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Transcriptional activity of human papillomavirus type 16 variants having deletions in the long control region

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Abstract

Transcription of the *E6* and *E7* viral oncogenes of human papillomavirus (HPV) type 16 is regulated by the P97 major early promoter, and enhancer and silencer elements found in the long control region (LCR). In this study, we tested the transcriptional activity of natural HPV 16 variants having long deletions in the LCR. The HPV 16 LCR regions were amplified from invasive cervical cancer specimens, and cloned into the reporter vector pALuc. Transcriptional activity of the different clones was measured by luciferase test after transient transfection into HeLa cells. The deletions found in the LCR encompassed parts of the enhancer and either the YY1-specific silencer alone or together with the CDP-specific silencer. The transcriptional activity of these deletion variants were usually reduced compared with that of the corresponding full-length clones. However, a deletion variant lacking the whole enhancer and both silencer regions retained substantial enhancer activity on the P97 promoter. These results point to the existence of a novel context-dependent enhancer element in the 5′ LCR of HPV 16.

Keywords: HPV; LCR; Deletions; Transcription; Enhancer; Silencer

1. Introduction

Human papillomavirus (HPV) type 16 is the most prevalent genital HPV type, which has an important role in the establishment of cervical cancer [1]. The *E6* and *E7* oncogenes of high-risk genital HPVs have been shown to inactivate the p53 and pRB cellular tumour suppressor proteins, respectively, which is thought to be important in both the *in vitro* immortalisation of keratinocytes and *in vivo* carcinogenesis [2,3].

Transcription of the *E6* and *E7* viral oncogenes of human papillomavirus (HPV) type 16 is regulated by the P97 major early promoter, and enhancer and silencer elements found in the long control region (LCR) [4]. The central part of the LCR contains an epithelial-specific enhancer, with several binding sites for positively acting transcription factors (Nuclear Factor 1 (NFI)), activating protein-1 (AP-1), octamer-binding factor-1 (Oct-1), transcription enhancing factor 1 (TEF-1)) [4–6]. Between the enhancer and P97 promoter regions, two adjacent silencer elements have been identified. The promoter-distal silencer is regulated negatively by the transcription factor YY1 [7]. The other silencer, which overlaps the binding site of the E1 replication protein, is downregulated by the differentiation-specific transcription factor CCAAT displacement protein-1 (CDP)/Cut [8].

Sequence variation in the LCR and *E6–E7* regions of the HPV 16 genome may have an impact on the oncogenicity of the virus. Sequence analysis revealed the existence of five major geographical variant classes of HPV 16: one European (E), two African (Af1 and Af2), one Asian (As), and one Asian-American (AA) [9]. Infections caused by European HPV 16 variants were found to have a lower risk of progression to cervical intraepithelial neoplasia than those caused by other variants [10]. In addition, *in vitro* studies also revealed some functional differences between certain HPV 16 variants. The E6 protein of an Asian–American HPV 16 isolate was found to induce more differentiation-resistant colonies than the reference E6 protein in human foreskin keratinocytes [11]. However, the LCR of the Asian-American HPV 16 variant was found to have enhanced transcriptional activity compared with that of the European variant [12,13].

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Certain HPV 16 variants from cervical cancers were found to have deletions of variable length in the LCR. Such deletions were frequently found in the YY1-specific silencer region, with consequent elevated transcriptional activity [14]. Grassmann and colleagues reported deletions in either the central enhancer or the 5′ LCR of HPV16. These deletion variants had either lower or only slightly higher transcriptional activity than the full-length LCR clone [15].

During previous work with HPV 16 variants from cervical cancer samples [12], we also isolated some clones which apparently carried relatively long deletions in the LCR. In this study, we analysed the sequences of these deletion variants and tested their transcriptional activities in parallel with full-length variants isolated from the same patients.

2. Materials and methods

2.1. Patients

The HPV 16 variants analysed in this study were isolated from 4 patients suffering from squamous cervical cancer who carried episomal, integrated or both episomal and integrated HPV 16 DNA in their tumours. The methods used to determine the physical state of HPV 16 DNA in these cervical cancer specimens were described earlier in Ref. [16].

