Acta Biochimica Polonica

Vol. 52 No. 4/2005, 823–832

on-line at: www.actabp.pl

The HPV16 E2 transcriptional regulator mode of action depends on the physical state of the viral genome

Marcin T. Schmidt[1]✉, Agnieszka K. Olejnik[1] and Anna Goździcka-Józefiak[2]

[1]*Department of Biotechnology and Food Microbiology, August Cieszkowski University of Agriculture, Poznań, Poland;* [2]*Department of Molecular Virology, Adam Mickiewicz University, Poznań, Poland;*
✉*e-mail: mschmidt@au.poznan.pl*

Received: 25 April, 2005; revised: 22 July, 2005; accepted: 06 October, 2005
available on-line: 07 November, 2005

Human papillomavirus (HPV) infection is a major risk factor for the development of cervical cancer. The HPV-induced immortalization of epithelial cell usually requires integration of the viral DNA into the host cell genome. The integration event causes disruption of the E2 gene and this is followed by overexpression of the E6 and E7 oncoproteins. The E2 protein is a transcription factor that regulates expression of the E6 and E7 oncoproteins by binding to four sites within the viral long control region. We used an *in vitro* cell culture model to explore the role of the E2 protein in the transcriptional control of the HPV16 long control region. Employing transient and stable transfection experiments we simulated the episomal and integrated states of the viral genome, respectively. We show that the E2 protein up-regulates E6/E7 transcription from episomal DNA but represses it in the case of integrated DNA. The activator function of the E2 protein seems to counteract the repressive chromatin structure formed over episomal DNA. Steroid hormones and retinol also modulate oncogene transcription differently depending on the physical structure of the viral DNA. Our data suggest regulatory mechanisms involving interactions between the E2 protein and nuclear hormone receptors.

Keywords: HPV, LCR, E2, chromatin, steroid hormones, retinol, transcription

Human papillomaviruses (HPV) are epitheliotropic small dsDNA viruses with a circular genome of approximately 8 kbp. More than 80 different HPV types are known and new ones are currently being isolated. These viruses can cause benign proliferative lesions (e.g., skin warts and anogenital condylomata) and anogenital cancers (especially cervical cancer). HPV types have been classified as either high risk (e.g. HPV16 and -18) or low risk (e.g. HPV6 and -11) on the basis of the clinical lesions they are associated with and the relative predisposition of these lesions to progress to cancer (zur Hausen, 1996; 2000; Longworth & Laimins, 2004). The viruses encode two oncoproteins, E6 and E7, whose expression extends the life span of the normal host cell (squamous epithelial cells). Overexpression of these oncoproteins leads to immortalization and transformation of the host cell. The main transforming properties of the E6 and E7 oncoproteins are their ability to inactivate the p53 and retinoblastoma (pRB) tumor suppressor proteins, respectively. HPV-induced carcinogenic progression is frequently related to integration of the viral genome into the human chromosome in cancer cells. Such an event often results in the loss of expression of the viral E2 gene but preserves E6/E7 expression or even increases it (Barbosa & Schlegel, 1989; Choo *et al.*, 1987; Longworth & Laimins, 2004; Nakagawa *et al.*, 2000; Sashiyama *et al.*, 2001).

E2 is a regulatory protein able to either activate or repress transcription from the promoter directing expression of the E6 and E7 oncogenes (Cripe *et al.*, 1987; Thierry & Yaniv, 1987; Nishimura *et al.*, 2000; Bouvard *et al.*, 1994). This 42-kDa protein is composed of an N-terminal transcriptional activation domain, a hinge region and a C-terminal DNA-binding and dimerization domain. The viral regulatory protein binds to multiple copies of the $ACCN_6GGT$ motif that occur in the long control region (LCR) of all HPVs (McBride & Myers, 1997; Antson *et al.*, 2000; Hou *et al.*, 2002). The LCR regulatory sequence contains also multiple binding sites

Abbreviations: CBP, CREB-binding protein; HPV, human papillomavirus; LCR, long control region; MAR, matrix attachment region; ORF, open reading frame; PBS, phosphate-buffered saline; SH/R, steroid hormone or retinol; TSA, trichostatin A.

for cellular transcription factors that modulate its action positively (TFIID, SP-1, AP-1, NF1, Oct-1, TEF-1, steroid receptors) or negatively (YY1, CDP, NF-κB, C/EBPβ). The transcription factors interacting with their specific motifs form a keratinocyte-specific enhancer and silencer, respectively (O'Connor *et al.*, 1995).

Correlation studies (de Villiers, 2003) and *in vitro* experiments have shown that steroid hormones and retinol influence cervical cancer cells (Khare *et al.*, 1997; Narayanan *et al.*, 1998; Webster *et al.*, 2001). The effect is mainly attributed to the presence of hormone response element sequences in the viral LCR (Khare *et al.*, 1996), but HPV-independent actions have also been observed (Khan *et al.*, 1997).

The research reported here has three objectives. First, we wish to determine whether there is a difference in the chromatin structure over episomal and integrated reporter constructs resembling the different states of the HPV16 LCR. Second, we wish to analyze the effects of the E2 protein on the reporter constructs in these two physical states. Finally, we examine the influence of steroid hormones and retinol on HPV16 oncogene transcription in the presence and absence of the E2 protein. Our results indicate that the HPV16 genome in the episomal state undergoes severe repression by chromatin and that this repression is partially relieved by the E2 protein. After integration the chromatin over the HPV16 DNA goes through structural changes rendering it more open but totally altering the effect of the E2 protein. Furthermore, our work with steroid hormones and retinol indicates that the HPV16 genome is differently influenced by these factors depending on the physical structure of the viral DNA.

MATERIALS AND METHODS

Plasmids and constructs. The LCR (nt 7003–104) and the E6 matrix attachment region (E6MAR, nt 88–560) of the HPV16 reference clone (sequence identical to that published in the GenBank under the accession number NC_001526) was PCR-amplified with *Pfu* polymerase and specific primers containing restriction enzyme recognition sequences for *Sac*I (GGAGGAGAGCTCTGCAGACCTAGATCAGTTTCC), *Xho*I (GCAGCCTCGAGTTGCAGTTCTCTTTTGGTGC), *Bam*HI (GACTTAGGATCCAAAAGAGAACTGCAATGTTTCAG), and *Sal*I (CTCCAGTCGACATTACAGCTGGGTTTCTCTACGTG), respectively. Restriction-digested PCR products were cloned into pGL3-Basic vector (Promega) giving pGL3-LCR/E6MAR reporter vector constructs. The constructs were verified by DNA sequencing.

