DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
87

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87

THE DETERMINANTS FOR THE NATIVE ACTIVITIES OF THE BOVINE PAPILLOMAVIRUS TYPE 1 E2 PROTEIN ARE SEPARABLE

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LIST OF ABBREVIATIONS

aa	amino acid
AdMLP	adenovirus major late promoter
A_E	papillomavirus early polyadenylation signal
A_L	papillomavirus late polyadenylation signal
BPV1	bovine papillomavirus type 1
BS	binding site
CE	BPV1 constitutive enhancer
ChIF	immunofluorescence on mitotic chromosomes
COPV	canine oral papillomavirus
DBD	DNA binding domain
EBV	Epstein Barr virus
ELISA	enzyme linked immunosorbtion assay
EMSA	electrophoretic mobility shift assay
FISH	fluorescence *in situ* hybridisation
HPV	human papillomavirus
Mab	monoclonal antibody
MME	minichromosome maintenance element
ND10	nuclear domain 10
NLS	nuclear localisation signal
ORF	open reading frame
PML	promyelocytic leukemia protein
POD	promyelocytic oncogenic domain
PolyA	polyadenylation signal
PV	papillomavirus
PyV	mouse polyomavirus
TAD	transactivation domain
tk	tymidine kinase
URR	upstream regulatory region
VLP	virus-like particle
wt	wilde type

1. INTRODUCTION

This PhD thesis covers my work on bovine papillomavirus E2 protein. Papillomaviruses are a family of small DNA tumour viruses that induce warts (or papillomas) in a variety of higher vertebrates, including humans. Some papillomaviruses have also malignant potential for animals and humans. Papillomaviruses are efficient viruses which have arisen before the man stepped on the Earth. During this long time of evolution papillomaviruses have evolved efficient molecular mechanisms that regulate every side of their life within the cell. According to their small size, about 8 kb, the papillomaviruses have only limited capacity to encode the functions required for their propagation. However, the interplay between viral DNA replication, transformation and gene expression mechanisms seems to be amazingly complex and elegant. The virally coded E2 protein is one of the main regulators of several different viral activities. The E2 protein is directly involved in the regulation of transcription, initiation of viral DNA replication, efficient segregation of viral genomes etc. How E2 as a medium size protein can fulfil all these functions and how are these activities regulated? The data presented in this thesis try to clarify several important details on the functioning of E2, especially which determinants are responsible for the different activities and how different E2 activities are related to each other.

2. REVIEW OF LITERATURE

2.1. PAPILLOMAVIRIDAE — THE VIRUS, THE GENOME AND THE GENES

Papillomaviruses (PV-s) are the family of small double-stranded non-enveloped DNA viruses. Different PV types have been found from multiple species, from human to birds. The virus particles are 52–55 nm in diameter and have the sedimentation coefficient (S_{20}, W) of 300. The virion consists of a major capsid protein L1 and a minor capsid protein L2, and contains a single molecule of double stranded circular DNA about 8 kb together with at least four types of histones (H2a, H2b, H3 and H4). The viral capsid consists of 72 capsomers arranged on a T=7 surface lattice (60 hexavalent and 12 pentavalent). The Bovine Papillomavirus type 1 (BPV1) virions can infect cells with a "single-hit" mechanism, at least in a cell culture system (Dvoretzky *et al*., 1980). The PV virions or pseudovirions and Virus Like Particles (VLP-s) can bound to the cell surface of different cell types. However, productive infection is very specific to the organism and cell type. There exists a group of ungulate PV-s (including BPV1) causing benign fibropapillomas, which can infect and transform also other species than their natural host, but this is specific only for this group of PV-s.

For persistence, the PV must infect the basal cells of epidermis. This is possible, for example, owing to microlesions often found in the skin.

The PV genome is ~8 kb long (ranking from 7100 of HPV48 to 8607 of COPV). The genome contains a coding region and an about 1 kb long non-coding region, which contains regulatory elements for PV gene expression and replication (and at least in case of BPV1 — for proper segregation and partitioning) and is also called Long Control Region (LCR) or Upstream Regulatory Region (URR) (the later name is used through this thesis) (Fig. 1).

Over 100 viral genomes from at least 120 virus types from about 16 host species have been sequenced completely (according to HPV sequence database http://hpv-web.lanl.gov and Viral Genome Resource at Genbank http://www.ncbi.nlm.nih.gov/genomes/VIRUSES/151340.html). The general architecture of the genome (~1kb URR and ~7 kb coding region) is conserved almost in all of them. The Open Reading Frames (ORF) are found only in one strand. There are no data published hitherto, stating that the lower strand contains ORF-s or has been transcribed.

The position, size and function of many (but not all) ORF-s are well conserved among various PV types. The coding region is divided into two parts according to their location on the genome and their expression time during infection. The early region (E) contains up to 10 ORF-s and the late region (L) 2 ORF-s.

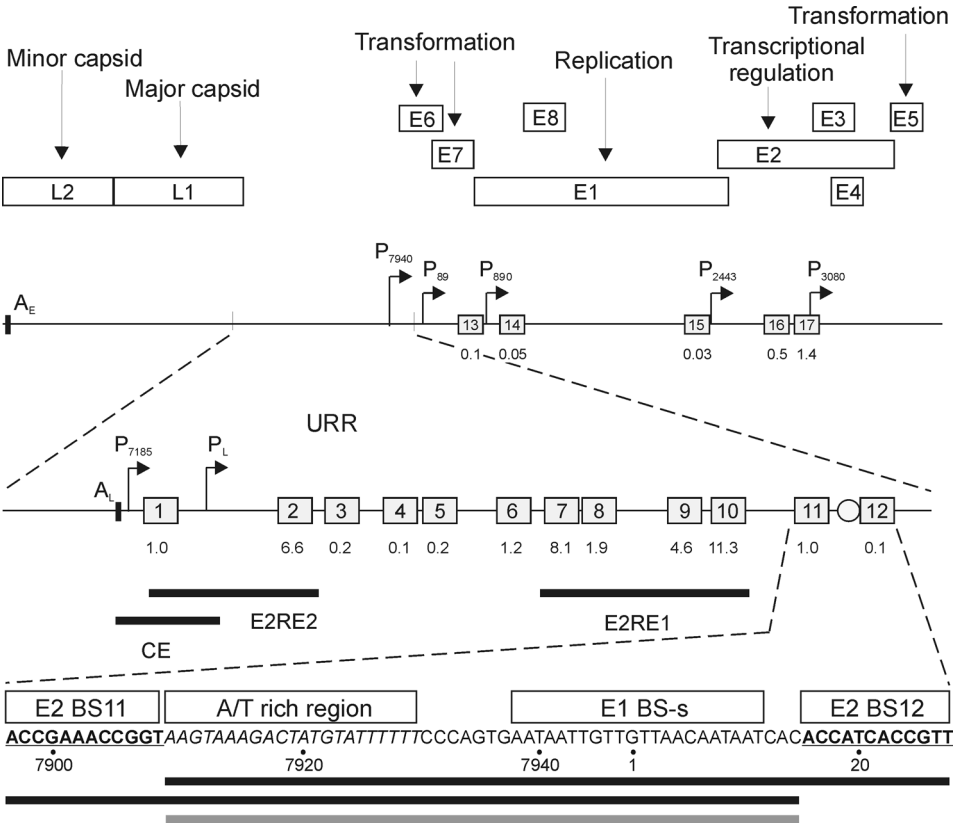


Figure 1. Map of the linearized BPV1 genome. Shaded boxes represent the 17 E2 BS-s found in the genome. The number inside the box shows the E2 BS number and the number below the box the relative strength of BS. The open bars lettered E1 to E8 and L1 and L2 represent the ORFs. The upstream regulatory region (URR) is enlarged below. The circle indicates the E1 BS-s. From the URR, the origin region is enlarged and shown in more details. The solid lines indicate minimal ori *in vivo* and the gray line minimal ori *in vitro*. P — promoter, A — polyadenylation signal, CE — constitutive enhancer, E2RE — E2 response element

The early region encodes the proteins required for the regulation of viral gene expression, viral DNA replication and host cell transformation. The late region encodes the viral capsid proteins L1 and L2.

The PV genome contains multiple promoters and two polyA sites, the early polyA (A_E), in BPV1 at nt. 4203 and late polyA (A_L), in BPV1 at nt. 7175 (Fig. 1). Both polyA signals are conserved between different PV types.

The **E1 ORF** of different PV-s is well conserved and encodes protein with length between 600 and 700 aa. E1 is a replication initiator protein with specific and unspecific DNA binding, helicase, ATPase, ATP binding and DNA un-

winding activities (for a review (Wilson *et al*., 2002)). According to structural, biochemical and bioinformatic data E1 can be divided into three domains:

a) N-terminal domain (aa1-130 in BPV1 E1) with NLS and phosphorylation sites;
b) DNA binding domain (DBD) — aa 142-308 in BPV1 E1;
c) ATPase/helicase domain — aa ~310-605 in BPV1 E1 or last ~300 C-terminal aa generally in PV-s.

The **E2 ORF** encodes the proteins involved in regulating viral transcription, viral DNA replication and segregation/partitioning. Three proteins with a common C-terminal part are found in BPV1 transformed cells — the full-length E2 activator protein and truncated repressor proteins E2C and E8/E2 (Fig. 2A in page 21). The different shorter forms of E2 sharing the hinge and DNA binding region with full-length E2 are also reported for some other PV types. The E2 proteins are described in more detail in Chapter 2.5. **Through the rest of this thesis, the E2 protein means full-length E2 protein.**

The **E3 ORF** is annotated in multiple PV-s as an ORF, however, it does not contain an AUG codon for the initiator methionine. The aa from the E3 ORF are not found in fusion with other PV proteins.

The peptide encoded by **E4 ORF** is found in some PV types in fusion with the E1 protein due to splicing. E4 is the only protein from the early region expressed not until the late stage of productive infection starts. The expression of the E4 protein in multiple HPV types coincides with PV vegetative replication in tissue samples (Doorbar *et al*., 1997) (Peh *et al*., 2002). The expression of E4 is not coincident with the expression of the capsid proteins and precedes the expression of L1. In cultured epithelial cells the E4 protein is associated with keratin cytoskeleton and induces the collapse of the cytokeratin network. The expression of HPV1a E4 protein leads to reorganizing ND10 nuclear bodies (Roberts *et al*., 2003). The expression of HPV18 and HPV16 E1:E4 leads to block in G_2/M transition (Davy *et al*., 2002) (Nakahara *et al*., 2002). The mutational analysis of BPV1 E4 has shown that E4 was not essential for viral transformation or viral DNA replication in C127 cells (were the vegetative replication does not take place) (Neary *et al*., 1987) (Hermonat and Howley, 1987). The HPV1 E4 protein expression is required for the onset of the late stage of the virus life cycle in rabbit species (Peh *et al*., 2004). The HPV1 unable to express full-length E4 is still able to replicate as an extrachromosomal plasmid and induce papilloma development (Peh *et al*., 2004).

The **E5 ORF** of BPV1 encodes a 44 aa hydrophobic protein which is one of the smallest oncogenes known. This protein can activate PDGFβ receptors by dimerizing them and as a result transform the cells. The E5 proteins also form a complex with vacuolar H+-ATPase and inhibit it, which leads to the acidification of the Golgi complex.

The function of HPV E5 is not well understood and not too much examined. The possible reason for this is the fact that HPV E5 is not expressed in most of the HPV positive cancers (the BPV1 and BPV2 E5 are expressed in naturally

occurring tumours, but not in all BPV containing samples (Nasir and Reid, 1999) (Borzacchiello *et al*., 2003)). However, both the BPV1 and HPV16 E5 protein affect antigene presenting pathways in cells, albeit in using different specific mechanisms (Ashrafi *et al*., 2002) (Marchetti *et al*., 2002; Zhang *et al*., 2003). Unlike for most HPV-s, the E5 protein is the major transforming protein for the BPV1.

The protein encoded by **E6 ORF** is the most conserved oncogene in PV-s. Like E5, the E6 exerts its activity through interactions with different cellular proteins. E6 together with E7 are the major transforming genes for HPV-s. The most studied (and most important) is the interaction between E6 of high risk HPV-s and p53, which promotes the degradation of p53 through the ubiquitin-dependent pathway. The E6 from low risk HPV-s also interacts with p53, but this does not induce the p53 degradation. A number of additional, p53 independent targets have been identified for E6, including several members of signal cascades that control the cellular proliferation (Mantovani and Banks, 2001). The E6 protein can modulate the expression of multiple genes, for example IL-8 and human telomerase reverse transcriptase subunit hTERT (Huang and McCance, 2002). For HPV16 E6, interaction with transcriptional co-activators CBP/p300 and Ada3 has been shown (Patel *et al*., 1999) (Zimmermann *et al*., 1999) (Kumar *et al*., 2002). Also the BPV1 E6 can bind to the CBP/p300 and downregulate transcription (Zimmermann *et al*., 2000).

The **E7 ORF** encodes a protein with the length of ~100 amino acids. The most studied effect of HPV E7 on cellular transformation is binding to the pRb protein. This binding inactivates pRb and promotes its proteolysis. Also the pRb independent activities of E7 have been described. The E7 interacts with the coactivator pCAF and regulates IL-8 expression (Huang and McCance, 2002). E7 can interact with cyclin-dependent kinase inhibitor $p27^{kip1}$ and inactivate it (Zerfass-Thome *et al*., 1996). Differently from HPV E7 proteins the BPV1 E7 does not contain an LXCXE motif for binding to pRb, suggesting that it does not share the pRb binding and inactivation properties with other HPV E7 proteins.

