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TORMI REINSON

Studies on the Genome Replication of Human Papillomaviruses

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Studies on the Genome Replication of Human Papillomaviruses

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications that will be referred to by their Roman numerals:

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

II. **Reinson T**, Toots M, Kadaja M, Pipitch R, Allik M, Ustav E, Ustav M. 2013. Engagement of the ATR-dependent DNA damage response at the human papillomavirus 18 replication centers during the initial amplification. J Virol 87(2): 951–964.

III. **Reinson T**, Henno L, Toots M, Ustav M Jr, Ustav M. 2015. The Cell Cycle Timing of Human Papillomavirus DNA Replication. PLoS One 10(7): e0131675.

My contributions to the papers are as follows:

I. I performed the experiment describing changes in viral gene expression during vegetative amplification of HPV genomes; I helped to analyze the data and helped write the manuscript.

II. I participated in the experimental design, and together with Mart Toots performed most of the experiments; I participated in data analysis and wrote most of the manuscript.

III. I designed and performed most of the experiments. I analyzed the data and wrote the manuscript.

LIST OF ABBREVIATIONS

HPV – human papillomavirus
PV – Papillomavirus
ORF – open reading frame
URR – upstream regulatory region
BM – basement membrane
BPV1 – Bovine papillomavirus type 1
HFK – human foreskin keratinocytes
BS – binding site
ORC – origin recognition complex
OBD – origin binding domain
MCM – minichromosome maintenance complex
Topo I – topoisomerase I
DDR – DNA damage response
UV – ultraviolet light
IR – ionizing radiation
PI3K – phosphoinositide 3-kinases
ATM – ataxia telangiectasia mutated
DSB – double-strand break
ATR – ataxia telangiectasia and Rad3 related protein
DNA-PKcs – DNA-dependent protein kinase catalytic subunit
HR – homologous recombination
NHEJ – non-homologous end joining
MRN – Mre11-Rad50-Nbs1
ssDNA – single-stranded DNA
RPA – replication protein A
dsDNA – double-stranded DNA
9-1-1 – RAD9-RAD1-HUS1
TopBP1 – DNA topoisomerase 2-binding protein 1
CHK1 – checkpoint kinase-1
BLM – Bloom syndrome protein
EBV – Epstein–Barr virus
HSV1 – Herpes simplex virus 1
MVM – Minute virus of mice
HU – hydroxyurea
MVC – Minute virus of canines
MCV – Merkel cell polyomavirus
HCMV – Human cytomegalovirus
LTag – large T antigen
BKPyV – BK polyomavirus
JCV – JC virus
RDR – recombination-dependent replication
TUNEL – terminal deoxynucleotidyl transferase dUTP nick end labeling
RT-PCR – reverse transcriptase PCR

FISH – fluorescence in situ hybridization
RDR – recombination dependent replication
wt – wild-type
FoSTeS – fork stalling and template switching
MMBIR – microhomology-mediated break-induced replication

INTRODUCTION

One in every six cancers diagnosed worldwide is caused by a viral infection. A significant fraction of cancers with a viral origin are caused by the human papillomavirus (HPV). HPV is a DNA virus that infects the skin and mucous membranes and that frequently causes no or only mild symptoms, such as papillomas, warts or other benign tumors, during its normal life cycle. However, in some cases, HPV infection can lead to malignant progression and the formation of cancer. While only a limited proportion of HPV infections lead to fatal consequences, the extremely high prevalence of HPV in the general population results in hundreds of thousands of new cases of HPV-related cancers being diagnosed each year. Cancers caused by HPV are characterized by the uncontrolled expression of viral oncogenes that directly contribute to the development of cancer cell properties. However, a more detailed characterization of the viral processes that lead to the uncontrolled expression of viral oncogenes is needed.

The broader objective of this study was to characterize aspects of HPV genome replication, including the properties of viral replication proteins. To achieve this objective, we developed a new cellular assay system and novel methods to study DNA replication in papillomaviruses. We applied these new tools to study viral interactions with cellular DNA damage response pathways and to characterize the timing of viral DNA replication in the cell cycle.

LITERATURE REVIEW

Introduction to papillomaviruses

Papillomaviruses (PV) are a diverse group of nonenveloped DNA viruses that infect epithelial cells in vertebrate species. Different Human PV (HPV) types infect either cutaneous or mucosal tissues and are highly tissue-specific. Infections with HPVs are widespread among human populations, in which they can cause benign lesions of skin and mucous membranes or remain completely asymptomatic. However, infection with certain human papillomavirus types can induce the formation of cervical or other epithelial cancers. Because it is responsible for causing around half a million new cases of malignant tumors each year, HPV is one of the most important cancer-causing agents, and this fact has brought this virus to the attention of many researchers around the world.

Genome Structure and Organization

HPVs have a relatively small circular and double-stranded DNA genome with a length of approximately 8000 base pairs. The genome contains eight or nine open reading frames (ORFs). The ORFs are divided into early and late regions based on the timing of their protein expression during the viral life cycle. Early region genes encode for non-structural proteins that are responsible for viral genome replication, transcriptional regulation and host cell modulations, while late region genes encode the structural proteins that form the viral capsid. In addition to these coding sequences, which take up most of the viral genome, there is a thousand base pairs long non-coding region (the upstream regulatory region, URR), and this sequence contains the origin of replication and transcriptional control elements (for review, see (Howley and Lowy 2007).

Functions of viral proteins

E1 is the most conserved protein and the only enzyme out of all of the PV proteins. It works as a replication initiator protein and a replicative helicase (Bergvall et al. 2013). In addition to E1, only one other viral protein, E2, contributes directly to HPV DNA replication. The E2 protein interacts with E1 and at the same time binds to the viral genome with very high sequence specificity to provide crucial help during the initiation of DNA replication (Stenlund 2003b).

In addition to viral DNA replication, the multifunctional E2 protein serves other crucial roles during HPV infection (McBride 2013). Another crucial task of the E2 protein is to regulate viral transcription by binding to its binding sites in the regulatory region of the genome, resulting in the activation or repression of viral promoters. During the latent phase of the HPV life cycle, E2 is

responsible for maintaining viral genomes in the nuclei of dividing cells by binding the viral genomes to mitotic chromosomes.

Another set of functions that are performed by viral proteins are related to cellular modulations. Because HPV relies heavily on cellular proteins for its genome replication and transcription, it must adjust to the cellular milieu to successfully complete its life cycle. The main transforming proteins of HPVs are E6 and E7 (Howie et al. 2009; McLaughlin-Drubin and Munger 2009). These two multifunctional proteins exert their effects on a broad array of cellular processes, including cell cycle progression, cellular differentiation, host cell gene expression, growth factor dependence and cell survival, through their binding partners. The most important and well-known cellular proteins with functions that are influenced by E6 and E7 are p53 and pRB, respectively.

Viruses must protect their genomes in the external environment and bind to susceptible cells to initiate a new infection. These roles are mediated by the HPV structural proteins L1 and L2, which form the viral non-enveloped icosahedral capsid (Conway and Meyers 2009).

Viral life cycle

The life cycle of HPV is directly linked to epithelial cell differentiation. Wounding the epithelium is necessary for HPV infection because it allows the virus particle to bind to the basement membrane (BM) of the epithelium, a crucial first step for viral cell entry (Kines et al. 2009). Binding to the BM leads to a conformational change in the viral capsid and the subsequent furin cleavage of the L2 protein, which allows the virion to bind to the cell surface. In this way, HPV guarantees that only cells that are permissive to infection are infected because cell cycle progression is required to establish HPV infection (Pyeon et al. 2009) and only the cells in the layer closest to the BM are mitotically active (Blanpain and Fuchs 2006).

After reaching the nucleus of a basal keratinocyte, the viral genome goes through the first phase of gene expression and the initiation of DNA replication (initial amplification). The initial amplification of the HPV genome increases the viral genome copy number to approximately 50–200 copies per cell (Doorbar et al. 2012). The initial amplification is dependent on the viral replication proteins E1 and E2, which are expressed from the early region of the HPV genome (Ustav and Stenlund 1991; Ustav et al. 1991). After infection has been established and the initial amplification has completed, the viral genomes are maintained at an approximately constant copy number in undifferentiated basal cells (Turek et al. 1982). During the stable maintenance phase, the viral copy number is doubled during S phase, and the viral genomes are divided equally between the resulting daughter cells during mitosis. This process maintains a stable number of viral genomes in each cell. The E2 protein plays a crucial role in the segregation process by tethering the viral genomes to mitotic

chromosomes, which results in them being divided equally between the two forming nuclei (Skiadopoulos and McBride 1998; Ilves et al. 1999).

To maintain homeostasis, epithelial tissues are constantly renewed by the proliferation of cells in the basal layer. Basal cells divide asymmetrically to give rise to a new basal cell and a cell that begins to move outward in the tissue and to differentiate (Lechler and Fuchs 2005). As a cell differentiates, it exits the cell cycle and dies before it is shed off from the epithelial surface (Fuchs and Raghavan 2002). Because HPV relies on cellular replication proteins, it must keep cells dividing to maintain the conditions required for its own genome replication processes. For this reason, HPV expresses the oncoproteins E6 and E7, which push cells toward S-phase and avoid the induction of apoptosis, which is otherwise activated as a result of unscheduled DNA replication (Howie et al. 2009; McLaughlin-Drubin and Munger 2009). Thus, viral oncoproteins preserve proliferation in HPV-positive cells, while regular differentiating keratinocytes exit the cell cycle.

During the differentiation of the host cell, viral late promoters are activated, and this causes an increase in the levels of the E1, E1^E4, E2 and E5 transcripts (Hummel et al. 1992; Ozbun and Meyers 1998b). The increase in the protein expression of the E1 and E2 proteins causes the amplification of viral DNA, which increases the viral genome copy number to several thousand per cell (Bedell et al. 1991; Ozbun and Meyers 1998a). The completion of the HPV life cycle requires the production of the viral capsid proteins L1 and L2. The elevation of late transcript levels is achieved by changing polyadenylation site usage from early to late sites via the expression of high levels of the E2 protein (Johansson et al. 2012). After viral genome encapsidation, virion maturation occurs in the uppermost layers of the epithelium, and the virion is eventually released into the environment after the normal disintegration of the host cells near the surface of the epithelium.

Papillomavirus DNA replication

Experimental systems for studying HPV DNA replication

Propagating papillomaviruses in cell culture models has proven to be difficult because the natural infection and viral life cycle takes place in the stratified squamous epithelium, which is not mimicked in monolayer cultures. For this reason, the full HPV life cycle, starting from infection and ending with progeny virion production, cannot be reconstructed in such cultures. Although the full viral life cycle can be studied in three dimensional raft cultures, some restrictions, such as low viral yields, limit the usefulness of this approach in viral DNA replication studies. Hence, most information concerning PV DNA replication mechanisms is obtained from works that involve the transfection of recircularized naked DNA genomes or expression constructs of viral proteins.

