Genomic instability of the host cell induced by the human papillomavirus replication machinery

Meelis Kadaja¹, Alina Sumerina¹, Tatjana Verst¹, Mari Ojarand¹, Ene Ustav²
and Mart Ustav^{1,2,*}

¹Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia and ²Institute of Technology, University of Tartu, Tartu, Estonia

Development of invasive cervical cancer upon infection by ‘high-risk’ human papillomavirus (HPV) in humans is a stepwise process in which some of the initially episomal ‘high-risk’ type of HPVs (HR-HPVs) integrate randomly into the host cell genome. We show that HPV replication proteins E1 and E2 are capable of inducing overamplification of the genomic locus where HPV origin has been integrated. Clonal analysis of the cells in which the replication from integrated HPV origin was induced showed excision, rearrangement and *de novo* integration of the HPV containing and flanking cellular sequences. These data suggest that papillomavirus replication machinery is capable of inducing genomic changes of the host cell that may facilitate the formation of the HPV-dependent cancer cell.

The EMBO Journal (2007) **26**, 2180–2191. doi:10.1038/

sj.emboj.7601665; Published online 29 March 2007

Subject Categories: microbiology & pathogens; molecular biology of disease

Keywords: cancer; DNA replication; genomic instability; HPV; papillomavirus

Introduction

Papillomaviruses are small species-specific DNA tumor viruses that establish latent infection in the basal cells of the differentiating epithelium with the help of viral oncoproteins and maintain their small, about 8 kb, circular double-stranded (ds) genomes as episomal multicopy nuclear plasmids in proliferating transformed cells (Howley and Lowy, 2001) via action of the viral replication proteins E1 and E2 (Ustav and Stenlund, 1991; Chiang *et al*, 1992; Sverdrup and Khan, 1994). The E1 protein is the replication origin recognition factor and viral helicase (Yang *et al*, 1993; Sedman and Stenlund, 1998), which in cooperation with E2 facilitates recognition and effective loading of the host cell replication complexes at the papillomavirus origin in the upstream regulatory region (URR) (Ustav *et al*, 1991, 1993; Remm *et al*, 1992; Russell and Botchan, 1995; Stenlund, 2003). Increase of E1 concentration above a certain level

results in overly frequent loading of the viral hexameric helicase and cellular replication complexes at the viral origin and induction of ‘onion skin’-type replication intermediates on the viral genome (Männik *et al*, 2002). Consequently, complicated topological structures of the ‘onion skin’-type replication intermediates as well as heterogeneous linear subgenomic fragments are generated.

Based on tissue tropism, more than 100 different types of human papillomaviruses (HPVs) are divided into subgroups of mucosal or cutaneous papillomaviruses; individual viruses from each group are designated ‘high risk’ or ‘low risk’ according to the propensity of the infected transformed cells for malignant progression (zur Hausen, 2002; de Villiers *et al*, 2004; Munger *et al*, 2004). ‘Low-risk’ HPVs (LR-HPVs) like HPV6 and HPV11 are considered as rather normal microflora of the human epithelia, as they are generally limited to low-grade lesions or genital warts that rarely progress to malignancy. However, moderate and severe cervical dysplasia as well as invasive cervical carcinomas contain predominantly the ‘high-risk’ type of HPVs (HR-HPVs) such as HPV16 and HPV18. Development of invasive cervical cancer is a stepwise process, which is associated with integration of the HR-HPV DNA into the host cell chromosome, switching off the functions of the two viral replication proteins and upregulating the expression of viral oncoproteins E6 and E7 (Corden *et al*, 1999; Kalantari *et al*, 2001; Peter *et al*, 2006). Cells carrying integrated HR-HPV sequences have selective growth advantage due to the increased cell immortalization (Romanczuk and Howley, 1992; Jeon *et al*, 1995). The trigger and mechanism of the integration is unclear, but it seems to be a critical event in cervical carcinogenesis preceding the development of chromosomal abnormalities that drive the malignant progression (Pett *et al*, 2004, 2006). In the case of HPV18, HPV31 and HPV35, nearly 100% of the viral sequences show integration into the cancer cell genome. Integrated and episomal viral genomes are commonly found in the HPV16 DNA-positive cancers (Cooper *et al*, 1991; Kristiansen *et al*, 1994; Peitsaro *et al*, 2002b; Andersson *et al*, 2005; Arias-Pulido *et al*, 2006). Application of sensitive methods has shown that mixed (episomal/integrated) pattern is the most prevalent physical state of HPV16 already in PAP smears with normal morphology and in LG-SIL (Kulmala *et al*, 2006). The experiments with the HPV16-positive W12 cell line demonstrate the initial coexistence of episomal and integrated HPV16 DNA in the same cells in early passages (Pett *et al*, 2006), resulting in the loss of HPV plasmids in later passages. Similar results were obtained with the cell line carrying HPV33 (Peitsaro *et al*, 2002a). Coexistence of the replicating episomal viral genome and integrated HPV with potential viral replication origin raises the question about the functionality of the integrated origins. The papillomaviruses do not follow once-per-cell cycle replication mode (Ravnan *et al*, 1992; Piirsoo *et al*, 1996); therefore, multiple unscheduled initiation events at the functional integrated HPV origin could extend into the adjacent genomic locus and trigger rearrangements

*Corresponding author. Department of Biomedical Technology, Institute of Technology, University of Tartu and Estonian Biocentre, Nooruse 1, Tartu 50411, Estonia. Tel.: +372 737 4800; Fax: +372 737 4900; E-mail: mart.ustav@ut.ee

Received: 27 September 2006; accepted: 5 March 2007; published online: 29 March 2007

like deletions or duplications of the sequences of the cellular genome by repair/recombination machinery. Amplification of integrated polyomavirus genome in the presence of large T antigen (Botchan *et al*, 1979; Syu and Fluck, 1997) followed by recombination of the sequences suggests that such processes may take place also in the case of HPV. Furthermore, the integrated HPV regulatory sequences have been shown to be transcriptionally active in cervical cancer cells (Schneider-Gadicke and Schwarz, 1986; Baker *et al*, 1987).

We studied the events that may contribute to the formation of invasive cancers in the case of HR-HPV infections by demonstrating the effective HPV E1- and E2-dependent DNA amplification of the integrated HPV18 and HPV16 origins in HeLa and SiHa cells, respectively. The replication forks initiated at the integrated HPV origins extend into the flanking regions of cellular DNA. These amplified genomic sequences, presumably resembling 'onion skin'-type DNA replication intermediates, could be targets for the recombination and repair system that causes excisions of these sequences, resulting in *de novo* rearrangements and recombinations within the cellular DNA. The data presented suggest that papillomavirus DNA replication machinery

should not be considered as passive virus-specific activity alone, but also as one that can actively induce irreversible changes in the genomic make-up of the cell at sites of HPV origin integration.