The **E8 ORF** encodes a short peptide which is often fused to E2 hinge and DBD coding regions by splicing. Recently it has been shown that the E8 domain of HPV31 E8/E2C is responsible for the general repression of extrachromosomal replication origins (HPV31 and EBV oriP) as well as for the repression of transcription (Stubenrauch *et al*., 2001) (Zobel *et al*., 2003).

The **L1 ORF** encoded protein is the major capsid protein of the PV virions. The L1 protein can self-assemble to VLP in the absence of other proteins and also bind DNA. The L1 is responsible for virus particle stability. This is achieved on the account of disulphide bridges between L1 molecules. Interaction of BPV1 virions with microtubules is also mediated by the L1 protein (Liu *et al*., 2001).

The **L2 ORF** encoded protein is the minor capsid protein. It favors the formation of VLP-s and is required for the formation of infectious virus

particles. Most probably, L2 is involved in the packaging of viral DNA into virions (Zhao *et al*., 1998). The L2 protein has been shown to localize in nucleus to the ND10 (or POD) bodies and also induce the localization of L1 and E2 to these bodies (Day *et al.*, 1998). The L2 of HPV16 has been shown to interact with β-actin and facilitate infection (Yang *et al*., 2003).

2.1.1. The PV life-cycle

In general, the primary infection which leads to the persistence of viral genomes in organism occurs in proliferating basal cells. The PV-s entry probably takes place via clathrin-dependent receptor-mediated endocytosis or caveola-mediated endocytosis depending on the virus type (Bousarghin *et al*., 2003; Day *et al*., 2003). The BPV1 virions enter via clathrin dependent endocytosis (Day *et al*., 2003). The receptors by which PV-s bind and enter cells have not been identified unequivocally. The $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins were the first receptors identified (Evander *et al*., 1997; McMillan *et al*., 1999). Later it was found that heparan sulfate proteoglycans are also responsible for virus binding and infectivity. The transport of viral particles towards nucleus has been shown to occur both by microfilaments and microtubules transport mechanism, whereas L1 interacts with tubulin and L2 with actin (Liu *et al*., 2001) (Yang *et al*., 2003). The desintegration of virions probably takes place in cytoplasm near or at the nuclear membrane, as no viral particles are observed in nucleus by electron microscopy in the early steps of infection (Zhou *et al*., 1995) (Liu *et al*., 2001). At the same time, L1 and L2 are found to be in the nucleus according to immunofluorescence study (Zhou *et al*., 1995). The transcription from the viral genome is most likely initiated by cellular factors, as there are no reports about the presence of early viral proteins in capsid. The transcription from viral promoters is strongly controlled by a cellular differentiation program (described in more detail in chapter 2.2.). The early proteins E1 and E2, as well as E5 (in case of BPV1), are expressed in basal cells. The expression of the replication proteins E1 and E2 leads to the replication of the PV genome, and the establishment of viral infection takes place. The initial replication of viral DNA is believed to occur faster than cellular replication, as in native infections which usually start with “single hit” mechanism, at the later step of infection the copy number of the genome ranges from 50 to 300 (Dvoretzky *et al*., 1980). The vegetative DNA replication starts in the differentiating cells and thereafter also the expression of late proteins and virion assembly are initiated. The mature virions are shed on the upper layers of the epidermis.

In general, different PV types have a similar differentiation-dependent expression pattern. However, the exact time of expression of each protein in different layers of epidermis depends on virus type (Peh *et al*., 2002).

The biology of PV-s as first DNA tumor viruses identified has been studied for a long time (Rous and Beard, 1935; Shope, 1933). However, the cell culture system for studying BPV1 transcription, transformation and replication was established in ~1980 and for HPV-s ten years later. There are still no systems to produce infectious virus particles in sufficient amounts to study the viral entry and escape steps of PV-s, mostly due to difficulties in getting terminally differentiated cells in the cell culture, which is a prerequisite for late gene expression.

The members of the PV family and certain proteins from different PV-s are in some aspects very similar, but they are very different in some other functioning. Thus, the data about the functioning of the proteins of one type of PV-s might not be valid for the others. **The focus of this thesis lies on BPV1 E2 protein. Therefore, the data presented in the next chapters deal with the functioning of BPV1 and its proteins, if not mentioned otherwise.**

2.2. REGULATION OF BPV1 GENE EXPRESSION

The regulation of BPV1 gene expression takes place at multiple levels — on the initiation of transcription, on splicing, on mRNA stability, on translation. The only level of regulation of viral gene expression which has been shown to be directly regulated by viral proteins is transcription.

The virus particles have been shown to contain also histones and the viral genome is packaged into nucleosomes (up to 30 nucleosomes per genome) (Favre *et al.*, 1977). As there are no reports on the presence of early viral gene products in virions, the expression of early genes immediately after infection must be initiated solely by cellular transcription factors. However, due to difficulties in getting native virus particles the early step of the regulation of BPV1 gene expression is poorly understood.

For the BPV1, seven promoters have been described: P_{7185}, P_{7940}, P_{89}, P_{890}, P_{2443}, P_{3080} and P_L with start site between nucleotides 7214 and 7256 (Fig. 1). Five promoters (P_{7185}, P_L, P_{89}, P_{2443} and P_{3080}) are also active in *in vitro* transcription assays, whereas P_{89} is the most active promoter (Linz and Baker, 1988).

The presence of different virus specific transcripts depends primarily on the differentiation status of the cells. Several different mRNA-s have been detected both in BPV1 transformed cells and productively infected wart tissues, but some mRNA species, including for example mRNA-s coding the L1 and L2 proteins, are present only in wart tissues (Baker and Howley, 1987). The late promoter (P_L) produces predominantly mRNAs from the transforming region (not the late region), the most abundant of these is the E4 mRNA (Baker and Howley, 1987). The P_L is strongly upregulated in the granular layer of the fibropapilloma (Barksdale and Baker, 1993). In wart tissue, the transcripts from P_L are very abundant: 10–100 times more abundant than the levels of mRNA derived from

any other BPV1 promoter (Baker and Howley, 1987). The E5 protein expression is observed both in basal cells and in highly differentiated keratinocytes (Burnett *et al*., 1992), however, spliced transcript from P_{2443} is found only in basal keratinocytes and fibromas (Barksdale and Baker, 1993). The E5 protein, expressed in highly differentiated keratinocytes, is most likely encoded by transcript starting from P_L but polyadenylated from A_E (Barksdale and Baker, 1995).

The promoter usage in cells transiently transfected with the BPV1 genome was qualitatively similar to that used in BPV1-transformed cell line, however, significant quantitative differences exist (Szymanski and Stenlund, 1991). P_{89} is the most active promoter in the C127 cells stably transformed with the BPV1 genome (Szymanski and Stenlund, 1991). In the cells transiently transfected with the BPV1 genome, P_{3080} is ~2.5 times more active than P_{89} (Szymanski and Stenlund, 1991). Transcription from P_{890} is also much more effective in transiently transfected cells as compared to that in stably transformed ones (Szymanski and Stenlund, 1991). Both promoters, more active in transiently transfected cells express the mRNA-s coding the repressor forms of E2 (see also Chapter 2.5).

The E2 protein regulates viral transcription by binding to the E2 BS-s present in genome (chapter 2.5.3.). Apart from the clear involvement of E2 and contradictory data about the role of E1 in viral transcription, no other viral protein has been reported to affect BPV1 transcription directly (Szymanski and Stenlund, 1991) (Le_Moal *et al*., 1994; Parker *et al*., 2000; Sandler *et al*., 1993; Zemlo *et al*., 1994).

The 5' region of URR contains a constitutive enhancer (CE), very active in bovine embryo fibroblasts. However, the absence of CE does not affect the P_{7940} and P_{89} promoters (Vande_Pol and Howley, 1990) but reduces strongly the expression from P_{890} and P_{2443} (Vande_Pol and Howley, 1992), the major promoters for E2 production.

Not too much is known about the cellular factors regulating the BPV1 transcriptional program. The URR (and the CE) contains consensus binding sites for different transcription factors, but the real binding and/or involvement of these factors in the BPV1 transcriptional regulation has been studied only in few cases. Sp1 has been shown to be required for both basal and E2 activated transcription from the P_{89} and P_{2443} promoters (Sandler *et al*., 1996; Spalholz *et al*., 1991). Near P_{2443}, the Sp1 BS but not the E2 BS15 is required for basal promoter activity (Spalholz *et al*., 1991) (Vande_Pol and Howley, 1992).

On P_{7185}, the E2 BS overlaps with the binding site for cellular factors and E2 represses this promoter (Stenlund and Botchan, 1990). The transcriptional regulator CBF can compete with E2 and regulate the activity of the P_{7185} promoter (Schmidt *et al*., 1997).

All promoters, except P_L, are shown to be regulated by the E2 protein, so E2 is the most important virally coded regulator of the BPV1 transcription.

2.3. BPV1 DNA REPLICATION

The BPV1 DNA replication can be described by three different modes depending on the temporal course of infection. The **first** mode, the **amplificational replication**, takes place during the initial infection of basal keratinocytes by the virus (which may occur by a "single hit" mechanism (Dvoretzky *et al*., 1980)) where the copy number of viral genomes increases to 50–100. In the **second** phase of viral DNA replication the viral genome is maintained as an episomal unit at a constant copy number — called also **stable replication** or **stable episomal maintenance**. This occurs in dividing basal cells and dermal fibroblasts. In these cells, the viral minichromosomes are maintained as stable multicopy plasmids, which replicate during the whole S phase, but not exactly once per cell cycle (Gilbert and Cohen, 1987; Ravnan *et al*., 1992), and are faithfully partitioned to daughter cells. This type of DNA replication ensures a persistent and latent infection in the stem cells of epidermis. The **third** type of DNA replication is **vegetative DNA replication** which occurs in the more differentiated epithelial cells of the papilloma. In these cells which no longer undergo cellular DNA synthesis one observes a burst of viral DNA synthesis, generating the genomes to be packaged into progeny virions. For this mode of viral DNA replication no good experimental systems exist.

2.3.1. Initial amplificational replication

Some aspects of amplificational replication can be studied in a transient transfection assay, which is also called "transient replication assay". For initial amplificational replication, the viral E1 and E2 proteins are required as *trans* factors and the origin of DNA replication (ori) as a *cis* factor. The minimal ori consists of E1 BS, A/T rich region and E2 BS11 or BS12 (Fig. 1). In general, the BPV1 ori is similar to other viral origins containing usually a binding site for initiator, A/T rich sequences and an auxiliary element, which very often is a binding site for a transcriptional regulator. In the presence of E1 and E2, the ori containing plasmid DNA replicates in multiple different mammalian cell lines, indicating that the unability of viral genomes to replicate in those cell lines is caused by unproper transcriptional regulation of viral promoters in these cell lines. The E2 BS near the origin can be substituted with multiple high-affinity BS-s at the distance (Ustav *et al*., 1993). In the presence of E1, E2 and ori containing plasmid, the test plasmid replication takes place more than once per cell cycle (Kivimae *et al*., 2001). In addition to minimal origin absolutely required for replication, also other regions have been shown to affect the efficiency of BPV1 transient replication (Pierrefite and Cuzin, 1995). Also the Purα factor can bind to the single stranded A/T rich regions and may affect the replication efficiency (Jurk *et al*., 1996). The E1 protein is loaded to the origin

by the E2 protein and works as a replicative helicase during elongation. At very high E1 levels *in vitro*, E1 itself can initiate replication, however, this is a largely origin independent initiation (Bonne-Andrea *et al*., 1995) (Yang *et al*., 1993). Adding E2 to the *in vitro* replication reaction, the ori-independent replication falls to the background level (Bonne_Andrea *et al*., 1997). The high level of E1 in cells *in vivo* leads to "onion skin" replication from the origin region (Mannik *et al*., 2002). However, the "onion skin" replication *in vivo* was not affected by the elevated E2 level (Mannik *et al*., 2002). Studies with purified proteins indicate that cellular factors required in BPV1 *in vitro* replication overlap to a large extent, but not entirely, with those required for SV40 replication (Melendy *et al*., 1995).

The replication can be modulated by the phosphorylation of the E1 and E2 proteins. The replication of the viral genome is likely to be affected by the phosphorylation of the E1 protein as the mutation of serine 109 (which is phosphorylated both *in vivo* and *in vitro* by protein kinase A and protein kinase C) to alanine leads to more effective replication (Zanardi *et al*., 1997). Phosphorylation of serine 48 by caseine kinase II has most likely a positive effect on replication, as the mutation of this residue to Gly leads to replication defective E1 (in transient replication as well as in the context of viral genome) and mutation to Asp or Glu has wt-like activity (McShan and Wilson, 2000). The mutation of Ser584 (phosporylated *in vitro* by CK2) to Ala also leads to a replication defective E1, but a number of biochemical activities of this mutant protein were unaltered (Lentz *et al*., 2002) (Lentz, 2002). Thus, at least in transient replication assays the phosphorylation of E1 at different positions by different kinases can both downregulate and upregulate replication.

