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PRESS

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which will be referred to by their Roman numerals:

I Lepik, D.,* **Ilves, I.**,* Kristjuhan, A., Maimets, T. and Ustav, M. (1998). p53 protein is a suppressor of papillomavirus DNA amplificational replication. *Journal of Virology*, 72, 6822–6831.

II **Ilves, I.**, Kivi, S. and Ustav, M. (1999). Long-term episomal maintenance of bovine papillomavirus type 1 plasmids is determined by attachment to host chromosomes, which is mediated by the viral E2 protein and its binding sites. *Journal of Virology*, 73, 4404–12.

III **Ilves, I.**, Kadaja, M., and Ustav, M. (2003) Two separate replication modes of the bovine papillomavirus BPV1 origin of replication that have different sensitivity to p53. *Virus Research*, 96, 75–84.

* Equal contribution

LIST OF ABBREVIATIONS

BPV1	bovine papillomavirus type 1
BrdU	5-bromo-2-deoxyuridine
CBP	CREB- binding protein
Cdk	cyclin-dependent protein kinase
CHO	chinese hamster ovary cells
CMV	cytomegalovirus
CREB	cyclic AMP-responsive element binding protein
DBD	DNA binding domain
E2TA	E2 transcription activator
E6AP	E6-associated protein
EBNA1	EBV nuclear antigen 1
EBV	Epstein-Barr virus
FACS	fluorescence-activated cell sorter
FISH	fluorescent *in situ* hybridisation
HPV	human papillomavirus
HSP	heat-shock protein
hTERT	human telomerase reverse transcriptase subunit
kb	kilobasepairs
kD	kilodalton
KSHV	Kaposi's sarcoma associated herpesvirus
LANA1	latency-associated nuclear antigen 1
LCR	long control region
lTAg	large T antigene
MHC	major histocompatibility complex
MME	minichromosome maintenance element
ORF	open reading frame
ori	origin of DNA replication
p/CAF	p300/CBP-associated factor
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pRb	retinoblastoma protein
PV	papillomavirus
RPA	replication protein A
RSV LTR	Rous sarcoma virus' long terminal repeat
ssDNA	single stranded DNA
SUMO-1	small ubiquitin-related modifier 1
SV40	simian virus 40
TAF	TBP-associated factor
TBP	TATA binding protein
TF	transcription factor

TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling
URR	upstream regulatory region

1. INTRODUCTION

Viral genomes are short, leaving viruses with only limited capacity to encode the functions required for their multiplication. In order to compensate this, the viruses have evolved several sophisticated means of utilising the molecular mechanisms of the cell for achieving their own goals. My studies in the lab and the resulting publications that form the basis of the current thesis have dealt mainly with virus-cell interactions that govern the establishing and maintenance of bovine papillomavirus type 1 (BPV1) genomes as episomal plasmids in latently infected cells. These studies are described in the results and discussion section of the thesis. In the literature overview section, I am trying to present some background information necessary for conceiving the specific objectives of these studies.

2. LITERATURE REVIEW

2.1. Papillomaviruses and BPV1 — general introduction

Papillomaviruses (PV) are a family of epitheliotropic viruses with a relatively small (~8kb) double-stranded circular DNA genome, which is packed into a small (55nm) icosahedral capsid with no surrounding envelope. The members of this family are species-specific and widely distributed in nature. Large variety of PV types has been identified from different bird and mammalian species, including more than 100 (and counting) from humans. The use of new sensitive detection methods (PCR) has revealed that PV infection may remain asymptomatic with a surprisingly high frequency and can be more common in host populations than previously thought (Antonsson *et al.*, 2000; Antonsson and Hansson, 2002; Astori *et al.*, 1998; Boxman *et al.*, 1997). Visible symptoms of the PV infection are mostly presented by periodic benign lesions of infected skin and mucosa. These lesions, generally referred to as papillomas, are also called 'warts', if found on the skin, and 'condylomas', if associated with genitalia. The interest in studies of PVs has been undoubtedly boosted by the fact that some of the human papillomavirus types, like HPV16, HPV18 and HPV31, have been demonstrated to associate with neoplastic alterations in epithelial tissues, most particularly with cervical cancer (zur Hausen, 2002; Walboomers *et al.*, 1999).

Several ungulate PVs form a distinct group in this otherwise epithelitropic family. Their infection may cause fibropapillomas, which have clear fibroblastic component in addition to epithelial one. Most well known representative of this group is bovine papillomavirus BPV1. In addition to less strict tissue tropism, these PVs have also somewhat more relaxed species specificity. For example, bovine papillomaviruses BPV1 and BPV2 may cause benign sarcoid tumours on horses, donkeys and mules (Campo, 2002). BPV1 can also non-productively transform rodent fibroblasts (Breitburd, 1987). The development of relatively simple and well-defined *in vitro* transformation assay of mouse cell-lines has been one of the main historic reasons why this bovine virus has emerged as one of the prototypes for the research of PV biology.

2.2. Papillomavirus life cycle

The BPV1-transformed mouse fibroblast cell lines have appeared to be a useful tool for studying the early steps in PV life cycle. The full life cycle of PVs cannot be followed in this model system, as its completion is tightly linked to the epithelial differentiation of host cells. The detailed studies on the later, productive stages of the infection were enabled only after the emergence of

more suitable laboratory model systems. These are based on HPV-carrying human keratinocyte cells, which are induced to differentiate either in the organotypic raft cultures or by suspension in methylcellulose (Dollard *et al.*, 1992; Flores and Lambert, 1997; Frattini *et al.*, 1996; Frattini *et al.*, 1997; McCance *et al.*, 1988; Meyers *et al.*, 1992; Meyers *et al.*, 1997).

PV particles are capable of recognising and binding to wide variety of cell types from several species (Muller *et al.*, 1995; Roden *et al.*, 1994; Volpers *et al.*, 1995). Thus, the narrow species specificity and tissue tropism of PVs are not determined by host cell recognition, but rather by post-entry steps of the viral life cycle. Successful establishing of PV genomic DNA takes place in proliferating undifferentiated basal epithelial cells. Viral entry into these cells initiates the expression of genes encoding for early viral proteins (including viral replication factors) and initial amplification of viral genome takes place. It is followed by eventual nuclear establishing of the viral genome as extra-chromosomal circular plasmid at stable copy number and viral latency. This process is likely to involve the switchover to more controlled genome replication mechanism and active partitioning of viral genomes in dividing cells. The proliferation of host cells leads to detachment of some of the daughter cells from the epithelial basement membrane. These are pushed further upwards by growing basal layer, start to stratify and differentiate. Terminal differentiation triggers the initiation of final, productive stage of PV life cycle inside the host cell. It is characterized by the vegetative amplification of viral genomes, the expression of capsid proteins, and subsequent formation of new viral particles. It should be noted, however, that even though the general build-up of the life cycle is believed to be the same in the case of all PVs (Howley, 2001), some differences are likely to exist in details. For example, the exact timing of specific life cycle related events relative to the host cell differentiation status has been shown to vary in the case of different PVs (Peh *et al.*, 2002).

2.3. Papillomavirus genome structure and genes

2.3.1. General features

PVs are well defined not only by their similar life cycle. The comparison of sequencing data from different PV types has revealed a remarkable conservation of the genomic organisation inside the family.

Typical PV genome consists of separate coding and non-coding regions. Up to 8 ORFs (E1–E8) can be found in the early transcribed part and two (L1, L2) in the late part of the coding region. The early ORFs correspond to non-structural and late ORFs to structural proteins, and all the genes are transcribed from the same strand of viral DNA. The extent of similarity on the primary

sequence level in certain parts of coding region has been chosen as a definition for new PV types. If it is less than 90% in the combined sequences of E6, E7 and L1 genes, the virus isolate is postulated to represent a new type (de Villiers, 1994). The non-coding region of PV genome is usually around 1kb long and consists of sequences vital for the regulation and control of the viral life cycle. It is named accordingly as upstream regulatory region (URR) or long control region (LCR) and contains the viral origin of replication, transcription enhancer elements, and binding sites for various cellular transcription factors as well as for viral multifunctional protein E2.

The transcription of PV genome is a complex process. It involves the use of different promoters as well as polyadenylation sites and extensive splicing. The exact use of these mechanisms in the course of transcription program as well as resulting transcripts vary to some extent in the case of different PV types, but the overall regulation of this process is always tightly linked to the differentiation of host cell. mRNA-s are translated into proteins that have considerable homology, both on the primary sequence level as well as on the functional level, in different members of the PV family. I will discuss the main features of some PV proteins relevant to the specific topic of the current thesis in the following sections of this chapter. In the present and the following chapters, I am concentrating on the bovine papillomavirus BPV1 as the main object of my studies. Even though much of the information is true also in the case of other PVs, some features specific to certain types are emphasised where appropriate.

2.3.2. E2 as a master regulator of the papillomavirus life cycle

The protein corresponding to BPV1 E2 ORF was first characterized as transcription activator (Spalholz *et al.*, 1985). Subsequent studies have revealed that E2 of BPV1 as well as other PVs is a multifunctional protein. In addition to the regulation of viral transcription, it participates also in several other important processes of the PV life cycle, like replication and stable extra-chromosomal maintenance (McBride and Myers, 1997; Ustav and Ustav, 1998).

The PV E2 proteins can be expressed in several alternative versions. Besides full-length 48kD protein, also two shorter forms — E8/E2 and E2C — are translated from alternative BPV1 mRNAs. They are also referred to as E2 repressor forms, owing to their ability to inhibit the transcription activation (as well as other activities) by the full-length E2 transactivator (E2TA). The inhibition can be achieved through competing for E2 cognate binding sites and through heterodimer formation (Lambert *et al.*, 1989; Lambert *et al.*, 1987). Both repressor forms have intact C-terminal DNA binding (DBD) and dimerisation domain, but their N-terminal domain is truncated (Giri and Yaniv, 1988; Haugen *et al.*, 1988; Hirochika *et al.*, 1987; Hubbert *et al.*, 1988; McBride *et*

al., 1989; McBride *et al.*, 1988). In addition, E8/E2 protein carries also a short peptide encoded by E8 ORF in its N-terminus. All three E2 polypeptides contain 'hinge' region, which links N- and C-terminal domains in the full-length protein and is the least conserved part on the primary sequence level of this otherwise relatively well conserved protein. Through DBD (which has to be in dimerised form), E2 binds to specific consensus sequence ACC(N6)GGT in DNA (Androphy *et al.*, 1987; Li *et al.*, 1989). BPV1 genome contains 17 such binding sites (BS) with varying exact sequence as well as E2 binding affinity; 12 of these are localised in URR region (Li *et al.*, 1989).

The binding of E2 to these sites is important for the regulation of viral life cycle in several aspects. To begin with, it can activate the transcription from viral promoters (Haugen *et al.*, 1987; Hermonat *et al.*, 1988; Spalholz *et al.*, 1987; Szymanski and Stenlund, 1991). This activity is required for the transformation of cells by BPV1 (Brokaw *et al.*, 1996; DiMaio, 1986; DiMaio and Settleman, 1988; Rabson *et al.*, 1986; Sarver *et al.*, 1984), but, interestingly, is not essential for carrying out the early and late stages in the life cycle program of HPV31 and possibly other oncogenic HPVs (Stubenrauch *et al.*, 1998). E2TA proteins of BPV1 and other PVs can bind numerous components of cellular transcription machinery, thus acting as recruitment factors contributing towards regulating the transcription from promoters adjacent to the E2 binding sites. These factors include the components of general transcription machinery, such as TATA-binding protein (TBP), TBP-associated factors (TAFs) in TFIID complex, TFIIB, and RNA polymerase II (Rank and Lambert, 1995; Steger *et al.*, 1995; Wu and Chiang, 2001); as well as several possible co-regulators for transcription, such as Sp1, Gps2/AMF-1, and TopBP1 (Boner *et al.*, 2002; Breiding *et al.*, 1997; Li *et al.*, 1991). E2 can also recruit the co-factors that possess histone acetyltransferase activity and are thus capable of remodelling chromatin structure in the promoter region (Lefebvre *et al.*, 1997). Examples include p300 and CREB-binding protein (CBP) as well as p300/CBP-associated factor (p/CAF) (Lee *et al.*, 2002; Lee *et al.*, 2000; Peng *et al.*, 2000). Efficient transcription activation by E2 requires at least two copies of binding sequence (Harrison *et al.*, 1987; Haugen *et al.*, 1987; Hawley-Nelson *et al.*, 1988; Spalholz *et al.*, 1985). The binding to two neighbouring sites is a cooperative process and the determinants for cooperative binding are located in the N-terminal domain (Monini *et al.*, 1991; Thierry *et al.*, 1990).

Full-length E2 proteins of different PVs can also repress the transcription from certain viral promoters, both passively, through sterically interfering with binding of the components of transcription initiation complex to the promoter (Demeret *et al.*, 1994; Dong *et al.*, 1994; Dostatni *et al.*, 1991; Tan *et al.*, 1994; Vande Pol and Howley, 1990) as well as through actively interfering with some steps after TBP or TFIID binding (Hou *et al.*, 2000). However, the significance of this activity in viral life cycle remains to be determined. At least in the case of HPV16, the repression of specific early promoter has been shown to occur in viral genomes that are integrated into host genome but not on normal episomal

copies, possibly as a result of alternative chromatin structure in the promoter region (Bechtold *et al.*, 2003). It is possible that both transactivation and repressor functions of E2 may be of secondary importance for efficient maintenance of certain PV types in host cells.

Perhaps one of the most important functions of the E2 protein in PV life cycle is its role as an auxiliary specificity factor in the initiation of PV replication. This process is especially well studied in the case of BPV1 E2. The binding of E2TA to its binding sites in the viral origin of replication (ori) is responsible not only for tethering of E1 helicase to ori (Mohr *et al.*, 1990; Sanders and Stenlund, 1998; Sedman and Stenlund, 1995; Yang *et al.*, 1991), it also unmasks the activity of E1 to bind specifically to ori sequences, through inhibiting the competing intrinsic non-specific binding activity of the protein (Stenlund, 2003). The N-terminal transactivation domain of E2 is essential for the interaction with E1 and for the functioning of E2 in replication initiation (Benson and Howley, 1995; Berg and Stenlund, 1997; Winokur and McBride, 1996). This domain is functional in the initiation of replication even if tethered to BPV1 ori as fused to heterologous DBD (Berg and Stenlund, 1997; Kivimae *et al.*, 2001). The interaction between C-terminal DBD of E2 and E1 can take place, however, if both proteins are bound to adjacent sites on DNA; such interaction is facilitating the binding between the N-terminal transactivation domain and E1 (Berg and Stenlund, 1997; Chen and Stenlund, 1998). Activation of transcription and replication are carried out by separate determinants of the N-terminal domain, as they can be genetically separated by single point mutations (Abroi *et al.*, 1996; Brokaw *et al.*, 1996; Ferguson and Botchan, 1996; Grossel *et al.*, 1996). E2 may stimulate the viral DNA replication perhaps also by additional means, like directly recruiting host replication factors (e.g. RPA) to the origin (Li and Botchan, 1993).

BPV1 E2 protein mediates the attachment of viral genomes to host mitotic chromatin and this process is believed to ensure proper nuclear maintenance and partitioning of viral genomes in dividing cells (see chapter 3.3). In addition, E2 proteins of several HPVs have been implicated in cellular processes, which may be directly relevant to carcinogenesis. In cervical carcinoma cells, the integration of high-risk HPV genomes into host genome almost exquisitely disrupts the E2 ORF. The ectopic expression of E2 from different PVs in such cells may block the cell proliferation (Desaintes *et al.*, 1997; Dowhanick *et al.*, 1995; Hwang *et al.*, 1996) and lead to replicative senescence (Goodwin *et al.*, 2000; Wells *et al.*, 2000) or apoptotic cell death (Desaintes *et al.*, 1997). Such growth inhibitory and apoptotic effects may involve multiple pathways. E2 can repress the transcription of viral E6 and E7 genes, the products of which inhibit the activity of cellular p53 and Rb tumour suppressors, respectively (see chapter 2.3.4). As a result, the ectopic E2 over-expression may reactivate growth inhibitory and apoptotic signals that are dependent on p53 and Rb. E2-dependent apoptosis may also occur independent of the regulation of PV gene expression, using both p53-dependent as well as independent pathways

(Webster *et al.*, 2000; Desaintes *et al.*, 1999). In this context, it is interesting to note that HPV16 E2 protein (HPV16) has been shown to interact with p53 (Massimi *et al.*, 1999) and HPV18 E2 is able to repress human telomerase reverse transcriptase promoter (Lee *et al.*, 2002).

2.3.3. Replication protein E1

E1 is the longest among PV ORFs. E1 proteins encoded by different PVs are well conserved both in sequence level as well as on the functional level (Wilson *et al.*, 2002). The E1 of BPV1, biochemically the best described from PV E1 proteins, has the molecular weight of 68kD. *In vitro* studies have demonstrated that it is required for both, initiation and elongation stages of the viral replication (Gillette *et al.*, 1994; Liu *et al.*, 1995), acting as a DNA-dependent ATPase and DNA helicase responsible for the unwinding of ori and subsequent replication fork progression (Bream *et al.*, 1993; Hughes and Romanos, 1993; Seo *et al.*, 1993; Yang *et al.*, 1993). E1 can bind to its binding site in the PV ori region, but the binding of E1 alone is relatively weak and insufficient to provide the specificity required *in vivo* conditions (Ustav *et al.*, 1991; Wilson and Ludes-Meyers, 1991; Yang *et al.*, 1993). However, the cooperative binding with E2 increases dramatically the efficiency and the ori-specificity of the process (Sanders and Stenlund, 2000; Sedman and Stenlund, 1995). As a reflection of this, the papillomavirus DNA replication requires both, E1 and E2 proteins *in vivo* (Ustav and Stenlund, 1991; Ustav *et al.*, 1991), even though E1 alone is sufficient to initiate the replication *in vitro* where the concentration of E1 protein is higher and the concentration of competing DNA lower than *in vivo* conditions (Bonne-Andrea *et al.*, 1995; Melendy *et al.*, 1995; Muller *et al.*, 1994). As a first step in the ori recognition process, the E1 and E2 proteins bind cooperatively to its adjacent binding sites in the ori, generating initial $E1_2E2_2$ complex (Sanders and Stenlund, 1998; Sedman *et al.*, 1997). E2 is then released from the complex in the ATP-dependent manner and additional E1 molecules are recruited, eventually leading to the formation of larger complexes that cause partial melting of the DNA duplex in ori region (Lusky *et al.*, 1994; Sanders and Stenlund, 1998). The final, active complex of E1 with ATPase and 3'> 5' helicase activity, is formed as a hexameric ring on the single-stranded DNA (Fouts *et al.*, 1999; Sedman and Stenlund, 1998). The direct interaction between E1 and E2 proteins, apart from assisting E1 in viral replication initiation, can also modulate the activity of E2. For example, the binding of BPV1 E1 can interfere with the transactivation function of E2 (Ferran and McBride, 1998; Sandler *et al.*, 1993) as well as with the binding of E2 to mitotic chromosomes (Voitenleitner and Botchan, 2002).

E1 can interact not only with E2 but also with a number of host cell proteins, which can be directly or indirectly linked to the PV replication. BPV1 E1 can bind to the DNA polymerase α-primase (Bonne-Andrea *et al.*, 1995; Park *et al.*,

1994) as well as to RPA (Han *et al.*, 1999), both have been shown to directly participate in BPV1 replication (Melendy *et al.*, 1995; Muller *et al.*, 1994; Park *et al.*, 1994). The interactions of E1 with S-phase specific kinase cyclinE/Cdk2 (Cueille *et al.*, 1998) and SUMO-1 conjugase Ubc9 (Rangasamy and Wilson, 2000; Rangasamy *et al.*, 2000) lead to the covalent post-translational modifications of the E1 protein, phosphorylation and sumoylation, respectively. The phosphorylation by cyclinE/Cdk2 seems to be important for the activation of the E1 activity in replication; the sumoylation is required for efficient nuclear accumulation of the E1 protein. In addition, the binding of HPV11 E1 to histone H1 (Swindle and Engler, 1998) and HPV18 E1 to chromatin modifying factor Ini 1/hSNF 5 (Lee *et al.*, 1999) could reflect the possible link between E1-dependent replication and modifications of the chromatin structure.

2.3.4. Transforming proteins E5, E6, and E7

Apart from E1 and E2 initiatory proteins, all the other PV replication proteins are 'borrowed' from host. As the activity of these proteins is exerted in S-phase of the cell cycle, the sustained proliferative state appears to be critical for efficient PV replication in differentiating epithelial cells that normally would exit from the cell division cycle. To answer these needs, PVs encode for three transforming proteins capable of promoting cellular proliferation- E5, E6, and E7. However, different papillomaviruses seem to utilise these proteins in somewhat different ways.

In contrast to the HPVs, the major transforming protein of BPV1 and other fibropapillomaviruses appears to be E5 (DiMaio and Mattoon, 2001). BPV1 E5, with its 44 amino acids, is probably one of the shortest oncogenes known. E5 protein is very hydrophobic and forms homodimers. It is associated with intracellular membranes, localising predominantly to the Golgi complex and endoplasmic reticulum (Burkhardt *et al.*, 1987; Burkhardt *et al.*, 1989; Burnett *et al.*, 1992; DiMaio *et al.*, 1986; Schiller *et al.*, 1986; Schlegel *et al.*, 1986). BPV1 E5 is sufficient for the transformation of rodent cell lines *in vitro* (DiMaio *et al.*, 1986; Groff and Lancaster, 1986; Rabson *et al.*, 1986; Schiller *et al.*, 1986). The functioning of E5 as an oncogene is achieved through the contacts with other cellular membrane proteins and modulation of their activity, as E5 itself does not possess any enzymatic activity. It has been shown that E5 protein can bind to and activate the platelet-derived growth factor (PDGF) ß receptor in the absence of exogenous ligand (Petti and DiMaio, 1992; Petti *et al.*, 1991). Such binding correlates well with transformation activity of E5 (Klein *et al.*, 1998). BPV1 E5 also binds to the 16-kDa transmembrane channel-forming subunit of the vacuolar proton ATPase (Goldstein and Schlegel, 1990; Goldstein and Schlegel, 1990) and this binding can lead to the alkalisation of the Golgi lumen. Again, the genetic analysis has demonstrated good correlation between Golgi alkalisation and transformation by E5 (Schapiro *et al.*, 2000). E5

may also function to protect the infected cells from host immune system through interfering with the expression and transport of the antigen-presenting MHC I complex to the cell membrane (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002).

The role of E6 and E7 proteins is more prominent in the transformation by different HPVs and both proteins are linked to the development of cervical cancer in the case of high-risk HPV types (Mantovani and Banks, 2001; Munger *et al.*, 2001; zur Hausen, 2002). Like E5, both E6 and E7 exert their activity through interactions with different cellular regulatory proteins. Probably the most studied and the most important is the binding of E6 and E7 to tumour suppressor proteins p53 and pRb, respectively, which both are capable of controlling the cell cycle progression. The binding of E6 to p53 in a complex with the cellular ubiquitin ligase E6-AP promotes the degradation of p53 protein in the ubiquitin-dependent pathway (Huibregtse *et al.*, 1991; Scheffner *et al.*, 1993; Scheffner *et al.*, 1990; Werness *et al.*, 1990). The binding of E7 inactivates the pRB (Cheng *et al.*, 1995; Dyson *et al.*, 1989; Martin *et al.*, 1998; Munger *et al.*, 1989) and promotes its proteolysis (Boyer *et al.*, 1996). However, both processes are efficient only in the case of E6 proteins of high-risk HPVs, e.g. HPV16 and 18. In the case of low-risk HPV6 and 11, the binding of E6 to p53 is unable to induce its degradation (Crook *et al.*, 1991), and E7 proteins bind to pRb with a lower efficiency, failing to promote transformation as efficiently as E7 of high-risk HPVs (Gage *et al.*, 1990; Munger *et al.*, 1989). In the case of BPV1 proteins, there is most likely no direct interaction between E6 and p53 as well as between E7 and pRb. It is also interesting to note that some of the bovine PV types, namely BPV3, 4, and 6, lack the E6 ORF entirely (Jackson *et al.*, 1991).

