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TOOMAS SILLA

Study of the segregation mechanism of the Bovine Papillomavirus Type 1

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My contributions to the papers are as follows:

Ref. I I designed and performed the experiments, except the flow cytometer experiments and long term experiments in mutated E2 cell lines. I analyzed the data, and I wrote most of the manuscript;

Ref. II I constructed lyciferase assay constructs, performed transactivation assays with following western blots and analyzed the data;

Ref. III I wrote the stable replication part of the manuscript;

Ref. IV I was actively involved in patent writing and discussions;

Ref. V I designed and performed the experiments and wrote the manuscript.

LIST OF ABBREVATIONS

BPV1	Bovine Papillomavirus Type 1
HPV	Human Papillomavirus
PV	papillomavirus
VLP	virus like particle
ORF	open reading frame
URR	upstream regulatory region
LCR	long control region
Rb	Rentinoblastoma protein
TAD	transactivation domain
DBD	DNA-binding domain
E2BS	E2 binding site
EBV	Epstein-Barr virus
PyV	mouse polyomavirus
BS	binding sites
MME	minichromosome maintenance element
FR	family of repeats
CTD	C-terminal domain
RNAP II	RNA polymerase II
hEF1α	human elongation factor 1 α
EBV	Epstein-Barr Virus
KSHV	Kaposi`s sarcoma associated human herpesvirus type 8
Brd4	Bromodomain-containing protein 4
P-TEFb	transcription elongation factor b

1. INTRODUCTION

A number of eukaryotic DNA viruses maintain their genomes as extrachromosomal multicopy nuclear plasmids in infected host cells. For instance, this type of episomal maintenance characterizes latent infection by papillomaviruses, Epstein-Barr virus (EBV) and Kaposi`s sarcoma-associated human herpesvirus type 8 (KSHV). For all these viruses, effective segregation of their genome into daughter cells and its nuclear retention during mitosis is mediated through a single viral protein that attaches the viral genome to the host's mitotic chromosomes. These linker proteins are E2 for papillomaviruses, LANA1 for KSHV and EBNA-1 for EBV.

Bovine papillomavirus type 1 (BPV1) and its encoded E2 protein is used as a prototype for papillomavirus stable maintenance studies. E2 protein itself has been described as a multifunctional protein that participates in viral replication, transcription and partitioning. Mutational analysis of E2 has shown that its N-terminal transactivation domain is very complex and is absolutely required for all these functions.

The first part of the present thesis gives a general overview about papillomaviruses, E2 protein and its functions in the papillomavirus life cycle. The research part of this dissertation focuses on the following areas: (i) studies of BPV1 and EBV segregation elements with heterologous replicons; (ii) discussion and characterization of stably maintained heterologous plasmids as tools for protein production; (iii) the use of chimeric E2 proteins to study the role of the transactivation domain of E2 in the chromatin attachment and in the segregation process and (iiii) characterization of the role of Brd4 in BPV1 replication and E2-mediated transactivation.

2. LITERATURE REVIEW

2.1. General introduction to papillomaviruses

Papillomaviruses (PVs) are a varied group of non-enveloped DNA viruses that have been found in more than 20 different mammalian species, as well as in birds and reptiles (Doorbar, 2005). PV genomes are circular double-stranded DNA close to 8 kbp in size. Although PV genomes have relatively little capacity, their molecular biology is very complex (de Villiers et al., 2004). The PV life cycle takes place exclusively in body surface tissues, such as the skin, or the mucosal surfaces of the genitals, anus and mouth (Doorbar, 2005). Visible symptoms of PV infection are benign tumors (warts, papillomas, condylomas) in their natural host and occasionally in related species (de Villiers et al., 2004). A wide variety of different types of PVs can be detected at random sites on healthy skin of humans and animals (Antonsson et al., 2003; Antonsson et al., 2000; Antonsson and Hansson, 2002). Thus, it appears that many PVs occur preferentially in a latent life cycle without causing any visible symptoms.

In the 1980s, the newly developed techniques of molecular biology led to the detection of dozens of human papillomaviruses (HPVs) in benign and malignant mucosal lesions (Gissmann et al., 1977; zur Hausen, 2002). The first HPV types isolated directly from biopsies of cervical cancer, indicating that HPVs may act as cancerous agents (Boshart et al., 1984; Durst et al., 1983). This observation initiated a rapid expansion of the field, and their medical importance has led to their being extensively studied. To date, the whole genomes of about 100 HPV types have been isolated and completely sequenced, but current data support the existence of more than 200 HPV types (Bernard, 2005). HPVs have been well established as sexually transmitted agents, and more than 50% of sexually active women have been infected by one or more genital HPV types at some point in time (Baseman and Koutsky, 2005; Bernard, 2005). Scientific studies have found that HPV infection is responsible for virtually all cases of cervical cancer (Munoz et al., 2003; Walboomers et al., 1999). Only a small number of HPV-infected women will go on to develop cervical cancer; nonetheless, cervical cancer is the second most common female cancer, with approximately 500,000 new cases and 290,000 deaths occurring worldwide per annum (Saleem et al., 2009). Fifteen HPV types, including types 16, 18, 31 and 45, are called "high-risk" types because they can lead to cervical cancer, as well as anal cancer, vulval cancer and penile cancer (Baseman and Koutsky, 2005; Munoz et al., 2003). However, HPV16 is the most prevalent type causing cervical cancer (Baseman and Koutsky, 2005; Woodman et al., 2001).

Due to scientific success in HPV molecular biology studies, two preventive HPV vaccines are currently on the market: Gardasil (Merck&Co) and Cervarix (GlaxoSmithKline). The vaccines that both companies have developed are subunit virus-like particle (VLP) vaccines composed of a single viral protein, L1, which is the major capsid protein of the virus and contains the immunodominant neutralization epitopes of the virus. Both vaccines protect against

initial infection with HPV types 16 and 18, which cause most of the HPV-associated cancer cases. Gardasil also targets HPV types 6 and 11, which are responsible for most of the genital warts (Psyrri and DiMaio, 2008). However, the current HPV vaccines do not offer any benefit to men and women who already have HPV 16 and 18 infection, and these vaccines do not target all the HPV types that cause cervical cancer (Psyrri and DiMaio, 2008; Wain, 2009). Therefore, even after vaccination, women should continue to undergo cervical screening (Psyrri and DiMaio, 2008).

For historical reasons, bovine papillomavirus type 1 (BPV1) is probably the best-studied representative of PVs. This literature review mainly focuses on the most extensively studied HPVs, HPV16 and -18, and on BPV1. BPV1 has been taken as a prototype for PV studies, and the molecular mechanisms that direct the replication and transcription of its genome have been studied thoroughly. From these studies, a general pattern of viral features has been revealed that, with slight modifications, can be applied to all PVs. Specific features of certain PVs are highlighted where appropriate.

2.2. The PV life cycle

PVs infect basal epithelial cells, which are the only actively dividing cells in the epithelial layer, and the subsequent life cycle of PVs is strictly dependent on the differentiation state of the infected epithelium (Fig. 1). Lack of appropriate epithelial tissue in *in vitro* models has been the main barrier to studying the PV life cycle. Nevertheless, BPV1 unique ability to infect and transform the mouse fibroblast cell line C127 has been a useful property for studying the early steps in the PV life cycle. To mimic differentiation of keratinocytes, organotypic (raft) cultures or semisolid methylcellulose systems have been used (Dollard et al., 1992; Flores et al., 1999; Meyers et al., 1992; Meyers et al., 1997; Ruesch and Laimins, 1998). Such systems have been a significant improvement for *in vitro* study of the viral life cycle. Most work on the PV life cycle has centered on the analysis of the high-risk HPV types, and in particular on HPV16.

Initial PV infection requires access of the infectious particles to cells in the basal layer of the epithelial tissue, which is thought to require a break in the stratified epithelium (Fig. 1). Such breaks or wounds may not be readily apparent, and these micro-traumas may occur in any environment and at any stage in the host's life. Controversy exists regarding the receptor used for the initial attachment of PV virions to the cell surface. Alpha integrin and heparan sulfate are two main candidates for the receptor (Giroglou et al., 2001; Oldak et al., 2006). The internalization of bound virions is believed to be a slow process that occurs through the endocytosis of clathrin-coated vesicles (Culp and Christensen, 2004; Day et al., 2003; Selinka et al., 2002).

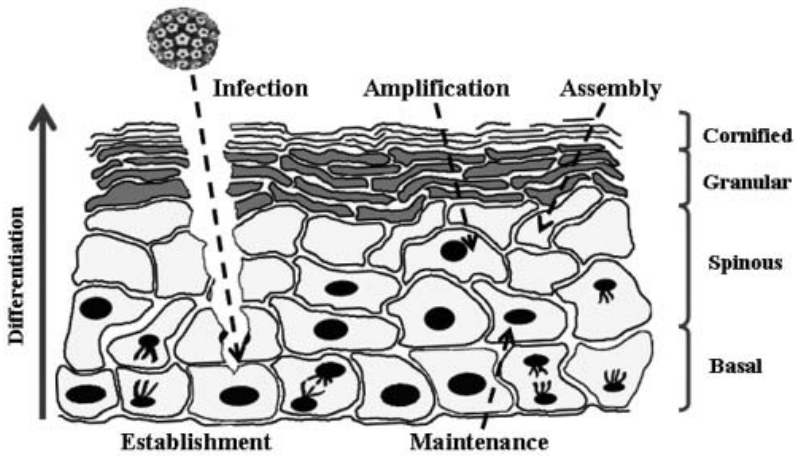

Figure 1. Cartoon of the PV life cycle. PV infection requires access to basal epithelial cells. This usually occurs through micro-abrasions within the skin or mucosa. After the infection is established, viral genomes are maintained at approximately 20–200 episomal copies per cell. In the maintenance phase, viral genomes passage up through the differentiating strata as the mitotically active basal cells divide. In the suprabasal layers, several viral genes are activated, leading to viral genome amplification and assembly of progeny virions. Particles are released at the highly keratinized cornified surface, allowing additional infected tissues to continue the viral life cycle. Figure adapted from Conway and Meyers, 2009.

After successful establishment of infection, the expression of viral early genes takes place, including that of the replication proteins E1 and E2. These two proteins recruit host DNA replication machinery to the viral origin of replication to ensure initial amplification of viral genomes (Chiang et al., 1992; Ustav and Stenlund, 1991). It has been suggested that the viral genome is maintained in the basal layer at around 20–200 copies per cell (Conway and Meyers, 2009). E1 and E2 are also responsible for establishment and maintenance of the viral genomes as extrachromosomal nuclear plasmids at stable copy numbers.

In normal epithelium, basal cells exit the cell cycle soon after migrating into the suprabasal cell layers and undergo a process of terminal differentiation (Fuchs, 2008). Although terminally differentiated epithelial cells are not normally able to support DNA synthesis, viral DNA replication takes place in these cells. The viral oncogenes E6 and E7 induce unscheduled re-entry into the S-phase of the cell cycle, with activation of the host replication machinery needed for viral DNA replication (Cheng et al., 1995). It has been shown that episomal expression of E6 and E7 is tightly regulated, with high-level expression only in suprabasal post-mitotic cells (Durst et al., 1992; Stoler et al., 1992).

For the production of infectious virions, PVs must amplify their genomes, and for this process high levels of the viral replication proteins E1 and E2 are required. The open reading frame (ORF) of E7 contains a late promoter, and it

is thought that its up-regulation leads to increased expression of E1 and E2 proteins (probably also E4 and E5 proteins) (Grassmann et al., 1996; Klumpp and Laimins, 1999; Ozbun and Meyers, 1998). Thus, E6/E7 and E1/E2 up-regulation leads to cell-cycle progression and dramatic viral DNA amplification, up to thousands copies in differentiated keratinocytes.

PVs encode two structural proteins, L1 and L2, that are expressed in the upper layers of infected tissue once viral genome amplification has been completed. Subsequently, this leads to the formation of new viral particles (Doorbar, 2005). PVs are non-lytic viruses that are not released until the infected cells reach the epithelial surface and are shed from the epithelial surface. A general build-up of the life cycle is believed to be the same in the case of all PVs (Fig. 1); however, some differences are likely to exist in the details.

2.3. PV genome organization and encoded proteins

PV genomes are circular dsDNA molecules approximately 8 kbp in size that share similar organization. Based on comparisons of sequencing data from different PV types, three distinct regions in the PV genome have been described (Fig. 2): (1) a non-coding upstream regulatory region (URR; also called the long control region, or LCR) that carries multiple regulatory elements required for viral replication and gene expression; (2) an early region (E) that contains translational open reading frames (ORFs) that encode proteins required for transcription, replication and transformation and (3) two ORFs in a late region (L) that encode the major and minor capsid proteins.

The early region of the PV genome typically contains eight ORFs, and the encoded proteins are named accordingly. The ORF E1 encodes a nuclear phosphoprotein, which in complex with the viral regulatory protein E2 co-operatively binds to the viral origin of replication. This binding is absolutely required for viral DNA replication (Blitz and Laimins, 1991; Lusky and Botchan, 1985; Lusky and Fontane, 1991; Ustav and Stenlund, 1991; Yang et al., 1991). E1 has ATP-dependent DNA helicase activity that forces apart the DNA strands and thereby prepares the viral genome for replication by cellular DNA replication factors (Stenlund, 2003b).