Results presented in the literature so far indicate that the initial amplificational replication is a prerequisite for the establishment of stable replication (Kim and Lambert, 2002).

2.3.2. Stable episomal maintenance

In order to be stably maintained as an episome in proliferating cells, the episomal plasmid must contain a replication function and a proper segregation function. For a stable replication of the BPV1 origin containing plasmids as episomal minichromosomes, the set of elements required for transient replication is insufficient (Piirsoo *et al*., 1996). In order to be stably maintained in cells where E1 and E2 are present, also an element called MME is required. The MME consists of multiple E2 BS-s which are responsible for the stable episomal maintenance of plasmids with minimal origin in the presence of E1 and E2 (Piirsoo *et al*., 1996). The effect of the E2 protein and its BS-s is based on the tethering of MME containing plasmids to mitotic chromosomes (Ilves *et al*., 1999). Also the viral genomes are tethered to mitotic chromosomes in an E2 dependent manner (Lehman and Botchan, 1998; Skiadopoulos and McBride,

1998). There are also data that E1 is required only for the establishment but not for the maintenance of stable replication (Kim and Lambert, 2002). If so, the viral DNA must be entirely replicated by cellular factors and, thus, replication must occur only once per cell cycle. This is not the case in the cells stably transformed with BPV1 genome and in the cell line stably maintaining URR containing plasmid (Gilbert and Cohen, 1987; Piirsoo *et al*., 1996; Ravnan *et al*., 1992). In addition, the E1 protein is probably involved in regulating stable maintenance through affecting the E2 chromatin attachment (Voitenleitner and Botchan, 2002). The transient replication and stable maintenance replication have also different sensitivities to p53 mediated replication repression (Ilves *et al*., 2003; Lepik *et al*., 1998). The transient replication can be repressed by p53 whereas the stable maintenance replication is not affected by p53 (Ilves *et al*., 2003).

2.3.3. Vegetative replication

Until recently, the lack of a good model system has hampered the studies on the PV replication during the vegetative stage. Some data indicate that the BPV1 uses the rolling circle mode of replication during the vegetative stage (Burnett *et al*., 1989; Dasgupta *et al*., 1992). This observation is complemented by the data from the 2D analysis of replication intermediates, showing that the mode of rolling circle replication may be involved in the vegetative replication of HPV16 and 31 (Flores and Lambert, 1997).

2.4. DNA PACKAGING AND VIRION ASSEMBLY

As there does not exist a good system for studying vegetative replication, there are no laboratory used *in vivo* systems to study viral DNA packaging, virion assembly and escape. Most of the data about virion assembly are obtained from *in vitro* experiments. These data show that infectious BPV1 particles can be assembled *in vitro* and that E2 has an effect on the packaging of DNA into capsids. There exists a mouse xenograft model for obtaining infectious viral particles, but in very low amounts. There are cell culture systems to obtain infectious HPV virions, but the problem is the same — a low yield of virions for detailed studies.

2.5. THE PAPILLOMAVIRUS E2 PROTEIN

The BPV1 E2 protein is a multifunctional factor involved in different aspects of viral nucleic acid metabolism — it is involved at least in transcriptional regulation, viral DNA replication and segregation. The BPV1 E2 gene encodes

three different proteins: full length E2, E2C (also called E2TR) and E8/E2, which share a common DBD (Fig. 2). The E2 protein can be expressed from P_{2443} or from spliced mRNA from upstream promoters by using a splice acceptor at nt. 2558 (Vaillancourt *et al*., 1990; Yang *et al*., 1985). The E2C is encoded by a transcript produced from the P_{3080} promoter and the initiator methionine corresponding to the Met162 in the full length E2, thus most of the transactivation domain (TAD) is not present. E8/E2 is translated from spliced mRNA from the P_{890} promoter (1235-3225) which encodes 11aa from E8 ORF followed by the hinge and DBD region of E2 starting from 206 aa. The relative abundance of these three E2 DBD containing forms is reported to be 1 E2: 10 E2C: 3 E8/E2 (Hubbert *et al*., 1988) or 1:21:6 (Lambert *et al*., 1989b) in C127 cells and 1:19:1.5 in NIH 3T3 cells (Hubbert *et al*., 1988). These data indicate that the ratio of the E2 gene products may vary in different cell lines. The ratio of different forms depends also on the cell cycle, being the highest in late S to M (E2/(E2C+E8/E2)=0.4-0.5) (Yang *et al*., 1991b). However, in this study E2 was immunoprecipitated with the Mab B202, recognizing the epitope between aa 285-310 (Prakash *et al*., 1992) which contains also major phosphorylation sites (S290, S298, S301 (McBride *et al*., 1989a)) for E2 and phosphorylation of E2 peaks at G_2/M (Voitenleitner and Botchan, 2002). Thus, the real ratio of the activator to repressors can be a little bit different. Recently it has been shown that the ratio of mRNA-s encoding full-length and repressor forms can be affected at transcription level by tyrosine kinase inhibitors (Baars *et al*., 2003). Thus, the ratio of the activator to repressor forms depends strongly on cellular environment. The half-lifes of E2 proteins in BPV1 transformed C127 cells are estimated as follows: 40 min for E2, 10 min for E2C and 15 min for E8/E2 (Hubbert *et al*., 1988). However, these data can be misleading, because the different forms of E2 may dimerize with each other when co-expressed (Lim *et al*., 1998) (McBride *et al*., 1989b) and the half-life was measured in the conditions where the heterodimers could form. Thus, the real half-life of homo-dimers or certain heterodimers in not known.

The mutation of the initiator codon for E2C leads to a virus with much higher transforming activity, elevated E5 expression (Lambert *et al*., 1990) and a higher copy number (5–20 times) in stably transformed cells (Lambert *et al*., 1990), (Riese *et al*., 1990). At the same time, the presence of at least one repressor form is required for efficient transformation and stable maintenance of a genome (Lambert *et al*., 1990). All the E2 proteins use the NLS located in the DBD and coincide with DNA binding helix (Skiadopoulos and McBride, 1996) (Allikas *et al*., 2001).

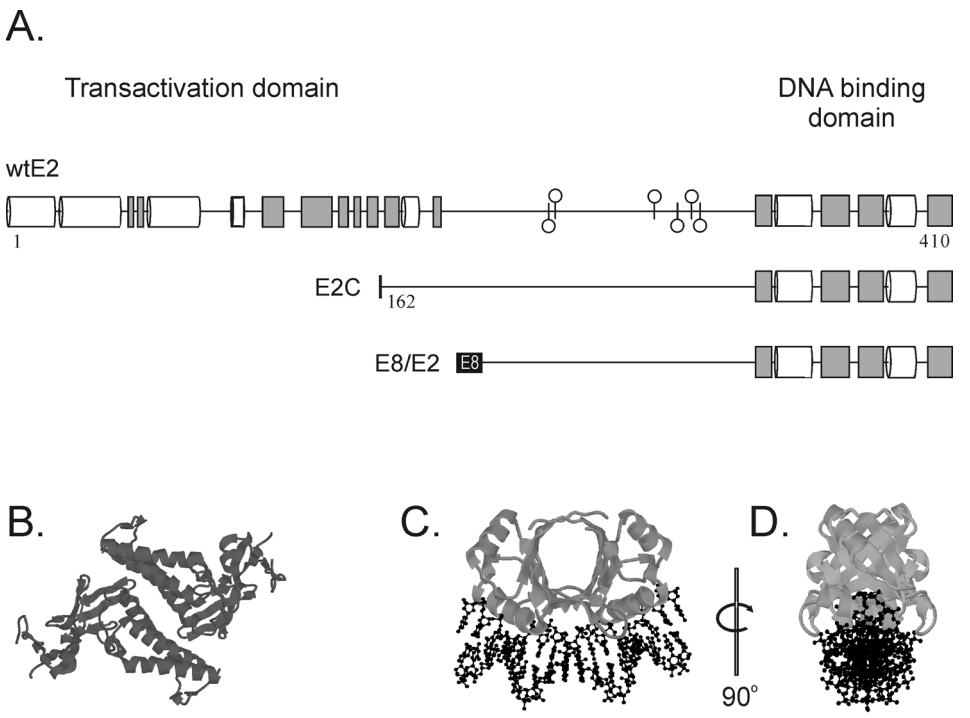


Figure 2. Structural and functional domains of the BPV1 E2 and its repressor forms. **A.** Secondary structure presentation of E2, E2C and E8/E2. Open box — alpha-helix, shaded box — beta-sheet. The small circles indicate E2 phosphorylation sites. **B**. The ribbon presentation of HPV16 E2 TAD (PDB code 1DTO). **C**. and **D**. BPV1 E2 DBD bound to its target (PDB code 2BOP). The E2 DBD is presented in ribbon mode and DNA in ball-and-stick mode.

2.5.1. The structural and functional domains of the E2 protein(s)

According to structural, functional and bioinformatics data, the E2 protein is composed of three different domains (Fig. 2). The first ~200 aa form the N-terminal domain which is usually called the transactivation domain (TAD). It was first defined according to E2 protein sequence alignments where the first ~200 and last ~100 aa form well conserved domains in contrast to unconserved intermediate sequences which are called the “hinge” region (Giri and Yaniv, 1988) (Haugen *et al*., 1988). Later, the functional as well as structural (for HPV16 and 11) independence of the E2 TAD has been demonstrated. The TAD can function both in the transactivation and initiation of BPV1 replication when linked to heterologous DBD (Berg and Stenlund, 1997; Breiding *et al*., 1996; Kivimae *et al*., 2001; Winokur and McBride, 1996). The C-terminal DBD has also been shown to form an independent DNA-binding and dimerization unit.

As already mentioned above, all the E2 derivatives dimerize through their common DBD-s. This dimerization is very stable because practically no free E2 monomers exist in solution (Prakash *et al*., 1992) (Abroi *et al*., 1996) (Knight *et al*., 1991) and the preformed homodimer does not exchange the subunits to form heterodimers without denaturation (Prakash *et al*., 1992) (Haugen *et al*., 1988).

2.5.1.1. The structure of the E2 TAD

The exact structure of the BPV1 E2 TAD is not known. However, the crystal structures of the E2 TAD-s from HPV16, HPV18 and HPV11 have been reported, showing that not all activation domains are disordered in the free state (Harris and Botchan, 1999) (Antson *et al*., 2000) (Wang *et al*., 2003). The E2 TAD consists of two sub-domains: a sub-domain containing curved anti-parallel β-sheets and an α-helical sub-domain containing three anti-parallel α-helices (Fig. 2B and Appendix 2 in www.ebc.ee/aabroi/thesis). In the crystal lattice, the HPV16 E2 TAD forms a dimer through the α-helical sub-domains (Antson *et al*., 2000). As the DBD-s from different PV-s are similar in spite of divergent sequence identity, the TAD from BPV1 has most likely also a structure similar to TAD from HPV-s.

2.5.1.2. The structure of the E2 DBD

The DBD of the E2 protein forms an eight-stranded anti-parallel β-barrel made up of four strands from each subunit (Fig. 2C). Upon dimerization, strands β2 and β4 at the edges of each subunit participate in a continuous hydrogen-bonding network which results in an 8-stranded β-barrel. The dimer interface is extensive, made up of hydrogen bonds between subunits and a substantial hydrophobic β-barrel core. A pair of α-helices symmetrically positioned outside barrel contains the amino acid residues that are required for specific DNA interaction. The structure of the DBD-s from different PV-s is very similar (for review (Hegde, 2002)). Moreover, the DBD of the Epstein-Barr virus EBNA1 protein has a very similar structure to the E2 DBD despite no sequence similarity between these two proteins (Bochkarev *et al*., 1995).

2.5.2. The DNA binding of the E2 protein

The E2 DNA-binding is required for most of the activities of the E2 protein. E2 usually binds most efficiently to the consensus sequence **ACC**GN_4C**GGT**. The E2 DBD binds to one face of the DNA double helix (Fig. 2D). The binding constants for E2 and E2C are quite identical, whereas the binding constant for

E2 DBD is slightly weaker ($1{,}9\times10^{-11}$, 1.5×10^{-11} and 40×10^{-11}, respectively). E2 can also bind to DNA unspecifically with a much lower affinity constant (about 5–6 orders of magnitude weaker depending on the nature of target DNA) (Monini *et al*., 1993). During the DNA binding slight conformational changes occur in E2 DBD (Hegde *et al*., 1998; Veeraraghavan *et al*., 1999) (Hegde *et al*., 1992). It has been shown for BPV1 and also for HPV31 E2 that in the absence of DNA, the DNA recognition helix of E2 is not as stable as a typical α-helix (Liang *et al*., 1996; Veeraraghavan *et al*., 1999). In the E2 DBD:DNA complex, the recognition helices are inserted into successive major grooves of the DNA (Hegde *et al*., 1992). Multiple aa of the recognition helix form a direct contact with the bases of the consensus half-site (GGT).