A number of additional, p53- and Rb-independent possible targets have been identified in the case of E6 and E7 proteins, including several members of the signal cascades that control the cellular proliferation (Mantovani and Banks, 2001; Munger *et al.*, 2001). E6 is also able to function through modulating the transcription of certain genes, like that encoding for human telomerase reverse transcriptase subunit (hTERT) (Gewin and Galloway, 2001; Klingelhutz *et al.*, 1996; Veldman *et al.*, 2001), and the demonstration of the interaction between transcriptional co-factor p300/CBP and HPV16 E6 has provided possible mechanism for such modulation (Patel *et al.*, 1999; Zimmermann *et al.*, 1999).

In summary, the cellular transformation by PV oncogenes E5, E6, and E7 is likely to represent a complex process, involving cellular tumour suppressor genes p53 and pRb as well as several alternative proteins that may be differently targeted by individual PVs in various cellular conditions.

2.4. BPV1 DNA replication

The basis of viral multiplication lies in the replication of its genome. All three stages of the PV life cycle — initial amplification, following stable extrachromosomal maintenance, and vegetative multiplication of the viral genome — require the replication of viral DNA to take place. Several studies have indicated that alternative replication mechanisms may be used during the different stages. In a sense, we can use the term "replication cycle" not only as synonymous to life cycle or multiplication cycle of PVs, but also specifically referring to the ordered transitions taking place in the replication mechanism of viral genome.

2.4.1. E1,E2-dependent replication and viral extrachromosomal establishing

It is widely acknowledged now, that during the initial extrachromosomal establishing stage, BPV1 as well as other PVs use E1,E2-dependent replication mechanism. The disruption of E1 or E2 ORF renders the BPV1 genomes unable to show any detectable extrachromosomal replication signal after the entry into the cell (DiMaio and Settleman, 1988; Ustav and Stenlund, 1991). In transient transfection assays, the replication of reporter plasmids that carry PV origin of replication (ori) can be achieved only when E1 and E2 are expressed in the cells (Del Vecchio *et al.*, 1992; Remm *et al.*, 1992; Ustav and Stenlund, 1991). The interactions between *cis*-and *trans*-elements for replication are surprisingly well conserved in the case of different PVs. For example, the E1 and E2 proteins of HPV11 and BPV1 can initiate the replication from ori sequences of the same viruses in freely mixed combinations in various cell lines, albeit with varying efficiency in different combinations (Chiang *et al.*, 1992).

Both *cis* and *trans* requirements for E1,E2-dependent replication as well as first steps of the initiation process have been studied extensively not only in the transient *in vivo* assays, but also in various *in vitro* systems assembled from cellular extracts and purified proteins. As already discussed in previous chapters, the only viral protein participating directly in the replication initiation and following elongation stages is hexameric E1 helicase. E2 works as auxiliary recruitment and specificity factor for E1. The cellular proteins that are required in E1,E2-dependent replication of BPV1 overlap in large extent, but not entirely, with those required for SV40 replication (Melendy *et al.*, 1995). The origin of replication for BPV1 as well as other PVs has been mapped into the non-coding URR (LCR) region (Chiang *et al.*, 1992; Del Vecchio *et al.*, 1992; Remm *et al.*, 1992; Ustav *et al.*, 1991). The ori functions as the initiation complex recruitment zone as well as the region of initial destabilisation of the DNA duplex (Howley, 2001). Minimal ori contains E1 binding site, at least one

binding site for E2, and adjacent A/T rich region (Ustav *et al.*, 1991). The function of the binding sites is the initial recruitment of E1 and E2 proteins, and the A/T rich-region facilitates the duplex melting (Stenlund, 1996).

2.4.2. Stable maintenance replication

It is not clear yet, if the E1,E2-dependent replication mechanism is required only for initial amplification and extrachromosomal establishing of the viral genomes, or is basically the same mechanism used also after extrachromosomal maintenance has been successfully established. E1 and E2 are clearly necessary for establishing of the extrachromosomal maintenance of the BPV1 URR reporter plasmids in the long-term assays (Piirsoo *et al.*, 1996). However, it has been reported recently that even though intact E1 is required for episomal establishing of the BPV1 genomes in mouse C127 cells, its continuing activity seems to be dispensable for already established stable extrachromosomal maintenance status (Kim and Lambert, 2002). In the same publication, Kim and Lambert also hypothesise that the papillomavirus stable maintenance replication might rely entirely on the same factors and complexes, including DNA helicase, that replicate host genomic DNA. Some indirect support to this hypothesis comes from two separate notions. First, the genomic DNA of BPV1 as well as some HPVs can apparently replicate stably also in *Saccharomyces cerevisiae* cells, and, at least in the case of HPV16, none of the viral ORFs (including E1) is essential for this process (Angeletti *et al.*, 2002; Zhao *et al.*, 2002). Second, such use of the cellular replication complexes has been already established in the case of two other DNA viruses with prominent latent extrachromosomal maintenance stage — Kaposi's sarcoma associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) (Chaudhuri *et al.*, 2001; Dhar *et al.*, 2001; Schepers *et al.*, 2001). Despite all this, additional and more conclusive evidence is clearly required to clarify this point. For example, the replication of BPV1 genome during its stable extrachromosomal maintenance does not follow the strict once-per cell-cycle initiation mechanism that would be expected if the cellular replication complexes were involved. Such mechanism is used during the replication of EBV oriP by cellular machinery, but more relaxed random-choice initiation mechanism is used by BPV1 (Gilbert and Cohen, 1987; Ravnan *et al.*, 1992).

The 2D analysis of replication intermediates (Schvartzman *et al.*, 1990; Yang and Botchan, 1990) as well as electron microscopy analysis of the initiation zone (Waldeck *et al.*, 1984) in stably transformed rodent cells has mapped the initiation region into the URR part of the BPV1 genome. Therefore, whatever the replication mechanism, the *cis*-sequences that participate in the initiation of the stable maintenance replication seem to map into the same, uncoding URR part of the PV genome, which is used also in the E1,E2-dependent replication initiation. In accordance with this, the URR region is

sufficient to ensure the establishing of stable extrachromosomal maintenance of reporter plasmids in E1,E2-expressing cells (Piirsoo *et al.*, 1996).

2.4.3. Papillomavirus vegetative replication

Until recently, the lack of good model systems has hampered the studies on the PV replication during the later, vegetative stage. 2D electrophoresis analysis of the replication intermediates has indicated that the rolling-circle replication mechanism may be involved in the vegetative replication of HPV16 and HPV31 (Flores and Lambert, 1997). There are indications that similar mechanism may be used also by BPV1 (Burnett *et al.*, 1989; Dasgupta *et al.*, 1992).

2.5. Concluding remarks

Despite almost two decades of concentrated efforts, there is still a lot to be clarified about the processes that direct the multiplication of BPV1 as well as PVs in general. The genome structure and specific properties of the viral proteins have been characterised quite extensively. On the other hand, there are noticeable gaps in the present understanding of how the different viral regulatory sequences and protein activities interact with the molecular processes operating inside the host cell, and how the proper order and interplay of all these interactions is achieved in the viral replication cycle. The continuous emergence of new laboratory techniques and better *in vitro* models for studying the full viral replication cycle should provide additional means to find the answers to these questions.

3. RESULTS AND DISCUSSION

3.1. Objectives of the present study

Together with my colleagues, I have tried to find answers to some of the questions that concern the virus-cell interactions in the replication cycle of BPV1 and papillomaviruses in general. More specifically, we have made efforts to understand the molecular processes that govern the establishing and efficient preservation of the stable episomal maintenance of BPV1 genomes in the proliferating host cells. The main objectives of the studies that have lead to the publications forming the basis of this thesis can be very shortly summarised as follows:

1. To look for possible effects of the tumour suppressor protein p53 on the papillomavirus replication;
2. To use p53 as a tool for testing the possible utilisation of different replication mechanisms during the early stages of BPV1 life cycle;
3. To investigate the viral factors that determine the efficient nuclear maintenance and mitotic partitioning during the stable extrachromosomal maintenance of BPV1 genomes.

3.2. Studies on the effects of p53 on the papillomavirus replication (I,III)

3.2.1. p53 and viruses — short introduction

Tumour suppressor protein p53 is a central coordinator of the defence mechanisms that ensure genetic stability in the metazoan cells. It is built up as a typical eukaryotic transcription activator, containing the DNA binding and transactivation domains and possessing the ability to activate or repress the transcription of certain genes. By modulating the transcription of genes of several regulatory proteins in response to different genotoxic stress conditions, p53 can prevent the fixation of genetic errors by blocking the transition of cells in the cell cycle or inducing the apoptotic death of the damaged cells (Ko and Prives, 1996; Vousden and Lu, 2002). For successful propagation, several DNA viruses encode for proteins that bind and effectively modulate the activity of p53 protein (including PV E6, see chapter 2.3.4), thus counteracting its effects on the viral multiplication. It has been shown, for example, that the transcriptional activity of p53 is abrogated by the viral oncoproteins lTAg from simian virus SV40, E1B 55kD from adenovirus Ad5, and high-risk HPV E6 proteins (Mietz *et al.*, 1992; Yew and Berk, 1992). p53, in turn, can modulate

the functions of viral proteins, like the helicase activity of SV40 lTAg (Sturzbecher *et al.*, 1988; Wang *et al.*, 1989). p53 can also suppress the replication of DNA viruses, like SV40 (Braithwaite *et al.*, 1987) and polyomavirus JCV (Staib *et al.*, 1996).

3.2.2. p53 suppresses the amplificational replication of the papillomaviruses — what could be the mechanism?

We have studied the effect of p53 expression on the replication of papillomavirus origin of replication and found that the over-expression of human p53 can suppress the E1,E2-dependent amplificational replication of BPV1, HPV11, and HPV18 ori reporter plasmids in the transient co-transfection assay (I, Fig.1A; III, Fig.1A). This effect is not dependent on the endogenous p53 background, occurring also in the human Saos2 cell-line that does not express any p53 (I, Fig.4). The genetic mapping indicated that the parts of p53 protein that are required for this activity include the intact central DNA-binding domain, the oligomerisation region from C-terminal domain, and part of the N-terminal domain containing the RPA-binding and proline-rich sequences. In contrast, the first N-terminal 39 amino acids that carry main transactivation activity and also C-terminal regulatory domain in the extreme C-terminus (30 amino acids) are dispensable for the suppression of PV replication (I, Fig.2; III, Fig.1).

Due to our assay conditions, the inhibition of papillomavirus replication by p53 could have also been achieved by rather trivial means — through reducing the expression levels of the essential viral replication proteins (E1 and E2), or inducing either cell-cycle block or apoptosis. However, we performed several control assays with CHO4.15 cells and its daughter cell-line CHOBgl40, which excluded this possibility. Firstly, none of the three approaches (FACS, TUNEL, and BrdU labelling), which we used to analyse the cells processed in the same assay conditions as in the parallel transient replication assays, indicated any apparent correlation between p53-dependent replication suppression and changes in the cell growth or viability (I, Fig.6 and Table 1; III, Fig.2). As for the p53-dependent changes in the E1 or E2 expression levels, the p53 constructs that are capable of inhibiting the PV replication seem also to inhibit the expression from the strong promoters of the heterologous viral origin, such as cytomegalovirus immediate early promoter (Lepik and Ustav, 2000) and SRα (III, Fig.3A). As we found out, this could have lead to the diminished levels of E1 protein in our assay conditions (III, Fig.3A). However, the restoration and even the several-fold rise of the E1 expression above normal endogenous level due to the additionally transfected expression construct caused only slight rescue of the p53-dependent inhibition of the amplificational replication (III, Fig.4). Therefore, the sensitivity of PV transient amplificational replication to the p53 over-expression cannot be explained solely by reduced level of the essential viral replication proteins and reflects most likely the direct effect of the

p53 protein on the replication. To support this conclusion, the human p53 protein is able to suppress the E1-dependent replication of BPV1 ori reporter constructs also in the cell-free *in vitro* system (Kadaja, M., and Ustav, M., personal communication).

We can presently only speculate, what is the exact mechanism of the suppression. We know that according to the deletion analysis, this mechanism does not require the transcription activation by p53. The inhibitory effect of p53 on the expression of some cellular replication factor(s) cannot be excluded, but is highly unlikely. First of all, such effect of p53 seems to be specific to the expression from strong heterologous viral promoters, but not form weaker ones, like HSP70 promoter (see the E2 expression on I, Fig.5A; and on III, Fig.3A) or RSV LTR (Lepik and Ustav, 2000). Moreover, it seems feasible to assume that significant decrease in the levels of general replicator factors should have lead to the changes also in the host genome replication and cell cycle, but no such changes were apparent. In addition, p53 also fails to inhibit the EBNA1-dependent once-per-cell-cycle replication of EBV oriP in the conditions where it efficiently inhibits the papillomavirus amplificational replication (I, Fig.3). Therefore, the effect of p53 on the PV replication is likely to be direct and specific, involving both the recognition as well as following steps that are aimed to interfere with the replication mechanism. Number of reports has provided hints about the activities and interactions of the p53 protein that could be potentially involved in this process. p53 can bind to the cellular replication factors, like replication protein A (RPA) (Dutta *et al.*, 1993; He *et al.*, 1993; Li and Botchan, 1993) and DNA polymerase α (Kuhn *et al.*, 1999). p53 can recognise and bind to the lesions in genomic DNA, like single-stranded regions (Bakalkin *et al.*, 1995), that are likely to appear not only after various genotoxic stresses but also during intensive amplificational replication process. p53 has also a 3'>5' exonuclease (Mummenbrauer *et al.*, 1996) and ssDNA reannealing activities (Bakalkin *et al.*, 1994; Brain and Jenkins, 1994; Oberosler *et al.*, 1993). In addition, the possibility of direct interaction of p53 with viral replication proteins cannot be overlooked, as E2 proteins from HPV16 and HPV8 have been shown to interact with p53 (Akgul *et al.*, 2003; Massimi *et al.*, 1999). However, according to electromobility shift analysis, neither the binding of BPV1 E2 to its specific binding sites on DNA nor the mobility of E2-DNA complex are affected by p53 in our assay conditions (I, Fig.5C, and data not shown). It has been also suggested that in the case of HPV8, the p53-dependent inhibition of replication may be achieved through competitive binding of p53 and E2 to the overlapping binding sites in viral ori (Akgul *et al.*, 2003). Such mechanism is highly unlikely in the case of BPV1, as there are no apparent p53 binding sites present in the viral minimal ori region.

In addition to the human p53, we have tested also the mouse p53 in the transient assays and found that it can function as the suppressor of the PV amplificational replication (I, data not shown). We have not tested the effect of bovine p53 in these conditions. According to BLAST alignment

of the respective amino acid sequences from NCBI database (http://www.ncbi.nlm.nih.gov), the full-length human p53 carries approximately 80% amino acid sequence identity with bovine and 77% with mouse p53. Therefore, we can expect significant conservation of these p53 proteins also on the functional level, including the ability to suppress the amplificational replication of PVs. On the other hand, the results presented by the colleagues from our lab have demonstrated that both human and mouse p53 proteins that suppress the replication of BPV1 ori in human and hamster cell-lines, are unable to do so in several mouse cell-lines (Lepik and Ustav, 2000). The kinetics of E1,E2-dependent accumulation of the newly replicated BPV1 ori reporter plasmid in mouse cells is roughly similar to that in the human and hamster cells. Therefore, the inability of p53 to function in mouse cells most likely does not reflect the differences in the replication mode or in the replication intermediates, but rather the inability of p53 protein to interact with specific target proteins participating in the replication initiation or regulation mechanisms.

3.2.3. Two separate replication modes of the BPV1 origin of replication that have different sensitivity to p53 — possible implications of this observation on the BPV1 life-cycle

According to our transient replication assays, p53 is unable to interfere with the EBNA1-dependent once-per-cell-cycle replication of EBV oriP in the conditions where it efficiently inhibits the papillomavirus amplificational replication (I, Fig.3). This suggested us that only the 'uncontrolled' amplificational replication is sensitive to p53 action. We became curious about the effect of p53 on BPV1 stable maintenance replication, which has characteristics of both its own amplified replication (random choice initiation) as well as the replication of oriP (the overall synchrony with host genome duplication). In our lab, we have established a CHOBgl40 cell-line as a simplified model system for studying the basic processes of replication and nuclear maintenance during BPV1 latency (Piirsoo *et al.*, 1996). This cell-line expresses constitutively viral E1 and E2 proteins from chromosomally integrated cassettes and maintains stably episomes of the BPV1 URR reporter plasmid pNeoBgl40. Thus, only very minimal viral *cis* (URR) and *trans* (E1, E2) determinants required for the establishing of the BPV1 stable maintenance are present in this well-defined model.

Despite of the already present stable maintenance replication of the BPV1 URR reporter plasmid in these cells, the transient transfection of similar reporter into CHOBgl40 cells leads to the initial amplificational replication of the newly transfected reporter. Therefore, using the panel of p53 mutant versions already tested in previous transient replication assays (I, Fig.2), we could make sure that p53 is able to function as a repressor of the BPV1 ori-dependent replication in

this cell-line (III, Fig.1). Note that we have disained the input URR reporter to contain one additional HindIII site compared to the stably maintained one. Because of that, the digestion with HindIII linearises stably maintained reporter, but gives ~1kb shorter fragment in the case of amplificationally replicating input reporter. The respective fragments can be separated on the agarose gel electrophoresis and analysed by Southern hybridisation after transferring to nylon membrane.

We then proceeded to analyse the possible effect of p53 on the BPV1 stable maintenance replication in these cells, using the transient transfection assay that was slightly modified in order to get rid of the 'background' signal coming from non-transfected cells lacking p53 expression. The results of these experiments demonstrated that the truncated version of p53 (N39C362), which contains a minimal set of domains required for replication suppression activity, fails to inhibit the stable maintenance replication of BPV1 URR reporter in CHOBgl40 cells (III, Fig.3). This construct inhibits efficiently the simultaneous transient amplified replication of the input BPV1 URR reporter in these cells. Therefore, these two BPV1 URR-dependent replication modes — initial transient amplificational replication and stable maintenance replication — have different sensitivity to p53.

These data raise two main questions: first, what significance could have the observed phenomenon in the BPV1 life cycle; and second, what are the differences between these replication mechanisms that are reflected in their different sensitivity to p53. The transient E1,E2-dependent replication assay mimics the initial amplificational replication stage, which is required for successful extrachromosomal establishing of the BPV1 genomic DNA after infection. We can assume that the uncontrolled over-replication is not favourable for virus, as it could induce the cellular responses leading to cell-cycle block or apoptosis. The period of initial quick rise of the copy number per cell is apparently considerably shorter in the case of full-length BPV1 genome in C127 cells than it is in the case of BPV1 ori plasmids with E1 and E2 additionally co-expressed from strong heterologous promoters (Ustav and Stenlund, 1991). The possible reason for that is a tight control of the levels of E1 and E2 transcription from viral promoters upon the initial amplification, which in turn could normally limit the frequency of replication initiation. It could also explain, how BPV1 could establish itself in p53-expressing cells, even though its E6 protein, unlike that of high-risk HPVs, is unable to induce the degradation of p53 protein in ubiquitin-dependent proteolysis pathway (see chapter 2.3.4). The ability of p53 to suppress the amplificational replication process might be used by virus in order to apply some additional control on its initial amplification, if usual viral control mechanisms fail. In addition, p53 might also suppress the replication of already established stably maintained viral genome molecules that for some reason re-initiate uncontrolled amplification.

Our data also suggest that the mechanisms of initial transient amplificational replication and following stable maintenance replication of BPV1 are likely to

differ from each other. We found that the simultaneous amplificational replication of the BPV1 URR reporter does not affect the efficiency of the stable maintenance replication of similar reporter in the same CHOBgl40 cells. Assuming that stably maintained URR plasmids are picked randomly from total pool for replication initiation (Piirsoo *et al.*, 1996), we can conclude that the transiently replicating URR plasmids are not included into stably replicating pool. It means, that stably replicating URR reporter plasmid episomes form a population that is somehow separated from amplificationally replicating input reporter population. This separation is bound to more tightly regulated replication initiation mechanism and requires certain establishing stage to be passed in order to occur. The differences between control mechanisms of amplificational and stable maintenance replication are clearly emphasised by altered sensitivity towards p53. The E1-independent replication mechanism, discussed in chapter 2.4.2, could be one of the potential explanations to such mechanistic differences between BPV1 amplificational and stable replication. The fact that neither the p53-dependent down-regulation of the E1 expression nor the additional over-expression of E1 (III, Fig.4.) in CHOBgl40 cells affected the copy number of stably maintained BPV1 URR reporter in our experiments seems to support this speculation. However, there is also an alternative explanation to this observation — the replication of stably maintained reporter may depend on E1, but its level in CHOBgl40 cells is much higher than actually required for the replication of the reporter. Consequently, the frequency of replication initiation and the copy number of stably maintained reporter might be determined not by E1 level in these cells, but by other factors.

The long-term stable maintenance of BPV1 replicon is likely to be dependent on its non-covalent attachment to host chromatin, and this process has been linked to efficient partitioning and nuclear retention of the viral genomes during mitosis (see chapter 3.3). We can speculate that the sub-nuclear localisation of BPV1 genomic DNA could be important also for the regulation of its replication initiation, as the association with chromatin could enable the access to the replication control mechanisms operating during the host cell genome replication. Different p53 sensitivity could appear as an indirect consequence of the altered sub-nuclear localisation, as it may be responsible for the changes in replication mechanism of the viral DNA that are differentially recognised and affected by p53. In addition, the sub-nuclear localisation of the viral replication process could perhaps be directly linked to the altered p53 sensitivity, as different sub-nuclear compartments could have different accessibility to p53.

In conclusion, our data suggest that BPV1 can use different replication modes for initial transient amplification and during the following stable maintenance stage. As these two replication modes have different sensitivity to p53, the elucidation of specific targets of the p53 protein could provide a convenient tool for defining the differences between these replication modes on the molecular level.

3.3. Studies on the viral factors that determine the long-term episomal maintenance of the BPV1 genome (II)

Efficient extrachromosomal maintenance of the DNA viruses in proliferating host cells requires the viral genome replication and partitioning between daughter cells to proceed in the overall synchrony with the maintenance cycle of the host genome. Because of their relatively small genomes, the episomal DNA viruses, unlike the cellular chromosomes, cannot afford to carry long and complex centromeric regions that could ensure the proper partitioning and nuclear retention functions during mitosis. Some other strategy has to be used instead.

In the case of BPV1, as already discussed in previous sections, the URR region carries all the *cis*-elements required for the establishing of stable extrachromosomal maintenance in E1,E2-expressing cells. These elements consist of the minimal origin of replication and additional E2 binding sites, the latter forming a so-called minichromosome maintenance element (MME). Sufficient number of high affinity E2 binding sites is critical for proper MME function (Piirsoo *et al.*, 1996). The function of E2 binding sites (MME) in the stable episomal maintenance process was not clear in the beginning. The first insights were provided by two publications, which showed that BPV1 genomes, as well as E2 protein, are tethered to the host mitotic chromosomes in C127 mouse fibroblasts, and that mutations in E2 and E1 encoding regions are able to affect such localization (Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). These data gave us the idea to test if the chromosome localisation of BPV1 is mediated by its E2 protein and MME, and if such localisation can be linked to the stable maintenance function of the MME.

We used the fluorescent *in situ* hybridisation (FISH) to test the localisation of the stably maintained BPV1 URR reporter plasmid in mitotic CHOBgl40 cells. The results of these experiments demonstrated that the URR reporter is distributed on mitotic chromosomes following speckled and obviously random pattern (II, Fig.2 panel A), which resembles the chromosomal localisation pattern of full-length BPV1 genomes according to earlier studies (Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). The number of URR reporter-specific dots in the CHOBgl40 cells was approximately in the same range (10–50 per nucleus) as its previously determined copy number in this cell-line (Piirsoo *et al.*, 1996), suggesting that the FISH analysis was sensitive enough to detect single plasmid molecules. Similar pattern was observed also in the case of CHO4.15 and CHO49 cells (expressing E1 and E2, or E2 only, respectively) that were transiently transfected with URR reporter (II, Fig.2 panel B; Fig.4 panel A). No signal was observed if CHO cells, which do not express E2, were transfected with URR reporter. These data demonstrated first of all, that BPV1 URR carries all the necessary *cis*-elements for chromatin attachment,

and E2 protein is the only viral *trans*-factor that is necessary for this process. Another interesting observation from these studies was that the E2 and URR dependent chromatin attachment does not seem to require a prolonged establishing period in order to occur. The analysis was performed around 48hrs after transfection, by which time the cells had barely passed through one cell cycle. We could also say that the chromatin attachment is not affected by moderate E1 co-expression. Later it was shown, however, that E1 can titrate E2 out of chromatin, but only on much higher E1:E2 ratios (Voitenleitner and Botchan, 2002).