2.2. Plasmids

The long control region (LCR) of HPV 16 was amplified from cervical cancer samples using the primers 16-007, 5′-TT*CTGCAG*ACCTAGATCAGTTTCC-3′ (nucleotide (nt) 7003-7026; underlined is a natural *PstI* site), and 16LCR-R, 5′-GCGC*GGTACC*TTGCAGTTCTCTTTTGGTG-3′ (nt 86–104; an artificial *KpnI* site is underlined). Amplification was performed with *Pwo* polymerase (an enzyme with proof-reading activity), according to the manufacturer's recommendations (Boehringer-Mannheim, Germany). The amplification products were purified with the Qiaquick polymerase chain reaction (PCR) purification kit (Qiagen, USA), digested by *PstI* and *KpnI*, purified again and cloned into pUC19 vector digested by *PstI* and *KpnI*. The resulting clones were sequenced by the T7 sequencing kit (Amersham Pharmacia, UK), and those selected for functional analysis were subcloned into the promoterless *luciferase* reporter vector pALuc [14] digested by *PstI* and *KpnI*. The reporter vector pALuc-pt16LCR was constructed using a similar strategy, but here the reference full-length HPV 16 clone was used as template in the PCR. In order to construct the reporter vector pALuc-16P, the P97 early promoter region was amplified (nt 14–104) using the primers 16-014K, 5′-GCGC*GGTACC*-ATGTATAAAACTAAGGGCGT-3′ (nt 14–33, artificial *KpnI* site underlined) and 16LCR-R. The resulting amplimer was digested by *KpnI*, purified and cloned into pALuc. The tissue-specific enhancer region was amplified (nt 7527-7854) using the primers 16-7527, 5′-GCGCCTGCAG-AACTTGTACGTTTCCTGCTT-3′ (with *PstI* recognition sequence) and 16-7854, 5′GTCAGGATCC-CACACCCATGTGCAGTTTTA-3′ (with *BamHI* binding site) and cloned into pALuc-16P in front of the promoter region using *PstI* and *BamHI* restriction enzymes. The resulting construct was named pALuc-16PENH. The vectors pALuc-16P and pALuc-16PENH were also confirmed by sequencing. The pCMV1-pt16E2 expression vector and the reporter construct pC18Sp1-Luc were described earlier in Ref. [12].

2.3. Cell culture, transient transfection and luciferase test

HeLa (ATCC CCL-2) cervical cancer cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were transfected with 2 μg of the reporter vectors and 1 μg of pRSVβ-Gal in 6-cm-diameter dishes using Lipofectamine (GIBCO BRL). The cells were harvested 48 h after transfection by the addition of 500 μl Reporter Lysis Buffer (Promega, USA) and one freeze–thaw cycle. The Luciferase Assay System of Promega was used to measure the luciferase activity of cell extracts. β-Galactosidase assay was also performed to standardise for transfection efficiency.

In co-transfection experiments, HeLa cells were co-transfected with 2 μg of the reporter construct pC18Sp1-Luc, 1 μg of the eukaryotic expression vector pCMV1-pt16E2, and 1 μg of pRSVβ-Gal. The amount of DNA transfected was kept constant by the addition of the empty expression vector pCMV1. The effects of HPV16 E2 on different LCR variants were also tested in cotransfection experiments using HeLa cells transfected with 2 μg of the reporter vector pALuc containing different HPV16 LCR variants, 1 μg of the expression vector pCMV1-pt16E2, and 1 μg of pRSVβ-Gal.

3. Results

During a previous study, we isolated HPV 16 LCR variants from cervical cancer samples [12]. After amplification of the LCR region and electrophoresis, in addition to bands corresponding to the full-length LCR, some shorter bands were also found in a few cases. In this study, two samples (nos. 33 and 936) with episomal, one with integrated (no. 990), and one with both episomal and integrated (no. 926) HPV 16 genomes were

chosen for detailed analysis of these putative deletion variants. The physical state of HPV DNA in these samples and the methods used to determine it were described recently in Ref. [16]. Amplification products were not gel purified, but instead purified by the Qiaquick PCR purification kit (Qiagen, USA), digested by restriction enzymes and cloned into pUC19. The different variants were then sequenced and those which proved to contain HPV 16 sequences were subcloned into the promoter-free luciferase reporter vector pALuc in the forward orientation. The transcriptional activity of the different LCR variants was then analysed by luciferase test after transient transfection into the cervical cancer cell line HeLa.

The short variants isolated from the samples with integrated (no. 990) or episomal and integrated (no. 926) HPV 16 genomes turned out to contain unidentified and human genome specific sequences, respectively, and these were not analysed further.

