Journal of General Virology (2000), **81**, 2969–2982. Printed in Great Britain

Identification and functional analysis of sequence rearrangements in the long control region of human papillomavirus type 16 Af-1 variants isolated from Ugandan penile carcinomas

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Human papillomavirus type 16 (HPV-16) is the predominant HPV isolate found in malignancies of male and female lower genital tracts. However, only a small percentage of individuals infected with high-risk HPVs develop a genital neoplasia, suggesting that additional events at both the cellular and the virus level are necessary for the progression to cancer, including genetic mutations/rearrangements of viral sequences involved in the oncogenic process. In this study, the genetic stability of the long control region (LCR) (nt 7289–114), which regulates expression levels of oncoproteins E6 and E7, was analysed in HPV-16 isolates from penile carcinoma (PC) biopsies of patients recruited from Uganda, one of the countries with the highest incidence of genital cancers in both men and women. Nucleotide changes within the LCR region typical of the African-1 (Af-1) lineage were observed in all HPV-16 isolates. Two out of five samples showed further rearrangements of the enhancer region. The functional activity of LCR with Af-1 mutations and/or rearrangements was evaluated by cloning each LCR into CAT expression vectors, followed by transfection in several epithelial and non-epithelial cell lines. CAT expression levels driven by a rearranged LCR were significantly higher than those driven by Af-1 or European prototype LCRs. Furthermore, in the NIH3T3 focus formation assay, the transforming activity of E6 and E7 genes, driven by a mutated or rearranged LCR, was 1·4- to 3·0-fold higher, respectively. These results indicate that rearrangements within the LCR of HPV-16 isolated from African PCs are frequently found (2 out of 5, 40%). It is also shown that increased HPV LCR activity is associated with an increased E6/E7-mediated *in vitro* transforming activity, suggesting that natural variants can play a major role in the pathogenesis of genital carcinomas.

Introduction

Human papillomaviruses (HPVs) form a large group (> 80 types) of epitheliotropic double-stranded DNA tumour viruses (reviewed by IARC, 1995). A subset of HPVs, referred to as 'high-risk' HPVs, have been associated with the development of human anogenital tumours such as cervical cancers in women and penile cancers in men (zur Hausen, 1989, 1991, 1996). HPV-16 and HPV-18 are the most frequently identified HPV types found in genital tumours (Wagatsuma *et al.*, 1990; zur Hausen, 1991) and are present in 40–60% and 10–20% of cervical carcinomas (Riou *et al.*, 1990; Van den Brule *et al.*, 1991; Higgins *et al.*, 1992; Lorincz *et al.*, 1992) and in 20–50% and 3–15% of penile cancers, respectively (McCance *et al.*, 1986; Tornesello *et al.*, 1992; Wiener *et al.*, 1992).

Prevalence of HPV infection in men may be similar to that observed among women. The natural evolution of genital HPV infection in men, however, is less well understood. Up to 70% of male partners of women with cervical HPV infections and intraepithelial neoplasia are diagnosed with subclinical

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HPV infections or HPV-associated diseases (Barrasso *et al.*, 1987; Schneider, 1994). The incidence of HPV-associated penile cancer, however, is much lower, suggesting that additional events and/or cofactors are involved in the malignant evolution of HPV infection in men.

HPV-transforming activity seems to be mainly associated with E6 and E7 expression (Schwarz *et al.*, 1985; Smotkin & Wettstein, 1986; Hsu *et al.*, 1993). These oncogenes are transcribed at high levels in tumour tissues and in tumour-derived cell lines (Higgins *et al.*, 1992) and are also required for the maintenance of the transformed phenotype (von Knebel Doeberitz *et al.*, 1992). The E6 and E7 oncoproteins of high-risk HPVs contribute to cell immortalization by association with oncosuppressors, such as p53 and pRb family members (p110 Rb, p107 and p130), which play a central role in signal transduction pathways and mediate G_1 arrest after DNA damage (Dyson *et al.*, 1989; Werness *et al.*, 1990; Davies *et al.*, 1993; Scheffner *et al.*, 1993). The p53 oncosuppressor has been shown to induce the cyclin-associated protein $p21^{cip1}$, which in turn inhibits the *in vitro* cyclin complex-mediated phosphorylation of pRb (Slebos *et al.*, 1994). During HPV infection, the HPV E6 and E7 oncoproteins may undermine this cell-cycle checkpoint, contributing to the accumulation of the genetic alterations observed in the progression of malignancy (Demers *et al.*, 1994; Morozov *et al.*, 1997; Jones & Munger, 1997; Martin *et al.*, 1998).