Many early works on papillomavirus DNA replication mechanisms were performed using Bovine papillomavirus type 1 (BPV1) because it was observed that BPV genomes transform mouse C127 cells and replicate in these cells as an extrachromosomal multicopy nuclear plasmid (Law et al. 1981). HPV genomes were also shown to replicate in keratinocyte cell lines that were infected with virions that were purified from warts (LaPorta and Taichman 1982) and to transform mouse C127 cells following calcium phosphate precipitation transfection (Watts et al. 1984).

Another source of cell lines that contain HPV genomes is patient biopsy specimens. The most widely used of these cell lines are CIN612 (Bedell et al. 1991) and W12 (Stanley et al. 1989), which contain 50–100 episomal copies of HPV31 and HPV16, respectively. While patient cell lines are useful for studying the properties of wild-type viruses during the stable maintenance and differentiation dependent amplification, these cellular systems do not allow genetic engineering of HPV genomes. Genetic analysis of the HPV genome became possible when stable transfection methods were developed for human foreskin keratinocytes (HFKs) (Frattini et al. 1996; Frattini et al. 1997). To isolate HPV genome-containing cell lines, cloned HPV genomes are excised from cloning vectors, re-circularized via self-ligation, and then transfected into HFKs. Although only a low number of HFKs are transfected with viral genomes during this process, co-transfection with an antibiotic selection marker allows the elimination of untransfected cells and the isolation of HPV-containing cell lines. Although the process is time consuming, established cell lines that contain episomal HPV genomes are valuable tools for studying the stable maintenance and vegetative amplification phases of the viral life cycle. Continuously passaging these cells enables the study of the stable maintenance phase, while inducing cellular differentiation in HFK cultures triggers the vegetative amplification phase of HPV genomes. This approach has been widely used to study high-risk alpha-HPVs; however, it has not worked very well in low-risk HPVs and cutaneous beta-HPVs.

Several methods have been established that can be used to trigger the differentiation-dependent amplification of viral genomes in HFK cultures. HFKs can be induced to differentiate by increasing the calcium concentration in the growth medium or by seeding the cells into semisolid agar or methylcellulose (Fehrmann et al. 2003; Moody et al. 2007). Another method for inducing differentiation in HPV-containing keratinocytes is growing them on collagen raft cultures. This method is a very useful cell culture model for PV because it resembles the normal differentiation pattern of the epidermis by allowing the cells to grow three dimensionally on an air-liquid interface (McCance et al. 1988). Executing an epidermal differentiation program in this cell culture model allows the reproduction of the full viral life cycle and the production of infectious virions (Dollard et al. 1992; Meyers et al. 1992).

In addition to using full viral genomes to study PV replication, a substantial amount of information about these processes has been gained from experiments in which viral replication proteins are expressed from expression vectors in the

presence of viral origin-containing DNA in mammalian cell lines. Such DNA begins to replicate in the cells in which the stable or transient expression of the PV replication proteins E1 and E2 is ensured. This system was used to characterize the viral proteins and cis-sequences that are necessary for transient DNA replication of BPV and HPV (Ustav et al. 1991; Chiang et al. 1992a; Chiang et al. 1992b; Remm et al. 1992; Ustav et al. 1993; Russell and Botchan 1995). PV DNA replication can also be reconstructed in cell-free system *in vitro* using purified E1 and E2 proteins that are complemented with cell lysates or purified host replication factors (Yang et al. 1991; Kuo et al. 1994; Muller et al. 1994; Melendy et al. 1995).

HPV Replication origin

Approximately 1000 bp long non-coding region (URR) is present in PV genomes. This sequence contains the elements that are responsible for viral DNA replication, transcriptional regulation and genome partitioning during mitosis. All of these functions are crucial for the effective replication of viral genomes after infection or the transfection of cells. Because the sequences in the URR that perform these functions are overlapping, it is difficult to study their functions separately in the context of full viral genomes. For this reason, the transfection of expression constructs of viral replication proteins has been used to identify the sequences in the viral genome that are involved in DNA replication.

The PV replication origin in the viral URR consists of an approximately 100 bp sequence (Remm et al. 1992) that contains elements that contribute directly to the initiation of PV DNA replication. These elements include a binding site (BS) for the E1 protein, a stretch of AT-rich sequence and two or more E2 binding sites, depending on the viral type. One high affinity E2 binding site is absolutely necessary to induce the in vivo replication of BPV (Ustav et al. 1993) as well as HPV11 DNA (Remm et al. 1992; Lu et al. 1993; Russell and Botchan 1995). However, there are differences between PVs in whether or not an E1 BS is required for viral DNA replication. While an E1 BS is necessary for BPV1 DNA replication (Ustav et al. 1991), HPV11 does not require an E1 BS (Lu et al. 1993). The elements in the viral DNA replication origin must guarantee the binding of the E1 and E2 proteins to the viral genome, and while the E2 BS appears to be the major contributor to this function, the E1 BS helps to increase overall binding affinity, which is necessary for viral DNA replication during natural infections.

Functions of viral replication proteins E1 and E2

The virus relies heavily on host proteins to execute viral life cycle processes. Viral DNA replication is one of the processes in the viral life cycle in which host proteins play a major role. The host replication machinery is responsible for synthesizing new viral genomes. However, viral replication proteins E1 and

E2 are crucial because they play the major role in initiating DNA replication and subsequently recruiting host factors to the viral DNA.

The first step in the initiation of DNA replication is the recognition of the replication origin – the region at which dsDNA is first melted (opened) to start the synthesis of new DNA strands (Mechali 2010). This activity is performed by the heterohexameric origin recognition complex (ORC) during the replication of eukaryotic genomes. The PV replication origin is recognized by the viral replication proteins E1 and E2 (Stenlund 2003b). In mammals, 30000 – 50000 replication origins with no described consensus sequence can be active during a single cell cycle (Mechali 2010). In comparison, the initiation of PV DNA replication is like finding a needle in a haystack: at the beginning of viral infection, E1 and E2 must find the one and only replication origin (one viral genome per cell) within an enormous amount of competing cellular DNA. For this reason, very high sequence specificity is needed to accomplish this task.

The E1 protein, as the major replication protein of PV, is the only viral protein that is needed in an HPV cell-free replication system, in which the protein concentration is high, and no competing DNA is present (Yang et al. 1993). However, in a cellular environment, E1 and E2 are both essential for HPV DNA replication (Ustav and Stenlund 1991). The E2 protein acts as a specificity factor during HPV DNA replication, and it assists in loading the E1 protein onto the viral origin (Mohr et al. 1990; Sedman and Stenlund 1995). One way in which E2 contributes to viral DNA replication initiation is by binding to its binding site and thereby directing E1 to the viral origin. At least one E2 binding site is necessary for the initiation of viral DNA replication *in vivo* (Ustav et al. 1991; Lu et al. 1993). However, E2 also plays a role in modulating E1 DNA binding activity. The E1 protein is a truly multifunctional protein. It works in sequence-specific recognition of the viral origin in addition to later steps of DNA modification during replication initiation and elongation. To perform these distinct tasks, the E1 protein has two different DNA binding activities: sequence-specific binding, which is mediated by the origin binding domain (OBD), and non-specific DNA binding, which is mediated by a helicase domain (Stenlund 2003a). Although OBD alone can bind DNA with high sequence specificity, the non-specific binding to random sequences by the helicase domain masks this specificity and results in the overall low sequence specificity of the protein. Therefore, the other role of the E2 protein during the initiation of DNA replication is to block the non-specific binding of the E1 protein to host DNA by interacting with the E1 helicase domain (Stenlund 2003a; Abbate et al. 2004).

After recognition of the replication origin, the next step in the initiation of DNA replication is melting the dsDNA to convert it into a replication fork (Gai et al. 2010). In the first step of PV DNA replication, two E1 and E2 molecules bind to the viral origin as a double dimer (Chen and Stenlund 1998). During the following ATP-dependent steps, E2 proteins are excluded from the origin, and additional E1 molecules are loaded, resulting in the formation of E1 double trimer. The double trimer is the functional complex that uses ATP hydrolysis

energy to melt dsDNA at the viral origin (Chen and Stenlund 2002; Abbate et al. 2004; Schuck and Stenlund 2005).

A region of single-stranded nucleic acid is needed to load and activate most helicases (Patel and Donmez 2006). The generation of ssDNA during the initiation of PV DNA replication is therefore necessary for the subsequent loading of active double hexameric replicative helicase onto the DNA, which occurs by adding additional E1 molecules to the complex (Schuck and Stenlund 2005; Schuck and Stenlund 2011). Both of the E1 hexamers in the double hexamer encircle one ssDNA strand and unwind the DNA via allosteric exclusion of the complementary strand while translocating on the DNA using energy obtained from ATP hydrolysis (Enemark and Joshua-Tor 2006; Lee et al. 2014). Therefore, during the elongation phase of PV DNA replication, E1 plays the same role that the replicative helicase minichromosome maintenance complex (MCM) performs in the DNA replication of a eukaryotic cell (Bochman and Schwacha 2009).

In its function as a replicative helicase, the E1 hexamer unwinds DNA strands to prepare a template for the synthesis of a new DNA strand. The synthesis of new viral DNA is mediated by the cellular replication machinery, which the E1 protein recruits to the PV genome. Replication proteins, including topoisomerase I (Topo I) (Clower et al. 2006), single stranded DNA-binding protein (replication protein A – RPA) (Han et al. 1999), and the DNA polymerase alpha-primase complex (Park et al. 1994), have been shown to directly interact with the E1 protein. These E1 protein interactions are important in linking DNA unwinding to leading strand synthesis. An important provider of this function at the replication forks of human chromosomes is the four protein complex GINS, which interacts with the MCM helicase complex (Labib and Gambus 2007). For example, GINS binds to and stimulates DNA polymerase alpha-primase, and this behavior is analogous to that of the E1 protein (De Falco et al. 2007).

In conclusion, the E1 protein recognizes and binds to the viral replication origin, melts dsDNA, functions as a replicative helicase and recruits the cellular replication machinery to replicate PV genomes. Therefore, E1 is a truly multifunctional replication protein that performs tasks that involve tens of proteins when the DNA of a eukaryotic cell is replicated. The distribution of these tasks between so many proteins in a cellular context is probably needed to ensure an extremely high level of control over the process. This is necessary to duplicate the entire cellular genome while avoiding the detrimental re-replication of any genomic region and responding to different cell growth conditions or DNA damage (Fragkos et al. 2015). On the contrary, PV does not need to have as high a level of control over the replication process. Instead, it benefits from having a short genome that encodes multifunctional proteins like E1.