The following genetic dissection demonstrated that the E2 binding sites (MME) from URR determine its ability to be tethered to chromatin in E2 protein dependent fashion (II, Fig.2). The E1 binding site (and thus the replication ability) as well as other sequences in the URR are dispensable for the chromatin attachment function (II, Fig.2). Our data provided also evidence about the link between MME, chromatin attachment, and efficient stable maintenance, as none of the reporter constructs lacking intact MME and unable for stable episomal maintenance was tethered to mitotic chromosomes (II, Fig.2; Fig.7). In conclusion, we demonstrated that MME exerts its role in episomal minichromosome maintenance of the BPV1 genome through the viral E2 protein mediated association with the host cell chromatin. Therefore, the BPV1 stable episomal maintenance consists of two main functions- chromatin attachment, which is likely to provide proper partitioning and nuclear retention to the viral genomes, and replication, which is responsible for compensation of the plasmid loss during host cell divisions.

It is easy to imagine that the tethering to host chromatin could provide the means for viral genome partitioning and nuclear retention, through a 'piggyback ride' during the mitotic separation of host chromosomes to daughter cells. In addition, as already discussed in the chapter 3.2.3, the chromatin association could perhaps also provide the access to cellular replication control mechanisms that help to establish the viral genome copy number control during stable episomal maintenance. It should be noted, however, that the BPV1 MME-dependent chromatin attachment has been so far conclusively demonstrated only in mitotic cells and the data are lacking about the maintained chromatin localisation during S-phase. The utilisation of chromatin attachment to ensure the stable episomal maintenance is not unique to papillomavirus family, but, paradoxically, is also not necessarily common mechanism inside this family. Two gammaherpesviruses with prominent latent phase in their life cycle — EBV and Kaposi's sarcoma associated Herpesvirus (KSHV) — use similar means for their extracromosomal maintenance in dividing cells. Like in the case of BPV1, the single viral protein acts as a molecular linker between viral genome and host chromatin: EBNA1 in the case of EBV (Kanda *et al.*, 2001), and LANA1 in the case of KSHV (Ballestas *et al.*, 1999). On the other hand, the minimal number of E2 binding sites in BPV1 URR sufficient to provide the minichromosome maintenance function (Piirsoo *et al.*, 1996) exceeds the

number of these sites generally found in URR of different HPV types. It is possible, therefore, that these HPVs may use additional cellular factors to ensure the chromatin attachment of their genomes, or use maybe entirely different strategy for their efficient extrachromosomal maintenance. Further studies are clearly needed to clarify this point.

CONCLUSIONS

1. p53 suppresses the transient amplificational replication of different papillomavirus ori constructs in various human and hamster cell lines. The suppression reflects most likely the direct effect of p53 on the papillomavirus amplificational replication and does not require N-terminal transactivation as well as C-terminal regulatory domains of the protein. On the other hand, p53 is unable to interfere with the once per cell-cycle replication of Epstein-Barr virus oriP in similar transient transfection assays.
2. The transient amplificational replication of BPV1 URR reporter plasmid can take place in cells that already maintain stably extrachromosomal copies of similar reporter plasmid. The copy number of stably replicating plasmid is unaffected by the simultaneous transient amplification of similar reporter plasmid and stable maintenance replication is also insensitive to p53. It suggests that the initial amplificational- and following long-term stable maintenance replication of BPV1 are using two clearly different mechanisms.
3. The BPV1 URR reporter plasmids are tightly associated with the mitotic chromosomes if maintained as stable episomal plasmids as well as in transient transfection experiments. E2 binding sites inside BPV1 URR have been shown to form a minichromosome maintenance element (MME), which, together with the minimal origin of viral replication, are sufficient *cis*-requirements for establishing of the BPV1 stable maintenance in E1, E2-expressing cells. We found that the role of MME in this process is to ensure the E2-dependent chromatin tethering. The E1 binding site (and thus the replication ability) as well as other sequences in the URR are dispensable for the chromatin attachment. There is a clear link between MME, chromatin attachment, and efficient stable maintenance, as none of the reporter constructs lacking intact MME and unable for stable episomal maintenance was tethered to mitotic chromosomes. The E2-mediated association of the viral genomes with nuclear chromatin is likely to guarantee the proper partitioning and nuclear retention of BPV1 genomes in dividing cells; it can perhaps also ensure the optimal exposure of viral DNA replication process to the cellular control mechanisms during S-phase.
4. The successful establishing of the BPV1 stable extrachromosomal maintenance involves the E2-dependent partitioning mechanism as well as specific changes in the replication mechanism of the viral DNA. Therefore, the BPV1 stable episomal maintenance seems to consist of two main functions — chromatin attachment, which provides proper partitioning and nuclear retention to the viral genomes, and controlled replication function, which is responsible for sufficient compensation of the plasmid loss occurring as a result of host cell divisions.

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KOKKUVÕTE

Papilloomiviirused (PV) on perekond väikeseid kaheahelalise DNA genoomiga epiteeliomaseid viirusi. Neid iseloomustab pikaajalise vaikioleku võime alusepiteeli paljunevates rakkudes ja nad on suutelised perioodiliselt põhjustama nakatunud kudedes healoomulisi vohandeid- soolatüükaid ja kondüloome. Mõned inimese PV tüübid on seotud ka pahaloomuliste emakakaela kasvajate tekkega.

Käesoleva doktoritöö aluseks olevates teadusartiklites olen koostöös oma kaastöötajatega uurinud seda, milliseid vahendeid kasutavad PVd tõhusaks peremeesrakus püsimiseks. Uurimismudelina oleme kasutanud veise papilloomiviirust BPV1. BPV1 genoomi sisenemisele rakku järgneb tema võimendatud replikatsioon peremeesraku tuumas, mille käigus valmistatakse kiiresti terve hulk viiruse genoomi koopiaid. Osa neist koopiatest seab end jagunevates peremeesrakkudes püsivalt sisse kromosoomiväliste rõngasmolekulidena, mille koopiaarv raku kohta hoitakse ühtlastes piirides. Püsiva koopiaarvu tagamiseks peab viirus kontrollima nii enda genoomi juurdekopeerimist kui tagama värskelt paljundatud viirusgenoomide võrdse jaotamise tütartuumade vahel peremeesraku pooldumise käigus. Meie katsete tulemused näitavad, et BPV1 esialgne võimendatud replikatsioon on alla surutav kasvajasuppressorvalgu p53 poolt; samas stabiilse kromosoomivälise püsimise käigus toimuv replikatsioonimehhanism ei ole p53-le tundlik. Esialgne võimendatud replikatsioon on suuteline toimuma rakkudes kus põhimõtteliselt samasugune viiruspõhine replikon juba stabiilselt replitseerub. Selline koosreplikatsioon ei mõjuta stabiilselt püsiva replikoni koopiaarvu. Nende tulemuste põhjal võib järeldada, et üks ja seesama BPV1 replikon on võimeline kasutama oluliselt erinevaid replikatsioonimehhanisme oma sisseseadmise käigus peremeesraku tuumas ja sellele järgneva stabiilse püsimise tarbeks.

Nii meie kui teiste teaduslaborite jõupingutuste tulemusena on selgunud, et BPV1 genoomide enam-vähem võrdselt tütartuumade vahel jagunemine poolduvas peremeesrakus toimub peremehe kromosoomidele kinnitumise kaudu. Kromosoomidele kinnitunud viirusgenoomi molekulid transporditakse mitoosis koos peremehe kromosoomidega tütarrakkude tuumadesse. Meie uurimused selgitasid, et selliseks kromosoomidele kinnitumiseks on viiruse poolt vajalikud ja piisavad viirusvalk E2 ja küllaldane arv tema seondumiskohti viiruse genoomis. Seega avaldub E2 seondumiskohtadele eelnevalt omistatud roll BPV1 stabiilses peremeesrakus püsimises just läbi tema võime vahendada viirusgenoomide seondumist tuumakromatiinile. Võimalik, et sellise kinnitumise abil tagatakse ka viiruse replikatsiooni kontroll, kuna tekib parem ligipääs raku enda genoomi replikatsiooni kontrollmehhanismidele.

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PUBLICATIONS

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p53 Protein Is a Suppressor of Papillomavirus DNA Amplificational Replication

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p53 protein was able to block human and bovine papillomavirus DNA amplificational replication while not interfering with Epstein-Barr virus oriP once-per-cell cycle replication. Oligomerization, intact DNA-binding, replication protein A-binding, and proline-rich domains of the p53 protein were essential for efficient inhibition, while the N-terminal transcriptional activation and C-terminal regulatory domains were dispensable for the suppressor activity of the p53 protein. The inhibition of replication was caused neither by the downregulation of expression of the E1 and E2 proteins nor by cell cycle block or apoptosis. Our data suggest that the intrinsic activity of p53 to suppress amplificational replication of the papillomavirus origin may have an important role in the virus life cycle and in virus-cell interactions.

Human papillomaviruses (HPVs) are small DNA viruses clearly associated with the induction of cancer. The papillomavirus life cycle can be divided into three stages (7, 20). First, following initial entry, the papillomavirus genome is amplified in the nucleus and viral copy number is increased up to 1,000 per haploid cell genome. During the second, maintenance stage, the viral DNA replicates in synchrony with the cellular DNA, at a constant copy number per cell. The third, vegetative replication stage of the viral genome occurs in the terminally differentiated cells. Papillomaviruses have developed an efficient system for modulating the activity of cellular tumor suppressor genes. HPV type 16 (HPV-16) and HPV-18 E6 proteins are capable of interacting with p53 and directing its degradation (50), while the E7 protein forms a complex with retinoblastoma protein (pRB) (15). These events lead to the loss of cell control over crucial events—DNA replication, repair and apoptosis—therefore creating favorable conditions for rapid viral DNA amplification and establishment of infection. In addition, expression of the E6 and E7 proteins may be an indication that some stages of papillomavirus replication during the three-step life cycle are susceptible to the action of p53 or pRB.

The tumor suppressor protein p53 is believed to be one of the key players in the control of the genomic stability of the cells (25, 27, 32). It is structured as a typical eukaryotic transcription activator which contains DNA-binding and transactivation domains and is able to activate or repress the transcription of certain genes (for a review, see reference 25). Exposure of normal cells to different stress conditions induces both an intracellular increase in the steady-state level of p53 and direct activation of the protein (23). As a result, the transition of cells in the cell cycle may be prevented, and apoptotic death of the cells with damaged DNA may be induced (reviewed in reference 32).

Several studies found that the mutation or loss of one or both alleles of p53 was sufficient to allow gene amplification to occur in the cells (36, 67), thus indicating that the p53 protein is involved in the control of events leading to the amplification of genomic sequences. The p53 protein seems to be directly involved in the control of DNA replication and repair (for reviews, see references in reference 25). It has been demonstrated that the p53 protein is capable of interacting with several proteins and enzymes involved in DNA repair or replication, such as single-stranded DNA (ssDNA)-binding replication protein A (RPA) (14, 33), cellular DNA helicases (47), and homologous recombination factor RAD51/RecA (53). The p53 protein lacking its C-terminal regulatory part blocks nuclear DNA replication in the transcription-free *Xenopus* egg extracts (13). Immunostaining studies show colocalization of the p53 protein with proliferating cell nuclear antigen (PCNA), DNA polymerase α, DNA ligase, and RPA at the sites of DNA replication in herpes simplex virus-infected cells (62). Replication of simian virus 40 (SV40) DNA can be prevented by binding to and inactivating the large T antigen by the p53 protein (52, 60). Replication of the polyomavirus origin is inhibited by p53 in vitro when up to 16 copies of the p53-specific binding sites have been inserted into the plasmid (39), while replication of the polyomavirus origin in vivo is activated by the same protein in a sequence-dependent manner (22).

We studied the effect of the p53 protein on the replication of papillomavirus origins in vivo in different cell lines and found that the p53 protein is a potent repressor of bovine and human papillomavirus amplificational replication. The repression of replication was dependent on the p53 protein concentration in the cells. We show that the intact central DNA-binding domain and the oligomerization domain of the p53 protein, as well as a part of the N-terminal domain containing the RPA-binding and proline-rich sequences, are essential for this activity. In the same time, the p53 protein and its mutants were unable to interfere with the once-per-cell cycle replication of Epstein-Barr virus (EBV) oriP. Repression of papillomavirus DNA amplification is neither an indirect consequence of the p53-dependent cell cycle block or apoptosis nor mediated by the transactivation or transrepression activities of the p53 protein. Possible implications of the observed phenomena on virus-cell interactions will be discussed.

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MATERIALS AND METHODS

Plasmids. Bovine papillomavirus type 1 (BPV-1) E1 expression vector pCGEag, E2 expression vector pCGE2, minimal replication origin plasmid pUCAlu, HPV-11 E1 expression vector pMT2-E1, HPV-11 E2 expression vector

pMT2-E2, and HPV-11 upstream regulatory region (URR)-containing plasmid p7072-99 have been described previously (11, 57). pNeoBgl40 contains the BPV-1-URR from nucleotides 6946 to 63 and has been described previously (44). The BPV-1 origin constructs pUC12B and pUC18A have been described previously (55). The HPV-18 E1 and E2 expression vector pCGE1B and origin plasmid pLCR have been reported earlier (45). Plasmid p994 harboring the EBV latent oriP is a kind gift of B. Sugden (24). Bcl-2 expression plasmid pcDBCL2 has been described by Mah et al. (37).

Human p53 cDNAs were cloned into expression vector pCG (54). pCGwtp53 and pCGtrp248 encode wild-type (wt) and Arg248Trp mutant p53 proteins, respectively. The mutant Arg248Trp p53 cDNA was kindly provided by Bert Vogelstein. All deletion mutants were created by PCR and expressed from the pCG vector. pCGΔN39 encodes wt p53 protein with deletion of the first 39 amino acids. pCGΔC362 and pCGΔ305 encode truncated proteins with stop codons at positions 362 and 305, respectively. pCGΔ324-355 encodes p53 with deletion of amino acids at residues 324 to 355. pCGΔN39ΔC362 and pCGΔN39ΔC362trp248 encode wild-type or Arg248Trp mutant p53 starting from amino acid 40 and containing a stop codon at position 362. ΔN61ΔC362 and ΔN92ΔC362 lack the first 61 and 92 N-terminal amino acids, respectively, and contain a stop codon at position 362. ΔProΔC362 and ΔN39ΔProΔC362 lack amino acids 63 to 91 and contain the stop codon at position 362; ΔN39ΔProΔC362 lacks also the N-terminal 39 amino acids. The correctness of the endpoints and all mutated sites of the p53 coding regions were verified by sequencing.

Cells and transfections. The cell line CHO and its derivatives CHO4.15 (expressing BPV-1 E1 and E2 proteins), CHOBgl40 (in addition containing latent BPV-1 origin plasmid), and CHO212 (expressing BPV-1 E1) (44) were maintained in Ham's F12 medium supplemented with 10% fetal calf serum. Human osteosarcoma 143 (66), Cos7, and SAOS-2 cells were maintained in Iscove's modified Dulbecco's medium with 10% fetal bovine serum. Electroporation experiments were carried out as described earlier, using an Invitrogen ElectroPorator at capacitance setting 960 μF. Voltage settings were 230 V for CHO, CHO4.15, and CHOBgl40 cells, 170 V for human osteosarcoma 143 cells, 180 V for Cos7 cells, and 210 V for SAOS-2 cells. Transfection efficiencies were determined by in situ staining of the cells transfected in parallel with the β-galactosidase-expressing plasmid pON260 (56). Transient replication assays were performed as described previously (56).

Immunoblotting and DNA binding assays. The expression level of p53 mutant proteins was estimated by Western blot analysis of CHO4.15 cells transfected with 500 ng of p53 expression plasmid and processed 24 or 48 h after transfection according to standard methods (48). Equal amounts of total protein were analyzed in each experiment. Antibodies pAb240, pAb421, and pAb1801 were used for detection of p53 proteins. The E2 protein level in CHO4.15 cells in the presence of expressed p53 constructs was analyzed in the same way, using a mixture of purified monoclonal E2-specific antibodies 1E2, 3F12, 1H10, 1E4, and 3C1 (2). Goat anti-mouse antibody conjugated to alkaline phosphatase was used as a secondary antibody.

The effect of p53 expression on E2-specific DNA-binding activity in CHO4.15 cells was measured as described earlier (2). Analysis was performed 48 h after transfection with p53 expression plasmids. p53-specific DNA binding was tested by an analogous protocol, using the artificial p53-binding double-stranded oligonucleotide 5'AGACATGCCTAGACATGCCT3' (21). Monoclonal antibodies pAb421 and 3F12 were added for supershifting the p53-specific and E2-specific complexes, respectively. Monoclonal antibody HO7.1 was used for p53 deletion mutants lacking the pAb421 epitope.

Northern blotting of E1 mRNA. CHO4.15 cells were transfected with 500 ng of p53 expression constructs, and 48 h later the total RNA was extracted by using an RNeasy Total RNA kit supplied by Qiagen. Northern blot analysis of the extracted RNA was performed according to standard methods (48). E1-specific radioactive probe was generated by random priming using the 1.8-kb *Xba*I-*Eco*91I BPV-1 E1-encoding fragment from pCGEag (57) as a template. E1-specific signals were quantitated on a PhosphorImager SI (Molecular Dynamics), and the results were normalized to S7- and β-actin-specific signals. Human ribosomal protein S7 (3) and β-actin cDNA plasmids used as a templates to generate radioactive probes were kind gifts of Tarmo Annilo and Mati Reeben, respectively.

Analysis of cell cycle distribution and sub-G_1 DNA content of p53-transfected CHO4.15 cells. Both floating and adherent cells were collected 48 h posttransfection, washed once with phosphate-buffered saline (PBS), and fixed in 5 ml of ice-cold 70% ethanol for flow cytometric analysis. The propidium iodide fluorescent staining of nuclei was analyzed in an ATC3000 flow cytometer (Odam-Brucker, Wissembourg, France) equipped with a Spectraphysics argon laser. Cells were pelleted prior to the analysis, washed once in PBS, suspended in 500 μl of PBS with 1 mM $MgCl_2$ and 30 μg of RNase A per ml, and incubated at 37°C for 1 h to digest cellular RNA. Propidium iodide was added to a final concentration of 10 μg/ml, and samples were incubated on ice for at least 15 min to stain the nuclear DNA. The signals from 50,000 cells were collected from each sample and analyzed by the method of Dean and Jett (13a), using the standard software provided by the manufacturer of the flow cytometer. Cells for the parallel replication assay were processed as described above. The terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed as described in reference 17.

RESULTS

p53 protein inhibits amplificational replication of papillomavirus origins. We have developed an efficient model system to study the replication of papillomavirus origins in tissue culture (11, 44, 45, 56). To determine whether the p53 protein has any effect on the replication, we performed transient replication assays in CHO-K1 cells, where BPV-1 and HPV origin-containing plasmids replicate in the presence of homologous and heterologous E1 and E2 proteins (11, 57). CHO cells have been used extensively for DNA amplification studies and have been shown to carry the defective p53 gene with substitution Thr211Lys (28). Inspection of these cells with a mixture of p53-specific antibodies did not reveal any detectable endogenous expression of the p53 protein in our hands (data not shown).

Cotransfection of the BPV-1 E1 and E2 expression plasmids with the BPV-1 origin plasmid into CHO cells resulted in robust replication (Fig. 1A, lane 1). Coexpression of human wt p53 protein suppressed BPV-1 origin replication almost completely in this system (Fig. 1A, lane 2). The extent of suppression was proportional to the amount of introduced p53 expression plasmid (Fig. 1B) and was detected at 25 ng of the cotransfected plasmid DNA. The effects of p53 protein expression on the replication of the HPV-11 (Fig. 1A, lanes 3 and 4) and HPV-18 (lanes 5 and 6) origin plasmids in the presence of the homologous E1 and E2 replication proteins were identical. The replication signal of the HPV origins in CHO cells decreased for the third time point (96 h posttransfection), possibly as a result of the less intense replication and the loss of E1 and E2 expression plasmids from the cells upon cell division. Cotransfection with the vector carrying no p53 sequences did not affect replication of the papillomavirus origin (Fig. 1A, lane 7), which indicates that the block of replication is not caused by promoter competition between the p53, E1, and E2 expression cartridges. Experiments carried out with mouse wt p53 protein gave identical results (data not shown).

In the next step, we studied the effect of p53 on the replication of different BPV-1 origin deletion mutants in the cell line CHO4.15. This cell line exhibits constitutive expression of BPV-1 E1 and E2 replication proteins from the integrated expression vectors (44). Figure 1C represents replication of the BPV-1 full-length origin plasmid pNeoBgl40 and of origin deletion variants pUCAlu, pUC12B, and pUC18A in the absence and presence of overexpressed p53 protein. Our data show that the replication of plasmids pNeoBgl40, pUCAlu, pUC12B, and pUC18A (depicted schematically in Fig. 1D) is efficiently blocked by the overexpressed p53 protein (Fig. 1C, lanes 2, 4, 6, and 8) and suggest that there are no defined p53-specific *cis* elements in the BPV-1 origin of replication that could mediate the effect, unless it is the minimal replication origin itself: A/T-rich region and E1- and E2-binding sites.

Structural determinants of the p53 protein responsible for inhibition of amplificational replication of the BPV-1 origin. To map the domains of the p53 protein responsible for the inhibition of papillomavirus replication, a set of p53 mutants was constructed (schematically depicted in Fig. 2A). The stability, expression level, and activity of the mutant proteins were tested in CHO4.15, Cos7, and SAOS-2 cell lines by Western blot and specific DNA band shift analysis. The mutant proteins with the deleted N-terminal activation domain were expressed at an approximately fivefold-higher level than proteins with the intact N terminus, wt p53, ΔC305, ΔC362, and ΔProΔC362. The N-terminal activation domain contains the binding site of the Mdm2 protein, which has been shown to facilitate degradation of the p53 protein in vivo and therefore reduce the

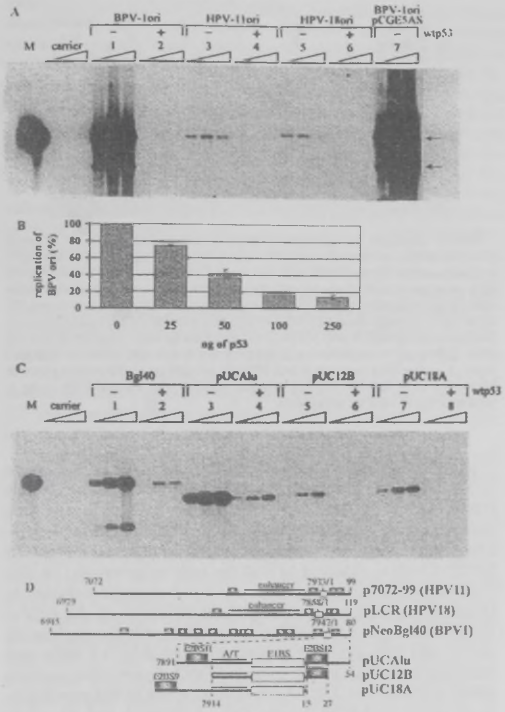

FIG. 1. Southern blot analyses. p53 suppresses the amplificational replication of different papillomavirus replication origins. Episomal DNA was extracted from cells at 48, 72, and 96 h after transfection and digested with restriction endonucleases *Pst*I and *Dpn*I. Filters were probed with radiolabeled HPV-11 URR containing plasmid p7072-99. M, 200 pg of the linear HPV-11 origin plasmid marker; carrier, mock-transfected cells. Arrows indicate the bands generated after digestion of the episomal BPV-1 origin plasmid with *Pst*I. (A) Effect of wt p53 expression on the transient replication of BPV-1, HPV-11, and HPV-18 full-length origin plasmids in CHO cells. In this assay, 100 ng of BPV-1 origin pNeoBgl40 (lanes 1 and 2), HPV-11 origin p7072-99 (lanes 3 and 4), or HPV-18 origin pLCR (lanes 5 and 6), with (+) or without (−) 100 ng of wt p53 expression construct pCGwtp53, was transfected. pCGE5AS, control with plasmid producing no p53 (lane 7). Amounts of E1 and E2 expression vectors used were 250 ng for BPV-1 (pCGEag and pCGE2), 500 ng for HPV-11 (pMT2-E1 and pMT2-E2), and 650 ng for HPV-18 (pCGE1B). (B) The inhibition of replication of the BPV-1 replication origin is proportional to the amount of introduced p53. The replication signals of two independent experiments (72 h posttransfection) were quantified with a PhosphorImager, and signals from cells transfected with origin plasmid only were used as a control to normalize the results. (C) Effect of wt p53 expression on the transient replication of plasmids containing different BPV-1 origin constructs in CHO4.15 cells. In this assay, 100 ng of wt p53 expression plasmid and 100 ng of each BPV-1 replication origin construct were transfected into the cells. Lanes: 1 and 2, replication of full-length BPV-1 origin plasmid pNeoBgl40; 3 and 4, origin plasmid pUCAlu; 5 and 6, origin plasmid pUC12B; 7 and 8, origin plasmid pUC18A. (D) Schematic representation of the papillomavirus replication origin inserts used. The specific transcription enhancer region in HPV replication origins (enhancer), the BPV-1 origin A/T-rich region (A/T), and E1 protein (E1BS; unfilled boxes)- and E2 protein (E2BS9, E2BS11, and E2BS12; shadowed boxes)-binding sites are indicated. Numbers indicate positions on the HPV-11, HPV-18, or BPV-1 nucleotide sequence.

half-life and steady-state level of the p53 protein in cells (19, 26). All of the mutants except those with point mutation Trp248 and deletions ΔC305 and Δ324-355, gave a specific complex with the double-stranded oligonucleotide corresponding to the artificial p53-binding site (21). The intensity of the band shift correlated with the expression level of the p53 proteins in the extract (data not shown).