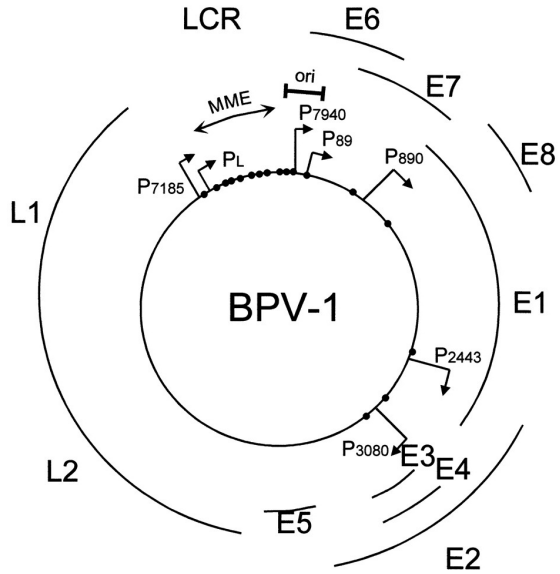

Figure 2. A schematic representation of the BPV1 genome. The locations of eight early (E) and two late genes (L) are indicated. Promoters are represented by arrows. Localization of E2BS are shown by small black circles. The LCR (long control region) contains regulatory elements for transcription and DNA replication such as the origin and the minichromosome maintenance element (MME). Figure adapted from Skiadopoulos and McBride, 1998.

The E2 ORF encodes a family of nuclear regulatory proteins that regulate viral transcription, replication and partitioning/segregation. The full-length product of the ORF, the E2 protein (also called E2TA), is a transcription activator; however, HPV E2 proteins have also been shown to be transcriptional repressors of the viral oncogenes E6 and E7 (Howley, 2007). E2 protein also loads replication protein E1 onto the origin of viral replication (Stenlund, 2003b). E1 and E2 together are absolutely required for the initiation step of viral DNA replication *in vivo*. It has been shown that E2 protein attaches to the host's mitotic chromosomes and also tethers viral DNA to the chromosomes, thereby ensuring viral genome segregation to daughter cells after mitosis (McBride, 2008). Two shorter spliced forms of E2, E2C and E8/E2, are known as transcriptional repressors. The functions of E2 proteins are described in more detail in chapter 2.4.

The E3 ORF exists only in a few PV types and does not contain an AUG start codon; it therefore does not appear to serve any function.

The E4 ORF is entirely located within the E2 ORF, but due to different reading frames, the E4 protein's amino acid sequence is different from that of E2. The transcript of the E4 protein contains five codons corresponding to amino acids from the E1 ORF, hence the encoded protein is frequently called

E1-E4. The expression of E4 increases dramatically during the late phase of infection in the differentiating layer of epithelium, where viral vegetative replication is ongoing but capsid proteins are not yet synthesized (Doorbar et al., 1997). The E4 protein interacts with the keratin cytoskeleton and causes it to collapse, and this may be required for the release of mature viral particles (Doorbar et al., 1991). E4 also causes cell-cycle arrest in G2/M, which may limit cell-cycle progression and facilitate efficient replication during the productive cycle (Davy and Doorbar, 2007).

The E5 ORF encodes a small, very hydrophobic protein. The 44–amino acid E5 protein of BPV1 is a transmembrane protein that is the smallest known oncoprotein (Talbert-Slagle and DiMaio, 2009). E5 has been shown to be the main transforming protein of fibro-papillomaviruses (BPV1, BPV2) (Yang et al., 1985) because it activates the cell growth-promoting signaling of PDGF-β growth receptors (Talbert-Slagle and DiMaio, 2009). BPV1 E5 has also been shown to interact with the transmembrane channel forming subunit of H^+-ATPase and thereby impairs the acidification of the Golgi apparatus, but it has not yet been directly shown that this plays a role in cellular transformation (Schapiro et al., 2000).

The functions of HPV E5 proteins are less well known, although HPV E5 proteins have been shown to interact with PDGF-β growth receptors and H^+-ATPase. However, it should be noted that the E5 gene is not expressed in most HPV-positive cancers, suggesting that it presumably functions in benign papillomas and not in cancer (Howley, 2007).

ORFs E6 and E7 encode major transforming proteins for HPVs. Expression of high-risk E6 leads to the transformation of NIH 3T3 and human mammary epithelial cells, but efficient immortalization of human keratinocytes requires the expression of both E6 and E7 oncogenes (Hawley-Nelson et al., 1989; Longworth and Laimins, 2004). The primary transforming activity identified for the high-risk E6 protein is inactivation of the tumor suppressor protein p53, a property not possessed by low-risk HPV E6 proteins (Werness et al., 1990). E6 forms a complex with a ubiquitin ligase called E6AP, which then binds to p53 and leads to its degradation through the ubiquitin-dependent pathway (Huibregtse et al., 1991). p53 is a well-characterized tumor suppressor protein that regulates the cell cycle and apoptosis. For viruses, it is important to prevent host cell apoptosis. Therefore, many viruses have evolved mechanisms to avoid the induction of apoptosis, for instance by down-regulating the pro-apoptotic properties of p53. Some studies have shown that E6 can induce transformation and immortalization of human cells through a p53-independent pathway, probably by interacting with PDZ family proteins (Kiyono et al., 1998; Liu et al., 1999).

The high-risk E6 proteins interact with a number of other cellular proteins. Another important function of high-risk E6 proteins for transformation and immortalization is the activation of the catalytic subunit of telomerase, hTERT (Klingelhutz et al., 1996). The lack of telomerase activity results in a shortening of telomeres that finally leads to senescence. Activation of telomerase is linked

to cell immortalization and is a characteristic of most tumor cell lines (Liu, 1999). E6 activates hTERT transcription by interacting with the transcription factor Myc (Veldman et al., 2003).

E7, the second important transforming protein, is primarily known to inactivate members of the retinoblastoma (Rb) family of tumor suppressor proteins (Dyson et al., 1989). E7 protein interaction sequesters Rb protein away from the E2F transcription factor complex, resulting in activation of genes required for cell-cycle progression from G1 to S-phase. This allows productive replication of PV genomes in differentiated suprabasal cells (Chellappan et al., 1992; Longworth and Laimins, 2004; Weintraub et al., 1995). Both high-risk and low-risk HPV E7 proteins are capable of binding to Rb family members, although low-risk E7 proteins are not able to activate E2F-inducible genes or transform rodent cells and many low-risk E7 proteins show much lower affinity for Rb (Ciccolini et al., 1994). In addition to binding to Rb family members, E7 proteins also associate with cyclins, cyclin-dependent kinases and histone deacetylases, and they have been shown to induce genomic instability in host cells (Duensing et al., 2000; Longworth and Laimins, 2004).

In some cases, the activities of E6 and E7, together or independently, may allow for the development of malignancies; however, the primary role of E6 and E7 in the viral life cycle is not to induce transformation or immortalization, but more likely involves facilitation of some aspect of viral replication.

The E8 ORF of several PV types encodes a short peptide that is fused to the E2 hinge and DBD by splicing (Choe et al., 1989; Doorbar et al., 1990). The E8 domain encoded by HPV31 consists of only 12 residues. E8-E2C acts as a transcriptional repressor from both promoter-proximal and promoter-distal E2BS (Stubenrauch et al., 2001). HPV31 genomes carrying mutations within the E8 gene replicate at high copy number, suggesting a critical role for the E8 domain in limiting viral genome replication (Zobel et al., 2003).

The L1 ORF encodes the major capsid protein of PV virions. L1 can spontaneously self-assemble into pentameric capsomeres (Kirnbauer et al., 1992; Kirnbauer et al., 1993). Seventy-two capsomeres of L1 make up the capsid. It is postulated that the C-terminal H4 helixes in each L1 molecule are responsible for capsomere maturation through forming disulfide bonds (Pereira et al., 2009). Compared to other PV genes, the amino acid sequences of most portions of L1 are well-conserved between different types and have therefore been used for the identification of new PV types (de Villiers et al., 2004).

The L2 ORF-encoded protein is a minor capsid protein. The stoichiometry of L1:L2 is speculated to be 30:1; however, there are 72 potential sites in the capsid in which L2 could be found (Pereira et al., 2009). L2 plays important roles in viral entry into cells, localization of viral components to the nucleus, DNA binding, capsid formation and capsid stability (Pereira et al., 2009).

2.4. E2 as a master regulator of the PV life cycle

2.4.1. General structural features of E2 protein

The E2 of BPV1 was first characterized as a transcriptional activator (Spalholz et al., 1985; Yang et al., 1985). It is now well known that PV E2s are multi-functional proteins (Fig. 3). The contributions of E2 to the transcription (Androphy et al., 1987; Spalholz et al., 1985; Thierry and Yaniv, 1987), replication (Chiang et al., 1992; Stenlund, 2003a; Ustav et al., 1993; Ustav and Stenlund, 1991; Ustav et al., 1991), maintenance and segregation (Abroi et al., 2004; Ilves et al., 1999; Lehman and Botchan, 1998; Piirsoo et al., 1996; Skiadopoulos and McBride, 1998) of viral genomes have been well characterized. In addition, it has been shown that E2 also participates in cell-cycle progression (Hwang et al., 1993), apoptosis (Blachon et al., 2005) and senescence (Goodwin and DiMaio, 2001).

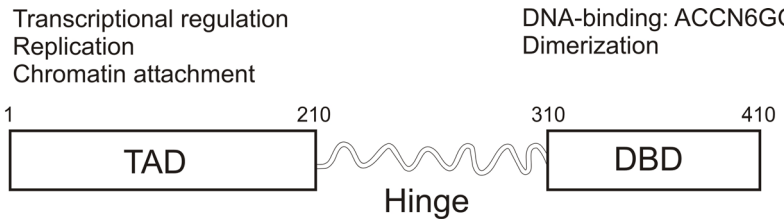

Figure 3. Map of the full-length BPV1 E2 protein. Key functions of the N-terminal transactivation domain (TAD) and the C-terminal DNA-binding domain (DBD) are indicated above. The amino acid numbers that delimit the domains correspond to those of BPV1 E2.

The full-length 48-kDa E2 protein is expressed from the entire E2 ORF, and it contains three conserved functional domains (Fig. 3): an N-terminal trans-activation domain (TAD) of ca. 200 amino acid residues, a C-terminal DNA-binding/dimerization domain (DBD) of ca. 100 residues and a hinge region of ca. 70 amino acid residues linking these domains (Giri and Yaniv, 1988). The repressor forms of E2, E2C and E8-E2C, contain DBD, but they lack N-terminal TAD (Howley, 2007).

The crystal structure of E2 TAD-s have been reported for HPV16, HPV18 and HPV11 (Antson et al., 2000; Harris and Botchan, 1999; Wang et al., 2004). All these structures show similarities; therefore, this structural information is most likely valid for other PV species. The TAD contains two sub-domains; an N-terminal subdomain of anti-parallel α-helices and a C-terminal β-sheet subdomain (Antson et al., 2000; Harris and Botchan, 1999).

The TAD appears to be extremely sensitive to conformational disruption. Deletions or even single amino acid substitutions within the TAD may

inactivate E2's functions in DNA replication, interaction with the E1 protein, transcriptional regulation and segregation (Abroi et al., 2004; Baxter and McBride, 2005; Baxter et al., 2005; Breiding et al., 1996; Zheng et al., 2005; Winokur and McBride, 1992). E2 protein acts as a dimer, and it is widely accepted that dimerization occurs through the E2-DBD (McBride et al., 1989b). But it has been shown that the HPV16 E2 TAD also forms a dimer both in the crystal and in solution (Antson et al., 2000). By contrast, the HPV11 E2 TAD has been shown to be a monomer in solution (Wang et al., 2004). It has been suggested that TAD-mediated oligomerization could play a role in interactions between E2 molecules bound at distant E2BSs on the viral genome, mediating DNA looping that may serve to relocate distal DNA-binding transcription factors to the site of HPV transcription initiation (Antson et al., 2000; Hernandez-Ramon et al., 2008).

The structures of the E2-DBDs from several viral types have also been reported. These include the crystal structures of the E2-DBD from HPV16 (Hegde and Androphy, 1998), HPV18 (Kim et al., 2000), HPV31 (Bussiere et al., 1998) and BPV1 (Hegde et al., 1992). The E2-DBD forms a dimeric β-barrel, and it is the prototype for a novel structural class of DNA-binding proteins (Hegde, 2002). The other DNA-binding protein known to have a dimeric β-barrel structure is the Epstein-Barr virus (EBV) replication protein EBNA-1, which is remarkably similar to the structure of the E2 protein, despite a complete lack of sequence conservation (Bochkarev et al., 1995). The tertiary structures of all characterized E2-DBDs are similar. However, sequence conservation among the various E2-DBD ranges from 77% similarity among the closely related strains HPV16 and HPV18 to 51% similarity between the distant strains HPV16 and BPV1 (Hegde, 2002).

Some structural information is also available for the hinge region that connects the DBD and the TAD. It has been shown that this region is non-conserved and unstructured (Gauthier et al., 1991). Analysis of the BPV1 E2 protein revealed that the hinge region contains two major Ser residue phosphorylation sites at positions 298 and 301 (McBride et al., 1989a). Later studies have shown that phosphorylation at these sites may be necessary for the regulation of viral DNA replication (Lusky and Fontane, 1991; McBride and Howley, 1991). Mutational analyses in the hinge region of low-risk HPV11 E2 have shown that a cluster of basic amino acid residues, which are conserved among many mucosotropic papillomaviruses, are required for efficient nuclear localization and nuclear matrix association (Zou et al., 2000). This HPV11 E2 NLS is not conserved in the high-risk HPV16 and HPV18 E2 proteins, in which an NLS is described to be located in the DBD (Klucevsek et al., 2007). A functional NLS has also been shown to be located in the DBD of BPV1 E2 protein (Skiadopoulos and McBride, 1996).