However, performing this many activities using a single polypeptide can also result in undesirable side effects. For example, we previously found that expression of the E1 protein induces DNA damage in HeLa cells. HeLa is a cervical cancer cell line that contains integrated HPV subgenomic fragments. These

sequences contain a viral replication origin and ORFs of the E6 and E7 oncogenes but not the E1 and E2 genes. The expression of viral replication proteins in these cells, either from a viral genome or by heterologous expression vectors, initiates E1- and E2-dependent replication from the integrated viral origin, which activates the DNA damage response and causes genomic instability. However, the expression of E1 alone, without the initiation of replication from the integrated viral origin, also caused the activation of the DNA damage response. Although E1 expression alone induced lower level of DDR activation than was induced by E1 and E2 co-expression, these data indicated the possibility that E1 alone can potentially interfere with cellular DNA replication or repair pathways or directly damage host chromosomes (Kadaja et al. 2007; Kadaja et al. 2009).

The DNA damage response

The cellular genome is constantly being challenged by assaults from both internal and external factors. DNA can be damaged by both physical and chemical factors in the environment, including ultraviolet light (UV), ionizing radiation (IR), and chemical agents in cigarette smoke. In addition to the DNA damaging agents that originate from the external environment, our own metabolism constantly generates compounds such as reactive oxygen or nitrogen species that can react with and damage the cellular genome (De Bont and van Larebeke 2004). Failures in DNA replication and transcription can also cause damage to host chromosomes. Finally, limited chemical stability causes spontaneous lesions in DNA as a result of hydrolysis, oxidation or non-enzymatic methylation (Lindahl 1993). These factors are responsible for a range of DNA lesions, such as single- and double-stranded breaks, pyrimidine dimers, aromatic DNA adducts and a variety of oxidative base and sugar products.

It has been estimated that tens of thousands of lesions are generated in every cell each day (Hoeijmakers 2009). These lesions can interrupt transcription or genome replication, and this can threaten the viability of the cell or the whole organism if the damage is left unrepaired or is repaired incorrectly. DNA damage can be mutagenic or cytotoxic, depending on the type, location and number of lesions. While an insufficient response to cytotoxic damage leads to the apoptosis or senescence of the cell, mutagenic damage can threaten the whole organism by introducing carcinogenic alterations in cellular genomes. At the same time, DNA is the only biomolecule in cells that is repaired rather than replaced with a new copy when it is damaged. Therefore, because it is an extremely important molecule that is a target of continuous physical and chemical assaults, cells invest heavily in DNA repair mechanisms that maintain the proper functionality of the genome.

This extensive protein network, which is responsible for safeguarding the genome, is called the DNA damage response (DDR) (Harper and Elledge 2007; Jackson and Bartek 2009). The DDR responds to DNA lesions by first

recognizing the damage using sensor proteins. This is followed by the activation of transducers, which mediate and amplify the signal and recruit effector proteins. The role of effector proteins in the DDR is to repair the DNA and protect the genome from experiencing further damage. These tasks are accomplished by activating and relocating proteins, switching on checkpoints and modulating signaling or metabolic pathways (Shiloh and Ziv 2013). If the damage cannot be removed, the cell death or senescence pathways are activated to prevent the proliferation of cells with damaged genomes.

The DDR is divided into separate arms based on the type of DNA damage that occurs. In the centers of these pathways lay a group of closely related phosphoinositide 3-kinases (PI3Ks). These kinases include ataxia telangiectasia mutated (ATM), which is a key protein that is activated in response to DNA double-strand breaks (DSBs) (Shiloh and Ziv 2013), and ataxia telangiectasia and Rad3 related protein (ATR), which is primarily activated by replication stress (Cimprich and Cortez 2008). The third PI3K protein is DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which has a smaller number of substrates than ATM and ATR and regulates a smaller group of proteins that participate in a specific form of DSB repair. Although these are the main pathways of the three kinases, the DDR is more complex than three straight lines. It is instead an intricate network of proteins that are often able to interact with proteins outside their canonical pathways to fine-tune responses to all of the different types of DNA lesions that can occur in a myriad of possible cellular circumstances. The vast extent of the DDR became evident after proteomic approaches were used to identify ATM and ATR substrates (Matsuoka et al. 2007; Bensimon et al. 2010). Hundreds of proteins were found to be phosphorylated, and a large number of cellular pathways were affected by the activation of ATM and ATR kinases.

DNA double-strand breaks

Although DSBs are relatively rare compared to some other types of DNA lesions, they are highly cytotoxic and difficult to repair because both of the DNA strands are damaged simultaneously. Homologous recombination (HR) and non-homologous end joining (NHEJ) are the two main mechanisms that are used to repair DSBs. While HR is less error-prone than NHEJ, it is only accessible during the S and G2 phases of the cell cycle, when sister chromatids can be used for homology-directed repair (You and Bailis 2010).

The two main sensors of DSBs are the Mre11-Rad50-Nbs1 (MRN) and Ku70/Ku80 complexes. Ku70/Ku80 recruits DNA-PKcs to repair broken DNA using the NHEJ pathway (You and Bailis 2010). The MRN complex, on the other hand, engages ATM (Lee and Paull 2005), which is activated by autophosphorylation (Bakkenist and Kastan 2003) to promote repair by HR. A crucial mediator in the ATM pathway is CHK2 kinase, which spreads the signal throughout the nucleus (Zannini et al. 2014). Local signaling induced by the

phosphorylation of histone H2AX is important because it helps to recruit DNA repair factors and chromatin-modifying components that allow the efficient repair of the lesion (Huen and Chen 2008).

Many cellular processes are reorganized as a result of ATM activation by targeting DNA repair proteins, transcription factors, cell cycle regulators, and the apoptosis machinery. One of the most well-known targets of ATM is the tumor-suppressor protein p53, which is phosphorylated by both ATM (Banin et al. 1998) and CHK2 (Hirao et al. 2000). The p53 transcription factor has a central role in responses to a diverse array of stress signals, and it coordinates cellular responses by inducing transient cell cycle arrest or by triggering the apoptosis or senescence of the cell. Other ATM pathway targets include Cdc25A and Cdc25C, which participate in G1/S and G2/M checkpoint activation to coordinate cell cycle progression with DNA repair activities (Matsuoka et al. 1998; Falck et al. 2001).

Response to DNA replication stress

The second important part of the DDR is the response to DNA replication stress (Lopez-Contreras and Fernandez-Capetillo 2010). Replication stress is manifested as stalled replication forks, which can be caused by many things, including unrepaired DNA lesions (such as interstrand cross-links), mis-incorporated ribonucleotides, DNA secondary structures, collisions with the transcription machinery or a shortage of nucleotides (Zeman and Cimprich 2014). The role of the DDR in this situation is to stabilize and help to restart stalled replication forks so that DNA duplication can be successfully completed without generation of any further damage to the cellular genome.

During the DSB response, the activating DNA structure that is recognized by DDR components is a free DNA end. During replication stress, the DNA structure that is recognized is a stretch of single-stranded DNA (ssDNA) that is bound by RPA adjacent to double-stranded DNA (dsDNA)(MacDougall et al. 2007). RPA is required for processes that involve ssDNA intermediates, including DNA replication, during which it helps to protect ssDNA from nucleases and prevents the formation of hairpin structures (Fanning et al. 2006). The structure that activates the DDR forms when replicative helicase is uncoupled from DNA polymerase and long stretches of ssDNA are generated next to newly synthesized dsDNA (Byun et al. 2005). The DDR response is initiated after a dimer of ATR and its obligate partner ATRIP, recognizes and localizes to the site of DNA damage (Zou and Elledge 2003). This is followed by the recruitment of the RAD9-RAD1-HUS1 (9-1-1) complex, which brings the crucial activator DNA topoisomerase 2-binding protein 1 (TopBP1) to the site and triggers ATR kinase activity (Kumagai et al. 2006; Delacroix et al. 2007).

The ATR kinase plays a central role in the following DDR responses by transmitting a signal to a large number of substrate proteins. One of the best-studied ATR substrates is checkpoint kinase-1 (CHK1). CHK1 arrests the cell

cycle at the G2/M checkpoint by regulating Cdc25 proteins and thereby preventing cells with damaged or incompletely replicated chromosomes to enter into mitosis (Furnari et al. 1997). Another goal of ATR signaling is to stabilize replication forks to maintain fork integrity during replication arrest. Finally, ATR substrates include proteins that directly participate in DNA repair, including BRCA1 and Bloom syndrome protein (BLM) (Tibbetts et al. 2000; Davies et al. 2004).

Viruses and DNA damage response

The DDR plays such an important role in eukaryotic cells that viruses cannot ignore it while replicating their own genomes. The relationship between viruses and the DDR is two-fold. First, the DDR is a potent antiviral defense mechanism that the virus must inactivate to successfully replicate its genome. On the other hand, the DDR is a set of powerful pathways that are able to control many cellular processes, and it therefore represents a valuable opportunity for the virus to hijack for its own benefit.

The DNA damage response as an antiviral mechanism

It can be argued that there are two general themes to explain how DDR activation can hinder viral replication and why viruses must inactivate parts of the DDR for successful infection. First, the DDR can work directly as an antiviral mechanism by recruiting DNA repair proteins to inactivate the viral genome. For example, in the context of an adenovirus infection, viral proteins block the DDR by targeting MRN proteins. Viral mutants defective in MRN inactivation exhibit severe replication deficiency resulting from the intrinsic antiviral activity of the MRN complex (Shah and O'Shea 2015). The second reason for inactivation of the DDR is to eliminate its indirect negative effects on viral replication. DDR activation can cause extensive changes in the cellular environment, including the activation of cell cycle checkpoints or apoptosis, which might be detrimental for the virus. An example of this approach is the HPV oncoprotein E6, which deregulates DDR by inducing the degradation of the p53 protein, which is a central player in cellular response to stress.

Viruses counteract pathways that limit their replication by degrading, inactivating or relocating cellular proteins. Adenovirus, which has a linear dsDNA genome, must inactivate part of cellular DDR to avoid concatemerization of its genome by DNA repair proteins. This is achieved by viral oncoproteins that reorganize and degrade members of the MRN complex and thereby block the ATM pathway (Stracker et al. 2002). The Epstein–Barr virus (EBV), which is an oncovirus in the Herpesviridae family, inactivates ATR by increasing the expression of STAT3, which activates caspase 7 and induces the degradation of claspin, a crucial Chk1-regulatory protein (Koganti et al. 2014). Another herpes virus, herpes simplex virus 1 (HSV1), shuts down ATR signaling using viral

replication proteins that obscure the access of the 9-1-1 complex to DNA substrates that would normally activate the ATR pathway (Mohni et al. 2010; Mohni et al. 2013). Therefore, different from what has been observed in the previously mentioned viruses, HSV1 achieves the inactivation of a DDR pathway not by inducing protein degradation but by blocking the necessary relocation of DDR proteins. However, HSV1 uses a protein degradation strategy to inactivate the DNA-PK pathway by causing the active degradation of DNA-PKcs through the actions of the viral transactivator ICP0 (Lees-Miller et al. 1996). While members of Herpesviridae use several strategies to inactivate the ATR pathway, various parvoviruses have developed even more divergent relationships with this pathway. On the one hand, the parvovirus Minute virus of mice (MVM) inhibits ATR signaling, as was demonstrated by the impaired induction of Chk1 phosphorylation as a result of hydroxyurea (HU) treatment during late viral infection (Adeyemi and Pintel 2014). However, some other members of this small ssDNA virus family, including the Minute virus of canines (MVC) (Luo et al. 2011a) and the human parvovirus B19 (Luo et al. 2011b), activate ATR signaling.