For stable transfection the LCR-*luc*-E6MAR reporter cassette was cut out from pGL3-LCR/E6MAR reporter vector with *Sac*I and *Sal*I followed by agarose gel electrophoresis and purification from the gel using QIAquick Gel Extraction Kit (Qiagen).

The HPV16 E2 expression vector pCMV-pt16E2 and empty vector pCMV1 (Veress *et al.*, 1999) were a generous gift of Dr. Gyorgy Veress.

Plasmids were isolated and purified with the Mobius 1000 Plasmid Kit (Novagen).

Cell culture. The HPV-negative human cervical carcinoma cell line C-33A was cultured in Dulbecco's Modified Eagle's medium (Sigma) supplemented with 10% foetal calf serum (Gibco) and gentamycin (Sigma) at 37°C and 5% CO_2.

Cell transfections. The day before transfection, 3×10^5 cells per well were seeded in a 12-well plate. The cells were transiently transfected using Tfx-20 Reagent (Promega) and 2 μg of pGL3-LCR/E6MAR plasmid DNA at a 2:1 ratio in serum free medium for 1.5 h. In the case of co-transfections 2 μg of pGL3-LCR/E6MAR reporter vector and 1 μg pCMV-pt16E2 expression vector (or pCMV1 empty expression vector as a negative control) were used.

For the generation of stably transfected C-33A lines linear LCR-*luc*-E6MAR reporter cassette with the neomycin resistance gene expression cassette (Neo^R) at a 10:1 ratio was introduced to cells using the Effectene Transfection Reagent (Qiagen) according to the procedure recommended by the manufacturer. Transfectants were selected using the neomycin homologue G418 antibiotic (ICN) added gradually to the culture medium to the final concentration of 0.8 mg/ml and maintained for 3 weeks. The stably transfected C-33A cells represent a mixed population. A cell line stably transfected with LCR-*luc*-E6MAR/Neo^R was transiently transfected with 2 μg pCMV-pt16E2 or pCMV1 expression vector using Tfx-20 Reagent (Promega).

Cell treatment and luciferase assays. Cell cultures were supplemented, where necessary, with dexamethasone (100 nM), 17β-estradiol (100 nM), progesterone (100 nM) or retinol acetate (3 μM) in the form of cyclodextrin-encapsulated water-soluble complexes (Sigma), and trichostatin A (TSA, 300 ng/ml; ICN) 24 h after transfection. Forty-two hours after transfection the cells were washed with PBS and lysed in 100 μl Cell Culture Lysis Reagent (Promega). Cell lysate (20 μl) was mixed with 100 μl luciferin substrate (Promega) and the activity of luciferase was measured in a Biocounter M1500 (Lumac) with 2 s delay and 10 s integration period. Luciferase activity was standardized to total protein content of the cell lysates. Protein concentration was measured using the modified Lowry's method (Total Protein Kit; Sigma) after protein precipitation with trichloroacetic acid.

DNA extraction and real-time PCR copy number determination. Cells were washed three times with TEN buffer (10 mM Tris/Cl, pH 7.4, 5 mM EDTA, 0.9% NaCl) to remove extracellular

plasmid DNA prior to cell lysis in the case of transiently transfected cells. Total DNA from stably and transiently transfected cells was isolated using the Genomic Mini Kit (A&A Biotechnology, Poland). The quantitative real-time PCR assay was based on primers that specifically amplify the *luc* luciferase gene (forward: 5′-TGTGGACGAAGTACCGAAAG-GT-3′ and reverse: 5′-CCTTCTTGGCCTTTATGAG-GATCT-3′) and a TaqMan probe (5′-6FAM-CCG-GAAAACTCGACGCAAGAAAAATCAG-TAMRA-3′; IBB PAS, Warszawa, Poland). Using the Smart-Cycler (Cepheid) the PCR was performed in a total volume of 25 μl in capillary tubes containing 300 nM of each primer, 100 nM of TaqMan probe, 25 ng of genomic DNA, and 1× Smart Reaction Buffer (Smart Kit, Eurogentec). The PCR program was initiated with a step of 50ºC for 2 min and 95ºC for 10 min, followed by 45 cycles of 95ºC for 15 s and 60ºC for 1 min. Real-time PCR quantitation was performed three times. The quantification of the *luc* gene (integrated transgene copies or plasmid molecules that entered cells) was performed to self prepared standards. The standard was made by serial dilutions of pGL3-Basic plasmid in 5 mM Tris buffer pH 8.0, containing genomic C-33A DNA.

RESULTS

Chromatin represses the HPV16 LCR to different extents in episomal and integrated states

Nucleosomal organization of eukaryotic DNA is required to store the bulky genome and to facilitate gene regulation. Most often the effect of chromatin is suppressive for transcription (van Holde & Zlatanova, 1996; Berger, 2002). To test how the chromatin structure influences transcriptional activity of the p97 promoter in episomal and integrated state we made use of the histone deacetylase inhibitor trichostatin A (TSA). Transiently transfected C-33A cells containing pGL3-LCR/E6MAR and stably transfected C-33A cells containing LCR-*luc*-E6MAR simulating the episomal and integrated states of the viral genome, respectively, were treated with TSA. A significant increase in luciferase activity was observed in both cases, about 8.5-fold for the episomal and about 3.3-fold for the integrated reporter ($P<0.01$, Fig. 1). The transiently transfected cells contain over 1000-fold more copies per cell of the reporter construct plasmid than the stably transfected line (about 3.8 reporter construct copy/cell genome) as measured by real-time PCR (not shown).

These data indicate that the chromatin structure in the LCR-E6MAR region in the episomal and integrated states represses the p97 promoter-driven transcription to very different extents. These findings

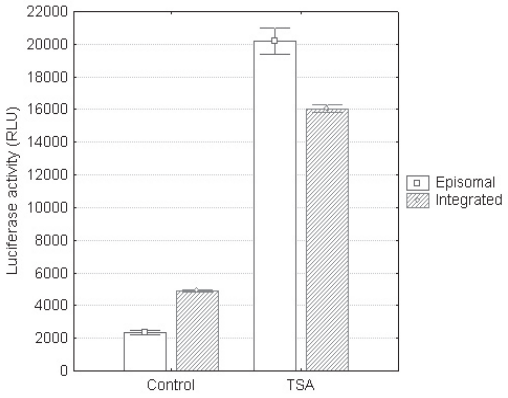

Figure 1. The effects of TSA on HPV16 p97 promoter activity in the episomal and integrated states.
The HPV16 LCR-E6MAR region was cloned into reporter vector pGL3-Basic. The reporter constructs were transiently or stably transfected into C-33A cells, simulating the episomal and integrated states of the viral genome. p97 transcriptional activity in different physical states and in the presence or absence of TSA were determined using luciferase assays. The data represent the averages of four independent experiments, with error bars indicating ±S.D.

suggest that the HPV16 episomal genome undergoes much higher repression by chromatin structure than the integrated genome.