2.4.2. E2 as a transcriptional regulator

The first evidence that E2 protein is involved in transcriptional regulation of viral promoters via enhancer elements derived from studies of BPV1 E2 protein made in the first half of 1980s (Lusky et al., 1983; Spalholz et al., 1985; Yang et al., 1985). Further studies showed that these viral enhancer elements contain E2 binding sites (E2BS) located in the URR region of the viral genome and that E2 binds as a dimer to these specific sequences (Androphy et al., 1987; McBride et al., 1989b; Moskaluk and Bastia, 1987). A consensus sequence has been established for the E2BS: $ACCN_6GGT$, but internal and flanking nucleotides have been shown to influence the affinity of E2 for these cognate sites (Androphy et al., 1987; Hawley-Nelson et al., 1988; Hines et al., 1998; Li et al., 1989; Moskaluk and Bastia, 1987). There are 17 E2BSs located within the BPV1 genome, but in the case of HPVs the number of E2BSs is restricted to only four (Howley, 2007). HPV16 E2 binds with increased affinity to E2BSs that contain A:T-rich spacers (Bedrosian and Bastia, 1990; Hines et al., 1998). However, BPV1 E2 does not display any significant ability to discriminate between E2BSs with different spacer sequences (Hines et al., 1998). Several studies have shown that E2 binding to BSs occurs cooperatively, thus, two BSs in proximity display enhanced E2 occupation (Lambert et al., 1989; Monini et al., 1993; Monini et al., 1991). Two copies of the E2BS are sufficient to function as an E2-dependent enhancer in mammalian cells (Harrison et al., 1987; Hawley-Nelson et al., 1988; Spalholz et al., 1988)

The BPV1 genome contains seven promoters (Fig. 2): six of them (P_{89}, P_{890}, P_{2443}, P_{3080}, P_{7185}, P_{7940}) are active in transformed cells, and the major late promoter (P_{7250}) is active in productively infected keratinocytes (Howley, 2007). It has been shown that E2 itself can activate transcription from viral promoters $P_{7940,}$ $P_{89,}$ $P_{890,}$ P_{2443} and P_{3080} (Haugen et al., 1987; Hermonat et al., 1988; Prakash et al., 1988; Spalholz et al., 1987; Stenlund et al., 1985; Vaillancourt et al., 1990). The P_{7185} promoter, on the other hand, is negatively regulated by E2 (Stenlund and Botchan, 1990). It has been shown that E2 acts as transactivator even in yeast cells (Lambert et al., 1989), and it activates transcription from heterologous promoters, whereas transactivation required the presence of a minimum of two E2BSs in close proximity to the promoter or five BSs at a distance (Thierry et al., 1990).

In addition to E2BSs, the URR of PVs contains a number of binding sites for cellular transcription factors, including TFIID, Oct-1, AP1, SP1, YY1 and AP-2 (Longworth and Laimins, 2004). E2 is unable to activate transcription from minimal promoters containing only a TATA box; additional promoter proximal elements that bind transcription factors such as Spl are also required (Ham et al., 1991).

Interestingly, the N-terminal TAD of E2 can activate transcription from heterologous promoters without DNA binding activity, suggesting that the mechanism of trans-activation involves a direct interaction between the N-terminal TAD of E2 and common transcription factors at the promoter (Haugen et

al., 1988). To date, it has been reported that PV E2 interacts with several transcriptional activators or co-activators, including Sp1 (Li et al., 1991), TBP/TFIID, TFIIB (Benson et al., 1997; Rank and Lambert, 1995; Yao et al., 1998), p300 (Peng et al., 2000), AMF1 (Breiding et al., 1997), C/EBP (Hadaschik et al., 2003), hNAP-1 (Rehtanz et al., 2004) and Brd4 (You et al., 2004).

As mentioned above, E2 proteins can also repress transcription. E2-dependent transcriptional repression has mostly been studied in the context of HPVs, where E2 represses transcription of the viral oncogenes E6 and E7 through repression of the viral early promoter (Thierry and Yaniv, 1987). In the case of HPV16 and HPV18, two E2BSs are located just upstream from the TATA box of the early promoter, and binding of E2 to these sites may sterically hinder the binding of cellular factors such as SP1 and TFIID, which prevents the formation of the transcriptional initiation complex (Thierry, 2009). In a similar situation, an E2BS located downstream from the BPV1 P_{7185} promoter overlaps an Spl-binding site. This Spl site is essential for P_{7185} activity, and binding of E2 can repress expression from this promoter, probably by interfering with Spl binding (Stenlund and Botchan, 1990; Vande Pol and Howley, 1990). Interestingly, the same E2-recruited factor (for example, the chromatin component Brd4) may participate in transcriptional activation or repression (Lee and Chiang, 2009; Schweiger et al., 2007; Smith et al., ; Wu et al., 2006). Because Brd4 binds preferentially to acetylated chromatin, epigenetic modifications have been taken into consideration in the study of PV E2-dependent transcriptional activation and/or repression processes. It has also been proposed that looping of the regulatory sequences through interaction of the E2 amino-terminal domains could be involved in the mechanism of repression (Hernandez-Ramon et al., 2008).

2.4.3. Stable genome maintenance via E2 chromatin attachment and tethering functions

In the beginning of the 1980s, several independent groups showed that the BPV1 genome replicates exclusively as an extrachromosomal multicopy plasmid in transformed cells and within tumors (Amtmann et al., 1980; Binetruy et al., 1982; Law et al., 1981; Sarver et al., 1981). Viruses that infect actively dividing cells must ensure retention of their genomes within the nuclear envelope to prevent genome loss during cell division. Some viruses integrate their genome into the host genome; in this case, the viral genome becomes a part of the infected cell's DNA, which ensures viral genome replication and segregation along with cellular DNA. However, the above-mentioned extrachromosomal state of BPV1 is characteristic of all PVs. Thus, PVs must have some other mechanism that ensures viral genome retention in the nuclear envelope and that promotes roughly even distribution of episomal DNA molecules to each daughter cell.

Initially it was shown that the URR of BPV1 contains two partially overlapping *cis*-regulatory control elements for stable replication, termed plasmid maintenance sequence 1 (PMS-1) (Lusky and Botchan, 1984) and negative control of replication 1 (NCOR- 1) (Roberts and Weintraub, 1986). Later experiments from our lab demonstrated that these sequences are not required for long-term episomal maintenance (Piirsoo et al., 1996). Further studies indicated that stable replication of PBV1 genomes requires a minimal replication origin (MO) and multiple E2BSs (which together are defined as a minichromosome maintenance element; MME) in *cis* and viral E1 and E2 proteins in *trans* (Piirsoo et al., 1996). In addition, plasmids with less than six E2BSs failed to replicate in a long-term replication assay (Piirsoo et al., 1996). These results indicated that E2 protein and its BS have key function in stable BPV1 maintenance; however, the exact mechanism remained uncertain.

Further experiments showed that BPV1 E2 protein is associated with mitotic chromosomes in dividing cells, where E2 protein was observed as punctate dots (Skiadopoulos and McBride, 1998). The shorter E2C and E8/E2 repressor proteins do not bind to mitotic chromatin, indicating that the N-terminal TAD of E2 is crucial for this association (Skiadopoulos and McBride, 1998). Additional studies confirmed that the E2 TAD alone attaches to chromosomes and that the DNA binding function of E2 is not required for chromatin association (Bastien and McBride, 2000; Skiadopoulos and McBride, 1998). E2 is associated with cellular chromosomes at all stages of mitosis and probably also in interphase (Bastien and McBride, 2000; Kurg et al., 2005). The hinge region of E2 contains phosphorylation sites at positions 235, 298, 301 and 290 (Lehman et al., 1997; McBride et al., 1989a). The Botchan group showed that in the background of the entire viral genome, E2 protein that carries alanine substitutions at these positions does not attach to chromosomes, and this protein does not support stable BPV1 genome maintenance (Lehman and Botchan, 1998). However, in the absence of other viral proteins, the E2 protein containing alanine substitutions was detected on mitotic chromosomes similarly to wild-type E2 protein (Bastien and McBride, 2000; Voitenleitner and Botchan, 2002). Moreover, E1 protein can dislocate E2 protein from chromosomes, and the phosphorylation mutant was much more sensitive to chromosomal dislocation, indicating that E1 protein may regulate chromosomal attachment of E2 in a way that may be dependent on the phosphorylation status of E2 (Voitenleitner and Botchan, 2002). E2 proteins with amino acid substitutions in conserved residues of the TAD showed the remarkable complexity of this domain (Abroi et al., 2004; Baxter et al., 2005; Zheng et al., 2005). Just a single amino acid substitution may abrogate the ability of E2 to associate with mitotic chromosomes (Abroi et al., 2004; Baxter et al., 2005; Zheng et al., 2005).

It has been shown that the several E2 proteins from the alpha group of HPVs (e.g., HPV11, -16, -31 and -57) can stably interact with mitotic chromosomes (Donaldson et al., 2007; Oliveira et al., 2006). Compared to BPV1 E2, the association of alpha group E2 proteins to chromosomes seems to be much more dynamic and unstable (Oliveira et al., 2006). Studies with a member of the beta

group of HPV indicated that E2 protein of HPV8 binds as large speckles at the pericentromeric region of chromosomes, in contrast to BPV1 E2, which binds to all chromosomes as small speckles (Poddar et al., 2009). Furthermore, HPV8 E2 protein binds to the repeated ribosomal DNA genes that are found on the short arm of human acrocentric chromosomes, and the C-terminal DNA binding domain of HPV8 E2 is necessary and sufficient for interaction with mitotic chromosomes (Poddar et al., 2009; Sekhar et al., 2009). The Chow group has shown a further difference in the case of HPV11, -16 and -18, the E2 proteins of which were found to be attached to the mitotic spindle during mitosis (Dao et al., 2006; Van Tine et al., 2004). It seems that targeting of host chromosomes by viral E2 protein is characteristic of many PVs; however, the localization determinants required for this process differ.

It is widely accepted that E2 protein does not associate directly with host DNA. It is believed that E2 protein chromosomal binding is mediated through cellular proteins that are bound to chromosomes. Finding those factors has been an interesting challenge for many groups. To date, several potential E2 receptors on host chromosomes have been identified, including Brd4, ChlR1, TopBP1 and MKlp2 (Donaldson et al., 2007; Parish et al., 2006; You et al., 2004; Yu et al., 2007). The bromodomain family member Brd4 was the first candidate identified and is therefore the most extensively studied (You et al., 2004). It has been shown that Brd4 is required for the attachment of HPV31, HPV16 and BPV1 E2 to mitotic DNA (Abbate et al., 2006; Baxter et al., 2005; You et al., 2004). In support of this, overexpression of the C-terminal domain of Brd4 (Brd4-CTD) or a small peptide shown to block the interaction between E2 and Brd4 prevents the association of E2 with host cell chromosomes and enhances genome loss in BPV1-transformed cells (Abbate et al., 2006; McPhillips et al., 2005; You et al., 2004). On the other hand, it has been shown that Brd4 is not required for the maintenance of all PV types (McPhillips et al., 2006). Moreover, depletion of Brd4 using RNA interference has no effect on the association of BPV1 or HPV11 E2 proteins with mitotic chromosomes (Parish et al., 2006). These data strongly suggest a role for Brd4 in the E2 chromatin attachment of at least some PV types.

In addition to E2 protein, the BPV1 genome itself is also closely associated with mitotic chromosomes (Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). Experiments by Ilves et al. showed that the URR of BPV1 contains sequences required for chromatin attachment (Ilves et al., 1999). Further analysis of viral factors revealed that the E2 protein in *trans* and its multiple BSs in *cis* are both necessary and sufficient for tethering viral genomes to chromatin (Ilves et al., 1999). Additional functional experiments demonstrated that effective chromatin attachment of E2 protein tethers E2BS-containing plasmid DNA to chromosomes and is required for its partitioning function (Abroi et al., 2004). In the case of HPVs, it has been shown that HPV11 URR-containing plasmids are localized to the mitotic spindle, and this recruitment is E2-dependent (Van Tine et al., 2004). These data provide firm evidence that the partitioning/segregation function of the BPV1 genome is

dependent on the chromatin attachment process mediated by viral E2 protein and its multiple BSs. This conclusion is likely to be valid for HPVs generally, with some subtle differences.

According to data provided above, a model has been proposed for all PVs regarding viral genome segregation and maintenance (Fig. 4). The E2 protein forms a link between the viral genome and host DNA. In general, the E2 C-terminal DBD is specifically bound to the E2BS located in the viral genome, and the N-terminal TAD of E2 ensures association with mitotic chromosomes via interaction with cellular chromatin-associated protein(s). This E2-mediated chromatin attachment assures viral genome tethering to the host DNA, which is a prerequisite for the nuclear retention and segregation of the viral genome during host cell mitosis.

