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ANALYSIS OF HPV 18 E6/E7 ONCOGENES EXPRESSION IN ASSOCIATION WITH DNA AMPLIFICATION FROM INTEGRATED HPV SEGMENTS IN HELA CELLS

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CONTENTS

REVIEW OF THE LITERATURE 6
HUMAN PAPILLOMAVIRUSES 6
GENOMIC ORGANIZATION OF HPV 6
LIFE CYCLE OF HPV 8
PAPILLOMAVIRUS DNA REPLICATION 9
TRANSFORMING PROTEINS OF HPV 11
Functions of the E6 oncoprotein 11
Functions of the HPV E7 oncoprotein 13
E5 protein 15
HPV AND CERVICAL CARCINOGENESIS 16
MATERIAL AND METHODS 20
CELL LINES AND TRANSFECTION 20
PLASMIDS 20
REPLICATION ASSAY 21
WESTERN AND NORTHERN BLOTS 21
IMMUNOFLUORESCENCE AND FLOW CYTOMETRY 22
RESULTS AND DISCUSSION 24
INTEGRATED VIRAL ORIGIN DNA IS REPLICATION COMPETENT IN HELA CELLS 24
EFFECT OF OVER-REPLICATION ON THE EXPRESSION OF VIRAL ONCOPROTEINS 28
Western blot analysis of E7 and p53 expression levels in the context of over-replication 28
Western blot analysis of E7 and p53 expression levels performed in time 30
EFFECT OF OVER-REPLICATION ON E6/E7 RNA EXPRESSION IN HELA CELLS 32
EFFECT OF OVER-REPLICATION ON CELL GROWTH 35
SUMMARY 39
KOKKUVÕTE 41
ACKNOWLEDGEMENTS 43
REFERENCES 44

ABBREVIATIONS

7-AAD - 7-aminoactinomycin

aa - amino acid

bp – base pair

BPV 1 – Bovine Papillomavirus 1

cdk – cyclin dependent kinase

CMV – cytomegalovirus

CR - conserved region

DNA – deoxyribonucleic acid

DTT – dithiotreitol

GFP - green fluorescent protein

E1BS – binding site for the E1 protein

E2BS – binding site for the E2 protein

E6-AP – E6-associated protein

EDTA – ethylenediaminetetraacetic acid

EGFP - enhanced green fluorescent protein

FACS - fluorescence activated cell sorter

FCS – fetal calf serum

FITC – fluorescein isothiocyanate

HDAC - histone deacetylase

HFK - human foreskin keratinocyte

HPV – Human Papillomavirus

HRP - horseradish peroxidase

IMDM - Iscove's Modified Dulbecco's Medium

kb – kilobase pair

ori – origin of replication

ORF – open reading frame

PBS – phosphate buffered saline

Rb – retinoblastoma protein

SDS – sodium dodecyl sulphate

SV40 - simian virus 40

TAD - transactivation domain

URR – upstream regulatory region

INTRODUCTION

High-risk human papillomaviruses (HPV) infection is thought to be the primary cause of cervical cancer. Two early HPV genes, E6 and E7, play crucial role in tumor formation, interacting with the cellular tumor supressor proteins p53 and Rb, respectively. The HPV genome is maintained in HPV-infected cells as a supercoiled closed circular molecule, termed episome. However, in cervical cancer cells, HPV DNA is often found as integrated form, suggesting that integration into the host chromosome is important step in HPV-induced tumorigenesis.

HeLa, a cervical carcinoma cell line, contains several copies of integrated HPV 18 DNA. Integrated viral segments include URR, which contains the origin of viral DNA replication, and additionally intact E6 and E7 open reading frames (ORFs). The full-length E1 and E2 replication proteins are not expressed in HeLa cells because of the disruption of their ORFs during the integration process. However, it has been shown that the amino-terminal portion of the HPV 18 E1 protein is expressed in HeLa cells (Ma et al., 1999) and has a putative role in inhibition of HPV 11 DNA transient replication by sequestering cyclin/cyclin dependent kinase (cdk) complexes (Lin et al., 2000).

In our laboratory it has been recently shown that additional expression of both E1 and E2 replication proteins in HeLa cells results in viral DNA replication, which is initiated from integrated viral origins and progress in both directions into the flanking cellular sequences. Elimination of either E1 or E2 protein prohibited any initiation of replication from the integrated origins in HeLa cells. At high levels of the E1 protein, over-replication of the integrated viral regions results in forming of DNA puffs at integration sites and various replication products broken off from these sites. Such over-replication of integrated HPV DNA and generation of vast amounts of aberrant replicational intermediates may have an additional role in the pathogenic course of infection by high-risk papillomaviruses. In order to investigate this phenomenon, the following tasks of present study were established.

Firstly, it is required to investigate whether the intense amplification of integrated viral DNA is correlated with intense transcription from generated subgenomic fragments for the over-production of the E6 and E7 oncoproteins.

It is known that ectopical expression of the E2 proteins of different HPV types and BPV 1 results in transcriptional repression of integrated E6/E7 oncogenes in HeLa cells (Thierry and Yaniv, 1987). This in turn leads to reactivation of dormant tumor suppressor proteins p53 and Rb, and as a consequence leads to the cell cycle arrest and

senescence. The second task of this work is to analyse the expression levels of the p53 and Rb proteins in the context of the over-replication of integrated HPV DNA.

E2 alone is able to cause a growth arrest and over-replication of integrated viral DNA and flanking cellular DNA sequences upon addition of the E1 protein could lead to significant genomic instability, triggering the cellular defense mechanisms. The third task of this work is to carry out the parallel cell cycle analysis, fluctuations of which could be the cause of over-replication in concert with E2 effect.

REVIEW OF THE LITERATURE

HUMAN PAPILLOMAVIRUSES

Human papillomaviruses (HPV) are small double-stranded DNA viruses, which infect basal epithelial cells. There are more than 100 different HPV types, which can be devided into those infecting cutaneous and those infecting mucosal epithelial tissues. *Epidermodysplasia verruciformis*, a rare genetic disorder, and skin warts are caused by cutaneous HPV types. The viral types that infect the genital tract fall into two categories: high risk and low risk. The high-risk types, such as HPV 16 and HPV 18 cause lesions that can progress to cervical carcinoma. In contrast, low-risk types (HPV 6 and HPV 11) induce benign genital warts and are rarely found in cancers (zur Hausen, 2000).

HPV infection is one of the most common sexually transmitted diseases. The majority of HPV infection leads to mild disease, which usually will spontaneously regress in less than a year. The mechanisms by which the cellular immune response clears HPV infections are still not clearly understood. However, some of the lesions, especially caused by high-risk HPV types, are not cleared by immune system and persistence of HPV infection is the greatest risk factor for development of cervical cancer (zur Hausen, 1996).

GENOMIC ORGANIZATION OF HPV

HPVs are nonenveloped DNA viruses with icosahedral capsids that replicate their genomes within the nuclei of infected host cells. The circular DNA genomes of all HPV viruses are approximately 8 kilobases (kb) in size and have remarkable similarity in genomic organization. The genomic map of HPV 18 DNA is shown in Figure 1. All of the open reading frames (ORFs) are located on one strand of viral DNA. Transcriptional studies of the RNAs encoded by the papillomaviruses indicate that only one strand serves as a template for transcription.

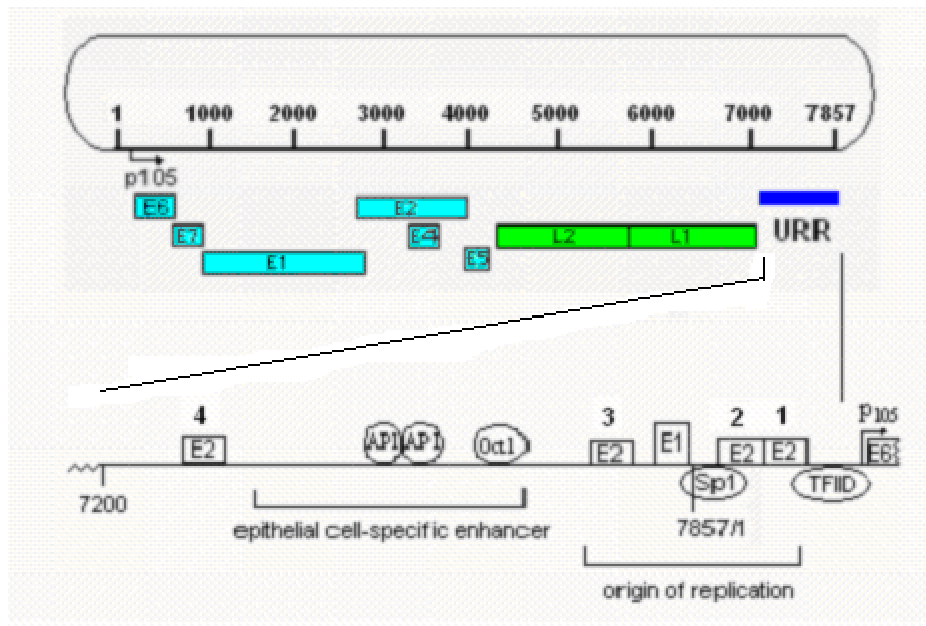


Figure 1. Genomic organization of HPV 18. Early ORFs are indicated in blue, while capsid genes are shown in green. Major promoter (P_{105}) is designated by arrow. In magnified lower part only noncoding region (URR) is shown. E1 and E2 binding sites are represented as rectangles. E2 binding sites are numbered from 1 to 4 from the most proximal to the P_{105} promoter to the farthest. Binding sites for various cellular transcription factors are shown by ovals.

The coding strand for HPV 18 contains 8 ORFs that are classified as either early (E) or late (L) ORFs. The early region (E) of viral genome encodes viral regulatory proteins, including those viral proteins that are necessary for initiating viral DNA replication. The L1 and L2 ORFs encode the viral capsid proteins and are expressed only in productively infected cells (Baker and Howley, 1987). The individual ORFs whose functions have been well characterized are described later in following sections of this thesis. There is a region of about 1 kb in all papillomavirus genomes in which there are no ORFs. This control region, termed as Upstream Regulatory Region (URR), localized directly upstream of the early region, contains the regulatory elements for viral transcription and viral DNA replication (Figure 1).

The major early promoter, which in case of HPV 18 is referred as p105, drives the expression of early viral proteins prior to amplificational DNA replication. It has been indicated that in some HPVs there is an additional strong promoter specific to the vegetative phase of HPV life cycle. In mucosal type of HPVs, the late phase promoter is found within the E7 gene (Chow, 1987a; Hummel et al., 1992). This promoter drives the expression of all downstream genes while E6 and E7 genes are expressed in smaller amounts in upper layers of epithelium (Higgins, 1992; Iftner, 1992).

LIFE CYCLE OF HPV

The papillomavirus life cycle differs from all other virus families: productive infection by HPV is linked to the differentiation program of epithelial host cells, keratinocytes (Figure 2). Because the basal cell of the epithelium is the only cell capable of dividing, the virus must infect the basal cell to induce a lesion that can persist. HPV infection of these cells leads to the activation of a cascade of viral gene expression that results in the production of approximately 20 to 100 extrachromosomal copies of viral DNA per cell (amplificational replication).

