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The Role of E2 Proteins in Papillomavirus DNA Replication

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1. Introduction

Papillomaviruses are small, nonenveloped, double-stranded DNA viruses, which infect a wide variety of vertebrate species and induce proliferative lesions in their host. The viruses are species-specific and to date, more than 150 different types of papillomaviruses have been identified; each virus infects a specific region of cutaneous or internal mucosal epithelium in its host (McLaughlin-Drubin and Münger, 2009). Papillomaviruses are widely spread in nature and in most cases, papillomavirus infections are cleared by the host immune system, however, sometimes papillomaviruses establish persistent infections. Papillomavirus classification is based on nucleotide sequence homology (de Villiers et al., 2004). Human papillomavirus (HPV) types that infect the genital epithelia belong to subgroup A (alpha-papillomaviruses), and are classified into high- or low-risk types, depending upon their oncogenic potential. Persistent infection with high-risk HPVs (HPV16, 18, 31, 33, 45) can lead to cervical cancer, the second most common cancer in women (Durst et al., 1983; zur Hausen, 2000). Low-risk HPVs, such as HPV6 and 11, can infect genital tract as well as oral sites where they are generally associated with benign papillomas. The second major group of HPVs, supergroup B, also known as beta-papillomaviruses, infect skin epithelia and may develop skin cancers at the site of HPV infection (McLaughlin-Drubin and Münger, 2009).

2. Papillomavirus life cycle

Papillomaviruses, with the help of only few genes, can achieve a complete replication cycle in the epidermal and mucosal keratinocytes. Most viral types are predominantly trophic for one or the other cell types, but certain genotypes can infect and persist in both. Papillomavirus virions enter the epithelial tissue through microwounds and infect a subset of basal cells, probably stem cells, at low copy number (Egawa, 2003). HPV virions migrate to the cell nucleus and establish their genomes as episomes. Next, the HPV early promoter is activated and the viral early proteins, E1 and E2, are transcribed, the synthesis of viral DNA is initiated and the copy number of viral episomes is raised up to 20-100 genomes per cell. During this amplification stage, rapid viral replication is required to quickly reach an optimal copy number. As basal cells divide, HPV genomes are replicated and distributed evenly between daughter cells in mitosis. This is a phase of episomal maintenance with minimal viral gene expression, viral replication proceeds at moderate level and is

synchronized to cellular proliferation (De Geest et al., 1993). Papillomavirus persistence is set up through the maintenance of a constant copy number of extrachromosomal viral genomes in the nuclei of dividing host cells. The expression of late genes encoding the capsid proteins and virus assembly are tightly coupled to the differentiation of epithelial tissue (Longworth and Laimins, 2004). When the infected keratinocyte enters the differentiating compartment, exiting the cell cycle, the vegetative phase of the HPV life cycle leading to amplification of the viral genome is initiated. This phase includes amplification of viral copy number to at least 1000 copies per cell and expression of late transcripts encoding proteins for viral capsid. The viral DNA is packed into virions, which are released with dead cells after the infected cells reach the epithelial surface (for review, see Chow et al. 2010; Doorbar, 2005).

The dependence of papillomavirus life cycle on epithelial differentiation and difficulties in reproducing this process in cell culture has complicated the studies of papillomavirus replication. Short-term replication assays closely mimic the initial amplification phase of replication, while long-term replication assays mimic viral maintenance in undifferentiated basal cells. DNA amplification and virion assembly can be analyzed only in differentiating epithelial cells. The most extensively studied papillomavirus is bovine papillomavirus type-1 (BPV-1) because of its capacity to transform rodent cell lines in culture (Law et al., 1981). The mouse fibroblast cell line C127 transfected with the BPV1 genome maintaining the viral DNA as extrachromosomal plasmid with the constant copy number was historically the first model system for studying mechanisms of papillomavirus DNA replication. Our knowledge of initiation of papillomavirus DNA replication and maintenance of virus genomes has primarily derived from studies with BPV1. However, BPV-1 belongs to delta-papillomaviruses and is evolutionary distinct from the human papillomaviruses.
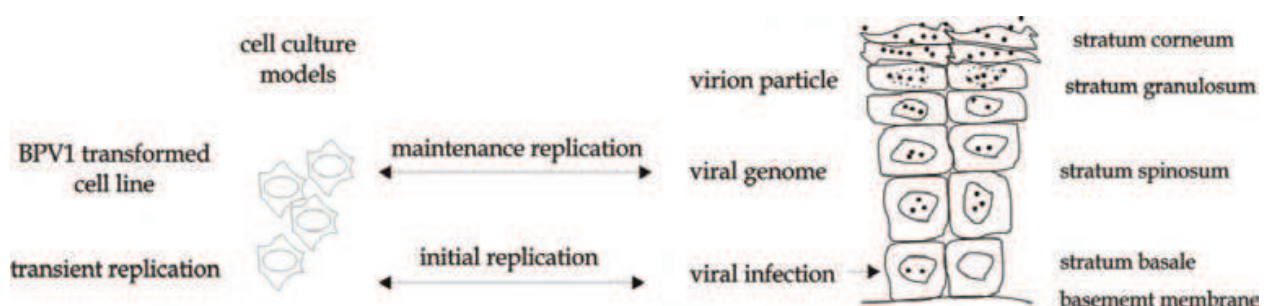

Fig. 1. Schematic representation of epithelia showing various differentiated layers and PV replication, and cell culture models mimicking papillomavirus DNA replication.

The limited availability of appropriate cell culture systems supporting HPV DNA replication has hampered research of HPV replication and regulatory pathways involved in these processes. Primary keratinocytes, transfected with circulized HPV genome, replicate HPV genomes at low copy number. Productive HPV replication needs culture systems where epithelial cells are able to differentiate. Adaptation of organotypic culture techniques, „raft" culture systems, provide the basis for propagation of HPVs, although the yield of virions is very low (Dollard et al., 1992; McCance et al., 1988; Meyers et al., 1992). Introduction of adenovirus recombinants carrying the HPV genome flanked by loxP sites and Cre recombinase into PHK cells grown in raft cultures results in efficient establishment of cells harboring thousands of copies of the HPV18 genome. Virions obtained from these

cultures efficiently infect PHKs at a multiplicity of infection (MOI) of <50, resulting in expression of spliced viral mRNAs (Wang et al., 2009a). However, organotypic culture of HPV-infected cells is not a very convenient system for long-term viral persistence and latency studies. Cell lines from mild dysplasia harboring episomal HPV genomes (W12 and CIN-612) have been very useful tools for studying stable episomal HPV replication, and also loss of episomes and integration of HPV into the host genome, a process accompanied by tumourigenesis (Pett and Coleman, 2007). At early passages in monolayer culture, the W12 cells retain HPV16 episomes at 100 to 200 copies per cell, but in long-term cultivation in the absence of feeder cells, the spontaneous loss of episomes and integration of HPV16 occurs (De Geest et al., 1993; Pett et al., 2004; Stanley et al., 1989). The genomes of both high- and low-risk alpha-papillomaviruses as well as cutaneous beta-papillomaviruses replicate in human osteosarcoma cell line U2OS, and are able to establish persistent replication in these cells. These cell lines might be a valuable and efficient tool for studying fundamental properties of HPV DNA replication, especially the long-term viral persistence, and for the development of inhibitors of HPV genome replication (Geimanen et al., 2011).

