al. 2005; Luo et al. 2011).

When HPV-positive cells reach the spinous layer, viral replication proteins are expressed at a much higher rate. Therefore, the viral DNA copy number increases and this step is called second amplification (reviewed in (Bodily and Laimins 2011)). Lastly, in the upper layers of the epithelium, amplified viral genomes are packaged into viral particles produced from the major (L1) and minor (L2) virus late coat proteins. Because late proteins induce a strong immune response, their expression is delayed until the infected cell reaches the upper epithelial layer. As E1^E4 protein constructs induce the collapse and apoptosis of differentiated host cells in the uppermost layers of the epithelium, they play an important role in the detachment of viral particles (Nakahara et al. 2005). Virions only detach from the upper epithelial layer when the host cells are almost collapsed because papillomaviruses are not lytic, which is another reason to avoid the immune response of the host organism (reviewed in (Bodily and Laimins 2011)).

Second amplification can be mimicked *in vitro* by cultivating HPV-positive keratinocyte cell lines in methylcellulose (Green 1977), a high-calcium medium (Boyce and Ham 1983; Berghard et al. 1990) or high cell confluence (Poumay and Pittelkow 1995; Minner and Poumay 2009). Organotypic raft cultures of keratinocytes are capable of supporting the entire life cycle of HPVs (Dollard et al. 1992; Cheng et al. 1995; Flores et al. 1999; Fehrmann et al. 2003).

New model system to study HPV genome replication

Several cellular systems that mimic layered epithelial structures have been created to study HPV genome replication mechanisms. With the aid of these systems, one can follow and analyze viral genome replication in both basal and differentiated cells. The most suitable cellular system for mimicking HPV replication cycles utilizes primary foreskin or cervical keratinocytes, which can reconstruct the viral life cycle in raft cultures when transfected with high-risk HPV genomes (Chow et al. 2010). Though, experiments that utilize this kind of system are labor-intensive, expensive, and complicated. However, less

complicated systems, such as monolayer cultures of human transformed cell lines, cannot successfully mimic the entire HPV life cycle.

Our research group at the Institute of Technology within Tartu University has developed a novel model system that is based on transfecting viral genomes into U2OS cells (Geimanen et al. 2011). These cells provide efficient replication of not only high-risk HPVs (HPV16, HPV18) but also low-risk (HPV11, HPV6b) and cutaneous HPVs (HPV5, HPV8, HPV38). In addition, this model system can mimic the transient, stable, and vegetative phases of the HPV replication cycle. The U2OS model system is less labor-intensive, cheaper, and easier to use compared with other model systems (Geimanen et al. 2011).

Because HPV genomes exist in nuclei as covalently closed circular plasmids (episomal molecules), we conduct our experiments using similar, but bacterially grown, minicircle genomes (Kay et al. 2010; Henno et al. 2017). The transcription pattern of HPV18, HPV11, and HPV5 genome replication in U2OS cells is identical to the pattern of transcription in keratinocytes (Wang et al. 2011; Sankovski et al. 2014; Toots et al. 2014; Isok-Paas et al. 2015). This result gives us confidence that our model system mimics the initial transient, stable maintenance, and vegetative amplification replication phases of HPVs.

The role of oligomers in the HPV replication cycle

Using our model system, which is based on transfecting different HPVs into U2OS cells, we are able to identify linked molecules that consist of several HPV genomic copies – oligomers (also known as multimers) from extracted HPV DNA. HPV genomic oligomers contain several viral genomes in a head-to-tail orientation (Geimanen et al. 2011; Orav et al. 2013). HPV genomes begin to generate oligomers as soon as 24 h post transfection, and the proportion of monomers and multimers shifts over time such that the number of oligomers increases (Geimanen et al. 2011; Orav et al. 2013).

Studies performed in HPV-positive U2OS cell lines that mimic the stable phase of replication show that HPV genomes only exist in them as oligomers (Geimanen et al. 2011). We propose that these oligomers have only one active origin and can therefore replicate several genomes while utilizing only one replication complex. This arrangement enables HPV to maintain a large number of viral genomes while using a small number of host and viral proteins. The phenomenon of utilizing one active origin in an oligomer has been shown in BPV-1 (bovine papillomavirus type 1) (Schvartzman et al. 1990).

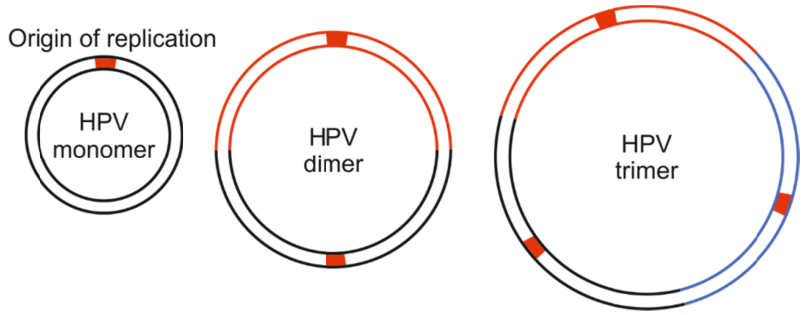

Figure 3. Schematic representation of oligomers that consist of HPV monomers in head-to-tail orientation. Each HPV monomer consists of one genome and one origin of replication; thus, dimers and trimers have two and three genomes and origins of DNA replication, respectively.

We propose that oligomer generation is dependent on homologous recombination (HR) related processes (Orav et al. 2013). The results and discussion section of this dissertation provides further insights into the mechanism of HPV genome replication and oligomer generation and the possibility of one active origin in an oligomer. The next section provides an overview of the various mechanisms proposed for HPV DNA replication.

Mechanisms proposed for circular DNA replication

Several different mechanisms have been proposed for circular DNA replication, some of which can also be related to PV DNA replication. This section presents the most important differences between them.

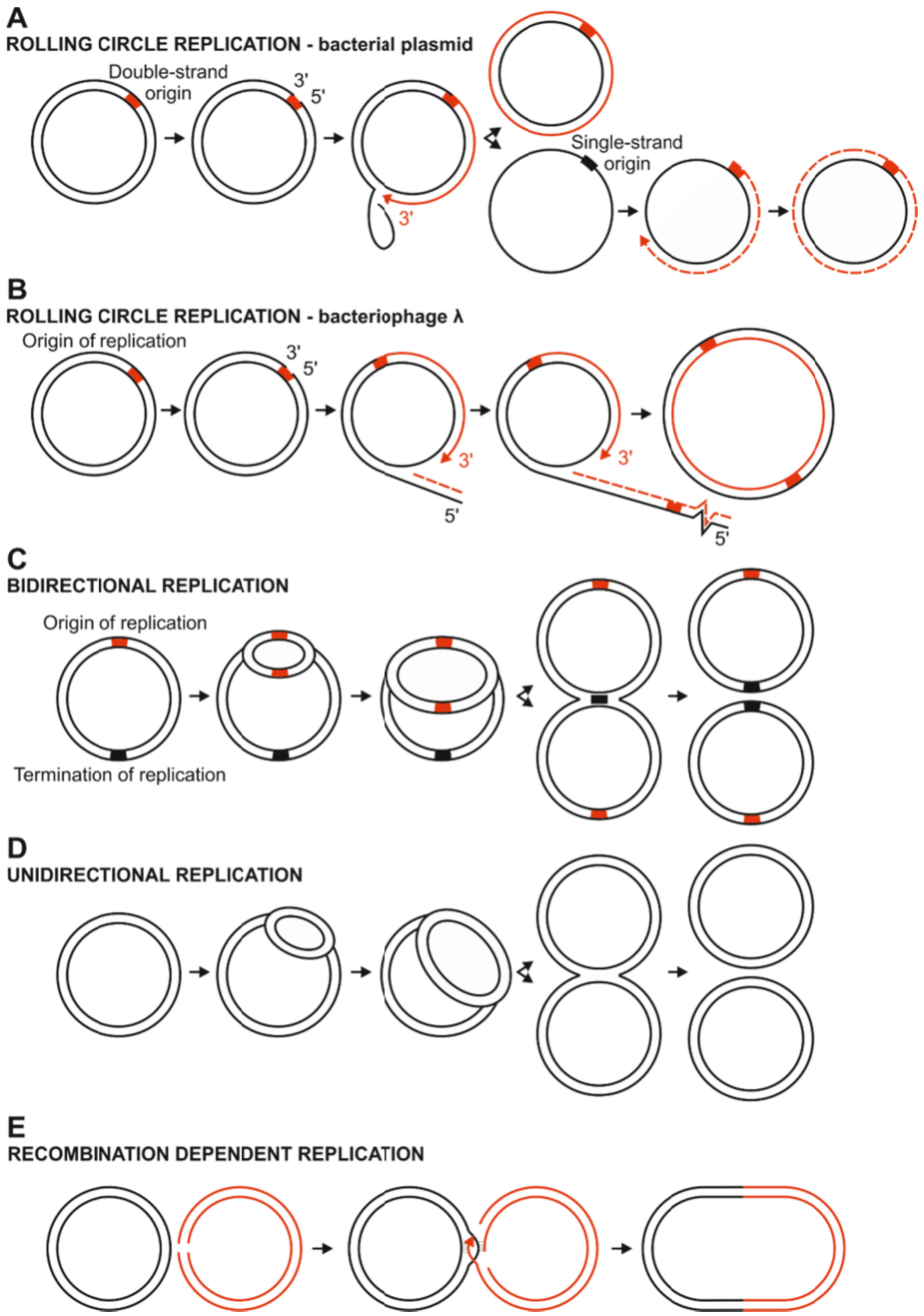

Figure 4. Five different kinds of mechanisms of circular dsDNA replication. A) Rolling circle replication of bacterial plasmids is initiated when a sequence-specific ssDNA break is introduced into the dsDNA origin, the double helix is unwound and elongation of the 3' strand occurs in a way that the non-template single strand is displaced. The ssDNA then acts as a template for synthesis of the lagging strand. Okazaki fragments are indicated with a fragmented line on the lagging strand. **B)** Rolling circle

replication of bacteriophage λ results in RIs with long tails of linear concatemeric λ DNA. Okazaki fragments are indicated with a fragmented line on the lagging strand. **C)** Bidirectional theta replication is initiated at the origin of replication. Two replication forks progress until they meet opposite of the origin. This meeting results in the separation of dsDNA daughter molecules. **D)** In unidirectional replication, a single replication fork progresses in one direction until it reaches the initiation site, which results in the separation of dsDNA daughter molecules. **E)** The likely mechanism for recombination dependent replication and the generation of oligomeric circular molecules of HPV.

Rolling circle replication

Rolling circle replication (RCR) is a replication mechanism adopted by several organisms as it is the simplest to initiate. RCR of bacterial plasmids is initiated by a sequence-specific nick in the dsDNA origin (Fig. 4A, marked with a red square), which generates a primer (3' DNA) for leading-strand initiation and a priming site for lagging-strand synthesis. The 3' DNA end is then extended by a DNA polymerase. Elongation of the leading strand occurs as the double helix is unwound and the non-template DNA strand is simultaneously displaced. Termination of a round of leading-strand replication occurs when it reaches the nick site, which is followed by cleaving of the new molecule and release of the non-template ssDNA. This single strand then serves as a template for synthesis of a lagging strand ((del Solar et al. 1998), reviewed in (Khan 2005; Ruiz-Maso et al. 2015)). A schematic representation of circular dsDNA RCR of a bacterial plasmid is provided in Fig. 4A.