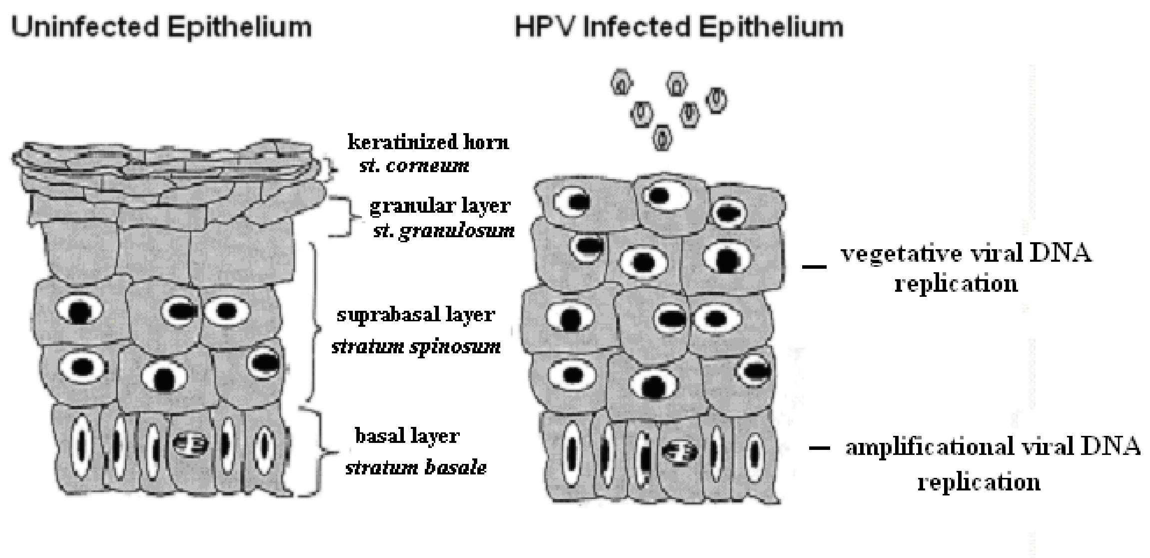


Figure 2. Cartoon of uninfected (left) and HPV-infected (right) epithelia showing various differentiated layers and virion production (Longworth and Laimins, 2004).

In the next phase of viral DNA replication copy number of viral genome is stable e.g. HPV genome is dividing more or less synchronously with cellular genome ("stable viral replication"). This occurs in dividing basal cells, where viral genomes are partitioned into daughter cells, one of which remains in the basal layer while the other moves up and starts to terminally differentiate.

In normal uninfected epithelia, cells exit the cell cycle as they leave the basal layer, and this often results in the loss of nuclei in suprabasal cells (Figure 2). As infected cells leave the basal layer, they remain active in the cell cycle due to the expression of viral proteins (E6 and E7), which are capable of stimulating G1 to S-phase progression (Cheng et al., 1995). However, at the same time the virus does require a certain level of differentiation because the shift from the early to late promoter is mediated by differentiation-induced transcription factors, resulting in the synthesis of large quantities

of structural proteins in terminally differentiating keratinocytes. The mode of viral DNA replication switches to a “rolling-circle” mode, which allows high amplification of the viral genome (vegetative DNA replication) (Flores and Lambert, 1997). In the upper layer of infected epithelia, there are about a thousand viral genomes per cell and assembly of viral particles occurs (Stanley, 2001).

PAPILLOMAVIRUS DNA REPLICATION

The replication of HPV DNA occurs in all three stages of viral life cycle according to differentiating status of epithelial host cells. Molecular analysis of the papillomavirus DNA replication machinery has first been addressed with bovine papillomavirus 1 (BPV1). It has allowed the identification of the essential elements, which have been later confirmed to be similar for HPVs. The E1 and E2 proteins are the only virus-encoded gene products that are necessary to initiate replication from the viral origin of DNA replication (ori) (Ustav and Stenlund, 1991; Ustav *et al*., 1991), which in HPV 18 is comprised of three highly homologous E2 binding sites (E2BS) flanking a less conserved E1BS (Figure 1) (Demeret et al., 1995). The host cell provides all of the other necessary components of replication machinery and substrates.

The HPV18 encodes a 73-kDa phosphoprotein E1, which has DNA helicase activity, DNA-dependent ATPase activity and shares amino acid sequence and functional homology with the related helicase, large T antigen, of simian virus 40 (SV40) (Mansky, 1997). As an initiator protein E1 can recognize and bind small repeat motifs within the origin of DNA replication and subsequently unwind the origin region and the DNA ahead of the moving replication fork (Gillette et al., 1994; Liu, 1995). The E1 protein binds to origin of DNA replication with low affinity, but in the presence of E2 protein, E1 binds to E1BS more efficiently (Russell and Botchan, 1995).

The papillomavirus E2 protein plays critical role in the viral life cycle, as it regulates both viral DNA replication and transcription of the viral E6/E7 oncogenes. The HPV18 E2 ORF encodes a 365 amino acid (aa) protein that can be divided into three distinct domains. The first 206 amino-terminal residues constitute the transactivation domain (TAD), which is followed by a nonconserved hinge domain and a DNA binding domain (DBD) of 80 aa (Figure 3.). The DBD domain is also responsible for the dimerization (Giri and Yaniv, 1988). There are four E2 binding sites localized within the HPV 18 URR, to which E2 protein binds as a dimer. Three of these sites, E2BS 1 to 3,

are confined to the proximal region of the promoter. E2BS 1 lays only 3 nucleotides (nt) upstream of the P_{105} TATA box, and E2BS 2 lies only 1 nt downstream of an Sp1 binding site. Binding of Sp1 and the TATA box binding protein (TBP) is essential for promoter activity (Dong et al., 1994; Hoppe-Seyler and Butz, 1992). A fourth site, E2BS 4, is located far from the other ones, upstream of the transcriptional enhancer (Figure 1).

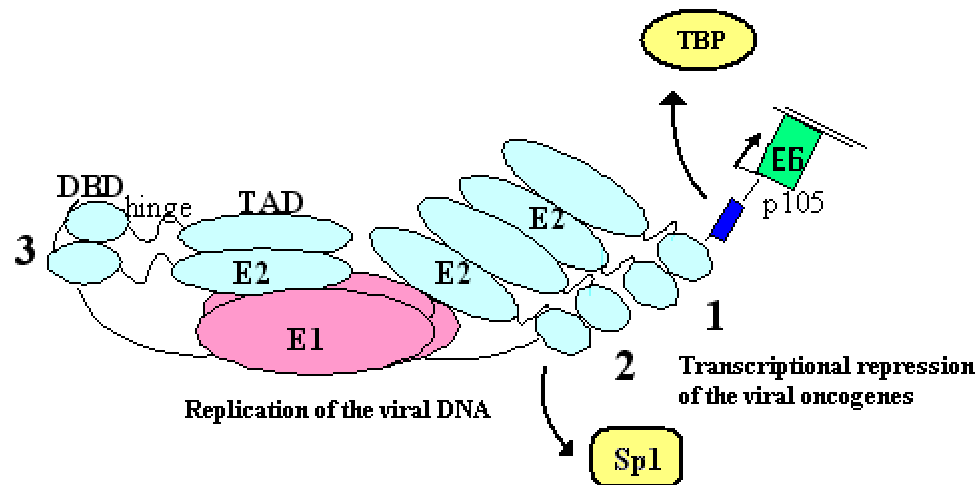


Figure 3. Schematic representation of the molecular consequences of HPV 18 E2 binding to the three binding sites of the origin of DNA replication.

Replication of the viral DNA is initiated by the cooperative binding of E1 and E2 proteins to their binding sites in the origin of DNA replication (Figure 3.). E2 interacts with the E1 protein through its TAD domain and helps to bring the low specificity E1 protein to the proper binding site. These interactions are essential to form an efficient replication initiation complex on the viral genome *in vivo* (Remm et al., 1992; Demeret et al., 1995). E2 also functions in preinitiation complex assembly but is not required during the elongation phase (Liu et al., 1995). In contrast, E1, the initiation helicase, interacts with Hsp40 (Liu et al., 1998) and DNA polymerase α (Park et al., 1994) and is required throughout elongation (Liu et al., 1995).

As noted above, the E2 protein is also a transcriptional regulator. HPV 18 E2 has been shown to repress transcription of the E6 and E7 viral oncogenes by binding to the same sites as those used for DNA replication. This E2 binding sterically hinders the access of cellular transcription factors (SP1, TBP) and the forming of transcription initiation complex (Demeret et al., 1997; Dostatni et al., 1991). At low levels, E2 activates the early promoter, probably through binding to the E2BS 4. At high concentrations through the binding to the E2BS 1, 2 and 3, HPV 18 E2 represses E6/E7

promoter simultaneously activating viral DNA replication. This regulation of viral expression contributes to copy number control in undifferentiated cells. On differentiation, there is a switch to the late promoter, which is not repressed by E2, resulting in increased E1 and E2 expression levels and subsequent vegetative viral DNA replication (Klumpp and Laimins, 1999; Steger and Corbach, 1997, Demeret et al., 1997).

TRANSFORMING PROTEINS OF HPV

The most dangerous aspect of the human papillomavirus is its potential to cause cancer. The E6 and E7 proteins have been shown to be primarily responsive for the transforming activity of the virus. They are independently able to immortalize various human cell types in tissue culture, but efficiency is increased when they are expressed together (Munger et al., 1989). With the help of these proteins the virus has evolved complex mechanisms to functionally inactivate the tumor suppressor proteins, p53 and Rb, resulting in the loss of cell cycle checkpoints and in accumulation of genetic abnormalities.

Functions of the E6 oncoprotein

The E6 proteins of both high- and low-risk types are approximately 150 amino acids in size and contain two zinc binding domains with the motif Cys-X-X-Cys (Barbosa et al., 1989). Expression of high-risk E6 alone leads to the transformation of NIH 3T3 cells as well as the immortalization of human mammary epithelial cells (Kiyono et al., 1998). However, efficient immortalization of human keratinocytes requires as mentioned above, the expression of both E6 and E7. Interestingly, it was found that E7 primarily caused tumor promotion, whereas E6 contributed weakly to the early stages, acting more strongly during tumor progression, accelerating the malignant conversion of benign tumors (Song et al., 2000). This finding suggests that E6 may be responsible for the malignant progression of HPV induced tumors *in vivo*.

The first cellular target of E6 to be identified, and probably still the most important, is p53. p53 is a well-characterized tumor suppressor that regulates the expression of proteins involved in cell cycle control, including the cyclin dependent kinase (cdk) inhibitor, p21. On exposure to DNA damage, p53 becomes activated and induces high-level expression of p21, resulting in cell cycle arrest and apoptosis. To

overcome the proapoptotic activities of p53 and allow for cell cycle progression, the high-risk HPV E6 protein has employed a major strategy to abrogate p53`s oncosupressive functions, inducing its degradation through the ubiquitin-proteasome pathway (Scheffner et al., 1990). As a consequence, the steady state levels of p53 are 2-3-fold lower in E6 immortalized cells or in HPV-positive cervical carcinoma cells compared with primary cells (Kessis et al., 1993; Foster et al., 1994). E6 accelerates degradation of p53 by recruiting an ubiquitin ligase called the E6-associated protein (E6-AP). Ubiquitin ligases are specificity factors in an enzymatic cascade that marks proteins for degradation by covalently tagging them with ubiquitin. Multiubiquitinated proteins are then recognized and degraded by the 26S proteasome (Hershko and Ciechanover, 1998). In normal cells, p53 is ubiquitinated by Mdm2 protein, but in cervical cancer cells the mdm2-mediated p53 degradation is inactivated and the E6/E6AP complex is the sole functional p53 specific ubiquitin protein ligase in such cells (Hengstermann et al., 2001). E6 can also retain p53 in the cytoplasm, blocking its translocation to the nucleus and thus inhibiting its function independently of degradation (Mantovani and Banks, 1999).