2.5.2.1. E2 DNA binding sites

According to footprint and EMSA studies, E2 has 17 BS-s on the BPV1 genome (Fig. 1). Some of the sites identified have a single nucleotide deviation from canonical $ACCN_6GGT$. The relative strength of the BS-s varies up to 300 times (BS10 vs. BS15) (Li *et al*., 1989). It should be noted that the binding affinity depends not only on the presence of the consensus sequence but also on the nonconserved nucleotides between conserved half-sites (N_6) and on 5' and 3' flanking nucleotides (Li *et al*., 1989). The dissociation rates between different BS-s vary also more than 50 times. The location of different BS-s and their relative strength is shown on Fig. 1. The DNA of the E2 BS in complex with DBD is wrapped smoothly around the protein barrel and approximates a circle with a 45 Å radius (Hegde *et al*., 1992). One of the factors determining the binding affinity — the flexibility of N_4 in consensus BS ($ACCgN_4cGGT$) — has a higher impact for HPV E2 DNA binding compared to that for BPV1 E2 binding (Hines *et al*., 1998) (Kim *et al*., 2000).

2.5.2.2. E2 cooperative DNA binding

When the two E2 BS-s are located in proximity, the E2 binding to these BS-s occurs better than just by chance (Lambert *et al*., 1989a). The cooperative binding parameter was measured for E2 (purified from Sf9 extract) and was found to be 8.5, which is roughly similar to that found for other transactivators (Monini *et al*., 1991). The same parameter for the E2 DBD (purified from bacteria) is 1.9 and for the E2C 2.9 (purified from Sf9 extract) (Monini *et al*., 1991) (Monini *et al*., 1993).

The cooperativity of E2 binding has also been shown to take place during the occupation of E2 BS16 and BS17 near P_{3080} which locate 167 nt from each other. BS17 has the equilibrium binding-constant 3 times higher than BS16.

When both BS-s are intact, both of them are protected from DNase attack. However, when BS17 is mutated, neither BS-s are protected. At the same concentration, the E2C occupied only the BS17 (Knight *et al*., 1991). The cooperative binding to the neighboring BS-s leads probably to a much lower macroscopic off-rate from this double BS. The long distance cooperativity is probably achieved by DNA looping activity which also depends on the presence of E2 TAD (Knight *et al*., 1991). The DNA looping occurs also when three E2 BS-s are located more than 500 bp from each other and all three E2 dimers form a single particle on electron microscope (Knight *et al*., 1991). The cooperative binding of E2 to different E2 BS-s configurations was also reported by Hou et al (Hou *et al*., 2002).

2.5.3. E2 as a transcription regulator

Historically, the transcriptional activation and enhancer binding was the first activities of the E2 protein discovered. The E2 protein activates transcription from different BPV1 promoters (P_{7940}, P_{89}, P_{2443} and P_{3080}) as well as different heterologous promoters in mammalian and yeast cells. The E2 protein can also activate transcription *in vitro* (Hou *et al*., 2002). E2 can act also as a repressor — for example E2 represses the P_{7185} promoter in C127 cells (Stenlund and Botchan, 1990). Experiments with cells transfected with BPV1 genome carrying translational termination mutation for all E2 gene products and an increasing amount of exogenous E2 expression vector indicate that P_{2443} is more dependent on the E2 level than the others (P_{89}, P_{890}, P_{3080}) and that the absolute level of transcription is the highest for P_{2443} and P_{3080} in these conditions (i.e. in the absence of endogenous E2 proteins and in the presence of exogenous E2 and before the replication starts) (Szymanski and Stenlund, 1991). Still, using a genome defective for all E2 proteins, transcripts from P_{89}, P_{890}, P_{2443} and P_{3080} can be observed in transiently transfected C127 cells (Szymanski and Stenlund, 1991).

The deletion analysis of URR indicates that no individual E2 BS-s or pair of sites located in URR are absolutely required for the promoters to respond to E2. Thus, the specific E2 BS-s in URR contribute to the activity of all promoters similarly and do not display preferences for certain promoters (Szymanski and Stenlund, 1991). The E2 BS-s near promoters P_{2443} (BS15) and P_{3080} (BS16 and BS17) (Fig. 1) are not absolutly required for activation by E2, these regions have rather some regulatory effect on the E2 response (Szymanski and Stenlund, 1991).

E2 activates efficiently transcription from the heterologous promoters when at least two neighboring E2 BS-s are found near the transcription initiation site. When more than 4 E2 BS-s are located together, the E2 dependent enhancer works quite independently from its positioning to basic promoter elements (Thierry *et al*., 1990) (Forsberg and Westin, 1991). However, the effect of the

same number of binding sites is stronger when these BS-s locate near the promoter elements (Thierry *et al*., 1990). In mammalian cells as well as in yeast, the increase in the number of E2 BS-s enhances also the E2 dependent activation (Thierry *et al*., 1990) (Morrissey *et al*., 1989). The E2 can also activate promoters without having an E2 BS on the plasmid; however, this activation is at least 10 times lower compared to E2 BS dependent activation and does not require the intact DBD (Haugen *et al*., 1988). In this context it is interesting to note that HPV8 E2 facilitates the p300 dependent transcription independently from E2 BS-s on reporter plasmid (Muller *et al*., 2002). For full transcriptional activation by E2 the TATA box and, to a lower extent, Inr elements are required in the context of HPV18 p105 promoter (Ham *et al*., 1994). The TATA box and Inr can be substituted with two Sp1 BS in the context of AdMLP. The cooperative effect of Sp1 and E2 is shown also in multiple promoter configurations (Ham *et al*., 1991) (Ushikai *et al*., 1994) (Li *et al*., 1991). The overexpression of TBP extends the fold of E2 activation in the case of TATA box from constitutively weak promoters (Ham *et al*., 1994). For such activation, a core domain of human TBP is required (Ham *et al*., 1994). The length of the hinge region is not important for transactivation (Winokur and McBride, 1992). For the BPV1 E2, interactions with multiple proteins from the transcription machinery — transcriptional activators and coactivators TFIIB, TBP, Sp1, AMF1 (Breiding *et al*., 1997), p300 (Peng *et al*., 2000) (Muller *et al*., 2002), C/EBP (Hadaschik *et al*., 2003) — have been identified (Fig.3). The functional effect on *in vivo* transcription has been shown for TFIIB (Yao *et al*., 1998), TBP, AMF1 (Breiding *et al*., 1997) and p300 (Muller *et al*., 2002). The TAD is defined to be absolutely required for transcriptional activation. Interestingly, very few functional interactions with transcription factors and cofactors are mapped to the E2 TAD. Only functional interactions with p300, TFIIB and AMF1 are TAD specific for BPV1 E2 (Fig. 3); CBP and TopBP1 can be added to this list when interactions with HPV E2 TAD-s are included. For each of them, the effect on transcription is rather small: ~1.5–4 fold.

At least for some promoters, E2 has the highest activity in epithelial cells indicating that the quantitative effect of E2 on transcription depends strongly on cellular environment (Vance *et al*., 1999).

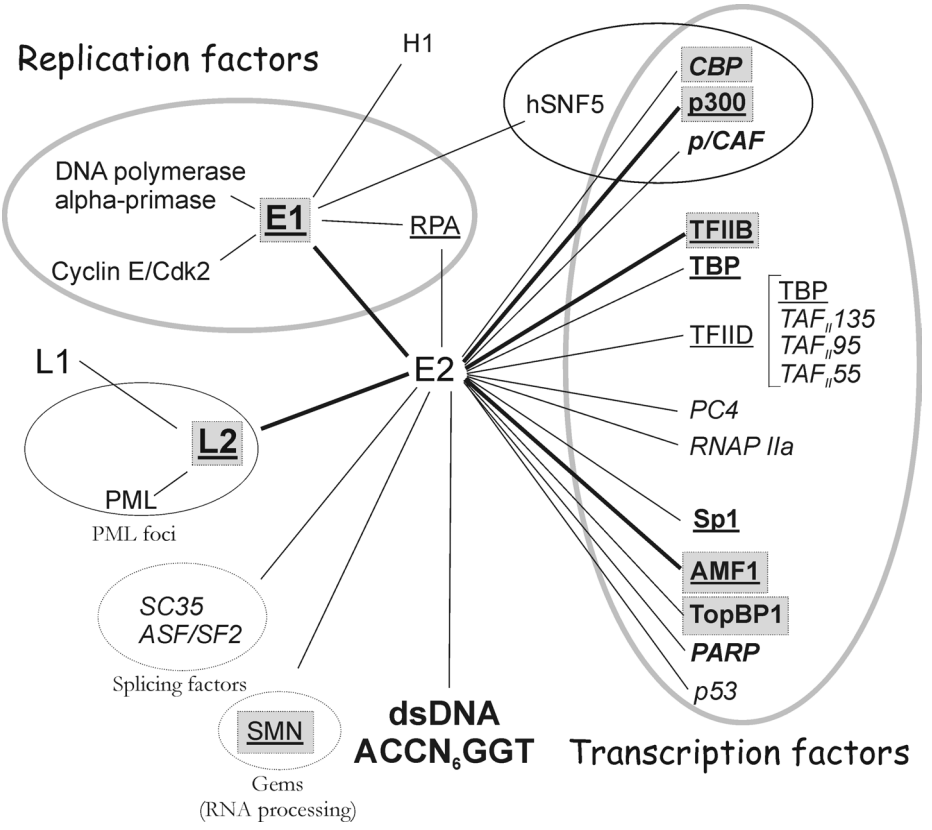


Figure 3. Interaction partners of the PV E2 protein. Proteins interacting with BPV1 E2 are underlined; **bold** indicates the proteins, whose the **functional** effect is verified and shaded proteins interact (physically or functionally) with the TAD of the E2 protein. The bold lines correspond to the interactions which are BPV1 E2 TAD specific and **functionally** proved.

2.5.4. E2 as a replication factor

On the initiation of replication the E2 protein has multiple roles. Some of them have been studied to greater details (for a review (Stenlund, 2003b)). For the initiation of replication *in vivo* the E2 is absolutely required. In *in vitro* experiments E1 alone has the ability to initiate replication. At high E1 concentrations E1 can initiate replication also ori-independently (Yang *et al*., 1993) (Bonne-Andrea *et al*., 1995). In the presence of E2, the ori-independent replication, taking place at high E1 concentrations, becomes ori dependent (Bonne_Andrea *et al*., 1997). The *in vitro* replication system becomes E2 dependent when a high level of competitor DNA is added and the E1 level is

lowered, thus mimicking more closely the native conditions (Sedman and Stenlund, 1995). Thus, the E2 protein works as a specificity factor on the loading of E1 to the ori region. This is achieved by forming a complex between E1 and E2, which has very high specificity for the origin binding, thus ensuring that E1 binds only to the viral origins and not to cellular A/T rich sequences (Sanders and Stenlund, 2000; Sedman and Stenlund, 1995). During the loading of E1 to the origin through BS12, E2 DBD interacts with E1 DBD and E2 TAD with the helicase domain of E1. The interaction between DBD-s leads to a sharp bending of DNA, which is required for interaction between E2 TAD and the helicase domain of E1 (Gillitzer *et al*., 2000). E2 abolishes also the E1 helicase domain's ability to bind unspecifically to the neighboring DNA (Stenlund, 2003a). After the ori binding by E1:E2 complex, E2 will be displaced from E2 BS12 near the ori in an ATP dependent manner (Sanders and Stenlund, 1998). The E2 protein must leave the complex, otherwise it inhibits the E1 hexamer assembly on the origin. This explains also why BS12 is a weak BS (Fig. 1). Finally, the (double)hexameric E1 complex is formed with ATPase and helicase activity (Sedman and Stenlund, 1998) (Stenlund, 2003b). When both the E2 BS11 and BS12 are present on the origin also the E2:E2C heterodimer can activate replication at least in an *in vitro* replication system and can load the E1 to the origin (Lim *et al*., 1998). It has been shown that E2 BS11 and BS12 have a different role on the initiation of replication from the genome of BPV1 when E1 and E2 are expressed from their native context (from the virus genome and in the presence of repressor forms) (Gillette and Borowiec, 1998). When both BS-s (BS11 and BS12) are mutated in the context of a viral genome, the genome is unable to replicate in C127 cells (Gillette and Borowiec, 1998). In contrast, when E1 and E2 are expressed from heterologous promoters, multiple E2 BS-s at a distance activate the E2 BS11 and BS12 defective origin (Ustav *et al*., 1993). There is also a quantitative difference in the replication assays between the ori constructs containing only BS12 or both BS11 and BS12 at their native position when E2 and E1 are expressed from heterologous promoters (Lepik *et al*., 1998). The ATP-dependent displacement of E2 occurs only from BS12 and not from BS11, and this displacement is position, not BS dependent (Sanders and Stenlund, 2000).

In native ori configuration where ori contains E2 BS12 and no other BS-s the intact E2 DBD is required for replication. However, when the BS for E2 is located at a distance the E2 DBD can be replaced by heterologous DBD-s (Berg and Stenlund, 1997) (Kivimae *et al*., 2001). In contrast to transcription activation by E2 where the length of the hinge region is not important, the length of the hinge region seems to be critical for the functioning of E2 in replication (Berg and Stenlund, 1997) (Allikas *et al*., 2001) (Haugen *et al*., 1988; Winokur and McBride, 1992).

In vitro studies show that transcription *per se* is not required for the initiation of replication as the replication occurs also in the presence of α-amanitin, the inhibitor of RNA Pol2 (Yang *et al*., 1991a). As shown by multiple independent

studies, the E2 ability to transactivate is not required for the activation of the replication from PV origin (Abroi *et al*., 1996) (Brokaw *et al*., 1996; Ferguson and Botchan, 1996) (Grossel *et al*., 1996b).