The HPV16 E2 protein regulates p97 transcription differently depending on the chromatin structure over the HPV16 LCR

To study the effects of the E2 protein on the activity of the LCR in the episomal and integrated states the stably transfected LCR-*luc*-E6MAR C-33A cells were transiently transfected with the E2 expression construct pCMV-pt16E2, and C-33A cells were transiently co-transfected with pGL3-LCR/E6MAR and pCMV-pt16E2. Additional TSA treatment allowed an assessment of the influence of E2 on chromatin.

Expression of the HPV16 E2 protein increases (about 1.19-fold, $P<0.01$) the transcriptional activity of the episomal reporter and decreases (about 1.19-fold, $P<0.01$) the activity of the integrated reporter (Fig. 2). As in the previous experiment TSA treatment increases the transcription about 8.5-fold and about 3.3-fold for the episomal and integrated reporter, respectively ($P<0.01$). Expression of E2 protein in conjunction with TSA treatment brings about a further enhancement of the reporter activity in the case of the episomal reporter (about 11-fold, $P<0.01$). However, E2 and TSA together have little additional effect over TSA alone in the case of the integrated reporter (about 3.5-fold, $P<0.01$).

These results demonstrate that the effect of the E2 protein depends on the chromatin structure formed on the HPV16 LCR. In the episomal state E2 protein acts as a transcriptional activator but after

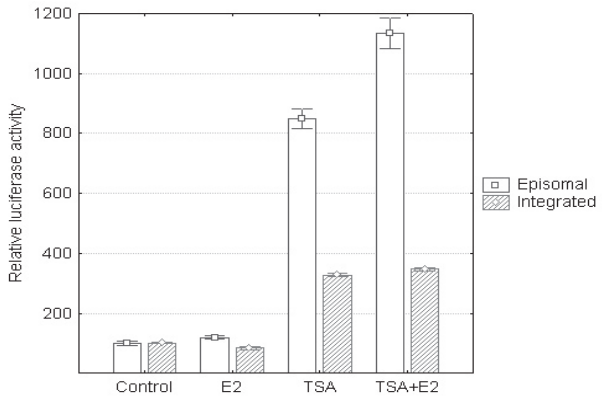

Figure 2. The effects of the chromatin structure and the HPV16 E2 protein on the p97 promoter activity in the episomal and integrated states.
The reporter constructs were transiently or stably transfected into C-33A cells, simulating the episomal and integrated states of the viral genome. p97 transcriptional activity in the different physical states and in the presence and absence of the HPV16 E2 protein (cotransfection with expression vector) and/or TSA were determined using luciferase assays. All luciferase activities were calculated in relation to control samples for transient (episomal) and stable (integrated) transfection experiments, respectively. The data represent averages of four independent experiments, with error bars indicating ±S.D. Control, indicates C-33A cells transiently transfected with pGL3-LCR/E6MAR and stably transfected with LCR-*luc*-E6MAR cotransfected with empty pCMV1 vector; E2, C-33A cells transiently transfected with pGL3-LCR/E6MAR and stably transfected with LCR-*luc*-E6MAR cotransfected with the pCMV-pt16E2 expression vector; TSA, and TSA+E2, as above but trichostatin A-treated.

integration E2 represses the p97 promoter. Trichostatin A treatment relieves the repressive action of chromatin and allows a further increase of the p97 transcriptional activity by E2 in the episomal state. In contrast, the activity of the integrated reporter construct does not respond significantly to E2 in the presence of TSA.

The HPV16 E2 protein is a potent activator of the p97 promoter in the episomal state

HPV16 is a steroid hormone-responsive virus. There are three steroid response motifs within the HPV16 LCR sequence and these are able to bind with different affinities both steroid and retinol receptors (Khare *et al.*, 1997; Yokoyama *et al.*, 2001). To examine the effects of steroid hormones and retinol combined with the HPV16 E2 protein on the HPV16 p97 promoter activity in the episomal state we used the above-described experimental model.

In the absence of the E2 protein retinol increases about 1.13-fold ($P<0.05$) and progesterone decreases about 1.12-fold ($P<0.10$) p97 promoter activity, whereas dexamethasone and estrogen have no significant effect (Fig. 3). In the presence of E2 and steroid hormones or retinol p97 activity is increased significantly (Fig. 3). The combined action of estrogen, progesterone or retinol with the E2 protein statistically significantly increases the transcriptional activity of the viral promoter. The data analysis shown in Table 1 shows that dexamethasone appears to maintain p97 transcriptional activity at an unchanged level despite the presence of E2. This suggests that dexamethasone blocks the activity of E2. Estradiol has no effect on the p97 promoter either in the presence or absence of the E2 protein. In contrast, E2 counteracts the repressive effect of progesterone, changing the transcription level from about 0.89 ($P<0.10$) to about 1.17 ($P<0.05$) that of control, thus causing an overall about 1.30-fold increase ($P<0.05$) in the reporter activity. Retinol (about 1.13-fold increase, $P<0.05$) and E2 (about 1.19-fold increase, $P<0.05$) seem to operate in an additive manner giving together about 1.33-fold ($P<0.05$) upregulation of the p97 promoter activity.

These data show that the E2 protein is a transcriptional activator of the episomal reporter construct. E2 modulates positively the HPV16 LCR p97 promoter activity in the presence of progesterone and retinol, but does not function in the presence of dexamethasone. The episomal reporter construct is not responsive to estradiol treatment in these experimental conditions but neither does estradiol treatment block the effect of E2.

The p97 promoter in the integrated HPV16 LCR state is repressed by E2

To investigate the effects of E2 in the case of an integrated reporter construct, we used stably transfected LCR-*luc*-E6MAR C-33A cells transiently transfected with the empty pCMV1 vector or the E2 expressing pCMV-pt16E2 construct and measured the transcriptional activity in the presence or absence of steroid hormones and retinol (Fig. 4).