Another biological activity of high-risk HPV E6 proteins that is clearly independent of p53, is its ability to activate the expression of the catalytic subunit of telomerase, hTERT (Klingelhutz et al., 1996). Telomerase is a four-subunit enzyme that adds hexamer repeats to the telomeric ends of chromosomes. Telomerase activity is usually restricted to embryonic cells and is absent in somatic cells. Without telomerase, telomeres shorten with each cell division, until they reach a critically short length and cell senescence is initiated (Liu, 1999). Analysis of E6 mutants that discriminate between the ability to degrade p53 and to activate hTERT demonstrated that the latter activity is most important for immortalization. However, immortalization of human foreskin keratinocytes (HFKs) also requires inactivation of the Rb pathway through either mutation of cellular genes or the presence of E7 (Flores et al., 2000). It was shown that despite E6-mediated increases in telomerase activity in fibroblasts, telomere lengths do not increase. Instead, telomeres are maintained at a short, but apparently sufficient, length to allow for cell survival (Filatov et al., 1998).

A number of additional cellular targets of high-risk E6 proteins have been identified. It was demonstrated that E6 protein can bind the transcriptional co-activators p300/CBP (Patel et al., 1999), which play important role in activating a great number of genes involved in the regulation of cell cycle, differentiation and immune response. HPV 16 E6 was shown to inhibit the transcriptional activity of p300/CBP on both p53- and NF-κB-responsive promoter elements (Goodman and Smolik, 2000). A striking feature of

all E6 proteins derived from the high-risk HPV types is the presence of a highly conserved C-terminal domain, which is not involved in p53 binding and degradation, but which nonetheless contributes to E6 transforming activity (Kiyono et al., 1997). This region contains a PDZ-binding motif (XT/SXV), a short stretch of amino acids which mediates the specific interaction with proteins containing PDZ domains (Doyle et al., 1996). These include hDlg (the human homologue of the *Drosophila melanogaster* tumor suppressor protein discs large), MUPP1 (a multi-PDZ domain protein with a putative role in signal transduction) and hScrib (the human homologue of the *Drosophila melanogaster* tumor suppressor Scrib), which controls formation of cell junctions and inhibition of epithelial cell growth (Kiyono et al., 1997; Nakagawa and Huibregtse, 2000). The binding of PDZ family members to the high-risk E6 proteins results in the degradation of the PDZ protein. The fact that the PDZ binding site is not present in the low-risk HPV E6 proteins and some PDZ containing proteins are involved in negatively regulating cellular proliferation it is conceivable that interaction with PDZ proteins may contribute to the transforming activities of high-risk HPV E6 proteins.

Functions of the HPV E7 oncoprotein

The second HPV oncoprotein that is important for both immortalization and viral pathogenesis is E7. E7 proteins of both high- and low-risk types are small predominantly nuclear polypeptides of approximately 100 amino acids. They bind zinc through their carboxyl terminal domain, which contains two copies of a Cys-X-X-Cys domain that is related to those that make up the E6 protein (Munger et al., 2001). The E7 proteins are phosphorylated by casein kinase II (CKII) at an amino terminal domain and by an unidentified protein kinase in the C-terminus. E7 is a short-lived protein and its degradation both *in vitro* and *in vivo* is mediated by the ubiquitin-proteasome pathway (Reinstein et al., 2000).

Central to the action of the E7 proteins is their ability to associate with the retinoblastoma (Rb) ”pocket protein” family, which include Rb, p107, and p130 (Munger et al., 1989; Dyson et al., 1989). The binding of Rb is mediated through one of three conserved regions present in all high-risk E7 proteins: conserved region 1 (CR1) at the N-terminus; CR2, which contains an LXCXE motif that binds the Rb family members; and CR3, which contains two zinc finger-like motifs (Barbosa et al., 1990). The Rb tumor suppressor protein plays a central role in the regulation of the eukaryotic cell cycle and its activity is modulated by phosphorylation in a cell-cycle-dependent manner (Chen et al.,

1989). At least two cyclin-cdk complexes phosphorylate Rb, cyclin D-cdk4 or -cdk6 and cyclin E-cdk2 (Ewen, 1994). In hypophosphorylated state, Rb can bind to transcription factors such as the E2F family members and Rb/E2F complexes function as transcriptional repressors (Figure 4). As cells progress from G1 into S phase, Rb family members become progressively hyperphosphorylated by cyclin dependent kinases (cdk), releasing the transcription factor E2F, which in turn upregulates the transcription of the cyclins (D, E and A) that allow for transition from G1 to S phase (Dyson, 1998). E7 protein is able to bind to hypophosphorylated Rb and induce its degradation through the ubiquitin-proteasome pathway (Boyer et al., 1996). The E7-Rb interaction results in the release of E2F. It was shown that Rb family members are the major regulators of the cell cycle exit that occurs during epithelial differentiation. So, the abrogation of Rb function by E7 thus allows for productive replication in differentiated suprabasal cells (Chellappan et al., 1992).

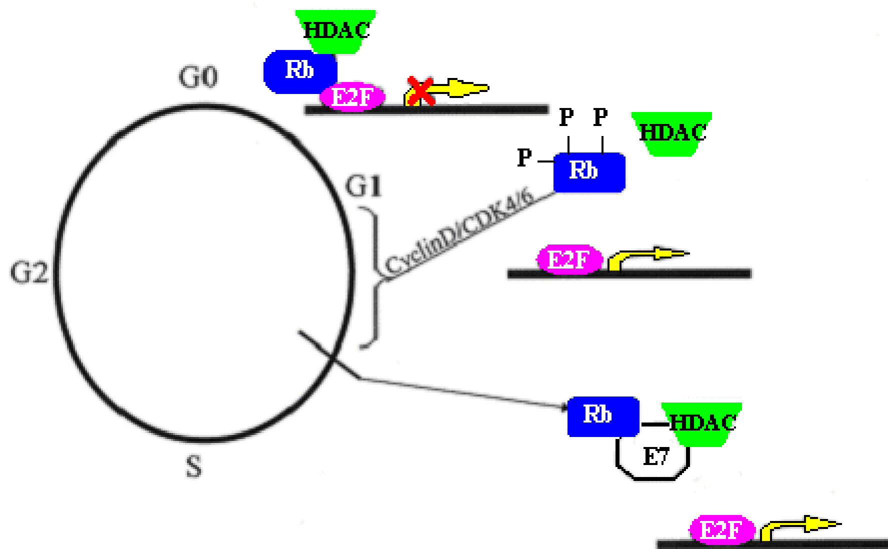


Figure 4. Cartoon of cell cycle regulatory activities mediated by Rb, HDAC, and E2F proteins. The effects of E7 on Rb and HDAC binding are indicated. E7 binds Rb and HDACs independently, resulting in the constitutive activation of E2F-inducible genes (Longworth and Laimins, 2004).

Genetic studies indicate that complex formation between E7 and Rb family proteins is not sufficient to account for its immortalization and transforming functions, suggesting that there are likely to be additional cellular targets of E7 that are relevant to cellular transformation. It was demonstrated that E7 can associate with histone deacetylase (HDAC) -1 and -2 through its zinc finger-like motif (Brehm, 1998) and this association is important for the role of E7 in immortalization (Longworth and Laimins, 2004) (Figure 4). HDACs modulate gene transcription indirectly through deacetylation of

histones and act directly to deacetylate cell cycle regulatory proteins such as p53 and E2F, resulting in loss of their function. Among the genes negatively regulated by HDACs are those involved in cell cycle progression, and E7 could act to sequester HDACs away from these promoters (Thiagalingam, 2003).

One of the intriguing properties of the high-risk E7 proteins is their ability to induce genomic instability. Expression of E7 alone was shown to be sufficient to induce an increase in abnormal centrosome numbers in primary human keratinocytes (Duensing et al., 2000). Centrosomes are major microtubule-organizing centers and coordinate segregation of chromosomes into daughter cells during cell division. Abnormal centrosome duplication rapidly results in genomic instability and aneuploidy, one of the hallmarks of a cancer cell. It is speculated that the ability of E7 to induce centrosome abnormalities may be related to the ability of this oncoprotein to dysregulate cdk2 and/or E2F activities (Jones et al., 1997).

E5 protein

The HPV E5 protein is a small hydrophobic membrane protein and is located just downstream of the E2 ORF. In contrast to bovine papillomavirus type 1 E5, which has been shown to encode the primary transforming function, little is known about the biological activity of HPV E5. HPV 16 E5 can activate the epidermal growth factor (EGF) receptor and MAP kinases (Crusius et al., 1997). Ectopic expression of HPV 16 E5 has been reported to induce transformation-related cellular changes in mouse fibroblast lines or mouse epidermal keratinocytes in the presence of epidermal growth factor (EGF) (Leechanachai et al., 1992, Leptak et al., 1991). HPV E5 is likely to be expressed primarily during the late phase of the life cycle in the differentiated epithelial cells and acts to augment the activity of E6 and E7 in modulating progression through cell cycle in differentiated cells (Fehrmann et al., 2003). However, the E5 protein is inessential for immortalization and transformation of human keratinocytes and it is not expressed in most HPV-positive cancers.

HPV AND CERVICAL CARCINOGENESIS

Cancer is the result of the loss of control over normal cell growth. Infection with the human papillomavirus has been established as a cause of cervical cancer. Of the oncogenic HPV types, HPV 16 and HPV 18 are found with the highest frequency in invasive cervical carcinomas in most populations (Bosch et al., 1995). Accumulating research suggests that HPV 18 infection is associated with a more aggressive form of cervical neoplasia than is HPV 16 infection. However, carcinoma develops infrequently even after infection by these HPV types, and it typically occurs years to decades after the initial infection.

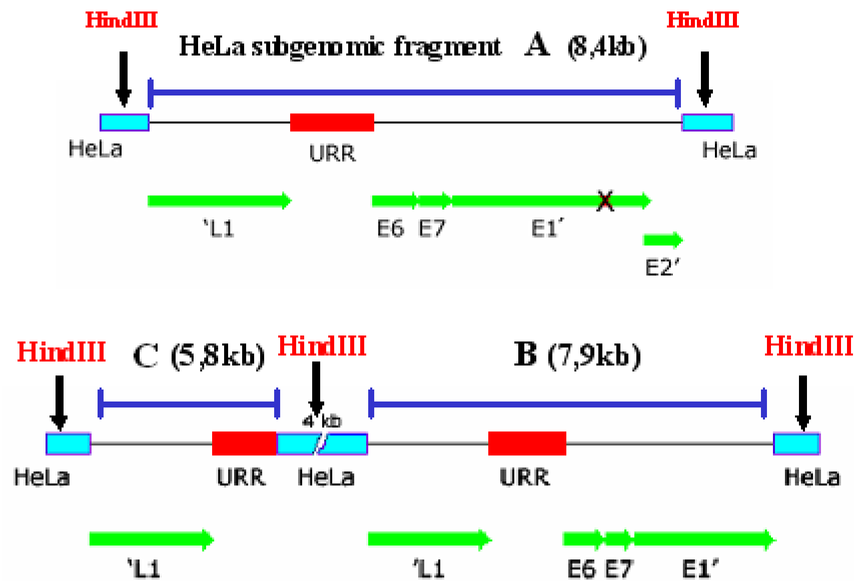
In HPV positive cervical cell populations, viral DNA is often found integrated into the cellular genome. Integration appears to occur more frequently in HPV 18-associated cervical cancer than in HPV 16-assotiated cervical cancer (Badaracco et al., 2002). To date, no preferential integration sites within the host cell genome has been identified (Wentzensen et al., 2002). Nevertheless, it has been repeatedly observed that integration of HPV DNA sequences into chromosomal regions takes place near the *c-myc* gene in some HPV-associated cervical cancers (Durst et al., 1987; Couturier et al., 1991; Ferber et al., 2003). However, no clear association between the virus and *c-myc* was found at the molecular level (Sastre-Garau et al., 2000). These results indicate that target-directed insertional mutagenesis or the activation of nearby oncogenes apparently plays a minor role for the development of HPV-assotiated cervical cancers.