2.1 Papillomavirus genome

All papillomaviruses have a similar genomic organization. The papillomavirus genome of approximately 8000 base pairs in length contains a non-coding upstream regulatory region (URR), early (E) region of open reading frames (ORFs) and late (L) region of ORFs. The circular papillomavirus genome encodes roughly eight ORFs from a single DNA strand. The URR alone is 600-900 base pairs in length and contains the origin of replication and binding sites for transcription factors and enhancers (Fig. 2). All transcription takes place in the same direction (clockwise on the circular map) using multiple promoters. The E region and L region are both followed by a poly-A addition site. Within the virions, the papillomavirus genome is associated with cellular histones forming chromatin-like complexes.

Papillomavirus transcription is complex and involves the usage of different promoters, multiple splice patterns and differential production of mRNA species in different cells. In BPV1, seven different promoters, six of which being active in undifferentiated cells, have been identified (Ahola et al., 1987; Baker and Howley, 1987). In the high-risk HPV types, transcripts are initiated at two major viral promoters, one for early and the other for late transcripts. In contrast, early genes of low-risk HPVs are expressed from two independent promoters (Chow et al., 1987). The HPV early promoter (p105 in case of HPV18) is activated by binding of cellular transcription factors; however the factors that determine the cell specificity are still not fully understood. Among the main players involved in PV replication are the ubiquitous transcription factors Sp1 and AP1 (Gloss and Bernard, 1990; Hubert et al., 1999; Thierry, 2009; Thierry et al., 1992). In addition to the binding sites for the cellular transcription factors, the URR contains binding sites for the virally encoded E2 regulatory proteins and E1 helicase. Early region proteins, E6, E7, E1 and E2 are transcribed from the HPV early promoter active in basal, nonproductively infected cells and in transformed cells, and all four early proteins are translated from the same polycistronic mRNA (Hummel et al., 1992). E1 and E2 are directly involved in viral DNA replication; the other early proteins - oncoproteins E6 and E7 - support viral DNA amplification indirectly by inactivating major tumor suppressor proteins and activating signal transduction pathways (for review, see (McLaughlin-Drubin and Münger, 2009; Moody and Laimins, 2010).
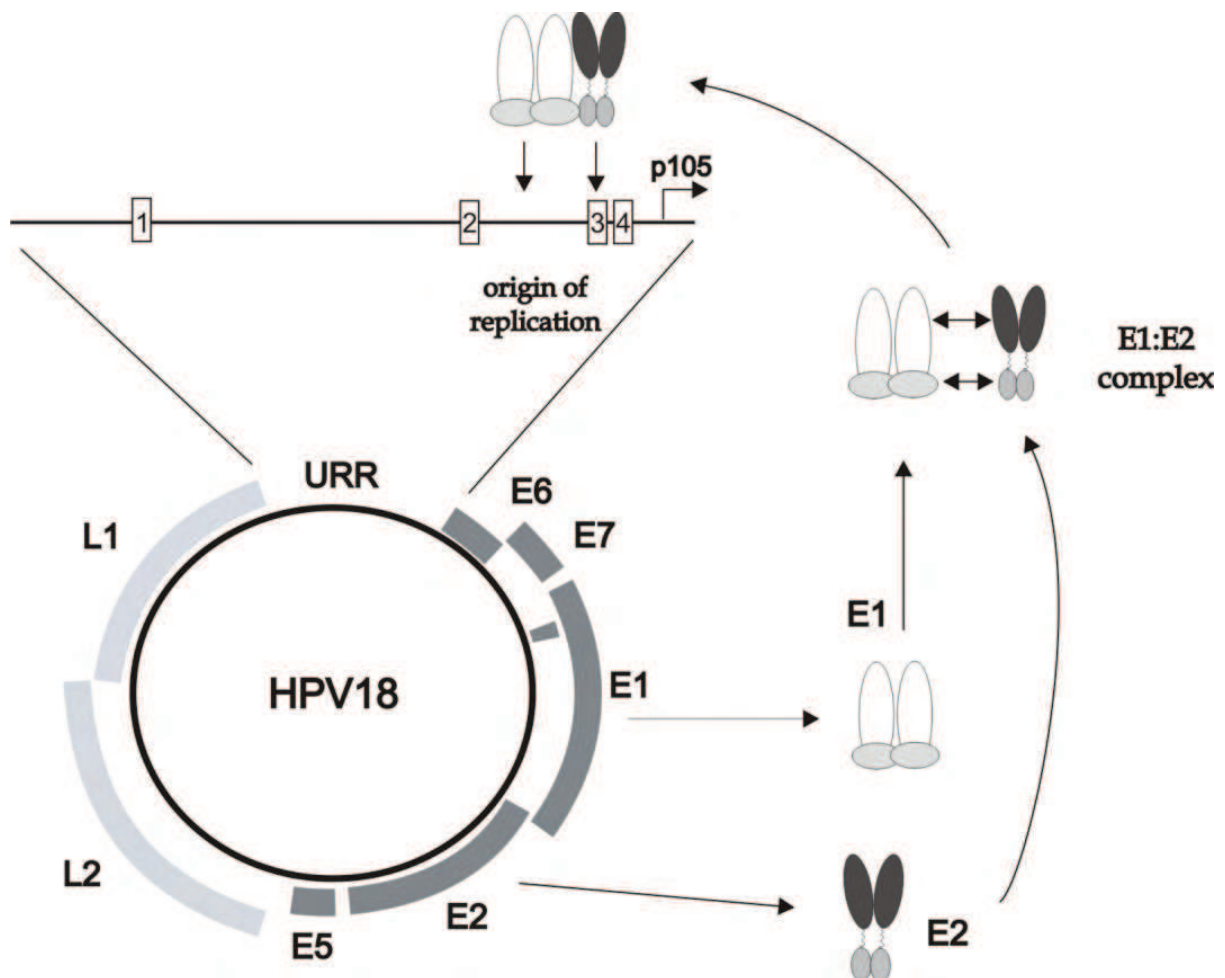
Fig. 2. Organization of the HPV18 circular genome showing the *cis*-sequences and viral proteins required for initiation of papillomavirus DNA replication. Open reading frames are indicated by dark grey (early region) and light grey (late region) boxes. The origin of replication, early promoter (p105) and E2 binding sites (boxes with numbers) are shown on the scheme of URR. Papillomavirus E1 and E2 proteins, transcribed from the early promoter, form a complex and bind cooperatively to their binding sites within the origin of replication locating in the URR.