Results

Regulation of the HPV E1 protein expression

Expression of the HPV E1 protein is regulated by the activities of the promoters, pre-mRNA splicing, stability of the mRNA, unusual translation mechanism of the polycistronic mRNA, nuclear export and import, and by stability of E1 protein (Remm *et al*, 1999; Malcles *et al*, 2002; Deng *et al*, 2003). Based on preliminary experiments (M Kadaja *et al*, unpublished), the expression vectors for producing the HPV6b, -11, -16 and -18 E1 proteins in a wide range in host cells such as COS, CHO, C33A, 293, HeLa and SiHa, were constructed. The E1 protein expression was evaluated by Western blot (Materials and methods) using HA-tag-specific antibody in HeLa cells (Figure 1D) and in SiHa cells (Figure 3C). Expression of the 3F12 epitope-tagged E2 proteins, directed from pQM vectors under the CMV promoter, was also mon-

Figure 1 HPV E1 and E2 proteins initiate the replication from episomal and integrated HPV origins in HeLa cells. (**A**, **B**) HeLa cells were cotransfected with plasmids for expression of homologous E2 and E1 from HPV6b (lanes 1–4), HPV11 (lanes 5–8), HPV16 (lanes 9–12) and HPV18 (lanes 13–16) together with 0.5 µg of pUCURR-6b, -11, -16 and -18, respectively. As controls, either 5 µg pMHE1-18 and 5 µg pQMNE2-18 (lanes 17 and 18) or 0.5 µg pUCURR18 alone (lanes 19 and 20) was transfected. A 3 µg measure of total DNA extracted 24 and 48 h after transfection was digested with *HindIII*/*DpnI* and analyzed by Southern blotting with radiolabeled pUC probe (**A**) or all four HPV URR sequences (**B**). (**C**) Schematic presentation of the HPV6b, -11, -16 and -18 E1 expression constructs. (**D**) Analysis of expression of HA epitope-tagged E1 of HPV6b (lanes 1 and 2), HPV11 (lanes 3 and 4), HPV16 (lanes 5 and 6) and HPV18 (lanes 7 and 8) 48 h after transfection with 3F10-HRP antibody. (**E**) Schematic representation of the episomal HPV18 genome and the integrated HPV18 in HeLa cells (Lazo, 1987; Meissner, 1999). Cellular DNA is shown as dashed line and HPV18 DNA as solid line. Open arrows represent the viral ORFs and noncoding URR is shown as an open box.

Figure 2 Replication of integrated HPV18 is dependent on E1 protein concentration, while E2 has no effect. (A, E) Increasing amounts of HPV18 E1 expression plasmids (from 0.5 to 10 µg) were cotransfected with 1 µg of HPV18 E2 expression vector. Total cellular DNA was extracted 24 h and 48 h after transfection and 3 µg of DNA was digested with *Hind*III (A) or *Bam*HI (E) and analyzed by Southern blotting with ³²P-labeled HPV18 URR probe. (B) Southern blot analysis of HeLa cells transfected with HPV18 E1 vector (5 µg) and an increasing amount of HPV18 E2 vector (0.5–10 µg). *Dpn*I was used to remove input plasmids. (C) Western blot analysis of HPV18 E1 protein expression in HeLa cells 48 h after transfection with 3F10-HRP antibody. (D) Western blot analysis of HPV18 E2 protein with 4E4 antibody. (F) Total cellular DNA was extracted at various time points from cells transfected with 1 µg pQME2-18 and 10 µg pMHE1-18 (lanes 1–6) or from mock-transfected cells (lanes 7–12); 3 µg from each DNA sample was digested with *Bam*HI/*Dpn*I and subjected to Southern blotting analysis with ³²P-labeled HPV18 URR-specific probe.

itored using a monoclonal antibody of 4E4 (Figure 2D). Tagging of E1 and E2 did not interfere with the functions of the proteins (data not shown) and enabled comparable expression levels of all proteins (HPV6b, -11, -16 and -18).

Expression of E1 and E2 proteins induces amplification of episomal and integrated HPV18 origins in HeLa cells

Replication of HPV plasmids in cervical carcinoma-derived cell lines has been shown to be strongly influenced by the presence of integrated HPV DNA, as these cells poorly support E1- and E2-dependent HPV-transient DNA replication (Chiang *et al*, 1992; Lin *et al*, 2000). To test our E1 expression constructs and to identify the conditions conducive to viral origin replication, the origin plasmids (pUC/URRs) of all HPVs, together with the homologous expression vectors for E1 and E2, were cotransfected into HeLa cells. The *Dpn*I-resistant replication signal from the transient assays was examined by Southern blot analysis of total DNA with common plasmid probe (Figure 1A). Replication of all origin plasmids was clearly detected in HeLa cells. Western blot analysis to monitor E1 protein levels in transfected cells (Figure 1D) confirms its effective and comparable expression

in HeLa cells. E2 levels were kept constant and considerably low in order to support replication without suppression of E6 promoter. HPV16 and HPV18 E1 proteins seem to be the most efficient in initiation of DNA replication within the used expression range, while HPV6B and HPV11 E1 proteins are clearly less efficient. In addition to the unit-sized plasmid, a smear of newly synthesized products with considerably larger and smaller size than input was detected, especially in the case of HR-HPV replication proteins (Figure 1A, lanes 9–12 and 13–16). The same kind of smearing characterized the ‘onion skin’ type of replication mode, as identified by 2D analysis (Männik *et al*, 2002). Probing of the same blots with URR-specific probes showed additional replication signals of different intensity in every lane across the panel (Figure 1B, compare with panel A). These signals may result from the multiple integrated HPV18 sequences located in at least five different sites of the host genome (Lazo, 1987; Meissner, 1999). It has been demonstrated that the URR with origin is present in addition to the coding sequences for viral oncoproteins E6 and E7 (schematically shown in Figure 1E). According to Lazo and Meissner, the *Hind*III digest generates three fragments of 8.4 kb (1A), 7.9 kb (1B) and 5.8 kb (2),

carrying HPV18 sequences (Figure 1E). The URR-specific probe for HPV18 detected amplification of exactly these fragments (Figure 1B). The intensities of these bands correlated with the E1 expression levels and were the strongest in the lanes of HR-HPV E1 expression constructs (Figure 1B, lanes 9–18). LR-HPV E1 proteins were less efficient for replicating integrated HPV18 origin, which may be explained by lower specificity toward the HPV18 origin, although expression level of the E1 proteins was comparable. Intensive replication signal was detected also in the control lanes, where only the viral expression vectors were transfected, suggesting that integrated HPV18 origin is fully competent for initiation of DNA replication (Figure 1B, lanes 17 and 18). These results show that papillomavirus replication proteins are capable of mobilizing integrated HPV origin and that simultaneous DNA replication of episomal and integrated HPV origins may occur in HeLa cells.

E1 protein is the major determinant of the efficiency of DNA replication

Titration of the HPV18 E1 and E2 proteins in the transient assays showed that the efficiency of DNA replication initiation depends on the E1 protein concentration (Figure 2A), while modulation of E2 concentration in a quite wide range had little effect on the efficiency of initiation of the DNA replication of the integrated HPV (Figure 2B). The replication reached a plateau at 0.5 μ g of transfected E2 expression plasmid (Figure 2B, lanes 6 and 7) and changed little at higher vector concentrations. Therefore, replication assays were run at low E2 levels to avoid suppression of E6 and E7 mRNA transcription from integrated copies and induction of apoptosis as well as cellular senescence (Desaintes *et al*, 1997; Wells *et al*, 2000). The E2 expression levels in our replication assays did not induce senescence or apoptosis of the transfected cells, as analyzed by different methods (data not shown). High E1 levels caused smearing of the integrated HPV18 origin replication signals characteristic of the ‘onion skin’ type of replication mode.