The BPV-1 origin plasmid and the different mutant p53 protein expression plasmids were cotransfected into CHO4.15 cells; episomal DNA was harvested and analyzed by Southern blotting (Fig. 2B). wt p53, the C-terminal regulatory domain-defective mutant ΔC362, the N-terminal deletion mutant ΔN39 lacking the transcription activation domain, and the double-

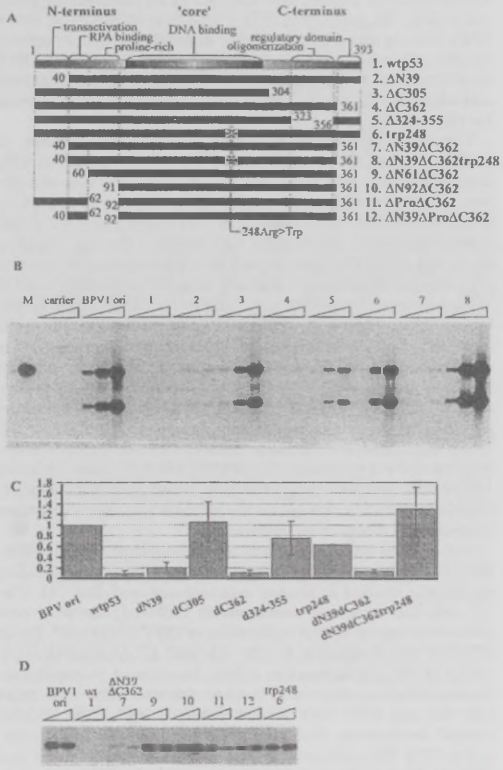

FIG. 2. Mapping of the p53 domains necessary for suppression of papillomavirus amplificational replication. (A) Schematic representation of p53 mutants. Numbers indicate positions on the amino acid sequence. (B) Southern blot analysis of the transient replication of BPV-1 origin plasmid pNeoBgl40 in the presence of different p53 mutants in the CHO4.15 cell line. Episomal DNA was extracted from cells at 48, 72, and 96 h after transfection and digested with restriction endonucleases *Pst*I and *Dpn*I. Filters were probed with radiolabeled HPV-11 URR containing plasmid p7072-99; 100 ng of pNeoBgl40 together with 250 ng of p53 expression plasmid was transfected into the cells. Lanes 1 to 8 correspond to the transfections with p53 mutants in the same order as depicted in panel A. Carrier, mock-transfected cells; BPV1 ori, control with no added p53. (C) Relative inhibition of replication of the BPV-1 replication origin by different p53 mutants. The replication signals of three independent experiments (72 h posttransfection) were quantified with a PhosphorImager and signals from the cells transfected with origin plasmid only were used as a control to normalize the results. (D) Southern blot analysis of transient replication of the BPV-1 origin plasmid pUCAlu in the presence of additional N-terminal p53 deletion mutants in the CHO4.15 cell line. Episomal DNA was extracted from cells at 72 and 96 h after transfection and digested with restriction endonucleases *Pst*I and *Dpn*I. Filters were probed with radiolabeled pUCAlu plasmid. Lanes 1, 7, 9, 10, and 11 correspond to transfections with p53 mutants as depicted in panel A.

deletion mutant ΔN39ΔC362 all retained the ability to suppress replication (Fig. 2B; compare lanes 1, 2, 4, and 7 with lane BPV1 ori). The replication signals from three independent experiments were measured with a PhosphorImager, and the data are presented in Fig. 2C. The mutants with a deleted oligomerization domain (Δ324-355) or the whole C-terminal part of the protein up to amino acid 305 (ΔC305) (Fig. 2B, lanes 3 and 5; Fig. 2C) had little or no effect on replication. The point mutation Arg248Trp in the DNA-binding domain of the p53 protein abolished the suppressor activity of the full-size p53 protein (Fig. 2B, lane 6) and even seemed to convert the double-deletion mutant ΔN39ΔC362 to an activator of replication (Fig. 2B, lane 8; Fig. 2C). These data indicate that intact DNA-binding and oligomerization domains are both necessary for the p53 protein activity to suppress papillomavirus DNA amplificational replication, while the N-terminal transcription activation and C-terminal regulatory domains are dispensable for this activity.

The active p53 deletion mutant ΔN39ΔC362 contains, in addition to the DNA-binding core region (residues 100 to 300), flexible linker region (residues 301 to 320), and oligomerization domain (residues 320 to 360) (25), also the RPA-binding domain (residues 40 to 60) (1, 14, 30) and a proline-rich putative binding site for proteins with the SH3 domain (residues 61 to 91) (59). We constructed four additional p53 deletion variants and tested their stability and DNA-binding activity. The constructed mutants were stable in CHO4.15 cells and bound DNA sequence specifically, as measured by DNA gel shift assay (data not shown). These mutants were used for the suppression of replication of the minimal origin plasmid pUCAlu in CHO4.15 cells (Fig. 2D). None of the newly constructed deletion mutants was able to block replication of the pUCAlu origin plasmid comparably to wt p53 or ΔN39ΔC62. These data indicate that four domains of the p53 protein—oligomerization (residues 320 to 360), DNA-binding (residues 100 to 300), proline-rich (residues 61 to 92), and RPA-binding (residues 40 to 61) domains—are necessary for the replication suppressor activity of the protein.

p53 protein suppresses only amplificational DNA replication. The action of p53 and its mutants on different replication modes was studied in human osteosarcoma cell line 143. The 143 cell line expresses constitutively EBNA-1, the only viral protein necessary for the replication of EBV latent oriP. These cells are also permissive for the E1- and E2-dependent replication of the papillomavirus origin. In contrast to papillomaviruses, which quickly amplify their genome after viral entry into the cell, EBV oriP probably makes use of the cellular control mechanisms that guarantee once-per-cell cycle replication (65). We cotransfected the plasmids encoding p53 and HPV-11 E1 and E2 proteins together with the HPV-11 origin plasmid and EBV oriP plasmid into the 143 cells and studied their replication by Southern blot analysis. The replication assay conditions were adjusted so that relative replication signals of oriP and HPV-11 origin had comparable intensities on the same Southern blot. Once-per-cell cycle replication of the oriP-containing plasmid was not suppressed by wt p53, while amplificational replication of the papillomavirus origin was abolished in the same cells (Fig. 3; compare lanes 1 and 2). The mutant p53 proteins affected papillomavirus replication similarly in the 143 cells and CHO cells. Mutants ΔN39 and ΔC362, which suppressed replication of the BPV-1 full-length origin in CHO4.15 cells, also blocked replication of the HPV-11 origin in the 143 cell line and at the same time had little effect on the replication of oriP (lanes 3 and 5). Mutants ΔC305 and Δ324-355 influenced the replication of neither HPV-11 origin nor oriP (lanes 4 and 6).

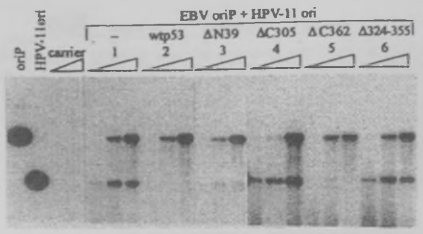

FIG. 3. Southern blot analysis of coreplication of oriP and HPV-11 origin plasmids in human osteosarcoma 143 cells. p53 blocks replication of the papillomavirus origin but not EBV oriP. Episomal DNA was extracted at 48, 72, and 96 h posttransfection, digested with *Bam*HI and *Dpn*I, and probed with radiolabeled origin plasmid p7072-99. One microgram of oriP plasmid p994 and 250 ng of HPV-11 origin plasmid p7072-99 together with HPV-11 E1 and E2 expression plasmids pMT-E1 and pMT-E2 (1 μg of each) were transfected into the cells; 250 ng of wt or mutant p53 expression plasmid was added as indicated (lanes 2 to 6). Other lanes: oriP and HPV-11 ori, 200 pg of the marker plasmids linearized with *Bam*HI; carrier, negative control with carrier DNA only; 1, positive control with no added p53.

p53 inhibits amplificational replication of the BPV-1 origin in SAOS-2 cells. Replication of the papillomavirus origin was tested also in human osteosarcoma SAOS-2 cells that lack endogenous p53 and pRB expression. The expression of exogenous wt p53 and several transactivation-competent mutants in SAOS-2 cells is sufficient to lead the cells to apoptosis (10, 68). To avoid these side effects, we used p53 mutants deficient in transcription activation activity. Cotransfection of the BPV-1 E1 and E2 expression plasmids together with the replication origin and p53 expression plasmids into SAOS-2 cells and subsequent analysis of the episomal DNA showed that mutants ΔN39 and ΔN39ΔC362 inhibited replication of the papillomavirus origin in SAOS-2 cells (Fig. 4, lanes 3 and 4), while mutants Trp248, ΔN39ΔC362 Trp248, and Δ324-355 (lanes 2, 5, and 6, respectively) had no effect on replication. These data are similar to the results of experiments with the cell lines CHO4.15 (using BPV-1 origin) and 143 (using HPV-11 origin) and suggest that the suppression of papillomavirus replication is a direct intrinsic property of the exogenously expressed p53 protein and is neither influenced by the endogenous p53 nor achieved through the pRB-regulated pathways.

p53 does not cause downregulation of expression of the E1 and E2 proteins. The E1 and E2 proteins are absolutely nec-

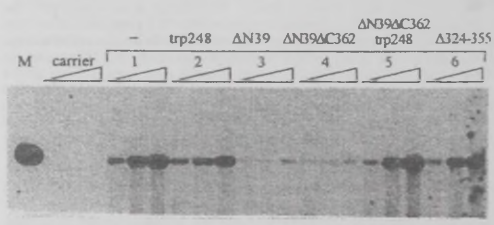

FIG. 4. Southern blot analysis of the BPV-1 origin plasmid pUCAlu in SAOS-2 cells (radiolabeled origin plasmid pUCAlu used as a probe). p53 mutant proteins inhibit replication of the BPV-1 minimal origin in SAOS-2 cells lacking endogenous p53 and pRB proteins. BPV-1 minimal origin plasmid pUCAlu (500 ng) together with BPV-1 E1 and E2 expression plasmids pCGEag and pCGE2 (1 μg of each) was transfected into the cells; 500 ng of p53 mutant proteins was cotransfected as indicated (lanes 2 to 6). Other lanes: M, 200 pg of the pUCAlu marker linearized with *Pst*I; carrier, control transfection with carrier DNA only; 1, positive control with no p53 construct added.

essary for papillomavirus replication. The p53 protein has been shown to possess transcription repressor activity in certain cases. Therefore, the inhibition of papillomavirus replication could, in principle, be achieved by downregulation of the expression level or activity of these proteins. We studied the expression level and activity of the BPV-1 replication proteins in CHO4.15 cells in the presence of the overexpressed wt and mutant p53 proteins. E2 protein expression is directed by the *HSP70* promoter, and E1 protein expression is directed by the SRα promoter in CHO4.15 cells (44). These cells are very efficiently transfected by electroporation (about 70%, based on parallel β-galactosidase expression vector pON260 transfections), and this fact served as a rationale for the measurements described below. The transfected CHO4.15 cells were studied for the expression level of the E2 protein by Western blot analysis of the cell lysates. Transfection efficiencies were determined in parallel in all experiments. Western blot analysis did not reveal any reproducible effects of the expression of wt or mutant p53 proteins on the steady-state level of the E2 protein in CHO4.15 cells (Fig. 5A). A possibility remained that p53 could modulate the activities of the E2 protein, for example, the ability to bind DNA.

We performed a DNA mobility shift assay of CHO4.15 cell lysates transfected with p53 expression constructs. The lysates were tested for E2-specific DNA binding with the oligonucleotide corresponding to E2-binding site 9 of the BPV-1 genome (34). To increase the specificity of the assay, we supershifted the E2-DNA complex with an excess of the E2-specific monoclonal antibody 3F12. E2-specific radioactive signals were measured with a PhosphorImager, and the results were normalized to the total amount of protein in the lysate, as determined by the Bradford assay (8). As in the case of measuring the steady-state level of the E2 protein, we were unable to detect any significant changes in the levels of active E2 protein in response to the expression of wt or mutant p53 proteins in CHO4.15 cells (Fig. 5C).

The low expression level of the E1 protein in CHO4.15 cells made it impossible to detect E1 by quantitative Western blot analysis or immunoprecipitation. Instead, we performed Northern blot analysis and analyzed the steady-state level of the E1 mRNA in response to p53 expression (Fig. 5B). The transcription level of the E1 protein coding sequence was determined relative to β-actin and ribosomal protein S7 mRNA levels on the same blots (Fig. 5B and C), and E1 mRNA-specific hybridization signals were measured with a PhosphorImager. Quantitation of the E1 mRNA level normalized to β-actin and S7 mRNA levels showed no downregulation of the E1 mRNA level in response to wt and mutant p53 expression in CHO4.15 cells (Fig. 5C).

These data suggest that the effect of p53 on papillomavirus amplificational replication is not caused by downregulation of expression of the E1 or E2 proteins, although these experiments do not exclude the possibility that p53 interferes with E1 or E2 (or both) activities at some stage of initiation or elongation of replication.

The inhibition of papillomavirus replication is not the consequence of p53-induced cell cycle block or apoptosis. p53 is a mediator of cell cycle block and apoptotic cell death. To examine the possibility that the suppression of papillomavirus amplification is an indirect consequence of any (or both) of these effects, we analyzed the p53-transfected CHO4.15 cells by flow cytometry. Overexpression of wt p53 protein in CHO4.15 cells induced detectable apoptosis in the culture, as shown by the appearance of the sub-G_1 DNA-containing fraction in the cell cycle profile 48 h posttransfection (Fig. 6A, panel 4). To examine the possible connection between p53-induced apoptosis and the suppression of replication, we made use of the ability of the Bcl2 protein to prevent the p53-induced apoptosis of cells (51). Increasing amounts of the Bcl2 expression plasmid were transfected into the cells. The expression of Bcl2 considerably reduced the amount of cells in the sub-G_1 DNA-containing fraction of the cells transfected with wt p53 (compare panels 4, 5, and 6). The cells transfected with p53 deletion mutant ΔN39ΔC362 as well as with the same deletion mutant with the Arg248Trp point mutation had some small sub-G_1 fraction, probably induced by electroporation, which was not influenced by the expression of Bcl2 in the cells (panels 7 to 12). The percentage of the apoptotic cells in these experiments was measured also by the TUNEL assay (Table 1), which gave essentially the same result and showed that Bcl2 expression in CHO4.15 cells reduced considerably the number of the p53-induced apoptotic cells in the culture. The distribution of CHO4.15 cells in G_1/G_0, S, and G_2/M stages of the cell

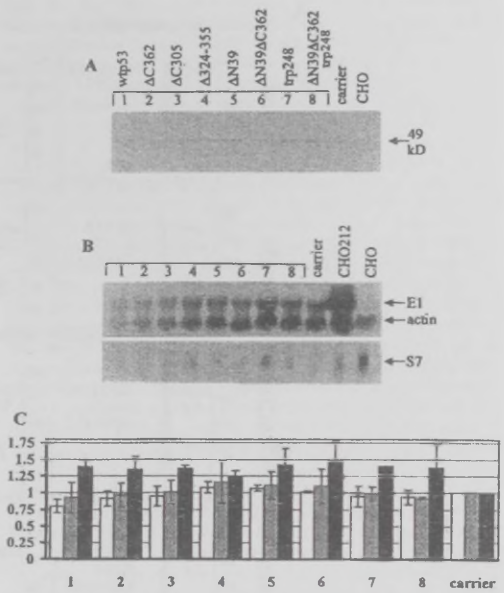

FIG. 5. Expression of p53 does not affect the level of E1 and E2 expression. A p53 expression construct (500 ng) was electroporated into CHO4.15 cells. In panels B and C, lanes and columns 1 to 8 represent transfections with different p53 mutants in the same order as in panel A. carrier, control with carrier DNA only; CHO, mock-transfected CHO cells (lacking both E1 and E2 expression). All analyses were performed 48 h posttransfection. (A) Western blot analysis of the E2 protein levels in p53-transfected CHO4.15 cells, using a mixture of five different E2-specific monoclonal antibodies (see Materials and Methods). (B) Northern blot analysis of the endogenous E1 mRNA levels in total RNA preparations from transfected CHO4.15 cells. CHO212, total RNA from E1-expressing cell line CHO212. The same filter was probed first with radiolabeled E1- and β-actin-specific probes and then reprobed with ribosomal protein S7-specific probe. Approximate lengths for mRNAs are 700 bp for S7, 2.0 kb for β-actin and 2.3 kb for E1. (C) Quantitation of the E1 Northern blots and E2 gel shift assay with a PhosphorImager. The E1 mRNA-specific signals in the total RNA preparations were normalized to the β-actin (open columns) and ribosomal protein S7 (shaded columns) mRNA signals in the RNA samples. Black columns represent the E2 gel shift data. The E2-specific signal in the lysates of the mock-transfected cells and the normalized E1 mRNA-specific signal from carrier-transfected control cells were set at 1.0 in each experimental series. Each column represents the average of two independent experiments.

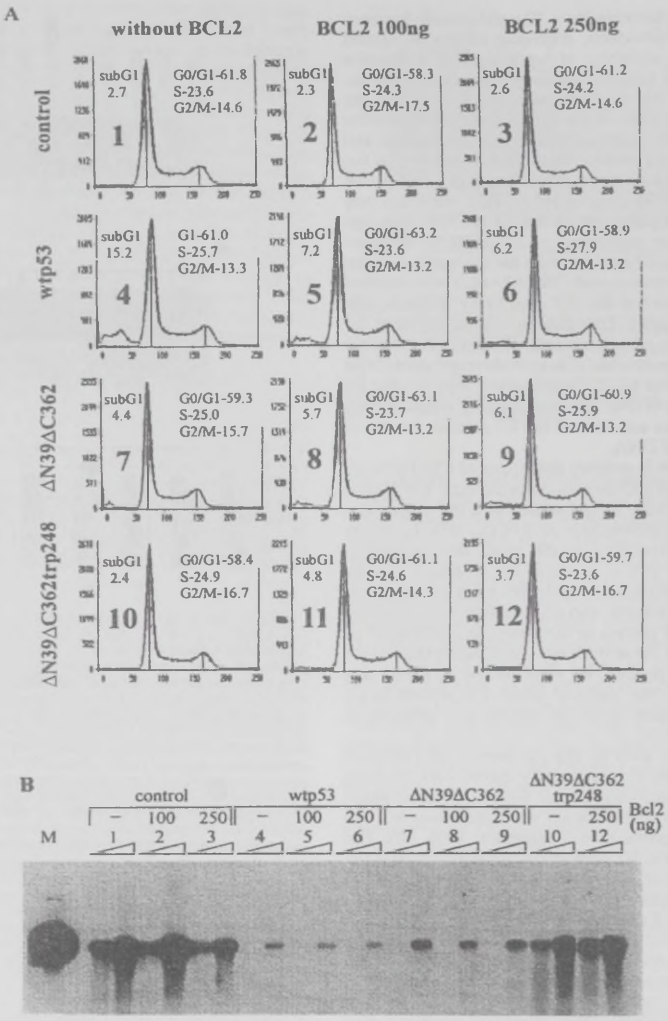

FIG. 6. Suppression of BPV-1 amplificational replication by p53 proteins is not the consequence of the p53-induced apoptosis or cell cycle block. (A) Flow cytometric analysis of the cell cycle distribution and the sub-G_1 DNA-containing apoptotic fraction of the p53-transfected CHO4.15 cells. In this assay, 250 ng of the p53 expression constructs without Bcl2 or together with 100 or 250 ng of the Bcl2 expression plasmid pcDBCL2 was transfected into the CHO4.15 cells; 100 ng of BPV-1 full-length origin plasmid pNeoBgl40 was used in each transfection. control, cells with no p53 expression constructs added. Cells were fixed 48 h after transfection. The percentage of apoptotic sub-G_1 DNA-containing signals and the calculated percentages of cells in G_0/G_1, S, and G_2/M phases (from total of 50,000 cells) are indicated on the each graph. *y* axis, cell number; *x* axis, DNA content. The sub-G_1 DNA fraction was not considered in the cell cycle calculations. Standard software provided by the manufacturer (Odam-Brucker) was used for the cell cycle calculations. (B) Southern blot analysis of the episomal DNA in the cells cotransfected with p53, Bcl2, and the BPV-1 origin plasmid pNeoBgl40. Episomal DNA was extracted at 72 and 96 h after transfection, digested with *Hin*dIII and *Dpn*I, and probed with radiolabeled origin plasmid pUCAlu. Lanes: M, 200 pg of the marker plasmid linearized with *Hin*dIII; 1 to 12, transfections 1 to 12 in panel A.

cycle was not influenced by the expression of Bcl2 or p53. We also analyzed if the Bcl2 rescues the replication suppression induced by p53 or its mutants. Expression of Bcl2 in CHO4.15 cells did not influence the replication of the BPV-1 origin itself, nor did it abrogate the inhibitory effects of wt p53 and deletion mutant ΔN39ΔC362 on replication of the origin (Fig. 6B, lanes 1 to 9). These data support the conclusion that the effect of the p53 protein on papillomavirus amplificational replication is not an indirect consequence of cell cycle block or apoptotic cell death.

TABLE 1. Apoptotic fraction in total population of CHO4.15 cells transfected with p53 and Bcl2 constructs (as measured by TUNEL assay)

Cells	Apoptotic fraction (%)	
	No Bcl2 cotransfected	250 ng of Bcl2 cotransfected
Control (no p53 transfected)	ND[a]	2.9
Transfected with p53 construct:		
wt p53	19.2	6.0
ΔN39ΔC362	2.3	6.7
ΔN39ΔC362Trp248	4.8	ND

[a] ND, not determined.