3. Papillomavirus DNA replication

3.1 Initiation of replication

The double-stranded circular DNA replicates as a multi-copy extrachromosomal plasmid in the nucleus of infected cells. The replication of papillomavirus DNA is initiated from the origin of replication consisting of binding sites for E1 and E2 proteins (Del Vecchio et al., 1992; Ustav et al., 1993; Ustav et al., 1991). Transient, short-term replication requires the origin of replication *in cis* and the expression of viral E1 and E2 proteins, all other replication enzymes and proteins are supplied by the host cells (Remm et al., 1992; Ustav and Stenlund, 1991). Papillomavirus DNA replication is not cell-type specific, despite of high degrees of host and cell-type specificity for infection. The BPV1 genome can replicate and is maintained in murine C127 cells. HPV11 and HPV18 genomic DNA can replicate not only in its natural host cells, primary foreskin keratinocytes, but also in the human tongue squamous cell

carcinoma cell line SCC4 and osteosarcoma cell line U2OS (Del Vecchio et al., 1992; Geimanen et al., 2011; Mungal et al., 1992). Transient replication of BPV1 and HPV origin-containing plasmids can take place in a number of mammalian cells if E1 and E2 proteins are provided from heterologous expression vectors (Chiang et al., 1992). HPV transcription exhibits stringent cell specificity and the lack of regulated gene expression of viral early proteins from HPV genomes could be the reason that restricts HPV genome replication to certain cell types. BPV1 DNA replication *in vitro* with purified proteins and cell extracts from murine, simian and human cells has been reconstituted showing that all cellular factors essential for papillomavirus DNA replication are provided by the host cell (Liu et al., 1995; Muller et al., 1994; Yang et al., 1991a). The cellular replication factors required for papillomavirus DNA replication include replication protein A (RPA), a single-stranded-DNA-binding protein, replication factor C (RFC), proliferating-cell nuclear antigen (PCNA) and DNA polymerase α–primase. It has been shown that E1 recruits Topo I to the papillomavirus replication fork and Topo I specifically stimulates the origin binding activity of papillomavirus E1 protein (Clower et al., 2006; Hu et al., 2006; Melendy et al., 1995; Park et al., 1994).

3.2 Origin of replication

The papillomavirus origin of replication consists of three E2 binding sites (E2BS), from which only one is absolutely required for replication, and an A/T-rich region containing an array of E1 protein binding sites (E1BS) (Remm et al., 1992; Ustav et al., 1991). The overall structure of BPV1 and HPV origins is conserved and various combinations of E1 and E2 proteins from different papillomaviruses can initiate DNA replication from several papillomavirus origins (Chiang et al., 1992; Del Vecchio et al., 1992; Kadaja et al., 2007). A relationship appears to exist between the affinity of the E2BS and the ability to function at a distance from the binding site for the E1 protein. A low-affinity site appears to be functional only when located close to the E1 binding site, while for function at greater distances higher affinities are required. In multimerized form, the high affinity E2BSs are able to function even when placed at a distance of one kilobase from the rest of the origin (Ustav et al., 1993). High-risk HPV genomes have been found integrated into host chromatin in tumors and cervix dysplasia biopsy specimens of patients (Dall et al., 2008; Kristiansen et al., 1994). Integration of the HPV genome is often accompanied by the disruption of E2 open reading frame and the loss of E2 activity, leading to enhanced expression of virus oncogenes E6 and E7. The HPV regulatory sequences are active in cervical cancer cells as expression of E7 promotes cell survival (Jiang and Milner, 2002). In patients, the HPV16 genome may exist at the same time in episomal as well as in an integrated form. Co-existence of the replicating episomal viral genome expressing the viral E2 protein, and integrated HPV with viral replication origin raises the question about the functionality of the integrated origins. Recent works have shown that expression of E1 and E2 proteins from expression vectors or from different HPV genomes can induce replication of the genomic integrated HPV origin. The replication forks initiated at the integrated HPV origins extend into the flanking regions of cellular DNA, and these amplified genomic sequences could be targets for the recombination and repair system. This suggests that replication induced from the papillomavirus integrated origin may induce genomic changes of the host cell (Kadaja et al., 2009; Kadaja et al., 2007).
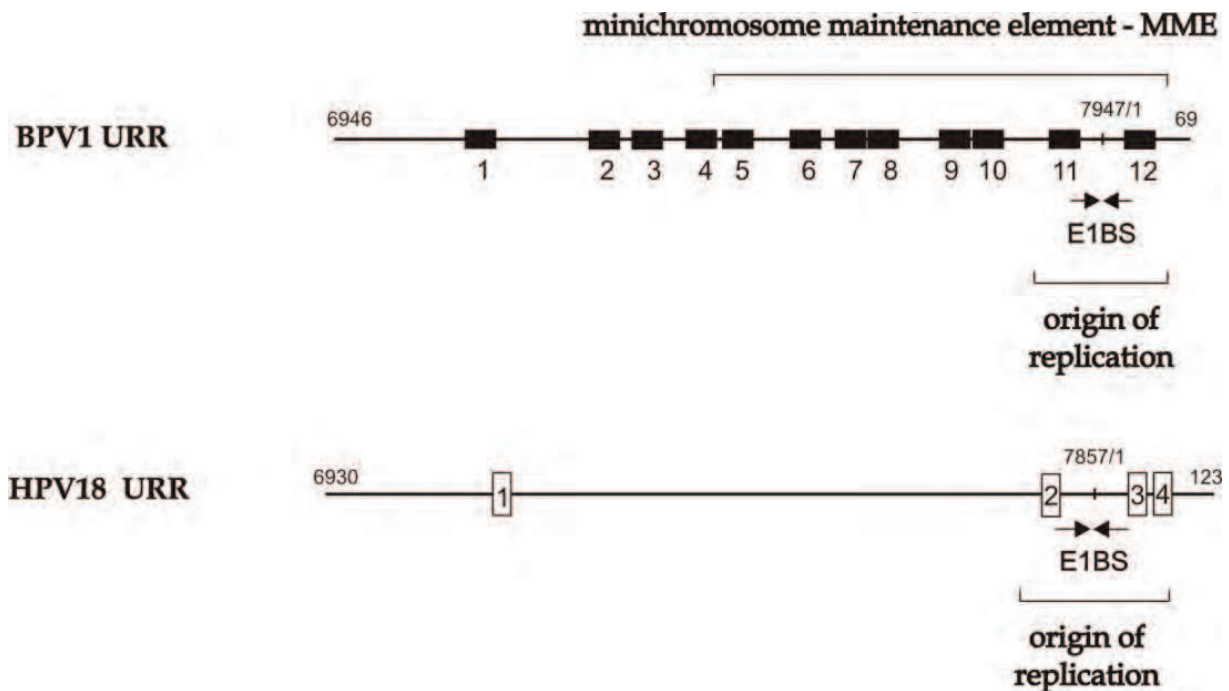
Fig. 3. Origin of replication of BPV1 and HPV18. E1 binding sites are depicted with arrows and E2 binding sites with boxes. The minimal origin of replication consisting of E1BS and E2BSs is shown for BPV1 and HPV18, and the minichromosome maintenance element MME, required for maintenance and segregation of viral DNA, for BPV1.