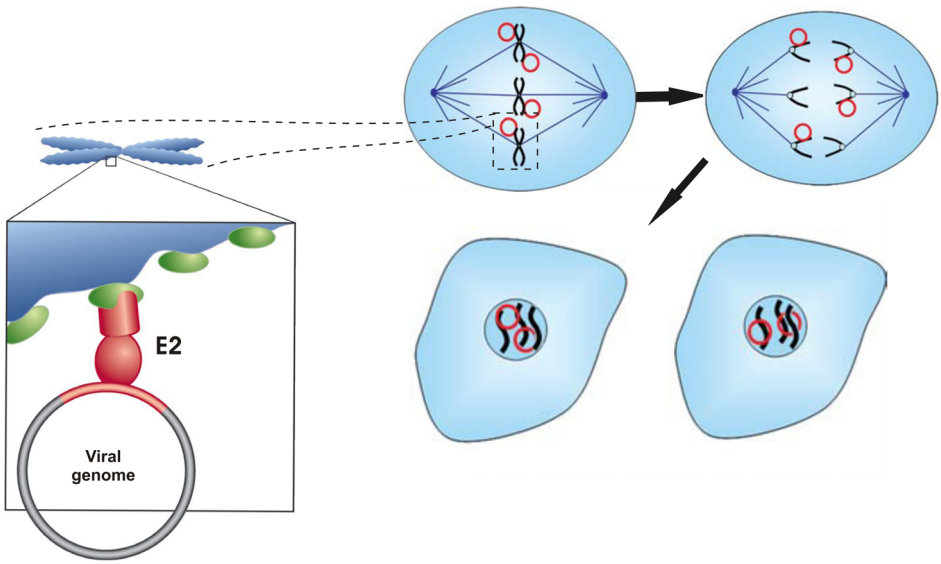

Figure 4. Viral genome tethering during mitosis ensures nuclear retention and viral genome segregation between daughter cells after cell division. The PV-encoded DNA-binding protein E2 (red) associates with specific sequences within the viral genome (circle) while simultaneously associating with chromatin (blue)-bound cellular protein(s) (green). This robust tethering mechanism ensures that episomal DNA molecules are more evenly distributed between daughter cells and remain in the nuclear compartment, thus ensuring genome maintenance in dividing cells and persistent viral infection. This figure is modified from Feeney and Parish, 2009.

2.5. Chromatin attachment and tethering proteins of other viruses

The strategy of maintaining and partitioning extrachromosomal viral genomes by tethering them to cellular chromosomes is not limited to PVs. A similar strategy has also been described for the gamma herpesviruses EBV, KSHV, Herpesvirus saimiri (HVS) and murine gamma herpesvirus-68 (MHV-68). All these viruses are known as DNA viruses that cause persistent infections (McBride, 2008)

Similarly to PVs, each of these viruses encodes a DNA-binding protein that binds specifically to repeated sites in the viral DNA and tethers the viral genome to cellular mitotic chromosomes to facilitate partitioning. The best-studied of these herpesvirus tethering proteins are EBNA-1 and LANA1 from EBV and KHSV, respectively.

Deletion studies have verified that three independent domains of EBNA1 are involved in mitotic chromosome binding (Marechal et al., 1999). It has been shown that histone H1 and EBP2 may be the EBNA1 targets on host chromosomes (Marechal et al., 1999; Wu et al., 2000). In addition to cellular protein-mediated EBNA1 binding to chromosomes, it has also been proposed that EBNA1 may directly interact with cellular DNA through its AT hooks (Sears et al., 2004). Similarly to E2 and EBNA1, LANA1 interacts with a number of different mitotic chromosome-binding proteins, such as histone H1, DEK, methyl CpG binding protein, Brd2, Brd4 and an H2A/H2B histone dimer (Barbera et al., 2006; Cotter and Robertson, 1999; Krithivas et al., 2002; Platt et al., 1999; You et al., 2006). Interestingly, all these viral tethering proteins interact with Brd4, which currently is the only known common interacting partner. In addition to Brd4-binding, EBNA-1 and LANA have many features in common with E2. Both proteins regulate viral transcription and are involved in the initiation of viral DNA replication (McBride, 2008). Thus, diverse viral types have evolved tethering proteins that have common features to ensure the episomal maintenance of their genomes, and the segregation processes used by these viruses are very similar (Fig. 4).

2.6. BPV1 DNA replication

The productive life cycle of PVs is exclusively linked to the stratified epithelium of skin or mucosa. During normal skin development, uninfected keratinocytes exit the cell cycle and are committed to terminal differentiation, ultimately leading to programmed cell death. Therefore, PVs have evolved mechanisms to adapt to the normal cellular growth control pathways and to adjust their DNA replication and maintenance cycle to contend with all different cellular differentiation states. A triphasic life cycle model has been proposed for PVs: initial amplification, subsequent stable maintenance and vegetative multiplication. Successful PV infection requires replication of viral DNA at all these stages.

2.6.1. Initial amplification replication

The successful infection of basal cells (Fig. 1) leads to an initial amplification of PV DNA copy number. Much of the knowledge about the early steps in the PV replication cycle is based on studies of BPV1 using its ability to replicate and maintain in the mouse fibroblast cell line C127. To date, it is widely acknowledged that the initial amplification of BPV1 and other PV genomes is dependent on the viral E1 and E2 proteins, which recruit host replication factors to the viral origin of replication. The initial amplificational replication can be modeled transiently in cell culture by co-transfecting a viral origin-containing plasmid with E1, E2 expression plasmids (Del Vecchio et al., 1992; Remm et al., 1992; Ustav and Stenlund, 1991). Interestingly, mixed combinations of E1 and E2 proteins from HPV11 and BPV1 are able to initiate replication from origin sequences of the same viruses in various cell lines, indicating that the interactions among *cis* and *trans* factors of PVs are conserved (Chiang et al., 1992).

The origin of replication of PVs has been mapped to the URR region (Chiang et al., 1992; Del Vecchio et al., 1992; Remm et al., 1992; Ustav et al., 1991). The minimal origin of replication contains at least one binding site for E1 and E2 and an A/T rich region (Remm et al., 1992; Ustav et al., 1993; Ustav and Stenlund, 1991).

Several groups have reconstituted PV origin-dependent replication in cell-free systems with purified E1 and E2 proteins and extracts from mammalian cells (Bonne-Andrea et al., 1995; Kuo et al., 1994; Melendy et al., 1995; Muller et al., 1994; Yang et al., 1991). *In vitro* experiments have demonstrated that E1 protein is the only viral protein participating directly in replication initiation and subsequent elongation. E1 is a hexameric ATP-dependent DNA helicase that is responsible for melting the DNA at the viral origin as well as for subsequent unwinding of the DNA double helix during replication fork progression (Sedman and Stenlund, 1998; Yang et al., 1993). E2 protein has an auxiliary role during the initiation of DNA replication, where it acts as a specificity factor for E1 (Bonne-Andrea et al., 1997; Sedman and Stenlund, 1995). E1 protein has low sequence specificity and therefore can also initiate DNA replication *in vitro* from non-specific DNA sequences (Bonne-Andrea et al., 1995; Bonne-Andrea et al., 1997; Sedman and Stenlund, 1995). Replication inside the cell, however, absolutely requires E2 in addition to E1 (Sedman and Stenlund, 1995; Ustav and Stenlund, 1991).

2.6.2. Stable maintenance replication

The initial amplification of viral genomes is believed to be rapid and transient. After a quick increase in copy number, the viral DNA is stably maintained at an almost constant copy number during subsequent divisions of the basal cells. This is thought to be achieved by maintenance replication, wherein the viral

genomes approximately double during S-phase of the host cell cycle and are almost equally segregated into the two daughter cells.

There are two scenarios that could in theory maintain the viral DNA at roughly the same copy number throughout the proliferative phase of the host cell: (i) viral DNA replicates once per S-phase or, (ii) replication initiation is not so rigidly regulated and multiple initiations from same origin may occur. Once-per-cell-cycle replication is strictly controlled by host cellular factors and has been described in the case of EBV (Adams, 1987). Once-per-S-phase replication has been described in the case of HPV16 in W12 cells. However, HPV31 and HPV16 replicate in a random-choice way in an alternative keratinocyte cell line NIKS. It seems that the switch between different replication modes is dependent on the level of expression of the E1 protein. This assumption is supported by the fact that in the case of higher expression levels of the E1 protein, HPV16 DNA replicates randomly in W12 cells (Hoffmann et al., 2006). It is widely accepted that the DNA replication initiation process during stable maintenance of BPV1 genomes as well as of URR reporter plasmids is guided by relaxed random-choice control mechanisms (Gilbert and Cohen, 1987; Piirsoo et al., 1996; Ravnan et al., 1992).

Two-dimensional electrophoretic analysis of replication intermediates from cells that stably maintain the genome of BPV1, HPV11 and HPV16 have mapped the origin of replication to the same part of the URR fragment that is used for E1/E2- dependent initiation of DNA replication (Auborn et al., 1994; Flores and Lambert, 1997; Schvartzman et al., 1990; Yang and Botchan, 1990). E1 and E2 proteins are also necessary for the extrachromosomal maintenance of BPV1 URR plasmids in a long-term replication assay (Piirsoo et al., 1996). Thus, it seems that the same *cis* sequences located within URR used in E1/E2 dependent replication initiation are also required for stable maintenance replication. However, it has been proposed that E1 is required only to establish BPV1 genomes as nuclear plasmids, and it is not required later on in the maintenance stage of the viral life cycle (Kim and Lambert, 2002). On the other hand, experiments in the budding yeast *Saccharomyces cerevisiae* have demonstrated that some HPVs can replicate and stably maintain in the absence of any particular viral protein, including E1 and E2 (Angeletti et al., 2002; Kim et al., 2005; Rogers et al., 2008). BPV1 can also replicate in *Saccharomyces cerevisiae*, but the BPV1 genome is not stably maintained (Angeletti et al., 2002). HPV E1/E2-independent replication and maintenance have also been demonstrated in mammalian cells, and the *cis* sequences required for stable maintenance were mapped to outside of the URR region (Pittayakhajonwut and Angeletti, 2008). Despite all this, more conclusive evidence is needed to clarify E1/E2-independent replication.

2.6.3. Vegetative replication

The third stage of viral replication is called vegetative DNA replication. *In vivo* it occurs only in the differentiating layers of the epithelium, where viral genomes are amplified to a high copy number to be packaged into the capsids of progeny virions (McBride, 2008). Vegetative replication requires that genomes be maintained extrachromosomally at earlier stages of the infection (McBride, 2008).

Little is known about the mode of vegetative replication. Even though bidirectional replication is widely accepted, some studies have indicated that in the vegetative stage the rolling circle mode of replication may be used (Dasgupta et al., 1992; Flores and Lambert, 1997). The rolling circle mechanism could be an efficient mechanism for creating large numbers of viral genomes that later could be packaged into viral capsids. Switching between different types of replication has been demonstrated in the case of EBV, where replication switches from the latent origin oriP to the lytic origin oriLyt for vegetative replication (Hammerschmidt and Sugden, 1988). Initiator proteins involved in rolling circle replication usually associate with rolling circle replication motifs and have nuclease activity to cleave the concatemeric intermediates produced by rolling circle replication (Ilyina and Koonin, 1992). E1 has been shown to be structurally related to the Rep initiator protein of adeno-associated virus, which initiates rolling hairpin replication (Hickman et al., 2004). However, despite the structural analogy, the catalytic residues of Rep are not conserved in E1. Therefore, it is still unclear whether PVs switch to a different replication mechanism for vegetative replication. More solid data are needed to clarify this point.

It has been proposed that vegetative replication is regulated at the transcriptional level and is triggered due to up-regulation of the levels of E1 and E2 (Bedell et al., 1991). It has been shown that BPV1-induced papillomas and cells that sustain ongoing genome amplification have high levels of E2 protein (Burnett et al., 1990; Penrose and McBride, 2000). In addition, mRNA species that are predicted to express the E1 and E2 proteins are induced at this stage of infection in HPV31-infected cells (Ozbun and Meyers, 1998). It is believed that high levels of E1 and E2 result in a switch from the E2-regulated early promoter to the E2-independent late promoter located within the E7 ORF (Grassmann et al., 1996; Klumpp and Laimins, 1999)

The final vegetative PV amplification is followed by the expression of the L1 and L2 structural proteins and virion assembly in the upper layers of infected tissue. Because PVs are non-lytic, they are not released until the infected cells reach the epithelial surface.

3. RESULTS AND DISCUSSION

3.1. Objectives of the present study

Together with my colleagues, I have tried to find answers to some of the questions that concern the stable maintenance process of PVs. We have used BPV1 as a model for these studies. More specifically, we have tried to understand which activities of viral tethering proteins are needed to establish efficient segregation, which cellular proteins may interfere with this process, and to use this knowledge to create new type of vectors for protein expression in mammalian systems. The main results of this thesis have been published (or are submitted for publishing) in international journals or as a patent, and they can be briefly summarized as follows:

1. To use *cis* and *trans* factors from BPV1 and EBV to provide replication activation and segregation functions to vectors of hybrid origin, and to use this knowledge to set up new types of vectors for large-scale protein expression.
2. To study the effect of the cellular protein Brd4 on E2-dependent replication and transcription.
3. To use chimeric E2 proteins as tools to identify E2 activities that are required for effective segregation.

3.2. Viral segregation determinants and development of episomal expression vectors for mammalian cells (Ref I, IV, V)

3.2.1. BPV1 E2 protein and its BSs as replication activation determinants for the polyomavirus core origin

Our lab has shown that both the BPV1 E2 protein-dependent MME (Abroi et al., 2004) and the EBV EBNA1-dependent FR segregation/partitioning and chromatin attachment activities may occur independently from plasmid replication (Ilves et al., 1999). In addition to E2's role in the segregation process, it is known that E2 of BPV1 transiently activates replication from its native origin as well as from heterologous origins in an E2 BS-dependent manner (Nilsson et al., 1991; Ustav et al., 1993). Nilsson et al. have shown that replacement of an enhancer region of the mouse polyomavirus (PyV) origin with two E2BSs activates replication of a reporter plasmid in an E2-dependent manner (Nilsson et al., 1991). First, we were curious whether E2 protein in *trans* and its BS in *cis* are able to activate replication as efficiently from the wt PyV replication origin as it does the enhancerless PyV origin.