E2 can also activate heterologous origins *in vivo*, for example Py virus origin (Nilsson *et al*., 1991) (Abroi, manuscript in preparation; Silla, 2001; Hääl, 2002).

For stable maintenance replication the oligomerized E2 BS-s can functionally replace the URR (E2 BS-s 1-11) in stable replication assays in the presence of E2 (Piirsoo *et al*., 1996).

2.5.5. E2 as chromatin attachment protein

The E2 protein has been shown to be closely associated with mitotic chromosomes (Skiadopoulos and McBride, 1998). The E2 repressor forms, E2C and E8/E2, are not associated with mitotic chromosomes. Specific DNA binding activity of the E2 protein is not required for mitotic chromosome binding (Skiadopoulos and McBride, 1998). In addition, the E2 TAD alone can be attached to mitotic chromosomes (Bastien and McBride, 2000). E2 seems to be associated with mitotic chromosomes during all phases of mitosis (Bastien and McBride, 2000). The phosphorylation in positions 235, 290, 298 and 301 is not required for the E2 chromatin attachment as mutation of this aa to Ala leads to a chromatin attachment active protein (Bastien and McBride, 2000) (Voitenleitner and Botchan, 2002). The E2 protein can be displaced from mitotic chromosomes at a very high level of the E1 protein, this displacement depends on the intact phosphorylation site of the E2 protein at position 235 (mutated form is dislocated more easily when also serine at positions 290, 298 and 301 are mutated to Ala) (Voitenleitner and Botchan, 2002). The fact that the non-phosphorylated form of E2 is displaced more easily by E1 is in good agreement with the fact that in solution, E1 forms complex preferentially with the non-phosphorylated form of E2 (Lusky and Fontane, 1991) (Voitenleitner and Botchan, 2002). Thus, the chromatin attachment of the E2 proteins depends on the E1:E2 ratio and on the phosphorylation status of E2.

The E2 attached to the chromosomes can tether viral genomes to mitotic chromosomes. The BPV1 mutant genome with mutation in E2C initiator codon is still able to associate with mitotic chromosomes. In the cell line stably expressing E1 and E2 proteins and maintaining extrachromosomally the URR containing plasmid, this plasmid is also attached to mitotic chromosomes, indicating that URR is responsible for BPV1 attachment to mitotic chromosomes. Deletion analysis of URR shows that the ability to be stably maintained and to be attached to the mitotic chromosomes are in good correlation (Ilves *et al*., 1999). In addition, replacement of URR to oligomerized E2 BS-s leads also to chromatin attachment in E2 dependent manner (Ilves *et al*., 1999). The replication function is not required for E2 and E2 BS-s

dependent chromatin attachment (Ilves *et al*., 1999). Thus, in mitosis the E2 protein is attached to chromosomes by binding to some chromosomal proteins through TAD and simultaneously, the DBD can bound to E2 BS-s present in viral genome or in reporter plasmid and tether the episomal DNA to the chromosomes.

2.5.6. Post-translational modifications of E2

The phosphorylation/dephosphorylation of proteins is a very important regulatory event in eukaryotic cells. Also the functioning of the E2 protein is regulated by phosphorylation at multiple sites. In the Sf9 and Cos1 cells both E2 and E2C are phosphorylated and E2 from Sf9 is phosphorylated mostly at serine but also at threonine residues (McBride *et al*., 1989a) (Lehman *et al*., 1997). When expressed in Sf9 cells, there are no major phosphorylation sites in the amino terminus unique to the full-length E2 and most of the phosphorylation sites located in the region between aa 227-322 (McBride *et al*., 1989a). A more detailed analysis has identified serines at position 290, 298 and 301 as phosphorylation sites (E2 with mutation of all these three Ser to Ala is called A3). The mutation of Ser301 to Ala, when tested in the context of viral genome, leads to a 2–4 times higher transactivating activity, wt level of transforming activity, 20 times higher copy number in transformed cells and up to 2–5 times higher transient replication, and these effects are trans-dominant (McBride and Howley, 1991). The A3 mutant genome can also stably replicate and transform cells (C127) in the absence of both E2C and E8/E2 whereas the genome with "wt" E2 cannot (Lehman *et al*., 1997) (Lambert *et al*., 1990). The effect of E2 301A is largely based on the longer half-life of mutated (S301A) E2 proteins (Penrose and McBride, 2000). The half-life of the E2 present in the cell lysate of soluble fraction of 0.15 molar salt increases from 50 min (wtE2) to 160 min (S301A) at the conditions where only E2 was expressed; thus only homodimers of full-length E2 may form. Earlier the half-life of E2 in high salt lysate was measured to be 45 min in the presence of heterodimers (Hubbert *et al*., 1988). However, there have to exist some additional phosphorylation sites as the E2 protein mutated in all three positions is still phosphorylated (McBride *et al*., 1989a). Also the additional phosphorylation sites in the hinge region are identified at position 235, 240 and 277 for E2 expressed in insect cells (Sf9) (Lehman *et al*., 1997). Mutation of this serine 235 to alanine results essentially in wt activity in transcription, replication and transformation assays; however, introduction into A3 mutant leads to a negative phenotype in transformation and stable replication assays but it still has wt activity in transient replication and transcription assays (Lehman *et al*., 1997).

However, in mammalian cells the E2 protein contains primarily phosphoserines (BHK-21 cells) and no phospho-threonines (Meneguzzi *et al*., 1989). In mammalian cells transformed with BPV1 genome (in ID14 cells) E2 exists as a

phosphoprotein and the repressor forms are not phosphorylated (Meneguzzi *et al*., 1989).

The effect of phosphorylation is characterized for the E2 protein and not for repressor forms or heterodimers of the E2 present in BPV1 transformed cells. Thus the formation of the net effect of the phosphorylation in the context of viral genome is still unclear.

2.5.7. Other activities of the E2 protein

The E2 protein can increase the efficiency of packaging DNA into capsids in some (Zhao *et al*., 2000), but not in other systems (Buck *et al*., 2004). E2, when coexpressed with L2, localizes to the PML bodies (Day *et al*., 1998). The BPV1 E2 affects also cellular proliferation of HPV positive cell-lines by p53 dependent and independent pathways (reviewed in (Dell and Gaston, 2001)). There are hints that PV E2 might be involved also in apoptosis as the HPV16 and HPV31 E2 alone can induce apoptosis in the absence of other PV proteins (Frattini *et al*., 1997; Webster *et al*., 2000).

The BPV1 E2 protein can alter the chromatin structure *in vivo* when at least two E2 BS-s are present (Lefebvre *et al*., 1997).

2.5.8. Summary for E2

All these data together indicate that E2 is a multifunctional and complex protein. E2 has a lot of properties — modular structure, clustered BS-s, synergistic effect when more than one BS is present, interactions with multiple factors — common to typical eukaryotic sequence-specific transcription activators (Kadonaga, 2004). The E2 protein has also properties not very usual to sequence-specific transcription activators. E2 is involved in different aspects of viral nucleic acid metabolism: in addition to the regulation of transcription, E2 is also involved in viral DNA replication and segregation. Moreover, unlike a lot of other TAD-s, the E2 TAD is highly structured. The last property adds a new dimension in studying the interrelationship between different E2 activities, especially studying E2 as a transcriptional activator as there are only a few TAD-s which structure is known.

3. RESULTS AND DISCUSSION

The E2 protein has multiple functions. It interacts with several cellular and viral proteins to fulfil its functions. The TAD of the E2 is required for a lot of E2 activities including replication, transcription activation, chromatin attachment, cooperative DNA binding etc. The studies on the structure-function relationship of the E2 TAD were initiated when no structural information about PV E2 TAD was available. The question about E2 TAD was whether it is organized to have such multiple functions. How are all these activities accomodated into 200 aa? Are these activities actually a different activies or just various expression forms of the one single activity?

The main objective of the studies summarized here was to investigate the structure-function relationship of E2 TAD, in particular how the E2 TAD is structurally organized and how separable are the different activities of the E2 protein. This includes also the question about the surfaces or sub-regions of E2 responsible for the different activities.

3.1. THE E2 TAD HAS REMARKABLE STRUCTURAL INTEGRITY (I AND II)

At the beginning of the 1990-s it was believed that transcriptional activators do not have a highly ordered structure (for a review (Triezenberg, 1995)). Later this hypothesis was verified at least for some TAD-s (for a review (Dyson and Wright, 2002)). However, the data on E2 indicate that E2 TAD is not a typical TAD in terms of structural organization. In a lot of other aspects, E2 is very similar to typical transactivators — it has a modular structure with TAD, hinge and DBD regions; it activates viral as well as different heterologous promoters in different mammalian and yeast cells. Later it was shown that TAD is able to activate transcription when linked to heterologous DBD (Breiding *et al*., 1996) (Winokur and McBride, 1996). The multiple sequence alignment, secondary structure prediction and in frame linker insertion analysis indicate that proteins with linker insertions retain some activity on transcription only when the insertions are introduced into loop regions or regions with low secondary structure probability (Giri and Yaniv, 1988; Haugen *et al*., 1988)(Abroi, unpublished). The antipeptide antibodies against the HPV16 E2 protein also indicate that E2 TAD is not unstructured (Gauthier *et al*., 1991). In order to study the structural organization of BPV1 E2, we made a panel of monoclonal antibodies against the E2 protein and started also a mutational analysis of E2. The use of purified full-length E2 as an antigene in mouse resulted in 22 different hybridoma cell lines positive to E2 in ELISA. Seventeen of these Mab-s recognized linear epitopes on western blot and another five had conformational epitopes. All five Mab-s with conformational epitopes recognized the DBD. The

region ~180-218 appears to be a highly immunogenic hotspot, since 12 of the linear epitopes from 17 different Mab-s fall into this region. Additionally, the polyclonal antibodies from rabbit recognized very easily E2C but very poorly E8/E2 (Abroi unpublished). No Mab-s recognizing linear epitopes between aa 1-180 have arisen when full-length E2 was used for immunization.

Therefore, for the second series, purified E2 TAD (E2 aa 1-208 plus 8 extra non-E2 aa) was used for immunization. Thirteen different hybridoma cell-lines were positive in ELISA in the initial screen and six of them recognized again the region in the C-terminus of the E2 TAD (aa. 180-208). Only two Mab clones recognized the linear epitope in the N-terminal region of E2 TAD. More detailed mapping identified the epitope to aa 86-110 while mutation E90A avoids E2 recognition by these antibodies in the western blot. This region is the longest unstructured region in the first N-terminal 180 aa (Fig. 4 on www.ebc.ee/~aabroi/thesis). One antibody recognized the C-terminal non-E2 aa and the other 3 had a conformational epitope (1 Mab had just a very weak affinity). None of the antibodies arose against TAD and recognizing epitopes outside the "hot-spot" region recognized the E2:DNA complex when analyzed in supershift assay (data not shown). All antibodies arose against full-length E2 except for 1E2 and 5H4 supershifted E2:DNA complex easily (II, Fig. 2A). These two antibodies have a very interesting behavior when tested in the "supershift assay" of the E2:DNA complex. The Mab 5H4 is able to dissociate the preformed E2:DNA complex (II, Fig. 2B). Although 1E2 could not recognize the full-length E2 in complex with DNA, it easily supershifted the E2C complex at the same concentration (II, Fig. 2C). Removal of already the first α-helix from the E2 N-terminus of the protein opens the epitope for 1E2 again (II, Fig. 2C). Thus, using this antibody it is possible to distinguish between the wt-like and E2C-like conformation of the epitope. As the E2C and E2(Δ1-23) are inactive in transcription and replication, the different accessibility of the epitope may reflect the functional status of the protein (active or inactive). The crystal structures of HPV16 and HPV18 E2 TAD-s do not give obvious explanation for the different accessibility of this epitope.

All tested antibodies, but not 1E2 and 5H4 recognized also the E1:E2:DNA complex. Therefore, one may assume that there are no drastic differences in the E2 structures in the E2:DNA complex compared to the $E1_2$:$E2_2$:ori complex.

From all these antibody studies we conclude that BPV1 E2 TAD has a complex and relatively rigid structure. This conclusion has been supported by the studies of E2 TAD from HPV16, HPV18 and HPV11 which show that these E2 TAD-s have very similar structures despite the proteins are free, truncated or bound to the low molecular weight inhibitor, respectively (Antson *et al*., 2000; Harris and Botchan, 1999; Wang *et al*., 2003).

3.1.1. The oligomerization status of the E2 protein can be affected by single point mutation

In order to study the structure-function relationship in E2 TAD we made 15 different single "charged to alanine" substitutions in positions with high probability to be on the surface of the protein (clustered-charged to alanine scan). When the template for homology modeling became available, we made a model for BPV1 E2 TAD, and all mutated aa, but not K111 appeared to be exposed to solvent in monomeric TAD (Fig. 4; and Fig. 4 and Appendix to Fig. 4 on www.ebc.ee/~aabroi/thesis) indicating that this approach is very effective to find aa located on the surface of the protein. All mutants are expressed at the level comparable to wt E2, indicating that overall stability is not affected by these mutations. However, three of them — K111A, K112A and E176A — do not enter the nucleus (I, Fig. 5) and form higher order oligomers or aggregates in the soluble fraction of the cell lysate (I, Fig. 6). These three mutants were also unable to form the specific E2:DNA complex characteristic for wtE2 (I, Fig. 4). After treatment of the cell lysate containing these mutants with pronase, the protease resistant core of the DBD from these mutants forms a complex with DNA as efficiently as wtE2 (I, Fig. 4C). These data indicate that the oligomerization status of the E2 protein can be affected by single point mutation and that DBD is not involved in the oligomerisation.