Virus replication and E6/E7 gene transcription are regulated by the long control region (LCR), which contains a large number of *cis*-responsive elements. The LCR sequence can be divided into three functionally distinct regions, namely the 5′, the central and the 3′ segment. The 5′ segment of the LCR, with an extraordinarily high A+T content (up to 85%), contains a negative regulatory element acting at the level of late mRNA stability (Mittal *et al.*, 1993) and a nuclear matrix attachment region that represses the oncoprotein expression (Tan *et al.*, 1998; Stunkel & Bernard, 1999). The central segment (ca. 400 bp) seems to function as an epithelial-specific transcriptional enhancer (Cripe *et al.*, 1987; Gloss *et al.*, 1989; Sibbet *et al.*, 1995). Multiple cellular transcription factors have been shown to bind *in vitro* to more than 20 sites within the HPV-16 enhancer sequence, including NF-1, NF-IL6, AP-1, AP-2, TEF-1, Oct-1, glucocorticoid receptor, YY1 and papillomavirus silencing motifs (Gloss *et al.*, 1989; Chan *et al.*, 1990; Ishiji *et al.*, 1992; Apt *et al.*, 1994; O'Connor & Bernard, 1995; O'Connor *et al.*, 1996, 1998; Khare *et al.*, 1997). The 3′ segment of the LCR contains the origin of replication and the E6 and E7 promoter, P97 (Smotkin & Wettstein, 1986; Dürst *et al.*, 1992). Thus the regulation of P97 is a complex process involving the equilibrium of many transcription factors that exert either positive or negative effects.

An important biological question that arises is which events modify the natural progression of HPV infection, where the final outcome is genital cancer along with increased E6 and E7 transcription levels. Several events could be involved, for example, nucleotide changes/rearrangements of viral and cellular regulatory genes or mutations of virus sites within the enhancer/promoter regions that bind nuclear transcription factors. One such event is the disruption of the E2 gene during integration of HPV into the host genome, an event observed in cervical and penile carcinomas (PCs) but not in pre-malignant lesions (Schwarz *et al.*, 1985; Cullen *et al.*, 1991; Tornesello *et al.*, 1997). The E2 protein in its truncated form is unable to displace either Sp1 or TFIID from their cognate sites, resulting in an increased expression of the E6 and E7 genes (Tan *et al.*, 1994). Mutations of cellular genes following HPV integration have also been described. In ME180 tumour cells carrying an integrated HPV-68 DNA, the recently described human gene APM-1, which encodes a protein with a BTB/POZ domain and four zinc fingers, is cotranscribed with the HPV-68 E6 and E7 genes as the 3′ sequence of the ME180 viral–cellular fusion transcripts (Reuter *et al.*, 1998). Recently, elevated levels of E6/E7 transcription, observed in a small number of independent primary tumours or metastases carrying only HPV-16 episomes, have been due to deletions or point mutations affecting one or more binding sites of the negative transcription factor YY1 (Dong *et al.*, 1994; May *et al.*, 1994).

The aims of the present study were to analyse the HPV-16 variants and their mutants in penile cancer biopsies of patients from one of the countries with the highest incidence of PC (Uganda), to evaluate the functional activity of the LCR with Af-1 mutations and/or rearrangements in a transient expression system (CAT assay) and to characterize the biological properties of these viruses in a morphological transformation assay.