RCR of bacteriophage λ has been shown to occur after bidirectional theta replication, and the resulting intermediates have concatemeric linear tails; thus, these RIs resemble the Greek letter σ (Skalka et al. 1972; Bastia and Sueoka 1975). Therefore, it is also termed both sigma and late replication. Regulation of the switch from theta to sigma replication of λ DNA remains unknown. Sigma type RCR replication produces concatemeric molecules that are several λ DNA units long (Taylor and Wegrzyn 1995). These long linear multi-genome length RCR sigma replication products are then cut at specific sites, and it is possible that the resulting dsDNA breaks (DSBs) become the entry sites for recombination (reviewed in (Szczepanska 2009)). A schematic representation of circular dsDNA RCR of bacteriophage λ is provided in Fig. 4B.

Bidirectional theta replication

Bidirectional theta replication is initiated at the origin of replication. Two replication forks assemble at the origin and progress in opposite directions until they meet opposite of the origin (termination of replication). In Fig. 4C, the origin is marked with a red square and termination is marked with a black square. After the replication forks have converged, the replicating molecule is separated into circular dsDNA daughter molecules. A schematic representation of bidirectional theta replication is provided in Fig. 4C.

Unidirectional replication

Unidirectional replication proposed for HPV may (Yang and Botchan 1990) or may not (Orav et al. 2015) have a specific initiation site. One replication fork progresses around the circular genome in a unidirectional manner until it reaches the initiation site, therefore, one of the two ends of the replication eye is stationary and the other moves with replication. A schematic representation of unidirectional replication is depicted in Fig. 4D.

Recombination dependent replication

Recombination dependent replication (RDR) has distinct special characteristics in different organisms. The initiation of RDR is similar to the initiation of HR, which leads to the synthesis of a minimal amount of DNA necessary for dsDNA break repair; however, RDR leads to the replication of extensive DNA sequences (Malkova and Ira 2013). In phage SSP1 with circular dsDNA genome, replication is initiated by the theta mode (circle-to-circle); but, after at least one round of replication, a switch to the σ type of RDR occurs in which the generated DSBs may trigger RDR at different locations (reviewed in (Flores et al. 1999)). RDR has been shown to be necessary to produce a high yield of DNA (Taylor and Wegrzyn 1995). Initiation by theta replication, followed by RDR, might be a characteristic replication mechanism in not only SPP1 and many other phages but also HSV-1 (Skalka 1977; Strang and Stow 2005; Shutt and Gray 2006; Szczepanska 2009). However, the exact mechanism determining when and how RDR is used to replicate viral DNA remains to be elucidated (reviewed in (Lo Piano et al. 2011)). A schematic representation of RDR is provided in Fig. 4E. RDR has many similar characteristics to break-induced replication (BIR), which is a pathway of HR. BIR contributes to the repair of broken replication forks and is initiated by invasion of a ssDNA into a homologous DNA molecule, resulting in the formation of a D-loop. This step is followed by extension of DNA synthesis.

Various replication mechanisms have been proposed to occur in different stages of the HPV life cycle, yet some are inconclusive and possibly even contrary. It has previously been shown that PVs use bidirectional replication during initial amplification and at least to some extent during the stable maintenance phase of viral DNA replication (Schvartzman et al. 1990; Flores and Lambert 1997). Data obtained from the second amplification phase is, however, inconclusive because it is suggested in (Auborn et al. 1994) that bidirectional replication is involved in the vegetative amplification replication of PV genomes, yet (Flores and Lambert 1997) proposes that RCR is the mechanism used in this phase. It has also been suggested that HPV is replicated in differentiated cells by RDR, which is supported by the induction and activation of HR-related DDR pathways (Sakakibara et al. 2013; Gautam and Moody 2016).

Our understanding from the experiments conducted in the U2OS cell-line with HPV18 genomes during the initial amplification phase confirm the occurrence of bidirectional theta replication. However, we cannot see the presence of RIs from rolling circle replication and our results suggest the involvement of another mode, possibly RDR, during the initial amplification phase (Orav et al. 2013; Orav et al. 2015). The key to understanding the mode of HPV DNA synthesis is understanding the structures of replicating molecules, and for this purpose, 2D AGE is one of the best methods of characterizing the RIs that are generated during the replication of HPV genomes.

DNA damage response

DNA damage lesions are a natural part of a cell's life, and their causes can be either endogenous or exogenous. Endogenous damage is caused by replication errors, unrepaired single-strand lesions, base deamination, or loss. Environmental lesions are caused by exposure to ultraviolet, infrared, reactive oxygen species, S-adenosyl methionine, dietary nitrosamines, and tobacco smoke. For a normal life cycle of a cell, cellular DNA must defy these internal and external agents, which can cause single- and/or double-strand DNA breaks, DNA mutations and crosslinking, formation of pyrimidine dimers, aromatic DNA adducts, and oxidative base and sugar products (reviewed in (Kastan and Bartek 2004; Jackson and Bartek 2009)). Proper DNA damage repair is necessary for genome stability, prevention of transformation, and tumor suppression. In response to DNA damage, cells mount finely coordinated responses and activate DNA damage repair pathways, cell cycle arrest, regulation of cellular gene expression and cellular DNA replication.

DNA breaks that have been induced in any way give rise to DDR signal cascades. The activation of DDR can result in cell cycle arrest; however, when DNA damage is massive, the cell triggers apoptosis. DDR is a network of proteins that regulate the preciseness of replication via detecting, signaling and repairing DNA lesions (Jackson and Bartek 2009). Ataxia-telangiectasia mutated kinase (ATM) and ATM- and Rad3-related kinase (ATR) are the main kinases that regulate DNA damage detection and repair via inhibiting cell cycle progression after damage (Matsuoka et al. 2007; Bensimon et al. 2010).

Different viruses use host cell DDR pathways to up- or down-regulate their DNA replication (EBV, SV40, and polyomavirus) (Zhao et al. 2008; Kudoh et al. 2009; Tsang et al. 2014). Similar to recruitment by these viruses, DDR components are also recruited to HPV DNA replication foci (Fradet-Turcotte et al. 2011; Sakakibara et al. 2011; Gillespie et al. 2012; Reinson et al. 2013). These viral replication compartments are very similar to cellular DNA damage nuclear foci. HPV can mimic DNA damage during viral genome replication and induce DDR pathways, which in turn enables the replication of viral DNA (Hong and Laimins 2013b; Wallace and Galloway 2014; Wallace and Galloway 2015). However, the exact mechanism by which the virus takes advantage of

these pathways remains unknown. It has been suggested that HPV E1 protein can induce DSB, activating the DDR pathway, which in turn contributes to DNA replication machinery ((Moody and Laimins 2009; Sakakibara et al. 2011; Reinson et al. 2013), reviewed in (McBride 2017)).

Double-stranded DNA breaks are the most cytotoxic and difficult lesions to repair. Two main mechanisms are used to fix DSBs in eukaryotes – HR repair and non-homologous end joining (NHEJ). It has been shown that HPV recruits proteins involved in HR repair (NsbI, Rad51, BRCA1, pRPA S33) to the viral replication centers; however, proteins involved in NHEJ are not recruited (Gillespie et al. 2012; Anacker et al. 2014).

Techniques to study HPV DNA replication

To analyze the RIs that result from HPV DNA replication, we use 2D and 3D AGE and visualize the results using the Southern blot method (Henno et al. 2017).

Agarose gel electrophoresis

After HPV genomes have replicated in the transient, stable, or vegetative replication phases, both the viral DNA products and RIs can be analyzed via AGE. DNA electrophoresis in agarose gel (AG) is an essential molecular biology technique used to separate DNA molecules of different sizes and shapes. DNA molecules move in the electric field from cathode to anode due to their negative charge, which is provided by the phosphate group in their sugar-phosphate backbone. The rate of movement is dependent on the agarose gel matrix and the percentage of AG; a tighter matrix results in slower DNA movement.

The voltage used in AGE is expressed in volts per centimeter (V/cm) (distance between the electrodes in the gel tank in cm); however, this voltage is not uniform because the rate of DNA movement is also dependent on the running buffer's content, volume and temperature.

To identify the replication mode of HPV genomes, we have conducted experiments mainly via 2D N/N and N/A and 3D N/N/A AGE. The text below provides an introductory overview of the conditions of these experiments and the resulting RIs patterns.

Two-dimensional AGE of DNA is used to detect and analyze DNA intermediates that arise through DNA replication and repair. Neutral/neutral (N/N) and neutral/alkaline (N/A) 2D AGE are two methods used to detect and analyze DNA intermediates of DNA replication. N/N 2D AGE maintains the double-stranded structures of DNA RIs throughout the experiment, whereas N/A resolves the dsDNA into its ssDNA components after the first dimension by applying denaturing conditions. By applying neutral/neutral/alkaline (N/N/A) 3D AGE, one can resolve the DNA duplex after running the second dimension of

N/N AGE and can then separate single strands in the third alkaline dimension. This method can be used to locate the parental and nascent strands.

To better understand the interpretation of HPV DNA replication signals, the following section provides examples of common RIs and their patterns in 2D N/N, N/A and 3D N/N/A AGE.

Neutral/Neutral 2D AGE conditions and patterns of replication intermediates

N/N 2D AGE enables determination of the replication initiation region and the direction of replication fork (RF) migration and differences in its speed, and it enables the detection of possible recombination events between two DNA molecules. In the first dimension, the DNA molecules are separated mostly by molecular weight (the complexity of the RI has little to no effect on its electrophoretic mobility). In the second dimension, the DNA molecules are separated mainly by shape (structural complexity). These specific separations are achieved by using a low agarose percentage and applying a low voltage for DNA separation in the first dimension; using a high agarose percentage, adding EtBr, and applying high voltage for DNA separation in the second dimension. EtBr intercalates DNA molecules and makes them more rigid, thereby slowing down the migration process in the gel in the electric field (Bell and Byers 1983; Brewer and Fangman 1988; Friedman and Brewer 1995).

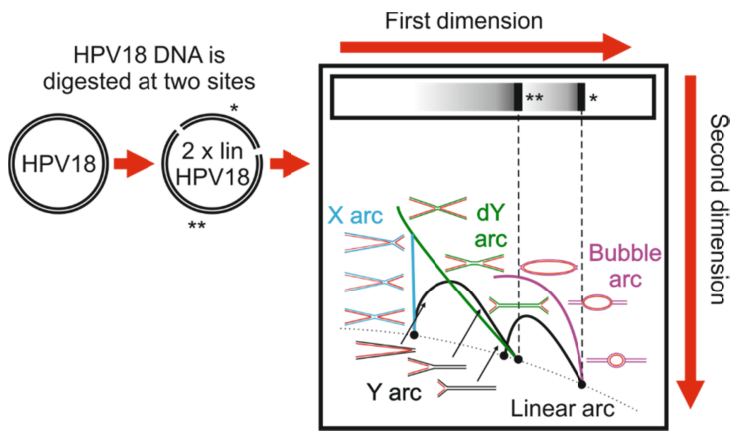

Figure 5. Schematic representation of the distribution of theta RIs in 2D N/N AGE. First, HPV DNA is digested at two sites; then, it is run on AGE to separate molecules with different sizes. Following this step, a gel slice containing separated HPV DNA is placed perpendicular to the first dimension, and the second dimension of 2D N/N AGE is conducted. The conditions of the second dimension enable the HPV RIs to be separated based on structural complexity. Figure adapted from (Pohjoismäki 2008).