Such a strong effect of single amino acid substitution is rather an indication of a highly ordered structure than of an unstructured protein, as in unstructured proteins all the potential binding epitopes would be more or less exposed. For example, the point and deletion mutations in the hinge region of the E2 do not have effect on E2 activity, indicating that these E2 proteins are not aggregated or oligomerized (McBride *et al*., 1989b).

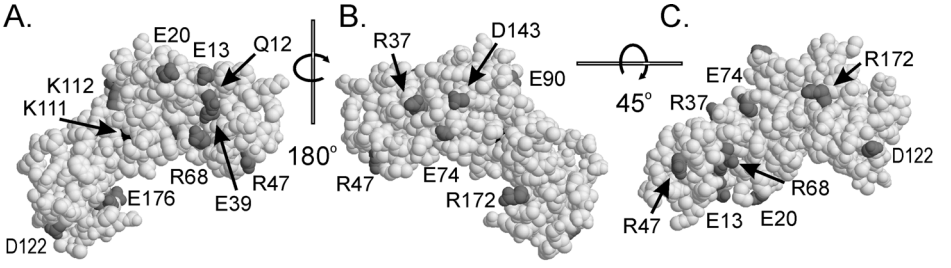


Figure 4. Localisation of mutated aa on the homology modeled structure of BPV1 E2. Mutated aa-s exposed on the surface are colored dark gray, K111 — a mutated aa not exposed is colored in black. (see also Fig. 4 on the www.ebc.ee/~aabroi/thesis).

3.2. THE ABILITY TO TRANSACTIVATE IS NOT REQUIRED FOR E2 TO ACTIVATE REPLICATION FROM BPV1 ORIGIN (I)

A lot of replication origins working in eukaryotic cells contain binding sites for transcription factors. This is true also in the case of BPV1 origin, which contains BS for the transcriptional regulator E2. Transcriptional activators may have different roles in activating replication (for review (Murakami and Ito, 1999)) and there are no common rules whether the ability to transactivate is required for activating replication. To address the question whether the trans-activation activity of E2 is necessary for E2 function in BPV1 replication, mutant E2 proteins were employed. The activity of these mutants was tested in a transient replication assay and in a transcription assay by using two different promoters. One of them was a complex promoter, namely viral URR, containing P_{7940} and P_{89} (pP2CAT in I). Another was a low complexity heterologous promoter, SV40 early promoter where the 72bp enhancer was replaced with three E2 BS9 (the promoter still has three 21bp repeat, working as a BS for Sp1)(pSV3BS9CAT in I). Most of the mutants (except for R37A and E90A) behaved like a wtE2 in the replication assay (I, Fig. 2), however, in the transcription assay the relative activity varied from less than 20% to ~140% compared to wt (I, Fig. 4B). Notably, mutants completely inactive in transcription (E74A and D/R) had almost wt activity in replication. So, we conclude that replication activity does not depend on the E2 abilities to transactivate as illustrated also on the blot on Fig. 5A and B. The *in vitro* studies show that transcription *per se* is not required for BPV1 ori replication and our study as well as some other similar studies shows that E2 ability to transactivate is not required for the initiation of replication *in vivo* (Brokaw *et al*., 1996) (Ferguson and Botchan, 1996) (Grossel *et al*., 1996b). Thus, the E2 is not only a transactivator but also a direct replication activator.

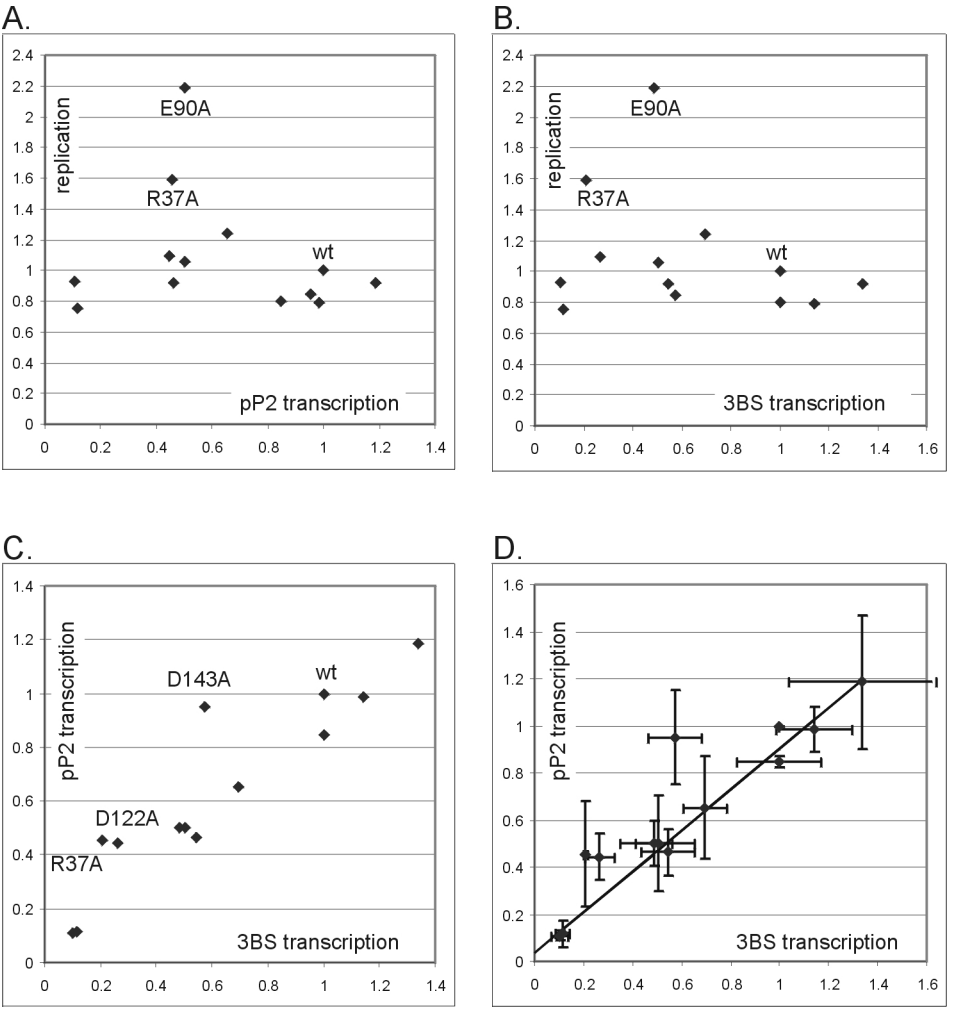


Figure 5. Comparison of the **relative** activities of the mutant E2 protein **normalized** to wt E2 (data from **I**). **A.** The replication activity of the E2 mutants (y-axis) is compared to the transcription activity from native BPV1 promoter(s) (pP2CAT reporter plasmid, a native BPV1 URR with P_{7940}+P_{89}) 'pP2 transcription' in x-axis. **B.** The replication activity of the E2 mutants in y-axis is compared to activity in transcription from simple artificial promoter p3E2BS9CAT, '3BS transcription' in x-axis. **C.** The transactivating activity of the E2 mutants from the native (and complex) BPV1 promoter (pP2CAT) 'pP2 transcription' in y-axis compared to transactivating activity from artificial and simple promoter (p3E2BS9CAT) '3BS trascription' in x-axis. **D.** The same as in panel C. but with respective average deviation. For numerical data see Table 1.

3.3. INTERACTIONS WITH MULTIPLE PARTNERS ARE PROBABLY REQUIRED FOR THE ACTIVATION OF TRANSCRIPTION (I)

As mentioned in the previous chapter, the ability of E2 mutants to activate transcription varied from basal level (the E2 independent transcription) to a level higher than wtE2 (Figure 5; Tabel 1; I, Fig.3 and 4). The analysis of the location of these mutants on the homology modeled structure of the E2 TAD indicates that mutations with clearly decreased activity in transactivation locate on multiple surfaces of the E2 TAD (Fig. 6A and 6B on the http://www.ebc.ee/~aabroi/thesis). In general, there are two possible mechanisms how the mutation of single residues to alanine can lead to a specific effect on one activity and not another. The first is the direct altering of the interaction surface (binding epitope) or eliminating interaction partner for some active group of interacting partner. The second is an allosteric effect on the interaction surface(es) of the protein. It is hard to believe that mutation in position 90 affects the surface around 122 or 12/39 cluster and *vice versa*. If these aa are not directly involved in interactions with transcriptional machinery, they may affect allosterically other protein surfaces responsible for protein-protein interactions. However, it is very unlikely that all these mutations affect allosterically the same surface or lead to similar conformational changes. In any case, our data, especially when complemented with data from other similar studies, show that mutations affecting specifically transcription are located on multiple surfaces (Brokaw *et al*., 1996; Ferguson and Botchan, 1996; Grossel *et al*., 1996b) (Fig. 6F on the www.ebc.ee/~aabroi/thesis). The hypothesis is that mutations at different surfaces influence different interactions and thus interactions with several different factors might be involved in transcriptional activation. Data presented on Fig. 6F on www.ebc.ee/~aabroi/thesis demonstrate some clustering of mutations of affecting transcription more or less specifically. The cluster including aa 122, 127 and 181 is most likely the region responsible for interaction with TFIIB. Namely, the E2 mutant N127Y, which is severely defective in transcription (less than 50% of the wt activity) and TFIIB binding both *in vivo* and *in vitro*, is strongly activating by over-expression of TFIIB (Yao *et al*., 1998). Another cluster can be found on the E2 surface enclosing aa 24, 90, 93 and 96. Also a clustering of transactivation defective mutants at or near the dimeriztion surface can be envisioned. Thus, these data indicate that the interaction of E2 with transcriptional machinery is very complex and multiple interactions are required for efficient activation.

Indeed, the E2 subdomains responsible for interactions with a single protein from the transcription machinery (TFIIB) cannot activate transcription, indicating that the recruitment of a single factor is not sufficient for the activation of transcription (Yao *et al*., 1998).

Thus, in activating transcription, the E2 protein recruits most probably the complex of proteins required for transcriptional initiation (and reinitiation) to the promoter.

It has been shown that HPV11 E2 interacts *in vitro* with TFIIB, TBP, TFIID, TAF135, TAF95, TAF55, RNAP2a, PC4 (Fig. 4) and recruits holoenzyme to promoter (Wu and Chiang, 2001). HPV11 E2 and BPV1 E2 work similarly in the *in vitro* transcription assay (Hou *et al*., 2002). *In vivo*, the BPV1 E2 is even a more potent transactivator than HPV11 E2 (Kovelman *et al*., 1996). Thus, the BPV1 E2 recruits most probably also Pol2 holoenzyme to the promoter and the interactions of E2 with multiple components of this holo-complex are responsible for effective recruitment. The Pol2 holocomplex has been purified from the mammalian cells in multiple laboratories and in addition to common components there also exist differences in the composition of these complexes. In some holocomplexes the coactivators p300 and CBP have been found. HPV16 and HPV18 E2 respond to p/CAF more efficiently than 6 and 11 (Lee *et al*., 2002). A similar phenomenon was observed for CBP (Lee *et al*., 2000). *In vivo*, BPV1 E2 and HPV16 work quite similarly in different cell lines.

The TBP dissociation from the TATA box is reduced by the E2 (Steger *et al*., 1995). During transcription initiation, TBP (as well as TFIID) remains bound to promoter, TFIIB dissociates and must be assembled on the next round of re-initiation. Thus, defects of E2-TBP interaction mostly have an impact on the first initiation and not so much in re-initiation. Small defects in the interactions between the activator and TFIIB most likely lead to a significant defect on transcription as it affects every re-initiation event.

The interaction of holocomplex with promoter is not very specific, and sequence-specific binding of transactivators determines the promoter where the holocomplex lands. The fact that the E2 mutants with crippled DNA-binding activity (D/R, C340A) are inactive in transcription also support this hypothesis (I, Fig. 5, (Grossel *et al*., 1996a)). However, in the initiation of replication, cooperative binding of E1 and E2 to the origin occurs which compensates the E2 defects on DNA binding to some extent and these proteins work on replication (I Fig.4, (Grossel *et al*., 1996a)).

All these data together support the hypothesis that E2 interacts with Pol2 holocomplex and targets it to the E2 BS-s containing promoter. Thus, *in vivo* the E2 is responsible for E2 BS-s dependent recruitment of the TFIID and/or RNAPol2 holocomplex. This hypothesis is also supported by the fact that at higher E2 concentrations the transcription activity decreases (I, Fig 3; (Ferguson and Botchan, 1996; Kovelman *et al*., 1996; Rank and Lambert, 1995)). This is dependent on the E2 protein level, not the E2 expression vector level (Kovelman *et al*., 1996). Most probably, at higher concentrations, the E2 protein, which is bound to holocomplex cannot be bound to E2 BS-s as the BS-s are already occupied by other E2 molecules. The fact that p53:E2 and VP16:E2 do not have such an effect may reflect differences in targets on the transcriptional machinery (I, Fig. 3). There exist experimental evidences that E2

and VP16 have both overlapping and also different targets in the transcriptional machinery as transactivation by E2 and E2 TAD of BPV1 and HPV16 is E1A sensitive but VP16 is not (Peng *et al*., 2000) and VP16 over-expression reduces the E2 dependent activation (Rank and Lambert, 1995). In addition, TAD-s of p53 and VP16 are unstructured when not bound to other proteins (Ayed *et al*., 2001) (Grossmann *et al*., 2001).