To prove that in HeLa cells high E1 levels do not cause unspecific initiation of DNA replication (Bonne-Andrea *et al*, 1995, 1997) and that origin-specific replication initiation is assured at any E1 concentration, we further analyzed the replication products. Cleavage of the integrated HPV18 sequences in HeLa cells (Figure 2E and F) with *Bam*HI generates a 1 kb URR fragment, which includes the complete functional HPV replication origin and 5.5 kb URR fragment containing nonfunctional origin linked to cellular sequence. The kinetics of accumulation of amplification products with time was studied from 24 h to 96 h after transfection (Figure 2E and F). The amplification level of functional HPV18 URR was estimated up to 90 times at the 72 h time point compared with controls. Moderate amplification of 5.5 kb fragment was detected at later time points, which most likely indicates that the replication forks that initiated from functional origins reach the nonfunctional origins in the 5.5 kb fragment. Interestingly, the 5.5 kb band is three times stronger than the 1 kb fragment in mock-transfected cells, which indicates that less than a quarter of the HPV18 URRs in HeLa cells carry functional replication origins.

The amplification of 6–8 kb viral/cellular fragments in the presence of E1 and E2 (Figure 2A) implies that DNA replication from HPV origin can extend into the flanking cellular

sequences. However, it was difficult to assess the size of viral amplicon in HeLa cells because of multiple integration sites and limited information about the surrounding sequences of the different HPV18 integration locuses. Therefore, the alternative cell line, SiHa, carrying a single copy of the integrated HPV16 genome, was further used to study the replication of the integrated HPV origin.

HPV E1 and E2 proteins efficiently initiate replication from integrated HPV16 origin in SiHa cells

SiHa cell line derived from the cervical carcinoma of a 55-year-old Japanese female is aneusomic and has been found to contain 66–72 chromosomes. However, this cell line has been shown to be disomic with respect to chromosome 13, which contains one copy of the HPV16 genome (Meissner, 1999; Suzhai *et al*, 2000). Integration of the HPV16 genome has occurred, with disruption in the E2 and E4 ORFs at nucleotides 3132 and 3384 of HPV16 genome (Figure 3A).

Expression plasmids for the E1 and E2 proteins were transfected into SiHa cells and replication of integrated HPV16 origin DNA was analyzed using HPV16 URR probe. The E2 expression plasmid concentration was kept constant at the level where replication of integrated HPV16 had reached a plateau (data not shown). Increasing amounts of

Figure 3 Amplification of integrated HPV16 URR in SiHa cells transfected with HPV E1 and E2 expression vectors. (A) Schematic presentation of the integrated HPV16 genome in chromosome 13 in SiHa cells. Viral ORFs are shown as open arrows and URR is indicated by an open box. Numbers in italics indicate the nucleotides of viral genome at the junction with cellular DNA. *Acc65I* restriction sites at nt 880 and nt 5378 of the viral genome are shown (B) SiHa cells were transfected with expression vectors for E1 and E2 as follows: 5–20 μ g of HPV6b E1 (lanes 1–3); 5–20 μ g of HPV11 E1 (lanes 4–6); 1.2–5 μ g of HPV16 E1 (lanes 7–9) and 1.2–5 μ g of HPV18 E1 (lanes 10–12). A 5 μ g measure of homologous E2 expression vectors was added for each transfection. For controls, either 5 μ g pMHE1-16 alone (lane 13) or 5 μ g pQMNE2-16 alone (lane 14) was transfected into the cells. The total DNA was isolated 24 h after transfection and 3 μ g was digested with *Acc65I*/*DpnI* and analyzed by Southern blotting using radiolabeled HPV16 URR probe. (C) Analysis of E1 protein expression in SiHa cells 24 h after transfection using Western blot.

E1 expression plasmids were used in replication assays. In order to reach comparable levels of E1 expression, much higher amounts of HPV6b and HPV11 E1 expression plasmids had to be used in the transfection mixture (Figure 3B and C). The results were interpreted using a previously derived physical map of the integration site of HPV16 (Meissner, 1999), shown schematically in Figure 3A. Untransfected SiHa cells as well as cells transfected with HPV16 E1 or E2 plasmids alone gave the faint, similar-strength signal of 3.4 kb after the digestion of an equal amount of total DNA samples with *Acc65I* (Figure 3B, lanes 13–15). Cotransfection of the E2 plasmid with increasing amounts of the E1 vector resulted in a significant increase in the HPV16 URR-specific signal in the case of all four HPVs. Expression vectors for the HPV16 (lanes 7–9) and HPV18 E1 proteins (lanes 10–12) induced most efficient initiation of DNA replication of the integrated HPV16 origin when compared with LR-HPV (lanes 1–3 and lanes 4–6). Remarkably, at higher concentrations of E1 protein of HR-HPVs (lanes 8 and 9, 11 and 12), a heterogeneous mixture of fragments that did not migrate in the gel as a specific band was generated. Analysis of the replication products by 2D gel indicated formation of ‘onion skin’-type replication intermediates and linear dsDNA fragments as shown for BPV1 (Männik *et al*, 2002) and HPV16 in SiHa (data not shown). This confirms that integrated replication origins of HR-HPV types are functionally active for the HPV E1- and E2-dependent initiation of replication.

Estimation of the size of the integrated HPV replicon

The replication competence of the integrated HPV origins directed by viral replication proteins brings up an intriguing possibility that flanking cellular sequences on both sides of viral integration could be coamplified. To test this possibility, we determined the size of the replicon in SiHa cells by measuring the amplification levels of cellular sequences at various distances from integrated viral DNA replication origin. The HPV16 genome is integrated at chromosome 13q21–31 in SiHa cells. The integrated HPV16 as well as the flanking cellular DNA have been sequenced (Baker *et al*, 1987; Meissner, 1999). The 5′ and 3′ flanking cellular DNA sequences, determined by Baker *et al*, were subjected to BLAST search against the NCBI 36 assembly of human genome (released in November 2005). According to the results, 5′ viral–cellular junction in SiHa was located at 72 686 871 bp and 3′ viral–cellular junction at 72 984 815 bp of chromosome 13. This suggests that the distance between these junctional sequences is normally ~300 kb, indicating that considerable loss of genomic DNA has taken place in the process of integration of HPV16 DNA. Coordinates of the viral–cellular junctions, determined by BLAST search, were used to identify and amplify cellular sequences at various distances from integrated HPV16 using PCR. Sequence blocks with the furthestmost nucleotide at 5.4 and 12.6 kb upstream (SL1, SL2 in Figure 4A) and 4.7 and 7.9 kb downstream of HPV16 ori (SR1, SR2 in Figure 4A), together with URR sequence itself, were used as probes in the following quantitative Southern blot analysis. The goal was to determine the amplification level of genomic sequences at different distances on both sides of the integrated HPV origin in SiHa cells, cotransfected with expression plasmids for E1 and E2 proteins of either HPV16 (Figure 4B, row 2) or HPV18 (Figure 4B, row 3). The control transfection was performed

with the carrier DNA alone (Figure 4B, row 1). The cellular DNA from transfected cells was digested with appropriate combinations of enzymes (lanes 1–5 in Figure 4B; location of restriction sites is depicted in the drawing in Figure 4A) and three analogous blots (with SiHa DNA, + HPV16 E1 and E2, and + HPV18 E1 and E2) were probed with four appropriate radiolabeled cellular sequences or URR probe (shown at the bottom of lanes 1–5 in Figure 4B). The hybridization signals of two independent experiments were quantified by Phosphorimager and normalized to the carrier-transfected SiHa cells (Figure 4A). The data show that DNA replication, initiated from the HPV16 origin, will extend into the flanking cellular sequences on both sides and that the DNA replication fork travels a distance of at least 12.6 kb from the HPV16 origin, on average, as analyzed at 24 h after transfection. That makes the total size of the amplicon to be more than 25 kb. We estimated that, under the experimental conditions used, the transfection efficiency was approximately 40%. This means that more than half of the signal comes from the cells that do not have E1- and E2-induced amplification. This suggests that at the cellular level amplification as well as the replicon size is likely to be more than the estimated values.