From case 936, in addition to a full-length LCR clone (936LCR) belonging to the European variant group, two deletion variants were isolated (Table 1 and Fig. 1). One variant (936LCRd1) contained an insertion of 'TA' just after nt 7521 and a 211 bp deletion (nt 7522–nt 7732) in the tissue-specific enhancer region. The other variant (936LCRd2) had a 378 bp deletion (nt 7564–nt 35) encompassing a great part of the enhancer and both 3′ silencer regions (the YY1-dependent and the CDP/Cut-dependent), and the Sp1 binding site in the promoter region.

From the other case (33), again a full-length clone (33LCR) and two deletion variants were isolated (Table 1 and Fig. 1). On the basis of nucleotide changes relative to the reference sequence, these isolates belonged to the Asian–American HPV 16 variant group [9,12]. A large part of the enhancer region and the total YY1-dependent silencer region were deleted (292 bps, nt 7566–nt 7857) in isolate 33LCRd1. The other deletion variant (33LCRd2) contained a 563 bp deletion (nt 7363–nt 19) encompassing the whole enhancer and both 3′ silencer regions. Cloning and sequencing of the deletion variants were repeated from independent amplification reactions, with the same results as described above.

In order to analyse the transcriptional activity of these HPV 16 LCR variants, we subcloned them into the luciferase reporter vector pALuc and transfected them into HeLa cells by Lipofectamine (GIBCO). After 2 days incubation, luciferase tests were performed with extracts prepared from the transfected cells (Fig. 2). For comparison, the transcriptional activities of the reporter constructs pALuc (empty vector), pALuc-16P, pALuc-16PENH and pALuc-pt16LCR were also tested. The plasmid pALuc-16P contains the P97 major early promoter (nt 14–104) in front of the firefly *luciferase* gene. In analysing the results of the luciferase tests, the activity of this plasmid was set to 1. The vector pALuc-16PENH, which was used as a positive control in the functional tests was constructed by cloning the epithelial-specific enhancer region of HPV 16 LCR (from nt 7527 to nt 7854) in front of the P97 promoter into the vector pALuc-16P. It must be emphasised that this construct contains the YY1-specific, but not the CDP/Cut-dependent promoter-proximal silencer region of the

Table 1
Nucleotide alterations of different HPV16 LCR variants compared with the reference HPV 16R sequence

Reference nucleotide	936LCR	936LCRd1	936LCRd2	33LCR	33LCRd1	33LCRd2	Binding site[b]
7193 G	T	T	T	T	T	T	
7233 A	.	.	.	C	C	C	
7339 A	.	.	.	T	T	T	
7394 C	.	.	.	T	T	–	GRE/1
7395 C	.	.	.	T	T	–	GRE/1
7485 A	.	.	.	C	C	–	GRE/2
7489 G	.	.	.	A	A	–	GRE/2
7521 G	A	ATA[a]	A	A	A	–	
7669 C	.	–	–	T	–	–	
7689 C	.	–	–	A	–	–	TEF-1
7716 G	T	–	–	.	–	–	NF1
7729 A	.	–	–	C	–	–	
7743 T	.	.	–	G	–	–	
7764 C	.	.	–	T	–	–	
7786 C	.	.	–	T	–	–	
7886 C	.	.	–	G	G	–	

HPV, human papilloma virus; LCR, long control region. Only those positions are given in which sequence alterations were found. The first column gives the nucleotides found at these positions in the HPV 16R sequence (http://hpv-web.lanl.gov), and the sequence alterations are given relative to this. The absence of nt change is marked by (.) while (–) indicates the deletion of the corresponding nucleotide.

[a] Insertion of TA after nt 7521 was found in this variant.

[b] GRE: glucocorticoid/progesterone response element; TEF-1, transcription enhancing factor 1; NF1, nuclear factor 1.