Activation of DDR pathways for viral benefit

While there are many examples of inhibited DDR pathways during viral infections, there are just as many cases in which cellular stress pathways are activated during a viral infection. Measurements of the phosphorylation status of DDR components and results showing their co-localization with viral replication compartments have shown that many DNA viruses trigger DDR signaling. However, the activation of the DDR is not always detrimental to viral replication, but it can in certain infections be beneficial to it. The impact of DDR activation on viral DNA replication can be studied using small molecule kinase inhibitors or siRNAs to switch off specific pathways. This approach has been used to identify several viruses that depend on DDR proteins for the optimal replication of viral genomes, including Merkel cell polyomavirus (MCV) (Tsang et al. 2014), human cytomegalovirus (HCMV) (E et al. 2011), EBV (Kudoh et al. 2009), SV40 (Zhao et al. 2008), and HPV (Moody and Laimins 2009; Anacker et al. 2014).

While the number of viruses that have been shown to depend on DDR activation is growing, the exact mechanisms by which these pathways contribute to viral replication are in many of these cases not yet known. However, one of the best described examples is SV40 DNA replication. It has been proposed that this virus triggers the DDR through several different mechanisms. On the one hand, SV40's replicative helicase and its oncoprotein large T antigen (LTag) have been shown to cause damage to cellular DNA and to induce γH2AX formation when overexpressed (Hein et al. 2009). However, under native viral infection conditions, both ATM and ATR are activated not by LTag but by aberrant viral replication intermediates (Sowd et al. 2013). Both of these kinases

appear to be important for SV40 DNA replication because ATR helps to restart stalled viral replication forks, and ATM activity directs the choice of DSB repair pathway and thereby inhibits the concatemerization of viral genomes by the NHEJ (Sowd et al. 2013; Sowd et al. 2014).

Studies of SV40 indicate that DDR activation can increase the fidelity of viral DNA synthesis during phases of intense amplification of viral genomes and orchestrate the repair of replication-induced or other types of damage to viral DNA. However, in addition to protecting viral DNA, the role of the DDR in safeguarding the host genome may be equally important for a successful viral infection. Both the ATM and ATR kinases are activated during infection with BK polyomavirus (BKPyV), and inhibiting these pathways causes severe damage to host chromosomes and leads to a decrease in viral progeny (Jiang et al. 2012). DDR activation during a viral infection could therefore help by preventing detrimental effects to the host cell and thereby avoiding adverse consequences for the virus.

A final type of beneficial property conferred by viral-induced DDR activation is the adjustment of the cellular environment. Because DDR pathways can control many cellular processes, fine-tuning its activation state provides a useful opportunity for viruses to affect their host in favorable ways. As discussed above, some viruses might need to block DDR signaling to avoid detrimental cell cycle checkpoints, but there are also examples in which an arrested cell cycle is exactly what a virus needs for efficient replication. Many viruses have been shown to trigger G2/M cell cycle arrest (Davy and Doorbar 2007), and although the consequences of this activity for the virus life cycle are not always well defined, there are examples in which a clear beneficial effects have been shown for viral DNA replication. For example, the JC virus (JCV), a human polyomavirus, induces ATM- and ATR-mediated G2 checkpoint signaling, which clearly promotes viral genome replication (Orba et al. 2010).

DDR activation in the context of the HPV life cycle

The cellular environment is thoroughly reshaped during an infection with papillomaviruses. The HPV oncoproteins E6 and E7 play a major role in reshaping the environment by triggering cellular proliferation, blocking keratinocyte differentiation and promoting immune evasion (Moody and Laimins 2010). Among other cellular processes, HPV infection also affects DDR pathways. E7 expression deregulates cell cycle control and activates proteins in both the ATM and ATR pathways (Rogoff et al. 2004; Banerjee et al. 2011), while E6 expression prevents negative consequences, such as apoptosis, that would otherwise result from the uncontrolled DNA replication and the activation of DDR pathways.

The activation of DDR pathways is not a passive byproduct of HPV infection but is instead a crucial component of effective viral replication (McKinney et al. 2015). Inducing the inhibition of the ATM pathway using small molecule

inhibitors blocks HPV-productive amplification (Moody and Laimins 2009). However, inhibiting ATM did not affect stable replication, suggesting that different viral replication phases have distinct requirements for DDR components. Instead, the ATR pathway was shown to play an important role in controlling HPV episome levels during stable replication (Edwards et al. 2013).

HPV, like many other DNA viruses, replicates in distinct compartments of the nucleus that are called replication centers or foci (Swindle et al. 1999). These foci recruit cellular replication proteins and are the sites of viral DNA synthesis. Organizing these components into foci provides the advantage of allowing the recruitment of necessary proteins at high concentrations for efficient replication of viral genomes. In addition to virally induced global changes in the DDR state, HPV replication directly recruits DNA damage-sensing and repair proteins to its replication foci. A wide range of DDR proteins have been shown to co-localize with viral replication centers. For example, proteins including ATM, CHK2, MRN, BRCA1, pNBS1, Rad51 and γH2AX localize into stable or productive replication centers of the HPV genome (Moody and Laimins 2009; Gillespie et al. 2012; Anacker et al. 2014). The DDR is also involved in viral replication centers where the heterologously expressed E1 and E2 proteins replicate the viral origin-containing plasmid (Sakakibara et al. 2011) indicating that HPV replication proteins or replication intermediates are also potent activators of DDR pathways.

Evidence supporting the involvement of homologous recombination proteins, such as Rad51, in HPV replication centers suggests the intriguing possibility that recombination-dependent replication (RDR) might be used to synthesize new viral genomes (Sakakibara et al. 2013). Cells use RDR to restart replication forks and to thereby guarantee genome stability. Many dsDNA viruses have evolved the ability to hijack this mechanism for the origin-independent assembly of replisomes on viral DNA (Lo Piano et al. 2011). This strategy is beneficial for the viruses because it allows the initiation of new replication forks without requiring viral initiator proteins. The oligomerization of viral genomes in the U2OS cell line during transient replication provided further evidence showing that RDR can be employed to replicate HPV DNA (Orav et al. 2013). However, the mechanism by which HPV switches from E1-driven replication to RDR-driven replication is not known.

AIMS OF THIS STUDY

DNA replication of papillomaviruses has been thoroughly studied using the heterologous expression of viral replication proteins E1 and E2. These studies have described the E1-dependent initiation of viral DNA replication in a great detail. However, HPV genome replication has not been studied as thoroughly because it has a very complex viral life cycle and there is no available easy-to-use model system for this virus.

The general aim of this study was to develop a novel means for studying HPV genome replication and to use these methods to characterize aspects of viral genome replication, including the involvement of DDR components and the timing of viral DNA replication during the cell cycle. The specific aims of the study were as follows:

- To find and describe a monolayer cell line that can be used in HPV genome replication studies.
- To describe the DNA-damaging activity of the HPV replication protein E1 in a heterologous expression system and in a viral genome context.
- To characterize the DDR components that are recruited into HPV genome transient replication centers.
- To determine the timing of HPV DNA replication in the cell cycle during the initial amplification and stable replication phases of the viral life cycle.

MATERIALS AND METHODS

We used a monolayer cell line (U2OS) in these experiments. Cells were transfected with bacterially produced wild type (wt) or mutant viral genomes or plasmids that contained viral subgenomic regions. Mutant viral genomes that did not express certain viral proteins as a result of a frameshift mutation in the corresponding ORF were produced to characterize the involvement of individual viral proteins in the studied processes. In addition to viral genomes, plasmids containing viral non-regulatory sequences (URR) or expression constructs encoding the E1 or E2 proteins were also used in this study.

U2OS cells provide a suitable environment for the activation of HPV gene expression, and they can be transfected with very high efficiency using electroporation. This allows for the characterization of viral DNA replication in cells transfected with viral genomes. Geimanen et al. (publication I) was the first report to use U2OS cells for HPV genome replication studies. The benefits and drawbacks of this system are discussed in the results and discussion section of this thesis. Because U2OS cells provide suitable conditions for viral gene expression and genome replication, they can also be used to study the properties of single viral proteins. The transfection of expression constructs of viral proteins was used to characterize the properties of viral proteins alone and in combinations.

To achieve the aims of this thesis, a wide range of molecular biology techniques were used to characterize the levels and localization of viral and cellular proteins and viral DNA replication products and to analyze cellular consequences, such as the generation of DNA damage or changes in cell cycle profiles, in U2OS cells that were transfected with HPV genomes or viral protein expression vectors. The detailed descriptions of all the experimental protocols are given in the materials and methods sections of publications I, II and III. While most of the protocols used in this thesis have been widely used by the scientific community, we developed a novel method for the quantification of levels of newly synthesized DNA. In this method, replicating DNA is labeled with the nucleoside analogue EdU and subsequently purified from non-replicating DNA based on the presence of the EdU label. The labeled DNA is then quantified via qPCR to measure the level of newly synthesized DNA and to thereby estimate how actively viral DNA sequences are replicating at a given time point. A detailed description of the method can be found in publication III. The possible uses of this method in HPV research are discussed in the results and discussion section of this thesis.

RESULTS AND DISCUSSION

Characterization of a monolayer cell line that supports the efficient replication of HPV genomes

Many cell lines support the replication of HPV origin-containing DNA when the viral replication proteins E1 and E2 are expressed by heterologous expression vectors. However, no or only very low levels of viral DNA replication can be detected upon transfection of full viral genomes into most cell lines. The lack of a monolayer cell line that supports the efficient replication of the viral genome has complicated the research of HPV genome replication. For this reason, we looked for a cell line that would allow the detection of HPV genome replication in transient assays within a matter of days after DNA transfection.

We found that the human osteosarcoma U2OS cell line, which has a flat epithelial morphology and carries wild-type p53 and pRB genes, supported very efficient replication of HPV genomes in transient assays (I, Fig. 1). A strong DpnI-resistant signal was detected using Southern blot analysis within a matter of days following the transfection of HPV genomes into U2OS cells (I, Fig. 1). DpnI digests only bacterially methylated DNA and therefore allows differentiation between the DNA that was synthesized by the eukaryotic cells from the transfected DNA. The genomes of high-risk (HPV16 and HPV18), low-risk (HPV11 and HPV6B), and beta (HPV5 and HPV8) HPV types were shown to replicate with high efficiency in transient assays. The ability of these cells to support efficient genome replication of several HPV types representing divergent viral subgroups is a remarkable feature of the U2OS cell line (Fig. 1).