The replication proteins of HPV18 were more efficient in initiating DNA replication of the HPV16 URR compared with HPV16 proteins (solid line compared with dashed line in graph in Figure 4A). However, there are almost no differences in the amplification of distal cellular sequences (SL1, SL2, SR1 and SR2) between these sets of replication proteins. Furthermore, we observed that even if the replication of integrated HPV16 origin is highly dependent on E1 concentration (Figure 4C and D), the E1 concentration dependence of amplification of distal sequences decreases with the distance from the replication origin. There was a 10 times difference in the amplification signal of the HPV16 URR when 2.5 or 10 µg of HPV18 E1 plasmid was used, but less than 1.5 times difference in amplifying cellular sequences that are about 10 kb away (SL2 and SR2). These data suggest that overexpression of E1 protein may generate replication intermediates, locked for elongation, owing to the topological constraints in the ‘onion skin’ structures that would prevent traveling of replication forks further into the genome. Therefore, elongation of replication forks at considerable distances from the initiation site is determined presumably more by proper configuration of the template and replication complex functioning at it and less by the E1 level. These data also suggest that low E1 and E2 protein levels, for example from episomal HPV genome, and respective extension of the E1-driven replication into the flanking sequences may be highly relevant for induction of amplification of the HPV integration locus in HPV-infected cells.

Coreplication of integrated and episomal HPV18 in HeLa cells

HeLa cells were transfected with the plasmid carrying functional full-size HPV18 genome cloned into pUC19 plasmid. Extrachromosomal supercoiled plasmids from the transfected cells were extracted at certain time points by alkaline lysis. Analysis of the episomal replication products (provided in Figure 5A) shows that *DpnI*-resistant and *HindIII*-linearized HPV18 viral genome replication could be detected in HeLa cells at low level (Figure 5A, lanes 2 and 3), indicating that viral genome is basically functional and directs the

Figure 4 Amplification of the integrated HPV16 origin and flanking cellular sequences induced by HR-HPV E1 and E2 proteins. **(A)** Graphical representation of amplification of HPV16 URR and flanking sequences of two independent experiments, as described in **(B)**. A 5 µg portion of pMHE1-16 and 5 µg pQMNE2-16 (dashed line in panel A and second row in panel B) or 5 µg pMHE1-18 and 5 µg pQMNE2-18 (solid line in panel A and third row in panel B) were transfected into SiHa cells. A 3 µg portion of total cellular DNA was extracted 24 h after transfection, digested with different enzyme combinations (indicated at the top in panel B, restriction sites shown in panel A) and then subjected to Southern blot analysis. Filters were probed with either radiolabeled HPV16 URR (lane 3 in panel B) or with cellular sequences (panel B, lanes 1, 2, 4 and 5) from various distances from both sides of the viral integration (SL1, SL2, SR1 and SR2). The replication signals were quantified on PhosphorImager and normalized to the signal from the mock-transfected SiHa cells. The average increase in copy numbers of different sequences is calculated (shown by vertical italic numbers in the graph in panel A). **(C)** Graphical representation of the results from two independent experiments, where 5 µg pQMNE2-18 together with increasing amounts of pMHE1-18 (2.5 µg, dotted line; 5 µg, dashed line; 10 µg, solid line) was transfected into SiHa cells. The replication signals were analyzed as described above and the average copy numbers corresponding to different sequences (URR-16, SL1, SL2, SR1, and SR2) are shown in the table **(D)**.

expression of E1 and E2 replication proteins in some cells, although at low level. Cotransfection of HPV18 plasmid with E2 expression vector did not increase replication signal (data not shown); however, cotransfection of E1 expression vector with HPV18 genome considerably increased the replication signal of episomal HPV18 genome as well as showed a clear increase in the signal from integrated HPV18 sequences, especially at later time points (Figure 5A, lanes 4 and 5). This suggests that expression of E2 protein from the viral genome is sufficient to support E1-driven initiation of DNA replication of episomal as well as integrated HPV18 replication origins in HeLa cells. Detection of a clear, strong and increasing-in-time replication signal of the integrated HPV18 *HindIII* fragments in the extrachromosomal fraction suggests that excision of replicating integrated HPV18 sequences from the chromosomal DNA occurs, which is further followed by extrachromosomal replication of these plasmids. Cotransfection of HPV18 genome with E1 and E2 expression vectors tremendously increased HPV18 DNA replication (Figure 5, lanes 6 and 7). The smear detected in the Southern blots is presumably caused by single-stranded DNA fragments extracted from the ‘onion skin’ replication intermediates during alkaline extraction. The control experiment, where pUCURR18 plasmid was used, showed replication signal only

in the presence of E1 and E2 proteins (Figure 5, lanes 8 and 9 compared with 10 and 11), as expected. Analysis of total genomic DNA from the same transfection after 48 h is presented in Figure 5B. A relatively smaller fraction of cells was analyzed in this blot and it shows that without isolation of episomal DNA, the replication of HPV18 DNA is detected only in the case of cotransfection with E1 and E2 expression vectors (Figure 5B, lanes 2, 3 and 5).

In order to evaluate the ability of the HPV18 genome to mobilize the integrated replication origin, we cotransfected cloned HPV18 genome together with EGFP expression vector (pEGFP-N1) into HeLa cells. The EGFP-positive cells were isolated using cell sorter FACS-ARIA 48 h after transfection. This allowed us to analyze only the transfected cells and remove background of integrated HPV sequences of untransfected cells. Total cellular DNA was digested with *HindIII*/*DpnI* and analyzed by Southern blotting. First, we showed an increase in the average apparent copy number of input episomal HPV18 plasmid by four-fold, from 40 to 170 per cell, as estimated by the intensity of the URR containing *DpnI* fragment (Figure 5C). We reproducibly succeeded in detecting the replication of episomal HPV18 plasmid at the level of eight copies per cell. Most importantly, we also detected amplification of integrated HPV18 sequences by two-fold in