Replication of the PyV origin requires large T-antigen (LT) as the only viral replication factor (Clertant and Cuzin, 1980; Francke and Eckhart, 1973). To study E2's impact on PyV origin-dependent replication, we constructed a COP5-based cell line that constitutively produces LT from an integrated replication-defective PyV genome and BPV1 E2 protein from an integrated expression plasmid. As expected, in a transient assay, E2 protein-dependent activation of replication was clearly detected when the PyV enhancerless core origin was linked to multiple E2 BSs. However, addition of one E2 BS had no replication activation effect (I, Fig. 3A and 4A). Our results are in agreement with previous results by Nilsson et al. and Abroi et al. (A. Abroi doctoral thesis) that the replication of the PyV enhancerless origin can be activated by BPV1 E2 and its BS, and it requires at least two E2BSs (Nilsson et al., 1991). Interestingly, addition of one E2 BS to the wt PyV replication origin had a slight inhibitory effect (I, Fig. 3A). Thus, it seems that replication from episomal viral replication origins has a certain maximal threshold level, and further enhancement of replication is not possible even if more enhancer elements are added. Experiments from our lab by A. Abroi have shown that the maximal level of replication by the wt PyV origin is achieved within ~24 h post-transfection and that there is no replication increase at later time points; instead, a decrease is observed (A. Abroi, doctoral thesis). This could be explained by limiting levels of cellular replication factors or by the saturation of the nucleus with active genetic elements in the form of replication intermediates. On the other hand, we observed many floating dead cells after transfection with PyV wt origin constructs; thus, over-replication of episomes may have a toxic effect on the cell.

3.2.2. Tethering by E2 and its multimeric BSs provides partitioning function to PyV origin plasmids

Efficient partitioning/segregation of BPV1 and EBV genomes between daughter cells is determined by the viral-encoded tethering protein and its BSs located in the viral genome (Feeney and Parish, 2009). Both viral tethering proteins and their multimeric BSs, E2/MME for BPV1 and EBNA1/FR for EBV, provide an anchoring function for viral genomes to the mitotic chromosomes (Feeney and Parish, 2009). This linkage between the viral genome and host chromatin ensures that the viral genomes are distributed between daughter cells and maintained in the nucleus when the nuclear membrane is reassembled during mitosis.

The stable-maintenance function of EBNA1/FR has been used to provide long-term episomal maintenance for plasmids containing large fragments of mammalian DNA (Krysan and Calos, 1993; Wade-Martins et al., 1999). The E2/MME-dependent stable-maintenance function has not been tested with heterologous replication origins. The previous chapter described that PyV origin- and E2BS-containing plasmids show effective transient replication in an

LT-positive cell line (I, Fig. 3 A and B). Thus, we decided to go further and study the stable maintenance of different reporter plasmids that combine the E2/MME based stable maintenance function of BPV1 with different variants of the PyV replication origin. We followed the episomal maintenance of different PyV origin-containing constructs without any selective pressure and under puromycin selection after co-transfection of the origin plasmids together with the plasmid pBabePuro, which encodes the puromycin resistance marker. The episomal persistence of the PyV origin-containing plasmids was analyzed by southern blotting. Plasmids that contained wt PyV origins were lost from the cells under selective and nonselective conditions very rapidly (I, Fig. 3A and B). On the other hand, hybrid origins consisting of the PyV core origin and five or ten E2BSs were capable of long-term persistence, at least for 27 doublings (I, Fig. 3A). However, after 21 or 34 days without selective pressure, the only origin construct that was efficiently maintained as an episome was the hybrid of the core origin with ten E2BSs (I, Fig. 3A, 21- and 34-day time points without selection, lane 5). In addition, effective long-term maintenance under selective and nonselective conditions was also seen when a eukaryotic selection cassette was added to the same plasmid with the PyV core origin and ten E2BSs (I, Fig. 4B).

To exclude the possibility that the Southern blot signals at later time points were from integrated material, the episomal state of the stably maintaining plasmids was checked by using the nicking enzyme Nb.Bpu10I and by rescue into bacteria (I, Fig. 5). The results of both assays indicated that the majority of the stably maintained plasmids are not integrated and exist in an episomal state. However, analysis of the rescued plasmids showed that some plasmid rearrangements had occurred in roughly one-half of the analyzed colonies (I, Fig. 5C). Nonetheless, it is important to note that all the rearranged plasmids still carried the PyV minimal origin and the BPV1 maintenance element (I, Fig. 5D and E). Thus, our experiments proved that the E2/MME provides stable episomal maintenance not only to a BPV1-based replicon, but also to chimeric PyV origin constructs that are otherwise lost from the cell population during cell growth.

Somewhat unexpectedly, we found that when the BPV1 segregation/ partitioning element was linked to the PyV wt origin, these replicons were not stably maintained despite their high level of replication (I, Fig. 3A, lanes 1 to 4). This could be due to the over-replication of the intact enhancer-containing origin plasmid, which could lead to cell death. Indeed, inspection of the transfected culture indicated that the wt PyV origin plasmids induced extensive cell death at later time points.

3.2.3. Effective chromatin attachment of E2 protein is required to provide the partitioning function to PyV origin plasmids

A. Abroi et al. from our lab have made several N-terminal amino acid substitutions to the TAD of E2 and have characterized these mutant E2 proteins in various assays (Fig. 5) (Abroi et al., 2004). This study showed that the TAD of E2 is very complex. A single amino acid substitution may lead to the loss of E2 functions. We decided to take advantage of this work and study the requirement for E2 protein chromatin attachment in the segregation process of heterologous PyV origins. The E2 mutants E39A and R68A were inactive in the chromatin attachment function and failed to mediate the segregation/partitioning of BPV1 URR reporter plasmids, but they are still active in initiation of transient replication and in transactivation, where their relative activity was comparable to wt E2 (Fig. 5) (Abroi et al., 2004). We constructed COP5-based cell lines that stably express these mutant forms of the E2 protein. These cell lines were used to study the short- and long-term replication of PyV-based origin plasmids. Both E2 mutants activated PyV core origin replication in an E2BS-dependent fashion in established cell lines, suggesting that the E2 mutant forms R68A and E39A behave as efficiently in replication activation as wt E2 protein (I, Fig. 4A). The same transfected cells were followed for time periods up to 2 months in medium with and without selection for the transfected PyV origin plasmids. By this time, only the reporter plasmid with ten E2BSs added to the PyV the core origin was efficiently maintained in the wt E2-expressing cells (I, Fig. 4B). On the other hand, in the case of the E2 mutant forms E39A and R68A, only a very weak replication signal of the same reporter was observed in cells grown under selection for the origin plasmids (I, Fig. 4B). These results showed that the chromatin attachment function of E2 protein is required to ensure stable maintenance of chimerical PyV origins and that the replication activation function alone is not sufficient for stable episomal maintenance.

As mentioned above, the E2 mutants E39A and R68A are inactive for chromatin attachment, URR plasmid tethering and segregation/partitioning assays (Abroi et al., 2004). In contrast to the work of Abroi et al., Baxter et al. have shown that the same mutants have wt E2-like activity for chromatin attachment (Baxter et al., 2005). The apparent contradiction in the features of these E2 mutants may be attributable to a difference in the species of the cells used or to different cell fixation methods used to measure the chromatin attachment of the E2 mutants. Despite the contradictory results regarding chromatin attachment, we have shown that these mutant E2 proteins are nonfunctional in supporting long-term episomal maintenance of the hybrid MME/PyV core origin plasmid in C127 mouse cells. Our results show clearly that the replication initiation and transactivation activities of E2 protein are not sufficient to provide stable maintenance to chimerical origins. In addition, effective chromatin attachment is needed. We conclude that MME-mediated partitioning in conjunction with the PyV origin or its natural BPV1 origin is achieved by using the same strategy, i.e., through chromatin attachment.

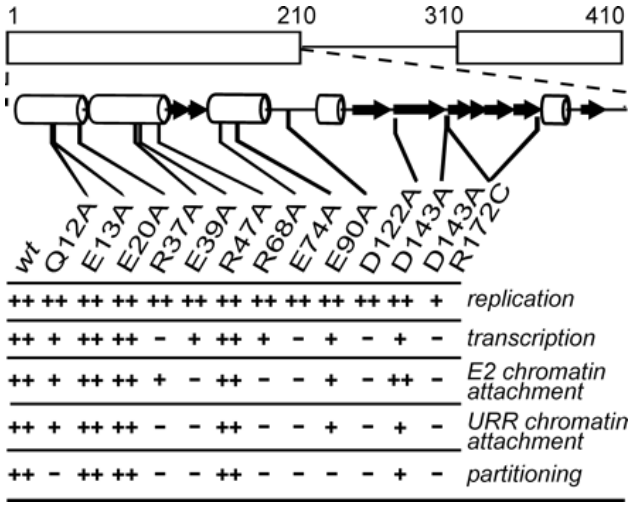

Figure 5. The data from ChIF (E2 chromatin attachment), FISH (URR chromatin attachment) and partitioning assays are compared to the activity of the respective mutant E2 proteins in transcriptional activation and transient replication assays. Activity less than 30% of wt is indicated by -, activity more than 80% of wt is indicated by ++, and activity between 30 and 80% of wt is indicated by +. Figure adapted from Abroi et al., 2004.

3.2.4. Comparison of the partitioning activities provided by the BPV1 MME and EBV FR elements to the PyV core origin plasmid

To compare the effects of the BPV1 MME and EBV FR-based elements on the segregation/partitioning of the PyV replication origin construct, we constructed reporter plasmids that contain the PyV core origin, FR or MME sequences, a Geneticin selection marker and an expression cassette for d1EGFP (I, Fig. 6A). The long-term maintenance of transfected reporter plasmids in cells expressing either E2 or EBNA-1 protein was monitored by flow cytometry. Transfected cells were grown in continuous culture in the presence or absence of Geneticin for up to several months. Under selective pressure, the percentage of dEGFP-positive cells stayed constant for more than 20 cell generations, indicating that these cells are capable of long-term maintenance of episomal genetic elements (I, Fig. 6C and D). As expected, after removal of Geneticin selection the percentage of dEGFP-fluorescent cells started to decline (I, Fig. 6C and D). In the absence of selection, the rate of episomal loss was ~6% per cell division for MME and ~13% for FR (I, Table 1). Our results showed that EBNA1/FR and E2/MME confer comparable segregation/partitioning functions on PyV core origin reporter plasmids in the analyzed cell model.

Replication of the BPV1 and PyV origins is initiated multiple times in a single S phase of the host cell cycle during amplificational replication. However, these viruses have different time courses of productive infection. Because PyV is a lytic virus, it does not need the stable maintenance phase that is characteristic of PVs. During stable maintenance replication of the BPV1 genome or the URR, replication initiation from the origin is not limited to one initiation in each cell cycle (Piirsoo et al., 1996; Ravnan and Cohen, 1995; Ten Hagen et al., 1995). At the same time, the EBV latent origin OriP replicates strictly once per cell cycle just as the chromosomal DNA does. Thus, in these terms, the replication mode of OriP is completely different from those of PyV and BPV1. As we showed, the BPV1 E2/MME and the EBV EBNA1/FR element can provide a stable maintenance function to the PyV core origin plasmids in the presence of viral trans factors. Our data suggest that the stable episome maintenance provided by the MME or the FR element is not connected to the mode of replication of the episome. Additionally, the replication function is not connected to the stable-maintenance function of the virus; replication origins of different viruses can be combined with heterologous stable-maintenance elements without the loss of either function.

The cellular receptors of BPV1 E2 protein and EBV EBNA1 protein, which link the episomes to mitotic host chromatin and therefore provide the stable-maintenance function, are most likely different (Kapoor and Frappier, 2003; Kapoor et al., 2001; You et al., 2004). Consequently, E2/MME- and EBNA1/FR-dependent plasmids display different localization on chromosomes. However, as we have shown, the different locations of the episome on mitotic chromosomes does not interfere with the replication of the PyV minimal replication origin.

3.2.5. Chromatin attachment and the TAD in the segregation process

The results represented above showed that the transactivation activity of E2 is not sufficient to provide stable maintenance. On the other hand, the studies of Abroi et al. with E2 mutants revealed that the transactivation and chromatin attachment/partitioning activities of E2 are not very clearly separable. Mutations that eliminate chromatin attachment and partitioning always have impact on E2 transactivation (Fig. 5) (Abroi et al., 2004). Therefore, we speculated that the segregation/partitioning of viral genomes may depend on two different types of interactions: first, with specific chromatin receptors, and second, with the transcription machinery. To test this hypothesis, we engineered a set of chimeric E2 proteins where E2 TAD was replaced or complemented with the chromatin attachment domain from LANA1 of KSHV or with the TAD from herpes simplex virus type 1 (HSV1) protein VP16. These recombinant proteins allowed us to individually assess the requirements for each of these domains and activities in the segregation process.

Similarly to E2, LANA1 of KSHV serves as a linker protein between viral genomes and host chromosomes. The chromatin attachment domain of the LANA1 protein is well defined as the first 22 amino acids from the N-terminus of LANA1 (Barbera et al., 2004; Barbera et al., 2006). Our strategy was to use this well-defined region in place of the E2 TAD and thereby create a chimeric E2 protein that retains E2's chromatin attachment and plasmid tethering function but lacks transactivation ability. Indeed, our chimerical LANA:E2C protein was defective for transactivation (V, Fig. 9F), it bound effectively to mitotic chromosomes and it also tethered an E2 BS-containing plasmid to chromosomes (V, Fig. 3). To our surprise, LANA:E2C did not support plasmid segregation in functional assays (V, Fig. 4), indicating that despite capacities for chromatin attachment and plasmid tethering, LANA:E2C lacked some activity essential to its role in the segregation process.