A typical result from a 2D AGE experiment is schematically represented in Fig. 5. HPV DNA extracted from cells is digested and separated by size in the first dimension and by shape in the second dimension. As a result, arcs of RIs are visualized via the Southern blot method. The specific conditions used to conduct 2D N/N AGE are described in (Henno et al. 2017). The resulting RI patterns are discussed in (Henno et al. 2017) and in the next paragraphs.

If replicating molecules with bubble structures in the center of the fragment are run via 2D N/N AGE, they form a bending arc that represents RIs with different extents of replication (Fig. 6A, labelled RI, the positions of the RIs that contain bubble structures are indicated above each RI arc). The smallest RIs have a minor bubble and are therefore located near non-replicating linear molecules referred to as 1n (Fig. 6A, labelled 1n). As replication proceeds, the RI structures become more complexed, the bubble increases, and the movement in the second dimension becomes slower until the molecule almost reaches the size of fully replicated, but not yet separated, molecules. These molecules are referred to as 2n (Fig. 6A, bubble structures closest to 2n).

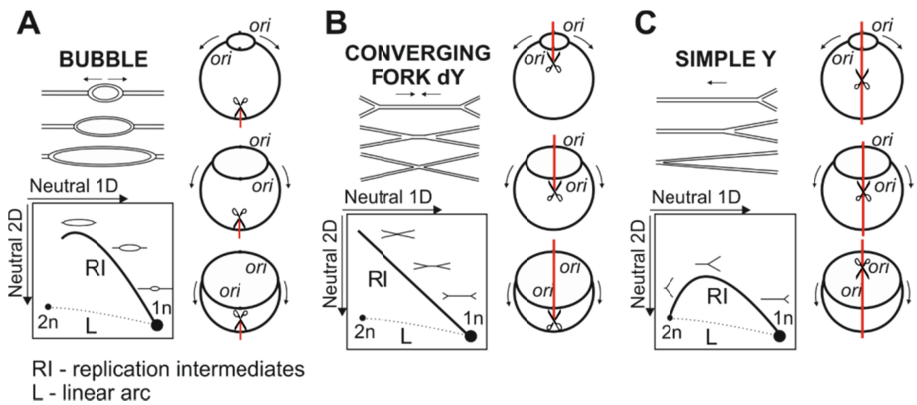

Figure 6. Schematic representation of the distribution of theta RIs in 2D N/N AGE. **A)** Distribution pattern of RIs containing bubble structures. **B)** Distribution pattern of RIs having converging fork structures. **C)** Distribution pattern of RIs containing simple Y structures. Figure adapted from (Henno et al. 2017).

Replicating molecules with converging fork structures form a straight arc that represents RIs that have different extents of replication (Fig. 6B, labelled RI, positions of RIs containing different dY structures are indicated above each RI arc). The smallest molecules are near 1n. As replication proceeds, the RI structures become more complexed, the forks at both ends of the molecule near each other, and movement in the second dimension slows down until the molecule almost reaches the size of a 2n fragment (Fig. 6B, dY structures closest to 2n).

Replicating molecules with simple Y structures form a bending arc from 1n to 2n (Fig. 6C, the labelled RI and the positions of RIs that contain different Y

structures are indicated above each RI arc). The smallest molecules with one minor fork structure are near the 1n point. As the replication proceeds, the fork becomes larger, the RI structures become more complexed, and the movement in the second dimension is slower. When all three arms of the molecule are almost equal length, the structural complexity is at its highest; however, when the replicating molecules approach the size of 2n, the complexity of the RI decreases and the arc starts to bend downwards (Fig. 6C, simple Y structures closest to 2n).

Neutral/Alkaline 2D AGE conditions and patterns of replication intermediates

Huberman and co-workers developed a different 2D technique that uses an alkaline second dimension (Huberman et al. 1987). The DNA molecules separated via a neutral first dimension are then soaked in alkaline to separate dsDNA strands, and the second dimension is carried out under alkaline conditions. These conditions resolve the dsDNA into its component single strands and separate nascent and parental strands while running the second dimension. Therefore, 2D N/A AGE separates DNA molecules by molecular weight in the first dimension and by strand composition in the second dimension. Because nascent strands are typically shorter than parental strands, they run faster in the second dimension, and thus, we can identify their structure based on their size. However, the nature of the nascent strand arcs also depends on the shape of nascent strands as molecular structure determines the electrophoretic mobility. 2D N/A AGE enables detection of not only the sites of termination and origin but also DNA structures that are not apparent under native conditions, such as single-strand nicks, partial duplexes, and hairpins (Schwacha and Kleckner 1994; Wahls et al. 2005; Lao et al. 2008; Zakharyevich et al. 2010). This technique can also determine the direction of RFs and their pause sites (Huberman 1997).

The detection of RFs and the regions of termination and origin can be achieved by using specific hybridization probes spread throughout the DNA under investigation because these probes enable measurement of the length of nascent strands. One rule of thumb is that the hybridization probe that detects the shortest nascent strands is closest to the origin. Therefore, the probe detecting only a small subset of nascent strands almost the size of parental strand is located farthest from origin (termination region).

If replicating molecules that contain bubble structures are run via 2D N/A AGE, their nascent strands form a straight arc that represents ssDNA molecules with different sizes (Fig. 7A, labelled N). Nascent strands that originate from bubble RIs vary in size depending on the extent of replication, and they range in size from very small strands (located farthest from parental strand arc) released from the smallest RIs to nearly full-size nascent strands (located close to parental strand arc) released from the largest RIs. This arc reaches the size of the parental strands near the 2n point (Fig. 7A, see the small structures near the bubble arc).

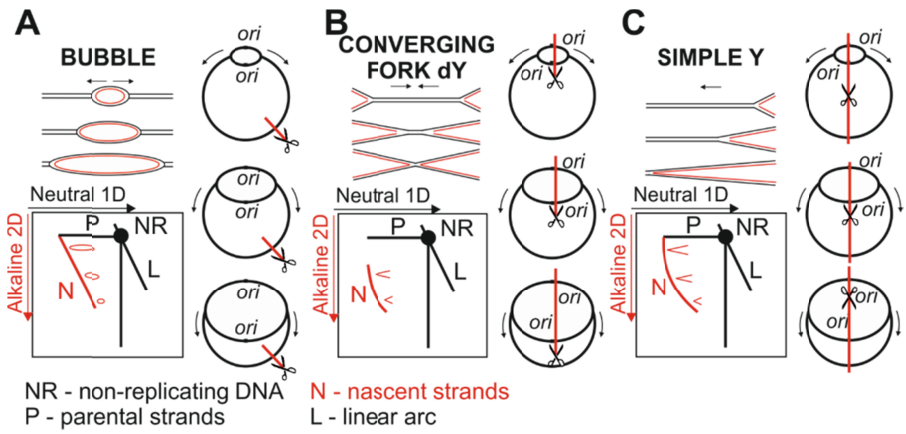

Figure 7. Schematic representation of the distribution of theta RIs in 2D N/A AGE. **A)** Distribution pattern of nascent strands released from RIs containing bubble structures. **B)** Distribution pattern of nascent strands released from RIs containing dYs. **C)** Distribution pattern of nascent strands released from RIs containing simple Ys. The full-length DNA under investigation is used for hybridization to achieve these patterns. Figure adapted from (Henno et al. 2017).

When replicating molecules with converging fork structures are run via 2D N/A AGE, their nascent strands form a bending arc that represents ssDNA molecules of different sizes (Fig. 7B, labelled N). Because the structure of dY molecules consist of two RFs, none of the nascent strands can be the same size as the parental strands. Therefore, when RFs of equal size converge, the resulting largest nascent strands are half the size of full-length parental strands and cannot reach the arc of the parental strands (Fig. 7B, compare the position of P and N arcs). Smallest nascent strands locate farthest from the parental strands and largest nascent strands locate closest to the parental strands (Fig. 7B, see small structures near the N arc).

Nascent strands released from replicating molecules with simple Y structures form a bending arc that represents ssDNA molecules of different sizes (Fig. 7C, labelled N). Smallest nascent strands locate farthest from the parental strands and largest nascent strands locate close to the parental strands (Fig. 7B, see small structures near the N arc).

Both 2D N/A and 2D N/N have their advantages and disadvantages. For example, less DNA is required to obtain a sufficient signal via 2D N/N because the parental strands are not separated from nascent strands, thereby increasing the local concentration. Additionally, a single restriction analysis can be obtained from a single hybridization via 2D N/N, whereas analysis of a single fragment using N/A electrophoresis requires at least two hybridizations (one probe for both ends of the fragment). However, 2D N/A provides information about the direction of each RF, whereas 2D N/N only provides that kind of information when utilizing in-gel restriction before applying the second dimen-

sion. In addition, the regions of origin and termination can be more accurately defined via 2D N/A because the sizes of nascent strands can be more accurately compared to parental strands. A good strategy to analyze unknown RIs is to use both 2D N/N and 2D N/A because they compensate for one another by discriminating between replication intermediates with identical masses, but subtle structural differences. Furthermore, to confirm the results obtained via 2D N/N and 2D N/A assays, we also conduct 3D N/N/A AGE.

Neutral/Neutral/Alkaline 3D AGE conditions and patterns of replication intermediates

The technique of applying 3D N/N/A AGE was introduced by (Liang and Gerbi 1994). This method enables the separation of DNA fragments by size in the first dimension and by shape in the second dimension and finally resolves the intermediates into parental and nascent strands in the alkaline third dimension. The first two dimensions are carried out in the same manner as 2D N/N AGE. The gel is then soaked in alkaline to separate dsDNA strands, and the third dimension is carried out in an alkaline electrophoresis buffer in the same direction as the first dimension or the second dimension. One possibility is to soak the entire AG obtained from the second dimension in alkali and then run the gel under alkaline conditions (Lucas and Hyrien 2000; Orav et al. 2015; Henno et al. 2017); another possibility is to cut out narrow gel slices perpendicular to the first dimension and then conduct the assay in the same manner as the second dimension of 2D N/A AGE (Liang and Gerbi 1994; Kalejta and Hamlin 1996).

RIs separated in the second dimension form arcs of common structures, such as forks and bubbles that are resolved into parental and nascent strands in alkaline conditions prior to the third dimension. The third dimension of 3D N/N/A separates the nascent and parental strands. Because parental strands have retained their size, they migrate the same distance and therefore maintain approximately the same shape of the original dsDNA arcs as after the second neutral dimension. However, nascent strands form unique arcs due to their faster migration because of their smaller size (Orav et al. 2015; Henno et al. 2017).