Figure 5 Coreplication of integrated and episomal HPV18 in HeLa cells. (A) Low-molecular-weight DNA was extracted from HeLa cells at 48 and 96 h after transfection and (B) total DNA isolated from transfected HeLa cells at 48 h was analyzed by Southern blotting. A 3 μ g portion of pUCHPV18 alone (lanes 2 and 3 in panel A and lane 1 in panel B), together with 10 μ g pMHE1-18 (lanes 4 and 5 in panel A and lane 2 in panel B) or 10 μ g pMHE1-18 and 2.5 μ g pQMNE2-18 (lanes 6 and 7 in panel A and lane 3 in panel B), was transfected into HeLa cells. As controls, HeLa cells were transfected with 1 μ g pUC-URR18 and 10 μ g pMHE1-18 (lanes 8 and 9 in panel A and lane 4 in panel B), or together with 2.5 μ g pQMNE2-18 (lanes 10 and 11 in panel A and lane 5 in panel B). A 5 μ g portion of total DNA or half of the material from 60 mm dish (in case of low-molecular-weight DNA) was digested with *HindIII*/*DpnI* followed by Southern blot analysis with labeled HPV18 URR probe. Mock transfections (lane 1 in panel A and lane 6 in panel B) as well as 100 pg of *HindIII*-digested pUCHPV18 and pUC-URR18 are shown (lane 12 in panel A and lane 7 in panel B). (C) Southern blot analysis of the total population (lane 2) and sorted GFP-positive cells (lane 3) cotransfected with 10 μ g pUCHPV18 plasmid and 0.5 μ g pEGFP-N1. Sample from mock transfection is shown in lane 1. Markers of pUCHPV18 plasmid are shown in lanes 4–7; numbers indicated in the figure correspond to the copy number per cell. The total cellular DNA was extracted 48 h after transfection and 1.6 μ g of it (corresponding to 2.5×10^5 diploid cells) was analyzed in each lane as described above. The results from two independent experiments are presented in (D).

these cells (Figure 5C, lane 3 and D, third bar). These data suggest that E1 and E2 proteins, expressed from the native context of the HPV18 genome in HeLa cells, initiate episomal DNA replication and are sufficient for mobilization of the integrated HPV origin locus for unscheduled replication. These data provide experimental proof for the hypothesis that similar amplification of the integrated HR-HPV sequences could occur in HPV-infected cells, as in LG-SIL and HG-SIL cells, if episomal HPV genomes producing replication proteins are present.

Replication from integrated HPV16 origin will cause chromosomal rearrangements in SiHa cells

Amplified HPV16 locus in SiHa cells is the potential target for repair/recombination machinery that may result in chromosomal alterations. To test this hypothesis, the cells transfected with low amounts of HPV16 E1 and E2 expression plasmids were single cell subcloned 72 h after transfection. The subclones were expanded, total DNA was extracted and purified, and the HPV16 integration patterns of subclones

were analyzed by Southern blot analysis using URR probe. Alterations in the restriction pattern of the integrated HPV16 locus could be detected only in cases when at least one of the cleavage sites for specific restriction enzyme is outside of the DNA fragment that was involved in rearrangement. Therefore, *Bam*HI cleavage, which generates 21.5 kb DNA fragment from 1.8 kb upstream to ~20 kb downstream of HPV16 URR, was used (Figure 6C). Analysis of 43 tested subclones, generated after transfection with the E1 expression plasmid alone, did not reveal any changes in the *Bam*HI restriction pattern (Figure 6A, upper panel). However, eight out of 44 subclones isolated from E1- and E2-transfected cells contained a novel HPV16 restriction pattern (lower panel in Figure 6A, lanes 3, 8, 13, 15, 24, 27, 36 and 39), representing either an internal rearrangement or reintegration at a novel site. Additional analysis of these subclones with *Hind*III and *Bgl*II (sites in the scheme of Figure 6C) confirmed the rearrangement of the HPV16 URR containing sequences and showed that every subclone resulted from the individual event of repair/recombination (Figure 6B). Thus, as

Figure 6 Chromosomal rearrangements caused by the replication of integrated HPV16. **(A)** Southern blot analysis of subclones from SiHa cells transfected with 10 µg pMHE1-16 alone (upper panel, lanes 1–43) or together with 5 µg pQMNE2-16 (lower panel, lanes 1–44). Single cell subcloning was performed 72 h after transfection. Total cellular DNA was extracted from each subclone and 3 µg of DNA was digested with *Bam*HI and analyzed by Southern blot with radiolabeled HPV16 URR probe. Total DNA samples from SiHa cells were used for control (lane 44, upper panel; lane 45, lower panel). **(B)** *Hind*III, *Bgl*II and *Bam*HI restriction analysis of subclones with rearrangements. Restriction pattern of control cells is shown in lanes 25–27. **(C)** Restriction map of SiHa chromosome 13 close to the HPV16 integration site. Lengths of the appropriate restriction fragments between two sites are depicted in parentheses. **(D)** Schematic representation of rearrangements at the HPV16 integration site in the third subclone as determined by DIPS-PCR. See Supplementary data for DNA sequence.

subclones were picked in random without any specific selection, and considering the fact that transfection efficiency of SiHa cells was below 50%, we conclude that cells can survive the considerable changes induced by the HPV DNA replication machinery, which results in effective rearrangement of the genomic DNA. More precise analysis of one of the subclones (Figure 6B, lanes 1–3) by DIPS-PCR suggests that *in situ* duplication of HPV16 genome together with 3' cellular DNA (Figure 6D) has occurred. We determined that the nucleotide 190 of chromosome 13 from the 3' end of initially integrated HPV16 has been linked to nucleotide 3852 (within the E2 stop codon) of the HPV16 genome. Sequence homology could not be found between these sites, indicating that illegitimate recombination has taken place.

Discussion

In the latently infected basal cells, the HR-HPV genomes have to persist as episomal multicopy circular nuclear plasmids in order to support the viral life cycle. However, coexistence of the integrated and the episomal forms of HPV DNA in the same cells has been reported (Cooper *et al*, 1991; Kristiansen *et al*, 1994; Alazawi *et al*, 2002; Peitsaro *et al*, 2002a,b; Andersson *et al*, 2005; Arias-Pulido *et al*, 2006; Kulmala

et al, 2006; Pett *et al*, 2006). The trigger for HPV integration is unclear, but linear fragments generated by dsDNA breaks of the genome or by 'onion skin' type of defective viral replication mode (Männik *et al*, 2002) may serve as substrates for integration (Figure 7, steps 1 and 2). The current study demonstrates that integrated HR-HPV origin is effectively mobilized for replication by E1 and E2 produced from respective expression vectors, and more importantly, from episomal HPV genomes transiently replicating in these cells. As a result, amplification of the integrated HR-HPV genome and flanking cellular DNA sequences occurs (Figure 7, step 3), which could serve as targets for repair/recombination machinery. This will result in rearrangements, including deletion as seen by the excision of HPV18 sequences or *de novo* integration of amplified sequences, as we have demonstrated by the analysis of the subclones (Figure 7, step 4). The exact outcome of rearrangements is difficult to predict, but it is reasonable to assume that modifications of the genome providing gain of the function result in transformation of cells and will give a growth advantage to the cells after the loss of episomal viral genome. The genomic changes may contribute to the transformation directly or indirectly by increasing expression of viral oncogenes E6/E7 as well as nearby host genes. Many studies show that HR-HPV E6 and

Figure 7 Genomic instability of host cell induced by the HPV replication machinery. Papillomavirus DNA is shown in blue, host cell DNA is colored in red and different regions are represented in black. See text for details.