3.3 Assembly of the DNA replication initiation complex

E1 is an initiator protein of papillomavirus DNA replication. E1 is responsible for recognition of the replication origin, melting of the DNA at the origin as well as for subsequent unwinding of the double helix during progression of the replication fork. The viral E1 protein is an ATP-dependent helicase which binds as a dimer to pairs of its binding sites (Yang et al., 1993). The binding sites for the E1 replication helicase are short sequences, 5-6 base pairs in length, arranged as two pairs of inverted repeats (Chen and Stenlund, 1998; Chen and Stenlund, 2001). E1 has low sequence specificity and therefore it can initiate DNA replication in vitro, and from non-specific DNA sequences (Bonne-Andrea et al., 1995). Within the cells, the E1 and E2 proteins form a complex through multiple protein-protein interactions and bind cooperatively to adjacent binding sites in the origin of replication (Berg and Stenlund, 1997; Mohr et al., 1990). E1 by itself binds to the origin with low degree of sequence specificity, but in the presence of E2 the sequence specificity is increased (Sedman and Stenlund, 1995; Sedman et al., 1997). In this process, E2 functions transiently and "catalytically", providing sequence specificity for the formation of the E1-ori complex. In addition, E2 enhances E1 binding to DNA through the DNA-binding domain by inhibiting the non-specific DNA binding of the E1 helicase domain (Stenlund, 2003a). The E1-E2-ori complex is able to bind DNA with high specificity but lacks other biochemical activities. In the next step, additional E1 molecules are added by displacing E2 from the DNA-bound complex in an ATP-dependent manner (Sanders and Stenlund, 1998; Sanders and Stenlund, 2000). Two additional E1 molecules are recruited to the origin, which results in the formation of two E1 trimers on the ori, followed by formation of two hexamers in the presence of ATP. E1 hexameric complex has the DNA helicase activity which is able to

unwind the DNA and initiate the papillomavirus DNA replication (Fouts et al., 1999; Schuck and Stenlund, 2005; Sedman and Stenlund, 1998).
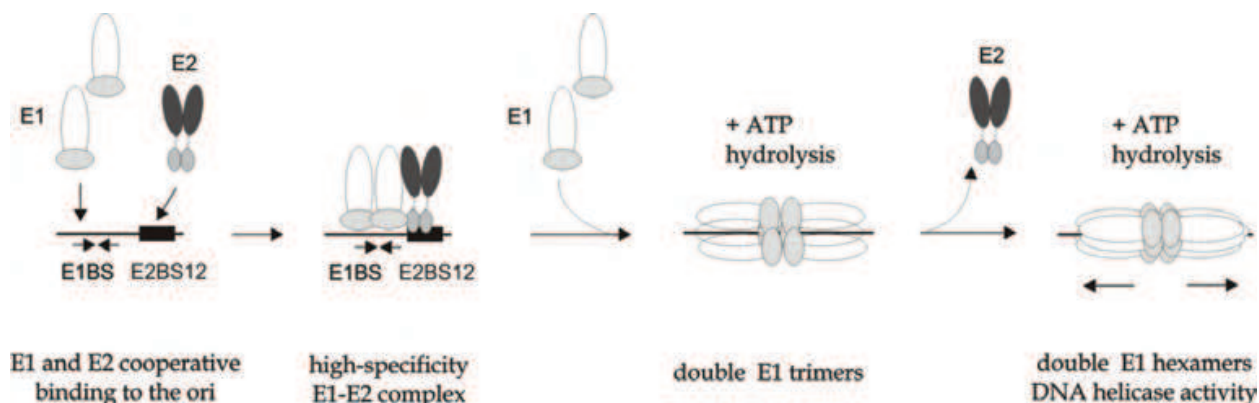

Fig. 4. Assembly of the replication initiation complex. The E1 and E2 proteins bind cooperatively to the origin of replication, which contains binding sites for both proteins (E1BS and E2BS). The resulting E1:E2 complex binds DNA with high affinity and specificity. In the next step, additional E1 molecules are recruited to the complex and E2 is displaced in the presence of ATP. ATP hydrolysis is also required for conversion of E1 complex into a double hexamer possessing DNA helicase activity (for review, see Stenlund, 2003b).

3.4 Stable maintenance replication

Papillomaviruses have the capacity to establish a persistent infection in mammalian epithelial cells. The BPV1-transformed C127 cells maintain the viral genome as a multicopy nuclear plasmid thus being a valuable tissue culture system for investigating the establishment and maintenance of papillomavirus genomic DNA. The studies with BPV1-C127 cells have revealed that during the maintenance of virus genomes BPV1 DNA is replicating by a conventional bi-directional (theta-type) replication mode throughout S phase of the cell cycle in a random-choice fashion (Gilbert and Cohen, 1987; Ravnan et al., 1992). Some molecules replicate once per cell cycle, some replicate more than once, and others do not replicate at all during a given cell cycle, resulting in statistically "once per cell cycle" replication of the viral DNA. Stable HPV DNA replication seems to be more complicated. This has been studied in human keratinocytes, where the HPV DNA is stably maintained at high copy number over several passages. HPV16 and HPV31 DNA can replicate randomly or in an ordered once-per-S-phase fashion depending on the cell line in which it is located. In W12 cells, which are derived from a natural cervical lesion, the HPV16 DNA replicates only once per S phase, but in another immortalized keratinocyte cell line, NIKS, it replicates randomly (Hoffmann et al., 2006). At later stages of the papillomavirus life cycle, there is a shift from the theta replication mode in the proliferating keratinocytes to the rolling-circle replication mode after the cells start to differentiate (Flores and Lambert, 1997).

Studies of subclones of BPV1-transformed C127 cells as well as U2OS cells maintaining HPV genomes have demonstrated cell to cell variation in the extent and state of genomic DNA. In addition to the monomeric form of genomic DNA, dimeric and sometimes higher oligomeric forms of BPV1 and mucosal and cutaneous HPV genomes are detected. Oligomeric forms of papillomavirus DNA are organized in a head-to-tail configuration and replication is initiated at only some of the origins (Geimanen et al., 2011; Schvartzman et al., 1990). In

patient samples, HPV16 DNA, which is maintained in carcinoma cells as episome, is always multimeric, suggesting that oligomerization of HPV genomes is common during viral infections *in vivo* (Cullen et al., 1991; Kristiansen et al., 1994).