DISCUSSION

p53 as a suppressor of papillomavirus amplificational replication: possible implications for virus-cell interactions. Amplificational replication of papillomavirus DNA is initiated after entry of the viral genome into the cell nucleus, which leads to a rapid increase in copy number of the virus genome during S phase (20). Papillomaviruses rely on cellular replication factors and enzymes (40) and coordinate the initiation of replication by two viral origin recognition and initiation proteins, E1 and E2 (11, 45, 56, 57, 64). The same viral proteins are used at the following latent replication stage. The mechanism of switching from amplificational to controlled-maintenance replication is unknown. Our data show that amplificational replication of bovine and different human papillomaviruses in the short-term replication assay can be suppressed by the p53 protein in all cell lines studied. It seems not to require any response elements in the origin of replication. It also does not require any activities carried by the C-terminal regulatory and N-terminal transactivation domains of the p53 protein, including the ability to activate transcription. The DNA-binding domain of p53 has been shown to be the target for most of the missense mutations which inactivate the tumor suppressor function of this protein in cells (12). Incidentally, the very same mutations inactivated p53 in the replication system studied.

p53 has been shown to block the replication of SV40 by interacting with large T antigen. The binding of SV40 large T antigen by the p53 protein downregulates the helicase function of the T antigen (52); in addition, p53 competes with DNA polymerase α for the binding of SV40 large T antigen at the initiation of SV40 DNA synthesis (16). Mouse polyomavirus replication was shown not to be inhibited by the p53 protein (22, 39) unless additional (up to 16) p53-specific RGC sites were included in the plasmid (39). This shows that sensitivity of the viruses within the papovavirus family to the action of tumor suppressor protein p53 is variable and obviously reflects the differences in the viral life cycles and different strategies for the utilization of cellular control mechanisms by these viruses. Papillomaviruses must infect basal epithelial cells in order to establish productive infection of basal and suprabasal epithelial cells. Amplificational replication of the viral genome in these cells is essential for the establishment of infection. The oncoproteins encoded by the E5, E6, and E7 open reading frames of papillomaviruses are essential for providing the cellular environment for the replication of viral DNA. However, amplificational replication has to be controlled in order to avoid overreplication and unscheduled death of basal or suprabasal cells, because the synthesis of late genes and the production of infectious particles takes place only in the terminally differentiated epithelial cells. It is tempting to speculate that the ability of p53 to block the papillomavirus amplificational replication characterized in the model system studied is actually used by the virus to control the productive infection of basal cells. The E6 proteins of the high-risk (50) and low-risk (35) HPVs have been shown to interact with p53; however, only E6 from the high-risk HPVs directs p53 to degradation (50). It can be speculated that the binding of p53 by the E6 proteins of either high-risk or low-risk human and animal viruses reduces the replication suppressor activity of p53. Other important players in this regulatory mechanism are the replication proteins E1 and E2, which determine the efficiency of initiation of replication. The expression level of these proteins would certainly depend on the copy number of the viral genome, therefore providing the positive feedback for amplification. The papillomavirus replication proteins E1 and/or E2 have been shown to repress the promoter which is closest to the replication origin and directs E6 expression (31, 38, 49, 58). Therefore, higher levels of the E1 and E2 proteins would reduce the level of E6, which in turn results in the higher level of the active p53 protein capable of suppressing replication. These interrelationships among p53, E6, E1, and/or E2 proteins could provide a regulatory loop which can be used by some papillomaviruses to keep viral genome amplification in optimal limits (Fig. 7). The proposed regulatory loop could further serve as one of the mechanisms for the copy number control of the replication of papillomavirus genome during the latent infection of the basal cells. However, the mechanism may be different with different papillomavirus types, as, for example, attempts to find any interaction between BPV-1 E6 and p53 have appeared to be unsuccessful. It is still possible that in this case some other step in the cellular control pathways, up- or downstream of p53 itself, may be neutralized by viral regulatory proteins.

The putative mechanism of action of the p53 protein. The p53 protein, in principle, could suppress papillomavirus DNA replication in vivo by a number of different mechanisms, such as by arresting the cell cycle, inducing apoptotic death of cells, downregulating the expression or activity of the E1 and E2 proteins, or interfering with viral and cellular replication proteins at the stages of initiation or elongation of DNA replication.

The induction of apoptosis or cell cycle block is an unlikely mechanism for the apparent suppression of replication by p53 or its mutants in the cells studied, as shown by the measure-

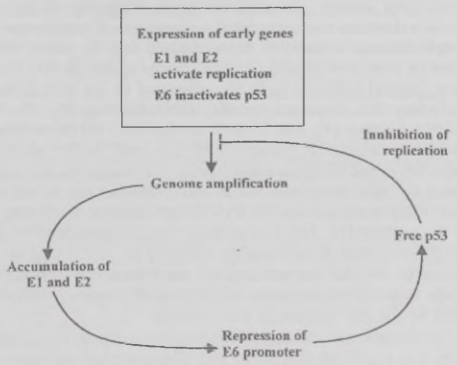

FIG. 7. A putative p53-controlled regulatory loop in the amplificational replication step of the papillomavirus life cycle.

ment of apoptosis and distribution of cells in the cell cycle. In addition, the efficient rescue of CHO4.15 cells from the wt p53-induced apoptosis by Bcl2 expression did not affect the suppression of BPV-1 origin replication in the same cells. Mutant ΔN39ΔC362 efficiently blocked replication of the papillomavirus origin in all of the studied cells but was unable to induce any detectable apoptosis. Additional convincing data come from the coreplication assay of the EBV and HPV-11 origins, which show that in the same cells two origins have differing sensitivity to the expression of p53 or its mutants. Replication of EBV oriP (65) and the papillomavirus origin (18) takes places during the S phase of the cell cycle, and intensive apoptotic death of the cells or cell cycle block should have also considerably reduced the replication of EBV oriP. Therefore, these experiments exclude several indirect and obvious explanations for the observed suppression of papillomavirus amplificational replication. It also seems unlikely in the light of these data that the replication block could have been achieved through the inactivation of general replication factors such as RPA, PCNA, and others by the expression of p53 or its mutants, because those factors are presumably used for the replication of EBV oriP and chromosomal DNA as well.

Another simple explanation is that the p53-induced suppression of papillomavirus replication could have been achieved through negatively modulating the activity of essential viral replication proteins (similarly to the case of SV40 virus) or through downregulating the expression of these proteins. However, we could not detect any significant p53-induced drop of the expression level and DNA-binding activity of the E2 protein and transcription level of E1 in CHO4.15 cells. Also, there are no data in the literature showing the interaction of the p53 protein with the E1 or E2 proteins of any papillomaviruses or demonstrating the modulation of activities of these proteins by p53. Therefore, the p53 protein has to act at later stages of replication initiation process, i.e., loading of the replication complex on the origin, unwinding of DNA, or elongation of the replication fork.

Our findings are substantiated by the fact that the C-terminally truncated form of the p53 protein (analogous to our mutant ΔC362) is able to block nuclear DNA replication in vitro in the transcription-free DNA replication extract from *Xenopus laevis* activated eggs (13). As for the suppression of amplificational replication of papillomavirus origin in the somatic cells, the DNA-binding activity of p53 was needed for the block of nuclear DNA replication in the transcription-free *Xenopus* extracts. It is possible that these two replication systems have similar p53-sensitive steps. Mapping of the p53 protein domains necessary for the repression of papillomavirus amplificational replication demonstrated that the intact DNA-binding core and oligomerization domains are clearly necessary. Several activities have been mapped to the core domain, including the sequence-specific DNA-binding (6, 43, 61), ssDNA-binding (4), and 3′-to-5′ exonuclease (41) activities of the p53 protein. All these activities, as well as the ability to suppress papillomavirus amplificational replication, are inactivated by point mutations which either abolish the direct contact of the protein with DNA or induce inactive conformation of the protein (12, 41). It is unlikely that the sequence-specific double-stranded DNA-binding function of p53 could be responsible for the suppression of amplificational replication, while sequence-nonspecific ssDNA-binding activity could be used by the p53 protein in this process.

Full-length p53 protein DNA-binding activity is regulated, sterically or allosterically, by the C-terminal domain of the protein (for a review, see reference 25). In addition, the C-terminal domain binds to DNA bulges resulting from DNA deletion/insertion mismatches (29) and also to the ends of short ssDNA molecules (5), promoting the reannealing of complementary strands (9, 42). Deletion of the last 30 residues, which has previously been shown to remove the above-mentioned activities of the p53 protein, did not affect its ability to suppress papillomavirus amplification in our assays. However, it is possible that both the ssDNA-binding and reannealing functions of the C terminus additionally contribute to the amplification suppressor activity of p53 in the case of the full-length protein.

Core and oligomerization domains, though necessary, are not sufficient for the replication suppressor activity. An additional N-terminal sequence that has been shown to contain two intriguing determinants, RPA-binding (residues 40 to 60) and proline-rich (residues 61 to 90) domains, is also needed. Deletion of any or both of these domains crippled the p53 protein in the replication suppression assay. It is highly likely that p53 coordinates its replication suppressor activity with other proteins bound on the ssDNA, such as through the interaction with RPA (14). RPA facilitates DNA unwinding and DNA synthesis in the initiation and elongation stages of DNA replication (63). The interaction of p53 with RPA could be important in two respects. First, ssDNA-bound RPA could be the target for p53 action, and its interaction with p53 could sequester RPA from the ssDNA; second, interaction between RPA and p53 on the stabilized ssDNA facilitates recognition of the amplifying DNA by p53. Interaction of p53 and RPA in solution does not require an intact DNA-binding domain (1, 14, 30), while it is needed for the suppression of replication. This suggests the possibility that p53-RPA interaction takes place on the ssDNA. Deletion mapping of p53 activity showed that also the proline-rich putative signalling domain in the N-terminal part of the protein is required for the suppression of papillomavirus replication. This domain contains several copies of the PXXP motif (P represents proline; X represents any amino acid), which constitute a binding site for the proteins with the SH3 domain (59). It has been suggested that this domain plays a critical role in the transmission of transactivation-independent antiproliferative signals and presumably links p53 directly to the appropriate signal transduction pathways (46, 59).

However, despite the findings provided here pointing to an attractive putative mechanism, additional experimental data are needed to determine in detail the mechanism of action of p53 in the suppression of papillomavirus amplificational replication.

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Long-Term Episomal Maintenance of Bovine Papillomavirus Type 1 Plasmids Is Determined by Attachment to Host Chromosomes, Which Is Mediated by the Viral E2 Protein and Its Binding Sites

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Papillomavirus genomes are stably maintained as extrachromosomal nuclear plasmids in dividing host cells. To address the mechanisms responsible for stable maintenance of virus, we examined nuclear compartmentalization of plasmids containing the full-length upstream regulatory region (URR) from the bovine papillomavirus type 1 (BPV1) genome. We found that these plasmids are tightly associated with the nuclear chromatin both in the stable cell lines that maintain episomal copies of the plasmids and in transiently transfected cells expressing the viral E1 and E2 proteins. Further analysis of viral factors revealed that the E2 protein in *trans* and its multiple binding sites in *cis* are both necessary and sufficient for the chromatin attachment of the plasmids. On the other hand, the BPV1 URR-dependent plasmid replication and chromatin attachment processes are clearly independent of each other. The ability of the plasmids to stably maintain episomes correlates clearly with their chromatin association function. These data suggest that viral E2 protein-mediated attachment of BPV1 genomes to the host cell chromatin could provide a mechanism for the coupling of viral genome multiplication and partitioning to the host cell cycle during viral latent infection.

Precise maintenance of the cellular genome requires exact doubling of the genome once and only once during the S phase and proper partitioning of the chromosomes between the daughter cells during the M phase of the cell cycle (26). Some DNA viruses, like papillomaviruses and Epstein-Barr virus (EBV), replicate as episomal multicopy nuclear plasmids in the host cells' nuclei during a latent infection (11, 13). In order to be successfully maintained in host cells during latency, these viruses have to possess certain control mechanisms that couple multiplication of the viral genome and partitioning to the host genome maintenance cycle. The relatively small size of the papillomavirus genome puts certain limits on the use of these maintenance mechanisms. It is clear, for example, that episomal DNA viruses, unlike the cellular chromosomes, cannot afford to possess long and complex centromeric regions in their genomes that could ensure the proper partitioning and nuclear retention functions during mitosis. Therefore, some other strategy has to be used instead.

Papillomaviruses infect basal epithelial and mucosal cells in a wide range of different hosts. The infection can cause benign or malignant lesions; the most known example is common skin warts. Papillomavirus genome replication can be generally described as a three-step process (11). After entry into the basal cells, the viral genomes are quickly amplified in the host cell nucleus. Initial amplification is followed by a viral latency period, during which the viral genomes are maintained extrachromosomally at a constant copy number in the proliferating host cells. The final, vegetative amplification stage, where the formation of new infectious particles occurs, takes place only after the host cells have terminally differentiated into keratinocytes.

The process of initiation of papillomavirus DNA replication has been extensively studied, focusing mainly on bovine papillomavirus type 1 (BPV1) as a model. Only two viral proteins—E1 and E2—are required for this process, and all other necessary components are derived from the host replication machinery (5, 16, 38–40). E1 has been shown to be a viral origin recognition factor and helicase (12, 33, 41). E2, apart from being a central viral transcription regulator (9, 23), also acts as an auxiliary factor that binds to E1 and to the replication origin in a cooperative manner, thus facilitating the formation of replication initiation complex (2, 21, 24, 32, 35). The origin of papillomavirus replication has been located to the noncoding upstream regulatory region (URR). The minimal part of the URR, sufficient for the initiation of viral replication (minimal origin of replication), is composed of an A/T-rich region, binding site for E1, and one binding site for E2 (37, 39). URR sequences of different papillomaviruses contain a different number of E2 binding sites that also play an important role in viral latency. The URR of BPV1 contains 12 E2 binding sites that together form a BPV1 minichromosome maintenance element (MME). This element, in addition to the minimal origin of replication, is required for long-term episomal maintenance of BPV1 replicator in cells expressing the E1 and E2 proteins. A sufficient number of high-affinity E2 binding sites is critical for proper MME function (27). However, the function of E2 binding sites in the stable maintenance of the viral genome has been unclear until lately. Two recent publications provided the first insights, showing that BPV1 genomes, as well as E2 protein, are localized to host cell mitotic chromatin in C127 mouse fibroblasts and that mutations in E2 and E1 coding regions are able to affect such localization (18, 34).

In this study, we demonstrate that MME is likely to exert its role in episomal minichromosome maintenance of the BPV1 genome through the viral E2 protein-mediated association with the host cell nuclear chromatin. Viral E2 protein in *trans* and MME, comprised of multiple E2 binding sites, in *cis*, are

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both necessary and sufficient for chromatin attachment of the plasmids in our model system. On the other hand, the E1 protein or its binding site as well as the plasmid replication function can be removed without affecting the plasmid association with the chromatin. These data suggest that E2-mediated association of the viral genomes with nuclear chromatin is likely to guarantee the proper partitioning and nuclear retention of papillomavirus genomes in dividing cells as well as the optimal exposure of papillomavirus replicon to cellular replication control mechanisms during S phase. Therefore, the BPV1 stable episomal maintenance consists of two main functions—chromatin attachment, which provides proper partitioning and nuclear retention to the viral genomes, and replication function, which is responsible for compensation of the plasmid loss during host cell division.

MATERIALS AND METHODS

Plasmid constructs. Plasmid pNeo10E2BS9 contains 10 oligomerized head-to-tail copies of high-affinity E2 binding site 9 and was constructed by inserting the *Bam*HI-*Ecl*136II fragment (containing the synthetic E2 binding site oligomer) from plasmid Msp/15+10×BS9 (37) between *Bam*HI and *Hpa*I sites of pNeo vector. All other BPV1 URR plasmids (27) and BPV-1 E1 expression vector pCGEag (38) were described previously.

Cells and transfections. Chinese hamster ovary cell line (CHO) derivatives CHO49 (expressing BPV1 E2 protein), CHO4.15 (expressing BPV1 E1 and E2), and CHOBgl40 (CHO4.15 cells that maintain BPV-1 full-length URR plasmid pNeoBgl40 episomally) (27) were maintained in Ham's F12 medium supplemented with 10% fetal calf serum. Electroporation experiments were performed with a Bio-Rad Gene Pulser with capacitance and voltage settings of 975 μF and 230 V, respectively. The transfection efficiencies were determined by in situ staining of the cells transfected in parallel with a β-galactosidase-expressing plasmid pON260 (38). The extraction of episomal DNA from cells and its analysis by Southern blotting were performed as described previously (38).

Cytogenetic analysis. Chromosome preparations were done by standard methods. Briefly, cells were exposed to Colcemid added at a final concentration of 0.1 μg/ml for 1 to 4 h to enrich the mitotic fraction. Colcemid-treated cells were harvested by trypsin treatment and suspended in a 0.075 M KCl solution, incubated at room temperature for 15 min, and fixed in ice-cold methanol-glacial acetic acid (3:1 [vol/vol]). The spread-out chromosomes at metaphase and nuclei at interphase for cytogenetic or fluorescence in situ hybridization analysis were prepared by dropping the cell suspension on wet slides. Chromosome analysis was performed by standard staining methods. CHO cells were karyotyped by G-banding analysis as described previously (4).

FISH. Cells were harvested and prepared for analysis as described above. Hybridization probes were generated by nick translation, using biotin-16-dUTP as a label and pNeoBgl40 plasmid as a template. The final size of probe fragments was adjusted to 100 to 300 bp by DNase I digestion in all cases. Fluorescence in situ hybridization (FISH) was performed essentially by the protocol of Tucker and coauthors (36). Briefly, chromosome preparations were denatured at 70°C in 70% formamide (pH 7.0 to 7.3) for 5 min, then immediately dehydrated in a series of washes (70, 85, and 96% ice-cold ethanol washes [for 3 min each]), and air dried. The hybridization mixture (18 μl per slide) was composed of 50% formamide in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10% dextran sulfate, 160 ng of a biotinylated plasmid probe DNA, and 10 μg of herring sperm carrier DNA. After 5 min of denaturation at 70°C, probe DNA was applied to each slide, sealed under a coverslip, and hybridized for 2 days at 37°C in a moist chamber. The slides were washed in three changes of 2× SSC containing 50% formamide, 2× SSC, and 2× SSC containing 0.1% IGEPAL CA-630 (Sigma Chemical Co.) at 45°C. Prior to immunofluorescence detection, slides were preincubated for 5 min in PNM buffer (PN buffer [25.2 g $Na_2HPO_4 \cdot 7H_2O$, 0.83 g $NaH_2PO_4 \cdot H_2O$, and 0.6 ml of IGEPAL CA-630 in 1 liter of H_2O] with 5% nonfat dried milk and 0.02% sodium azide). After that, the probe was detected with fluorescein isothiocyanate (FITC)-conjugated extravidin. The signal was amplified with biotinylated antiavidin antibody and a second round of extravidin-FITC treatment. Between each of the steps, the slides were washed in PN buffer containing 0.05% IGEPAL CA-630 at room temperature for 15 min. Chromosomes were counterstained with propidium iodide and mounted in *p*-phenylenediamine antifade mounting medium. Slides were analyzed with a Olympos VANOX-S fluorescence microscope equipped with appropriate filter set. All FISH experiments were coded, and the chromosomes from at least 50 cells at metaphase and at least 200 interphase nuclei were analyzed on each slide. In addition, two slides from each sample were prepared, hybridized, and scored on different dates. Fuji Fujicolor and Agfa Agfacolor films for color prints were used for photomicrographs.

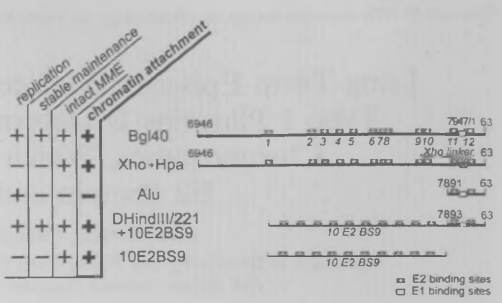
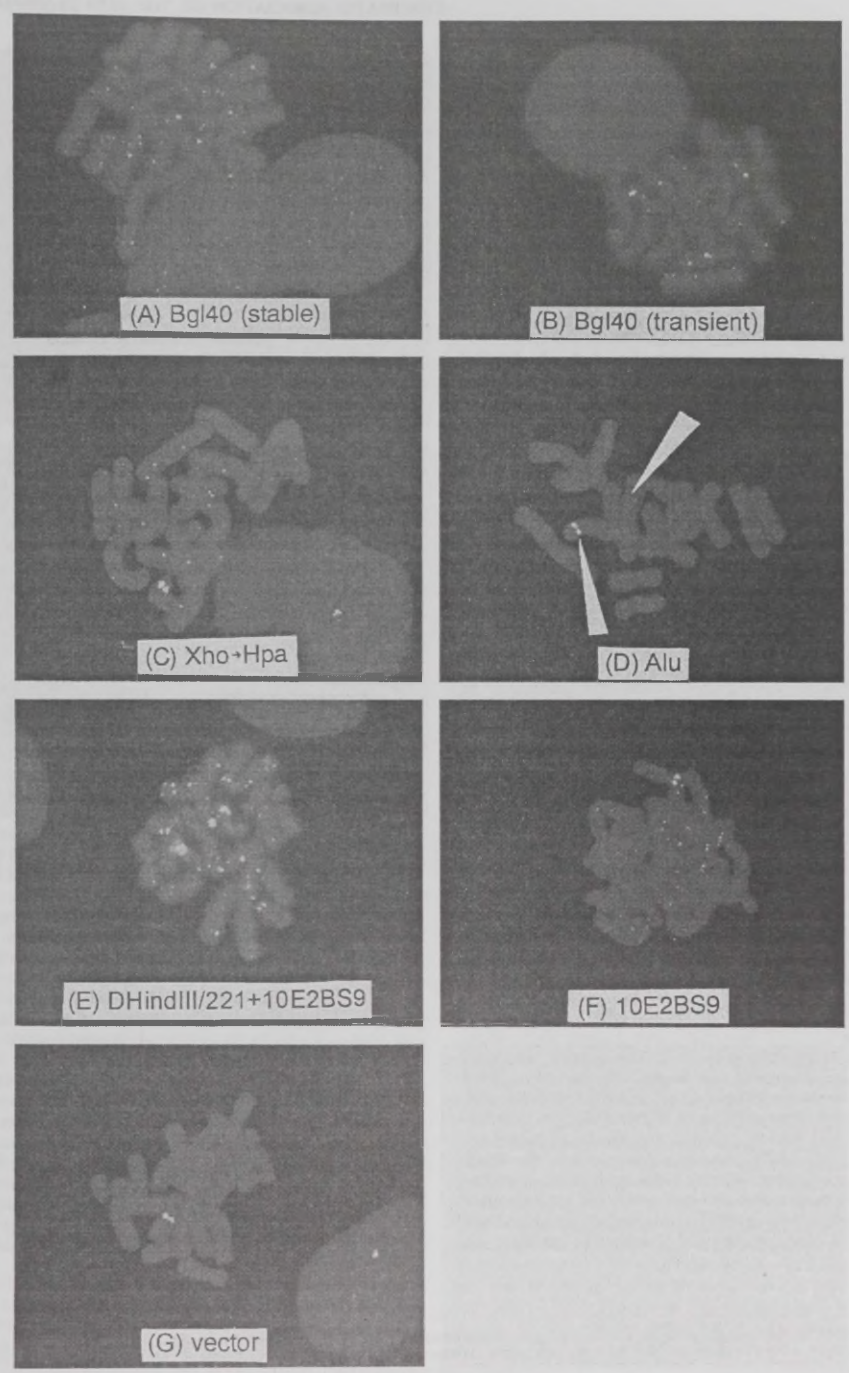


FIG. 1. Schematic representation and some relevant properties of the BPV1 URR constructs used in this study. The presence (+) or absence (−) of intact replication origin (replication), intact MME (sufficient number of E2 binding sites), competence for stable episomal maintenance in the long-term assay (stable maintenance), and competence of the construct for attachment to the host cell chromatin as determined by FISH analysis are indicated to the left of the schematic representations. The numbers in the schematically represented DNA sequences are the nucleotide positions in the BPV1 genome.