3.4. THE ACTIVATION OF THE E2 DEPENDENT POLYOMAVIRUS REPLICATION DEPENDS ON E2 TRANSACTIVATING ABILITIES BOTH IN *CIS* AND IN *TRANS* LEVEL (IV)

Using inframe insertion mutation analysis it has been shown that E2 can activate Polyomavirus (PyV) DNA replication when the PyV enhancer is replaced by two E2 BS-s (Nilsson *et al*., 1991) and that qualitative correlation between E2 activities in transactivation and PyV replication exists. However, the behavior of these inframe insertion mutants in the BPV1 replication system is unknown (at least — not published). One may hypothesise that the inframe insertions inactive in transactivation disturb the structure of the E2 protein. Since the E2 ability to transactivate was not required for the initiation of BPV1 replication, we asked whether this is the case also for activating replication from heterologous replicons and whether and how the E2 abilities to activate different replicons correlate with each other. To test this we examined the E2 protein in an E2 dependent Py replication assay. First we tested the cooperativity of E2 BS-s near the PyV ori. As shown in IV, Fig. 2A, the E2 activates the replication of reporterplasmid with single E2 BS very weakly, less than two-fold. When two E2 BS-s are present, the activation is more than ten-fold. Thus, similarly to the activation of transcription, for efficient activation of PyV ori at least two E2 BS-s are required and the effect of adding a second BS is higher than additive. Next we tested the effect of E2 point-mutations on the activation of E2 dependent PyV ori replication. For this purpose, we used the co-replication assay of PyV and PV origin in the same cell population. As shown in IV, Fig. 2B, the E2 mutants activate PyV replication to a different extent whereas the PV replication is more or less constant. The quantified data show that E2 activates PyV and PV ori differently (IV, Fig. 2C). The effect of E2 mutants on PyV replication varied from 0.1 to 1.5 from that of wtE2, *i.e.* over 15 times. Excluding the D/R which is defective in the DNA binding, and R37A which is discussed in chapter 3.6, the effect of E2 mutants in PV replication varied from 0.7 to 1.4 compared to wtE2. Thus, the E2 mutants have a different effect on the replication of these two replicons, and most probably activate different replicons using different pathways.

The E2 effect on PyV replication correlates with its transactivating properties very strongly (with slope ~1 and with correlation coefficient R^2 =0.9)

(IV, Fig. 2D). Therefore, one may assume that the E2 protein uses the same activities for activating transcription and PyV replication. As already discussed in the previous chapter, mutations affecting transcription locate on the different surfaces of E2. As we were unable to detect a significant effect of E2 on LT DNA binding and failed to detect the E2:LT interaction on far-western, we supposed that E2 activates PyV replication by the recruitment of complexes involved in chromatin altering and transcription activation.

3.5. THE E2 FUNCTION IN CHROMATIN ATTACHMENT AND PARTITIONING

The E2 protein can be attached to the mitotic chromosomes, this attachment allows E2 to tether the MME containing plasmid (including viral genome) to mitotic chromosomes (Bastien and McBride, 2000; Ilves *et al*., 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). The TAD of the E2 protein is responsible for this attachment. Next we wanted to know, which subdomains or surfaces are responsible for E2 attachment and MME tethering to the mitotic chromosomes.

3.5.1. The E2 has no linear epitopes responsible for chromatin attachment and MME tethering

To test whether the E2 contains linear epitopes in its TAD responsible for E2 chromatin attachment and MME chromatin tethering we tested a set of E2 deletion mutants in a respective assay. As shown in III, Fig. 3, none of the TAD deletion mutants have activity in these assays. These data show that E2 has no linear epitopes responsible for chromatin attachment. According to this, the E2 protein is clearly different from LANA1 and EBNA1 proteins, mediators of chromosome tethering of viral genomes in Kaposi sarcoma herpesvirus and Epstein Barr virus respectively, which have linear epitopes responsible for chromatin attachment. Most likely, the chromosomal partners of E2 are also different from LANA1 and EBNA1.

As already discussed in chapter 3.1, all these deletion mutants have an exposed epitope to the 1E2 antibody which is not accessible in wtE2. The data from deletion mutant analysis together with point-mutation analysis (see next chapter) indicate that overall structural integrity, rather than linear sub-sequences, is required for E2 chromatin attachment.

3.5.2. Chromatin attachment, MME tethering and partitioning can be disturbed by multiple point-mutations

As there was no linear subsequence responsible for E2 chromatin attachment, we tested a set of point-mutants in their activity to be attached to chromatin and to tether MME to the chromatin. The effect of E2 point mutations varied strongly (III, Fig. 4A) — some mutants are more active than wt and some have more than 10 times lower activity. These data indicate that chromatin attachment and MME tethering can be disturbed by single point mutations. In general, good correlation exists between E2 chromatin attachment and MME tethering with two exceptions, R37A and D143A, which will be discussed in chapter 3.6. All mutants were tested also in functional segregation assay. According to their behavior in segregation assay, the E2 mutants clustered to three different clusters (III Fig. 6). Three mutants, E13A, E20A and R47A behave like a wt in this assay. The same mutants have wt-like activity in all other activities tested (III, Fig. 7). Mutant D143A has intermediate activity and all other mutants (Q12A, R37A, E39A, R68A, E74A, E90A, D122A and D/R) have activity at background level. Thus, good correlation exists between the activities of the E2 mutants in MME chromatin tethering and the partitioning function. Only two mutants (Q12A and E90A) fall out of this correlation. The E2 proteins with Q12A and E90A mutations are at least partially active in the URR tethering but are essentially inactive in the partitioning assay. Thus, the E2-mediated tethering of the URR containing reporter plasmid does not necessarily lead to efficient partitioning. Two reasons for this behavior can be envisioned. First, even the relatively modest defects in chromatin attachment could perhaps have a cumulative effect on the long-term extrachromosomal DNA retention in the course of multiple cell divisions. Second, in addition to the binding to chromatin components required for URR tethering, some additional activities of E2 are required for an efficient BPV1 partitioning process.

3.5.3. The determinants for the partitioning and the transcriptional activation are separable

All E2 mutants essentially inactive in partitioning (Q12A, R37A, E39A, R68A, E90A, E74A, D122A and D/R) have still significant activity in transient replication assay (III, Fig. 7). Thus, the transient replication and MME chromatin tethering are clearly different activities of the E2 protein. Four partitioning defective mutants (Q12A, E39A, R68A and E90A) have significant activity (~50% from wt) in transcription (III, Fig. 7). Two of them (E39A and R68A) are also defective in MME tethering. These data become more significant when the structural information is included. Three mutants specifically defective in partitioning — Q12A, E39A and R68A (and two

mutants specifically defective in MME tethering E39A and R68A) locate on the continuous patch on the surface of E2 (III, Fig. 8; and Appendix 2 on http://www.ebc.ee/~aabroi/thesis/). All these data together show that the determinants for the partitioning and the transcriptional activation are separable.

3.6. THE DIMERISATION INTERFACE OF THE E2 TAD

The TAD of the HPV16 E2 forms a dimer in solution as well as under crystallization conditions (Antson *et al*., 2000). The dissociation constant was measured to be $K_d=8.1\times10^{-6}$ M at 20°C. Very recently the structure of HPV11 E2 N-terminal domain was published (Wang *et al*., 2003). For the HPV11 E2 TAD, no evidence on the formation of dimer was found neither by crystallographic nor by biophysical methods (Wang *et al*., 2003). Thus, the question arises about the dimerization status of the BPV1 E2 TAD.

To examine this question, the BPV1 E2 N-terminal domain was modeled using HPV16 N-terminal dimer as a template. The final model contains 12 interchain hydrogen bonds and 6 of them involve R37. One inter- and one intrastrand hydrogen bond of R37 are conserved between the BPV1 E2 model and the template structure 1DTO. Two mutated amino acids locate directly on the N-terminal dimerisation surface of the E2 — R37 and D143. These two mutants have also some “unusual” behavior in several aspects discussed below.

Analyzing the quantitative differences between E2 abilities to activate simple, SV40 early promoter and complex URR promoters, three mutants behave significantly differently from others (Fig. 5C and D; Table 1). The mutants D122A and D143A were about 1.7 times and R37A ~2.2 times more active in transactivating the URR containing reporter compared to SV40 early promoter. When we visualize the location of these aa on the E2 structure, then two of them, R37 and D143 locate on the dimerisation surface. The D122 locates on a very accessible region, not far from N127, which is supposed to be involved in the interaction with TFIIB (III, Fig. 8; Fig. 7 on the http://www.ebc.ee/~aabroi/thesis) (Yao *et al*., 1998).

Thus, the intact dimerisation interface is more important for a simple promoter than for a complex promoter. Similar data have been obtained for the HPV16 E2 protein in C33A cells where mutations in positions 37 and 73 are more active in transcription from URR containing SV40 early promoter (with 4 E2 BS) compared to 6 E2 BS-s containing tk promoter (Sakai *et al*., 1996). These aa also locate at or near the dimerisation interface.

The mutant R37A seems to be specifically active in PV replication as relative activity in other assays (transcription, PyV replication, chromatin attachment) was much lower (Table 1, Appendix 1). The activity of R37A in PV replication is also much higher than the wt E2 has, especially apparent on IV, Fig. 2. The E1:E2 interaction surface for HPV11 E2 has been suggested to locate on the helical face opposite to R37 (Wang *et al*., 2003). As the HPV11

E2 TAD, when linked to BPV1 hinge and DBD regions, is fully functional in the recruitment of BPV1 E1 to the origin and in BPV1 replication (Berg and Stenlund, 1997) we can conclude that the E1:E2 interaction surface on the BPV1 E2 TAD locates in the same region. Accounting that, two explanations for the increased activity of R37A can be visioned. First, the mutation can subtly affect the packing of three helices in TAD in a way that slightly alters the E1 binding surface allowing stronger interaction between E1 and E2. Second, the monomeric form of E2 TAD may work better in *in vivo* replication, as the E2:E2C heterodimer loads E1 to the origin and works in *in vitro* replication as well as wtE2 (Lim *et al.*, 1998). Such behavior of E2 suggests also a productive function for a large amount of E2:E2C heterodimers found in virus transformed C127 cells (Sepp and Kurg, unpublished).

Only two mutations — R37A and D143A, fall out of the proposed functional relationship between E2 chromatin attachment and MME chromatin tethering, and both of them are located on the proposed dimerisation surface (III, Fig. 4; Fig. 8D on the http://www.ebc.ee/~aabroi/art3). The exact nature of this discrepancy is not known. However, the cooperative binding of the E2 to the two neighboring BS-s depends on the TAD and might be required for efficient MME tethering. The fact that R37A has a much smaller co-operativity parameter than wt E2 supports this hypothesis (IV, Fig. 3).

All these data together support the assumption that BPV1 TAD forms homodimers in solution. However, additional experimental evidence is required to finally determine the oligomerization status of BPV1 E2 TAD.

4. CONCLUSIONS

During the course of investigation of the structure-function relationship of the E2 TAD we have characterized several important aspects on the functioning of E2. The most important of them are summarized below.