It is widely accepted that the E1 and E2 proteins are essential for long-term stable replication. However, some studies suggest that E1 is necessary to establish the viral genome as a nuclear plasmid, but is not required at the maintenance stage. The replication of the BPV1 genome containing temperature-sensitive mutation in the E1 gene was first initiated at the permissive temperature and then switched to the non-permissive temperature, where ts-E1 genomes were found to persist as nuclear plasmids for multiple cell generations (Kim and Lambert, 2002).

The functions of viral oncoproteins E6 and E7 are also essential for the maintenance of the extrachromosomal forms of HPV DNA (Thomas et al., 1999). Both E6 and E7 stimulate cell cycle progression and may create a cellular environment permissive to HPV maintenance and abrogate the checkpoints that would block the long-term retention of viral DNA (Garner-Hamrick et al., 2004). Oncogene expression in basal epithelial cells is shown to inhibit cellular differentiation thus promoting long-term persistence of HPV episomes (Hudson et al., 1990; Jones et al., 1997; Sherman and Schlegel, 1996). The E6 and E7 proteins of high-risk HPV types act as viral oncogenes. E6 binds the p53 tumor suppressor protein, which regulates the expression of proteins involved in cell cycle control, leading to degradation of p53 (Scheffner et al., 1990; Werness et al., 1990). Another important function of the high-risk E6 protein is the activation of telomerase in infected cells. High-risk HPV E6 has been shown to increase telomeric length by activating the catalytic subunit of the telomerase hTERT. This extends the life of epithelial cells containing HPV genomes (Oh et al, 2001; Stoppler et al., 1997). The function of the high-risk E7 protein is the binding and degradation of the RB family proteins, which are the major regulators of the cell cycle (Boyer et al., 1996; Schmitt et al., 1994). E7 interaction with histone deacetylases HDACs plays also an essential role in the viral life cycle, as cells harboring HPV genomes with mutations abolishing the E7-HDAC interaction display slower growth and a loss of episomal maintenance (Longworth and Laimins, 2004a). Integration of high-risk HPV into the host genome and loss of E2 expression leads to constitutive activation of the viral oncogenes. Reintroduction of E2 into HPV-associated cervical carcinoma cells, resulting in reactivation of p53 and pRB pathways, has shown to suppress cellular growth, because of cell cycle arrest in G1, apoptosis and senescence (Desaintes et al., 1997; Goodwin and DiMaio, 2000; Goodwin et al., 2000; Hwang et al., 1996; Thierry et al., 2004; Wells et al., 2000).

4. E2 as a viral regulatory protein

The proteins encoded by the papillomavirus E2 ORF play a crucial role in the viral life cycle and serve as major viral regulators of transcription, replication and segregation of the viral genome in the infected cells. The E2 protein is a modular protein consisting of three different structural as well as functional domains; the N-terminal transactivation domain (TAD) (aa 1-200), the C-terminal DNA-binding dimerization domain (DBD) (aa 310-410), and flexible unstructured "hinge region" which functions as a linker between the two domains. The carboxy-terminal DBD binds as a dimer to consensus sequence $ACCN_6GGT$ (Androphy et al., 1987) and the N-terminal activation domain is required for the replication, transactivation and segregation function of the protein (Abroi et al., 2004; Bastien and McBride, 2000; McBride et al., 1989; Ustav and Stenlund, 1991). The three-dimensional

structures of the C-terminal DBD and N-terminal transactivation domain of several E2 proteins have been reported revealing a tight dimer of the DBD bound to DNA and a L-shape structure of activation domain (Antson et al., 2000; Harris and Botchan, 1999; Hegde et al., 1992). The papillomavirus E2 proteins can function as activators or repressors of transcription depending on the concentration of proteins within the cell. At low concentrations, E2 activates, and at high concentrations, suppresses transcription from homologous and heterologous promoters containing E2 binding sites (Abroi et al., 1996; Schweiger et al., 2007). E2 has been demonstrated to be a transcriptional activator of early genes in BPV1 and a repressor of early genes in case of HPV16 (Soeda et al., 2006; Spalholz et al, 1985; Thierry and Yaniv, 1987).

E2 is a multifunctional protein. The transactivation domain of E2 is responsible for interactions with viral helicase E1 and with several cellular proteins. The transactivation and replication functions of E2 are separable by point-mutations in the N-terminal activation domain (Abroi et al., 1996; Brokaw et al., 1996; Ferguson and Botchan, 1996; Grossel et al., 1996). The X-ray crystal structures of a complex containing the activation domain of E2 and the helicase domain of E1, and the activation domain of E2 together with Brd4 have been solved and confirm that E2 interacts with E1 and Brd4 through different interaction surfaces (Abbate et al., 2004; Abbate et al., 2006). The cellular bromodomain protein Brd4 is the major cellular partner for E2, and interaction with Brd4 is crucial for both E2 transactivation and repression functions (Ilves et al., 2006; McPhillips et al., 2006; Schweiger et al., 2006). Brd4 is a component of the HPV11 E2 transcriptional silencing complex involved in repression of the E6/E7 promoter (Wu et al., 2006). Through the interaction of Brd4 and transactivation domain, E2 is associated with transcriptionally active cellular chromatin. This association is driving the E2-mediated tethering of viral genomes to host chromatin, and at the same time is retaining the viral genomes in transcriptionally active regions of the nucleus to escape silencing (Jang et al., 2009; Kurg et al., 2005). The transactivation domain mediates functional interactions with cellular transcription factors Sp1 and AP1, histone acetylase complexes containing CBP/p300 and pCAF, and with nucleosome assembly protein hNAP1 (Lee et al., 2002a; Lee et al., 2000; Li et al., 1991; Müller et al., 2002; Rehtanz et al., 2004; Thierry et al., 1992). E2-dependent activities are also modulated by interactions with Brm, a chromatin remodeling protein associated with SWI/SNF ATP-dependent chromatin remodeling complex, and with EP400, a component of the NuA4/TIP60 histone acetylase complex, and SMCX, also known as histone demethylase JARID1C , and Tax1BP1 (Kumar et al., 2007; Smith et al., 2010; Wang et al., 2009b).