Hybrid viral proteins have also been made in the context of LANA1 with the goal identifying regions in viral proteins that are involved in chromatin attachment and stable maintenance. Sinohara et al. showed that a chimera of ΔN-LANA with histone H1 rescued the chromosome association and long-term maintenance of an oriP episome (Shinohara et al., 2002). In contrast, a LANA1 chimera with H2B could not maintain oriP episomes, even though this chimeric protein attached to host chromosomes. These results together with our results demonstrate that chromatin association does not always guarantee plasmid segregation/partitioning.

The results described above show that at least five E2BSs are required for effective segregation of PyV replicon-based plasmids. Previously, in the context of the BPV1 origin it was demonstrated that six E2BSs are required for plasmid partitioning (Piirsoo et al., 1996). Therefore, we suspect that a complex containing at least 10–12 E2 molecules is necessary to form a segregation-competent complex. Next, we speculated that the failure of LANA:E2C to segregate could be due to ineffective formation of a segregation-competent complex, the maturation of which may require a higher dose of LANA:E2C. However, our western blot analysis showed that LANA:EC expression was significantly higher than that of wt E2, which in most cases is undetectable when expressed from the RSV LTR promoter (Fig. 6, compare lanes 1 and 5). However, we decided to increase the level of LANA:E2C by adding the first intron of human elongation factor 1 α (hEF1α) to the 5` end of the LANA:E2C coding sequence. Indeed, addition of the hEF1α intron notably increased the protein expression level (Fig. 6, compare lanes 1 and 2, 3 and 4 or V, Fig. 7A). Moreover, the increased expression of LANA:E2C restored its segregation properties (V, Fig. 7B). High-resolution image analysis of chromosome-bound proteins indicated that the E2 complex on chromosomes contained more nearby E2 molecules than did LANA:E2C (V, Fig. 6). However, LANA:E2C also gained an E2-like complex, but at a markedly higher protein dose (V, Fig. 6).

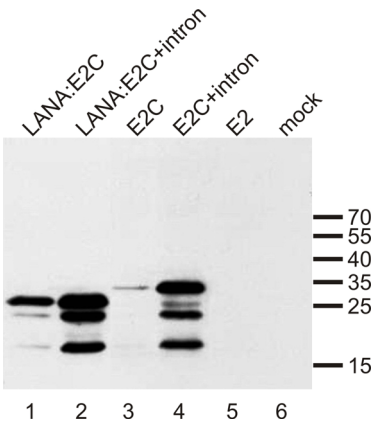

Figure 6. An hEF1α intron increases protein expression level. At 17 hours post-transfection of Jurkat cells, total protein was separated on a polyacrylamide- SDS gel and transferred to PVDF membrane. Western blotting was performed with monoclonal antibodies to 1E4 (Kurg et al., 1999) and peroxidase-conjugated secondary antibody signals were detected by using the ECL detection kit.

Thus, an E2 protein segregation-competent complex is formed very efficiently comparing to complexes made by LANA:E2C. We conclude from this that formation of such complexes requires overcoming a threshold, and in the case of E2 this threshold is exceeded very effectively already at low protein concentrations. On the other hand, these results showed that the TAD of E2 is responsible for segregation-competent complex formation, most likely through its interactions with cellular proteins. Our further studies with chimeric proteins and E2 mutants that contain the TAD of VP16 revealed that in addition to chromatin attachment, a functional TAD is required to obtain effective segregation (V, Fig. 8 and 9). The acidic TAD of VP16 can target many general transcription factors and chromatin-modifying coactivator proteins, such as TBP (Stringer et al., 1990), TFIIA (Kobayashi et al., 1995), TFIIB (Lin et al., 1991), transcription cofactor PC4 (Ge and Roeder, 1994; Kretzschmar et al., 1994), CBP (Hardy et al., 2002; Ikeda et al., 2002) and p300 (Kraus et al., 1999; Kundu et al., 2000). In addition to E2, EBNA1 and LANA1 are known to participate in transcription activation, and they all interact with several cellular proteins present in transactivation complexes. Therefore, we speculate that viral linker protein interaction with the transcription machinery is required for effective segregation-competent complex formation.

3.2.6. Production of recombinant therapeutic proteins – short introduction

The production of recombinant therapeutic biopharmaceuticals is one of the fastest-growing sectors of the pharmaceutical industry. Over 165 biopharmaceutical products have been approved globally with a market size of $33 billion in 2004 that is projected to reach $70 billion by the end of 2010 (Walsh, 2006). The vast majority of biopharmaceuticals are proteins, and this fact makes the efficient production of recombinant proteins extremely important for pharmaceutical companies. In addition to the pharmaceutical industry, many other fields are also relying intensely on recombinant proteins. Areas as diverse as agro-food technology, chemistry, detergent production, bioremediation, biosensing, petroleum and paper production all have received significant benefits from the use of recombinant proteins (Olempska-Beer et al., 2006).

Recombinant therapeutic proteins can be produced in microorganisms, plant cell cultures, insect and mammalian cell lines or transgenic animals. A number of recombinant therapeutic proteins, including recombinant human insulin, are produced in microbial expression systems established using bacteria or yeast (Grillberger et al., 2009). However, microbial expression systems have some limitations, particularly their lack of post-translational modifications, e.g., glycosylation. Thus, most microbial systems cannot perform all of the modifications necessary for proper protein structure and function, therefore these systems are not appropriate for most applications (Yin et al., 2007).

To overcome this disadvantage, mammalian expression systems have been the traditional approach for the large-scale manufacture of therapeutic proteins that require these critical post-translational modifications (Yin et al., 2007). An additional advantage of these mammalian cell systems is that the recombinant proteins are secreted into the medium in their natural form. *E. coli*-expressed proteins, on the other hand, mostly accumulate in a highly denatured form within the cell as inclusion bodies and require renaturation later in the manufacturing process (Yin et al., 2007). About 60–70% of all therapeutic recombinant proteins are produced in mammalian cells, primarily Chinese hamster ovary (CHO) cells (Wurm, 2004). CHO cells have many characteristics that have made them the most widely used mammalian host system. In addition to easy manipulation, CHO cells have proven safety profile in humans, and these proteins also have glycosylation patterns similar to those of human proteins (Wurm, 2004). Thus, it is likely that mammalian cells will remain the primary system for large-scale production of recombinant therapeutic glycoproteins.

The manufacturing process for a recombinant protein in mammalian cells usually follows a well-established scheme (Fig. 7C) (Wurm, 2004). Initially, the recombinant gene with the necessary transcriptional regulatory elements is transferred to the cells, together with a second gene that confers to the recipient cells a selective advantage. In the presence of the selection agent, which is applied a few days after gene transfer, only those cells that express the selection gene survive. Following selection, survivors are transferred as single cells to a

second cultivation vessel, and the cultures are expanded to produce clonal populations. Eventually, individual clones are evaluated for recombinant protein expression, the highest producers being retained for further cultivation and analysis. From these candidates, one clonal cell line with the appropriate growth and productivity characteristics is chosen to produce the recombinant protein. This traditional scheme is considered to be time-consuming (construction requires up to 12 months), and it requires copious manpower and finances. Therefore, pharmaceutical companies are very motivated to improve each step in this manufacturing scheme. Currently, the main focus is to obtain higher cell density by growing cells in large bioreactors in optimized growth medium. To reduce the timescale, many companies are starting to find solutions for large-scale transient gene expression (Fig. 7B). In conclusion, as the market for recombinant proteins expands, manufacturers are interested in new technologies and improvements that could be advantageous in this competitive field.

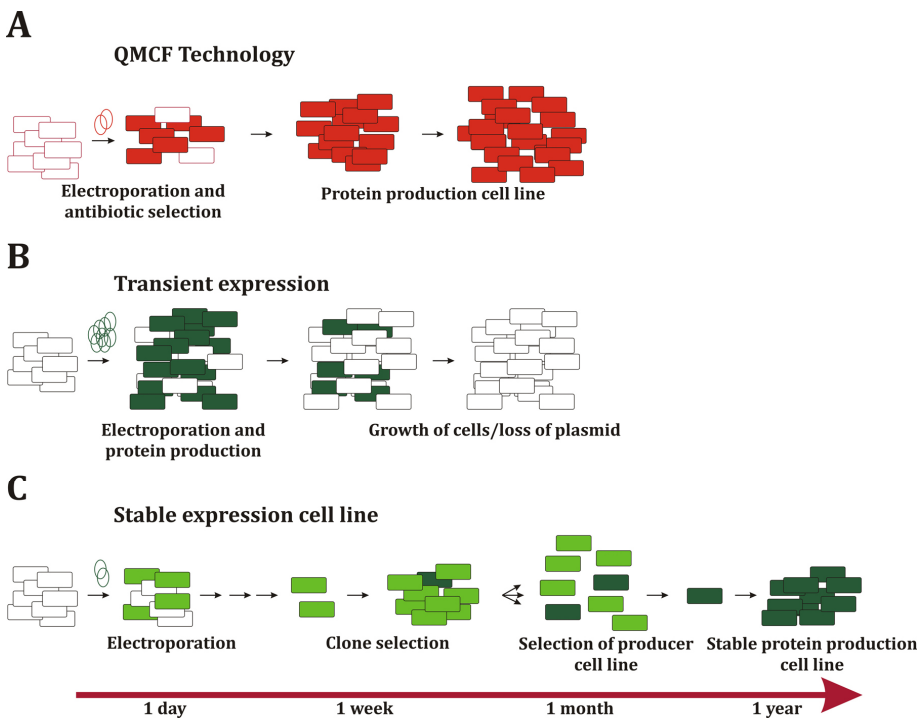

Figure 7. Comparison of different protein production technologies. (A) QMCF technology is a fast and efficient way to generate a protein production cell line in less than one month from a single electroporation into $6x10^6$ cells, antibiotic selection of plasmid-containing cells and generation of production cell banks in less than two weeks. (B) Transient expression technology is efficient for a short period of time. After one week, protein expression is reduced due to overgrowth of non-plasmid containing cells in the culture. (C) Stable cell line protein expression offers efficient production rates. Generation of a "superproducer" is a time-consuming process. Figure adapted from Icosagen Cell Factory OÜ.

3.2.7. Stably maintained heterologous plasmids as a tool for protein production

The traditional format for the production of biopharmaceuticals in mammalian cells requires construction of a clonal cell line that stably expresses the gene of interest (Fig. 7C). Construction of such a cell line is very time-consuming and requires abundant resources. Therefore, many companies (and not only pharmaceutical companies) are interested in producing large amounts of protein on a shorter timescale.

We have shown that plasmids that contain a viral replication origin and a nuclear retention element are stably propagated in cells that stably express viral replication and segregation proteins (Ref I, IV Figs. 3 and 6). In our study, we used plasmids that contain the Py DNA replication origin for plasmid amplification and EBV EBNA-1 or BPV1 E2 protein BSs for segregation. However, it should be noted that plasmids that contain the Py wt origin are not stably maintained, probably due to fatal over-replication of plasmid DNA (Ref I, IV Fig. 3). Thus, not all replication origin configurations are appropriate for long-term maintenance. In addition, we showed that our heterologous plasmids could maintain expression of a gene of interest (GFP in this case) for up to three months (Ref I, Fig. 6; Ref IV, Fig. 7). All of these data indicate that we will be able to construct vectors and cell lines that maintain and support the expression of a gene of interest for prolonged periods. Therefore, the vectors and cell lines we have constructed could be used for protein production.

Our findings are protected by patent applications in several countries (European Patent No. EP1851319; US patent application No. 11/351,809; patents pending in Australia, Canada and Japan). Patent applications are licensed to the Icosagen Group (formerly the Quattromed Group) for further development and commercialization.

Most therapeutic proteins are produced in CHO cells (Wurm, 2004). Therefore, a CHO-based suspension cell line was genetically modified for stable expression of the Py LT and EBV EBNA1 proteins (the cell line was named CHOEBNALT85). For protein expression, vectors containing multiple BSs for EBNA-1 and the Py minimal origin of replication were constructed (Fig. 8A). The cell lines and vectors constructed together form a technology platform called QMCF (Quattromed Cell Factory).

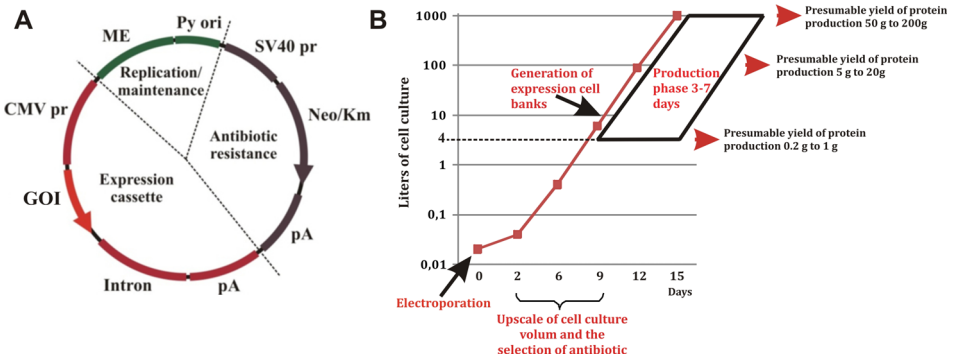

Figure 8. (A) Schematic representation of a QMCF plasmid. ME – maintenance element; Py ori – murine polyomavirus minimal origin of replication; SV40 pr – SV 40 promoter controlling expression of the Neo resistance gene; Neo/Km – Neomycin/ Kanamycin resistance marker; pA – polyadenylation sequence; CMVpr – cytomegalovirus promoter; GOI – gene of interest (B) Schematic representation of protein production using QMCF technology. Key steps in the production process are represented in red. Figure adapted from Icosagen Cell Factory OÜ.