Integration of viral DNA most often results in the disruption of the viral E2 ORF and the loss of E2 expression. Since E2 is a negative regulator of the E6/E7 promoter, the loss of E2 results in the deregulated expression of E6 and E7 (Thierry and Yaniv, 1987; Durst et al., 1992). The E6 and E7 proteins can immortalize cultured primary human keratinocytes, but these immortalized cells are not tumorigenic unless additional, undefined genetic events occur. In other words, up-regulated high risk HPV oncogene expression is a critical but not sufficient event in papillomavirus-associated carcinoma development.

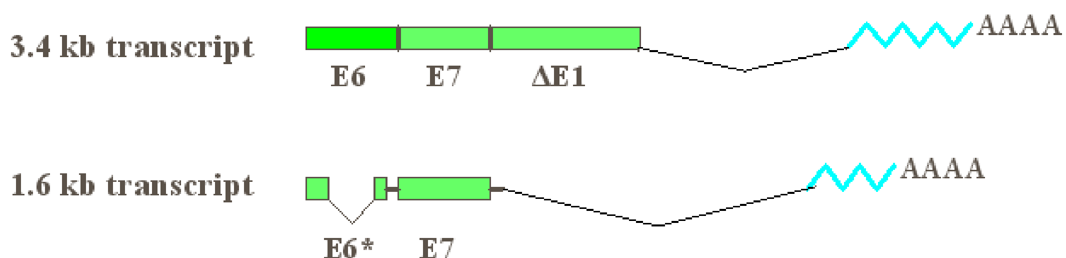
Cell lines derived from cervical carcinomas maintain papillomaviral DNA over long-term passaging and have therefore served as very useful models for the study of viral carcinogenesis. Human cervical carcinoma cell line used in this study is HeLa (Gey et al., 1952) and therefore will be discussed here in more detail.

HeLa cells have been demonstrated to contain 10 to 50 copies of HPV18 DNA sequences and three truncated forms of integrated HPV 18 DNA have been identified as

HindIII bands (Lazo, 1987). HPV-18 has no HindIII restriction site in its genome. The three integrated segments: A, 8.4 kb; B, 7.9 kb and C, 5.8 kb, have an incomplete viral genome (Scheme 1). The smaller HPV 18 subgenomic fragment, named C segment, has truncated L1 ORF, most of the HPV URR region and none of the genes of the early region. According to the published physical map of HPV 18 integration sites in HeLa cells (Scheme 1), during integration into the HeLa genome this segment has lost its 3´ part (ca 80 bp proximal to the P_{105} promoter) of sequence, which contains two binding sites for the E2 protein. It has been shown that these binding sites are very essential for HPV 18 DNA replication (Sverdrup and Khan, 1994). Because of the lack of viral P_{105} promoter and essential sites for viral DNA replication, the C segment is supposed to be inactive in both viral DNA replication and viral transcription in HeLa cells. The segments A and B contain complete URR region and intact E6 and E7 ORFs. It was shown that viral RNA transcripts in HeLa cells are derived from these segments (Lazo, 1987) and are transcriptionally active to produce the E6 and E7 proteins. The gene encoding the viral E2 protein, the transcriptional repressor of E6 and E7 gene expression, is disrupted or lost upon HPV DNA integration into the host genome, resulting in over-expression of the E6 and E7 oncogenes (Corden et al., 1999). Integration is also believed to stabilize E6/E7 mRNA transcripts by use of the cellular polyadenylation signal (Schwarz et al., 1985). Overexpressed E6 and E7 oncoproteins target the tumor suppressor proteins p53 and Rb, respectively, for accelerated proteosome-mediated degradation (Mantovani and Banks, 2001; Munger et al., 2001).



Scheme 1. Schematic representation of the three intergrated HPV 18 variant subgenomes present in HeLa cells. HeLa cellular DNA is shown in blue, the integrated viral URR regions are indicated in red and corresponding viral ORFs in green (Lazo, 1987).



Scheme 2. Splicing pattern of HPV 18 transcripts in HeLa cells. Two major transcripts- 3.4 and 1.6 kb presented in HeLa are shown schematically. E6, E6*, E7 ORFs and the 5´portion of E1 (ΔE1) are indicated in green. Zig-zag lines show flanking cellular sequences (Inagaki et al., 1988).

Two viral RNA transcripts of approximately 3.4 and 1.6 kb have been identified in HeLa, which are likely derived from segments A and B (Inagaki et al., 1988; Schwarz et al., 1985) (Scheme 2). The larger transcript contains E6, E7 and the 5` portion of E1, whereas smaller transcript carries alternatively spliced E6* and E7, both of them contain 3` human flanking sequences. In the 1997 Pim et al. showed that truncated HPV-18 E6* protein can interact both with the full-length E6 proteins from HPV-16 and HPV-18 and also with E6-AP, and subsequently blocks the association of full length E6 protein with p53. As a result of this block, E6* can inhibit E6-mediated degradation of p53 both *in*

vitro and *in vivo*, indicating a potential biological function for this polypeptide to modulate the activity of the full-length E6 during viral infection (Pim et al., 1997). It has been reported that HPV 18 DNA sequences in HeLa cells is located on chromosomes 5, 8, 9 and 22, where one integration site is within 40 kb 5`of the *c-myc* gene (Durst et al., 1987).

MATERIAL AND METHODS

CELL LINES AND TRANSFECTION

HeLa and C33A cells were cultured at 37°C in 5% CO_2 atmosphere, in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal calf serum (FCS), 100μg/ml penicillin and 100μg/ml streptomycin. Different amounts of plasmid DNA were transfected into HeLa cells by electroporation (975 μF, 220V, BioRad GenePulser). Cells were plated onto 100mm dishes and harvested 48 h and 72 h after DNA electroporation.

PLASMIDS

The basic constructs used in this work were HPV 18 E1 (EPIΔMB) and E2 (pCG18E2) expression vectors. The plasmid EPIΔMB contained HPV 18 sequences 105-2996 nt (including E6, E7 and E1 ORFs) inserted downstream from the cytomegalovirus (CMV) promoter in vector pCG (Tanaka and Herr, 1990). EPIΔMB also contains frameshift mutations within E6 and E7 ORFs and hemagglutinin (HA) epitope (12CA5) tag inserted after amino acid 4 of the E1 protein (Remm et al., 1999). The pCG18E2 expression vector was constructed by cloning a fragment containing the whole HPV 18 E2 ORF into the pCG vector (Tanaka and Herr, 1990). Plasmid pUCHPV18gen contains the wild-type HPV 18 genome, linearized with HindIII and inserted into the same site in pUC18 (Fermentas). HPV 18 URR containing fragment was obtained by restriction of pUCHPV18gen with BamHI and cloned into the same site in pUC18 vector, generating the pUC18URR. pEGFP-N1 was obtained from Clontech.

REPLICATION ASSAY

For transient replication assay HeLa cells were cotransfected with 1µg of the pCG18E2 and different amounts (0.5-10µg) of the EPIΔMB expression vectors. Total DNA from HeLa cell samples was isolated by the following protocol. Cells are washed twice on the dish with 1xPBS and lysed with 1 ml of Solution IV containing 100 mM NaCl, 10 mM EDTA, 0.2% SDS, 20 mM TrisCl (pH 8.0) and proteinase K (200 µg/ml). The very viscous lysate was digested overnight at 55°C and an equal volume of phenol-chlorophorm (1:1) was added, mixed vigorously and centrifuged at 13000rpm for 2 min. An agueous phase was placed into a new tube and precipitated with 96% ethanol. After incubation for 30 min at -20°C, the tubes were centrifuged at 13000 rpm for 15 min. The DNA pellet was washed with 70% ethanol, dried, re-suspended in TE buffer with 20µg/ml RNAse and stored at 37°C for 1 hour. After second precipitation with 96% ethanol plus 5M NaCl and washing step, DNA was dried and re-suspended in 50µl of TE buffer, pH 8.0. 5 µg of total DNA was digested with HindIII/DpnI enzymes and analysed by Southern analysis. Specific probes (labeled with [α-^{32}P]dCTP) for hybridisation were made by random priming using decalabel kit (Fermentas). Autoradiography was performed with Hyperfilm (Amersham) for several days at -80°C, depending on the intensity of the signal.

WESTERN AND NORTHERN BLOTS

For Western analysis of transfected HeLa cell extracts, transfected cells were washed and collected by scraping into PBS. About 20µl of re-suspended cells were used for manual counting in a Burker chamber, while the rest of cells were centrifuged at low speed and lysed in Laemmli sample buffer (2% SDS, 50 mM TrisHCl pH 6.8, 10% glycerol, 0.1% bromphenol blue, 100 mM DTT). Proteins were separated by 10% or 15% (for the HPV 18 E7 protein) SDS-polyacrylamide gel and transferred to PVDF membrane (Immobilon) by semi-dry electroblotting as described (Towbin et al., 1979). The unoccupied sites on the membrane were blocked with solution containing phosphate-buffered saline (PBS), pH 7.2, 0.05% Tween-20 and 5% w/v non-fat-milk powder at 4°C overnight. The membranes were probed with antibodies specific for the following proteins: p53 (DO-1, gift from Dina Lepik), HPV 18 E1 (horseradish peroxidase (HRP)-

conjugated monoclonal antibody directed against 12CA5 epitope tag), HPV 18 E2 (4E4) and E7 (10A8) from LabAs (Estonia). After incubation for 1 h at room temperature (RT) with 2% w/v non-fat-milk/PBS/0.05% Tween-20 and washing three times in wash solution (150 mM NaCl, 50mM TrisCl pH 7.5, 0.1% Tween-20), membranes then were incubated for 1 h RT in a 1:10,000 dilution of secondary antibody (HRP-conjugated goat anti-mouse IgG, LabAs, Estonia), washed as described above and incubated with ECL (Amersham). The signals were detected by Hyperfilm (Amersham).

Total cellular RNA was purified by using Trizol reagent (Life Technologies), and 20μg of RNA was subjected to 1% formaldehyde-agarose gel electrophoresis, transferred to a nylon membrane (AppiChem), UV cross-linked to the membrane with a Stratalinker (Stratagene) and hybridized with random prime-labeled probes (Decalabel kit, Fermentas) for the HPV 18 E6-E7 region. As loading control, a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene probe was used (kindly provided by Eva Zusinaite). Autoradiography was performed with Hyperfilm (Amersham) for several days at -80°C, depending on the intensity of the signal.

IMMUNOFLUORESCENCE AND FLOW CYTOMETRY

HeLa cells in 10 cm dishes were collected 48 h and 72 h after transfection by incubation for 5 min in PBS/3mM EDTA, pelleted by centrifugation, resuspended in 100μl of PBS and fixed by addition of 1 ml ice-cold 80% ethanol (-20°C for 15 min). Then 5 ml of 1% nonfat dry milk in PBS-0.05% Tween-20 was added, and the cells were collected by centrifugation (5 min, 300xg). The pellet was resuspended in 300 μl of primary antibody solution containing anti-E2 antibody 1E4 (1:500) or anti-E1 antibody against HA tag (1:1000; HA-7, product nr. H9658, Sigma) in PBS-Tween-20-5% nonfat dry milk and incubated for 1 h. 5 ml of 1% nonfat dry milk in PBS-Tween-20 was added, and the cells were pelleted by centrifugation as described above. The pellet was resuspended in 100 μl of anti-mouse FITC-conjugated secondary antibody solution (from DAKO, diluted 1:50 in PBS-Tween-20-5% nonfat dry milk supplemented with 1mM MgCl2 and 100 μg RNAse/ml) and incubated in the dark for 1 h. Finally, the cells were resuspended in 300 μl of PBS supplemented with 15 μg of DNA dye 7-aminoactinomycin D/ml (7AAD; Molecular Probes), incubated for 15 min and analyzed on a FACSCalibur cell sorter (BD Immunocytometry systems).