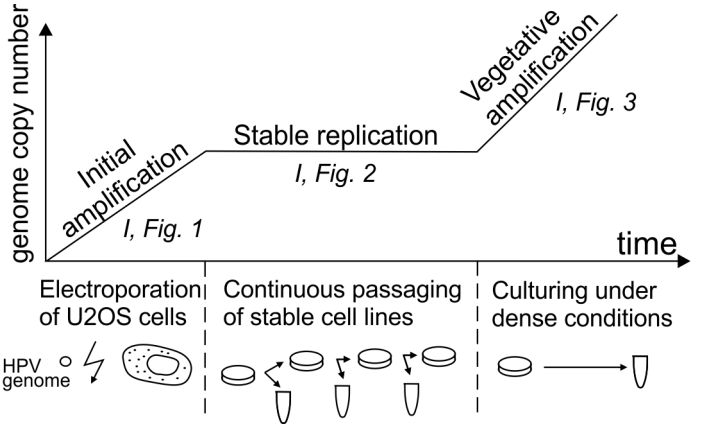

Figure 1. Schematic showing the U2OS cell line-based model system used to study the replication of HPV genomes. See the text for details.

During a natural viral life cycle, the initial amplification stage is followed by a stable maintenance phase, during which the viral genome copy number is maintained at a constant number for long periods of time. We isolated U2OS cell lines that contained episomal HPV DNA and observed that the viral genome copy number in these cells was maintained at a constant level for months during continuous passaging (I, Fig. 2). This finding indicates that U2OS cells provide suitable conditions for stable replication and for the segregation of HPV genomes, both of which are required for successful episomal maintenance. While the stable replication of high-risk HPV types can be studied in cell lines generated from HFK cells, HFK cells cannot be used to create cell lines that maintain low-risk HPV or beta-papillomavirus genomes. U2OS cells, on the other hand, support stable replication of HPV types representing both of these two viral groups. The opportunity that U2OS cells provide for designing experiments to compare stable replication of high- and low-risk HPV types is extremely valuable in terms of how much such experiments can increase our understanding of HPV-induced carcinogenesis.

Because U2OS cells supported the first two replication phases of HPV life cycle, the next logical step was to determine whether the last phase, vegetative amplification, could also be modeled using this system. We explored methods for triggering viral genome amplification in HPV-positive U2OS cell lines, and we found that growing the cells as confluent cultures without splitting for approximately ten days led to a significant increase in viral genome copy numbers (I, Fig. 3). In the HPV18-positive 18#1.13 cell line, the viral genome copy number increased from a stable level of 100 copies per cell to 1500 copies per cell over a 12-day growth period. This increase in the viral genome copy number closely resembles the increase observed during HPV vegetative amplification in HFK cells.

Viral gene expression is reorganized in differentiating keratinocytes, and this results in the increased expression of viral replication proteins and the induction of genome amplification (Bedell et al. 1991; Ozbun and Meyers 1998a). To test whether similar changes in viral transcript levels also take place in dense U2OS cell cultures, we used reverse transcriptase PCR (RT-PCR) with viral ORF-specific primers to quantify mRNA levels during amplification (I, Fig. 3F). This analysis demonstrated that viral transcription was indeed upregulated in confluent U2OS cultures, demonstrating that the viral genomes were transcriptionally active in the U2OS cell lines and that they responded to the cellular changes that accompanied growth under such dense conditions. Moreover, the dynamics of E1 and E2 ORF-containing mRNAs resembled those that were observed in HPV-positive cell lines that were allowed to differentiate in HFK raft cultures. We documented a 20-fold increase in E1-containing transcripts, while the levels of E2-containing mRNAs increased five-fold during a two week amplification experiment. Importantly, a similar increase in the E1 to E2 RNA ratio was also described during HPV vegetative amplification in HFK raft cultures (Ozbun and Meyers 1998a). The increase observed in E1 mRNA levels preceded a sharp increase in the viral genome copy number. These results indicate that HPV

genome amplification in U2OS cells, similar to what has been observed in HFK cells, is triggered by an increase in the levels of viral replication proteins.

Altogether, these experiments demonstrate that the U2OS cell line is suitable for modeling all the three phases of HPV genome replication. This is a valuable addition to the model systems that were previously available for HPV genome replication studies, and it has its own clear advantages over the others. Although HFK cells are natural hosts of HPV, there are clear drawbacks to using these cells rather than U2OS cells. First, unlike experiments involving U2OS cells, it is complicated to study genome replication of low-risk and beta HPV types in HFK cells. Second, because only low transfection efficiency can be achieved in HFKs, the initial amplification of the HPV genome cannot be examined. Finally, growing primary HFK cells is a more complicated and costly procedure than using fully transformed U2OS cells. However, the fully transformed nature of the U2OS cells is the biggest weakness of the cell line with regard to HPV research because it leaves open the possibility that some cellular pathways that are important during HPV infection might be altered and viral replication in this cell line may not accurately reflect what occurs in natural HPV host cells. However, we have shown that there are many similarities between HPV replication in U2OS cells and what has been described in HFKs. A careful analysis of the HPV18 transcription map in U2OS cells (Toots et al. 2014) showed almost complete overlap with the HPV transcription map in HFK cells (Wang et al. 2011). We have used this system to further characterize the replication and transcription properties of HPV11, a low-risk alpha PV (Isok-Paas et al. 2015), and HPV5, a beta-PV (Sankovski et al. 2014). Until now, genome replication of PV strains in these two groups has been difficult to study because there has been a lack of suitable cell lines in which to conduct such studies. All of these observations suggest that U2OS cells provide a valuable model system that can be used to explore and compare genome replication in divergent PV types.

The HPV E1 protein activates DDR pathways by causing DNA double-strand breaks

The DNA damage response has emerged as an important contributor to the life cycle of many DNA viruses. We showed that HPV can activate DDR by initiating replication from an integrated viral origin (Kadaja et al. 2009). However, this study also hinted that expressing the HPV replication protein E1 in HPV-positive HeLa cells could single-handedly activate the ATM pathway. Activation of the ATM pathway by the E7 protein was also shown to be crucial for the efficient replication of HPV genomes during vegetative amplification (Moody and Laimins 2009). These studies demonstrated the importance of DDR pathways in the viral life cycle and raised questions regarding the involvement of other potentially activated DDR pathways (besides ATM) and additional activation mechanisms. We decided to use an HPV-negative U2OS cell line to identify HPV E1 protein interactions with the cellular DDR machinery (II, Fig. 1–5).

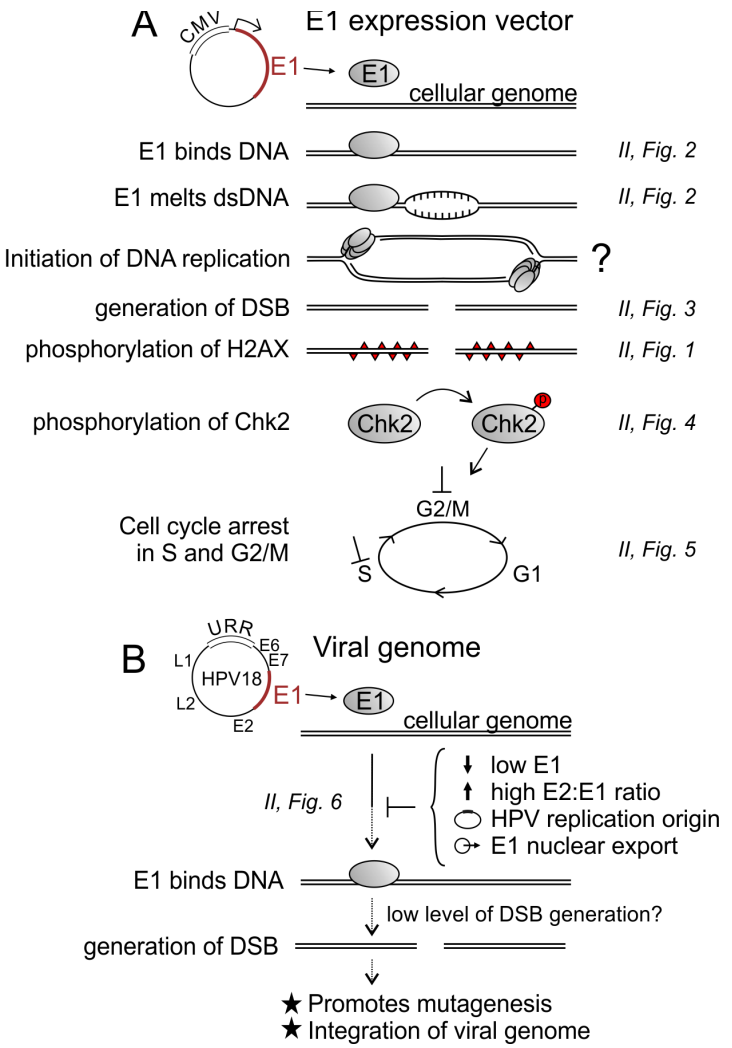

Figure 2. HPV E1 causes damage to the host genome. Heterologously expressed HPV E1 proteins caused DNA double-strand breaks in the host genome, possibly through initiation of uncontrolled DNA replication, resulting in the activation of the DDR and cell cycle arrest (A). However, when similar levels of E1 protein were expressed from a viral genome, they did not cause comparable levels of activation of DDR pathways (B). Low levels of the E1 protein, a high E2 to E1 ratio, the presence of an HPV replication origin or the nuclear export of the E1 protein could minimize the amount of DNA damage that was generated by the E1 protein in the context of a replicating viral genome. However, even a low level of DSB generation may promote mutagenesis or the integration of the viral genome, which can contribute to HPV-induced carcinogenesis.

We found that expressing the HPV E1 protein from expression constructs in the U2OS cell line caused the phosphorylation of histone H2AX, a marker of DNA damage on chromatin (Fig. 2). We observed a dose-dependent response when

E1 was expressed alone or with the E2 protein (II, Fig. 1). E1 proteins isolated from both high-risk types (HPV18 and HPV16) and low-risk types (HPV11 and HPV6B) induced the formation of γH2AX, indicating that this is a general feature of E1 proteins and that this property is not limited to cancer-causing high-risk virus types. We generated a series of E1 mutants that lacked certain core activities of the E1 protein to test which functions of the E1 protein are required to induce the formation of γH2AX (II, Fig. 2). The E1 protein was found to depend on its ability to hydrolyze ATP and melt dsDNA, while its sequence-specific DNA binding ability was not necessary to cause histone H2AX phosphorylation.

The formation of γH2AX indicates that high level of expression of the E1 protein caused DNA damage, most likely as DNA double-strand breaks (Bonner et al. 2008). The presence of γH2AX is strongly related to DSBs; however, other possible causes of histone H2AX phosphorylation, including DNA replication stress (Ward and Chen 2001) or the stable association of repair factors with intact chromatin (Soutoglou and Misteli 2008), have also been described. Thus, to confirm that DSBs are the cause of E1 protein-induced H2AX phosphorylation, we used single-cell gel electrophoresis assay to demonstrate the presence of these dangerous DNA lesions in E1-expressing cells (II, Fig. 3). This technique allows for a comparison of the amount of double-strand DNA breaks contained in cells from two cell populations. We found that cells expressing the E1 protein contained more double-strand DNA breaks than the mock-transfected cells, indicating that the expression of the HPV main replication protein can cause DSBs in the cellular genome. This experiment therefore confirmed previous suggestions based on the TUNEL assay that E1 expression causes DNA brakes into the host genome (Sakakibara et al. 2011).