E2 binds to the papillomavirus oncoproteins E6 and E7, leading to the modulation of their functions (Gammoh et al., 2006; Grm et al., 2005). E2 has activities that inhibit cell growth. High-risk, but not low-risk HPV E2 proteins can induce themselves growth arrest and apoptotic cell death in several HPV-negative carcinoma cell lines. Apoptosis can occur via a p53-dependent as well as independent pathways (Demeret et al., 2003; Parish et al., 2006b). E2 binds to the cellular protein p53. Expression of p53 can inhibit papillomavirus DNA replication and alter the transcriptional activity of E2 (Frattini et al., 1997; Lepik et al., 1998; Massimi et al., 1999). Interestingly, p53 inhibits the initial, amplificational replication, but not the stable, long-term replication of BPV1 (Ilves et al., 2003). E2 can modulate the activity of cellular proteins, like activators of the Anaphase Promoting Complex (APC) Cdh1 and Cdc20 inducing genomic instability (Bellanger et al., 2005). E2 is associated with transcriptionally active chromatin and as a transcription factor may directly regulate the expression of cellular genes. The HPV E2 proteins are reported to repress the hTERT promoter activity (Lee et al.,

2002b). HPV8 E2 protein suppresses β4-integrin expression and HPV16 E2 protein transcriptionally activates the promoter of a key cellular splicing factor SF2/ASF (Mole et al., 2009; Oldak et al., 2004). The expression of HPV8 E2 protein in transgenic mice can induce the formation of skin tumors through an unknown mechanism (Pfefferle et al., 2008).

The E2 proteins bind to inverted repeats with consensus sequence 5' $ACCGN_4CGGT$, where N_4 represents a 4 bp central sequence (Li et al., 1989). Although the base pairs in the central spacer sequence are not in contact with the protein, they affect protein binding. The E2 DNA binding is accompanied by bending of DNA and depends on DNA flexibility (Hines et al., 1998). HPV E2 proteins bind with higher affinity to sites with A/T-rich central spacer while BPV1 E2 has no preference (Dell et al., 2003). E2 binding can be inhibited by CpG methylation of the E2 binding site (Kim et al., 2003; Thain et al., 1996). The BPV1 genome contains 17 E2 binding sites from which 12 sites are located within the URR region. In genital HPV genomes, there are four E2 binding sites with conserved sequences and positions. E2 remains associated with the BPV1 URR throughout the cell cycle including mitosis (Melanson and Androphy, 2009).
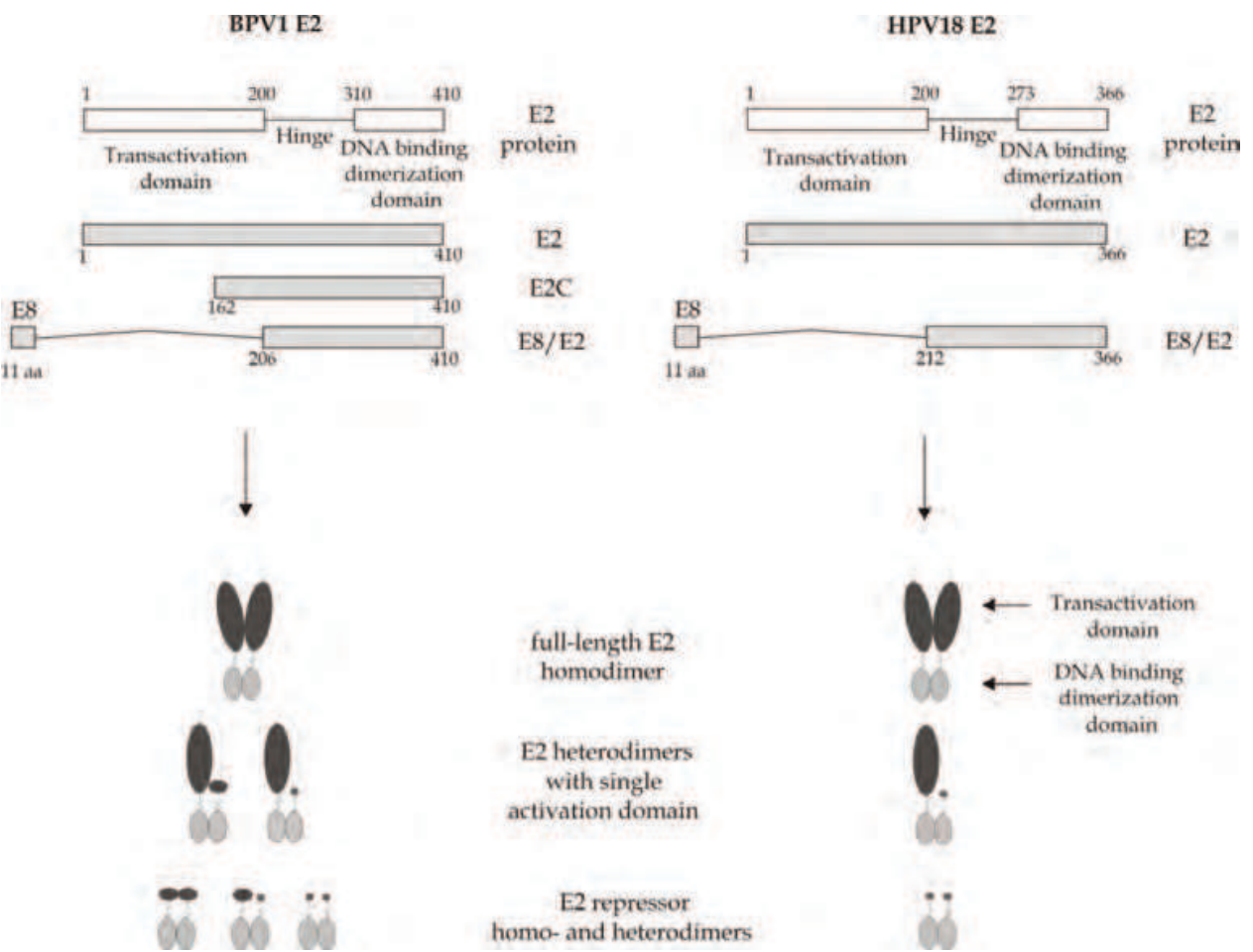

Fig. 5. Papillomavirus genomes encode multiple E2 proteins. In addition to the full-length E2 protein, BPV1 and HPV18 genomes encode truncated E2 proteins, which lack the activation domain and serve as repressors of replication and transcription. The full-length and truncated E2 proteins are able to form dimers through their common C-terminal DNA-binding-dimerization domain.

4.1 E2 repressors and heterodimers

In addition to the full-length E2 protein, the BPV-1 and mucosal HPV genomes encode truncated E2 proteins, which lack the activation domain, but maintain the DNA-binding-dimerization domain (DBD). For BPV1, mRNA for E2C is transcribed from a promoter internal to the open reading frame, and E8/E2 is created by splicing E8 ORF sequences to an acceptor located within the E2 ORF. The repressor proteins encoded by HPVs are similar to the BPV1 E8/E2 protein, since they contain a small conserved E8 ORF (HPV 11, 18, 31) or fragment of E1 ORF (HPV 11) fused to the C-terminus of E2. Transient over-expression assays have suggested that shorter E2 proteins act as negative regulators of E2 and function as repressors of transcription and replication (Chiang et al., 1991; Doorbar et al., 1990; Hubbert et al., 1988; Kurg et al., 2010; Lim et al., 1998; Stubenrauch et al., 2000). The shorter E2s antagonize the function of full-length E2 by competing for E2 DNA binding sites. In addition, the E8 of HPV 31 E8/E2 protein itself is a transcriptional repressor domain that functions independently of binding site competition inhibiting transcription and DNA replication by interacting with co-repressor molecules such as NCoR1/HDAC3, the histone methyltransferase SETDB1, and the TRIM28 protein (Ammermann et al., 2008; Powell et al., 2010).