E7 proteins are important for the transformation of cells. Independently from their ability to degrade p53 and pRB family members, HR-HPV E6 and E7 also induce numerical changes and structural chromosome instability. The ability of HPV-16 E7 to induce abnormal centrosome numbers is well known, which subsequently results in chromosome missegregation and aneuploidy (Duensing and Munger, 2002, 2004). HPV-16 E6 can induce abrogation of G2/M checkpoint control (Thompson *et al*, 1997; Thomas and Laimins, 1998) and reduce the efficiency of single-stranded break repair (Iftner *et al*, 2002). It has been demonstrated that the viral load of integrated HPV16 is related to cytological abnormalities (Kulmala *et al*, 2006) and the level of HR-HPV oncogene expression is associated with poor prognosis in cervical cancer patients (de Boer *et al*, 2007). Recently, it has been published that cellular regions at the MYC locus, encompassing HPV sequences, are amplified in several cell lines derived from tumors (Herrick *et al*, 2005). It was concluded that the amplification had taken place already in the primary tumors and the integration of viral DNA must have occurred before MYC gene amplification. The mapping of integration sites showed that the integrated HPV DNA sequences were located at the center part of the amplicon (Peter *et al*, 2006). Cytogenetic characterization of HeLa cells indicated dispersion and coamplification of c-MYC gene and HPV18 DNA (Macville *et al*, 1999). Data suggest that this has occurred after and was most likely triggered by viral insertion at a single integration site. Our study of the duplication of the HPV16 sequence in SiHa cells provides the mechanistic explanation of how the MYC amplification could have taken place if the replication forks initiated at the HPV replication origin are extended to longer distances.

Integrated HPV containing and viral oncoprotein-expressing cells could eventually be targets for superinfection by HR-HPVs and LR-HPVs. There are data indicating the presence of different papillomavirus subtypes in the same tissue in the clinical samples, suggesting that the frequency of coinfections may be quite high (Kulmala *et al*, 2006). Thus, also *de novo* infection of papillomaviruses could result in intercellular mixture of episomal and integrated HPV and subsequent amplification of integrated HPV DNA together with flanking cellular sequences (Figure 7, step 5). Therefore,

even the LR-HPVs could be dangerous, because of their ability to initiate DNA replication from the integrated HR-HPV origin. We can call it 'hit-and-run' mechanism as 'low-risk' or 'high-risk' HPV episome itself could be lost quickly after the infection, but the damage caused by the chromosome-associated HR-HPV amplification remains. It is especially remarkable since LR-HPVs by themselves are considered to be harmless owing to the lower oncogenic potential of their E6 and E7 proteins (Crook *et al*, 1991; Heck *et al*, 1992). Their role in HPV11 episomal maintenance has been demonstrated (Oh *et al*, 2004), which results in the extended lifespan but not immortalization of normal keratinocytes in monolayer cultures (Thomas *et al*, 2001). The inability of integrated E6/E7 to confer necessary growth advantage to the infected cells is probably also the reason why cells harboring integrated LR-HPVs are not found *in vivo*, although human foreskin keratinocytes transfected with the wild-type HPV-11 genome were found to contain also the integrated forms of viral genome (Oh *et al*, 2004).

In addition to amplification of regulatory sequences or genes driving the cell cycle, there is also a possibility for the deletion of genomic sequences. The SiHa cell line itself can be set as an example of such a process where cellular sequences flanking the HPV16 integration site (el Awady *et al*, 1987) lack, according to the 36th assembly of human genome (NCBI, released in November 2005), ~300 kb within the intact chromosome 13. Such deletion can also induce epigenetic changes in gene expression that could contribute to the transformation of cells.

We conclude that papillomavirus replication machinery could be active in changing cell genomic make-up, and it sets the stage for induction of genomic instability that could contribute considerably to the oncogenic transformation of HPV-infected cells.

Materials and methods

Plasmids

Plasmids pQMNE2-6b, -11, -16 and -18 were prepared by cloning E2 ORFs from HPV6b (2723–3829 nt), -11 (2723–3826 nt), -16 (2756–3853 nt) and -18 (2817–3914 nt) into the multicloning site of eukaryotic expression vector pQM-NTAg/Ai+ (Quattromed Ltd) followed by deletion of intron. The pUCURR-6b, -11, -16 and -18

plasmids were cloned by inserting viral sequences containing the URR region of HPV6b (7292–101 nt), -11 (7022–94 nt), -16 (6361–282 nt) and -18 (6929–124 nt) into the multicloning site of pUC-19 plasmid. pMHE1-6b, -11, -16 and -18 vectors contained E6, E7 and E1 ORFs from HPV6b (102–2781 nt), -11 (102–2781 nt), -16 (83–2814 nt) or -18 (105–2887 nt) that were directed by CMV promoter in the pQM-NTag/Ai+ vector with deleted intron and 3F12 epitope tag. Initiation codons for E6 and E7 oncogenes were deleted. The major splice donor site (AGGT) at the beginning of E1 ORFs was disrupted by inserting influenza hemagglutinin epitope tag (HA) in-frame into the E1 coding sequence. The inserted HA tag had no effect on the E1 protein activities (M Kadaja *et al*, unpublished). Additional point mutation was introduced into the splicing acceptor site of HPV11 E1 ORF (2622 nt), (ACA→ACC), and this did not change the coding capacity. pUCHPV18wt were cloned by inserting *EcoRV*-linearized HPV-18 genome (4670 nt within L2) into pUC19 *SmaI* site.

Cell lines and transfection

HeLa and SiHa cells were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal 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 220 V in all experiments. Cells were plated onto 60 mm dishes and harvested at different time points.

Transient replication assays

Low-molecular-weight DNA was purified by alkaline lysis (Ustav and Stenlund, 1991). Total DNA was extracted from cells (FM Ausubel *et al*, 1998). DNA digested with appropriate enzymes was resolved in 0.5 or 0.8% agarose gel, blotted and hybridized with appropriate ³²P-labeled probe generated by random priming (DecaLabel kit, Fermentas, Lithuania). The cellular sequences (SL1, SR2, SL1 and SL2) used in hybridization were amplified from SiHa genomic DNA with PCR using *Taq* polymerase and primers that were designed with the programs Primer3 (Rozen and Skaletsky, 2000) and GenomeTester (Andreson *et al*, 2006). Radioactive signals were quantified using ImageQuant software of PhosphorImager SI (Molecular Dynamics, Amersham Biosciences, UK).

References

- Alazawi W, Pett M, Arch B, Scott L, Freeman T, Stanley MA, Coleman N (2002) Changes in cervical keratinocyte gene expression associated with integration of human papillomavirus 16. *Cancer Res* **62**: 6959–6965
- Andersson S, Safari H, Mints M, Lewensohn-Fuchs I, Gyllenstein U, Johansson B (2005) Type distribution, viral load and integration status of high-risk human papillomaviruses in pre-stages of cervical cancer (CIN). *Br J Cancer* **92**: 2195–2200
- Andreson R, Reppo E, Kaplinski L, Remm M (2006) GENOMEMASKER package for designing unique genomic PCR primers. *BMC Bioinformatics* **7**: 172
- Arias-Pulido H, Peyton CL, Joste NE, Vargas H, Wheeler CM (2006) Human papillomavirus type 16 integration in cervical carcinoma *in situ* and in invasive cervical cancer. *J Clin Microbiol* **44**: 1755–1762
- Baker CC, Phelps WC, Lindgren V, Braun MJ, Gonda MA, Howley PM (1987) Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. *J Virol* **61**: 962–971
- Bonne-Andrea C, Santucci S, Clertant P (1995) Bovine papillomavirus E1 protein can, by itself, efficiently drive multiple rounds of DNA synthesis *in vitro*. *J Virol* **69**: 3201–3205
- Bonne-Andrea C, Tillier F, McShan GD, Wilson VG, Clertant P (1997) Bovine papillomavirus type 1 DNA replication: the transcriptional activator E2 acts *in vitro* as a specificity factor. *J Virol* **71**: 6805–6815
- Botchan M, Topp W, Sambrook J (1979) Studies on simian virus 40 excision from cellular chromosomes. *Cold Spring Harb Symp Quant Biol* **43** (Part 2): 709–719

Immunoblotting

Total protein from an equal number of cells was separated by electrophoresis on 10% polyacrylamide-SDS gels and transferred to Immobilon-P membrane (Millipore, USA). Antibodies 3F10-HRP (Roche) and 4E4 were used to detect E1 and E2 proteins using the enhanced chemoluminescence detection kit (Amersham Biosciences).