Based on our analysis of H2AX phosphorylation and single-cell gel electrophoresis experiments, we conclude that the E1 protein can damage host DNA by directly interacting with it to cause double-strand DNA breaks. A number of mechanisms could explain how the E1 protein damages the host genome. One way in which the E1 protein could cause genomic instability is by sequestering cellular replication proteins from chromatin through direct protein-protein interactions. However, the inability of an E1 dsDNA melting mutant to induce the formation of γH2AX demonstrates that this is not the case, and the viral protein must instead directly interact with the host DNA to activate the DDR. dsDNA melting refers to the prying open of the dsDNA into two single strands, which is the first task of the E1 protein during the initiation of DNA replication (Liu et al. 2007). Because this process is followed by the loading and activation of the full replicative helicase during the initiation of papillomavirus DNA replication, it is possible that double hexameric E1 helicase is also loaded onto the host DNA in E1-expressing cells. Moreover, the HPV E1 protein is capable of starting origin-independent replication in *in vitro* settings (Kuo et al. 1994), so one could argue that an already loaded E1 helicase could recruit cellular replication proteins to initiate uncontrolled DNA replication of the host DNA. This type of replication would be analogous to E1- and E2-dependent replication from an

integrated HPV origin, which causes genomic instability in HeLa and SiHa cells (Kadaja et al. 2007; Kadaja et al. 2009). The E1-dependent replication forks that are generated by the uncontrolled initiation of replication can have only limited processivity and will eventually stall. Stalled forks can be converted to DSBs either by spontaneous breakage or through cleavage by structure-specific nucleases, such as Mus81 (Hanada et al. 2007). Thus, it seems very likely that E1 causes DNA damage by initiating the uncontrolled replication of host DNA, which results in DNA breaks after stalling of the replication forks.

In eukaryotic cells, a strong cellular response is triggered as a result of the formation of a DSB. One of the important consequences that results from cellular DSB recognition is the arrest of the cell cycle. We were next interested in exploring the cellular consequences of E1-generated DNA damage. First, we demonstrated that global DNA damage signaling is activated by showing that the kinase Chk2 is phosphorylated in response to E1 protein expression (II, Fig. 4). Chk2 is the central messenger of the ATM pathway, and its function is to transmit the DNA damage signal to downstream effector proteins (Zannini et al. 2014). Effector proteins, including p53, Cdc25A and Cdc25C, are responsible for inducing the arrest of the cell cycle at the G1/S, G2/M or intra S-phase checkpoints. Two of these checkpoints, the G2/M and intra S-phase checkpoints, are activated in E1-expressing U2OS cells, as shown in flow cytometric analyses of cell cycle profiles (II, Fig. 5). This finding is consistent with previous findings of E1 induced cell cycle checkpoint activation (Fradet-Turcotte et al. 2011).

These experiments clearly demonstrate that the expression of the E1 protein from heterologous expression constructs damages the host genome and triggers the DNA damage response and cell cycle arrest. However, the level of E1 expression is considerably lower during a normal viral life cycle, and it is therefore not clear whether E1 activity has a role in natural infection. Our next goal was to explore the role of the DNA-damaging activity of the E1 protein during transient replication of HPV genomes. We transfected U2OS cells with wild type HPV18 or HPV18/E8- mutant genomes. The E8- mutant genome does not express the E8/E2 repressor and is therefore capable of approximately ten-fold higher levels of transient replication than the wt genome (Kurg et al. 2010). We used the E8- mutant because it displays very high genome replication levels and can therefore be used to identify the smallest of effects that the genome-expressed E1 protein might have on cellular DDR activation. However, the E1 protein that was expressed by the viral genomes did not appear to activate the cellular DDR at levels comparable to those observed following heterologous expression of the protein. We observed that transient replication of either the wild-type HPV18 genome or the E8- genome failed to induce cell cycle arrest (II, Fig. 6). Furthermore, E1-induced DDR activation was not necessary for viral DNA replication because the addition of the ATM small molecule inhibitor KU559933 did not decrease the replication levels of the wt or E8- genomes (II, Fig. 6E). However, most importantly, we observed that a similar level of E1 expression from replicating viral genome induced a much lower

level of γH2AX formation compared to the E1 expression that was obtained using heterologous vectors (II, Fig. 6D).

Our observation that viral genome-expressed E1 protein does not induce large-scale DDR activation indicates that the DNA damaging activity of the E1 protein is suppressed during the transient replication of the viral genome. The suppression of this E1 activity during the viral life cycle could be accomplished in several different ways. For example, coexpression of the E2 protein has been shown to reduce E1-dependent DDR activation (Fradet-Turcotte et al. 2011), and although we did not observe this effect in our experiments, this inhibitory effect might depend on precise expression levels and ratios of the viral proteins. Indeed, altering the relative amounts of E1 and E2 has been shown to affect the DNA binding pattern of viral replication proteins (Frattini and Laimins 1994), and E2 binding to E1 has been shown to shield the E1 protein domain that mediates non-specific DNA binding and to thereby increase its origin specificity and decrease non-specific binding to host DNA (Stenlund 2003a). We observed in our experiments that the presence of a viral origin in the cells moderately decreased E1 DNA-damaging activity (II, Fig. 1), and this could be another reason why E1-generated damage to the host DNA is not as prominent during viral genome replication. Finally, HPV reduces the detrimental effect of E1 on host cells by minimizing the time that the E1 protein stays in the nucleus by exporting it to cytoplasm for the G1 phase of the cell cycle (Fradet-Turcotte et al. 2011) and by maintaining the expression of the protein at very low levels.

Although the ability of the E1 protein to damage host cell DNA may be not necessary for viral DNA replication, even a small amount of E1-generated damage to the cellular genome could have a major effect on cancer formation. First, DNA double-strand breaks are extremely dangerous DNA lesions that can induce mutagenesis if left unrepaired or repaired incorrectly. Furthermore, DSBs in the host genome are thought to promote HPV genome integration (Winder et al. 2007); therefore, this E1 activity could increase the probability of this crucial event in HPV-induced carcinogenesis (Wentzensen et al. 2004; Pett and Coleman 2007). However, this E1 activity might also be important after the integration of viral genomes. The expression levels of E6 and E7 increase after viral genome integration (Jeon and Lambert 1995), and the same thing could happen to E1 expression, which would increase the amount of E1-generated damage to host DNA. While more DNA damage could result in a higher mutation rate and promote carcinogenesis, too much damage can be toxic and become a disadvantage during clonal selection. The latter is supported by observed inhibition of cellular proliferation as a result of E1 protein expression in cell culture (Fradet-Turcotte et al. 2011; Sakakibara et al. 2011). Mapping the viral genome brake points in cervical cancer specimens showed that the E1 ORF is a preferred brake point in viral integrated sequences (Hu et al. 2015), indicating that the elimination of E1 expression could confer a growth advantage to a pre-cancerous cell.

HPV genome replication centers engage DDR factors from the ATR pathway

HPV genome replication did not lead to a large-scale activation of the DDR that would be capable of causing an increase in overall γH2AX levels (II, Fig. 6). Nevertheless, the local activation of DDR pathways in viral replication foci might be triggered. Like many DNA viruses, genome replication of HPV is localized to distinct nuclear foci. It has been previously shown that HPV genome replication centers recruit activated members of the ATM pathway, including CHK2, MRN, BRCA1 and ATM, during the stable maintenance and vegetative amplification phases in keratinocyte cell lines (Moody and Laimins 2009; Gillespie et al. 2012; Anacker et al. 2014). We were therefore interested in whether HPV replication centers are present during the transient replication of viral genomes in the U2OS cell line and whether these foci also contain activated DDR components (II, Fig. 7). To increase the sensitivity of our detection methods, we used the HPV18/E8- genome, which replicates at higher levels than the wt genome, and employed fluorescence in situ hybridization (FISH) to visualize viral genomes during transient replication in U2OS cells. We found that the viral genome was concentrated into distinct foci in HPV-positive cells and that these centers also contained γH2AX (II, Fig. 7A). To confirm that the HPV genome- and γH2AX-containing centers were the sites of viral DNA replication, we performed co-immunostaining for the E1 protein and γH2AX and we used EdU incorporation to reveal the sites of ongoing DNA synthesis (II, Fig. 7B). We detected significant co-localization of E1 and γH2AX at foci in U2OS cells that were transfected with HPV18/E8- genomes. The E1 and γH2AX foci also contained the EdU signal, demonstrating that viral genome replication occurs at these sites.

The HPV oncoproteins E6 and E7 are known activators of the DDR (see (Moody and Laimins 2010) for a review). Expressing the E7 protein alone in HFK cells activates the ATM (Rogoff et al. 2004) and ATR (Banerjee et al. 2011) pathways. However, the observed activation of the DDR in HPV genome replication centers was not dependent on viral oncoproteins because mutant viral genomes, which do not express E6 or E7, continued to recruit γH2AX to their replication foci. These results indicate that viral DNA replication intermediates, and not viral oncoproteins, trigger DDR activation in HPV replication foci.

We did not detect changes in the cell cycle profiles of HPV18 genome-transfected U2OS cells (II, Fig. 6B and 6C) or the large-scale activation of H2AX (II, Fig. 6D). This suggests that H2AX is phosphorylated only on viral genomes and that no global DDR was activated. We argue that replication stress may cause γH2AX to form on viral genomes. H2AX is phosphorylated by ATR as a result of UV- or HU-generated replication stress (Ward and Chen 2001). Indeed, components of the ATR pathway appeared to localize to viral transient replication centers (II, Fig. 8). ATRIP and TopBP1, which are two crucial

partners for ATR activation, localized to HPV18/E8- genome foci, indicating that the ATR pathway was engaged in viral DNA replication (Fig. 3).

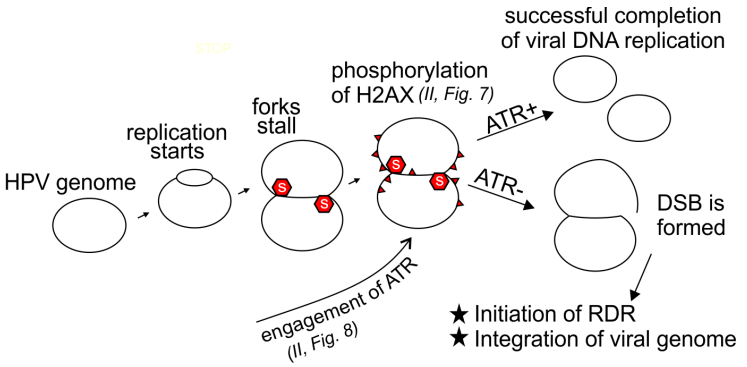

Figure 3. The ATR pathway is engaged at HPV genome replication centers. Transient replication centers of the HPV genome contain phosphorylated histone H2AX and the ATR pathway components ATRIP and TopBP1, indicating that DNA replication stress arises during viral DNA synthesis. We speculate that replication fork stalling, which is a major characteristic of DNA replication stress, causes the observed engagement of ATR. ATR engagement might be necessary to restart the forks to successfully complete the stalled replication or HPV may deregulate normal ATR signaling to produce the substrates that are necessary for initiating recombination-dependent replication.