The technique of N/N/A 3D gel electrophoresis enables determination of the size of forks and bubble intermediates and detects ssDNA in joint DNA molecules (Kalejta and Hamlin 1996; Lucas and Hyrien 2000). Because the migration of RIs in the first dimension is also influenced by molecular shape, RIs might not run at the same rate as linear molecules of the same mass even under conditions of low AG percentage and low voltage (Liang and Gerbi 1994). 3D N/N/A assays are able to solve this problem by separating forks and bubbles into single strands and resolving them solely by size in the third dimension. Additionally, in (Kalejta and Hamlin 1996), the researchers were able to detect broken bubbles using 3D AGE technology.

When replicating molecules with bubble structures are run via 2D N/N AGE and their nascent strands are separated from parental strands during an alkaline third dimension, these nascent strands form a bending arc that represents ssDNA molecules of different sizes (Fig. 8A, labelled N). Nascent strands that originate from bubble RIs vary in size depending on the extent of replication, and they range in size from very small strands (located farthest from parental strand arc) released from the smallest RIs to nearly the size of parental strands (located close to parental strand arc) released from the largest RIs (Fig. 8A, see the small structures near the bubble arc).

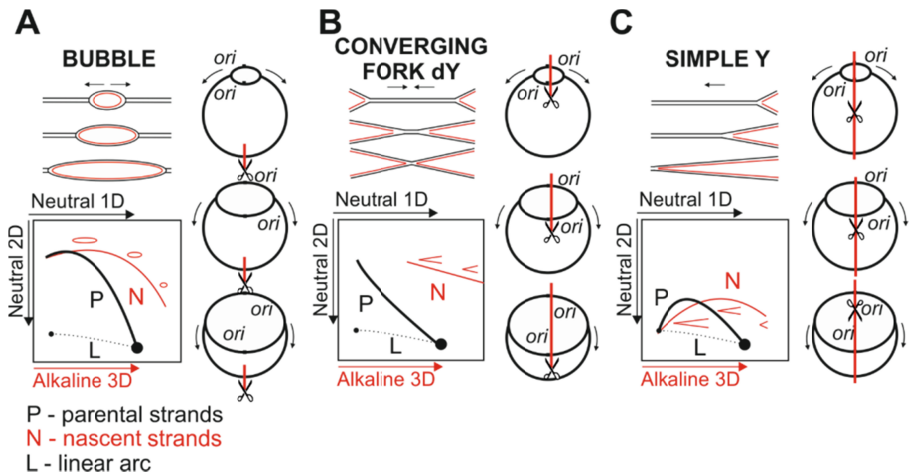

Figure 8. Schematic representation of the distribution of theta RIs in 3D N/N/A AGE. **A)** Distribution pattern of nascent strands released from RIs containing bubble structures. **B)** Distribution pattern of nascent strands released from RIs containing dYs. **C)** Distribution pattern of nascent strands released from RIs containing simple Ys. The full-length DNA under investigation is used for hybridization to achieve these patterns. Figure adapted from (Henno et al. 2017).

Nascent strands released during an alkaline third dimension from the arc of RIs with converging fork structures form a bending arc that represents ssDNA molecules of different sizes (Fig. 8B, labelled N). Because the structure of dY molecules consists of two RFs, no nascent strands can be the same size as their parental strands. Therefore, when RFs of equal sizes converge, the resulting largest nascent strands are half the size of the full-length parental strands and cannot reach the arc of the parental strands (Fig. 8B, compare the position of P and N arcs). Smallest nascent strands locate farthest from the parental strands and largest nascent strands locate closest to the parental strands (Fig. 8B, see small structures near the N arc).

Nascent strands released from the arc of replicating molecules with simple Y structures obtained from 2D N/N AGE form a bending arc that represents

ssDNA molecules of different sizes in the alkaline third dimension (Fig. 8C, labelled N). Smallest nascent strands locate farthest from the parental strands and largest nascent strands locate close to the parental strands (Fig. 8B, see small structures near the N arc).

OBJECTIVES OF THIS STUDY

HPV DNA replication mechanisms have been extensively studied; however, the results are often inconsistent. Because our group has previously developed a model system to study HPV genome replication (Geimanen et al. 2011), we conducted replication assays using different HPV types and inspected their RIs and replication mechanisms mainly via 2D AGE.

The general aim of this study was to analyze the replication mechanisms of HPVs by examining the RIs produced within U2OS cells using 1D, 2D, and 3D AGE methods. Specifically, this study focused on the following:

- Examining the oligomeric state of various HPVs within U2OS cells and in clinical samples obtained from patient tissues.
- Characterizing the necessary viral factors for HPV oligomer generation.
- Providing an overview of the important aspects to consider while interpreting the results obtained via N/N, N/A 2D and N/N/A 3D AGE.
- Characterizing HPV DNA replication intermediates using 2D and 3D methods in U2OS cells during the first amplification phase.
- Identifying possible replication mechanisms that could govern HPV18 genome replication in U2OS cells during the first amplification phase using the results obtained while characterizing the RIs.

MATERIALS AND METHODS

All experiments were conducted during the first amplification phase of HPV genome replication within a U2OS cell line based model system (Geimanen et al. 2011). The U2OS cell line (originally 2T) was derived from a 15-year-old girl's moderately differentiated osteosarcoma (Ponten and Saksela 1967). U2OS cells are HPV negative; however, they express pRB and p53 proteins and have a morphology similar to keratinocytes. These cells support HPV DNA replication very efficiently and can mimic the initial amplification of HPVs, the stable maintenance phase, and the second amplification phase of the HPV replication cycle. These cells, however, cannot support the productive phase of the HPV life cycle.

To improve the efficiency of conducting experiments with HPV genomes, we utilized the minicircle manufacturing system developed in (Kay et al. 2010). The minicircle DNA vector consists of a circular expression cassette devoid of the bacterial plasmid DNA backbone. As a result, minicircles mimic the natural physical state of HPV genomes after they enter the host cells. Exclusion of the bacterial vector and circularizing of the HPV plasmids prior to transfection reduces the initiation time, and thus, minicircle HPV DNA starts to replicate much faster in the U2OS cell line compared to HPV DNA that is provided as linear or loose circles (Ref. **I**). The precise protocol of the production of minicircles is provided in Ref. **III**.

In addition to using an efficient model system and HPV DNA production, we also utilized an HPV18 E8 mutant. This plasmid begins to replicate much more efficiently compared to HPV18wt in the U2OS cell line (Kurg et al. 2010; Reinson et al. 2013), which results in the same pattern with stronger signals and thus better visualization of HPV DNA RIs in 2D and 3D assays.

Most of the experiments are conducted with low molecular weight (LMW) DNA extracted from U2OS cells approximately 3–5 days after transfection with HPV genomes. We modified the common Hirt extraction protocol to preserve the fragile nature of DNA replication structures. The precise protocol of the LMW DNA extraction from U2OS cells is provided in Ref. **III**.

We analyzed HPV RI via 1D, 2D, and 3D AGE. Because multidimensional AGE experiments are technically challenging to carry out, Ref. **III** provides a thorough definition of the protocols used to conduct and analyze the outcome of these assays.

RESULTS AND DISCUSSION

The research group of human papillomavirus DNA replication within the Institute of Technology at Tartu University has developed an effective cellular model system to study the various genome replication methods carried out by different HPVs. The system is based on transfecting HPV genomes into human osteosarcoma cell line U2OS, where they are able to replicate (Geimanen et al. 2011). To mimic the circular covalently closed forms of HPV genomes in real life, we adopted minicircle technology (Kay et al. 2010), which makes using our model system even easier.

We use this novel model system to study the generation of oligomers of the viral genomes during the initial phase of replication. This short establishment phase of HPV genomes (initial amplification) can be mimicked by transfecting the U2OS cells with HPV genomes and extracting the replicated LMW HPV DNA several days later. We have identified that after transfection of the HPV molecules into U2OS cells, the viral DNA replicates and produces concatemeric genomic oligomers. The oligomer generation of viral genomes in host cells is a novel subject of research and not much is known about this phenomenon (Geimanen et al. 2011; Orav et al. 2013). It is not known how extensive the presence of oligomers is in nature, even though they were demonstrated in biopsies from cervical carcinomas over 30 years ago (Durst et al. 1985).

Using 2D N/N and N/A AGE, we conducted several assays to describe RIs of the HPV18 genomes during a transient DNA replication period.

Replication and oligomer generation of HPV genomes in U2OS cells (I)

After transfecting HPV genomes into U2OS cells and extracting LMW DNA at different time points, we observed that viral DNA replication yields oligomeric molecules that tended to become more prevalent over time (Ref. **I**, Fig. 1C). We showed that these oligomers are concatemeric circular head-to-tail tandems (Ref. **I**, Fig. 2B). The head-to-tail orientation was confirmed by partial digestion; digestion with a little concentration of enzyme yielded large linearized oligomers that cannot be obtained from catenated interlocking rings, which can also form oligomers (because partial digestion would only yield linear monomers and not higher oligomers) (Ref. **I**, Fig. 2B, see lanes 2–6 and 10–12). As the concentration of enzyme increased, these large molecules disappeared due to complete digestion.

Topology of input DNA

We have also shown that oligomers are generated independently of the topological form of the input DNA. This means that whether we transfect cells

with linear, open circle (oc) or covalently closed circular (ccc) HPV genomes, the outcome is the same: oligomers appear and become more prevalent over time (Ref. **I**, Fig. 1A and 1B). Only the onset of replication is dependent on the physical state (linear, oc or ccc) of input DNA, as transfection with linearized or ccc HPV18 E8 mutant genomes provides signals with different intensities (Ref. **I**, Fig. 1A, compare lanes 1–7 to 8–15 and 16–23).

To exclude the possibility that oligomer generation only occurs in U2OS cell lines, we have shown that this phenomenon also occurs in other cell lines, such as SiHa (Ref. **I**, Fig. 7A, lanes 1–3; Fig. 7C, lanes 1–9), HeLa (Ref. **I**, Fig. 7B, lanes 5–7; Fig. 7C, lanes 10–18), and C-33 (Ref. **I**, Fig. 7D, lanes 7–9; Fig. 7E, lanes 1–3 and 7–9), in addition to previous indications in other cell lines, such as primary human keratinocytes (Bodily et al. 2011), W12 (Alazawi et al. 2002), and CIN612 (Hong and Laimins 2013a). We also confirmed the presence of oligomers in HPV-infected patient tissue samples (Ref. **I**, Fig. 8A, lanes 17–19, Fig. 8B, lanes 13–15) in addition to previous indications of oligomers in clinical samples (Durst et al. 1985; Cullen et al. 1991; Kristiansen et al. 1994; Adachi et al. 1996). Therefore, we can conclude that the oligomeric state is part of the HPV life cycle.

Role of viral proteins in the process of formation of oligomers

We examined the role of HPV proteins during oligomer creation by generating HPV18 mutant genomes that lack one or more early open reading frames. We excluded the creation of L1 and L2 mutants from this experiment because as Ref. **I** Fig. 5B (lanes 1–4) indicates, an HPV18 sub-genomic construct without L1 and L2 ORFs is able to produce oligomers. Therefore, we only induced mutations that eliminate the expression of E1, E2, E6, E7, E8, E4, E1^E4, and E5 (Ref. **I**, Fig. 3).

E1 and E2 mutants were not able to replicate, as predicted, and thus were not able to generate oligomers (Ref. **I**, Fig. 3, lanes 10–12 and 16–18).