Cell sorting

For cell sorting, cells were cotransfected with pEGFP-N1 (Clontech) and pUCHPV18wt plasmids. Forty-eight hours after transfection, the transfected cells were sorted on the basis of EGFP fluorescence using the FACSDiva software and the FACSaria hardware (Becton Dickinson) equipped with a 13 mW argon ion laser set at 488 nm with a 530/30 nm filter. The purity of EGFP⁺ cells, when reanalyzed, was 90 ± 5%. Cell sorting experiments were performed in cooperation with Virology Core Facility (Tartu University, Institute of Technology).

DIPS-PCR

DIPS-PCR assay was performed as described previously (Luft *et al*, 2001). The ds adapter was constituted from AS1 (5'-PO₄-gattc-caacgtgtaagtctg-NH₂) and AL1 (5'-gggccatcagtcagcagtcgtacccggatc-cagacttacagttg-3') DNA oligos. The primers used in PCR were AP1 (5'-ggccatcagtcagcagtcgtg-3') and S1 (5'-agggatcccaatgaaggac-3'). PCR products were analyzed by 1.2% agarose gel electrophoresis followed by purification of the product of interest and sequence determination.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Dr Andres Männik, Dr Ivar Ilves and Kadrin Wilfong for their comments on the manuscript, Anne Kalling for helpful technical assistance, Agne Velthut for her assistance in constructing the E1 expression vectors and Kristi Kasemaa for assistance in beginning HeLa studies, Mihkel Allik for 4E4 Ab and Professor Stina Syrjänen for providing SiHa cell line. This work was supported in part by grant nos 5524, 5998, 5999 from the Estonian Science Foundation, target financial project 0182566 and grant INTNL 55000339 from Howard Hughes Medical Institute.

- Chiang CM, Ustav M, Stenlund A, Ho TF, Broker TR, Chow LT (1992) Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. *Proc Natl Acad Sci USA* **89**: 5799–5803
- Cooper K, Herrington CS, Stickland JE, Evans MF, McGee JO (1991) Episomal and integrated human papillomavirus in cervical neoplasia shown by non-isotopic *in situ* hybridisation. *J Clin Pathol* **44**: 990–996
- Corden SA, Sant-Cassia LJ, Easton AJ, Morris AG (1999) The integration of HPV-18 DNA in cervical carcinoma. *Mol Pathol* **52**: 275–282
- Crook T, Tidy JA, Vousden KH (1991) Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation. *Cell* **67**: 547–556
- de Boer MA, Jordanova ES, Kenter GG, Peters AA, Corver WE, Trimbois JB, Fleuren GJ (2007) High human papillomavirus oncogene mRNA expression and not viral DNA load is associated with poor prognosis in cervical cancer patients. *Clin Cancer Res* **13**: 132–138
- de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H (2004) Classification of papillomaviruses. *Virology* **324**: 17–27
- Deng W, Jin G, Lin BY, Van Tine BA, Broker TR, Chow LT (2003) mRNA splicing regulates human papillomavirus type 11 E1 protein production and DNA replication. *J Virol* **77**: 10213–10226
- Desaintes C, Demeret C, Goyat S, Yaniv M, Thierry F (1997) Expression of the papillomavirus E2 protein in HeLa cells leads to apoptosis. *EMBO J* **16**: 504–514