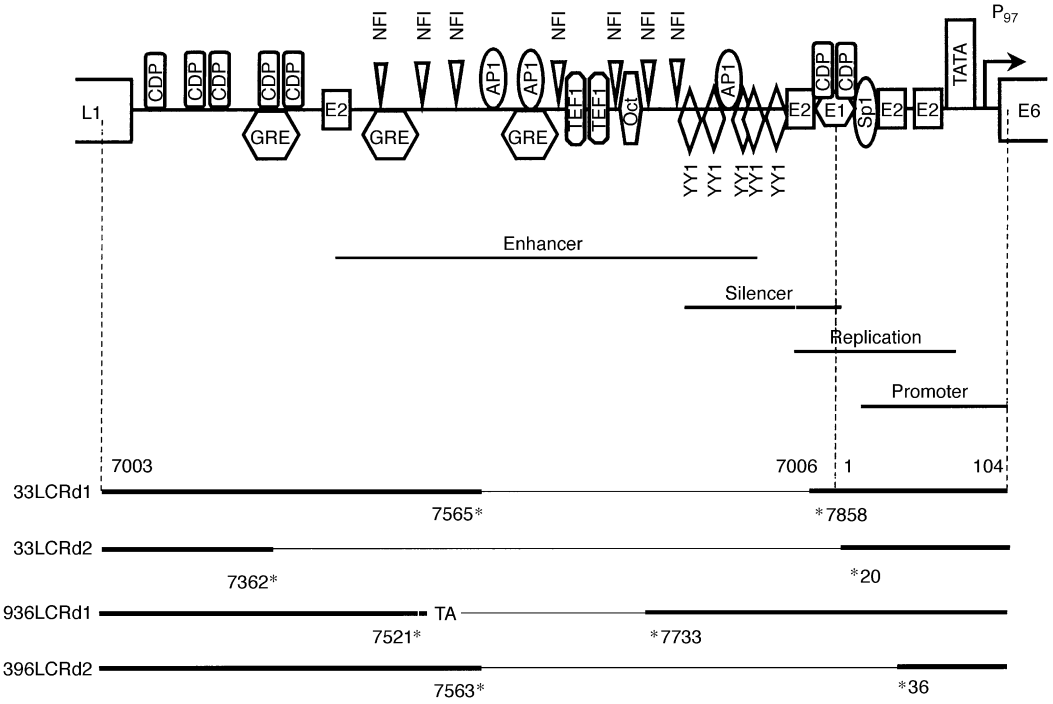

Fig. 1. Schematic representation of the human papillomavirus (HPV)16 long control region (LCR) deletion variants isolated from 2 cervical cancer patients. The full-length variants present in both cases are not shown. The deletions which are represented by thin lines are shown parallel with the different functional regions of the LCR. The nucleotides at the boundaries of deletions are marked by asterisks.

LCR. The control vector pALuc-pt16LCR contains the full LCR region of HPV 16 (from nt 7003 to nt 104) including both the YY1-specific and the CDP/Cut-specific silencer regions. In accordance with this, pALuc-16PENH had much higher transcriptional activity than pALuc-pt16LCR (Fig. 2).

In accordance with previous results [12,13], the full-length Asian–American LCR variant 33LCR had higher transcriptional activity than the European variants pt16LCR or 936LCR. The variants 33LCRd1 and 33LCRd2, containing deletions of a large part of the

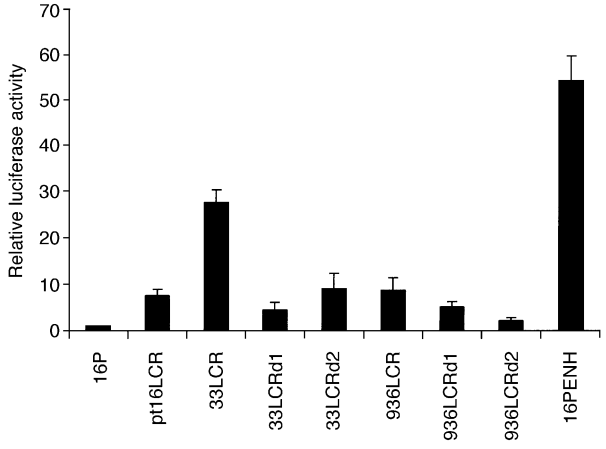

Fig. 2. Transcriptional activity of the different HPV 16 LCR variants determined by luciferase tests in HeLa cells. The data presented represent averages of at least three independent experiments, with error bars indicating standard deviations.

enhancer region and one or both silencer regions still had significantly higher activity than the construct containing the P97 promoter only (pALuc-16P). This was very impressive in the case of 33LCRd2, which had the largest deletion, but highest transcriptional activity of all the deletion variants tested. In addition to the P97 promoter region (nt 20–104), 33LCRd2 contains only the 3′ part of the *L1* gene and a 210 bp upstream LCR element (nt 7003–7362). We hypothesise that this 5′ LCR segment of the HPV 16 genome contains a transcriptional enhancer region similar to those described for HPV 11 and 18 [17–19].

The deletion variant 936LCRd1, which had a significant part of the enhancer region deleted had approximately 50% reduced activity compared with the full-length variant 936LCR. The other deletion variant isolated from the same sample (936LCRd2) had an even lower activity compared with the full-length clone which is not surprising as the deletion in this variant involved part of the promoter region also (the Sp1 binding site was deleted).