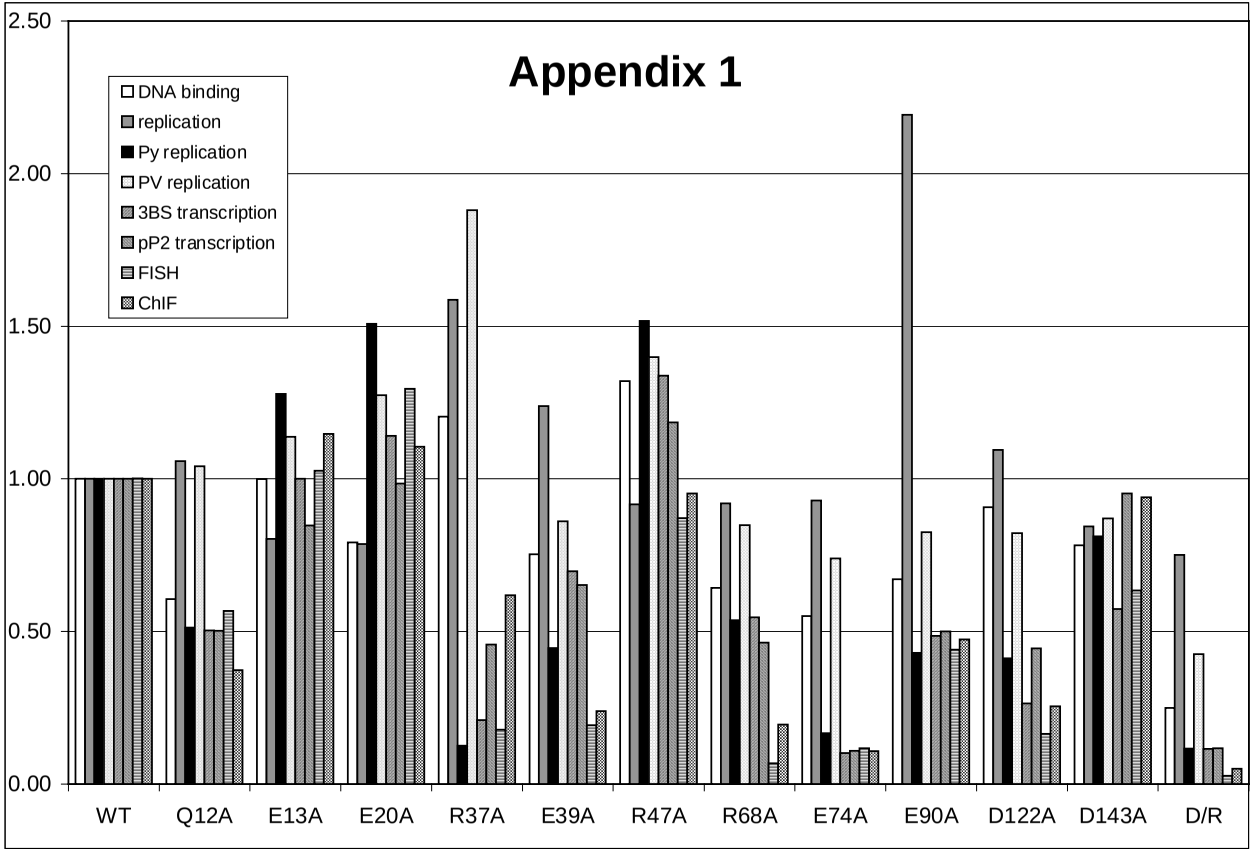
1. The E2 protein has a remarkable structural integrity which can be inferred by deletions and also by single point mutations. The single point mutations can lead to a completely inactive protein *in vivo* due to oligomerisation/ aggregation to the large aggregates unable to enter the nucleus.
2. The ability of E2 to transactivate is not required for the activation of the BPV1 origin of replication. Thus, E2 has clearly separable functions both as a transcription and a replication activator.
3. Interactions of E2 not only with DNA but also with multiple proteins or protein complexes are required for an E2 dependent activation of transcription.
4. The E2 protein activates PyV and BPV1 replication by different mechanisms, and E2 activities in PyV replication depend strongly on its transactivating properties. Thus, the E2 as a replication activator is PV specific.
5. The E2 protein, unlike chromatin attachment proteins from other viruses — LANA1 and EBNA1 — has no linear epitopes responsible for E2 chromatin attachment.
6. The E2 chromatin attachment, the E2 dependent MME tethering to chromosomes and the E2 mediated partitioning can be disturbed by several different single point-mutations.
7. The partitioning function of the E2 protein is most sensitive to point-mutations compared to other E2 activities tested.

The determinants for different E2 activities natively taking place in virus infected cells (transactivation, activation of replication, partitioning/segregation) are separable from each other. This might allow the differential regulation of these activities to ensure the proper completion of the viral live cycle. The above situation illustrates the very rational use of the coding capacity of the small viruses.

Table 1. Summary of the relative activities of mutant E2 in different assays.

	DNA binding			**replication**			**Py replication**			**PV replication**			**3BS transc.**			**pP2 transc.**				**FISH**			**ChIF**	
							in Py/PV coreplication																	
	A	AD	RE	A	AD	RE	A	AD	RE	A	AD	RE	A	AD	RE	A	AD	RE	A	AD	RE	A	AD	RE
-E2							0.11	0.05	0.46				0.09	0.05	0.60	0.07	0.01	0.21	0.01	0.02	1.67			
WT	1.00	0.00	0.00	1.00	0.03	0.03	1.00	0.15	0.13	1.00	0.17	0.16	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.02	0.02	1.00	0.04	0.04
Q12A	0.61	0.05	0.09	1.06	0.67	0.63	0.51	0.14	0.28	1.04	0.22	0.21	0.50	0.15	0.30	0.50	0.20	0.40	0.57	0.23	0.40	0.37	0.11	0.30
E13A	1.00	0.08	0.08	0.80	0.33	0.41	1.28	0.21	0.17	1.14	0.17	0.15	1.00	0.17	0.17	0.85	0.02	0.03	1.03	0.21	0.20	1.15	0.10	0.09
E20A	0.79	0.11	0.14	0.79	0.38	0.48	1.51	0.26	0.18	1.27	0.25	0.20	1.14	0.16	0.14	0.99	0.10	0.10	1.29	0.18	0.14	1.11	0.07	0.06
R37A	1.20	0.09	0.07	1.59	0.51	0.32	0.13	0.03	0.23	1.88	0.43	0.23	0.21	0.01	0.05	0.46	0.22	0.49	0.18	0.15	0.86	0.62	0.07	0.11
E39A	0.75	0.10	0.13	1.24	0.67	0.54	0.45	0.05	0.12	0.86	0.18	0.21	0.70	0.09	0.13	0.65	0.22	0.34	0.19	0.11	0.56	0.24	0.07	0.29
R47A	1.32	0.11	0.08	0.92	0.54	0.59	1.52	0.28	0.18	1.40	0.34	0.24	1.34	0.30	0.23	1.19	0.28	0.24	0.87	0.25	0.29	0.95	0.05	0.05
R68A	0.64	0.06	0.10	0.92	0.50	0.54	0.54	0.12	0.22	0.85	0.13	0.15	0.55	0.11	0.20	0.46	0.10	0.22	0.07	0.08	1.20	0.19	0.03	0.17
E74A	0.55	0.06	0.10	0.93	0.66	0.71	0.17	0.05	0.28	0.74	0.13	0.18	0.10	0.03	0.34	0.11	0.02	0.20	0.12	0.09	0.77	0.11	0.08	0.73
E90A	0.67	0.10	0.15	2.19	1.14	0.52	0.43	0.16	0.37	0.82	0.11	0.13	0.49	0.07	0.15	0.50	0.10	0.19	0.44	0.08	0.19	0.47	0.07	0.15
D122A	0.91	0.15	0.16	1.09	0.39	0.35	0.41	0.09	0.22	0.82	0.10	0.12	0.26	0.06	0.23	0.44	0.10	0.22	0.16	0.21	1.25	0.25	0.08	0.31
D143A	0.78	0.02	0.03	0.84	0.36	0.43	0.81	0.13	0.16	0.87	0.21	0.24	0.57	0.11	0.19	0.95	0.20	0.21	0.63	0.40	0.63	0.94	0.12	0.12
D/R	0.25	0.09	0.36	0.75	0.91	1.21	0.12	0.04	0.36	0.43	0.12	0.29	0.11	0.03	0.24	0.12	0.06	0.48	0.03	0.04	1.50	0.05	0.04	0.78
average	0.10			**0.50**			0.22			0.19			0.19			0.24			**0.59**			0.22		

A — average, AD — average deviation, RE — relative error (RE=AD/A)


Appendix 1
DNA binding
replication
Py replication
PV replication
3BS transcription
pP2 transcription
FISH
ChIF
2.50
2.00
1.50
1.00
0.50
0.00
WT
Q12A
E13A
E20A
R37A
E39A
R47A
R68A
E74A
E90A
D122A
D143A
D/R

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SUMMARY IN ESTONIAN

Veise näsakasvajaviiruse tüüp 1 valgu E2 natiivseid aktiivsusi määravad determinandid on lahutatavad

Käesolevas doktoritöös käsitletakse veise papilloomiviiruse valgu E2 erinevate aktiivsuste omavahelisi seoseid ning nende aktiivsuste struktuuri-funktsiooni vahelisi seoseid.

Papilloomiviirused on väikesed kaheahelalised rõngakujulised DNA-viirused, mis võivad mitmetel imetajatel tekitada näsakasvajaid. Papilloomiviirused on evolutsioneerunud koos imetajatega vähemalt kümneid miljoneid aastaid. Selle pika evolutsiooni jooksul on viirused välja arendanud molekulaarsed mehhanismid, mis reguleerivad viiruse iga eluavaldust raku sees. Kuna papilloomiviiruste genoom on väike, umbes 8000 alupaari, siis on piiratud ka tema kodeeriv potentsiaal, mida ta peab kasutama väga efektiivselt, et tagada kõik viiruse paljunemiseks vajalikud funktsioonid. Viiruse DNA replikatsiooni, transformatsiooni ja viirusgeenide ekspressiooni omavaheline seos näib olevat hämmastavalt kompleksne, kuid samal ajal väga ratsionaalne. Viiruse kodeeritud valk E2 on viiruse erinevate aktiivsuste üks peamisi regulaatoreid. Viirusvalk E2 on vahetult hõlmatud viiruse transkriptsiooni regulatsiooni, viiruse DNA replikatsiooni, viirus-genoomide efektiivsesse jaotumisse tütarrakkude vahel jne. E2 on keskmise suurusega valk, 410 aminohapet pikk ja molekulkaaluga 48 000. Arvestades valgu E2 keskmist suurust ja tema mitmeid funktsioone, tekib küsimus, kuidas ta suudab kõiki neid funktsioone täita ning kas ja kuidas on need aktiivsused omavahel reguleeritud. Probleemi esimene küsimus on, kas kõik need aktiivsused on tõepoolest erinevad aktiivsused või ühe ja sama aktiivsuse erinevad väljendusvormid. Ilmselt on viiruse jaoks vajalik, et ta saaks valgu E2 aktiivsusi eraldi reguleerida, see muudaks viiruse käitumise märksa paindlikumaks ja samas tagaks ka kodeeriva potentsiaali efektiivsema kasutamise. Selleks, et erinevaid aktiivsusi saaks erinevalt reguleerida, peavad need olema lahutatavad. Käesoleva doktoritöö peamiseks läbivaks küsimuseks on, kas ja kuidas on võimalik E2 erinevaid aktiivsusi lahutada. Antud küsimusele vastamiseks tehti E2 transaktivatsiooni domeeni mitmeid punkt-mutatsioone (laetud aminohape asendatud alaniiniga), mis eelduse kohaselt ei tohiks rikkuda valgu struktuurset terviklikkust, vaid peaks muutma ainult lokaalselt valgu pinda. Kui muteeritud pind on osaline valk-valk interaktsioonis, siis peaks mutatsioon mõjutama ka seda interaktsiooni. Sääraseid E2 punkt-mutante uuriti erinevaid aktiivsusi mõõtvates katsetes, mille lühikokkuvõte oleks järgmine.

1. Valk E2 omab märkimisväärset struktuurset terviklikkust, mida võivad rikkuda deletsioonid ja punkt-mutatsioonid. Üksikud punkt-mutatsioonid võivad viia valgu täielikult inaktiivsesse ja oligomeriseerunud/agregeernud

olekusse, kus selline E2 ei ole enam isegi võimeline liikuma oma normaalsesse asukohta raku tuumas.

2. E2 võime aktiveerida transkriptsiooni ei ole vajalik BPV1 replikatsiooni aktiveerimiseks. Seega on E2 selgelt ka **replikatsiooni aktivaator**, mitte ainult **transkriptsiooni aktivaator**.
3. E2 vahendatud transkriptsiooni aktivatsiooniks on vajalikud mitte ainult tema seondumine DNA-le, vaid ka interaktsioonid **mitmete** valkude või valkude kompleksidega.
4. E2 aktiveerib papilloomiviiruste ja polüoomiviiruste replikatsiooni erinevate mehhanismide kaudu. Erinevalt papilloomiviiruste DNA replikatsioonist korreleerub E2 võime aktiveerida polüoomiviiruse replikatsiooni hästi tema võimega aktiveerida transkriptsiooni. Seega on E2 ainult papilloomiviiruste spetsiifiline replikatsiooni aktivaator.
5. E2, erinevalt teistest kromatiini külge lõastavatest valkudest LANA1 ja EBNA1, ei oma lühikest lineaarset järjestust, mis vastutaks kromatiinile seondumise eest.
6. E2 kromatiini külge jäämist, E2 seostumiskohti sisaldava plasmiidi E2 sõltuvat lõastamist kromatiinile ning sellest tulenevat jaotumist tütarrakkude vahel võib rikkuda mitmete erinevate üksikute punkt-mutatsioonidega E2 aktivatsiooni domeenis.
7. E2-st sõltuv plasmiidide jaotumisfunktsioon tütarrakkude vahel on punkt-mutatsioonide poolt enim mõjutatud E2 aktiivsus, mida me oleme testinud (teised testitud aktiivsused: replikatsiooni aktivatsioon, transkriptsiooni aktivatsioon, E2 kromatiini külge jäämine, E2 seostumiskohti sisaldava test-plasmiidi kromatiinile lõastamine).

Kõik need tulemused kokku näitavad, et viirusega nakatunud rakus realiseeruvate E2 aktiivsuste (transkriptsiooni ja replikatsiooni aktivatsioon ning jaotumine) determinandid on lahutatavad. Selline olukord annab viirusele teoreetilise võimaluse neid aktiivsusi vastavalt viiruse elutsüklist tulenevatele vajadustele ka erinevalt reguleerida, mis omakorda tagab viiruse elutsükli korrektse lõpuleviimise. Samas on ka ülalkirjeldatud situatsioon ilmekas näide sellest, kui ratsionaalselt suudab viirus kasutada oma kodeerivat potentsiaali.

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