E6 and E7 mutants retained the capability of generating oligomers (Ref. **I**, Fig. 3, lanes 4–9), whereas an E7 mutant even produces a higher signal of replication and more intense oligomer formation compared with HPV18wt (Ref. **I**, Fig. 3, compare lanes 1–3 to lanes 7–9). The E8 mutant, as previously described, replicated much more efficiently than wt, leading to more efficient oligomer generation (Ref. **I**, Fig. 3, lanes 13–15). Mutations to E4 (Ref. **I**, Fig. 3, lanes 19–21), E1^E4 (Ref. **I**, Fig. 3, lanes 22–24), and E5 (Ref. **I**, Fig. 3, lanes 25–27) did not alter the pattern of oligomerization during viral DNA replication. Thus, we can conclude that although the replication efficiency and consequently the oligomer generation efficiency fluctuated, the pattern of oligomeric molecules remained unchanged.

In conclusion, the results indicate that oligomers are generated in all (E6, E7, E8, E4, E1^E4, and E5) mutants, except for E1 and E2 mutants, which are not able to replicate (Ref. **I**, Fig. 3, lanes 10–12 and 16–18). We can conclude that

no viral proteins, except for E1 and E2, are necessary for oligomer generation when replicating HPV18 genomes. Additionally, we can conclude that oligomer formation is a replication dependent process.

HPV18 URR region is capable of E1 and E2 dependent replication and oligomerization

To also investigate the potential overlap of replication and the formation of oligomers, we conducted an assay with an HPV18 URR construct that was generated from a BamHI-BamHI fragment obtained from the full-length HPV18 genome (Fig. 9). The URR construct contains the origin of HPV replication; however, replication can be achieved by co-transfecting it with the full HPV18 genome (schematically shown in Fig. 10A) or with E1 and E2 expression plasmids (schematically shown in Fig. 10B) as a source of viral replication proteins.

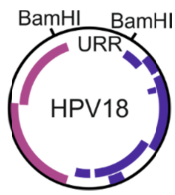

Figure 9. Schematic representation of HPV18 genome. Digesting the HPV18 genome with BamHI enzyme yields an approximately 1.1 kbp long URR construct.

To analyze the ability to both replicate and produce oligomers, U2OS cells were transfected with HPV18 URR minicircle DNA together with E1 and E2 expression vectors (Ref. **I**, Fig. 4B, lanes 5–10) or with a full-length HPV18 genome (Ref. **I**, Fig. 4B, lanes 1–4). However, when we co-transfected HPV18wt and URR genomes to characterize their replication patterns, we utilized a restriction enzyme that linearizes full-length HPV18 but leaves HPV18 URR undigested (schematically shown in Fig. 11A). As a result of these co-transfections, the URR region began to replicate and oligomers formed as a series of discrete bands. We suggest that the difference between the signal strengths of replication is due to differences in the amount of E1 and E2 proteins expressed. The levels of E1 and E2 expressed in these systems is much higher than those produced by the wt genome (Ref. **I**, Fig. 4B, compare lanes 1–2 to lanes 5–6). We observe that the pattern of oligomeric molecules that originate from URR regions is the same when using either co-transfection with E1 and E2 expression vectors (Ref. **I**, Fig. 4B, lanes 5–10) or full HPV18 genomes (Ref. **I**, Fig. 4B, lanes 1–4).

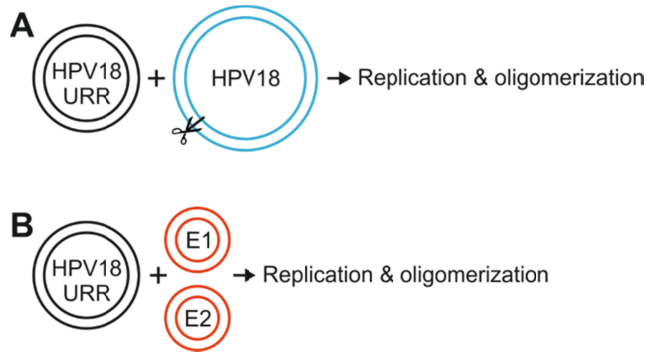

Figure 10. Schematic representation of the two possibilities for HPV18 URR replication. **A)** Co-transfection of HPV18 URR and HPV18wt results in replication and oligomerization of both constructs. **B)** Co-transfection of HPV18 URR and E1 and E2 expression plasmids results in replication and oligomerization of the HPV URR construct.

Taken together, we can conclude that the efficiency of oligomerization depends on the efficiency of replication (Ref. **I**, Fig. 4B, lanes 1–6) and that the pattern of oligomer formation remains unchanged, independently of the source or expression levels of viral replication proteins E1 and E2.

To further confirm the existence of oligomers, we conducted 2D gel electrophoresis (Ref. **I**, Fig. 4C and 4E). It is clear that arcs on linear, covalently closed circular (marked as 1xccc, 2xccc, etc.) and open circular DNA forms (marked as 1xoc, 2xoc, etc.) were present, both in the case of transfecting HPV18 URR with E1 and E2 expression vectors (Ref. **I**, Fig. 4C) or with HPV18 full genome (Ref. **I**, Fig. 4E). These arcs represent monomeric, dimeric and higher oligomeric molecules of HPV18 URR. In conclusion, we have demonstrated that the phenomenon of oligomerization can occur in a region even as small as the HPV origin.

In addition, the work presented in my Master's thesis (Henno 2013) showed that replication and oligomer generation can occur in much smaller regions of HPV18 URR when co-transfected together with E1 and E2 expression vectors. However, co-transfection of regions, excluding the origin of replication, with full-length HPV18 or E1 and E2 expression vectors did not yield replication or oligomerization (data not shown). In summary, oligomer generation is strictly dependent on the presence of the origin of replication, and because no open reading frame expression occurs with the URR construct, we can distinguish replication and oligomerization from transcription.

Mechanism(s) behind oligomer generation

To clarify the mechanism behind the generation of oligomers, we conducted separate assays with the full-length HPV18 genome, its sub-genomic fragments, and HPV11 E1 and E2 mutant genomes.

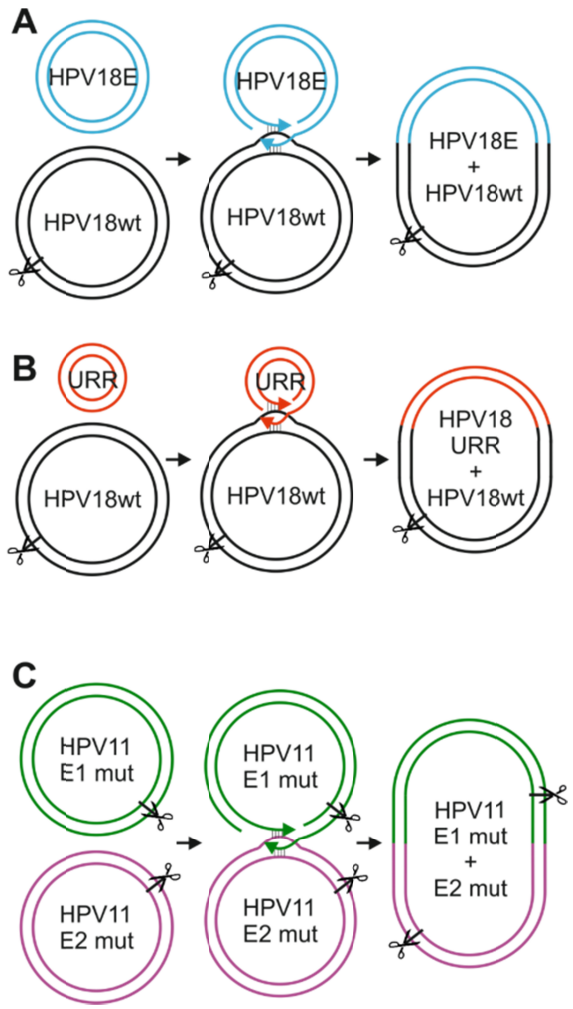

Figure 11. Schematic representation of the generation of hetero-oligomers. Scissors indicate the restriction sites in HPV DNA in the current experiment. **A)** Co-transfection of HPV18wt and HPV18E yields hetero-oligomers that can be seen using an enzyme that has a recognition site only in HPV18wt. **B)** Co-transfection of HPV18wt and HPV18 URR yields hetero-oligomers that can be seen using an enzyme that has a recognition site only in HPV18wt. **C)** Co-transfection of a HPV11 E1 mutant and a HPV11 E2 mutant yields hetero-oligomers that give specific digestion patterns in the AGE, where one restriction site has been mutated in both of the mutants.

First, we used the full-length HPV18 genome and its sub-genomic construct HPV18E, which lacks the late ORFs of HPV18 but has the ability to replicate and oligomerize. When HPV18 and HPV18E were transfected into the U2OS cell line and the resulting DNA was further analyzed, we observed a pattern of RIs and oligomers from both genomes (Ref. **I**, Fig. 5B, lanes 1–4, 13–16). In follow-up experiments, we separated the RIs of HPV18 from RIs of HPV18E using a restriction enzyme that linearizes full-length HPV18 but leaves HPV18E undigested (schematically shown in Fig. 11A). As a result, we obtained bands of linearized HPV18 (Ref. **I**, Fig. 5B, lanes 5–8, arrowhead indicating to linear fragment with a size of 8 kbp referred to as 1 lin) and monomers, dimers and higher oligomers of HPV18E. However, we also obtained linear fragments with the lengths of one full-length and one HPV18E genome (schematically shown in Fig. 11A) (Ref. **I**, Fig. 5B, lanes 5–8, arrow pointing to 13.4 kbp fragment). This result means that they have somehow formed joint molecules: hetero-oligomers (Ref. **I**, Fig. 5B, compare lanes 1–4 and 9–12 to lanes 5–8). In addition, we observed an 18.8 kbp joint molecule consisting of two HPV18E genomes and one HPV18 genome and a 24.2 kbp joint molecule consisting of three HPV18E genomes and one HPV18 genome (Ref. **I**, Fig. 5B, arrows point to hetero-oligomers).

Next, to identify possible joint molecules of HPV18 URR and full-length HPV18, URR was transfected into U2OS cells together with HPV18. The extracted HPV DNA was digested with an enzyme that linearizes full-length HPV18 but leaves HPV18 URR undigested (schematically shown in Fig. 11B). As a result, we obtained bands of linearized HPV18 (Ref. **I**, Fig. 4B, lanes 1–4, arrowhead indicating an 8 kb linear fragment) and oligomers of HPV18 URR (Ref. **I**, Fig. 4B, lanes 1–4, area marked as oligomers). To better visualize the HPV18 URR oligomers, we conducted 2D N/N AGE (Ref. **I**, Fig. 4E, long expo) where we could observe joint molecules in a short exposure of a film (Ref. **I**, Fig. 4E, short expo, arrows indicate hetero-oligomers). These oligomers represent molecules that have one HPV18 genome and one URR genome (9 kbp) (schematically shown in Fig. 11B), one HPV18 genome and two URR genomes (10 kbp), and one HPV18 genome and three URR genomes (11 kbp), among others. These experiments, once again, demonstrate the presence of hetero-oligomers. A schematic representation of one possible way to create hetero-oligomers is illustrated in Fig. 12.