In variant 33LCRd2, two of the four binding sites for the viral replication/transcription factor *E2* were deleted. We wanted to see if expression of HPV 16 *E2* had any specific effect on this variant different from that on the full-length clone. First, it was necessary to prove that transcriptionally active E2 protein is expressed in HeLa cells after transient transfection with the expression vector used. To do this, the *E2* expression vector pCMV-pt16E2 containing the reference HPV 16 *E2*

coding sequences was co-transfected into HeLa cells together with the luciferase reporter vector pC18-Sp1Luc. This construct contains 4 *E2* binding sites, 2 Sp1 binding sites, and the firefly *luciferase* gene driven by the adenovirus major late promoter, and it can be efficiently transactivated by papillomavirus E2 proteins [12]. When increasing amounts of the *E2* expression vector pCMV-pt16E2 were co-transfected with 2 μg of the reporter construct pC18-Sp1Luc, a dose-dependent transactivation effect of up to approximately 50-fold was seen (Fig. 3a).

Next, the effects of expressing the HPV16 *E2* gene on the transcriptional activity of certain HPV16 LCR variants were examined (Fig. 3b). It can be seen that expression of *E2* had little effect on the transcriptional activity of the control reporter vector pt16LCR in HeLa cells. In contrast, E2 inhibited transcription modestly from the variants 33LCR and 33LCRd1 and strongly from 33LCRd2. It should be emphasised that all 4 *E2* binding sites are conserved in 33LCR and 33LCRd1, while 2 promoter-distal *E2* binding sites are deleted in 33LCRd2. On the other hand, the two promoter-distal *E2* binding sites are thought to have positive effect on the P97 promoter, while the two binding sites within the promoter region (which are retained in 33LCRd2) were shown to inhibit the activity of the promoter [20]. Thus, it is not surprising that deletion of the two promoter-distal *E2* binding sites led to an increased inhibition of the P97 promoter by E2.

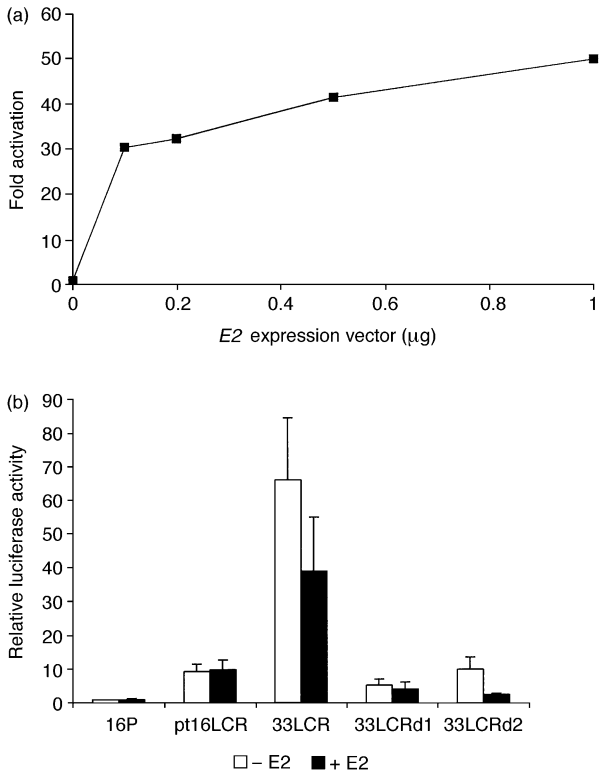

Fig. 3. Effects of the HPV16 E2 protein on different reporter vectors in HeLa cells. (a) Dose-dependent transactivation effect of HPV16 *E2* on the transcriptional activity of the reporter vector pC18Sp1-Luc containing *E2* binding sites. (b) Transcriptional activities of certain HPV16 LCR variants in the presence (+E2) or the absence (−E2) of the HPV 16 *E2* expression plasmid pCMV-pt16E2.

4. Discussion

In this study, we analysed the transcriptional activity of natural deletion variants isolated from cervical cancer cases carrying episomal HPV16 genomes. The deletions found in the long control region (LCR) of the viral genome encompassed parts of the epithelial-specific enhancer and the YY1-specific silencer alone or together with the CDP-specific silencer region, which overlaps the replication origin of the episomal HPV genome. The transcriptional activity of these deletion variants were usually reduced compared with that of the corresponding full-length clones. However, some of the deletion variants still had substantial transcriptional activity when compared with that of the control vector containing the P97 promoter alone without any enhancer elements. This was most impressive in the case of the deletion variant 33LCRd2, where the whole epithelial-specific enhancer and both the YY1-dependent and the CDP/Cut-dependent silencer regions were deleted. This variant clone contained only the 3′ end of the *L1* gene and a 210 bp upstream LCR element, in addition to the P97 promoter region, and still it had a transcriptional activity comparable to that of the full-length European LCR variants pt16LCR and 936LCR.