Dexamethasone treatment of the cells with the integrated reporter construct brings about downregulation of the promoter activity by 11% ($P<0.10$) and this is unaffected by the presence of the E2 protein (about 0.87-fold of the nominal activity, $P<0.05$; Fig. 4). Although estradiol treatment alone does not influence the activity of the integrated reporter construct, estradiol treatment partially relieves the E2-mediated repression (about 1.07-fold increase as compared to E2 action alone, $P<0.10$). Similarly, progesterone has little if any influence on the p97 promoter activity but also seems to partially relieve the E2-mediated repression. Finally, retinol treatment has little or no effect on the reporter activity in the absence of E2 but almost abolishes the E2-mediated repression.

In summary, the HPV16 E2 protein-mediated repression is affected by the steroid hormones in the case of the integrated reporter construct, although

Table 1. The effect of steroid hormones or retinol on the HPV16 LCR p97 promoter activity in the presence or absence of E2 protein.

Fold change in the HPV16 p97 activity in the episomal and integrated states of the reporter construct as influenced by the HPV16 E2 protein, steroid hormones and retinol. Values in bold designate statistical significance at $P<0.05$, and values with asterisks — at $P<0.1$. Control, represents C-33A cells co-transfected with empty pCMV1 expression vector, E2, cells co-transfected with the E2 protein expression vector pCMV-16ptE2, Dex, Est, Prg, and Ret — cells treated with dexamethasone, estradiol, progesterone and retinol, respectively. SH/R, depicts corresponding steroid hormone or retinol treated cells co-transfected with pCMV1 vector.

		Episomal state			Integrated state		
		Control	E2	SH/R	Control	E2	SH/R
Control			**1.19**			**0.83**	
E2		**1.19**			**0.83**		
SH/R	Dex	1.04			0.89*		
	Est	1.00			0.96		
	Prg	0.89*			0.99		
	Ret	**1.13**			1.02		
SH/R+E2	Dex+E2	1.08*	**0.90**	1.03*	**0.87**	1.04	0.97
	Est+E2	**1.22**	1.02	**1.21**	**0.89**	1.07*	0.93*
	Prg+E2	**1.17**	0.98	**1.30**	**0.87**	1.05	**0.88**
	Ret+E2	**1.33**	**1.12**	**1.17**	0.97	**1.16**	**0.94**

the protein shows no significant action in dexamethasone-treated cells when compared to the treatment alone. Retinol has little effect on the integrated reporter and it blocks repression by E2. The outcome of estradiol and progesterone treatment appears to be dependent on the chromatin structure over the reporter construct (physical state). Both treatments have little influence on the activity of the episomal reporter construct, but partially relieve the E2-mediated repression in the integrated state. These results together show that the effect of steroid hormone receptors and the HPV16 E2 protein depends on the chromatin structure over the HPV16 regulatory sequences.

DISCUSSION

The HPV E2 protein has been shown to both activate and repress transcription in transient re-

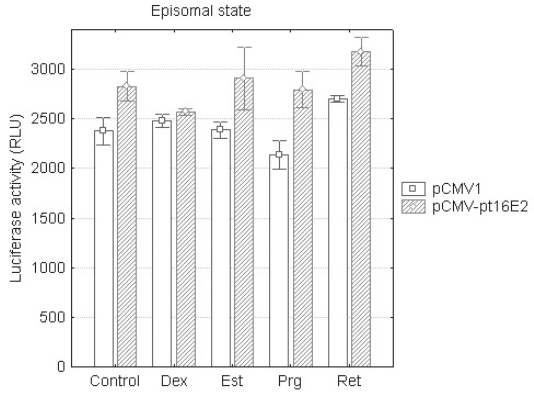

Figure 3. The effects of steroid hormones or retinol and the HPV16 E2 protein on p97 promoter activity in the episomal state.
The reporter construct HPV16 LCR-E6MAR was transiently transfected into C-33A cells, simulating the episomal state of the viral genome. p97 transcriptional activity in the presence of the steroid hormones or retinol and the HPV16 E2 protein (cotransfection with an E2 expression vector) were determined using luciferase assays. The data represent averages of four independent experiments, with error bars indicating ±S.D. Control, indicates hormone-untreated C-33A cells transiently transfected with pGL3-LCR/E6MAR cotransfected with empty pCMV1 or the E2 expressing pCMV-pt16E2 vector, and Dex, Est, Prg, and Ret, designate transfected cells additionally treated with dexamethasone, estradiol, progesterone and retinol, respectively.

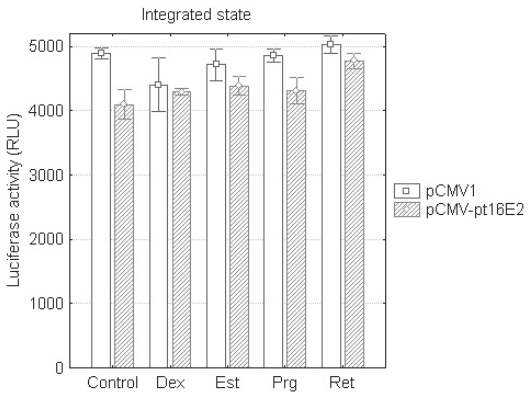

Figure 4. The effects of steroid hormones or retinol and the HPV16 E2 protein on p97 promoter activity in the integrated state.
The HPV16 LCR-*luc*-E6MAR reporter construct was stably transfected into C-33A cells, simulating the integrated state of the viral genome. p97 transcriptional activity in the presence of steroid hormones or retinol and the HPV16 E2 protein (cotransfection with an E2 expression vector) were determined using luciferase assays. The data represent averages of four independent experiments, with error bars indicating ±S.D. Control, indicates hormone-untreated C-33A cells transiently transfected with pGL3-LCR/E6MAR cotransfected with empty pCMV1 or the E2 expressing pCMV-pt16E2 vector, and Dex, Est, Prg, and Ret — designate transfected cells additionally treated with dexamethasone, estradiol, progesterone and retinol, respectively.