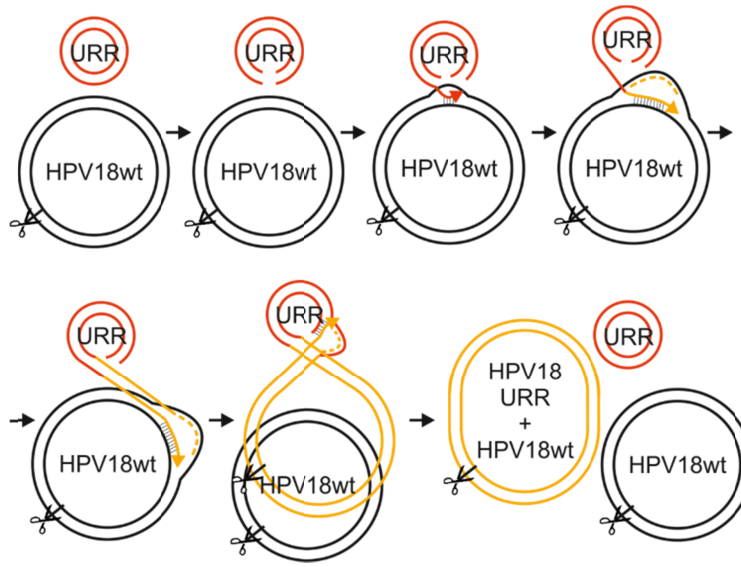

Figure 12. Schematic representation of one possible way of generating hetero-oligomers between HPV18 URR and full-length HPV18. Scissors indicate the restriction sites in HPV DNA in the current experiment and yellow indicates newly synthesized DNA. Co-transfection of HPV18wt and HPV18 URR yields hetero-oligomers that can be seen using an enzyme that has a recognition site only in HPV18wt.

Finally, we performed an additional experiment where we co-transfected two HPV11 E1 and E2 mutants not capable of replicating alone. Full-length HPV11wt has PaeI and SdaI restriction sites, the E1 mutant we used lacked PaeI and the E2 mutant lacked SdaI (schematically shown in Fig. 11C) (Ref. **I**, Fig. 6, lower panel showing restriction sites of monomers). Co-transfection of HPV11 E1 and E2 mutant genomes resulted in replication; therefore, they must have complemented each other's mutations (Ref. **I**, Fig. 6, upper panel, lanes 13–16, band of 8 kbp). In addition, oligomers of HPV11 E1 and E2 mutant genomes were generated (Ref. **I**, Fig. 6, upper panel, lanes 5–8). Oligomers consisting of only HPV11 E1 mutant genomes would have only SdaI restriction site, and oligomers consisting of only HPV11 E2 mutant genomes would have only PaeI restriction site. As a result, digestion with SdaI and PaeI yielded 8 kbp linearized monomers. However, when digesting the HPV DNA obtained from co-transfection of HPV11 mutants with SdaI and PaeI restriction enzymes, we also obtained linearized fragments with sizes of 6 kbp and 10 kbp (Ref. **I**, Fig. 6, upper panel, lanes 13–16, arrowheads indicating 6 kbp and 10 kbp). These fragments can be obtained from linearizing an oligomer consisting of both the HPV11 E1 and E2 mutant genomes, a hetero-oligomer that has one PaeI and one SdaI digestion site (schematically shown in Fig. 11C).

Several mechanisms are associated with replication and oligomer formation. Oligomers generated through RCR use a single molecule as a template to create

only homo-oligomers. However, creation of hetero-oligomers is possible only through HR-dependent replication, which occurs between two separate molecules that can be partially homologous or fully homologous with one acting as the donor and one as a template. As mentioned in the literature review above, papillomaviruses are very dependent on host factors; therefore, it is likely that HPVs utilize factors involved in host DDR and/or HR pathways to replicate and generate oligomers (reviewed in (Sakakibara et al. 2013)). In the process of HR, two different molecules that have a homologous sequence form a linkage followed by strand exchange, which results in a new combination of DNA strands. The end products of HR may contain crossover structures referred to as Holliday junctions (HJ) (Li and Heyer 2008), which might be resolved in a way that leads to the generation of oligomeric molecules. However, RDR can synthesize large amounts of concatemeric DNA that must later be resolved into unit-sized viral genomes (reviewed in (Lo Piano et al. 2011)). This process makes it probable that cellular repair factors involved in RDR can resolve HPV oligomeric structures into monomeric, circular genomes. The exact mechanisms of oligomer generation and how they are resolved must still be elucidated.

In conclusion, these experiments indicate that at least HPV genomic hetero-oligomers can arise through the homologous recombination step of replicating viral genomes. We have not yet identified which repair machinery related to viral DNA synthesis is involved; however, some have been predicted based on an analogy to some BIR-related mechanisms with unidirectionally moving RF (Malkova and Ira 2013). We suggest that HR-directed and repair-related replication mechanisms could also be responsible for the formation of standard HPV genomic homo-oligomers (oligomers consisting of identical genomes); however, the RCR mechanism cannot be excluded.

The reason why the oligomeric state has been taken into use by the virus can be explained by the necessity to keep a low profile in the host organism. BPV1 oligomers have been shown to have only one active origin (Schvartzman et al. 1990) that can replicate an entire oligomer consisting of many monomers using as many host and viral replication proteins as a monomer would. Therefore, much more viral DNA can be replicated while eluding the host adaptive immune system. This mechanism is also supported by the fact that viral genomes exist mainly as episomal oligomers in HPV-positive U2OS sub-clones that were designed by our research group (Geimanen et al. 2011).

Analysis of replication intermediates and the mechanism(s) behind HPV DNA replication (II and III)

We stated above that performing 2D gels is technically challenging and that interpretation of the results is even more difficult. Interpretation is based on a comparison of unknown RIs patterns with known, well-defined replication intermediates. Both Ref. **II** and **III** address the characterization of HPV18 RIs. Ref. **II** concentrates mainly on the mechanism behind replication and **III** in-

cludes an overview of the experimental procedures and an understanding of RI patterns obtained from 2D and 3D AGE.

Analysis of undigested HPV DNA via 2D N/N AGE

2D N/N AGE separates undigested replicating HPV genomes into patterns (specific arcs) of open circle, covalently closed circles, and linear HPV fragments (Ref. **II**, Fig. 1A, marked as oc, ccc and lin, respectively). These different topological forms of DNA were separated from each other due to their different mobility in AGE.

First, two days after transfection of HPV18 into U2OS cells, we observed only linear, oc and ccc monomeric molecules; however, at later time points, oligomers accumulated (Ref. **II**, Fig. 1A 2–9 days). To confirm the physical states of these RI signals, HPV genomes were digested with an enzyme that nicked the ccc molecules into oc structures so that we could identify their positions on 2D gels (Ref. **II**, Fig. 1B).

To obtain stronger signals of these RIs, we used an HPV18 E8 mutant that replicates more efficiently than HPV18wt (Kurg et al. 2010; Reinson et al. 2013). In addition to the arcs formed from different forms of HPV DNA molecules, we also observed arcs of replicating molecules that showed an increase in molecular weight in addition to changes in topological complexity (Ref. **II**, Fig. 1C, white bold arrow, black bold and narrow arrow; Ref. **III**, Fig. 14B.10.6B, black bold arrow). The scheme depicted in Ref. **II**, Fig. 1D indicates the locations of the arc of RCR and bidirectional theta replication (Belanger et al. 1996; Martin-Parras et al. 1998). In our analysis, we can clearly see the arc of bidirectional theta RIs originating from 1 ccc molecules (Ref. **II**, Fig. 1C, black bold arrow; Ref. **III**, Fig. 14B.10.6B, black bold arrow). The arc emanating from 2 ccc/1 oc has similar characteristics, but its origin is unclear (Ref. **II**, Fig. 1C, white bold arrow). Additionally, the diffuse arc between 1 ccc and 2 ccc has an unidentified origin (Ref. **II**, Fig. 1C, black narrow arrow). In addition, we observe that the arc of RCR intermediates is not present (Ref. **II**, compare Fig. 1C to Fig. 1D, marked as σRIs). To better visualize these RIs, we used restriction enzyme analysis to study linearized full-length HPV and linearized subgenomic fragments of the HPV genome via 2D AGE assays.

Analysis of single-digested HPV DNA via 2D N/N AGE

It has previously been stated that HPVs replicate using a bidirectional theta replication mechanism (Yang and Botchan 1990; Auborn et al. 1994). Bidirectional replication has been thoroughly described, including the resulting intermediates (Friedman and Brewer 1995). Patterns of common theta RIs, such as an arc of simple Ys, converging forks (dY), and bubble molecules, are schematically presented in Fig. 6 and Ref. **II**, Fig. 2B. If we linearize the HPV

genome near the origin of replication, we get an arc of converging fork molecules (Ref. **II**, Fig. 2B panel of dY intermediates). However, when we digest the HPV DNA opposite of the origin, it yields an arc of bubble intermediates (Ref. **II**, Fig. 2B, panel of replication bubble intermediates). Simple Ys originating from a bidirectional replication mechanism can be obtained if the HPV genome has been digested at two adjacent regions, whereas one digestion site is in the region of the origin (Ref. **II**, Fig. 2B panel of simple Y intermediates).

When analyzing the replication of HPV18 digested at the site of origin, we can see the common theta replication intermediates – dYs (Ref. **II**, Fig. 2A, black bold arrow). However, our assay also indicates the presence of yet another signal two days after transfecting the HPV18wt genome into U2OS cells (Ref. **II**, Fig. 2A, white bold arrow). These molecules must represent some sort of RIs because they show an increase in molecular weight coupled with structural complexity and they seem to accumulate as time passes (Ref. **II**, Fig. 2A, compare 2 days, 3 days and 5 days). We propose that these intermediates are generated through some other type of replication mechanism (and not the more commonly known bidirectional theta replication). Perhaps it is a homologous recombination directed and repair-related replication mode as seen during the generation of hetero-oligomers. The quite universal, but not clearly identified, RI patterns are present in every replication assay of once linearized HPV18 DNA. In this text, we refer to it as the novel replication mechanism. In addition to common theta structures and intermediates of the novel mechanism, we can also see the presence of large branched structures that migrate at the end of the signal of theta replication structures (Ref. **II**, Fig. 2A, black arrowhead). We propose that these signals represent late theta intermediates, where the replication forks have stalled before separation of the replicated molecule into the daughter genomes. This kind of accumulation of theta intermediates has also been noted in SV40 replication (Tapper and DePamphilis 1978; Seidman and Salzman 1979; Tapper and DePamphilis 1980).