The full-length and truncated E2 proteins are able to form dimers through their common C-terminal DNA-binding-dimerization domain (McBride et al., 1989). E2 heterodimers with single activation domain bind DNA sequence-specifically and serve as activators of transcription and replication in cell culture model systems (Kurg et al., 2006). E2 heterodimers can interact with viral helicase E1, and are able to recruit E1 to the origin of replication and activate the papillomavirus DNA replication in a cell-free system (Lim et al., 1998). Replacing the open reading frame of E2 with "single-chain" E2 in the context of BPV1 and HPV18 genome revealed that E2 heterodimer with single activation domain could support transient, but not long-term, replication in cell culture model systems (Kurg et al., 2009; Kurg et al., 2010). The full-length E2 protein is required for long-term papillomavirus DNA replication, the E2 heterodimer with single activation domain is crippled in this function. E2 requires two activation domains for interaction with Brd4, the cellular receptor for BPV1 E2 on mitotic chromosomes. Brd4 interacts efficiently with the BPV1 homodimer with two activation domains and with low affinity with the E2 heterodimer with single activation domain and with E2 mutants unable to form dimers between N-terminal activation domains (Cardenas-Mora et al., 2008; Kurg et al., 2006; You et al., 2004).

4.2 The role of E2 in initiation of DNA replication

The expression of E2 protein is required for initiation of papillomavirus DNA replication. The role of E2 in initiation of viral DNA replication is relatively well understood, E2 helps to recruit the viral helicase E1 to the viral replication origin by direct protein-protein and protein-DNA interactions as discussed above. The initiation step and interactions mediating the formation of the replication initiation complex are well studied and are conserved between BPV1 and alpha-papillomaviruses.

4.3 The role of E2 in stable maintenance

Segregation of papillomavirus genomic DNA is achieved through its attachment to mitotic host chromosomes during cell division. Non-covalent association of viral DNA with chromosomes is a general mechanism used by all papillomaviruses studied so far. This mechanism ensures that the replicated virus episomes are retained inside the nuclei of dividing host cells and faithfully partitioned to the daughter cells during mitosis (You, 2010).

Long-term replication and maintenance of BPV1 episomes requires the sequence of URR - the minichromosome maintenance element (MME) - consisting of at least six E2 binding sites and the minimal origin of replication as *cis*-elements. The plasmids containing BPV1 URR can be maintained as extrachromosomal elements in hamster CHO cells stably expressing the viral E1 and E2 proteins (Piirsoo et al., 1996). Extrachromosomal MME-containing plasmids containing ten oligomerized E2 binding sites segregate efficiently between daughter cells in the presence of E2 protein expressed from the same plasmid (Abroi et al., 2004; Silla et al., 2005). MME consisting of E2 binding sites and the E2 protein are responsible for anchoring of BPV1 genomes as well as URR reporter plasmids to host cell chromosomes (Ilves et al., 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). The functional organization of the HPV URR is significantly different from that of the BPV1. Alpha-papillomavirus genomes have four E2 binding sites in this region and only three of them are required for stable replication (Stubenrauch et al., 1998). The exact mechanism of segregation of HPV genomes is not yet known and needs further investigations.
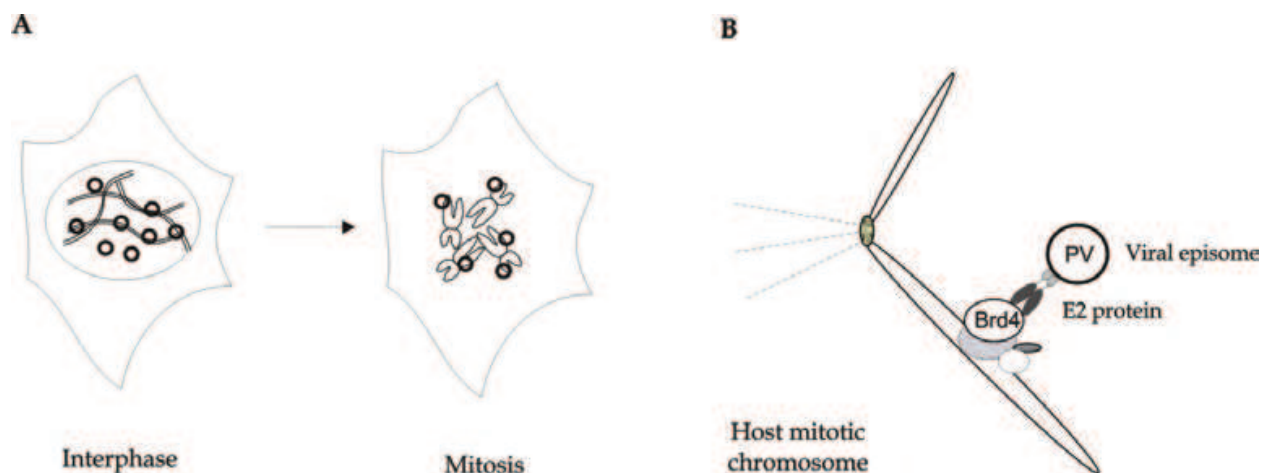

Fig. 6. Papillomaviruses establish persistent infection by maintaining viral genomes as episomes in host cell. (A) During mitosis, papillomavirus genomes are associated with cellular mitotic chromosomes. (B) For many papillomaviruses, association with mitotic chromosomes is mediated by the viral E2 protein and cellular bromodomain protein Brd4.