RESULTS

BPV1 URR-containing plasmids are associated with the host cell chromatin. It has been shown that the full-length BPV1 genomes are attached to the chromatin in C127 cells (18, 34). First we decided to examine whether the plasmids, which contain only the BPV1 URR sequences, possess the same ability in the Chinese hamster ovary (CHO) cell line-based model system, developed by us for the study of transient replication and stable maintenance of BPV1 (27, 39). We first analyzed the CHOBgl40 cell line, which expresses the BPV1 E1 and E2 proteins from integrated cassettes and maintains extrachromosomally the full-length BPV1 URR (Fig. 1) containing plasmid pNeoBgl40. A FISH analysis of both, prefixed mitotic metaphase spreads and interphase nuclei was performed with biotin-labelled BPV1 URR plasmid-specific DNA probe. The signals from hybridized probe were detected and amplified with FITC-conjugated extravidin and antiavidin antibodies, as described in Materials and Methods. The representative data are shown in Fig. 2A. The discrete yellow dots corresponding to plasmid-specific signals appeared as a merged yellow signal of the green FITC fluorescence on the red background of nuclear DNA stained with propidium iodide. The BPV1 URR plasmid signals were localized on the metaphase chromosomes with obviously random pattern distribution. Random distribution of plasmid signals was also observed in the interphase nuclei. Almost all (~90%) interphase nuclei and mitotic metaphase chromosomes from 180 analyzed cells contained BPV1 URR plasmid-specific signals, with overall number of plasmid signals from around 10 to 50 per nucleus in the majority of individual nuclei analyzed. This number is close to the estimated average of episomal plasmid copy number in CHOBgl40 cells (27), suggesting that FISH analysis was sensitive enough to detect every single plasmid copy in fixed nuclei. A small proportion of the cells from the total population contained higher number of signals (2% of cells containing more than 40 signals). However, the fractions of both high-copy-number and plasmid-negative phenotype nuclei varied significantly (up to 20% in both fractions) in several other CHOBgl40 subclones that were derived from the same long-term passage cell population. This apparent heterogeneity supports the earlier suggestion based on the similar phenotype in the case of long-term stable maintenance of


(A) Bgl40 (stable)
(B) Bgl40 (transient)
(C) Xho→Hpa
(D) Alu
(E) DHindIII/221+10E2BS9
(F) 10E2BS9
(G) vector

full-length BPV1 genomes (28, 29, 31), that the papillomavirus partitioning and replication processes are not subjected to very strict control mechanisms.

In addition to plasmid-specific randomly distributed single dots, two hybridization signals represented by double dots, one on both sister chromatids, were present on the spread-out chromosomes of CHOBgl40 cells. More-prominent hybridization signal on marker chromosome 8 (mar8) and much weaker signal on marker chromosome 4 (mar4) correspond to the genome-integrated E1 and E2 expression cassettes and appear as a result of cross-hybridization between bacterial plasmid backbones of the probe DNA and integrated expression cassettes. The same integrated markers were also present in the case of cell line CHO4.15, which is the BPV1 E1- and E2-expressing parental cell line used to generate the CHOBgl40 cells.

After verifying the attachment of stably maintained BPV1 URR plasmid to host mitotic chromatin, we next decided to examine whether similar attachment occurs in the case of transient-replication assay with the same plasmid. For this experiment, the BPV1 E1- and E2-expressing cell line CHO4.15 was transfected with plasmid pNeoBgl40, which contains the full-length URR of BPV1, cells were fixed 48 h after transfection, and the plasmid localization in interphase nuclei and on metaphase chromosomes was determined by FISH. Similar to the results obtained with stably maintained plasmid, randomly distributed BPV1 URR-plasmid specific signals were observed both on metaphase chromosomes and in interphase nuclei (Fig. 2B).

We conclude from these data that the BPV1 URR-containing plasmids are able to associate with host chromatin in the BPV1 E1 and E2 protein-expressing cells. The association with host chromatin is not dependent on the URR plasmid status, appearing both in the case of stably maintained and transiently replicating plasmid.

The multimerized E2 protein binding sites determine the chromatin attachment of the plasmids in the CHO4.15 cells. The data presented above showed clearly that chromatin attachment of the BPV1 URR plasmids could be studied in transient-transfection assay. A panel of different BPV1 URR-derived constructs (Fig. 1) in the same plasmid context, pNeo5′, was transfected into the BPV1 E1- and E2-expressing cell line CHO4.15. Half of the cells were fixed after 48 h, and FISH analysis of the plasmid localization with specific DNA probe was performed. Low-molecular-weight DNA was extracted from the other half of the cells and analyzed by Southern blotting to estimate the overall level and replication competence of the transfected plasmid DNA in cells. FISH analysis indicated that in addition to the intact full-length URR plasmid (Bgl40) (Fig. 2B), the plasmid containing URR with disrupted E1 binding site (Xho→Hpa) also displays the localization to mitotic chromatin (Fig. 2C). The fraction of nuclei considered plasmid positive was smaller (usually 10 to 20%) than the transfection efficiencies estimated in parallel with β-galactosidase expression plasmids (60 to 70%). These differences may be explained by different sensitivities of β-galactosidase staining and FISH protocols. Alternatively, the lower percentage of the positive cells by FISH analysis may indicate that not all plasmid molecules that reach the nucleus after transfection will be able to attach to the chromatin or perhaps they will require longer incubation periods. For example, the

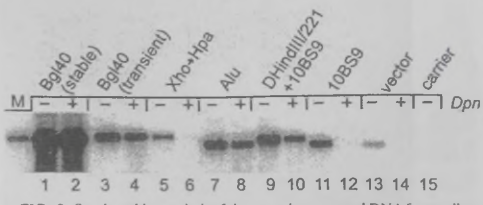


FIG. 3. Southern blot analysis of the extrachromosomal DNA from cells used for the parallel FISH analysis (see Fig. 2 for FISH results). Lane M contains 100 pg of linearized plasmid marker (pNeoBgl40). Lanes 1 and 2 contain extrachromosomal DNA from CHOBgl40 cells maintaining the BPV1 URR plasmid pNeoBgl40 episomally, and all the other lanes correspond to different transfections with BPV1 URR constructs (1 μg of each plasmid) in CHO4.15 cells. The mock-transfected control cells (carrier) are indicated. DNA preparations were digested with the appropriate restriction enzyme to linearize the plasmid DNA and with *Dpn*I where indicated (+ if added, − if not) which digests only bacterially methylated DNA, thus revealing the de novo-replicated plasmid pool.

successful establishment of the chromatin association may be dependent on passage of the cells through the particular cell cycle phase. One possible explanation could also be that chromatin attachment requires higher E2 levels than in some of the cells, but previous immunofluorescence analysis of the status of the E2 protein has not revealed any significant heterogeneity in the used subclones of CHO4.15 cell line (data not shown).

Plasmids with no BPV1 URR sequences (Fig. 2G) or containing essentially the minimal replication origin (Fig. 2D) with two E2 binding sites failed to give any plasmid retention in the interphase nuclei and on the metaphase chromosomes. On the other hand, parallel Southern blots indicated that the plasmid DNA was present in these cells at levels comparable to those detected in the case of plasmids that were able to localize to mitotic chromosomes (Fig. 3). For reasons that are not clear at this time, the vector molecule constantly gave lower signals upon harvesting in the transfected cells under the same transfection conditions (compare lane 13 with the other lanes with other input plasmids [Fig. 3]). *Dpn*I cleavage also demonstrated that minimal replication origin-containing plasmid pUCAlu, despite failing to associate with mitotic chromatin, at the same time replicated efficiently in transfected cells. These data suggest the possibility that BPV1 origin-dependent replication may take place both in the chromatin-associated form, as in the case of URR-containing plasmids, and freely in the nucleoplasm, as in the case of minimal replication origin plasmid pUCAlu. The presence of CHO4.15 cell line-specific cross-hybridization signals on marker chromosomes mar4 and mar8 (see above) served as an additional internal control verifying the success of the FISH procedure.

We conclude from these data that the chromosomal localization of plasmid-specific hybridization signals in the case of certain BPV1 URR constructs is not an indication of some unspecific feature of plasmid DNA but rather reflects the association with host mitotic chromatin that is dependent specifically on BPV1 URR sequences. Plasmids that are not bound to the chromatin are washed away from both the chromosome complexes at metaphase and the nuclei at interphase during fixation and hybridization procedures, as the lack of plasmid signal

FIG. 2. Multiple E2 binding sites determine the competence for chromatin attachment, but a functional replication origin is not necessary for this activity. The results of FISH analysis in the CHOBgl40 cell line that stably maintains a full-length BPV1 URR plasmid pNeoBgl40 (A) and of CHO4.15 cells transfected with plasmids (1 μg) containing different BPV1 URR inserts (B to G) that are depicted schematically in Fig. 1. Panel G shows the control experiment with plasmid containing no URR sequences E1 and E2 expression cassettes integrated into genome give cross-hybridization signals represented by double dots (indicated by arrowheads).

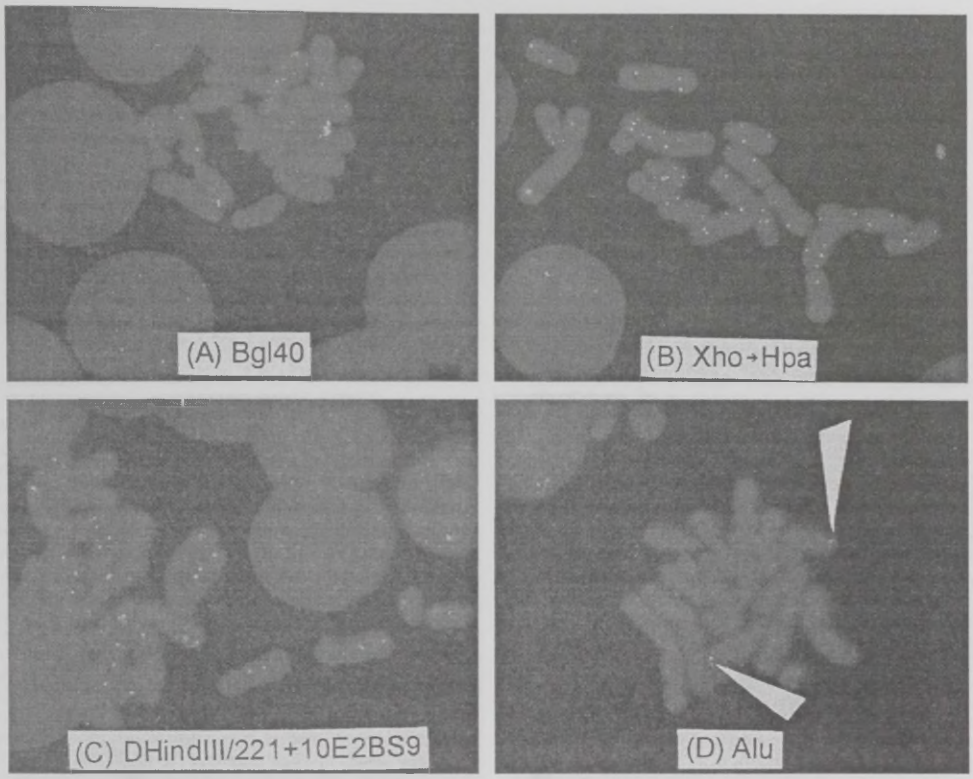


FIG. 4. The chromatin attachment of URR plasmids occurs also in the absence of E1 expression; E2 protein determines the attachment activity. The results of FISH analysis in the transient assay using cell line CHO49 that expresses only BPV1 E2 protein are shown. Cells were transfected with 1 μg of plasmids containing BPV1 URR inserts depicted schematically in Fig. 1. E2 expression cassette integrated into genome gives cross-hybridization signal represented by double dots (indicated by arrowheads).

on metaphase chromosomes was always accompanied by the lack or very low percentage of signals in the interphase nuclei.

The failure of replicating BPV1 URR deletion construct *Alu* to attach to the mitotic chromosomes leads us to the conclusion that replication and chromatin attachment are separate properties of the BPV1 replicon. This conclusion is further supported by the localization of the replication-deficient construct Xho→Hpa to mitotic chromatin (Fig. 2C). On the other hand, the attachment of plasmids to chromatin was dependent on the presence of a sufficient number of high-affinity E2 binding sites in *cis*. As shown above, BPV1 URR constructs with intact set of 12 E2 binding sites (Bgl40 and Xho→Hpa) were able to attach to chromatin. The addition of 10 oligomerized high-affinity binding sites was able to restore the chromosome attachment activity to the construct with only two E2 binding sites (Fig. 2E). Moreover, FISH analysis demonstrated that insertion of 10 oligomerized high-affinity E2 binding sites alone into the vector was sufficient to provide the chromatin attachment activity to plasmid DNA in the absence of any other additional BPV1 URR sequences (Fig. 2F). We conclude from these data that a sufficient number of high-affinity E2 binding sites determines the plasmid association to chromosomes.

Multiple E2 binding sites in *cis* and viral E2 protein in *trans* are the viral determinants of the chromatin attachment activity of the BPV1 URR-derived plasmids. The fact that oligomerized E2 binding sites were sufficient while functional replication origin and replication ability were unnecessary for chromatin attachment made us hypothesize that the attachment occurs only if viral E2 protein were provided in cells. In order to test that, we transfected the BPV1 E2-expressing CHO49 cell line with the same plasmids. Forty-eight hours after transfection, the cells were processed further for FISH analysis to demonstrate the plasmid localization in the nuclei and for parallel Southern blotting analysis to determine the plasmid levels in cells as described above in the case of CHO4.15 cells. As shown in Fig. 4, the E2 protein alone appeared to be sufficient in *trans* for providing the chromatin attachment activity for URR plasmids in the host cell nuclear background. Similar to the results obtained with E1- and E2-expressing CHO4.15 cells, all constructs containing a sufficient number of E2 binding sites, Bgl40, Xho→Hpa, and D234/221+10E2BS9 (Fig. 4A, B, and C), were attached to mitotic chromosomes. Of transfected cells, 10 to 20%, depending on the transfection, were clearly positive for plasmid signals, with

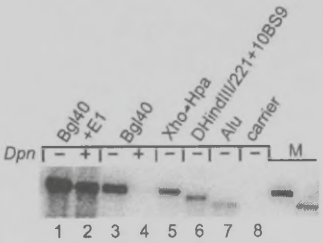


FIG. 5. Southern blot analysis of the extrachromosomal DNA from cells used for the parallel FISH analysis (see Fig. 4 for FISH results). Lane M contains 100 pg of linearized plasmid markers (pNeoBgl40 and pNeo). Lanes 1 to 8 correspond to different transfections with BPV1 URR constructs in CHO49 cells. BPV1 E1-expressing plasmid pCGEag (250 ng) was cotransfected on the panel Bgl40+E1 (lanes 1 and 2) as a control for E2 expression (ori plasmid replicates only if both E1 and E2 proteins are present). DNA preparations were digested with the appropriate restriction enzyme to linearize the plasmid DNA and with *Dpn*I where indicated (+ if added, − if not) which cuts only bacterially methylated DNA, thus revealing the de novo-replicated plasmid pool.

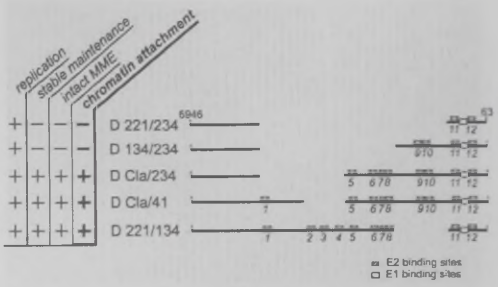


FIG. 6. Schematic representation of the structure and some relevant properties of the second panel of BPV1 URR deletion variants used in this study. See the legend to Fig. 1 for explanation of abbreviations, designations, etc.

transfection efficiencies estimated in parallel around 50%. The percentage of positive chromosomes at metaphase was again approximately equal to the percentage of positive nuclei at interphase in analyzed individual transfections, and the plasmid-specific hybridization signals followed an apparently random pattern. Control analysis with construct that contains only two E2 binding sites did not reveal any chromosomal localization of the plasmid (Fig. 4D), even though according to the Southern blotting analysis, the plasmid DNA was present in cells in case of this and other constructs used (Fig. 5, compare lanes 1 to 8). Cross-hybridization with chromosomally integrated E2 expression cassettes (one site on two different chromosomes) served as an internal control for the success of the FISH analysis.

No chromatin attachment of the same plasmids was observed if the CHO cell line, which does not express any BPV1 protein, was used in a similar experiment (data not shown). We conclude from these data that E2 protein in *trans* and its multiple binding sites in *cis* are the viral determinants of the BPV1 URR-dependent chromatin attachment.

The competence of BPV1 URR plasmids for stable episomal maintenance correlates with their ability to associate with host cell chromatin. A sufficient number of E2 binding sites form the MME which together with the viral minimal replication origin provides the long-term episomal maintenance property for the BPV1 replicator (27). Because of our results indicating that MME also determines the chromosomal attachment activity for BPV1 URR, we decided to further analyze the possible connection between the stable maintenance and chromosomal attachment activities. A panel of BPV1 URR deletion constructs with known stable maintenance properties was transfected into CHO4.15 cells (Fig. 6). Cells were processed 48 h after transfection for the FISH analysis to demonstrate the chromatin attachment of plasmids (Fig. 7) and for parallel Southern blotting analysis to detect the plasmid levels in cells (Fig. 8). The results of FISH analysis demonstrated clear correlation between the competence of each plasmid for stable episomal maintenance and its ability to associate with host cell chromatin. Only constructs with functional MME (DCla/234, DCla/41, and D221/134 [Fig. 7C, D, and E]), previously shown to be capable of stable maintenance (27), were tightly associated with the mitotic chromosomes at metaphase and in the nuclei at interphase. Constructs lacking functional MME and incapable of stable replication (D221/234 and D134/234 [Fig. 7A and B]), also failed to localize to chromosomes.

DISCUSSION

Two recent articles dealing with BPV1 chromatin attachment have reviewed the results of studies of full-length viral genome DNA in mouse fibroblasts (18, 34). This system has the disadvantage of being relatively complicated, because all viral early genes, including oncogenes, are expressed from episomal viral genome in these transformed cells. Therefore, the complex interplay between E1 and different E2 transactivation and repressor forms in the processes of regulation of viral transcription, transformation, replication, and genome copy number complicates unambiguous interpretation of the involvement of different viral gene products in chromatin attachment. We have used a different approach, trying to simplify the system as much as possible, looking for the minimal viral determinants for the chromosome attachment activity. In this regard, E1- and/or E2-expressing stable cell lines serve as good model systems. These cells do not express any other papillomavirus proteins and express constant E1 and E2 levels from the integrated constructs, thus providing a more defined system for comparative studies on the behavior of different BPV1-derived constructs.

Previous studies have pointed toward the E2 protein as being the best candidate for viral *trans* factor required for chromatin attachment. The genetic analysis in the full-length viral genome context by Lehman and Botchan (18) has suggested that in addition to E2, the viral E1 protein seems to participate in the tethering of viral genomes to chromosomes. Our data show that E2, in the absence of E1, can be sufficient for the chromatin attachment of BPV1 URR plasmids. It is possible that E1, as well as other viral (and cellular) proteins, does contribute, indirectly or directly through interaction with the E2 protein to the attachment process. However, E2 protein clearly appears to be the central viral *trans* determinant for this process.

This is also the first study of the viral *cis* elements that determine the chromatin attachment. We show that MME, which is composed of E2 binding sites and is necessary for stable episomal maintenance of BPV1 replicon, is also necessary and sufficient for chromatin attachment activity. The experiments with BPV1 URR deletion constructs demonstrated clear correlation between the competence for stable maintenance and chromosome association. Thus, MME is likely to exert its role in the stable maintenance of BPV1 episomes by providing access to necessary cellular control mechanisms through association with host cell chromatin, presumably providing access to those cellular mechanisms that grant the partitioning and nuclear retention functions to the viral genome.

It is interesting to note that chromatin attachment occurs both in the short-term transient-transfection and long-term

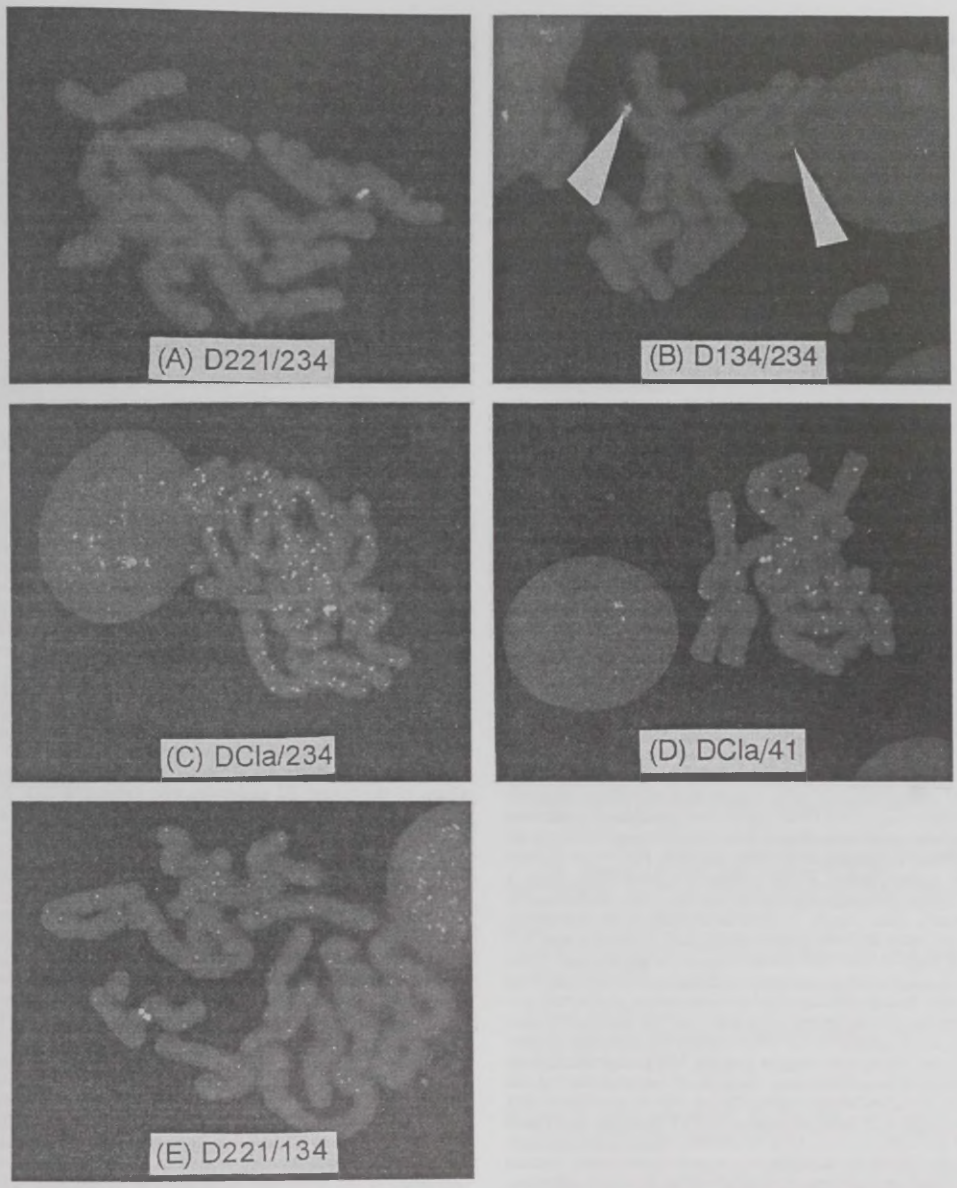


FIG. 7. The competence of BPV1 URR constructs for stable episomal maintenance correlates with their ability to associate with host chromatin. The results of FISH analysis in the transient assay using cell line CHO4.15 are shown. Cells were transfected with 1 μg of plasmids containing BPV1 URR inserts depicted schematically in Fig. 6. E1 and E2 expression cassettes integrated into the chromosomal DNA give cross-hybridization signals represented by double dots (indicated by arrowheads).

stable-maintenance assays. This fact supports the idea that the attachment of the viral genome to the chromatin may occur soon after sufficient levels of the E2 protein have been achieved in the cell and is not a result of the long-term selection process. It is possible that the establishment of the chromatin association is linked specifically to a certain stage of the host cell cycle as has been shown, for example, in the case of the formation of the preinitiation complex on the chromo-

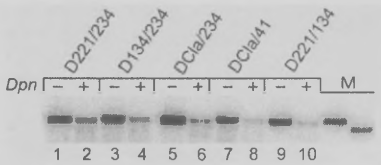


FIG. 8. Southern blot analysis of the extrachromosomal DNA from cells used for the parallel FISH experiments (see Fig. 7 for FISH results). Lane M contains 100 pg of linearized plasmid markers (pNeoBgl40 and pNeo). Lanes 1 to 10 correspond to transfections with BPV1 URR constructs in CHO4.15 cells in the transient assay. DNA preparations were digested with *Hin*dIII to linearize the plasmid DNA and with *Dpn*I (+ if added, − if not) which cuts only bacterially methylated DNA, thus revealing the de novo-replicated plasmid pool.

somal replication origins. However, no experimental data are available at the moment to clarify this point. The initial viral amplification during S phase probably creates and maintains the starting population of viral genomes large enough for subsequent finding and occupying of the optimal attachment sites on the chromatin. On the other hand, the analysis of different BPV1 URR deletion constructs demonstrated clearly that the replication and chromatin attachment functions are separate E2-dependent activities of the BPV1 replicator. Thus, the plasmid replication process itself is not directly linked to the chromatin attachment process. Resulting chromatin attachment is very likely to guarantee the viral genome partitioning and nuclear retention functions during host cell division, as was suggested previously (3, 18). In addition, chromatin association can also provide the cellular replication control function to the viral origin through the optimal exposure to chromatin-associated regulatory complexes. The latter may be needed in order to avoid undesired viral overreplication and therefore can provide the copy number control mechanism for the virus during latency.