QMCF protein production technology is optimized for high transfection efficiency, antibiotic selection of plasmid-containing cells, the ability to generate protein production cell banks and high-level production of recombinant proteins. QMCF protein production technology contains several optimized steps (Fig. 8B). It starts with transfection of CHOEBNALT85 cells by electroporation with 1 μg of plasmid DNA. The electroporation efficiency is usually more than 80%. The transfection step is followed by selection and scale-up of electroporated cells. This step lasts 9–10 days, and it involves selection of the cells with the antibiotic G418, which is added 48 h post-transfection. After selection and scale-up, it is possible to generate production cell banks, which can be stored and reused for protein production without electroporation of the expression plasmid. Protein production starts at 9–10 days after transfection or 5–6 days after inoculation of a culture from the production cell bank. The production phase extends from 3–7 days. In the production phase, the culture density is usually about $6x10^6$ viable cells/ml, and it requires a temperature shift to 30° C and additional feeding. By this procedure it is possible to scale up the initial culture to 1,000 liters within 15 days, and the presumable yield of expressed proteins is 50–200 g.

Initial production optimization studies have been made with several biologically active substances, such as recombinant proteins, recombinant antibodies and VLPs. As an example, two different human neurotrophic factors (NGFs) were produced (Fig. 9A and B). cDNA-s were cloned into the pQMCF-CMV promoter-containing expression plasmid (Fig. 8A) and proteins were produced in the CHOEBNALT85 cell line. The quality and level of NGFs production

were measured by western blotting (Fig. 9A and B) and quantified with an ImageQuant 300 imager (GE Healthcare). The production of the two NGFs in the supernatant of CHOEBNALT85 cell culture was 17 and 130 mg/l.

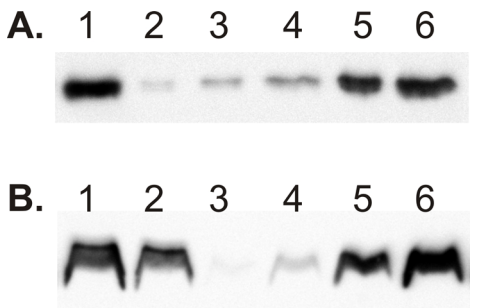

Figure 9. Quantitative western blot analysis of NGFs. (A) Quantitative western blot analysis of NGF1. Lanes: 1. 10 µl of NGF 1 in the supernatant produced by CHOEBNALT85 cells; 2–6 Dilutions of the positive control (5, 10, 20, 40, 80 ng). (B) Quantitative western blot analysis of NGF2. Lanes: 1 and 2. NGF 2 supernatant produced by CHOEBNALT85 cells, 2.5 and 1.25 µl, respectively; 3–6 Positive control (100, 200, 400, 800 ng). Figure adapted from Icosagen Cell Factory OÜ.

Although the basic protein production concepts have not changed since the mid-1980s, the productivity of recombinant cell lines has increased dramatically in the past two decades (Wurm, 2004). Researchers' efforts have led to improvements in vectors, host cell engineering, medium development, screening methods and process engineering and development. Today's fed-batch production runs last about 21 days with a maximum cell density of 10–15 million cells/mL. These bioprocesses typically have specific and volumetric productivities in the range of 50–90 pg/cell/day and 1–5 g/L, respectively (Hacker et al., 2009). However, it should be noted that such high product yield is typically achieved after numerous optimization steps and is largely dependent on the characteristics of the expressed protein. In comparison, Icosagen has produced more than 25 different monoclonal antibodies, including mouse IgG1 and IgG2a and human IgG1 subtype antibodies. The yield of the different antibodies produced by QMCF technology was 30–200 mg/l (data not shown). However, the production of another NGF protein in the supernatant of CHOEBNALT85 cells yielded more than 800 mg/l (data not shown). Thus, QMCF technology has the potential to achieve productivity levels on a par with those of stable cell lines, although it may require some further optimization of parameters such as cell culture density and medium.

Mammalian stable cell line expression systems have a long-standing and successful history in the production of recombinant proteins, but these expression systems are generally regarded as being cumbersome, tedious and expensive for generating recombinant proteins as tools for use in, for example, assay

development, early product analysis and high-throughput screening (Leder et al., 2007). As an alternative to stable cell line generation, large-scale transient expression of recombinant proteins in mammalian cells has been used to generate sufficient amounts of recombinant protein. The most widely used cell lines in conjunction with large-scale transient transfection approaches are EBNA-1 transformed HEK293 (HEK.EBNA) cells and CHO cells (Leder et al., 2007). The combination of an OriP containing expression vector and EBNA-1 contribute to elevated levels of transgene expression following transient transfection (Geisse and Henke, 2005; Pham et al., 2003). Transient protein production levels of 18–20 mg/l in HEK.EBNA cells using an OriP bearing vector have been reported (Durocher et al., 2002; Geisse and Henke, 2005; Pham et al., 2003). In contrast, transient recombinant protein yields in CHO cells tend to be significantly lower, 5–8 mg/l (Derouazi et al., 2004; Schlaeger et al., 2003; Tait et al., 2004).

Conventional large-scale transient protein expression includes transfection of an expression plasmid into cells with a chemical delivery agent like calcium phosphate or polyethylenimine (PEI). However, large-scale transfection requires abundant transfection reagent as well as high-quality DNA and cells, and there is a perception in the industry that large-scale transient gene expression is not reproducible (Hacker et al., 2009). To date, the largest volumes for transient protein expression have been about 100 l. Routine methods for volumetric scales in the 100–1,000 l range still need further development. Therefore, up to now, there has not been a therapeutic protein produced by transient protein expression that has gained regulatory approval (Hacker et al., 2009).

Comparison of QMCF technology to these above-described transient protein production systems shows that QMCF has several advantages over these widely used technologies. Transfection of QMCF cells is inexpensive and it requires only 1 µg of expression plasmid and $4x10^6$ viable CHOEBNALT85 cells. The subsequent up-scaling of production is very rapid, in theory allowing 1,000:l volumetric expansion within 15 days. During the up-scaling process it is possible create production cell banks, which enables the timescale to be shortened if new production is desired. QMCF technology has been licensed for research studies to several well-known pharmaceutical companies, such as Bayer Healthcare, Novartis, Wyeth and GE Healthcare. All these companies have declared QMCF technology to be a robust and efficient means of protein production, and the protein yields tend to be higher than those obtained with in-house technologies.

In conclusion, initial studies with the QMCF technology have proven that it has the potential to replace or to be an attractive alternative to conventional clonal cell line production and transient protein expression. Positive feedback from customers is very encouraging, and the QMCF technology will continue to be optimized. In addition, preliminary studies have shown that this technology can be used to develop cell-based assays. Thus, Icosagen has the challenge of improving its product portfolio and the QMCF technology.

3.3. Brd4 as a central regulator of the BPV1 life cycle (Ref II)

3.3.1. The inhibitory effect of the Brd4 CTD on viral DNA replication

The long-term task of identifying PV E2 protein partner(s) on host chromosomes revealed an interaction between E2 and the cellular protein Brd4 (You et al., 2004). Brd4 is a member of the BET family of proteins that attach to chromatin through their two bromodomains, which bind throughout the cell cycle to acetylated histones H3 and H4 (Dey et al., 2003). Brd4 has an essential roles in cellular growth, cell cycle control and DNA replication, and Brd4 gene rearrangements are associated with carcinomas (Wu and Chiang, 2007). Brd4 has also been found in several transcription complexes, including the general cofactor Mediator and the positive transcription elongation factor b (P-TEFb) (Jang et al., 2005; Yang et al., 2005). P-TEFb is an essential component of the pre-initiation complex that stimulates RNA polymerase II (RNAP II) transcription (Ping and Rana, 1999).

Initial studies about Brd4's role in the PV life cycle attempted solve its functions in E2 chromatin attachment and the PV stable maintenance process. It was shown that ectopic expression of the E2-binding C-terminal domain (CTD) of Brd4 in mammalian cells disrupts the interaction of E2 with cellular Brd4 and relocates E2 from mitotic chromosomes (You et al., 2004; You et al., 2005). Moreover, Brd4 can reconstitute BPV1 E2-dependent extrachromosomal plasmid maintenance in the yeast *Saccharomyces cerevisiae*, in which this process does not normally occur (Brannon et al., 2005). Later studies indicated a strong correlation between Brd4/E2 interaction and E2 chromosomal association (Baxter et al., 2005). As the Brd4-CTD is responsible for binding to the TAD of E2 (You et al., 2004), we decided to study the potential influences of Brd4 on E2-mediated transactivation and replication.

Overexpression or knockout of Brd4 in mammalian cells has been shown to cause severe alterations in cell growth (Houzelstein et al., 2002; Maruyama et al., 2002). Therefore, we used a dominant-negative truncated version of Brd4 as a useful alternative to manipulations with the full-length gene. Thus, for further studies we cloned the CTD (last 315 amino acids) of human Brd4 into a mammalian expression vector. Brd4 CTD efficiently coimmunoprecipitates with BPV1 E2, which confirms the interaction between these two proteins.

Mouse C127 cells have long been used as a model for studying the nonproductive part of the BPV1 life cycle. We tested the effect of the Brd4 CTD on transient BPV1 genome replication in these cells, which essentially mimics the initial step of BPV1 transformation. Analysis of low-molecular-weight DNA showed that replication of the BPV1 genome was inhibited in cells co-transfected with the Brd4 CTD expression vector (II, Fig. 1). We also tested effect of the Brd4 CTD on BPV1 DNA replication in a simple model that

consists only of the minimal *cis* and *trans* determinants of viral replication (II, Fig. 2A). Analysis of newly replicated reporter plasmid DNA showed clearly that the Brd4 CTD inhibited reporter plasmid replication in C127 and CHO cells (II, Fig. 2A, lanes 4, 5, 13, and 14). The results described above showed clearly that the Brd4 CTD has an inhibitory effect on BPV1 replication.

We next asked whether the inhibition is due to binding of the CTD to E2 by using a 37/73 mutant version (arginine 37 and isoleucine 73 replaced by alanine) of E2 in our transient-replication experiments. The 37/73 mutant E2 protein supported BPV1 DNA replication efficiently but did not bind to Brd4 (Baxter et al., 2005). Our results showed that the replication of BPV1 ori supported by 37/73 was equally sensitive to the Brd4 CTD in C127 and CHO cells (II, Fig. 2A). Therefore, the dominant-negative effect of Brd4 CTD on BPV1 ori-dependent replication does not require its binding to E2. The fact that inhibition of BPV1 DNA replication by the Brd4 CTD occurred independently of its binding to the virus-specific replication factor E2 led us to consider the possibility that the Brd4 CTD might affect the replication of other DNA viruses as well. We tested this idea on PyV DNA replication using a transient-replication assay. The hybridization signal corresponding to newly replicated PyV ori reporter DNA was significantly lower in cells expressing the CTD, and it was independent of the Py ori configuration (II, Fig. 2C).

Our results clearly showed that overexpression of the Brd4 CTD inhibited viral DNA replication and that this effect was independent of the binding of the Brd4 CTD to virally encoded replication initiator proteins (e.g., E2 and LT). Thus, the Brd4 CTD must target some cellular component(s) of the replication initiation complex. Brd4 has been shown to interact with the cellular replication factor C (RFC), but this binding requires the N-terminal bromodomain-containing region of Brd4, which is missing from the CTD (Maruyama et al., 2002). Our data thus indicate that Brd4 might be involved in steps of cellular DNA replication other than those linked to RFC binding. Considering the apparent insensitivity of the cell division cycle to the Brd4 CTD, likely the role of Brd4 is not critical for cellular DNA replication.

Interestingly, the inhibitory effect the Brd4 CTD seems to be of variable importance for BPV1 origin replication, as we noticed a lack of Brd4 CTD-dependent inhibition of replication in C33A cells (II, Fig. 2A). Simultaneously with our study, it was shown that the Brd4 CTD also fails to influence HPV16 origin replication in C33A cells (Schweiger et al., 2006). However, the CTD certainly not only binds to E2 in C33A cells but also can behave as a dominant-negative inhibitor of other Brd4-related activities of E2 in this cell line: its ectopic expression excludes E2 from chromatin (You et al., 2004), and as I describe in the next chapter and as also shown by Schweiger et al. (Schweiger et al., 2006), it inhibits E2-dependent transcription activation.