The most straightforward explanation for these observations is that the replication stress that arises during HPV genome replication engages the ATR pathway in viral DNA replication foci. DNA replication stress is characterized by stalled replication forks that are caused by problems in the movement of the replication fork (see (Zeman and Cimprich 2014) for review). Unrepaired DNA lesions, collisions with the transcription machinery, and a deficiency in nucleotides or components of the replication machinery can cause replication stress in the host genome. Some DNA sequences, such as trinucleotide repeats or GC-rich areas, are intrinsically challenging for the replication machinery to replicate. The circular form and small size of the HPV genome might complicate the replication process because more prominent topological tensions can arise during the unwinding of such a template. These factors might be responsible for causing replication fork stalling during HPV transient replication, which would result in the observed ATR engagement.

HPV may benefit in different ways from the engagement of ATR pathway proteins in its genome replication foci. First, ATR signaling might be activated, as is the case in SV40 genome replication. The activation of ATR is crucial for the replication of the small dsDNA genomes of SV40 because it is required to promote the repair of stalled forks (Sowd et al. 2013). ATR inhibition causes the converging replication forks of SV40 to stall and break, resulting in the

accumulation of aberrant replication products and unfinished genome replication. Thus, similar to what has been observed in SV40, the engagement of ATR in HPV replication foci may be necessary to protect the forks and to successfully complete the replication of the viral circular genome.

However, it is also possible that ATR components are recruited to HPV genome replication foci, but that ATR signaling is not activated. Several viruses, including HSV1 and MVM, recruit ATR to their replication centers but at the same time inhibit ATR signaling (Mohni et al. 2010; Adeyemi and Pintel 2014). The HPV16 E7 protein induces the degradation of claspin, which is an important regulator of ATR signaling (Spardy et al. 2009). It is therefore possible that HPV deregulates ATR signaling and thereby directs the choice of pathway used to restart replication in a way that initiates recombination-dependent replication (RDR) of the viral genome. The inhibition of ATR leads to the conversion of stalled replication forks to DSBs during SV40 genome replication (Sowd et al. 2013). Invasion of a resectioned ssDNA end, originating from a DSB, into an intact DNA molecule was proposed to initiate recombination-dependent replication of HPV genomes (Orav et al. 2015). ATR deregulation by HPV might therefore provide the substrates that are necessary for the initiation of RDR to replicate viral genomes.

The tendency of HPV DNA replication to cause stalling of replication forks might play a major role in virally induced carcinogenesis by promoting the integration of the viral genome. It was recently predicted that the main mechanisms that are responsible for HPV genome integration are microhomology-mediated DNA repair pathways, such as fork stalling and template switching (FoSTeS), and microhomology-mediated break-induced replication (MMBIR) (Hu et al. 2015). Stalled viral replication forks could provide substrates for these pathways, and if the corresponding microhomology is found in the host chromosome, the integration process could be initiated.

The timing of HPV18 DNA replication in the U2OS cell line

DNA replication in eukaryotic cells takes place during a specific time period in the cell cycle: the S- phase. During the S-phase, cellular replication proteins are active, and they duplicate the DNA genome. HPV genome replication depends on the same proteins that replicate cellular DNA. However, it has been shown that the most intensive DNA replication in the HPV life cycle, which occurs during vegetative amplification, does not take place during the S-phase, but instead takes place during the G2-phase (Wang et al. 2009).

In our experiments aimed at characterizing HPV18 genome transient replication centers, we observed that DNA replication (indicated by incorporated EdU signal) in HPV-positive cells took place only in viral replication foci (II, Fig. 7). Furthermore, the overall EdU signal in the HPV-positive cells was much weaker than that in the HPV-negative S-phase cells, which showed bright,

diffuse staining. These observations suggested that viral and cellular DNA replication did not take place concurrently in U2OS cells. We argued that similar to vegetative amplification, the initial amplification of viral genomes could take place during the G2-phase of the cell cycle.

Our next aim was to measure the timing of the transient and stable replication of the HPV genome in the U2OS cell line. Cell pools that contained stably replicating HPV genomes were created to study the stable maintenance phase of the viral life cycle. These cells contained mostly monomeric episomal viral genomes, and the viral genome copy number remained constant during a two-week growth period in these cultures (III, Fig. 1). The constant copy number and the episomal state of the viral genomes are characteristics of papillomavirus stable replication that demonstrate that these cell pools can be used to study the stable maintenance phase of the viral life cycle. The initial amplification phase was studied by transiently transfecting U2OS cells with HPV18 genomes (as in I, Fig. 1 and II, Fig. 6).

Until now, the only method used to measure viral replication activity was to determine the genome copy number either using Southern blot analysis or qPCR. This measure reveals how much viral DNA is in the cells, and by comparing several time points, an estimate for how actively the viral genome is replicating can be obtained. Usually, time points are taken in 24-hour increments to clearly detect the changes between them. We developed a method that allowed us to estimate the level of viral genome replication at a given time point that did not involve comparing two time points that were 24 hours apart. This method allowed us to measure viral DNA replication activity during different cell cycle phases by combining our protocol with cell cycle synchronization.

This assay relies on pulse labeling newly synthesized DNA with the nucleoside analogue EdU (assay scheme shown in III, Fig. 2A). Newly synthesized DNA is then biotinylated and purified from equal amounts of total DNA using streptavidin-conjugated beads and quantified using qPCR. We first validated this method during viral transient and stable replication and demonstrated its sensitivity to the replication inhibitor aphidicolin (Fig. 4 and III, Fig. 2C-D).

Pulse labeling with nucleoside analogues and the subsequent purification of labeled DNA have been used to purify replication fork-associated proteins (Kliszczak et al. 2011; Sirbu et al. 2012; Alabert et al. 2014) or to determine the timing of latent replication in herpes viruses (Vogel et al. 2010). The protocols used in these studies have relied on the same approach, including the pulse labeling and the purifying of the DNA that was synthesized just before cell lysis. However, other studies have not used EdU labeling in combination with qPCR to quantify newly synthesized DNA.

There are number of circumstances in which this assay could be useful for HPV replication studies. In the context of a viral genome, the DNA copy number is a function of segregation and replication efficiency, which makes it difficult to fully explain the changes observed in viral genome levels based on only this one measure. For example a mutation in a viral genome or the inhibition of a cellular protein could reduce the viral copy number either by decreas-

ing viral DNA replication levels or by making viral genome segregation less efficient. Our method helps in determining the reasons for the differences that can be observed in viral genome copy numbers between such samples. Another potential use for this method is to characterize the immediate effects of small molecule inhibitors on viral DNA replication. Some small molecule inhibitors can be toxic to cells when applied for long periods of time. The viral genome copy number can, in some cases, be lowered as a result of indirect effects even when the inhibited cellular proteins are not directly involved in viral DNA replication.

To measure the timing of the replication of the HPV genome, we combined this method with cell cycle synchronization. Transiently transfected U2OS cells and cell pools stably maintaining HPV18 wt genomes were synchronized into mitosis. After release, the cell populations progressed through one cell cycle in approximately 20 hours. We tested several time points during the 20 hour growth period to quantify the levels of newly synthesized viral and cellular DNA that were labeled during different cell cycle phases (III, Fig. 3). We found that the stable replication of the HPV18 genome followed the timing of host cell DNA replication, which take place during the S-phase of the cell cycle. However, transient replication clearly peaked at time points during which cellular DNA replication levels had already decreased. This finding suggests that the transient replication of HPV genomes starts in S-phase but continues during G2-phase, when it reaches its maximum level (Fig. 5). We confirmed this conclusion by co-immunostaining cells to identify viral replication centers and to label G2-phase cells using the marker cyclinB1. This analysis showed that large fraction of cells that contained HPV18 wt (III, Fig. 3G) or HPV18/E8- (II, Fig. 9) genome replication centers were in the G2-phase of the cell cycle. It is interesting that G2-phase is used for viral DNA replication during the amplification of viral DNA, while stable replication takes place only in S-phase. These results indicate that HPV uses different mechanisms for its genome replication during different phases of its life cycle. It is possible that stable replication is performed by solely cellular proteins and that no viral proteins are needed, as was previously suggested in a report that demonstrated that E1 protein is not required for the stable maintenance of HPV16 genomes (Egawa et al. 2012).

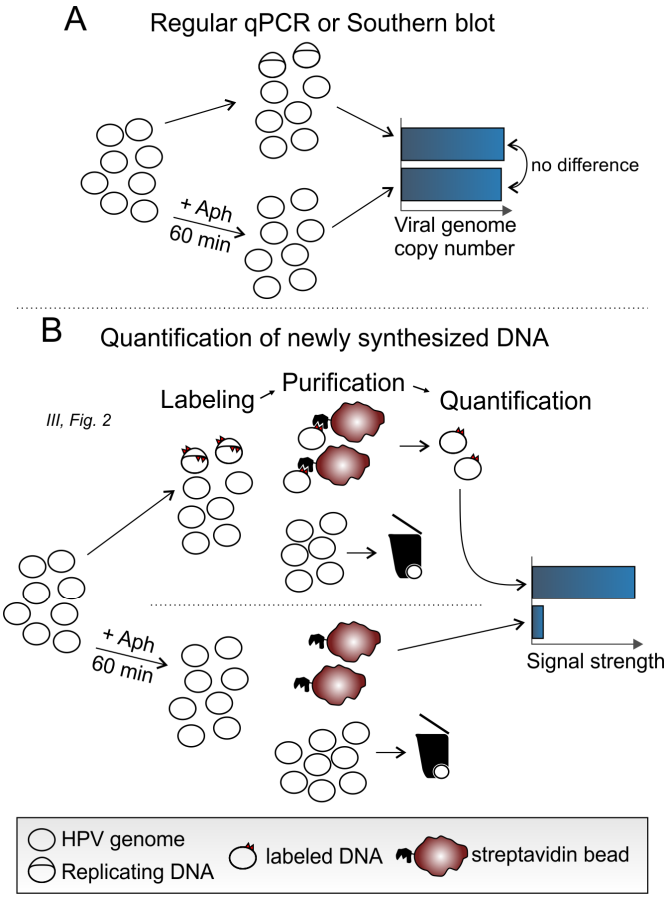

Figure 4. A novel assay for the quantification of newly synthesized DNA. The working principle of this assay is demonstrated using an example in which we measured the effect of the replication inhibitor aphidicolin. A short incubation time (60 minutes) does not cause a large enough difference in the viral genome copy number to be observed using conventional methods for estimating HPV replication levels (A). The new method involves pulse labeling newly synthesized DNA with the nucleoside analogue EdU (B). A short pulse time (usually 1 hour) guarantees that only the currently replicating DNA is labeled. The viral genome copies that are not replicating during the EdU pulse are not labeled and are therefore discarded during the purification process. The purified DNA is quantified using qPCR, and the signal strength indicates how actively the viral DNA was replicating in the cell population during the pulse.