According to results of our FISH analysis, the plasmids that failed to show any attachment to metaphase chromosomes also failed to show any staining in the interphase nuclei of the transfected cells. Replication-competent plasmids that failed to give any FISH signal were capable of replicating in the same cells according to Southern blotting analysis (e.g., pNeoAlu), confirming that these plasmids had to be present in the nucleus before FISH analysis was performed. Thus, it seems most likely that plasmids which were not attached to the chromatin were simply washed away both from metaphase chromosomes and interphase nuclei during fixation and following steps of the FISH procedure. On the other hand, in the case of attachment-competent plasmids, we could not observe any considerable difference in the percentage of plasmid-specific staining if the interphase nuclei and the mitotic chromosomes at metaphase in the same transfected populations were compared. These data suggest that the MME-dependent association with host chromatin could be maintained throughout the cell cycle, including S phase. It can also be speculated that the replication of stably maintained BPV1 replicator in S phase could take place on the host chromatin, where these genomes are well exposed to the replication control mechanisms that are utilized during host genome multiplication. However, additional and more-detailed studies are necessary to examine these possibilities.

The above-proposed possible access of chromatin-attached papillomavirus genomes to chromatin-associated cellular control mechanisms cannot be sufficient to grant the viral genome with very precise replication control. It is known that the papillomavirus genome is not replicating in a strict once per cell cycle mode during the viral latency that is used by host genome but rather follows a random-choice statistical initiation mechanism (7, 27, 29). On the other hand, an example of EBV indicates that once per cell cycle replication mode can still be achieved by episomal DNA viruses (42). EBV genome plasmids and viral latent replication origin (oriP) binding protein EBNA1 are associated with the host chromosomes (8, 10), and EBNA1 is able to provide nuclear retention function to the plasmids containing multiple EBNA1 binding sites (15). It is very likely that EBNA1, similar to E2 in the case of BPV1, mediates the attachment of viral genome to chromatin. Thus, chromatin attachment as a tool to exploit cellular control mechanisms for coupling the viral partitioning and replication to the host cell genome maintenance cycle may represent a more general feature for nonlytic episomal DNA viruses. The similar functional role for DNA binding proteins and their binding sites in partitioning function has also been reported for bacterial plasmids, bacterial chromosomes (19, 20), and *Saccharomyces cerevisiae* plasmids (1). These data seem to point toward general evolutionary similarities in different mechanisms of partitioning of the chromosomal and extrachromosomal elements.

E2 protein appears to be necessary and sufficient for linking of the MME-containing plasmids to the chromatin. As was discussed above, E2 protein has previously been shown to be capable of associating with the chromatin (18, 34). Two previous studies have indicated that the N-terminal transcription and replication activation domain of the E2 protein is crucial for the chromatin attachment activity of the protein itself. In addition, Lehman and Botchan (18) suggest that the hinge region between N- and C-terminal domains, which includes the major phosphorylation sites of the E2 protein, is also important for the attachment. Based on these and our data, it seems reasonable to assume that both the N-terminal chromatin-bound transactivation domain and the C-terminal MME-bound DNA binding domain, serve as necessary linkers for tethering MME-containing plasmids to the host chromatin. The E2 protein binding affinity to multiple oligomeric binding sites in MME would be remarkably high due to the cooperative interaction of the bound E2 molecules with DNA (14, 25). This would provide a tightly bound proteinaceous surface formed by multiple E2 N-terminal activation domains, which is responsible for the high efficiency of the interaction with the host chromatin. Efficient multicontact interaction with chromatin might explain why this survives a relatively harsh treatment, including DNA denaturation step, during the FISH procedures. The interaction with chromatin is sufficiently strong only in the case of E2 transactivation domain, because replacing it with the respective VP16 or p53 domain inactivates the hybrid protein's ability to tether the plasmids to the chromatin in CHO and human cells (22). The chromatin binding and the DNA binding, replication, and transcription activities of the E2 protein are possibly modulated through its phosphorylation and other posttranslational modifications. This could also explain the effect of the E2 protein linker region between N- and C-terminal domains in the regulation of the chromatin binding, as the modifications in hinge region may alter the placement of the protein domains in regard to each other (18). Also, the regulation of the full-length E2 protein by its repressor forms through heterodimer formation should be considered (6, 17). Altogether it could provide a complex regulatory mechanism to control the BPV1 genome multiplication and maintenance during viral latency.

It is still hard to guess which cellular factors from the chromatin side are required for the papillomavirus genome attachment. In the case of BPV1, the minimal number of E2 binding sites sufficient to provide the minichromosome maintenance

function exceeds the number of these sites generally found in upstream regulatory region of different human papillomavirus (HPV) types. Thus, in the case of the stable maintenance of the HPV genome in the transformed cells, some additional viral or cellular factors are probably necessary to provide the chromatin attachment activity. HPV URR sequences carry a so-called enhancer region, which contains numerous binding sites for different cellular transcription factors. It is tempting to speculate that certain cellular transcription activators or specific combinations of these activators, through some feature common with E2 protein, may compensate for the lack of sufficient contribution from HPV E2 binding sites. On the other hand, HPV E2 protein may provide some organizing function to these enhancer binding proteins. Interestingly, the EBNA1 protein, which is believed to be a possible mediator of the chromatin attachment of EBV genome, is also a viral transcription activator (30). It is possible that the target from the nuclear chromatin side, which allows the viral genome anchoring, may be identical in all these cases. However, the existence of such an attractive common mechanism in the case of different episomal DNA viruses remains to be proven in the future.

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III

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Two separate replication modes of the bovine papillomavirus BPV1 origin of replication that have different sensitivity to p53

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Abstract

We have shown previously that transient amplificational replication of reporter plasmids that carry the papillomavirus origin of replication is efficiently blocked by p53 protein in several cell lines. We demonstrate now that the replication of stably maintained episomal bovine papillomavirus BPV1 URR (upstream regulatory region) reporter plasmid is not sensitive to p53. In addition, these two replication modes—initial transient amplificational replication and stable maintenance replication of essentially the same BPV1 URR reporter plasmid—can take place in the same cells, where amplificational replication does not interfere with the stable maintenance replication. These data suggest that BPV1 replicons could follow two clearly separable replication mechanisms during initial amplification and during stable extrachromosomal maintenance.

Keywords: DNA replication; Papillomavirus; BPV1; p53

1. Introduction

Papillomaviruses (PV) are small DNA viruses that infect basal epithelial and mucosal cells in a wide variety of host species. Significant parts of the knowledge about early steps in the replication cycle of bovine papillomavirus BPV1 have been obtained from studies using the mouse C127 cell-line. The entry of BPV1 DNA into these cells leads to initial quick rise of the viral genome copy number per cell, which is followed by eventual establishment of the genome as a stable multicopy extrachromosomal plasmid (Howley and Lowy, 2001). The initial transient amplificational replication of BPV1 genomes (as well as the replication during later stages) relies heavily on host cell replication factors. Only two essential initiator proteins—E1 and E2—are encoded by the virus itself (Ustav and Stenlund, 1991). Therefore, this process can be efficiently modelled by transient transfection of E1 and E2 expressing cells with reporter plasmids, which carry sequences of the viral origin of replication (ori) from the non-coding upstream regulatory region (URR) (Ustav et al., 1993, 1991). The binding of E2 to its specific binding sites in ori facilitates the loading of E1 helicase and subsequent unwinding of DNA (Mohr et al., 1990; Sanders and Stenlund, 1998; Sedman and Stenlund, 1995; Seo et al., 1993; Yang et al., 1991, 1993). URR contains also a minichromosome maintenance element (MME), which consists of multiple additional E2 binding sites and is required for the establishment of stable extrachromosomal maintenance of BPV1 URR reporter plasmids in E1 and E2-expressing cells (Piirsoo et al., 1996). MME is responsible for the E2-mediated attachment of full-length BPV1 genomes as well as URR reporter plasmids to host cell chromosomes, and this process is believed to ensure the partitioning of viral extrachromosomal DNA molecules during host cell mitosis (Ilves et al., 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). Not much is known about the replication mechanism of

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BPV1 replicons during viral stable extrachromosomal maintenance. It is clear, however, that the replication initiation process during stable maintenance of full-length viral genomes as well as URR reporter plasmids is guided by relaxed random-choice rather than strict once-per-cell-cycle control mechanisms (Gilbert and Cohen, 1987; Piirsoo et al., 1996; Ravnan et al., 1992). These two critical features—chromatin attachment and relaxed replication control—are common for the stable episomal maintenance of both, URR reporter plasmids and intact BPV1 genomes.

Tumour suppressor protein p53 is a central coordinator of cellular defence mechanisms. By modulating the transcription of genes of several regulatory proteins in response to different genotoxic stress conditions, p53 can lead to the block of cellular proliferation or induction of apoptosis (Ko and Prives, 1996; Vogelstein et al., 2000; Vousden, 2000). For successful propagation, several DNA viruses encode for proteins that bind p53 and counteract its effects on the viral multiplication. These proteins include also papillomavirus E6 (Werness et al., 1990). Such binding could modulate the p53 activity (Lechner et al., 1992; Mietz et al., 1992), or, like in the case of E6 from high-risk human papillomaviruses (HPV), initiates the degradation of p53 protein (Scheffner et al., 1990).

We have previously demonstrated that p53 suppresses the transient amplificational replication of different papillomavirus ori constructs in various human and hamster cell lines (Lepik et al., 1998). In this paper, we show that the transient amplificational replication of a BPV1 URR reporter plasmid can take place in cells that already maintain stably extrachromosomal copies of similar reporter plasmids. The copy number of stably maintained plasmid is not affected in these conditions. More importantly, the stable maintenance replication of the BPV1 URR reporter plasmid, unlike its transient amplificational replication, is not sensitive to p53 expression in the same cells. These data suggest that the initial amplificational and following long-term stable maintenance replication of BPV1 may use two clearly separable mechanisms. Thus, the successful establishing of the BPV1 stable extrachromosomal maintenance could also involve, in addition to the E2-dependent partitioning mechanism, specific changes in the replication mechanism of the viral DNA.

2. Materials and methods

2.1. Plasmid constructs

Plasmids pE1-1 × 5, pNeoBgl40, and p53 expression constructs in pCG vector have been described previously (Lepik et al., 1998; Piirsoo et al., 1996). pNeoBgl40HIII was derived from pNeoBgl40 after insertion of additional *Hin*dIII linker into *Bam*HI site, thus placing ~1 kb URR fragment between two *Hin*dIII sites. pHook2 selection plasmid was obtained from Invitrogen. pHookN39C362 was constructed by inserting a *Xho*I (filled with Klenow)-*Eco*RI fragment containing entire p53 expression cassette from pCGN39C362 between *Ssp*I and *Mun*I sites of pHook2 vector.

2.2. Cells and transfections

CHOBgl40 cells (Piirsoo et al., 1996) were grown in Ham's F12 medium supplied with 10% foetal calf serum. Electroporation experiments were carried out as described earlier (Ustav and Stenlund, 1991), using the Bio-Rad Gene Pulser II apparatus supplied with a capacitance extender (Bio-Rad Laboratories, USA). Capacitance was set to 975 μF and voltage to 230 V in all experiments. Approximately 1×10^6 surviving cells were seeded to each 10 cm culture dish after transfection, which leaves enough room for cells to proceed through two doublings before reaching confluency.

2.3. Separation of transfected cells from total population

The Capture-Tec pHook selection system (Invitrogen Corporation, USA) was used to isolate transfected from non-transfected cells. Around 20 million cells (five confluent 10-cm dishes) were collected for each separate analysis. Cells were bound to magnetic hapten-coated beads, and bound cells were pulled down on magnetic stand and processed for further analysis according to protocol suggested by the manufacturer. According to control transfections with lacZ or green fluorescent protein test plasmids, the transfection efficiency was at least 60–70%, and roughly 10–15% of cells retained detectable expression levels from the plasmid by the time of selection (after two cell divisions). The yield of cell capture was between 5 and 30% from estimated pHook-carrying sub-population of cells. The non-specific background binding was less than 5% from the total selection yield, as estimated by parallel processing of the mock-transfected cells.

2.4. Replication assays

Total DNA was extracted from cells following standard protocol (Ausubel et al., 1998). Extraction of low molecular weight DNA from cells as well as analysis of reporter plasmid levels in both low molecular weight and total DNA preparations were performed as described previously (Piirsoo et al., 1996; Ustav and Stenlund, 1991). Radioactively labelled reporter plasmid specific probes (using pNeo plasmid backbone as a template) were generated by random priming using the DecaLabel kit (Fermentas, Lithuania), and respective radioactive signals on the blots were quantified on

PhosporImager SI using IMAGEQUANT software (Molecular Dynamics, Amersham Biosciences, UK).

2.5. Immunoblotting

Total protein from the same number of cells lysed in standard loading buffer supplemented with 100 mM DDT was separated by electrophoresis on 8 or 10% polyacrylamide-SDS gels and transferred to Immobilon-P membrane (Millipore, USA). Antibodies 1E4 (Kurg et al., 1999), and pAb240 (Gannon et al., 1990) were used to detect E2 and p53 proteins, respectively. The antibody against BPV1 E1 was a kind gift from Dr Arne Stenlund (Cold Spring Harbor Laboratory, USA). Peroxidase-conjugated goat-anti mouse antibody and the enhanced chemoluminescence detection kit (Amersham Biosciences) were used for subsequent developing of the blots, using a standard protocol provided by the supplier.

2.6. Cell cycle distribution analysis (FACS)

The preparation of cells for cell cycle distribution analysis has been described earlier (Lepik et al., 1998). Signals from 30 000 cells were collected using FACSCalibur flow cytometer and CELLQUEST software (BD Biosciences, USA); estimated fractions of cells in G0/G1, S and M phases were calculated using MODFIT LT program provided by FACS manufacturer.

2.7. BrdU labelling

Transfected cells were labelled with bromodeoxyuridine (BrdU) as described earlier (Yates and Guan, 1991). The cells were let to pass through two cell divisions and pHook-plasmid carrying cells were separated from total population using the Capture-Tec selection procedure described above. The non-specific background binding was somewhat higher in the case of BrdU-labelled compared to non-labelled cells (up to 15% from total selection yield). It can be probably explained by the enlarged phenotype of the BrdU-treated cells leading to increased cell surface and to relatively higher non-specific binding of the selection beads per cell. Total DNA extraction from the labelled cells, CsCl gradient separation, and following analysis of the genomic DNA distribution in different fractions was carried out as described previously (Piirsoo et al., 1996; Yates and Guan, 1991). Radioactively labelled CHO genomic DNA probe was generated using DecaLabel kit from Fermentas and respective radioactive signal on the blots was quantified on PhosporImager SI.

3. Results

3.1. Transient amplificational replication of BPV1 URR reporter plasmid is sensitive to p53 over-expression and can take place in CHOBgl40 cells that maintain stably extrachromosomal copies of the similar reporter

We have established a CHOBgl40 cell-line as a simplified model system for studying the basic processes of replication and nuclear maintenance during BPV1 latency (Piirsoo et al., 1996). This cell-line supports constitutive viral E1 and E2 protein expression from chromosomally integrated cassettes and maintains episomes of the BPV1 URR reporter plasmid pNeoBgl40 stably. We have shown previously that p53 is able to suppress transient amplificational replication of the PV ori plasmids in CHOBgl40 parent cell lines CHO and CHO4.15 as well as in human cells, but not in several mouse cell-lines (Lepik et al., 1998; Lepik and Ustav, 2000). These data indicated that the effect of p53 on PV ori replication may be dependent on cellular characteristics.

We transfected CHOBgl40 cells with pCG plasmid expressing different p53 mutant proteins (depicted schematically on Fig. 1B) and with BPV1 URR reporter plasmid pNeoBgl40HIII. The only difference between pNeoBgl40HIII that was transfected into cells and pNeoBgl40 that is stably maintained in CHOBgl40 cells lies in one *Hin*dIII restriction endonuclease site. Because of that, the digestion with *Hin*dIII linearises stably maintained reporter, but gives a ~1 kb shorter fragment (URR region is cut out) in the case of amplificationally replicating transfected reporter. The respective fragments can be separated on agarose gel electrophoresis and simultaneously analysed by Southern hybridisation after transferring to nylon membrane (Fig. 1A, top panel). Wt p53 expression construct was not included in our transfection panel, because of its strong apoptotic effect on CHO cells (Lepik et al., 1998). At 72 h after transfection, the low molecular weight DNA was extracted from cells, digested with *Dpn*I and *Hin*dIII, and analysed by Southern blotting. *Dpn*I digests bacterially methylated input DNA and retains only the molecules, which have passed through at least one round of replication in transfected cells. At the time of cell harvesting, the level of *Dpn*I resistant de novo replicated input reporter pool continued to grow exponentially and faster than the level of stably maintained reporter, which replicates in the overall synchrony with host genome (data not shown). The exponential growth of the Dpn-resistant input reporter level in E1 and E2 expressing cells appears as a result of several subsequent replication initiation events per ori in certain fraction of the input reporter molecules rather than due to the gradual addition of the unreplicated ori plasmids into replicating pool (Kivimae et al., 2001).

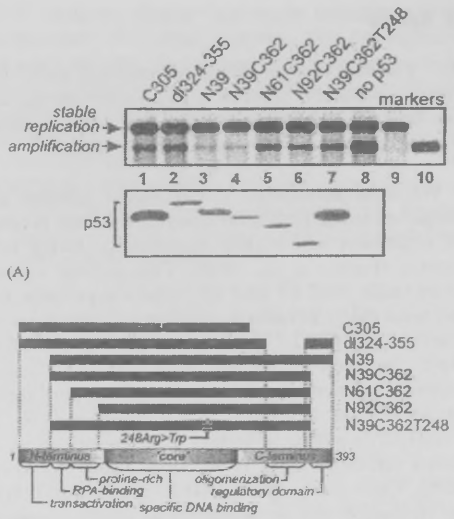


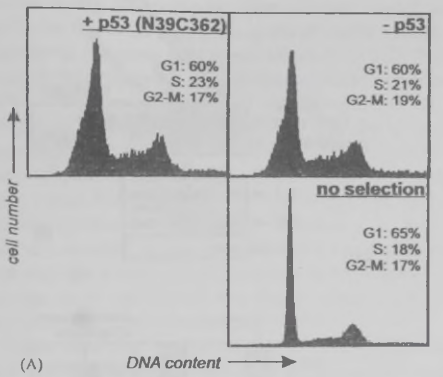
Fig. 1. The effect of different p53 constructs on amplification replication of the BPV1 ori in CHOBgl40 cells. (A) Cells were transfected with 250 ng of BPV1 URR reporter plasmid pNeoBgl40-HIII alone (lane 8), or together with 500 ng of the pCG plasmid expressing p53 mutant proteins (lanes 1–7) as depicted schematically on panel (B). 72 h after transfection, the effect of different p53 constructs on reporter plasmid replication was detected by Southern blotting analysis of the extracted low molecular weight DNA (A, top panel), and the p53 protein level was analysed by Western blotting (A, bottom panel). The position of bands corresponding to stably replicating endogenous pNeoBgl40 and to amplificationally replicating input reporter pNeoBgl40HIII is marked with respectively labelled legend arrows on the left. Lanes 9 and 10 on Southern blot correspond to 200 pg of the endogenous and input reporter plasmid markers, respectively.

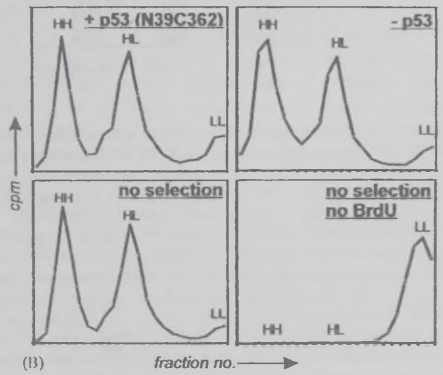
The results of these experiments demonstrate first of all that the amplificational replication of reporter plasmids carrying the BPV1 URR can take place in cells where the stable maintenance replication of essentially the same reporter plasmid has been already established (Fig. 1A, lanes 1–8). Secondly, p53 is an efficient inhibitor of the BPV1 ori-dependent amplificational replication in CHOBgl40 cells. This activity of the p53 protein does not require its N-terminal transactivation and C-terminal regulatory domains, as proteins which lack the first, or both, are efficient inhibitors of replication (Fig. 1A, compare lanes 3 and 4, respectively, to control transfection without p53 on lane 8). However, further deletion of any parts from either N- or C-terminus (Fig. 1A, lanes 1, 2, 5, 6) as well as the point mutation in central DNA binding domain (Fig. 1A, lane 7) relieves the block significantly. The effect of different mutations on the p53 activity is in good accordance with our earlier published results (Lepik et al., 1998). Parallel Western blotting analysis demonstrated that there was no apparent correlation between the expression level and the effect of different mutant p53 proteins on replication (Fig. 1A, lanes 1–7 on bottom panel). Therefore, we can conclude that p53 is able to function as a repressor of the BPV1 ori-dependent replication in CHOBgl40 cells.

3.2. Over-expression of p53 protein that is functional repressor of BPV1 URR-dependent replication does not lead to changes in the growth or viability of CHOBgl40 cells

Transient transfection assay and the analysis of total low molecular weight DNA by Southern blotting, used in experiments described above, are suitable for studying the effect of p53 on amplificational replication of the input BPV1 URR reporter. However, if we study the effect of p53 on the stably maintained reporter in CHOBgl40 cells, we have to bear in mind that all the cells contain the reporter, but only a certain fraction takes in the p53 expression construct during transfection. As a result, the non-transfected cells give rise to a 'background' signal, which could mask possible p53-induced changes in successfully transfected cells.