3.3.2. The Brd4 CTD inhibits E2-dependent transcriptional activation

The fact that Brd4 interacts with components of the Mediator coactivator complex and recruits the P-TEFb elongation regulating complex to promoter regions (Jang et al., 2005; Yang et al., 2005) led us to hypothesize that Brd4 might also interfere with E2-mediated transactivation, and this could be the reason for its inhibition of BPV1 genomic DNA replication. We transfected cells with expression constructs for E2 and the Brd4 CTD as well as with a reporter plasmid expressing firefly luciferase under control of the early viral promoter of the BPV1 URR. Our results showed that E2-activated transcription was efficiently down-regulated by the Brd4 CTD (II, Fig. 3A). To show that the effect of the Brd4 CTD was specific to E2-activated transcription, we performed experiments where increasing amounts of the Brd4 CTD expression construct were co-transfected with luciferase reporters as well as with a pCG vector expressing either BPV1 wt E2 or VP16E2 fusion protein (II, Fig. 3B). VP16E2 contains the BPV1 E2 DBD fused to the strong transactivation domain from the VP16 protein of human herpesvirus 1 (Li and Botchan, 1993), and it thus effectively activates transcription from promoters that contain E2BSs. We found that E2-activated transcription from the BPV1 native promoter reporter was effectively inhibited by CTD expression in a concentration-dependent fashion (II, Fig. 3B), while transcription activated from the same reporter by VP16E2 was not comparably affected. We conclude from these data that the negative effect of the CTD on E2-activated transcription requires the N-terminal TAD (and Brd4 binding) of E2, but not its C-terminal DBD, E2BSs in the promoter region or activated transcription in general.

At the same time with our study, Schweiger et al. also examined Brd4's role in E2-mediated transactivation (Schweiger et al., 2006). They showed that overexpression of the Brd4 CTD or knock-down of full-length Brd4 protein by siRNA significantly reduced E2-mediated transactivation, indicating that Brd4 is required for E2 transcriptional activation. These results are in good agreement with our results and conclusions. However, the exact mechanism of Brd4 in transcriptional activation remains unresolved. In addition to Brd4's role in transcriptional activation, it has been shown to be an important component in PV E2 protein-mediated transcriptional repression (Lee and Chiang, 2009; Smith et al.,; Wu et al., 2006). Both the transcriptional activation and repression activities of E2 are mediated through E2 BSs. It has been shown that Brd4 facilitates E2 binding to its BS, and this process requires at least one bromodomain of Brd4 and the CTD (Lee and Chiang, 2009; Wu et al., 2006). While Brd4 interacts the P-TEFb and Mediator complexes, it has been proposed that the E2/Brd4 complex recruits P-TEFb/Mediator compounds to promoter regions, which then leads to phosphorylation of serine 2 of the C-terminal domain of the largest subunit of RNAPII, which releases RNAPII from inhibitory transcriptional elongation factors, thus resulting in the transcriptional activation of E2-dependent promoters (Schweiger et al., 2007; Wu and Chiang,

2007). Interestingly, it has been shown that E2 transcriptional repression is also mediated through same Brd4/P-TEFb complex proposed to be required for transactivation (Yan et al., 2010). P-TEFb binds to the CTD of Brd4 and overlaps with the E2 binding site (Bisgrove et al., 2007; Yan et al., 2010). In a proposed transcriptional repression model, E2 competes with P-TEFb for Brd4 binding, and E2 interaction with Brd4 releases P-TEFb from the complex, which leads to reduced RNAPII activity and thus transcriptional repression (Yan et al., 2010). This model and the current data available succinctly explain E2-dependent transcriptional repression; however, it does not explain some aspects of E2-dependent transactivation. Thus, some other type of interactions or complexes may be involved in E2-dependent transactivation.

Genetic analyses of the activities BPV1 E2 have demonstrated relatively good general correlations between its transactivation, chromatin attachment and Brd4-binding activities, suggesting that the transactivation and chromatin attachment activities of E2 are likely to be executed by heavily overlapping Brd4-containing complexes (Abroi et al., 2004; Baxter et al., 2005). This means that these two activities of E2 have to be tightly coordinated with each other. However, the interaction of E2 and Brd4 is not required for genome partitioning of all PVs, because a number of PV E2 proteins associate with mitotic chromosomes independently of Brd4 binding (McPhillips et al., 2006). However, disruption of the E2-Brd4 interaction abrogates the transactivation function of E2, indicating that Brd4 is required for E2-mediated transactivation of all PVs (McPhillips et al., 2006). Thus, in the context of these results, we conclude that Brd4 is required for E2-mediated transactivation and repression, but its functions in the segregation process still require some further clarification.

In summary, we showed that the overexpression of the Brd4 CTD has a dominant-negative effect on the replication of the BPV1 genome *in vivo* and that this effect is likely to be carried out simultaneously through several E2-dependent and -independent pathways. We showed that the E2-dependent pathway includes repression of E2 transactivation by the CTD, which was later confirmed by other studies. Our results suggest a complex role for Brd4 in the BPV1 life cycle. Further studies have shown that Brd4 also has an important role in the HPV life cycle, especially in transactivation and repression. In addition, Brd4 is a critical component for E2 chromatin attachment and segregation. All this makes the E2-Brd4 interaction an important target for further studies.

4. CONCLUSIONS

To summarize the results of this study:

1. We constructed a series of heterologous plasmids containing different numbers of BPV1 E2BSs together with a minimal or wt PyV replication origin. Short-term replication assays showed that replication of the PyV minimal origin is very effectively enhanced by E2 and its BS. Long-term replication assays indicated that only plasmids that contain the PyV minimal origin and ten or five E2 BSs are successfully maintained in the episomal state in a cell line that constitutively expresses the proteins LT and E2. Further study of point-mutant E2 proteins demonstrated that efficient partitioning/segregation by E2 protein rather than its ability to activate replication is required for stable episomal maintenance. In addition to BPV1 segregation/partitioning elements, EBV FR elements showed similar properties in stable maintenance assays. This study was the first to assess the ability of BPV1 E2 plus its BS to stably maintain heterologous replication origins, and it revealed that maintenance functions from different viruses are fundamentally interchangeable and can provide segregation/partitioning function to heterologous origins in a variety of cells.
2. Based on our constructed heterologous plasmids carrying segregation elements from BPV1 or EBV and the minimal replication origin of PyV, the company Icosagen Group developed QMCF technology for large-scale protein production.
3. A cellular bromodomain protein was shown to be the main interacting partner of PV E2 protein. We demonstrated that Brd4 interferes with BPV1 and PyV replication and does so independently of its binding to E2. In addition, this study was the first to suggest a Brd4-dependent mechanism for E2-dependent transcriptional activation. Our data demonstrate the involvement of Brd4 in multiple early functions of the BPV1 life cycle.
4. Our studies with chimeric E2 proteins showed that effective chromatin attachment of the viral linker protein is not sufficient for efficient segregation. Efficient segregation requires formation of a segregation-competent complex that contains several viral linker molecules and probably several cellular proteins, likely transcription factors. In the case of BPV1 E2, this segregation-competent complex is formed very efficiently, and the activities required for this are located in the TAD of E2.

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SUMMARY IN ESTONIAN

Veise papilloomiviiruse tüüp 1 segregatsioonimehhanismi uurimine

Kõikide elusorganismide jaoks on määrava tähtsusega, et nende geneetiline materjal jaotuks rakkude jagunemise käigus võrdselt tütarrakkude vahel. Eukarüootide kromosoomidel aitab tütarrakkudesse jaguneda tsentromeer, mille suurus võib ulatuda kuni ühe megaaluspaarini. Viirused oma kompaktsuse tõttu ei saa selliseid suuri mittekodeerivaid alasid endale võimaldada. Kuid vaatamata sellele on ka paljude viiruste jaoks oluline, et pärast peremeesrakkude jagunemist säiliks nende genoom võimalikult paljudes rakkudes. Seega peavad viirused kasutama teistsuguseid mehhanisme oma genoomi säilitamiseks.

Mitmete viiruste (näiteks retroviiruste) genoom või selle cDNA koopia integreerub peale rakku jõudmist kromosoomi ja jääb sinna stabiilselt püsima ning satub tütarrakku peremeesraku kromosoomi ühe osana. Kuid on teada viiruseid, mille genoom ei integreeru peremehe kromosoomi, aga sellest hoolimata nad säilivad rakkude jagunemise jooksul. Sellisteks viirusteks on näiteks veise papilloomiviiruse tüüp 1 (BPV1), Epstein-Barri viirus (EBV) ja inimese herpesviirus 8 (KSHV). Need viirused replitseeruvad, kui ekstrakromosomaalsed plasmiidid ning nende genoom seostub peremeesraku kromosoomidega tagades seeläbi jagunemise tütarrakkudesse. Seejuures on seostumine peremeesraku kromosoomidega vahendatud viiruselise valgu ja tema seostumiskohtade (BS) kaudu. Nendeks viiruselisteks valkudeks on BPV1 puhul E2 ja EBV puhul EBNA1.

Käesoleva doktoritöö eksperimetaalse töö üks osadest keskendubki BPV1 ja ka EBV jagunemiseks vajalike faktorite uurimisele. Esmalt olime huvitatud kas nende viiruste segregatsiooni elemendid on funktsionaalsed ka heteroloogses süsteemis. Selleks konstrueerisime seeria plasmiide, mis sisaldasid BPV1 või EBV segregatsiooni elemente ja hiire polüoomiviiruse (PyV) metsik tüüpi või minimaalset replikatsiooni alguspunkti. Saadud konstruktide võimet replitseeruda ja säilida kontrolliti nii lühiajalistes kui ka pikaajalistest katsetes. Transientsed katsed näitasid, et PyV minimaalse *origin*`i replikatsioon on E2-st ja tema BS-st sõltuvalt aktiveeritav. Pikaajaline plasmiidide jälgimine rakkudes demonstreeris, et BPV1 E2 BS-id ja EBV segregatsiooni element FR suudavad tagada heteroloogse plasmiidi säilimise, vastavate viirusvalkude juuresolekul. Meie poolt tehtud töö oli esimene, mis näitas, et E2 ja tema BS-d on funktsionaalsed ka heteroloogses süsteemis.

Varasemad uurimused meie laboris on näidanud, et E2 kromatiinile seostumise ja transaktivatisooni funktsioonid ei ole lahutatavad. Kõik mutatsioonid E2 N-terminaalses osas, mis mõjutavad transaktivatsiooni häirivad ka kromatiinile seostumist. Sellest lähtuvalt püstitasime hüpoteesi, et lisaks kromatiinile seostumisele on ka E2 transaktivatsioon vajalik viiruse segregatsiooni protsessis. Meie katsed kimäärsete E2 valkudega näitasid, et tõepoolest, efektiivne kromatiinile seostumine ei taga veel segregatsiooni. Komplementatsioonikatsed

valgu VP16 transaktivatsiooni domeeniga tõestasid, et kromatiinile seostumine ja transaktivatsiooni domeen mõlemad on vajalikud, et tagada efektiivne segregatsioon.

Kaasaegne biotehnoloogia ja ravimitööstus vajab suures koguses puhastatud valke, et läbi viia prekliinilisi katsetusi ja skriininguid. Lisaks sisaldavad väga paljud maailmas kasutatavad ravimid valkkomponenti. Tööstuslikult kasutatakse valkude tootmiseks põhiliselt rakuliinide konstrueerimist või transientset transfektsiooni. Rakuliini valmistamise miinuseks on sellele kuluv pikk aeg, töö mahukus ja kallidus. Transientne lähenemine seevastu tagab ainult lühiajalise valgu produktsiooni. Meie poolt kirjeldatud heteroloogsed plasmiidid on rakkudes pikaaegselt säilivad, mistõttu sobiksid valgu tootmiseks ning oleks alternatiivne võimalus praegu laialt kasutatavatele meetoditele. Vastavad plasmiidid ja rakuliinid on patenteeritud ning litsentseeritud tootearenduseks firmale Icosagen Cell Factory OÜ. Nende poolt arendatud tehnoloogia QMCF on osutunud edukaks ning mitmed tuntud ravimifirmad nagu näiteks Bayer Healthcare, Novartis, Wyeth, GE Healthcare on ostnud QMCF tehnoloogia litsentsi, et seda kasutada oma uuringutes.

Pikka aega ei olnud teada, millise rakulise valgu vahendusel BPV1 on E2 peremeesraku kromosoomidele seotud. Aastal 2004 näidati, et rakuline bromodomeene sisaldav valk Brd4 võiks olla E2 partner mitootilistel kromosoomidel. Kuna E2 osaleb lisaks viiruse genoomi segregatsioonile ka viiruse transkriptsiooni ja replikatsiooni regulatsioonis, siis otsustasime uurida Brd4 mõju just nendele E2 funktsioonidele. Meie tulemused näitasid, et Brd4 võib mõjutada viiruse replikatsiooni, kusjuures see sõltub kasutatavast rakuliinist ja on E2-st sõltumatu. Teiseks meie uurimus demonstreeris, et Brd4 on vajalik E2 sõltuvaks transaktivatsiooniks. Seega, lisaks Brd4 rollile viiruse genoomi segregatsioonil ja kromatiinile seostumisel, õnnestus meil näidata, et Brd4 on ka väga oluline komponent teistes E2 valgu vahendatud aktiivsustes.

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II Ilves I, Mäemets K, Silla T, Janikson K, Ustav M. (2006) Brd4 is involved in multiple processes of the bovine papillomavirus type 1 life cycle. Journal of Virology, 80, 3660–5.

III Kadaja M, Silla T, Ustav E, Ustav M. (2009) Papillomavirus DNA replication – from initiation to genomic instability. Virology, 384, 360–8.

IV Silla T, Hääl I, Geimanen J, Janikson K, Abroi A, Ustav E, Ustav M, Mandel T. (2009) Vectors, cell lines and their use in obtaining extended episomal maintenance replication of hybrid plasmids and expression of gene products. European Patent: EP1 851 319 B1.

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II Ilves I, Mäemets K, Silla T, Janikson K, Ustav M. (2006) Brd4 is involved in multiple processes of the bovine papillomavirus type 1 life cycle. Journal of Virology, 80, 3660–5.

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2. **Enn K. Seppet**. Thyroid state control over energy metabolism, ion transport and contractile functions in rat heart. Tartu, 1991, 135 p.
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