porter assays (Phelps & Howley, 1987; Bernard *et al.*, 1989; Lees *et al.*, 1990; Parton *et al.*, 1990; Romanczuk *et al.*, 1990; Bouvard *et al.*, 1994; Tan *et al.*, 1994; Demeret *et al.*, 1994; 1997; Kovelman *et al.*, 1996; Alloul & Sherman, 1999; Lee *et al.*, 2000; DeFilippis *et al.*, 2003; Thierry *et al.*, 2004). The various findings of several research groups point to the dual action of the E2 protein, but can also be attributed to variations in the experimental models used, since transcriptional regulation is strongly context-dependent (reviewed in Fry & Farnham, 1999). However, when expressed in cells that hold integrated HPV genomes, E2 represses the transcription of E6 and E7 (Dowhanick *et al.*, 1995; Desaintes *et al.*, 1997; Francis *et al.*, 2000; Goodwin & DiMaio, 2000; Nishimura *et al.*, 2000). The repressor action of E2 on integrated HPV genomes is indirectly confirmed by the observation that in most cervical cancer cells the E2 ORF is disrupted (Schwarz *et al.*, 1985; Romanczuk & Howley, 1992; Chen *et al.*, 1994; Park *et al.*, 1997; Yoshinouchi *et al.*, 1999). Our experiments have shown that the E2 protein represses the integrated but activates the episomal HPV16 LCR-p97 promoter. These findings are to some extent in agreement with the results of the Beard group (Bechtold *et al.*, 2003), which used the pair of isogenic cell lines W12 and S12 (containing episomal and integrated HPV16 DNA, respectively). They found that the E2 protein represses transcription from the integrated HPV16 genome but has no influence on the episomal viral DNA transcription. The inconsistency can be ascribed to the possible difference in DNA conformation between the reporter plasmids and the viral minichromosome. Another possibility is that additional factors introduced in the course of recombinant adenovirus infection used to express the E2 protein have effects on HPV transcription (Leppard, 1997; Yew & Perricaudet, 1997; Steinwaerder *et al.*, 2001).

Organization of DNA into chromatin and the interaction of DNA with the nuclear matrix influence various aspects of the regulation of transcription and viral DNA copy number control and partition (van Driel *et al.*, 1995; Tan *et al.*, 1998; Stunkel & Bernard, 1999; Hancock, 2000; Stunkel *et al.*, 2000). The episomal HPV DNA inside the viral capsid is packed into nucleosomes forming a viral mini-chromosome (Favre *et al.*, 1977). Small circular DNA introduced into the cell acquires nucleosomes (Jeong & Stein, 1994) and interacts with the nuclear matrix (Mearini *et al.*, 2004), thus plasmids transfected into cells can simulate the episomal form of the viral DNA. A common hallmark of cell transformation by HPV is viral integration into the host cell genome. This occurs in a random fashion but preferentially into fragile and transcriptionally active sites (Choo *et al.*, 1996; Klimov *et al.*, 2002; Thorland *et al.*, 2000; 2003; Wentzensen *et al.*, 2004). Consequently, stable transfection can be used to model the integrated form of the viral genome (Matzner *et al.*, 2003). We used these approaches to study the role of chromatin structure in the control of HPV transcription. The reporter construct used for these experiments contains the entire HPV16 LCR (including 5′MAR) and the downstream E6MAR (the 3′MAR) flanking the enhancer and the p97 promoter (Tan *et al.*, 1998) and therefore resembles the structure of the HPV16 regulatory region. Our findings demonstrate that the chromatin structure on the episomal DNA is over 5-fold more repressing than on the integrated DNA. This is consistent with the data from Stunkel and coworkers who showed that MAR repressed HPV16 transcription in undifferentiated epithelial cells (Stunkel *et al.*, 2000). The E2 protein stimulates the HPV16 LCR-p97 promoter in the episomal state and appears to counteract the repressive effects of chromatin. This E2 activity can be attributed to its ability to interact with the CBP transcriptional co-activator, a protein that exhibits histone acetyltransferase activity (Lee *et al.*, 2000). E2 proteins of all HPV types probably have the ability to bind nuclear matrix as it has been shown for the HPV11 E2 protein (Zou *et al.*, 2000). We have also shown that the E2 protein has an opposite action on the integrated HPV16 LCR-p97 promoter. The repression of transcription in the integrated state may be involved in the maintenance of the differentiation state of the cells. Tumor cells are not productive in virus replication as they do not differentiate (Kaur & McDougall, 1989; Zhao *et al.*, 1997; Pei *et al.*, 1998; Flores *et al.*, 2000; McMurray *et al.*, 2001; Hadaschik *et al.*, 2003; Longworth & Laimins, 2004). An integration event leading to derepression of viral oncogenes may favor tumorigenic cells in the course of cervical cancer etiology (Jeon *et al.*, 1995; Badaracco *et al.*, 2002). However, after the initial integration event the remaining episomes can still express the E2 protein and repression of the integrated HPV genome may leave the cellular phenotype unaltered. Thus the total loss of E2 expression may lead to malignant lesions that contain only integrated HPV genomes (Daniel *et al.*, 1997; Vernon *et al.*, 1997; Tonon *et al.*, 2001; Pett *et al.*, 2004).

Our findings suggest that the E2 activator function is based on stimulation of the basal transcriptional machinery. The HPV16 LCR-p97 promoter on episomal DNA is activated by E2 both in the presence and absence of trichostatin A. The E2 repressive action on the HPV16 LCR p97 promoter on integrated DNA is probably mediated by preservation of chromatin acetylation. The E2 protein appears to be a transcriptional factor that regulates in a positive manner oncogene transcription in the case of episomal HPV16 genome. Our finding is supported by analyses of cervical lesions containing episomal HPV DNA (Crum *et al.*, 1989; Durst *et al.*, 1992; Higgins *et al.*, 1992). The activity may counteract the chromatin repressive form that develops on

exogenous DNA introduced into the nucleus that represents a cellular defense mechanism against viral infection.

Recently, it was demonstrated by Khare *et al*. (1996; 1997) that glucocorticoids up-regulated HPV oncoprotein expression. However, research by Khan *et al*. (1997) showed that growth stimulation of HPV-immortalized cells by glucocorticoids was independent of the E6/E7 mRNA level. We have shown that dexamethasone treatment does not increase reporter activity in either the episomal or integrated states. However, E2 activates the episomal reporter and represses the integrated one in untreated cells, and treatment with dexamethasone abolishes the effects of E2 in both cases. Our results thus seem to confirm the findings of Khan and coworkers but not those of Khare and collaborators.

Similarly, there are conflicting reports in the literature regarding the ability of retinoids to modulate the p97 promoter. According to the results of Narayanan *et al*. (1998), all-*trans*-retinoic acid is able to repress the E6/E7 promoter in CaSki cells. However, in experiments reported by Agarwal *et al*. (1994), all-*trans*-retinoic acid increased the viral E6/E7 mRNA levels in the same cell line. It has also been demonstrated that retinoids do not necessarily inhibit the proliferation of HPV-immortalized cervical cells *via* effects on HPV E6/E7 transcription but rather they can act through suppression of the EGF signaling pathway (Eckert *et al*., 1995; Sah *et al*., 2002). The latter, indirect mode of action is also in agreement with the finding that the response to retinoids (Narayanan *et al*., 1998) or progesterone (Yuan *et al*., 1999) are of the delayed type, requiring 3 to 7 days of treatment. Another finding that highlights an important issue in understanding the nature of the differences reported among various studies is that even closely related HPV16-immortalized cervical epithelial cell lines respond to all-*trans*-retinoic acid in different manners depending on the retinoids level (Choo *et al*., 1995). The HPV-driven immoralization of epithelial cells is a sophisticated process requiring several factors, which can operate *via* distinct pathways, adding further degrees of complexity to the well-known tumorigenesis model. Our observations suggest that E2 activity is dependent on chromatin structure and may be modulated by steroid and retinoid receptors.