To resolve this problem, we used the Capture-Tec selection system from InVitrogen. For performing our subsequent experiments, we cloned a truncated version of p53 (N39C362), which contains a minimal set of domains required for replication suppression activity, into the pHook2 selection plasmid. It is known that p53 over-expression can block the cell division cycle or initiate the apoptotic cell death program in certain conditions. As both of these processes would effectively interfere with interpretation of the data from reporter plasmid replication analysis, we first decided to check if any of these effects could occur in our experimental approach. We transfected 250 ng of the pHookN39C362 construct, or the same amount of empty pHook2 as a control, into CHOBgl40 cells. Appropriate amount of transfected p53-pHook expression plasmid was determined in advance experiments, taking into account both the relative efficiency of selection and optimal effect on transient amplificational replication of the BPV1 URR reporter (data not shown). 250 ng of BPV1 URR reporter plasmid pNeoBgl40HIII was added to both transfections, to mimic as closely as possible the conditions of following replication experiments. Total number of cells was let to rise at least four times after transfection, which corresponds to two doublings (~ 72 h). After that, the pHook carrying pool was separated from total population using Capture-Tec selection procedure and analysed on the flow cytometer (Fig. 2A). Control cells, which were transfected in parallel without any input plasmid DNA, were processed in identical conditions, excluding only the Capture-Tec selection. The results of these experiments demonstrated





	HH (%)	HL (%)	LL (%)
+p53 (N39C362)	41.0 ±2.1	48.6 ±1.3	10.4 ±1.1
- p53	46.3 ±1.6	48.1 ±2.3	5.6 ±0.7
no selection	43.7 ±1.2	49.3 ±1.3	7.0 ±1.1

(C)

Fig. 2. Analysis of the effect of p53 N39C362 expression on the growth and viability of transfected cells. CHOBgl40 cells were transfected with 250 ng of BPV1 URR reporter plasmid pNeoBgl40-HIII, together with 250 ng of pHookN39C362 ('+p53'), or with empty selection vector pHook2 ('−p53'). Transfected cells were let to pass through two cell divisions (72 h for FACS; 96 h for BrdU analysis) and pulled down from total population before cell analysis. 'no selection'-mock-transfected cells, processed in parallel without pull-down selection. (A) Flow cytometric analysis of the cells. The calculated percentage of cells in different cell cycle fractions is indicated on each separate panel. (B) (C) BrdU analysis of transfected cells. 'HH' (heavy–heavy), 'HL' (heavy–light) and 'LL' (light–light) indicate the fractions of genomic DNA with two, one, or none of the strands labelled, respectively. Panel 'no selection, no BrdU' represents non-labelled control cells and is included to indicate the approximate position of non-labelled genomic DNA peak. The percentage of different fractions from sum total, represented as a mean (± average deviation) of the data collected from three independent experiments, is indicated in table (C).

that the cell cycle profile of the p53-expressing cells was indistinguishable from that of the control cells carrying empty pHook2 selection plasmid (Fig. 2A, compare panel '+p53' to panel '−p53'), indicating that there are no p53 dependent effects on the growth of transfected cells. Despite the somewhat more diffuse histogram profile, the overall cell cycle distribution of Capture-Tec selected cells was close to that of the non-selected control cells (Fig. 2A, panel 'no selection'). This diffusion is not specific to p53 expression (Fig. 2A, compare panel '+p53' to panel '−p53') but rather appears as a result of the selection procedure, most likely because of the presence of cell-bound magnetic selection beads in Capture-Tec selected samples subjected to flow cytometer analysis. The expression of p53 N39C362 protein did not lead to the appearance of any significant apoptotic sub-G1 fraction on the cell-cycle profile (Fig. 2A, compare panel '+p53' to panel '−p53'), suggesting that the over-expression of p53 N39C362 protein does not induce significant changes in the growth or viability of CHOBgl40 cells in our experimental conditions.

For additional control of the growth characteristics of transfected and Capture-Tec selected cells, we also performed a BrdU labelling analysis. Transfection series of CHOBgl40 cells were performed essentially as in the case of cell cycle analysis described above, using the same plasmid constructs and quantities. The proliferation of cells through two cell doublings took slightly longer time in the presence of BrdU than without (96 h instead of 72 h). The pull-down of transfected cells was performed; total DNA from selected cells was fractionated on CsCl gradient, and analysed for relative BrdU labelling (Fig. 2B; the quantified results of three independent experiments are summarised on Fig. 2C). The results demonstrate that the Capture-Tec selection yields cells, the overall growth characteristics of which are similar to those in the total population. This is indicated by the BrdU labelling profile of the Capture-Tec selected, pHook2 transfected cells, which is almost indistinguishable from that of the mock-transfected total cells not passed through pull-down selection (Fig. 2B and C, compare panels '−p53' to panels 'no selection'). The BrdU labelling profiles of the pHook2 and pHookN39C362 transfected cells are also close to each other, indicating that the expression of p53 that is functional in replication suppression does not change the growth features of CHOBgl40 cells (Fig. 2B and C, compare panels '+p53' to panels '−p53'). The overall distribution profile of the labelled genomic DNA in all these experiments corresponds to that expected in the case of two cell division cycles having passed, with approximately equal percentage of DNA in heavy–heavy and heavy–light fractions (Fig. 2C, compare values in columns 'HH' and 'HL'). Low percentage of DNA in non-labelled light–light fraction, which corre-

sponds to the sub-population of cells that have not passed through genome replication phase, is essentially identical in both Capture-Tec selected and non-selected control cells, and is only slightly higher in p53 expressing selected cells (6–7% compared to 10.4%, Fig. 2C, compare values in column 'LL'). The small sub-fraction of non-dividing cells, as noted previously, most likely represents a population that is hypersensitive to the slight inhibitory effect of BrdU on the cell growth (Piirsoo et al., 1996), with p53 expression adding weak additional pressure.

In summary, both the flow cytometric and BrdU labelling analysis indicate that the p53 expression does not have any significant impact on the growth or viability of CHOBgl40 cells that could interfere with the replication analysis of the BPV1 URR reporter plasmids in our experimental conditions.

3.3. Transient amplificational replication of BPV1 URR reporter plasmid is efficiently suppressed by p53 N39C362 in CHOBgl40 cells, but the stable maintenance replication of essentially the same reporter is not detectably affected in the same cells

For the analysis of the effect of p53 on stable maintenance replication of BPV1 URR reporter plasmid in CHOBgl40 cells, the same experimental conditions were used as in the control experiments described above. We co-transfected the p53 expression plasmid pHookN39C362 together with BPV1 URR reporter plasmid pNeoBgl40HIII into CHOBgl40 cells. The latter plasmid was included as an amplificationally replicating internal control for the replication suppressor activity of p53. Parallel control transfections were performed with empty pHook2 instead of p53 expressing construct, and with no input plasmid DNA. After two doublings ($\sim$72 h), the pHook carrying cells were separated from total population using Capture-Tec selection protocol, and total DNA extraction was performed. Equal amounts of extracted total DNA, as a measure of equal cell number for the quantitative comparison of the data, were subjected to *Hin*dIII and *Dpn*I digestion, and processed further for Southern blotting analysis (Fig. 3A). The summarised data from four different experiments, quantified on PhosphorImager and normalised to results from pHook2 control transfections, are represented in Fig. 3B. It should be noted that in the case of stably maintained reporter, both the de novo replicated and non-replicated plasmid pools have similar eukaryotic methylation pattern. Consequently, the *Dpn*I digestion reveals de novo replicated fraction in the case of amplificationally replicating input reporter, but only the overall level in the case of the stably maintained internal reporter plasmid can be measured.

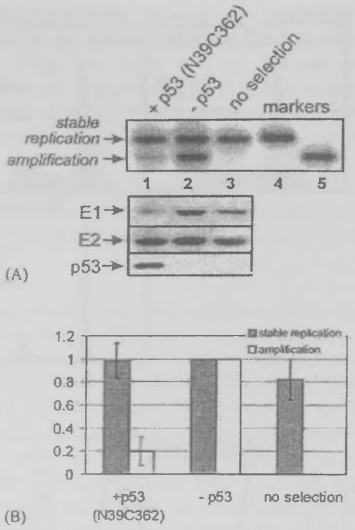


Fig. 3. p53 Expression has a different effect on the amplificational and stable maintenance replication of the BPV1 URR reporter plasmids. (A) 250 ng of BPV1 URR reporter plasmid pNeoBgl40HIII, together with 250 ng of the p53 N39C362 expressing (lane 1) or empty pHook2 selection vector (lane 2), was transfected into CHOBgl40 cells. After two cell divisions (72 h), the transfected cells were pulled down from total population and processed for subsequent analysis. The effect of p53 expression on the replication of BPV1 URR reporter plasmids was detected by Southern blotting (top panel), and the level of p53 as well as that of the endogenously expressed viral E1, E2 replication proteins was analysed by Western blotting (bottom panels). Lane 3 represents mock-transfected control cells processed in parallel without pull-down selection. In the case of the Southern blot, 0.5 μg of total cellular DNA was analysed on each lane, after digesting with *Hin*dIII and *Dpn*I. Positions of the stably replicating endogenous pNeoBgl40 and amplificationally replicating input reporter pNeoBgl40HIII are indicated with respectively labelled arrows. Lanes 4 and 5 represent 100 pg of endogenous and input reporter plasmid markers, respectively. For Western blotting, total protein from the same number of cells was analysed on each lane, 3.6×10^4 in the case of E1 and E2, and 4.5×10^4 on p53 blot. (B) Summarised results of Southern blotting analysis from four independent experiments quantified on PhosphorImager. Shaded columns represent the average steady state level of endogenous stably replicating BPV1 URR reporter pNeoBgl40 and open columns the average level of newly amplified input reporter pNeoBgl40HIII. Signals from the control transfections with empty pHook2 vector ('−p53') were set as 1.0 in every series. Column labels correspond to those on panel (A). The overall cell growth was estimated by counting the attached cells after transfection, and again before harvesting for pull-down selection. In experiments summarised on panel (B), the average rise in the cell number was 4.2±0.7 times, corresponding approximately to two cell divisions passed.

The results of these experiments show a clear p53-dependent inhibition of de novo amplificational replication of the input BPV1 URR reporter plasmid in CHOBgl40 cells (Fig. 3A, upper panel, compare lane 1

to lane 2; Fig. 3B, compare open column '+p53' to '−p53'). On the other hand, the level of stably maintained reporter in the same cells is not affected by p53. We can also see that the levels of endogenous stably replicating BPV1 URR reporter plasmid in Capture-Tec selected control and p53-expressing cells do not differ notably from the level in mock-transfected control cells that were not passed through the selection (Fig. 3A, compare lanes 1 and 2 to lane 3 on top panel; Fig. 3B, compare shaded column '−p53' to 'no selection'). It indicates that the selection procedure yields fractions that represent well the overall cell population in the regard of the average copy number of the stably maintained extrachromosomal reporter plasmid.

These data also supported our previous observation that both transient amplificational and stable maintenance replication modes of the BPV1 URR reporter plasmid can take place simultaneously in the same cells (Fig. 3A, lanes 1–2). Transient amplificational replication of the BPV1 reporter does not interfere with the simultaneous stable replication of similar reporter plasmid, as the copy number of the stably replicating reporter remains unchanged (Fig. 3B). Similar results were obtained from experiments where BPV1 URR was inserted into pHook selection plasmid and resulting construct was used both as the amplificationally replicating reporter and as a selection marker for pulling down transfected CHOBgl40 cells (data not shown). Thus, the initial transient amplificational replication and the replication of stably maintained URR reporter seem to be mechanistically different processes, and that fact is well reflected also in the different sensitivity to p53.

In order to exclude trivial explanations of the effects described above, we decided to check whether p53 had any effect on the expression of the key viral replication proteins in our experiment, E1 and E2. To examine this, we performed transfection and following Capture-Tec selection of transfected CHOBgl40 cells exactly as described above. The lysates from equal number of cells were subjected to Western blotting analysis using E1, E2, or p53 specific antibodies (Fig. 3A, bottom panels; respective blots are indicated by arrows). It was found that the levels of endogenously expressed E1 and E2 proteins are similar in both selected control cells carrying pHook2 vector, and in the mock-transfected control cells that were not passed through the selection (Fig. 3A, compare lane 2 to lane 3). The expression of p53 N39C362 protein (Fig. 3A, lane 1) does not affect endogenous expression level of the E2 protein in CHOBgl40 cells. However, the level of E1, which is expressed from different promoter, is somewhat decreased in response to p53 expression (Fig. 3A, compare lane 1 to lane 2 on respective blots).

To investigate whether the diminished E1 level could affect the evaluation of p53-dependent effects on the replication of BPV1 ori in CHOBgl40 cells, we tried to restore the E1 expression in p53 expressing cells to the initial level, and checked how it would affect the effect of p53 on transient amplificational replication of the reporter plasmid. We made a series of co-transfections of CHOBgl40 cells with the BPV1 URR reporter pNeoBgl40HIII, the pHook N39C362 expression plasmid (or empty pHook as a control), and rising amounts of the E1 expression plasmid pE1-1 × 5. The latter one is identical to the construct that is chromosomally integrated and responsible for the endogenous E1 expression in CHOBgl40 cells (Piirsoo et al., 1996). After two cell divisions (72 h), the efficiency of de novo amplification of the input BPV1 URR reporter was estimated by extracting low molecular weight DNA from the total transfected cells followed by its Southern blotting analysis (Fig. 4, top panel). Part of the transfected cells was passed through pull-down selection and lysed in SDS-loading buffer for following Western blotting detection of the p53 and E1 expression levels (Fig. 4, lanes 1–6 on bottom panels). The results of these experiments demonstrate, that the restoration and even the several-fold rise of the E1 expression above normal

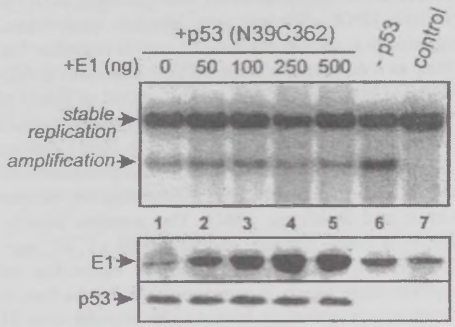


Fig. 4. The restoration and even several-fold rise of the E1 expression above its normal endogenous level does not restore the suppressing effect of p53 on the amplificational replication of the BPV1 URR reporter in CHOBgl40 cells. 250 ng of BPV1 URR reporter plasmid pNeoBgl40HIII, together with 250 ng of p53 N39C362 expressing (lanes 1–5) or empty pHook2 selection vector (lane 6), was transfected into CHOBgl40 cells. Rising amount (0–500 ng) of the E1 expression plasmid pE1 1 × 5 was co-transfected as indicated on top (lanes 1–5). After two cell divisions (72 h), low molecular weight DNA as extracted from total transfected cells, digested with *Hin*dIII and *Dpn*I, and subjected to Southern blotting analysis (top panel). Lane 7 corresponds to the mock-transfected control. Positions of the stably replicating endogenous BPV1 URR reporter pNeoBgl40 and amplificationally replicating input reporter pNeoBgl40HIII are indicated with respectively labelled arrows. Part of the transfected cells was pulled down from total population and total protein from 3×10^4 cells was analysed for E1 and p53 protein levels by Western blotting (bottom panel). No selection was applied to mock-transfected control cells on lane 7.

endogenous level caused only slight rescue of the p53-dependent inhibition of the amplificational replication (compare lanes 1–5 to lane 6). Parallel analysis showed that the p53 expression was insensitive to increasing levels of E1 expression (Fig. 4, lanes 1–5). Therefore, the sensitivity of BPV1 URR dependent transient amplificational replication in CHOBgl40 cells to the p53 over-expression cannot be explained solely by reduced level of the essential viral replication proteins.

4. Discussion

The results presented in this paper support our earlier observations, that the transient amplificational replication of the BPV1 URR reporter plasmids can be effectively suppressed by p53 expression. On the other hand, the already established, stable replication of extrachromosomally maintained BPV1 URR reporter is insensitive to p53. These data raise two main questions: first, what significance could the observed phenomenon have in the BPV1 life cycle; and second, what are the differences between these replication mechanisms that are reflected in their different sensitivity to p53.

Initial amplificational replication stage is required for successful extrachromosomal establishment of the BPV1 genomic DNA after infection. We can assume that the uncontrolled over-replication is not favourable for the virus, as it could induce the cellular responses leading to cell-cycle block or apoptosis. The period of initial quick rise of the copy number per cell is apparently considerably shorter in the case of full-length BPV1 genome in C127 cells than in the case of BPV1 ori plasmids with E1 and E2 co-expressed from heterologous promoters (Ustav and Stenlund, 1991). The possible reason for that is a tight control of the levels of E1 and E2 transcription from viral promoters upon the initial amplification, which in turn could limit the frequency of replication initiation. It could also explain, how BPV1 could establish itself in p53 expressing cells, even though its E6 protein, unlike that of certain cancer-associated high-risk HPVs, is unable to induce the degradation of p53 protein in ubiquitin-dependent proteolysis pathway (Scheffner et al., 1990; Thomas et al., 1999; Werness et al., 1990). In addition, p53 might also suppress the replication of viral genome molecules that for some reason re-initiate uncontrolled amplification during stable extracromosomal maintenance stage. Even though we have used human p53 for our studies, significant functional conservation of p53 proteins from different mammals can be expected on the basis of sequence homology, (http://www.ncbi.nlm.nih.gov) the full-length human p53 having approximately 80% amino acid sequence identity with bovine and 77% with mouse p53. Therefore, the ability of p53 to suppress PV replication can be attributed most likely not only to human and mouse p53 proteins, but also to those of other mammalian species, including cow.

On the other hand, both human and mouse p53 proteins that suppress the replication of BPV1 ori in human and hamster cell-lines, are unable to do so in several mouse cell-lines (Lepik and Ustav, 2000). The kinetics of E1, E2- dependent accumulation of the newly replicated BPV1 ori reporter plasmid in mouse cells is roughly similar to that in the human and hamster cells (Lepik et al., 1998; Ustav and Stenlund, 1991; Ilves and Ustav, unpublished). Therefore, the inability of p53 to function in mouse cells most likely does not reflect the differences in the replication mode or in the replication intermediates, but rather the inability of p53 protein to interact with specific target proteins participating in the replication initiation or regulation mechanisms.

The E1 and E2 dependent amplificational replication mechanism has been shown to be required for efficient establishing of the stable extrachromosomal maintenance of both BPV1 full-length genomes and URR reporter plasmids in C127 and CHO cells, respectively (Piirsoo et al., 1996; Ustav and Stenlund, 1991). Much less is known about the replication mechanism after the stable maintenance has been established. We show in this paper, that simultaneous amplificational replication of the BPV1 URR reporter does not affect the efficiency of stable maintenance replication of similar reporter plasmids in the same CHOBgl40 cells. Assuming that stably maintained URR plasmids are picked randomly from a total pool for replication initiation (Gilbert and Cohen, 1987), we conclude that the transiently replicating URR plasmids are not included into stably replicating pool. It means, that stably replicating URR reporter plasmid episomes have to be somehow differently 'imprinted' from amplificationally replicating input reporter plasmids. This 'imprinting' is bound to more tightly regulated replication initiation mechanism. The differences between control mechanisms of amplificational and stable maintenance replication are further emphasised by altered sensitivity towards p53. Unfortunately, we can only speculate at present, what exactly these differences are and which step in papillomavirus amplificational replication could be targeted by p53. Several reports have provided data about the activities and interactions of the p53 protein that suggest its direct involvement in the control of eukaryotic DNA replication process: p53 can bind to cellular replication factors, for example replication protein A (RPA) (Dutta et al., 1993; He et al., 1993; Li and Botchan, 1993) and DNA polymerase α (Kuhn et al., 1999), which might be differently used in different replication mechanisms. p53 can recognise and bind to the lesions in genomic DNA, like single-stranded regions (Bakalkin et al., 1995), that are likely to appear not only after various genotoxic stresses but also during intensive replication

processes. Such binding can regulate the activity of p53 protein (Jayaraman and Prives, 1995; Selivanova et al., 1996). The p53 has also a 3′→5′ exonuclease (Mummenbrauer et al., 1996) and ssDNA re-annealing activities (Bakalkin et al., 1994; Brain and Jenkins, 1994; Oberosler et al., 1993). One of the potential reasons for mechanistic differences between amplificational and stable replication of BPV1 could be that the stable maintenance replication might proceed independent of viral E1 helicase and rely on alternative cellular initiation factors and helicases. It has been reported recently, that even though intact E1 is required for episomal establishing of the BPV1 genomes in C127 cells, its continuing activity seems to be dispensable for already established stable extrachromosomal maintenance status (Kim and Lambert, 2002). The fact that neither the p53-dependent down-regulation of the E1 expression nor the additional over-expression of E1 (Fig. 4.) in CHOBgl40 cells affect the copy number of the stably maintained BPV1 URR reporter in our experiments seems to support this speculation. It cannot be excluded, however, that E1 is necessary, but expressed at much higher levels, for the replication of stably maintained reporter in CHOBgl40 cells. Consequently, the frequency of controlled E1-dependent replication initiation and the copy number of stably maintained reporter might be determined not by E1 level in these cells, but by other factors.

The long-term stable maintenance of BPV1 replicon is likely to be dependent on its non-covalent attachment to host chromatin, and this process has been linked to efficient partitioning and nuclear retention of the viral genomes during mitosis (Ilves et al., 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). We can speculate that the sub-nuclear localisation of the BPV1 genomic DNA could be important also for the regulation of its replication initiation mechanism. The association with chromatin could enable the access to the replication control mechanisms operating during the host cell genome replication. Different p53 sensitivity could appear as an indirect consequence of the altered sub-nuclear localisation, as it may be responsible for the changes in replication mechanism of the viral DNA that are differentially recognised and affected by p53. The sub-nuclear localisation of the viral replication process could perhaps also be directly linked to altered p53 sensitivity.

In conclusion, our data suggest that different replication modes of BPV1 for initial transient amplification and during the following stable maintenance stage have different sensitivity to p53. The elucidation of specific targets of the p53 protein could provide a convenient tool for defining the differences between these replication modes at the molecular level.

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Education and professional employment

1998–	research scientist in Department of Microbiology and Virology, IMCB, Tartu University
1995–2002	PhD student in Department of Microbiology and Virology, IMCB, Tartu University
1994	junior research scientist in Estonian Biocentre
1994	MSci in cell biology, Tartu University, the title of the thesis: "MAP-kinase in the slime mould Dictyostelium discoideum"
1993–1994	visiting graduate student in Exeter College and Department of Biochemistry, University of Oxford, UK; studies on the signal transduction of the slime mold Dictyostelium discoideum in the lab of Dr. Catherine Pears
1992–1994	MSci student in Department of Cell Biology, IMCB, Tartu University; studies on the tumour suppressor protein p53. Supervisor- Prof. Toivo Maimets
1988–1989	compulsory service in the soviet army
1987–1992	undergraduate studies in Tartu University, graduated *cum laude* as a cell biologist
1984–1987	studies in Pärnu Secondary School No.4, graduated with silver medal

Scientific work

Since 1994 I have been working in the lab of Prof. Mart Ustav, studying the replication and the virus-cell interactions of papillomaviruses.

CURRICULUM VITAE

Ivar Ilves

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Haridus ja erialane teenistuskäik

1998–	TÜMRI mikrobioloogia ja viroloogia teadur
1995–2002	TÜMRI mikrobioloogia ja viroloogia õppetooli doktorantuur
1994	Eesti Biokeskuse lepinguline nooremteadur
1994	Tartu Ülikooli teadusmagistri kraad rakubioloogias, magistritöö pealkiri: "MAP-kinase in the slime mould Dictyostelium discoideum"
1993–1994	külaliskraadiõppur Oxfordi Ülikooli Biokeemia õppetoolis ja Oxfordi Ülikooli Exeteri Kolledžis Suurbritannias, limaseene *Dictyostelium discoideum* biokeemiliste signaaliülekanderadade uuringud dr. Catherine Pears'i laboratooriumis
1992–1994	TÜMRI rakubioloogia õppetooli magistrant prof. Toivo Maimetsa juhendamisel, tuumorsuppressorgeeni p53 uuringud
1988–1989	ajateenistus nõukogude armees
1987–1992	õpingud Tartu Ülikooli bioloogiateaduskonnas, lõpetanud *cum laude* rakubioloogia erialal
1984–1987	õpingud Pärnu 4. Keskkooli bioloogia ja meditsiini eriklassis, lõpetanud hõbemedaliga
1976–1984	õpingud Tahkuranna 8-kl. Koolis, Tahkuranna Algkoolis ja Uulu 8-kl. Koolis

Teadustegevus

Tegelen papilloomiviiruste molekulaarbioloogiaga prof. Mart Ustavi juhitavas teaduslaboris, keskendudes viiruse replikatsiooni ja stabiilset peremeesrakus püsimist tagavate mehhanismide uurimisele.

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