The role of E2 in long-term stable replication is not yet fully understood. The maintenance of papillomavirus genomes during latency is achieved by tethering viral genomes to host the mitotic apparatus in dividing cells. In this, BPV1 and HPVs may use different targets to achieve their goal. In BPV1, the activation domain of E2 is attached to chromosomes and the DNA-binding-dimerization domain tethers viral genomes to achieve their accurate segregation during mitosis. The point-mutations in the activation domain of E2, disrupting the transcription activity of E2, affect the chromatin attachment, suggesting that this activity is required for efficient segregation and maintenance of MME-containing plasmids (Abroi et al., 2004). The cellular receptor for BPV1 E2 on mitotic chromosomes is the bromodomain protein Brd4 (You et al., 2004). However, E2 proteins from different papillomaviruses interact with Brd4 with different affinities. E2 proteins of alpha-papillomaviruses interact with Brd4 weakly and do not co-localize with Brd4 on host mitotic chromosomes, suggesting that HPV E2 proteins may use different cellular targets for tethering virus

genomes to host chromosomes (McPhillips et al., 2005; Oliveira et al., 2006). DNA helicase ChR1, a member of the mammalian cohesion complex, is a candidate partner for cellular receptor of HPV E2. ChlR1 and E2 co-localize at early stages of mitosis, however, during prometaphase, ChlR1 is localized to the spindle poles, suggesting that ChlR1 may help to load the papillomavirus E2 protein onto mitotic chromosomes during early mitosis (Parish et al., 2006a). HPV16 E2 co-localizes with TopBP1, a cellular protein involved in DNA damage response, on chromatin and centrosomes during late telophase, suggesting that TopBP1 could be the mitotic chromatin receptor for HPV16 E2 (Donaldson et al., 2007). On the other hand, E2 proteins of HPV11, HPV16 and HPV18 have been found to localize to centrosomes and mitotic spindles during cell division (Van Tine et al., 2004). MKlp2, mitotic kinesin-like protein 2, a kinesin-like motor protein of the central mitotic spindle, binds and co-localizes with papillomavirus E2 during mitosis (Yu et al., 2007). The beta-papillomavirus HPV8 E2 protein binds to the repeated ribosomal DNA loci that are found on the short arm of human acrocentric chromosomes. These speckles do not contain Brd4, the E2 protein co-localizes with UBF, the RNA polymerase I transcription factor (Poddar et al., 2009). A recent study using chimeric BPV1 E2 proteins has shown that attachment of the protein to chromatin is not sufficient for proper segregation. Successful partitioning of virus genomes during cell division is determined by effective formation of the segregation-competent complex which does not necessarily involve the Brd4 protein (Silla et al., 2010).

4.4 Regulation of replication by E2

The relative abundance of E2 proteins within the cell is an important factor regulating papillomavirus DNA replication. In BPV1-transformed cells, the relative ratio of E2 proteins is 1:5:1.5 for E2-E2C-E8/E2. The truncated E2 repressor proteins predominate in the steady state and the E2 heterodimers with single activation domain formed between the full-length and truncated E2 proteins are the preferential form for E2 (Kurg et al., 2006). The promoters for full-length E2 as well as for repressors are themselves controlled and differently regulated by E2, and furthermore, the ratio of the repressors to activators changes throughout the cell cycle. In G_1 cells, the repressors dominate, but in late S phase and G_2/M, the activator is present at about equal levels to that of the repressors. So, the level of E2 activators and repressors as well as E2 homo- and heterodimers is changing suggesting that the balance of different E2 proteins is a key event in the regulation of papillomavirus DNA replication (Szymanski and Stenlund, 1991; Yang et al., 1991b).

In papillomaviruses, there is a link between transcription and replication control, the protein that binds specifically to origin of replication also functions in control of transcription. BPV1 E2 protein levels are regulated by E2 itself and E2 activators and/or repressors have a positive or negative feedback to virus DNA replication. Initially the level of E2 activators is high to facilitate DNA amplification after infection, but later, the E2 expression is strictly controlled to avoid over-replication. Genetic studies have shown that the E8/E2 protein of HPV18, 31 and at least one of the BPV1 repressors are required for the long-term maintenance of virus episomes, demonstrating the important role of E2 repressors in the viral life cycle. Deletion of the E8 ORF results in robust initial replication of HPV genomes followed by rapid loss of virus episomes from dividing cells. The role of E2 repressors in the virus life cycle is to modulate the activity of full-length E2 protein by preventing the E2 binding to E2BS via binding site competition, and by acting as a repressor recruiting host co-

repressor molecules (Kurg et al., 2010; Lambert et al., 1990; Stubenrauch et al., 2000). However, the persistent replication and maintenance of virus genomes is not affected in E8 knock-out genomes of HPV16 and cotton-tail rabbit papillomavirus (CRPV), suggesting that E2 proteins of different papillomaviruses may regulate their expression and through this replication by different ways (Jeckel et al., 2003; Lace et al., 2008).

The efficiency of papillomavirus DNA replication is also controlled by the level of the E1 protein. E2 regulates E1 expression level through modulation of the activity of viral early promoters (Hubert and Laimins, 2002; Szymanski and Stenlund, 1991). However, it is still not clear to what extent HPV E2 proteins regulate HPV URRs. The HPV16 E2 protein does not repress HPV16 transcription when the URR is contained within an episomal HPV genome (Bechtold et al., 2003). Another group has shown that transcription activation function of the HPV31 E2 protein is not required for the viral life cycle (Stubenrauch et al., 1998). In addition to the transcriptional regulation by E2, the expression of E1 is regulated post-transcriptionally by mRNA splicing. In high-risk HPVs, E1 is translated by a discontinuous scanning mechanism and mRNA splicing within the E6 ORF is required for efficient expression of E1 (Hubert and Laimins, 2002)(Remm et al., 1999).

5. Conclusion

E2 is the master regulator of extrachromosomal replication of papillomaviruses. E2 regulates papillomavirus replication at multiple levels and through different mechanisms. First, E2 is essential for initiation of papillomavirus DNA replication. Second, E2 is required for long-term stable replication and is involved in maintenance and segregation of viral genomic DNA. Third, the abundance of E2 proteins and formation of homo- and heterodimers possessing different activities as well as the expression level of viral helicase E1 is regulated by E2. In addition to direct involvement in replication, E2 indirectly regulates papillomavirus replication through modulation of expression and activity of viral oncogenes E6 and E7, and the cellular environment.

6. Acknowledgment

7. References

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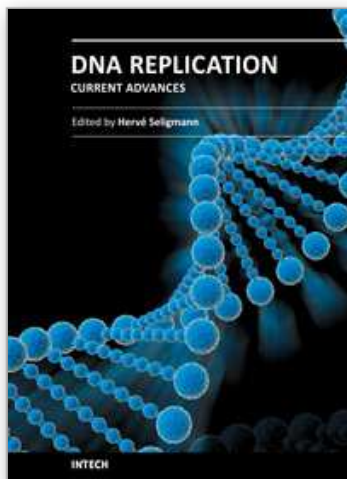
DNA Replication-Current Advances

Edited by Dr Herve Seligmann

The study of DNA advanced human knowledge in a way comparable to the major theories in physics, surpassed only by discoveries such as fire or the number zero. However, it also created conceptual shortcuts, beliefs and misunderstandings that obscure the natural phenomena, hindering its better understanding. The deep conviction that no human knowledge is perfect, but only perfectible, should function as a fair safeguard against scientific dogmatism and enable open discussion. With this aim, this book will offer to its readers 30 chapters on current trends in the field of DNA replication. As several contributions in this book show, the study of DNA will continue for a while to be a leading front of scientific activities.

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