Methods

■ **Tumour biopsies and cell cultures.** Biopsies of histologically confirmed penile squamous cell carcinomas were obtained from five Ugandan patients attending the New Mulago Hospital, Makerere University of Kampala (Uganda) and cryo-preserved in liquid nitrogen at −192 °C until further use for molecular hybridization analyses or establishment of long-term tumour cell lines (Gentile *et al.*, 1987). HeLa, SiHa, CaSki and HT3 cell lines derived from human cervical carcinoma as well as the human teratocarcinoma cell line NTERA-2 were obtained from the ATCC. The SiHa and CaSki cells contain 1 and 600 copies of HPV-16, respectively; HeLa cells contain ten copies of HPV-18. HT3 and NTERA-2 cell lines are HPV-negative. This was confirmed by PCR using MY09 and MY11 degenerated oligonucleotides, which enable amplification of most known and unknown genital HPV types (Ting & Manos, 1990). NIH3T3 murine cells were obtained from James McDougall (Fred Hutchinson Cancer Research Center, Seattle, USA). All cells were grown in minimal essential medium (MEM) supplemented with 10% heat-inactivated foetal calf serum (FCS).

■ **DNA isolation and Southern blot analysis.** DNA from frozen biopsies and cell lines was digested with proteinase K, extracted once with phenol, once with phenol–chloroform–isoamyl alcohol (1:1:40) and ethanol-precipitated. Genomic DNA (10 μg aliquots) was cleaved with the appropriate restriction enzyme, separated on a 0·7% agarose gel and transferred onto Hybond-N nylon membranes (Amersham Pharmacia

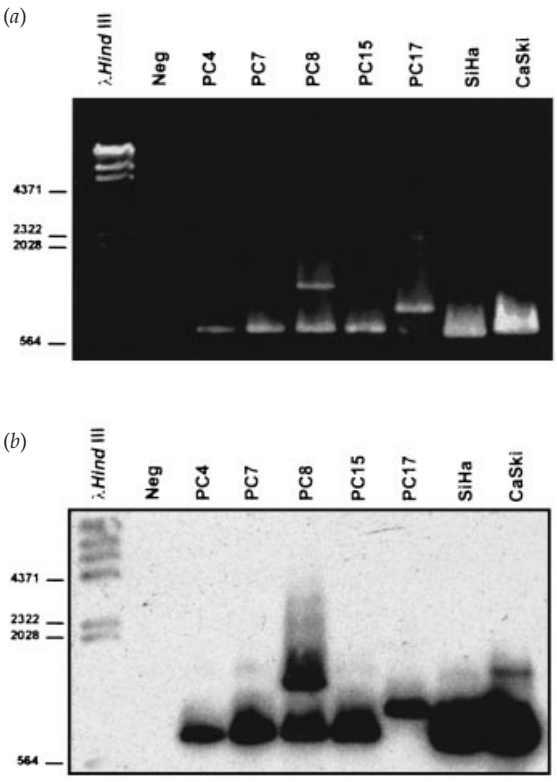

Fig. 1. (*a*) PCR-amplified LCR products analysed by electrophoresis through a 1% agarose gel. The lane labelled *λHind*III contains the molecular size marker *Hin*dIII-digested lambda DNA. The lane labelled Neg contains HPV-negative human placental DNA. (*b*) Southern blot analysis of the agarose gel (*a*) probed with an α-^{32}P-labelled HPV-16 fragment corresponding to the LCR region.

Biotech). Following pre-hybridization at 65 °C for 1 h, filters were hybridized with α-^{32}P-labelled HPV-16 probes (Church & Gilbert, 1984).