HeLa cells were transfected with 1 µg of a plasmid encoding enhanced green fluorescent protein (EGFP) in combination with different amounts of pCG18E2 and EPIΔMB expression vectors. Cells were washed twice with PBS, and then fixed with 0.5% paraformaldehyde (1:1) for 5 min RT, following ice-cold 80% ethanol fixation for 15 min at -20°C. After washing with PBS, cells were incubated with 10µg/ml RNAse/1mM $MgCl_2$ 1% non-fat-milk/PBS (1h, RT), stained with 7AAD and analyzed by FACS as described above. The GFP-positive cells were gated for analysis of their DNA content.

RESULTS AND DISCUSSION

INTEGRATED VIRAL ORIGIN DNA IS REPLICATION COMPETENT IN HELA CELLS

HeLa, a cervical carcinoma derived cell line, contains multiple copies of integrated HPV 18 DNA. Integrated segments in HeLa cells have the upstream regulatory region (URR) of HPV 18 DNA, which contains the viral regulatory elements, including the origin of viral DNA replication, the E6/E7 ORFs and the E1 ORF, which is non-functional either due to truncation or mutation. The expression of this non-functional HPV 18 E1 protein variant had been shown in HeLa cells (Ma et al., 1999). However, truncated E1 does not function as a replication initiator. On the contrary, it is possible inhibitor of HPV 11 transient DNA replication (Lin et al., 2000). The E1 and E2 proteins together are the only viral factors required for initiation of papillomavirus DNA replication (Ustav and Stenlund, 1991) and the rest of the essential factors for viral replication are supplied by the host cell. Although, previous results indicate that HeLa cells support the transient viral DNA replication poorly it was nevertheless decided by us to test the replication competence of HPV 18 integrated origins.

For transient replication assay the E1 (EPIΔMB) and E2 (pCG18E2) protein expression vectors of HPV 18 were used (described in Materials and Methods). The EPIΔMB construct (E1 ORF with upstream mutated E6 and E7 ORFs) was utilized to drive the expression of E1 protein since it is much more efficient than corresponding expression from the monocistronic E1 expression construct (for detail see Remm et al., 1999). Different amounts of E1 (0.5-10μg) and constant amount (1μg) of E2 protein expression vectors were transfected into HeLa cells by electroporation. The analysis of replication products was carried out by Southern blotting 48 and 72 h posttransfection (Figure 5A). The parallel Western blotting analysis of the exogenously expressed viral E1 and E2 replication proteins was performed (Figure 5B). The total cellular DNA was purified and digested with two enzymes: with DpnI, to remove *dam*-methylated DNA of expression vectors, in order to avoid formation of non-specific fragments during

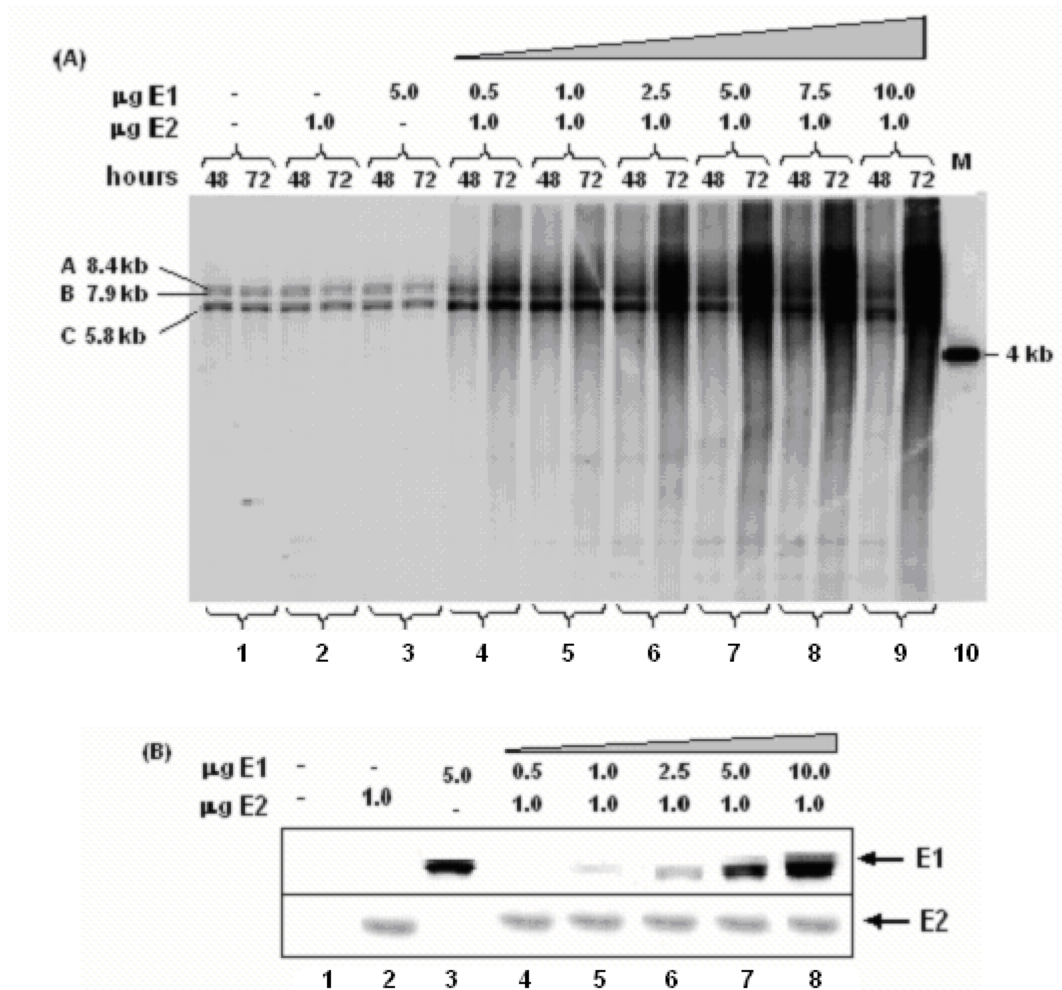


Figure 5. HeLa cells support the DNA replication of integrated viral origins in the presence of the E1 and E2 protein expression vectors. (A) 5µg of total DNA was digested with HindIII/DpnI and analyzed by Southern blot by using HPV 18 URR probe. Three viral DNA segments are indicated as A, B and C; E1 and E2 are EPIΔMB and pCG18E2 expression vectors, respectively; M, marker, pUC18URR linearized with HindIII. (B) Western blot analyses of expression levels of replication proteins E1 and E2 at 48 h after transfection in HeLa cells.

hybridization, and with HindIII, to analyse the replicated DNA containing viral origin in HeLa cells.

Due to the fact that all of integrated viral DNA fragments contain URR sequence, we used for hybridization radiolabeled HPV 18 URR fragment (ca 1 kb), which was derived from the pUCHPV18gen plasmid by BamHI restriction (see Materials and Methods and Appendix 1). The results were interpreted using the previously published physical maps of HPV 18 integration sites in HeLa cells (Meissner, 1999), shown also in Scheme 1 (see in Review of the literature).

Total DNA of non-transfected HeLa cells, digested with HindIII produce three basic fragments that can be visualized after Southern blotting and hybridization: A (8.4

kb), B (7.9 kb) and C (5.8 kb) (Figure 5A, lane 1). This is in agreement with the previously published data (Lazo, 1987). Transfection with E1 or E2 protein expression vectors alone did not reveal any changes in the signal intensity and was similar to the signal from non-transfected HeLa cells (compare lane 2 and 3 with lane 1), which is in accordance with the requirement of E1 and E2 proteins for initiation of replication. Western blot analysis reveals the presence of the E2 and E1 proteins produced from corresponding vector-vehicles transfected into HeLa cells (Figure 5B, lanes 2 and 3) and absence in non-transfected HeLa cells (Figure 5B, lane 1). This indicates the lack of expression of any endogenous viral replication proteins in HeLa system. Very strong replication signals have been detected in HeLa cells co-transfected with both E1 and E2 expression vectors and the rise of intensity of replication signal correlated with the E1 protein expression levels (Figure 5B, lanes 4-8), being more stronger at higher concentrations of the E1 protein and at later time points (Figure 5A, lanes 4-9). As the integrated HPV DNA has no HindIII restriction sites (Scheme 1), it is obvious, that replication, which is initiated from the integrated viral origins extends into the flanking cellular sequences. Many general mechanisms have been described for gene amplification in eukaryotic cells, and one of them is a disproportional replication mode, in which a portion of the genome is replicated more than once during a single cell cycle. Such replication- named onion-skin type replication- was described for integrated simian virus 40 (SV40) genomes by Botchan et al. in the 1979 (Botchan et al., 1979). According to this model new following replication complexes are formed on the origin of the viral locus in the chromosome before previous had completed their round of replication.

We suggest that replication in HeLa cells, induced by exogenous E1 and E2 viral replication proteins is similar to onion-skin type replication firstly described by Botchan et al. According to this model, the over-replicated viral origin-containing fragments are generated in HeLa cells as a result of multiple initiations of replication that take place at the integrated viral origins. As we tested, the newly replicated strands can either remain associated in a chromosome locus, where viral DNA is integrated, generating the DNA puff, or break off, resulting in extrachromosomal replication products which could be the subjects for many possible reactions, such as ligation, recombination, and others. Presence of such chromosomal and extrachromosomal fractions in HeLa cells in the context of over-replication was shown, using the modified Hirt method of DNA extraction (Hirt, 1967). This method allows the separation of high- (chromosomal) and low- (extrachromosomal) molecular-weight DNA. Extracted DNA was subjected to electrophoresis and hybridized with the same HPV 18 URR probe as described above.

The results have shown, that the newly replicated DNA is formed in the chromosomal fraction around the integrated viral origins, generating probably the DNA puff (schematically shown in Figure 6). The episomal (extrachromosomal) DNA fraction, containing presumably either linear fragments or circle molecules, is ranged in size from very small fragments of several hundred base pairs up to the 14 kb fragments (data not shown, lab experiments).

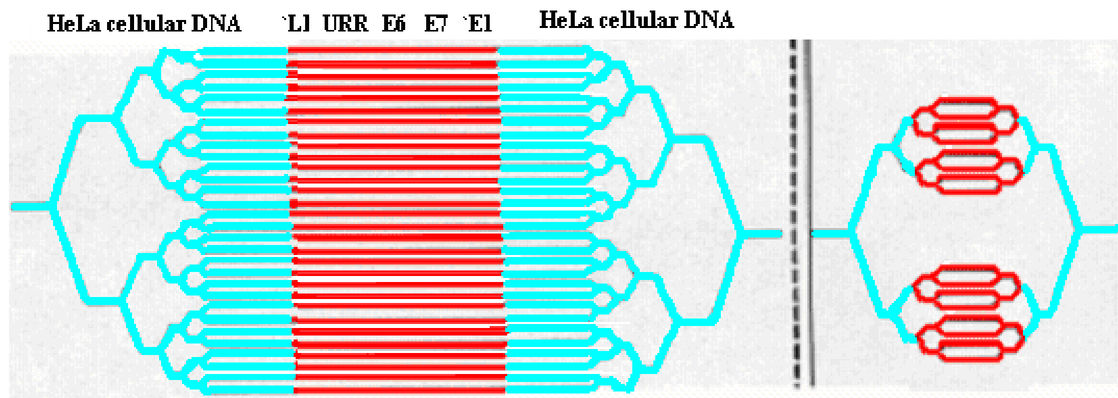


Figure 6 Schematic representation of DNA puff, which could be generated in result of over-replication of integrated viral and flanking DNA sequences in HeLa cells. The integrated HPV 18 DNA is shown in red, the genomic sequences are indicated in blue.