On the basis of these results, we suggest that the 5′ part of the HPV16 LCR contains a transcriptional enhancer region which can provide transcription of the *E6/E7* oncogenes in the absence of the central epithelial-specific enhancer region. This 5′-LCR segment of HPV16 was shown to represent a nuclear matrix attachment region (MAR) and to function as either a repressor or activator of the P97 promoter depending on the physical state of the viral DNA [21]. This 5′-LCR MAR was shown to function as a silencer when the reporter vector was present in an episomal form, while it functioned as an enhancer when the vector had been integrated in the host genome. In our study, the reporter vectors were tested in transient transfections (when they are present episomally), and still the 5′-LCR seemed to function as a weak enhancer. It should be considered that in the study by Stünkel and colleagues, the function of this LCR segment was studied in the presence of the central enhancer region, while in the variants tested here, this enhancer region was deleted partly or completely. This may explain the partly different results found in the two studies.

A detailed functional analysis will be necessary using homologous and heterologous promoters to confirm the transcriptional enhancer activity of the 5′ LCR segment

of HPV 16. If the enhancer activity is confirmed, one possible transcription factor responsible for it may be AP1, which has potential binding sites in this 5′ LCR region (at nucleotides 7305, 7332 and 7361). It is also interesting that similar 5′ LCR segments of HPV 11 and HPV 18 were found to function as transcriptional enhancers in both keratinocytes and fibroblast cells [17–19]. However, there are alternative explanations for the relatively high transcriptional activity of the deletion variant 33LCRd2. One such scenario may be that the large deletion in this variant altered the nucleosomal organisation of the LCR and relieved transcriptional repression by nucleosomes, CDP, and the histone deacetylase enzyme HDAC1 [21].

The effects of the viral transcription factor E2 on the transcriptional activity of certain LCR variants were also tested. This was done because these variants were isolated from cervical cancers containing episomal HPV16 genomes where the *E2* gene may be transcriptionally active. In cotransfection experiments, overexpression of the HPV16 E2 protein had different effects on the different HPV16 LCR variants. Notably, the transcriptional activity of the deletion variant 33LCRd2, which lacked two promoter-distal *E2* binding sites was quite strongly inhibited by E2. It must be noted, however that the level of active E2 protein was probably much lower in the original cancer cells than in the cotransfection experiments, where E2 was transcribed from a strong CMV promoter.

It was an interesting finding in this study that parts of the replication origin of the episomal HPV16 genome [6] (which overlap the CDP dependent silencer region) were found to be deleted in two variants isolated from specimens with episomal HPV genomes. It is theoretically possible that these deletion variants, which were present in small amounts in addition to the full-length variants were integrated into the host chromosome and replicated with it in the cancer cells where they were isolated from. Alternatively, other sequences (perhaps outside the LCR) may also function as origins of replication of the episomal viral genome. In this study, the replication activity of these deletion variants was not tested *in vitro*, so it is not known whether these isolated LCR variants could be replicated in the absence of other regions of the HPV16 genome.

It is difficult to determine the biological significance of the natural deletion variants described in this study. It must be emphasised that these deletion variants were present together with full-length variants in the cases reported here. However, others reported that similar deletion variants were sometimes present alone in cervical cancer specimens carrying episomal HPV genomes [15]. It can be speculated that such deletion variants may have a selective replicational advantage over the full-length episomal genomes and are able to overgrow them after a time. This hypothesis is supported by the results of Grassmann and colleagues, who found that after five *in vitro* passages of explanted cells from a VINIII (vaginal intraepithelial neoplasia grade III) lesion carrying both wild-type and LCR deletion mutant HPV 16 genomes, the deletion variant persisted in an episomal form, while the wild-type variant became undetectable [15]. However, the transcriptional activities of the deletion variants were not significantly higher than the activities of the corresponding full-length variants either in this study or in the study by Grassmann and colleagues. Thus, the biological significance of these LCR deletion variants may be that they have enhanced replication potential rather than enhanced transcriptional activity over the full-length variants.

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