■ **LCR amplification by PCR.** DNA samples were subjected to single-round PCR amplification of the LCR region (nt 7289–114). HPV DNA was amplified by PCR using the following set of oligonucleotides: 16-LCR-1, 5′ GCTTGTGTAACTATTGTGTCA 3′ (nt 7289–7310), and 16-LCR-2, 5′ GTCCAGAAACATTGCAGTTCT 3′ (nt 93–114). HPV-16 DNA nucleotide positions are numbered according to the published sequence of the reference clone (Seedorf *et al*., 1985), revised as described by Icenogle *et al*. (1991), Chan *et al*. (1992), Eschle *et al*. (1992) and Ho *et al*. (1993). The reaction mixture (50 µl) contained 200 ng target DNA, 20 pmol of each primer, 50 mM KCl, 2·5 mM $MgCl_2$, 100 mM Tris–HCl pH 8·3, 0·1% Triton X-100, 50 mM of each dNTP and 1·8 U thermostable DNA polymerase (Perkin Elmer). DNA was amplified in a GenAmp PCR System 9600 thermal cycler (Perkin Elmer) with the following steps: an initial 5 min denaturation at 94 °C, 30 cycles of 55 °C for 45 s, 72 °C for 60 s, 94 °C for 15 s and a final annealing at 55 °C for 45 s with 5 min elongation at 72 °C. PCR amplification products, extracted with phenol and chloroform–isoamyl alcohol and purified by precipitation with 10% polyethylene glycol (PEG 6000) in 1·25 M NaCl, were subjected to direct nucleotide sequencing and sequence analysis after cloning into *Sma*I-dephosphorylated pBS (Stratagene).

■ **Sequence analysis.** Recombinant plasmids containing LCR inserts were prepared from minicultures (5 ml Luria broth) using the Wizard minipreps DNA purification system (Promega). Sequencing reactions were performed using the dideoxynucleotide chain termination method (Sanger *et al*., 1973) using the Sequenase 2.0 kit according to the manufacturer's instructions (Amersham Pharmacia Biotech). Several primers have been used: the 17-mer universal (−20) sequencing primer, the 16-mer reverse M13 sequencing primer, 16-LCR-1, 16-LCR-2 and 16-LCR-3 (5′ CAAGCCAAAAATATGTGCCTAAC 3′, nt 7695–7717). Direct sequencing of the amplified and PEG-purified DNA was performed using a rapid method modified from Winship (1989). Briefly, DNA samples were denatured at 95 °C, in the presence of 10% DMSO, immediately cooled in liquid nitrogen and subsequently sequenced with the Sequenase protocol modified in the labelling step (3 min on ice). Amplification primers were also used in direct sequencing reactions. All samples were amplified and analysed in duplicate to identify possible point mutations originating from the PCR reaction. Sequences were analysed on a 6% polyacrylamide wedge sequencing gel.

■ **Plasmid constructs.** All constructs used in functional assays consisted of PCR-generated LCR fragments cloned into pCAT-based vectors (Promega). The complete LCR, containing both the HPV-16 promoter and enhancer (nt 7289–114), was cloned into the pCAT-Basic vector, which lacks eukaryotic promoter and enhancer sequences. The P97 promoter was removed from the LCR region by digestion with *Hpa*II, which removes the fragment from nt 57–114 prior to cloning into the enhancer-less pCAT-Promoter; this plasmid contains only the SV40 promoter upstream of the CAT gene. HPV-16 LCR sequences of the original isolate (Dürst *et al*., 1983) were also amplified from the HPV-16 clone pBR322/HPV-16 (kindly provided by Harold zur Hausen, Deutsches Krebsforschungszentrum, Heidelberg, Germany) and will be referred to as the prototype (solely to indicate that it is the first HPV-16 isolate). The plasmids pCAT-Control (Promega) and pSV-*β*gal (Promega), both containing the SV40 early promoter and enhancer upstream of CAT and *lacZ* genes, respectively, were used to monitor transfection efficiencies.

All constructs used in the NIH3T3 transformation assays consisted of the LCR/E6/E7 sequence (nt 7289–875), released with *Pst*I digestion from the HPV-16 Af-1 variants described in Tornesello *et al*. (1992, 1997) and from the HPV-16 prototype. Sequences were then cloned into the pRc/CMV eukaryotic expression plasmid (Invitrogen), which is depleted of the CMV promoter. The LCR/E6/E7 region isolated from PC8 was also digested with *Eco*RI in order to remove the entire rearranged sequence and to obtain the pRc/PC8Δ construct. This construct is depleted of the PC8 duplicated sequence and represents our Af-1 reference clone with only three point mutations from the Af-1 consensus sequence (Human Papillomaviruses Compendium, 1996).