Acknowledgements

The pCMV-pt16E2 and pCMV1 plasmids were a gift of Dr. Gyeorgy Veress, University of Debrecen (Hungary). We are grateful to Dr. Kevin Gaston for constructive discussions and comments on the manuscript.

This research was supported by a grant from the State Committee for Scientific Research.

REFERENCES

Agarwal C, Hembree JR, Rorke EA, Eckert RL (1994) Interferon and retinoic acid suppress the growth of human papillomavirus type 16 immortalized cervical epithelial cells, but only interferon suppresses the level of the human papillomavirus transforming oncogenes. *Cancer Res* **54**: 2108–2112.

Alloul N, Sherman L (1999) Transcription-modulatory activities of differentially spliced cDNAs encoding the E2 protein of human papillomavirus type 16. *J Gen Virol* **80**: 2461–2470.

Antson AA, Burns JE, Moroz OV, Scott DJ, Sanders CM, Bronstein IB, Dodson GG, Wilson KS, Maitland NJ (2000) Structure of the intact transactivation domain of the human papillomavirus E2 protein. *Nature* **403**: 805–809.

Badaracco G, Venuti A, Sedati A, Marcante ML (2002) HPV16 and HPV18 in genital tumors: Significantly different levels of viral integration and correlation to tumor invasiveness. *J Med Virol* **67**: 574–582.

Barbosa MS, Schlegel R (1989) The E6 and E7 genes of HPV-18 are sufficient for inducing two-stage in vitro transformation of human keratinocytes. *Oncogene* **4**: 1529–1532.

Bechtold V, Beard P, Raj K (2003) Human papillomavirus type 16 E2 protein has no effect on transcription from episomal viral DNA. *J Virol* **77**: 2021–2028.

Berger SL (2002) Histone modifications in transcriptional regulation. *Curr Opin Genet Dev* **12**: 142–148.

Bernard B, Bailly C, Lenoir MC, Darmon M, Thierry F, Yaniv M (1989) The papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. *J Virol* **63**: 4317–4324.

Bouvard V, Storey A, Pim D, Banks L (1994) Characterization of the human papillomavirus E2 protein: evidence of trans-activation and trans-repression in cervical keratinocytes. *EMBO J* **13**: 5451–5459.

Chen CM, Shyu MP, Au LC, Chu HW, Cheng WT, Choo KB (1994) Analysis of deletion of the integrated human papillomavirus 16 sequence in cervical cancer: a rapid multiplex polymerase chain reaction approach. *J Med Virol* **44**: 206–211.

Choo KB, Pan CC, Han SH (1987) Integration of human papillomavirus type 16 into cellular DNA of cervical carcinoma: preferential deletion of the E2 gene and invariable retention of the long control region and the E6/E7 open reading frames. *Virology* **161**: 259–261.

Choo CK, Rorke EA, Eckert RL (1995) Retinoid regulation of cell differentiation in a series of human papillomavirus type 16-immortalized human cervical epithelial cell lines. *Carcinogenesis* **16**: 375–381.

Choo KB, Chen CM, Han CP, Cheng WT, Au LC (1996) Molecular analysis of cellular loci disrupted by papillomavirus 16 integration in cervical cancer: frequent viral integration in topologically destabilized and transcriptionally active chromosomal regions. *J Med Virol* **49**: 15–22.

Cripe TP, Haugen TH, Turk JP, Tabatabai F, Schmid PG 3rd, Durst M, Gissmann L, Roman A, Turek LP (1987) Transcriptional regulation of the human papillomavirus-16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 transactivator and repressor gene products: implications for cervical carcinogenesis. *EMBO J* **6**: 3745–3753.

Crum CP, Symbula M, Ward BE (1989) Topography of early HPV 16 transcription in high-grade genital precancers. *Am J Pathol* **134**: 1183–1188.

Daniel B, Rangarajan A, Mukherjee G, Vallikad E, Krishna S (1997) The link between integration and expression

of human papillomavirus type 16 genomes and cellular changes in the evolution of cervical intraepithelial neoplastic lesions. *J Gen Virol* **78**: 1095–1101.

DeFilippis RA, Goodwin EC, Wu L, DiMaio D (2003) Endogenous human papillomavirus E6 and E7 proteins differentially regulate proliferation, senescence, and apoptosis in HeLa cervical carcinoma cells. *J Virol* **77**: 1551–1563.

Demeret C, Yaniv M, Thierry F (1994) The E2 transcriptional repressor can compensate for Sp1 activation of the human papillomavirus type 18 early promoter. *J Virol* **68**: 7075–7082.

Demeret C, Desaintes C, Yaniv M, Thierry F (1997) Different mechanisms contribute to the E2-mediated transcriptional repression of human papillomavirus type 18 viral oncogenes. *J Virol* **71**: 9343–9349.

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.

de Villiers EM (2003) Relationship between steroid hormone contraceptives and HPV, cervical intraepithelial neoplasia and cervical carcinoma. *Int J Cancer* **103**: 705–708.

Dowhanick JJ, McBride AA, Howley PM (1995) Suppression of cellular proliferation by the papillomavirus E2 protein. *J Virol* **69**: 7791–7799.

Durst M, Glitz D, Schneider A, zur Hausen H (1992) Human papillomavirus type 16 (HPV 16) gene expression and DNA replication in cervical neoplasia: analysis by in situ hybridization. *Virology* **189**: 132–140.

Eckert RL, Agarwal C, Hembree JR, Choo CK, Sizemore N, Andreatta-van Leyen S, Rorke EA (1995) Human cervical cancer. Retinoids, interferon and human papillomavirus. *Adv Exp Med Biol* **375**: 31–44.

Favre M, Breitburd F, Croissant O, Orth G (1977) Chromatin-like structures obtained after alkaline disruption of bovine and human papillomaviruses. *J Virol* **21**: 1205–1209.

Flores ER, Allen-Hoffmann BL, Lee D, Lambert PF (2000) The human papillomavirus type 16 E7 oncogene is required for the productive stage of the viral life cycle. *J Virol* **74**: 6622–6631.