To further confirm the presence of this novel replication mechanism, we conducted 2D assays using the HPV18 E8 mutant through which we obtained stronger signals of replication and therefore, clearer images. HPV18 E8 mutant DNA was extracted from U2OS cells three days post transfection. The theta replication mechanism gives rise to the familiar arcs of dY (Ref. **II**, Fig. 3A, BglI and Bpu1102I, black bold arrow; Ref. **III**, Fig. 14B.10.7C, BglI, black bold arrow) or bubble intermediates (Ref. **II**, Fig. 3A, XmaJI and PsyI, black bold arrow; Ref. **III**, Fig. 14B.10.7D, PsyI, black bold arrow) when digesting the HPV DNA near the origin or the termination regions, respectively. The bubble arc, however, shifts into an upward tilted diffuse signal when RIs occur near the 2n point because some molecules are transitioned into dY structures due to the choice of enzymes (BglI and Bpu1102I), which do not digest exactly at the origin of replication (Ref. **III**, Fig. 14B.10.7D and 14B.10.7G). The diffuseness of the signal could also be explained by the different speed of the replication forks. All images contain common arcs and structures (originating from a novel mechanism) that do not change when using different restriction enzymes (Ref.

II, Fig. 3A, white bold arrow; Ref. **III**, Fig. 14B.10.7C-D, white bold arrow), which were also present in an HPV18wt assay we conducted (Ref. **II**, Fig. 2A, white bold arrow). This kind of unchangeable pattern independent of the restriction sites indicates that the novel mechanism does not seem to have a certain origin for HPV18 DNA replication.

Analysis of single-digested HPV DNA via 2D N/A AGE

To better understand the structural complexity of novel RIs, we conducted a 2D N/A AGE assay (Ref. **II**, Fig. 4; Ref. **III**, Fig. 14B.10.8). This 2D AGE method applies a neutral gel electrophoresis separation step followed by another in the alkaline dimension. This divides the parental and nascent strands of molecules undergoing replication. As a result, the pattern of linearized RIs is entirely different from that obtained using N/N AGE conditions. The 2D N/A method helps to determine the existence of bi- and/or unidirectional forks and to detect nascent ssDNA originating from RIs with bubble, Y, and dY structures. Ref. **II**, Fig. 4A indicates the resulting patterns of single strands that originate from simple Y, bubble, and dY intermediates. A detailed explanation is provided in the literature overview section of this dissertation (Fig. 7).

To better understand the HPV18 RIs pattern obtained through 2D N/A, we conducted 2D N/N to locate the positions of previously described and well-identified intermediates. The conditions for running the first dimension were the same; thus, we can draw lines to see where intermediates migrated in first dimension (Ref. **II**, Fig. 4B and 4C, compare upper and lower panel). When digesting HPV18 genomes near the origin (BglI), we obtained dYs (Ref. **II**, Fig. 4B, black bold arrow) as previously described, whereas digesting opposite of the origin (XmaJI) yielded bubble intermediates (Ref. **II**, Fig. 4C, black bold arrow). Single strands originating from dY intermediates constitute a bending arc that does not reach the size of parental strands (due to having two replication forks) and single strands from bubble intermediates form a straight line that almost reaches the position of parental strands. This result is because XmaJI does not digest exactly at the termination of replication, and therefore, RIs contain a proportion of dY molecules in addition to bubble RIs (Ref. **III**, Fig. 14B.10.8B and 14B.10.8E). Both in the case of bubble and dY intermediates, we can identify that the largest molecules (late theta RIs) are situated in the top left corner of the gel (Ref. **II**, Fig. 4B and 4C, black arrowheads, compare upper and lower panel; Ref. **III**, Fig. 14B.10.8D-E, marked as 2n). In addition, we can see the arcs of single strands that originate from novel replication mechanism intermediates (Ref. **II**, Fig. 4B and 4C, white arrowheads and white bold arrows; Ref. **III**, Fig. 14B.10.8D-E, white and grey bold arrows). When analyzing the replication of HPV18 DNA using N/A AGE, we can verify that the signals of the novel replication mechanism do not differ when using two distinct restriction enzymes that digest at different regions of the HPV18 genome (Ref. **II**, compare Fig. 4B and 4C, white arrowheads and white bold arrows; Ref. **III**,

compare Fig. 14B.10.8D and Fig. 14B.10.8E, white and grey bold arrows). When we analyse these signals, we can see that the smallest single strands originating from RIs form a straight arc that is characteristic of nascent strands originating from bubble structures (Ref. **II**, Fig. 4B and 4C, white bold arrows; Ref. **III**, Fig. 14B.10.8D-E, white bold arrows). However, when replication continues, the arc of single stranded molecules starts to bend and resembles the pattern of ssDNAs that originate from simple Ys or severely asymmetrical dYs (Ref. **II**, Fig. 4B and 4C, white arrowheads; Ref. **III**, Fig. 14B.10.8D-E, grey bold arrows). Thus, we propose that when the novel replication mechanism is initiated, the intermediates resemble bubble structures because their arc of nascent strands partially converges with the ssDNA arc that originates from early bubble RIs of theta replication. However, they cannot be identical because they have different patterns in 2D N/N AGE. When the novel replication mechanism continues, we see that the ssDNAs that originate from RIs form an arc resembling ssDNA that originates from simple Y RIs because the arc starts to bend. This result can be explained by the digestion of novel RI bubble-resembling structures into simple Y structures (as the bubble has increased in size and therefore is more likely to be digested). Before the newly synthesized molecule is fully replicated, the structure is unknown (Ref. **II**, Fig. 4B and 4C, white arrowheads; Ref. **III**, Fig. 14B.10.8D-E, gray bold arrows).

Analysis of single-digested HPV DNA via 3D N/N/A AGE

To further confirm the composition HPV RIs, we conducted 3D N/N/A AGE assay (Ref. **II**, Fig. 3B; Ref. **III**, Fig. 14B.10.9). 3D N/N/A method involves soaking 2D N/N gel in alkali and separating parental and nascent strands in a third dimension (Ref. **III**, compare 2D N/N assay in Fig. 14B.10.9E to 3D N/N/A assay in Fig. 14B.10.9F, parental and nascent strands are marked black and red dotted lines). We can see that the intermediates of novel mechanism contain bubble or simple Y structures, as we cannot distinguish between them via 3D assay (schematic representation of the location of parental and nascent strands of bubble and simple Y is depicted in Ref. **III**, in Fig. 14B.10.9B-C). The smallest theta intermediates have a weak signal and this observation could be the reason why we cannot see their nascent strands. However, late theta intermediates have stronger signals and we can also detect their nascent strands (Ref. **III**, Fig. 14B.10.9F, marked with a black bold arrow). The arc of nascent strands implies that the RIs contain converged forks in different positions in the HPV genome (Ref. **III**, compare schematic representation in Fig. 14B.10.9D to 3D N/N/A assay in Fig. 14B.10.9F). This result supports the hypothesis that the replication forks are stalled before separation of the replicated molecule into the daughter genomes.

Analysis of linearized HPV18 sub-genomic fragments via 2D N/N AGE

To further analyze the composition of the novel RIs, we analyzed the sub-genomic fragments formed from the HPV18 genome. We used combinations of 2 to 4 different restriction enzymes (Bpu1102I, PsyI, XmaJI, and CfrI) to digest the HPV genome at different regions (Ref. **II**, Fig. 5, genome animation). This kind of digestion results in four approximately 2 kbp fragments. As a result, we could separately analyze various regions, including a region containing the origin (CfrI-Bpu1102I, ORI probe), a region that excludes the origin (Bpu1102I-CfrI, TERM probe), a region containing E1 (Bpu1102I-PsyI, E1 probe), a region excluding E1 (Psy-Bpu1102I, L1 probe), a region containing L1 (XmaJI-CfrI, L1 probe), a region excluding L1 (CfrI-XmaJI, E1 probe), a region containing the termination sequence (PsyI-XmaJI, TERM probe), and a region excluding termination (XmaJI-PsyI, ORI probe).

While analyzing these ~2 kbp sub-genomic fragments, we found the classical theta replication RIs that we expected to find (Ref. **II**, Fig. 5, maller fragments: Fig. 5A ORI probe, Fig. 5B TERM probe, Fig. 5C L1 probe, Fig. 5D E1 probe). The region containing the origin (CfrI-Bpu1102I, ORI probe) yielded a bubble, simple Y, and dY structures. We observed simple Ys in both the E1 (Bpu1102I-PsyI, E1 probe) and L1 regions (PsyI-XmaJI, L1 probe) and a simple Y and dY intermediate in the termination region (XmaJI-CfrI, TERM probe). Please refer to Ref. **II**, Fig. 5, smaller fragments: Fig. 5A ORI probe, Fig. 5B TERM probe, Fig. 5C L1 probe, and Fig. 5D E1 probe. The presence of dY intermediates in both the origin and termination regions confirms the phenomenon of having one active origin in an oligomer consisting of an even number of genomes. For example, bidirectional forks emerging from one origin of replication converge in the opposite site of origin, which in the case of a dimer would be the other origin of replication – possibly a passive origin. dY cannot originate from RIs from a novel replication mechanism because otherwise dY RIs would also be present in both the E1 (Ref. **II**, Fig. 5D E1 probe) and the L1 regions (Ref. **II**, Fig. 5C L1 probe), which they are not.

Coupled with every 2 kbp fragment, we also analyzed the rest of the HPV genome, excluding a 2 kbp sub-genomic fragment (in other words, ~6 kbp fragments) (Ref. **II**, Fig. 5, larger fragments: Fig. 5A TERM probe, Fig. 5B ORI probe, Fig. 5C E1 probe, Fig. 5D L1 probe). However, while observing the larger fragments, we found the same pattern of novel intermediates that we found while analyzing the linearized full genome (Ref. **II**, compare Fig. 4A and Fig. 5 larger fragments, white bold arrows). We see arcs from RIs that originate from classical theta replication, including dYs in CfrI-Bpu1102I with TERM probe (Ref. **II**, Fig. 5A TERM probe, black bold arrow) and a bubble to dY transition in XmaJI-PsyI with ORI probe (Ref. **II**, Fig. 5B ORI probe, black bold arrow). However, the simple Ys on Bpu1102I-PsyI with the L1 probe and XmaJI-CfrI with the E1 probe coincide with the pattern of novel intermediates (Ref. **II**, Fig. 5C and 5D, larger fragments, white bold arrows). This result

confirms the presence of the simple Y in the arc of novel intermediates structures that we previously observed using 2D N/A (Ref. **II**, Fig. 4B and 4C).

In summary, through the careful use of 2D N/N and N/A AGE, we obtained evidence of another genome replication method with unidirectional moving forks that operates in addition to the classical bidirectional theta type of HPV genome replication (Orav et al. 2015; Henno et al. 2017). We do not observe the involvement of RCR in any of the results we present here. It has been demonstrated that RCR intermediates form an eyebrow-shaped arc in uncut DNA analysis via 2D N/N GE (Belanger et al. 1996; Martin-Parras et al. 1998; Cohen et al. 2005), and our 2D assays do not display that kind of arc. Even though oligomers can be generated via either the RCR or RDR mechanisms, in our experiments, we observed intermolecular recombination of homologous sequences, and the only possible mechanism for generating hetero-oligomers could be through a HR-directed replication mode, excluding the possibility of RCR. However, RCR cannot be excluded entirely because oligomers consisting of a single molecular species could be obtained via the RCR mechanism.