Although, the goal of present work is not the studying of overreplication itself in HeLa cells, but the studying of the effect of this overreplication on the expression of the endogenous viral E6 and E7 oncogenes.

EFFECT OF OVER-REPLICATION ON THE EXPRESSION OF VIRAL ONCOPROTEINS

Western blot analysis of E7 and p53 expression levels in the context of over-replication

Replication assay analysis has shown (Figure 5A) that integrated viral and flanking cellular DNA are replicated in HeLa cells upon exogenous expression of the HPV 18 E1 and E2 proteins. We suppose that generated replication products could serve as templates for additional transcription of viral E6 and E7 and further protein synthesis. In order to investigate this, the expression levels of E6/E7 oncoproteins and p53/Rb tumor suppressor proteins were assessed. Unfortunately, some problems prevented us to carry out the complete analysis: western blot analysis did not show any HPV 18 E6 and Rb protein specific signals, using commercial antibodies MAB874 (Chemicon) and G3-245 (BD PharMingen) for the E6 and Rb proteins, respectively. Probably, the difficulty of detection of E6 and Rb proteins is due to their low expression levels in HeLa cells and poor affinity of their antibodies. However, analyzing the expression levels of E7 and p53, it is possible to determine how the E6 and Rb proteins are expressed. Transcription of the E6 and E7 genes is driven by the single P_{105} promoter and activation or inactivation of this promoter leads to increase or decrease in expression levels of both E6 and E7 simultaneously. The same happens to the p53 and Rb protein levels, which are in straight dependence on expression levels of E6 and E7. So, meaning in parallel the levels of the E7 we refer to the levels of the E6 protein. In analogy the correlation will be used for the p53 and Rb proteins.

At first, we have analysed the expression levels of the HPV 18 E7 and p53 proteins by Western blot. HeLa cells were transfected with different amounts of E1 (0.5-10μg) and constant amount (1μg) of E2 protein expression vectors (Figure 7). Using non-transfected HeLa cells as control we observed the presence of the signal of endogenous E7 protein (lane 1) and no detectable signal for the p53 protein (lane 8). Transfection with E1 protein expression vector alone did not change anything in the expression levels of studied proteins (lane 2 and 9). HeLa cells transfected with E2 protein expression vector alone showed the decrease of E7 protein expression level (lane 3) and simultaneous increase in p53 protein expression level (lane 10), which is correlated to previous published results about the E2-mediated repression of E6/E7 viral promoter (for example, Goodwin and DiMaio, 2000; Desaintes et al., 1997). Termination in the E6

protein synthesis resulted in reduced amount of p53 targeted to the ubiquitin degradation system, leading therefore to increased levels of p53.

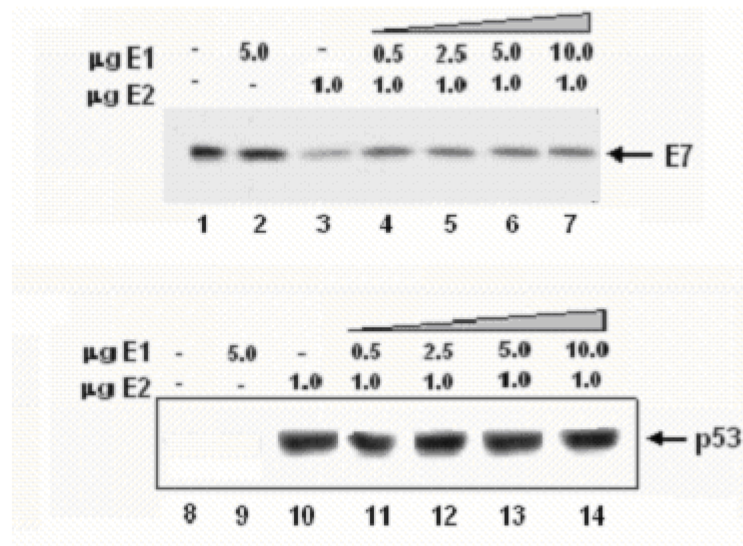


Figure 7. Effect of over-replication on the E7 and p53 proteins expression level. Proteins were harvested at 48 h after transfection, electophoresed, transferred, and probed with antibodies specific for HPV 18 E7 and p53 .

When E2 expression plasmid was co-transfected with different amounts (0.5-10µg) of E1 expression vector into HeLa cells, the E7 protein expression level became slightly increased (lanes 4-7), compared to the lowest expression level, which was caused by the influence of exogenous E2 (lane 3). The same E2 level was kept constant during the experiment (Figure 5B). We carried out several independent experiments using the same conditions, but results, concerning the E7 protein expression levels were not quite identical. In HeLa cells co-transfected with both E1 and E2 vectors the expression levels of the E7 protein slightly differed from each other, sometimes giving a little stronger or weaker signal at different concentrations of E1 vector, but nevertheless the signal was always lower compared to non-transfected HeLa cells (Figure 7, lane 1). However, the expression of p53 remained at the same increased level and did not depend on transfection of HeLa cells with either E2 vector alone or together with different amounts of E1 vector at this time point (compare lane 10 to lanes 11-14).

The major problem in establishing certain reproducible results is control of regulation of both viral DNA replication and E6/E7 oncogenes transcription regulation by the E2 protein. Replication of the viral DNA is initiated by the cooperative binding of E1 and E2 proteins to their binding sites to the origin of replication. E2 interacts with the E1 protein to form an efficient replication initiation complex on the viral genome (Remm et al., 1992; Demeret et al., 1995). E2 also represses the E6/E7 transcription by binding to

the same sites as those used for DNA replication (Demeret et al., 1997). Therefore, it is very difficult to separate these two processes in HeLa cells. Analysis of Western blots (Figure 7) showed, that the E2 repressive effect on E6/E7 transcription is stronger than effect from the over-replication. If suppose that at this time point (48 h) the E6 protein is expressed from DNA puff or from various extrachromosomal DNA, its amounts are nevertheless not enough to provide p53 degradation.

Western blot analysis of E7 and p53 expression levels performed in time

As previous analysis of expression of E7 and p53 was carried out only at 48 h posttransfection (Figure 7), we decided to investigate the expression of these proteins at later time points. HeLa cells were transfected with 1μg of E2 expression vector or co-trnsfected with 5μg of E1 expression vector alone. Cells were harvested at 24, 48, 96 and 120 h after transfection. The comparative analysis of expression of E1, E2, p53 and E7 is shown in Figure 3. All experiments have been done in transient system. It is obvious that E1 and E2 expression vectors introduced into HeLa cells, begin to disappear at forth or fifth day after transfection (Figure 8, lanes 4-7 for E1 and E2).

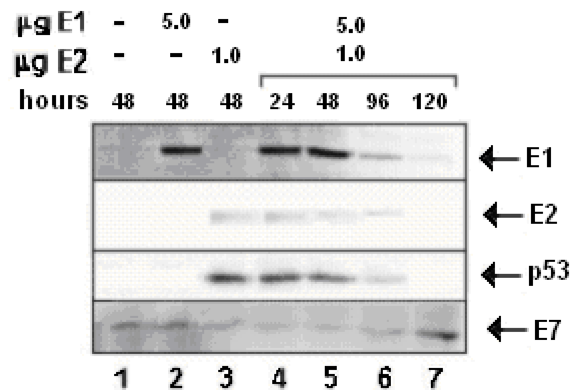


Figure 8. Western blot analysis of E1, E2, p53 and E7 protein levels at indicated hours after transfection with E1 and E2 vectors. E1, EPIΔMB; E2, pCG18E2.

Previous results, obtained in our lab, show that the probable DNA puff and extrachromosomal fraction are still maintained on fifth day after transfection with 2.5 μg of E2 and 10μg of E1 expression vectors into HeLa cells (Southern blot analysis, data not shown). Over-replication of integrated viral and flanking cellular sequences could induce the HeLa genome instability, which could in turn activate p53 through another pathway in parallel with E2-mediated stabilization of p53. But Figure 8 (rows for E2 and p53) illustrates well, that the expression level of p53 is tightly dependent on the level of E2 protein expression, which has been decreased in HeLa cells at 96 h posttransfection and completely disappeared at 120 h posttransfection (Figure 8, compare lanes 4-7 from E2 and p53 rows). The parallel Western blot analysis of the E7 protein expression level have also revealed the correlation between the co-transfected E1 and E2 expression vectors and endogenously expressed E7 (Figure 8, compare lanes 4-7 from E1, E2 and E7 rows). This correlation is rather due to the time-dependent loss of E2 expression vector from HeLa cells and further release of E7 transcriptional repression from integrated viral promoters. However, it can be supposed, that several days after inducing the over-replication in HeLa cells, when the transiently expressed E1 and E2 vectors disappear from the cells and E2 does not repress E6/E7 transcription anymore, the intensive viral transcription and consequent E6/E7 proteins synthesis would have become activated. However, further experiments should be done to clarify the situation.

EFFECT OF OVER-REPLICATION ON E6/E7 RNA EXPRESSION IN HELA CELLS

In addition to the protein analysis, we performed the analysis of the E6/E7 RNA transcripts in HeLa cells by Northern blot hybridization. There are found two major chimeric viral-cellular transcripts in HeLa cells (see Scheme 2 in Review of the Literature) that are composed of 5´-terminal HPV 18-specific sequences from the E6-E7-E1 part of the early region and 3´-terminal host cell sequences (Inagaki et al., 1988). The viral sequences are spliced to the 3´co-transcribed cellular sequences by using a splice donor signal located at the HPV 18 E1 ORF in combination with cellular splice acceptor sites.

HeLa cells were transfected with 1µg of E2 expression vector and 5 µg or 10µg of E1 expression vector alone, and also 1 µg of E2 vector together with different amounts (0.5-10 µg) of E1 vector. Total RNA was extracted from HeLa cells 48 h posttransfection, purified and subjected to formaldehyde-agarose gel electrophoresis. RNA was hybridized with a radiolabeled HPV 18 E6/E7 DNA fragment (Figure 9, upper panel). Reprobing the blot with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe shows that the equal amounts of RNA were loaded in each lane (Figure 9, lower panel).