Francis DA, Schmid SI, Howley PM (2000) Repression of the integrated papillomavirus E6/E7 promoter is required for growth suppression of cervical cancer cells. *J Virol* **74**: 2679–2686.

Fry CJ, Farnham PJ (1999) Context-dependent transcriptional regulation. *J Biol Chem* **274**: 29583–29586.

Goodwin EC, DiMaio D (2000) Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways. *Proc Natl Acad Sci USA* **97**: 12513–12518.

Hadaschik D, Hinterkeuser K, Oldak M, Pfister HJ, Smola-Hess S (2003) The papillomavirus E2 protein binds to and synergizes with C/EBP factors involved in keratinocyte differentiation. *J Virol* **77**: 5253–5265.

Hancock R (2000) A new look at the nuclear matrix. *Chromosoma* **109**: 219–225.

Higgins GD, Uzelin DM, Phillips GE, McEvoy P, Marin R, Burrell CJ (1992) Transcription patterns of human papillomavirus type 16 in genital intraepithelial neoplasia: evidence for promoter usage within the E7 open reading frame during epithelial differentiation. *J Gen Virol* **73**: 2047–2057.

Hou SY, Wu SY, Chiang CM (2002) Transcriptional activity among high and low risk human papillomavirus E2 proteins correlates with E2 DNA binding. *J Biol Chem* **277**: 45619–45629.

Jeong S, Stein A (1994) Micrococcal nuclease digestion of nuclei reveals extended nucleosome ladders having anomalous DNA lengths for chromatin assembled on non-replicating plasmids in transfected cells. *Nucleic Acids Res* **22**: 370–375.

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.

Kaur P, McDougall JK (1989) HPV-18 immortalization of human keratinocytes. *Virology* **173**: 302–310.

Khan MA, Canhoto AJ, Housley PR, Creek KE, Pirisi L (1997) Glucocorticoids stimulate growth of human papillomavirus type 16 (HPV16)-immortalized human keratinocytes and support HPV16-mediated immortalization without affecting the levels of HPV16 E6/E7 mRNA. *Exp Cell Res* **236**: 304–310.

Khare S, Kumar KU, Tang SC, Pater MM, Pater A (1996) Up-regulation of hormone response of human papillomavirus type 16 expression and increased DNA-protein binding by consensus mutations of viral glucocorticoid response elements. *J Med Virol* **50**: 254–262.

Khare S, Pater MM, Tang SC, Pater A (1997) Effect of glucocorticoid hormones on viral gene expression, growth, and dysplastic differentiation in HPV16-immortalized ectocervical cells. *Exp Cell Res* **232**: 353–360.

Klimov E, Vinokourova S, Moisjak E, Rakhmanaliev E, Kobseva V, Laimins L, Kisseljov F, Sulimova G (2002) Human papillomaviruses and cervical tumours: mapping of integration sites and analysis of adjacent cellular sequences. *BMC Cancer* **2**: 24.

Kovelman R, Bilter GK, Glezer E, Tsou AY, Barbosa MS (1996) Enhanced transcriptional activation by E2 proteins from the oncogenic human papillomaviruses. *J Virol* **70**: 7549–7560.

Lee D, Lee B, Kim J, Kim DW, Choe J (2000) cAMP response element-binding protein-binding protein binds to human papillomavirus E2 protein and activates E2-dependent transcription. *J Biol Chem* **275**: 7045–7051.

Lees E, Osborn K, Banks L, Crawford L (1990) Transformation of primary BRK cells by human papillomavirus type 16 and EJ-ras is increased by overexpression of the viral E2 protein. *J Gen Virol* **71**: 183–193.

Leppard KN (1997) E4 gene function in adenovirus, adenovirus vector and adenoassociated virus infections. *J Gen Virol* **78**: 2131–2138.

Longworth MS, Laimins LA (2004) Pathogenesis of human papillomaviruses in differentiating epithelia. *Microbiol Mol Biol Rev* **68**: 362–372.

Matzner I, Savelyeva L, Schwab M (2003) Preferential integration of a transfected marker gene into spontaneously expressed fragile sites of a breast cancer cell line. *Cancer Lett* **189**: 207–219.

McBride A, Myers G (1997) The E2 Proteins. In: *Human Papillomaviruses 1997 Compendium*. Myers G, Bernard HU, Delius H, eds. III: 54–73, Los Alamos National Laboratory Press, Los Alamos USA.

McMurray HR, Nguyen D, Westbrook TF, McAnce DJ (2001) Biology of human papillomaviruses. *Int J Exp Pathol* **82**: 15–33.

Mearini G, Nielsen PE, Fackelmayer FO (2004) Localization and dynamics of small circular DNA in live mammalian nuclei. *Nucleic Acids Res* **32**: 2642–2651.

Nakagawa S, Yoshikawa H, Yasugi T, Kimura M, Kawana K, Matsumoto K, Yamada M, Onda T, Taketani Y (2000) Ubiquitous presence of E6 and E7 transcripts in human papillomavirus-positive cervical carcinomas regardless of its type. *J Med Virol* **62**: 251–258.

Narayanan BA, Holladay EB, Nixon DW, Mauro CT (1998) The effect of all-trans and 9-cis retinoic acid on the

steady state level of HPV16 E6/E7 mRNA and cell cycle in cervical carcinoma cells. *Life Sci* **63**: 565–573.

Nishimura A, Ono T, Ishimoto A, Dowhanick JJ, Frizzell MA, Howley PM, Sakai H (2000) Mechanisms of human papillomavirus E2-mediated repression of viral oncogene expression and cervical cancer cell growth inhibition. *J Virol* **74**: 3752–3760.

O'Connor M, Chan SY, Bernard HU (1995) Transcription factor binding sites in the long control region of genital HPVs. In: *Human Papillomaviruses 1995 Compendium*. Myers G, Bernard HU, Delius H, eds. **III**: 21–40, Los Alamos National Laboratory Press, Los Alamos USA.

Park JS, Hwang ES, Park SN, Ahn HK, Um SJ, Kim CJ, Kim SJ, Namkoong SE (1997) Physical status and expression of HPV genes in cervical cancers. *Gynecol Oncol* **65**: 121–129.

Parton A, Grand RJ, Biggs P, Settleman J, DiMaio D, Gallimore PH (1990) Integrated HPV 1 genomes in a human keratinocyte cell line can be transactivated by a SV40/BPV1 recombinant virus which expresses BPV1 E2 proteins. *Virology* **175**: 508–517.