Novel replication mechanism

Because many 2D analyses have previously been performed with PVs, there must have been some evidence of the novel mechanism, otherwise it would only be an artifact of our system. However, as mentioned above, analyzing sub-genomic fragments of the HPV18 genome via 2D AGE provides a visualization of only common RIs, such as simple Y, dY, and bubble intermediates, because more complex structures have been digested into smaller and therefore less complex structures. Most of the 2D experiments performed with PVs analyze sub-genomic fragments of the full genome, and this approach explains why there is not much indication of RIs with extremely branched and complex structures (more complex than RIs originating from bidirectional theta replication). As described above, when we linearize the viral genome using a single cutter enzyme, we can visualize a much more complex replication pattern (Ref. **II**, Fig. 2–3). The results we obtain are difficult to interpret, which supports a mixture of active replication mechanisms.

Several works have indicated the presence of two different mechanisms during both stable maintenance and the second amplification phase of PV replication. There are some indications of intermediates from the novel mechanism in addition to the well-known bidirectional theta replication mechanism published by Yang & Botchan and Flores & Lambert, who analyzed large sub-genomic fragments of PV or the full-length PV genome, respectively (Yang and Botchan 1990; Flores and Lambert 1997). Yang & Botchan hypothesized that the 2D N/N AGE patterns obtained from BPV1 may represent a unidirectional replication mechanism. Flores & Lambert proposed that upon differentiation, both HPV16 and HPV31 begin to replicate their genomes via a unidirectional replication mode without a specific origin; however, they proposed RCR to be

that mode of replication (Flores and Lambert 1997). In addition, Flores & Lambert analyzed a full HPV16 genome, and as they digested the DNA near the origin, they observed a strong spike emanating near 1n spot (signal is weak near 1n) and proposed that it refers to dY intermediates of bidirectional replication, whereas I would propose that it resembles RIs from the novel mechanism. Therefore, I suggest that the difficult-to-interpret signals obtained from 2D assays originate from the novel replication mechanism and possibly may have been misinterpreted.

The exact mechanism of how PVs manipulate and use HR pathways remains to be elucidated; however, it could be similar to the model proposed for circular dsDNA phage SPP1 (reviewed in (Lo Piano et al. 2011)) as proposed by (Sakakibara et al. 2013). SPP1 replicates bidirectionally until the replication forks are arrested, which leads to a DBR. The DBR is recognized by the host organism and induces the recruitment of host repair-related replisomal components to the site. The repair machinery then generates a D-loop into which a new replisome is assembled. The D-loop forms a bubble that migrates unidirectionally around the viral genome, thereby generating long concatemers. This step leads to a recombination dependent RCR-type of replication of SPP1 DNA. Thus, replication switches from the theta mode to recombination dependent RCR. However, aspects of this mechanism still require further confirmation. This kind of replication resembles the BIR mode of the HR mechanism, where ssDNA, which often originates from collapsed replication forks, invades dsDNA and forms a D-loop that migrates in a unidirectional fashion around the genome.

However, none of the abovementioned mechanisms are identical to HPV replication, which is strongly dependent on the unique life cycle of differentiating keratinocytes. Based on our results (Ref. **I**, Fig. 4E, Fig. 5B and Fig. 6), together with information gained from the work of (Fradet-Turcotte et al. 2011; Sakakibara et al. 2011; Gillespie et al. 2012; Reinson et al. 2013; Sakakibara et al. 2013; McKinney et al. 2015; Gautam and Moody 2016), we propose that HR and DDR are utilized by HPV DNA replication and that bidirectional and novel replication depend on each other. It is possible that the late theta intermediates that originate from bidirectional replication contain stalled replication forks. These collapsed forks give rise to double-stranded breaks that recruit factors of HR and DRR pathways and may trigger the novel replication mechanism. HPV replication, however, retains the ability to use both the bidirectional and RDR mode of replication during initial amplification in contrast with SSP1, where once the novel mechanism is triggered, it becomes the only mechanism used to replicate SPP1 genomes.

Additional discussion and future perspectives

Due to the complexity of the data we have collected, many patterns and features have yet to be confirmed. Future work should continue to study the mechanisms we propose here to add weight to or possibly refute our hypotheses. This work

can be achieved by analyzing the RIs via electron microscopy or atomic force microscopy; however, the best approach may be to improve the resolution of these images. Nevertheless, visual observation of the intermediates alone cannot identify the mechanism that HPV uses in addition to bidirectional theta replication.

The mechanisms behind the creation of novel intermediates we observe can also be examined by knocking down/out the necessary proteins for cellular repair and recombination and analyzing the resulting replication pattern obtained via 2D N/N AGE. However, we do not yet know for sure whether bidirectional and recombinational replication are dependent on each other and if there is even a possibility to separate those two replication mechanisms.

We conclude from the data collected from experiments using 1D, 2D and 3D assays that HPV18 genomes utilize two different mechanisms for viral genome replication and therefore for the synthesis of oligomers. One is bidirectional theta replication, and the other is a separate novel mechanism. Our results indicate that both mechanisms are used during the transient amplification phase. Bidirectional theta replication initiated from the previously identified origin of replication likely depends on the availability of viral replication factors E1 and E2. However, we propose that the novel replication mechanism may be controlled by cellular factors. At this point, we can only suggest that the novel replication mechanism operates via a unidirectional mode.

CONCLUSIONS

- High-risk HPV18 is capable of forming monomeric, dimeric and oligomeric molecules upon an initial establishment period (transient assay).
- We characterized these oligomers as episomal head-to-tail concatemeric molecules. No other viral proteins, except for viral replication proteins E1 and E2, are necessary for oligomer formation. Additionally, we can conclude that oligomer formation is a replication dependent process.
- The phenomenon of oligomerization can occur in a region as small as the replication origin containing URR fragment of HPV18 when E1 and E2 are provided from expression vectors; thus, we can distinguish replication and oligomerization from transcription.
- We conducted a complementation assay in which the complete HPV18 genome is transfected into U2OS along with sub-genomic replicons. As a result, we detected joint molecules (hetero-oligomers) consisting of full-length and sub-genomic HPV18 DNA. These experiments indicate that at least HPV genomic hetero-oligomers can arise through the homologous recombination step in replicating viral genomes; however, this step can also be the method of formation of homo-oligomers.
- We propose that HPV18 genomes utilize at least two different mechanisms for viral genome replication during initial amplification: the replication intermediates that originate from bidirectional theta replication are apparent in addition to uncertain structures originating from an undetermined replication mechanism that likely involves pathways of homologous recombination and DNA damage response.
- We propose that the two mechanisms involved in HPV replication must therefore also participate in the formation of oligomers; however, the exact mechanisms of oligomer generation and resolvation into monomers must still be elucidated.

SUMMARY IN ESTONIAN

Inimese papilloomiviiruse genoomi replikatsiooni ja oligomeeride tekke analüüs

Papilloomiviirused (PV-d) on väikesed, ligikaudu 8000 aluspaari pikkused kaheahelalise DNA genoomiga viirused, mis nakatavad kihistunud epiteeli basaalseid keratinotsüüte. PV-d on äärmiselt liigi- ja koespetsiifilised ja nad nakatavad ainult imetajate, lindude ja roomajate keratinotsüüte. Inimese papilloomiviirused (HPV-d) on populatsioonis laialt levinud patogeenid, mille infektsiooniga kaasneb healoomuliste vohandite tekkimine epiteelil (tüükad, papilloomid). Mõned HPV tüübid aga võivad põhjustada ka halvaloomuliste kasvajate teket. Inimese papilloomiviiruse poolt põhjustatud emakakaelavähk on kolmas enimlevinud vähk naistel, mistõttu selle valdkonna uuringud on väga olulised.

Käesolevas töös leidis kasutust meie töögrupi poolt välja töötatud U2OS rakkudel põhinevat mudelsüsteemi, et uurida HPV18 genoomi lühiajalise replikatsiooni käigus tekkivaid viiruse genoomi oligomeere. Seda, et HPV moodustab replikatsiooni käigus oligomeere, märgati juba üle 30 aasta tagasi, aga nende tekkepõhjuseid ei ole siiani välja selgitatud. Üks võimalik seletus, miks viiruse genoom esineb multimeerses vormis, on see, et HPV-de puhul kasutatakse ühe multimeeri paljundamiseks vaid ühte replikatsioonikompleksi (oligomeeris on aktiivne ainult üks replikatsiooni alguspunkt). Sellega kaasneb olukord, et väheste peremeesraku ja viiruse valkudega saab paljundada suurt arvu HPV genoome. See võimaldab ära hoida või edasi lükata HPV tuvastamist peremeesraku immuunsüsteemi poolt.

Selgitamaks välja oligomeeride tekkemehhanismi viisin läbi katse täispika ja subgenoomse HPV DNA-ga, mille tulemusena tekkisid hetero-oligomeerid (oligomeerid, mis koosnesid täispikast ja subgenoomsest HPV DNAst). Selline kahe eri molekuli vaheline kombineerumine saab toimuda ainult läbi homoloogse rekombinatsiooni radade ning üks võimalus HPV oligomeeride tekkeks ongi homoloogsel rekombinatsioonil põhinev DNA sünteesimehhanism.

Töö üheks eesmärgiks oli läbi replikatsiooni vaheproduktide kirjeldamise uurida, mis mehhanisme kasutades HPV genoom replitseerub. Selle uurimise sobilikumaks meetodiks on kahedimensionaalne agaroos geelelektroforees (2D AGE). Tuvastasime, et lisaks varasemalt hästi kirjeldatud kahesuunalise *theta* replikatsiooni moodusele, esineb HPV-del ka teinegi siiani kirjeldamata replikatsioonimehhanism. Pakume, et teine replikatsioonimehhanism kasutab DNA kahjustuse radade (DDR) poolt rakendatavaid rakulisi valke ja faktoreid. Seda kinnitab ka asjaolu, et eelnevalt on näidatud DDR faktorite lokalisatsiooni HPV replikatsioonitsentritesse.

Mitmedimensionaalseid geelelektroforeese on keerukas läbi viia, aga veelgi keerukam on tulemuste analüüs. Selle tõttu andsin käesolevas töös ka ülevaate, kuidas analüüsida ja tõlgendada kahe- ja kolmedimensionaalsest AGE-st saadud tulemusi.

Kokkuvõtvalt näitasin, et HPV kasutab genoomi paljundamiseks ja oligomeeride sünteesiks vähemalt kahte eri replikatsioonimehhanismi. Üks mehhanismidest on kahesuunaline *theta* replikatsiooni ja teine ühesuunaline homoloogsel rekombinatsioonil põhinev ning DDR radadest sõltuv replikatsioonimehhanism.

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2015 Initial amplification of the HPV18 genome proceeds via two distinct replication mechanisms. Marit Orav, Jelizaveta Geimanen, Eva-Maria Sepp, **Liisi Henno**, Ene Ustav, Mart Ustav. *Scientific Reports* 5, 15952

2015 Engagement of the ATR-dependent DNA damage response at the human papillomavirus 18 replication centers during the initial amplification. Tormi Reinson, **Liisi Henno**, Mart Toots, Mart Ustav Jr, Mart Ustav. *Journal of virology*, 87, 951–964

2017 Analysis of human papillomavirus genome replication using two- and three-dimensional agarose gel electrophoresis. **Liisi Henno**, Eva-Maria Tombak, Jelizaveta Geimanen, Marit Orav, Ene Ustav, Mart Ustav. *Current Protocols in Microbiology*, 45, 14B.10.1–14B.10.37.

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Saksa	kõnes ja kirjas keskmine
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