When 1µg of E2 expression vector was transfected into HeLa alone, there was some reduction in HPV E6/E7 RNA expression level compared with used non-transfected HeLa cells (Figure 9, compare lane 2 with lane 1), indicating the repressive effect of the E2 protein on the E6/E7 promoter. The fact that E1 expression plasmid (EPIΔMB) contains the E6/E7/E1 ORFs (Figure 10) could disturb us in receiving the specific endogenous signals. We used HPV-negative cervical carcinoma cell line- C33A, transfected with 5µg of EPIΔMB as a negative control. Indeed, we obtained EPIΔMB-specific signal, which was situated exactly at the same height as 3.4 kb E6/E7 transcript from HeLa cells (Figure 9, lane 9). As a result, HeLa cells transfected with 5 µg and 10 µg of EPIΔMB plasmid alone showed an increasing EPIΔMB-specific signal, hiding the signal of 3.4 kb E6/E7 RNA expressed from HeLa genome (lanes 3 and 4). Comparing the signals obtained from HeLa, transfected with 5 µg and 10 µg of EPIΔMB vector alone (lanes 3 and 4) to signals from HeLa transfected with the same amounts of EPIΔMB vector plus 1µg of E2 expression plasmid (lanes 7 and 8), it was observed that the RNA signal of 5 µg of EPIΔMB (lane 3) was weaker than the RNA signal come from HeLa cells co-transfected with 5 µg EPIΔMB/1 µg pCG18E2 together (lane 7). The same type

correlation of RNA signals was observed in HeLa cells transfected with 10 µg of EPIΔMB alone or together with 1 µg of pCG18E2 (lanes 4 and 8, respectively). The question is whether the stronger signal is a result of over-replication of integrated viral and flanking cellular sequences with subsequent increasing in production of E6/E7 RNA or this is due to the EPIΔMB-specific signal, increased in the presence of 1µg of pCG18E2 vector.

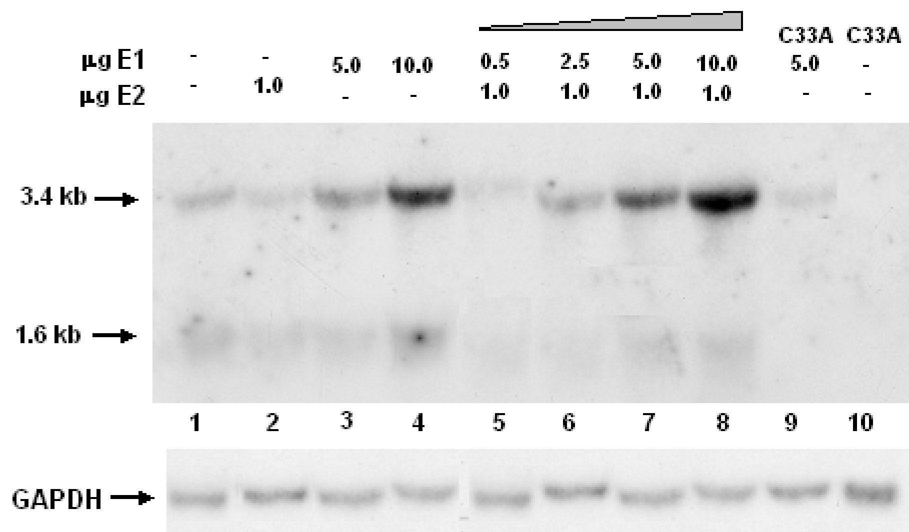


Figure 9. Northern blot analysis of HPV E6/E7 expression at 48 h posttransfection in HeLa cells (upper panel). GAPDH was used as internal control (lower panel). E2, pCG18E2, E1, EPIΔMB

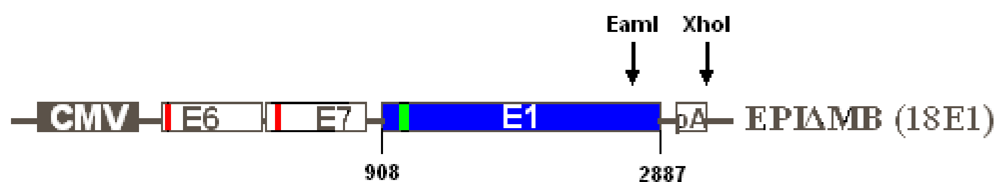


Figure 10. Schematic structure of EPIΔMB plasmid. Frameshift mutations in E6 and E7 ORFs are shown in red. 12CA5 tag inserted into 5′-terminal part of E1 ORF is shown in green. EamI and XhoI restriction sites are indicated. Nucleotide positions 908 and 2887 correspond to start and end nucleotide positions in the E1 ORF, respectively.

In order to investigate the nature of increased RNA signal, we reprobed the same blot with DNA, which is not present in HeLa derived transcripts, but specific to the EPIΔMB vector. According to the published results (Inagaki et al., 1988), the junction of viral and human sequences in 3.4 kb transcript is located next to the nucleotide 2497 of HPV 18 genomic sequence. For hybridization we used DNA fragment derived from

EPIΔMB vector by restriction with EamI (restriction site is located at 2660 nt in the E1 ORF) and XhoI enzymes (Figure 5). The results of hybridization are shown in Figure 6. As you see there is no HeLa specific signal and the 1.6 kb transcripts are not detectable. The only signal present is EPIΔMB-specific and the increasing intensity of signal, when EPIΔMB vector was co-transfected with 1μg of pCG18E2 is observed (Figure 11, compare lanes 7 and 8 with lanes 3 and 4). The reason why the presence of pCG18E2 plasmid promotes the expression of E6/E7/E1 RNA from EPIΔMB vector is not known.

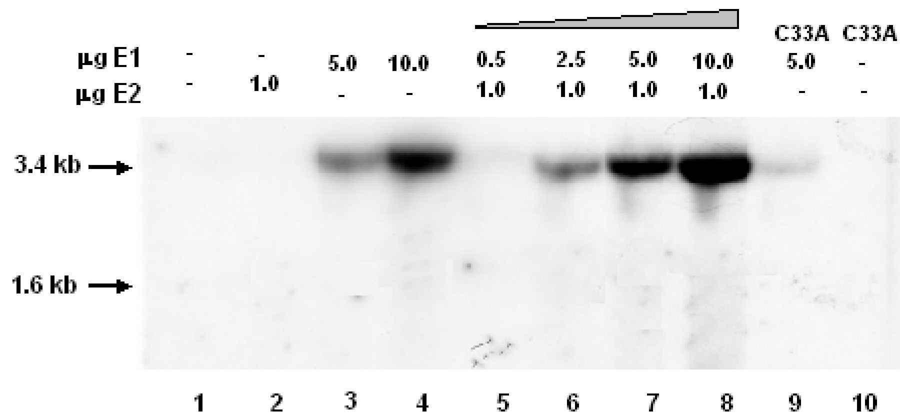


Figure 11. The same Northern blot used for analysis of HPV E6/E7 expression in HeLa cells (Figure 4) was stripped and reprobed with EPIMB specific DNA fragment.

Nevertheless, the real endogenous E6/E7 RNA signal is hidden behind the strong EPIΔMB-specific signal observed in Northern blot (Figure 9). However, it is likely, that at this time point (48 h) there is no increase in level of endogenous E6/E7 expression in HeLa cells co-transfected with both E2 and E1 expression vectors. The parallel Western blot analysis (Figure 8) has shown that at this time point (48 h) expression level of p53 is increased, indicating the prevalence of E2 repressive effect on E6/E7 expression in HeLa cells. Over-replication of viral/cellular sequences upon additional expression of the E1 protein in HeLa cells results in simultaneous repression of E6/E7 transcription because of E2 protein binding to the same sites required for both viral DNA replication and E6/E7 transcriptional repression, as described above. Taking this into consideration, it could be explained why we do not see any E6/E7-specific signal in addition to the EPIΔMB-specific signal (Figure 9). We suppose that on a fifth day posttransfection or later time points, when exogenously expressed viral E1 and E2 proteins disappear from HeLa cells (see Figure 8), but replication products are still present in a vast amounts (in accordance with copy number analysis performed in our lab), the expression of E6/E7 oncogenes

from the over-replicated viral sequences would take place. Therefore, further analysis of E6/E7 oncogenes expression is necessary to perform in time. It will be done in the near future.

EFFECT OF OVER-REPLICATION ON CELL GROWTH

The ability of papillomavirus E2 proteins to induce the changes in cell physiology is well documented by now. E2 has been shown to exhibit antiproliferative properties when ectopically expressed in HeLa cells, inducing either a G1 growth arrest or cell death by apoptosis (Demeret et al., 2003; Desaintes et al., 1997; Dowhanick et al., 1995). The G1 arrest is because of E2-mediated transcriptional repression of the resident E6 viral oncogene, which induces stabilization of p53, and results in p21 transcriptional activation. In contrast, apoptosis does not coincide with p53 activation (Desaintes et al., 1999), suggesting that the proapoptotic function of E2 might be independent from transcriptional repression of the viral oncogenes (Demeret et al., 2003). Therefore, we decided to check if such effects take place also in our experimental setup and if the additional expression of E1 and subsequent amplification of integrated viral genomes could cause any enhancement of these effects.

HeLa cells were transfected with 5 μg of E1 or E2 protein expression vectors alone or together. 48 and 72 h after transfection the E2 and E1 protein expressing cells were visualized by mixture of anti-E2 or anti-E1 monoclonal antibodies and FITC-conjugated secondary antibody. E2- or E1-positive cells were gated out and analysed for their DNA content by flow cytometry (Figure 12, shown are the data for a 48 h time point). As you can see, E2-expressing HeLa cells (panel b, middle) are mainly accumulated in the G1 phase of the cell cycle, confirming previously published results about the E2-induced G1/S arrest in HeLa cells (for example, Hwang et al., 1993; Dowhanick et al., 1995). Interestingly, relatively high percentage of E1-expressing cells can be observed in S phase of cell cycle (panel e, middle), which could indicate the possible cell-cycle arrest. This is unexpected result, because such effect of the E1 protein has never been established in HeLa cells. One possible explanation for the apparent accumulation of E1-positive cells in G1-S phase is removal of cytoplasmatic E1 during ethanol fixation following washing in PBS.