Pei XF, Sherman L, Sun YH, Schlegel R (1998) HPV-16 E7 protein bypasses keratinocyte growth inhibition by serum and calcium. *Carcinogenesis* **19**: 1481–1486.

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.

Phelps WC, Howley PM (1987) Transcriptional trans-activation by the human papillomavirus type 16 E2 gene product. *J Virol* **61**: 1630–1638.

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.

Romanczuk H, Thierry F, Howley PM (1990) Mutational analysis of cis elements involved in E2 modulation of human papillomavirus type 16 P97 and type 18 P105 promoters. *J Virol* **64**: 2849–2859.

Sah JF, Eckert RL, Chandraratna RA, Rorke EA (2002) Retinoids suppress epidermal growth factor-associated cell proliferation by inhibiting epidermal growth factor receptor-dependent ERK1/2 activation. *J Biol Chem* **277**: 9728–9735.

Sashiyama H, Shino Y, Kawamata Y, Tomita Y, Ogawa N, Shimada H, Kobayashi S, Asano T, Ochiai T, Shirasawa H (2001) Immortalization of human esophageal keratinocytes by E6 and E7 of human papillomavirus type 16. *Int J Oncol* **19**: 97–103.

Schwarz E, Freese UK, Gissmann L, Mayer W, Roggenbuck B, Stremlau A, zur Hausen H (1985) Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* **314**: 111–114.

Steinwaerder DS, Carlson CA, Lieber A (2001) Human papilloma virus E6 and E7 proteins support DNA replication of adenoviruses deleted for the E1A and E1B genes. *Mol Ther* **4**: 211–216.

Stunkel W, Bernard HU (1999) The chromatin structure of the long control region of human papillomavirus type 16 represses viral oncoprotein expression. *J Virol* **73**: 1918–1930.

Stunkel W, Huang Z, Tan SH, O'Connor MJ, Bernard HU (2000) Nuclear matrix attachment regions of human papillomavirus type 16 repress or activate the E6 promoter, depending on the physical state of the viral DNA. *J Virol* **74**: 2489–2501.

Tan SH, Leong LE, Walker PA, Bernard HU (1994) The human papillomavirus type 16 E2 transcription factor binds with low cooperativity to two flanking sites and represses the E6 promoter through displacement of Sp1 and TFIID. *J Virol* **68**: 6411–6420.

Tan SH, Bartsch D, Schwarz E, Bernard HU (1998) Nuclear matrix attachment regions of human papillomavirus type 16 point toward conservation of these genomic elements in all genital papillomaviruses. *J Virol* **72**: 3610–3622.

Thierry F, Yaniv M (1987) The BPV1-E2 trans-acting protein can be either an activator or a repressor of the HPV18 regulatory region. *EMBO J* **6**: 3391–3397.

Thierry F, Benotmane MA, Demeret C, Mori M, Teissier S, Desaintes C (2004) A genomic approach reveals a novel mitotic pathway in papillomavirus carcinogenesis. *Cancer Res* **64**: 895–903.

Thorland EC, Myers SL, Persing DH, Sarkar G, McGovern RM, Gostout BS, Smith DI (2000) Human papillomavirus type 16 integrations in cervical tumors frequently occur in common fragile sites. *Cancer Res* **60**: 5916–5921.

Thorland EC, Myers SL, Gostout BS, Smith DI (2003) Common fragile sites are preferential targets for HPV16 integrations in cervical tumors. *Oncogene* **22**: 1225–1237.

Tonon SA, Picconi MA, Bos PD, Zinovich JB, Galuppo J, Alonio LV, Teyssie AR (2001) Physical status of the E2 human papilloma virus 16 viral gene in cervical preneoplastic and neoplastic lesions. *J Clin Virol* **21**: 129–134.

van Driel R, Wansink DG, van Steensel B, Grande MA, Schul W, de Jong L (1995) Nuclear domains and the nuclear matrix. *Int Rev Cytol* **162A**: 151–189.

van Holde K, Zlatanova J (1996) What determines the folding of the chromatin fiber? *Proc Natl Acad Sci USA* **93**: 10548–10555.

Veress G, Szarka K, Dong XP, Gergely L, Pfister H (1999) Functional significance of sequence variation in the E2 gene and the long control region of human papillomavirus type 16. *J Gen Virol* **80**: 1035–1043.

Vernon SD, Unger ER, Miller DL, Lee DR, Reeves WC (1997) Association of human papillomavirus type 16 integration in the E2 gene with poor disease-free survival from cervical cancer. *Int J Cancer* **74**: 50–56.

Webster K, Taylor A, Gaston K (2001) Oestrogen and progesterone increase the levels of apoptosis induced by human papillomavirus type 16 E2 and E7 proteins. *J Gen Virol* **82**: 201–213.

Wentzensen N, Vinokurova S, von Knebel Doeberitz M (2004) Systematic review of genomic integration sites of human papillomavirus genomes in epithelial dysplasia and invasive cancer of the female lower genital tract. *Cancer Res* **64**: 3878–3884.

Yew P, Perricaudet M (1997) Advances in adenoviral vectors: from genetic engineering to their biology. *FASEB J* **11**: 615–623.

Yokoyama M, Nakao Y, Iwasaka T, Pater A, Sugimori H (2001) Retinoic acid and interferon-alpha effects on cell growth and differentiation in cervical carcinoma cell lines. *Obstet Gynecol* **98**: 332–340.

Yoshinouchi M, Hongo A, Nakamura K, Kodama J, Itoh S, Sakai H, Kudo T (1999) Analysis by multiplex PCR of the physical status of human papillomavirus type 16 DNA in cervical cancers. *J Clin Microbiol* **37**: 3514–3517.

Yuan F, Auborn K, James C (1999) Altered growth and viral gene expression in human papillomavirus type 16-containing cancer cell lines treated with progesterone. *Cancer Invest* **17**: 19–29.

Zhao W, Chow LT, Broker TR (1997) Transcription activities of human papillomavirus type 11 E6 promoter-proximal elements in raft and submerged cultures of foreskin keratinocytes. *J Virol* **71**: 8832–8840.

Zou N, Lin BY, Duan F, Lee KY, Jin G, Guan R, Yao G, Lefkowitz EJ, Broker TR, Chow LT (2000) The hinge of the human papillomavirus type 11 E2 protein contains major determinants for nuclear localization and nuclear matrix association. *J Virol* **74**: 3761–3770.

zur Hausen H (1996) Papillomavirus infections — a major cause of human cancers. *Biochim Biophys Acta* **1288**: F55–F78.

zur Hausen H (2000) Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *J Natl Cancer Inst* **92**: 690–698.