The expression levels of the E1 and E2 proteins are low during the stable maintenance phase, when viral DNA is replicated only in S-phase, and much higher during the initial and vegetative amplification phases, when HPV DNA replication occurs during the G2-phase. This suggests the hypothesis that the expression levels of HPV replication proteins control the timing of viral DNA replication. Indeed, when we increased the expression levels of the E1 and E2

proteins by transfecting stable HPV cell pools with expression constructs, the viral genome replication that previously took place only in S-phase extended into the G2-phase (III, Fig. 4). We also found that no other viral proteins were needed for viral DNA replication to occur during the G2-phase. If a viral replication origin-containing plasmid was cotransfected with E1 and E2-expressing constructs, a significant fraction of the cells that contained viral replication foci were also positive for cyclinB1 (II, Fig. 9).

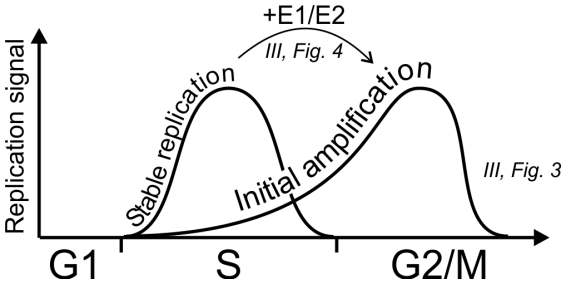

Figure 5. The timing of HPV DNA replication during the cell cycle. There is a difference in HPV replication timing between the stable replication and initial amplification phases. HPV stable replication takes place exclusively in S-phase, but the initial amplification begins in S-phase and reaches maximum levels in G2-phase. The timing of HPV replication is controlled by E1 and E2 expression. When E1/E2 expression was increased in cells that stably maintained viral genomes, the S-phase-only timing of the replication changed, and replication extended into the G2-phase.

HPV is one of many viruses that deregulate the cell cycle to cause cell cycle arrest by activating the G2/M checkpoint (Davy and Doorbar 2007). Viral manipulations of the cell cycle seem to be a useful adaption for efficient viral genome replication, but little is known regarding the exact mechanisms and consequences of viral DNA replication during the G2-phase of the cell cycle. The most straightforward explanation for viral replication taking place in the G2-phase is that it would avoid competition for DNA synthesis resources, including nucleotide pools and replication proteins. It has been suggested that the expression of the E7 protein induces the establishment of a pseudo-S-phase state by continuing the expression of S-phase genes in G2-arrested cells (Galloway 2009). Therefore, the E7 protein would counteract the decrease in the activity of cellular replication proteins, such as DNA polymerase alpha-primase (Voitenleitner et al. 1999), that occurs after the completion of the S-phase. Our finding that the E7 protein is not necessary for the creation of HPV replication foci in G2-phase indicates that the establishment of a pseudo-S-phase might not be needed to replicate viral DNA in the G2-phase. We suggest that HPV might instead use RDR during its genome replication in G2-phase because homologous recombination pathways are equally active during the S- and G2-phases of the cell cycle.

If RDR is used during G2-phase, and if the timing of DNA replication depends on E1/E2 expression levels, it could be argued that RDR depends on E1/E2 expression levels. We previously showed that ATR is engaged in HPV replication foci, indicating that replication stress arises during the replication of viral circular genomes. Based on these observations, we propose the following model. The E1 protein initiates HPV DNA replication during the S-phase, but the synthesis of the viral genome is not successfully completed and the replication forks stall. ATR is engaged into the replication centers and stalled forks are restarted using homologous recombination pathways. Viral DNA replication is thereby overtaken by RDR, which continues to synthesize new viral genomes during the G2-phase of the cell cycle.

CONCLUSIONS

1. The human osteosarcoma cell line U2OS supports the highly efficient replication of high risk, low risk and cutaneous HPV genomes.
2. The HPV replication protein E1 causes DNA double-strand breaks into host genome and this activity is dependent on direct interactions between the E1 protein and DNA. As a result of the activity of E1, dsDNA breaks are induced, the ATM pathway is activated, and the cell cycle is arrested. The DNA damaging activity of the E1 protein is probably not necessary for efficient viral genome replication, and it is suppressed in the context of transiently replicating viral genomes. However, even low levels of E1-induced DNA damage could play a role in viral-induced carcinogenesis.
3. The phosphorylation of H2AX and the recruitment of ATRIP and TopBP1 in viral DNA replication centers shows that the ATR-dependent DNA damage response is engaged at the initial amplification centers of HPV genomes. This ATR engagement suggests that DNA replication stress accompanies viral genome duplication. The viral response to DNA replication stress might play a major role in the initiation of recombination-dependent replication of HPV DNA or the integration of the viral genome into a host chromosome.
4. Combining pulse labeling with EdU with the subsequent purification of the labeled DNA can be used to measure newly synthesized viral DNA levels and to thereby estimate how actively viral genomes are being replicated at a given time point. This approach is an important addition to the conventional methods that are used to characterize viral DNA replication, which include measuring the DNA copy number.
5. During HPV stable replication, viral DNA replication takes place only in S-phase, but it starts in S-phase and is extended to G2-phase during the initial amplification of HPV genomes. The timing of viral replication is dependent on the expression levels of the viral replication proteins E1 and E2.

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SUMMARY IN ESTONIAN

Uurimistöö inimese papilloomiviiruse genoomi replikatsioonist

Inimese papilloomiviiruse (HPV) meditsiiniline tähtsus seisneb tema tõestatud võimes põhjustada pahaloomulisi kasvajaid. Hoolimata viimastel kümnenditel aset leidnud suurest edasiminekust HPV molekulaarbioloogilistes teadmistes, ei mõisteta täielikult HPV poolt põhjustatud vähkkasvajate tekkemehhanisme. HPV DNA replikatsioon on üheks oluliseks viiruslikuks protsessiks, mida oleks vaja vähitekke täpseks kirjeldamiseks paremini mõista.

HPV uurimise teeb keeruliseks tema suur koespetsiifilisus ja keeruline elutsükkel, mis on tihedas sõltuvuses HPV peremeesrakkude diferentseerumisest epiteelkoes. Käesoleva töö esimeseks eesmärgiks oli uudse U2OS rakuliinil põhineva mudelsüsteemi kirjeldamine HPV genoomi replikatsiooni uurimiseks. Selgus, et selles osteosarkoomi rakuliinis replitseeruvad erinevate HPV subtüüpide genoomid efektiivselt ja on võimalik uurida viiruse kõiki kolme replikatsioonifaasi. Seega osutus see HPV replikatsiooni uurimiseks varem kasutamata rakuliin oluliseks täienduseks olemasolevatele eksperimentaalsetele süsteemidele.

Järgnevalt uuriti uudset U2OS rakuliinil põhinevat süsteemi kasutades HPV DNA replikatsioonivalkude ja rakulise DNA kahjustuse vastuse vahelisi seoseid. Interaktsioonid DNA kahjustuse vastusega mängivad olulist rolli paljude viiruste elutsüklis ja on näidatud, et ka HPV elutsükkel sõltub nende radade aktivatsioonist. Käesolev töö näitab, et HPV replikatsioonivalk E1 põhjustab ekspressiooniplasmiidilt ekspresseerituna peremehe genoomi DNA katkeid ja seeläbi aktiveerib DNA kahjustuse vastuse. Kuigi antud E1 valgu omadus on viiruse replitseeruva genoomi kontekstis maha surutud, võib isegi minimaalne peremeesraku DNA kahjustamine E1 valgu poolt omada olulist rolli HPV poolt põhjustatud kartsinogeneesis.

Hoolimata sellest, et viiruse genoomilt ekspresseritud E1 valk ei põhjustanud märgatavat DNA kahjustuse vastust, olid need rajad HPV DNA replikatsiooni fookustes siiski aktiveerunud. Selgus, et rakulised valgud ATR rajast on lokaliseerunud rakutuuma piirkondadesse, kus toimub viiruse genoomi replikatsioon. ATR raja hõlmamine omakorda viitab, et HPV DNA replikatsiooniga kaasneb viiruse DNA replikatsioonistress, mis on põhjustatud probleemidest DNA replikatsioonikahvlite liikumisel. Peatunud replikatsioonikahvlite parandamiseks algatatud protsessid võivad aga viia HPV genoomi integreerumiseni peremeesraku genoomi ja seeläbi omada olulist rolli vähi tekkes.

Käesoleva töö eesmärk oli ka HPV DNA replikatsiooni ajastuse kirjeldamine rakutsüklis. Selgus, et HPV DNA replikatsiooni ajastus rakutsüklis on viiruse kahes esimeses elutsükli faasis erinev. Kui algse amplifikatsiooni käigus algab viiruse DNA replikatsioon S-faasis ja saavutab maksimumi G2-faasis, siis stabiilse säilumise ajal toimub kogu süntees ainult S-faasis. Erinevused replikat-

siooni ajastuses HPV elutsükli faaside lõikes viitavad erinevustele kasutatavates replikatsiooni mehhanismides.

Kokkuvõtvalt arendati käesolevas töös HPV genoomi replikatsiooni uurimise metoodikat ning kirjeldati viiruse DNA replikatsiooni seoseid rakulise DNA kahjustuse vastusega ja ajastust rakutsüklis.

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List of publications:

1. Geimanen J, Isok-Paas H, Pipitch R, Salk K, Laos T, Orav M, Reinson T, Ustav M Jr, Ustav M, Ustav E. 2011. Development of a cellular assay system to study the genome replication of high- and low-risk mucosal and cutaneous human papillomaviruses. J Virol 85(7): 3315–3329.
2. Reinson T, Toots M, Kadaja M, Pipitch R, Allik M, Ustav E, Ustav M. 2013. Engagement of the ATR-dependent DNA damage response at the human papillomavirus 18 replication centers during the initial amplification. J Virol 87(2): 951–964.
3. Reinson T, Henno L, Toots M, Ustav M Jr, Ustav M. 2015. The Cell Cycle Timing of Human Papillomavirus DNA Replication. PLoS One 10(7): e0131675.
4. Ustav M Jr, Castaneda FR, Reinson T, Männik A, Ustav M. 2015. Human Papillomavirus Type 18 -cis-Elements Crucial for Segregation and Latency. PLoS One 10(8):e0135770

Inventions:

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

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Publikatsioonide nimekiri:

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