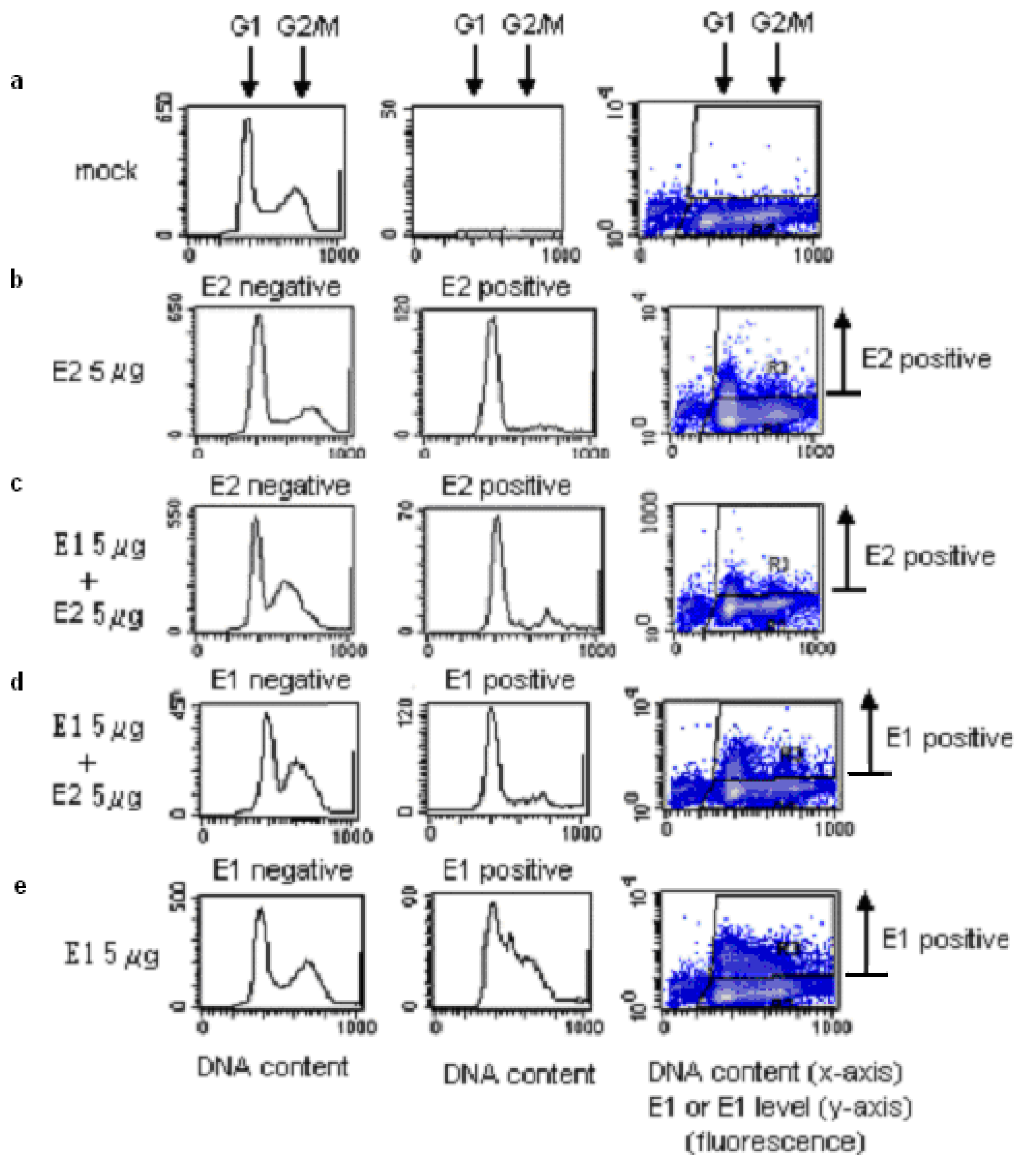


Figure 12. Analysis of the cell cycle profile in E1 and E2 expressing HeLa cells 48 h after transfection. On the density plots in the right column, the relative DNA content (x-axis) and the E1 or E2 level (y-axis) are analyzed for mock- or expression vector-transfected cells. The histograms in the left column illustrate the distribution of cells according to their relative DNA content in the population with no detectable protein expression, and those in the middle column illustrate the distribution of protein-positive cells from the same sample. Only the signals above the autofluorescence threshold were gated out to represent the E1- or E2-expressing cells (indicated at the top of the right column). The experiments were repeated at least three times with similar results. E1, EPIΔMB; E2, pCG18E2; mock, carrier DNA.

It has been shown by Deng et al., that nucleocytoplasmic localization of the HPV 18 E1 protein is regulated by cyclinE/cdk2 complex, which is required for entry into S phase (Deng et al., 2004). They showed also, that E1 is exported from the nucleus unless the nuclear export sequence (NES), located in E1, is inactivated by cyclinE/cdk2 phosphorylation. It is quite possible, that the E1 protein is located in the nucleus during the maximal activity of cyclinE/cdk2, e.g. in G1-S transition, and then is exported back to cytoplasm. Because of that, the fraction of cells that contain detectable E1 levels after ethanol fixation could be relatively over-represented in the S-phase compared to other cell cycle stages. To check this, we carried out another experiment, where the cells were cotransfected with 1 µg of EGFP and 5 µg of E1 expressing plasmids. 48 h after transfection the cells were fixed in 0.05% paraformaldehyde for 5 min follow by the ethanol fixation. The first fixation prevents the loss of cytoplasmatic fraction of proteins by cross-linking them to other cellular constituents. The reason we chose EGFP plasmid as a transfection marker is the independence of its expression upon the cell cycle. EGFP-positive cells were gated out and analysed for their DNA content, supposing at the same time, that cells expressing EGFP, express also E1. The flow cytometry analysis of E1/EGFP transfected cells did not reveal any E1- dependent change in the cell cycle distribution and the respective profiles were similar to mock-transfected cells (data not shown).

HeLa cells transfected with both expression vectors were divided into two parts, one of which was incubated with E1-specific antibody, and another with E2-specific antibody to analyze simultaneously the relative cell cycle distribution in E1- and E2-positive cells in the same sample. As it is shown on panels c and d (middle column), the additional expression of E1 and subsequent amplificational replication did not have any significant effect on the percentage of G1 arrested cells compared to the E2 protein expressing cells (compare panels c and d to panel b, middle column). The same accumulation in G1 was observed also at 72 h posttransfection (data not shown). Such result is expected, as E2-mediated p53 stabilization occurred already next day after E2 introduction into HeLa cells and remained at the same level for 3 days until E2 has its negative effect on E6/E7 transcription (Figure 8).

HeLa cells were also analysed for apoptosis. The cell cycle analysis of the HeLa cells transfected with 1 µg of E2 protein expression vectors alone revealed the presence of increased sub-G1 population, representing the cells that have passed an apoptotic cell death. The E2-expressing cells contained clearly higher percentage of apoptotic cells, around 20% compared to the 12% in the case of mock-transfected control cells. The

additional expression of E1 and subsequent amplificational replication did not increase the level of apoptotic cell death in transfected HeLa cells.

From this data, it can be concluded, that co-expression of HPV 18 E1 and subsequent amplification of integrated HPV 18 genome copies does not have any additional strong effect on cell growth or apoptotic cell death in addition to the E2-dependent effects. However, we cannot exclude that such effects are present, but too weak to be detected by our experimental approach.

SUMMARY

For quite a long time it has been acknowledged that human papillomavirus infection is the primary cause of the cellular changes leading to the formation of cervical cancer. HeLa, a HPV 18 positive cervical carcinoma derived cell line, was chosen as the model system for this study. HeLa contains multiple copies of integrated HPV DNA, which include the URR and E6 and E7 ORFs. Expression from these genes is absolutely necessary to maintain the transformed phenotype. We have been recently shown that integrated HPV 18 origins in HeLa cells are replication competent in the presence of viral E1 and E2 replication proteins. Additional expression of these proteins in HeLa cells gives rise to replication products, which seem to have arisen as a result of onion-skin type amplification process starting from integrated HPV 18 origins and extending to surrounding cellular sequences. The direct aim of the present study was to analyse the HPV 18 E6/E7 oncogenes expression from integrated HPV DNA amplified sequences and its effect on the cell growth.

The results of current study can be summarized as follows:

1. Expression of the HPV 18 E2 protein alone in HeLa cells results in repression of endogenous viral P_{105} promoter, leading to decrease in the level of E6/E7 protein expression and subsequent increase in p53 activity. At 48 h posttransfection E2-mediated repressive effect on E6/E7 transcription was dominating, therefore the additional expression of E1 and subsequent over-replication of integrated viral origins did not result in expression of E7 protein from newly synthesized viral/cellular sequences in HeLa cells. On the contrary, at later time point (120 h) the expression level of E7 has been increased due to the time-dependent loss of E2 expression vector from HeLa cells, resulting in further release of E7 transcriptional repression from integrated viral promoters and subsequent expression of E7 oncogene from amplified viral/cellular DNA sequences.

2. Upon E2 protein expression in HeLa cells, there was a reduction in E6/E7 RNA expression level, indicating the repressive effect of the E2 protein on the E6/E7 promoter once more. At 48 h time point from HeLa cells, co-transfected with both E1 and E2 expression vectors, the elevation of E6/E7 RNA expression levels was not detected, confirming the data from the Western blot analysis about the prevalence of E2 repressive effect on viral transcription at this time point and at chosen amount of E2 protein expression vector.

3. The analysis of the cell cycle has been shown that E2-expressing HeLa cells contain higher percentage of apoptotic cells compared to control cells and show the E2-induced G1/S arrest. The additional expression of E1 and subsequent over-replication clearly did not increase the level of apoptotic cell death and did not have any significant effect on the percentage of G1 arrested cells compared to HeLa cells expressing the E2 protein alone at 48 h and 72 h time points.

From all these data it could be concluded that the E1/E2 protein-mediated intense amplification from integrated viral and cellular sequences in HeLa cells takes place earlier than the expression of E6/E7 from corresponding over-replicated viral/cellular DNA. Further experiments should be done to confirm or deny this proposal.

KOKKUVÕTE

Vähkkasvaja arengu algetappidel integreeritakse kõrge riskiga HPV DNA tihti peremeesraku genoomi koosseisu. Inimese kartsinoomi rakuliinis HeLa on integreeritud HPV 18 regulatoorne piirkond (sisaldab viiruse DNA replikatsiooni origini) ja E6 ja E7 ORF-id. Viiruseline valk E2 on võimeline represseerima E6 ja E7 transkriptsiooni nende ühiselt promootorilt (P_{105}). Viiruse DNA integratsioonil HeLa raku kromosoomi on E2 ORF katkenud ning inaktiivne.

Hiljuti meie laboris näidati, et integreerunud HPV 18 originid on HeLa rakkudes replikatsioonivõimelised ja viiruselised replikatsiooni E1 ja E2 on suutelised initsieerima genoomiosas amplifikatsiooni. E1 replikatsioonivalgu taseme tõstmisel toimub integreeritud viiruselise DNA ning kõrvalasuvate rakuliste järjestuste intensiivne amplifikatsioon. Antud töö eesmärgiks oli analüüsida HPV 18 onkogeenide E6 ja E7 ekspressiooni tasemete muutusi HeLa rakkudes üleamplifikatsiooni kontekstis ning uurida selle mõju rakkude kasvule.

Eksperimentaalse töö tulemused võib kokku võtta järgnevalt:

1. HPV 18 E7 valgu ekspressiooni tase integreerunud viiruselistelt järjestustelt väheneb 48 tundi pärast E2 ekspressioonivektori transfektsiooni HeLa rakkudesse, põhjustades samal ajal tõusu p53 valgu ekspressioonis. Sellel ajamomendil (48 tundi peale transfektsiooni) E2-represseeriv toime oli domineeriv seda nii E2 üksi ekspresseerides kui ka koos E1 süsteemi lisamisega. Kuid hilisemal ajapunktil (120 tundi) E7 valgu tase oli tõusnud, näidates, et see võiks olla tingitud E2 ekspressioonivektori kadumisest HeLa rakkudest ajas ning selle järgneva E6/E7 transkriptsiooni käivitumisega. Kui palju annab juurde ülesvõimendatud transkriptiooni ühik E6/E7 ajas vaatamisel, vajab edasiseid uuringuid.
2. E6/E7 RNA tase oli samuti vähenenud HeLa rakkudest transfekteerituna E2 ekspressioonivektoriga üksi, tõestades E2-repressiooni efekti veel kord. 48 tundi pärast transfektsiooni, kasutades mõlemaid E1 ja E2 ekspressioonivektoreid E6/E7 RNA signaali tõusu ei andnud, mis on kooskõlas vähenenud E7 valgu tasemega E2 represseerivas süsteemis.
3. Rakutsükli analüüsil kinnitasime E2-vahendatud mõju rakkude kasvu peatumisele ning tõestasime suurenenud apoptootiliste rakkude hulka sel juhul. HeLa rakkudes,

kus olid ekspresseeritud mõlemad E1 ja E2 valgud, apoptootiliste rakkude hulk ei suurenenud ning G1/S faasi blokis asuvate rakkude protsent ei muutunud

Sellistest tulemustest lähtudes võiks oletada, et varasematel ajapunktidel domineerib E1/E2 põhine viiruseliste/rakuliste järjestuste replikatsioon HeLa rakkudes. Hilisematel ajapunktidel tekib aga võimalus E6/E7 onkogeenide ekspressiooniks, kuna E2 represseeriv efekt kaob rakkudest ajas. Lisakatsed tuleb teha , et kinnitada sellist oletust.

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