- Duensing S, Munger K (2002) The human papillomavirus type 16 E6 and E7 oncoproteins independently induce numerical and structural chromosome instability. *Cancer Res* **62**: 7075–7082
- Duensing S, Munger K (2004) Mechanisms of genomic instability in human cancer: insights from studies with human papillomavirus oncoproteins. *Int J Cancer* **109**: 157–162
- el Awady MK, Kaplan JB, O'Brien SJ, Burk RD (1987) Molecular analysis of integrated human papillomavirus 16 sequences in the cervical cancer cell line SiHa. *Virology* **159**: 389–398
- FM Ausubel RB, Kingston RE, Moore DD, Seidman JG, Struhl K (1998) *Current Protocols in Molecular Biology*. New York: John Wiley & Sons Inc
- Heck DV, Yee CL, Howley PM, Munger K (1992) Efficiency of binding of the retinoblastoma protein correlates with the transforming capacity of the E7 oncoproteins of the human papillomaviruses. *Proc Natl Acad Sci USA* **89**: 4442–4446
- Herrick J, Conti C, Teissier S, Thierry F, Couturier J, Sastre-Garau X, Favre M, Orth G, Bensimon A (2005) Genomic organization of amplified MYC genes suggests distinct mechanisms of amplification in tumorigenesis. *Cancer Res* **65**: 1174–1179
- Howley PM, Lowy DR (2001) Papillomaviruses and their replication. In: *Fields' Virology*, Knipe DM, Howley PM (eds) pp 2197–2230. Philadelphia: Lippincott Williams & Wilkins
- Iftner T, Elbel M, Schopp B, Hiller T, Loizou JI, Caldecott KW, Stubenrauch F (2002) Interference of papillomavirus E6 protein with single-strand break repair by interaction with XRCC1. *EMBO J* **21**: 4741–4748
- Jeon S, Allen-Hoffmann BL, Lambert PF (1995) Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. *J Virol* **69**: 2989–2997
- Kalantari M, Blennow E, Hagmar B, Johansson B (2001) Physical state of HPV16 and chromosomal mapping of the integrated form in cervical carcinomas. *Diagn Mol Pathol* **10**: 46–54
- Kristiansen E, Jenkins A, Holm R (1994) Coexistence of episomal and integrated HPV16 DNA in squamous cell carcinoma of the cervix. *J Clin Pathol* **47**: 253–256
- Kulmala SM, Syrjanen SM, Gyllenstein UB, Shabalova IP, Petrovichev N, Tosi P, Syrjanen KJ, Johansson BC (2006) Early integration of high copy HPV16 detectable in women with normal and low grade cervical cytology and histology. *J Clin Pathol* **59**: 513–517
- Lazo PA (1987) Structure, DNaseI hypersensitivity and expression of integrated papilloma virus in the genome of HeLa cells. *Eur J Biochem* **165**: 393–401
- Lin BY, Ma T, Liu JS, Kuo SR, Jin G, Broker TR, Harper JW, Chow LT (2000) HeLa cells are phenotypically limiting in cyclin E/CDK2 for efficient human papillomavirus DNA replication. *J Biol Chem* **275**: 6167–6174
- Luft F, Klaes R, Nees M, Durst M, Heilmann V, Melsheimer P, von Knebel Doeberitz M (2001) Detection of integrated papillomavirus sequences by ligation-mediated PCR (DIPS-PCR) and molecular characterization in cervical cancer cells. *Int J Cancer* **92**: 9–17
- Macville M, Schrock E, Padilla-Nash H, Keck C, Ghadimi BM, Zimonjic D, Popescu N, Ried T (1999) Comprehensive and definitive molecular cytogenetic characterization of HeLa cells by spectral karyotyping. *Cancer Res* **59**: 141–150
- Malcles MH, Cueille N, Mechali F, Coux O, Bonne-Andrea C (2002) Regulation of bovine papillomavirus replicative helicase e1 by the ubiquitin-proteasome pathway. *J Virol* **76**: 11350–11358
- Männik A, Runkorg K, Jaanson N, Ustav M, Ustav E (2002) Induction of the bovine papillomavirus origin 'onion skin'-type DNA replication at high E1 protein concentrations *in vivo*. *J Virol* **76**: 5835–5845
- Meissner JD (1999) Nucleotide sequences and further characterization of human papillomavirus DNA present in the CaSki, SiHa and HeLa cervical carcinoma cell lines. *J Gen Virol* **80** (Part 7): 1725–1733
- Munger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, Grace M, Huh K (2004) Mechanisms of human papillomavirus-induced oncogenesis. *J Virol* **78**: 11451–11460
- Oh ST, Longworth MS, Laimins LA (2004) Roles of the E6 and E7 proteins in the life cycle of low-risk human papillomavirus type 11. *J Virol* **78**: 2620–2626
- Peitsaro P, Hietanen S, Johansson B, Lakkala T, Syrjanen S (2002a) Single copy heterozygote integration of HPV 33 in chromosomal band 5p14 is found in an epithelial cell clone with selective growth advantage. *Carcinogenesis* **23**: 1057–1064
- Peitsaro P, Johansson B, Syrjanen S (2002b) Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. *J Clin Microbiol* **40**: 886–891
- Peter M, Rosty C, Couturier J, Radvanyi F, Teshima H, Sastre-Garau X (2006) MYC activation associated with the integration of HPV DNA at the MYC locus in genital tumors. *Oncogene* **25**: 5985–5993
- Pett MR, Alazawi WO, Roberts I, Dowen S, Smith DI, Stanley MA, Coleman N (2004) Acquisition of high-level chromosomal instability is associated with integration of human papillomavirus type 16 in cervical keratinocytes. *Cancer Res* **64**: 1359–1368
- Pett MR, Herdman MT, Palmer RD, Yeo GS, Shivji MK, Stanley MA, Coleman N (2006) Selection of cervical keratinocytes containing integrated HPV16 associates with episome loss and an endogenous antiviral response. *Proc Natl Acad Sci USA* **103**: 3822–3827
- Piirsoo M, Ustav E, Mandel T, Stenlund A, Ustav M (1996) *cis* and *trans* requirements for stable episomal maintenance of the BPV-1 replicator. *EMBO J* **15**: 1–11
- Ravnan JB, Gilbert DM, Ten Hagen KG, Cohen SN (1992) Random-choice replication of extrachromosomal bovine papillomavirus (BPV) molecules in heterogeneous, clonally derived BPV-infected cell lines. *J Virol* **66**: 6946–6952
- Remm M, Brain R, Jenkins JR (1992) The E2 binding sites determine the efficiency of replication for the origin of human papillomavirus type 18. *Nucleic Acids Res* **20**: 6015–6021
- Remm M, Remm A, Ustav M (1999) Human papillomavirus type 18 E1 protein is translated from polycistronic mRNA by a discontinuous scanning mechanism. *J Virol* **73**: 3062–3070
- Romanczuk H, Howley PM (1992) Disruption of either the E1 or the E2 regulatory gene of human papillomavirus type 16 increases viral immortalization capacity. *Proc Natl Acad Sci USA* **89**: 3159–3163
- Rozen S, Skaletsky H (2000) Primer3 on the www for general users and for biologist programmers. *Methods Mol Biol* **132**: 365–386
- Russell J, Botchan MR (1995) *cis*-Acting components of human papillomavirus (HPV) DNA replication: linker substitution analysis of the HPV type 11 origin. *J Virol* **69**: 651–660
- Schneider-Gadicke A, Schwarz E (1986) Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. *EMBO J* **5**: 2285–2292
- Sedman J, Stenlund A (1998) The papillomavirus E1 protein forms a DNA-dependent hexameric complex with ATPase and DNA helicase activities. *J Virol* **72**: 6893–6897
- Szuhaik K, Bezrookove V, Wiegant J, Vrolijk J, Dirks RW, Rosenberg C, Raap AK, Tanke HJ (2000) Simultaneous molecular karyotyping and mapping of viral DNA integration sites by 25-color COBRA-FISH. *Genes Chromosomes Cancer* **28**: 92–97
- Stenlund A (2003) Initiation of DNA replication: lessons from viral initiator proteins. *Nat Rev* **4**: 777–785
- Sverdrup F, Khan SA (1994) Replication of human papillomavirus (HPV) DNAs supported by the HPV type 18 E1 and E2 proteins. *J Virol* **68**: 505–509
- Syu LJ, Fluck MM (1997) Site-specific *in situ* amplification of the integrated polyomavirus genome: a case for a context-specific over-replication model of gene amplification. *J Mol Biol* **271**: 76–99
- Thomas JT, Laimins LA (1998) Human papillomavirus oncoproteins E6 and E7 independently abrogate the mitotic spindle checkpoint. *J Virol* **72**: 1131–1137
- Thomas JT, Oh ST, Terhune SS, Laimins LA (2001) Cellular changes induced by low-risk human papillomavirus type 11 in keratinocytes that stably maintain viral episomes. *J Virol* **75**: 7564–7571
- Thompson DA, Belinsky G, Chang TH, Jones DL, Schlegel R, Munger K (1997) The human papillomavirus-16 E6 oncoprotein decreases the vigilance of mitotic checkpoints. *Oncogene* **15**: 3025–3035
- Ustav E, Ustav M, Szymanski P, Stenlund A (1993) The bovine papillomavirus origin of replication requires a binding site

- for the E2 transcriptional activator. *Proc Natl Acad Sci USA* **90**: 898–902
- Ustav M, Stenlund A (1991) Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. *EMBO J* **10**: 449–457
- Ustav M, Ustav E, Szymanski P, Stenlund A (1991) Identification of the origin of replication of bovine papillomavirus and characterization of the viral origin recognition factor E1. *EMBO J* **10**: 4321–4329
- Wells SI, Francis DA, Karpova AY, Dowhanick JJ, Benson JD, Howley PM (2000) Papillomavirus E2 induces senescence in HPV-positive cells via pRB- and p21(CIP)-dependent pathways. *EMBO J* **19**: 5762–5771
- Yang L, Mohr I, Fouts E, Lim DA, Nohaile M, Botchan M (1993) The E1 protein of bovine papilloma virus 1 is an ATP-dependent DNA helicase. *Proc Natl Acad Sci USA* **90**: 5086–5090
- zur Hausen H (2002) Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* **2**: 342–350