■ **Cell transfection and CAT assay.** HeLa, SiHa, HT3 and NTERA-2 cells were cultured in MEM supplemented with 10% FCS using standard procedures. The cell lines were transiently transfected with 3 µg pSV-*β*gal and 5 µg of test DNA constructs using the Gene Pulser apparatus (BioRad Laboratories) and harvested 36–48 h after treatment.

To determine CAT activity, assays were performed using essentially the same procedure described by Seed & Sheen (1988). Protein (200 µg) was incubated in 100 mM Tris–HCl pH 7·0, 100 µM [^{14}C]chloramphenicol and 250 µM butyryl coenzyme A. After 1 h incubation at 37 °C, the reaction was terminated by extracting with 2 vol. mixed xylenes and counted in scintillation liquid. CAT activity was determined as pmoles chloramphenicol converted per min per mg protein (pmol/min/mg protein). Each reported value represents the average result, obtained in four to six independent transfections (in duplicate) using at least two different DNA preparations, normalized by the *β*-galactosidase activity.

The β-galactosidase activity of the control plasmid pSV-βgal was evaluated spectrophotometrically using the β-galactosidase enzyme assay system according to the manufacturer's instructions (Promega) and by *in situ* staining as described by Harper *et al*. (1988).

■ **Focus formation assay.** Long-term transfections were achieved by a modified calcium phosphate co-precipitation method. NIH3T3 cells (1×10^5 cells per 60 mm dish) were exposed to 5 μg of test DNA. After a 12 h incubation, the transfected cells were subcultured 1:3 and fed every 3 days. After 4–6 weeks, the cell culture dishes were fixed and stained with 50% ethanol, 10% Giemsa's stain and 40% H_2O. Each recombinant construct has been tested in triplicate with two different DNA preparations for a total of six independent transfections.

Results

Sequence analysis of HPV-16 LCR and E6/E7 genes from penile cancers

Specific oligoprimers have been designed to amplify a 730 bp fragment (nt 7289–114) that spans the entire enhancer and promoter region. The amplification product was detected by gel electrophoresis and confirmed by Southern blot analysis in all five PCs and in both CaSki and SiHa cell lines (Fig. 1). The expected 730 bp product was obtained in four out of five PCs and in the CaSki cell line. The PC8 sample shows, besides the expected product, an additional band greater than 1200 bp and the PC17 sample shows an amplification product greater than 900 bp. The PCR of SiHa cell DNA results in an amplification product of 693 bp containing a deletion of 37 bp (nt 7756–7793) as reported by Chan *et al*. (1992). Hybridization with an α-^{32}P-labelled HPV-16 LCR fragment showed that amplified products from samples PC8 and PC17, although larger than the expected size, are homologous to LCR sequences, suggesting that rearrangements within the LCR region could have occurred. These results indicate that PC8 contains two different LCR sequences, the rearranged LCR and the unit length region, while PC17 contains only a rearranged LCR region.

To confirm that rearrangements were present in the original tumour and did not represent an artefact of PCR amplification and also to determine the copy number of each LCR type, we have analysed the total cellular DNA from the original tumour by Southern blot analysis (Fig. 2*a*). The genomic DNA was digested with *Bam*HI and *Taq*I to release a fragment of 2259 bp (nt 6151–505) in the prototype sequence and was subjected to Southern blot analysis with a subgenomic fragment of HPV-16 LCR. Two bands of 2750 bp and 2259 bp have been identified by Southern blot analysis, indicating that the original tumour contained two different LCR variants. The ratio of the two bands was 1:2 as determined by quantification of their hybridization signal with the Phosphorimager Instrument (Packard Instrument).

Sequencing analysis of LCR regions amplified from the PC8 sample revealed that an insertion at nt 7831 had occurred and resulted in a 487 bp duplication within the LCR region (nt 7347–7833) (Fig. 2*b*). Therefore, the enhancer sequence (nt 7524–7755) was completely duplicated (Fig. 2*c*). In sample

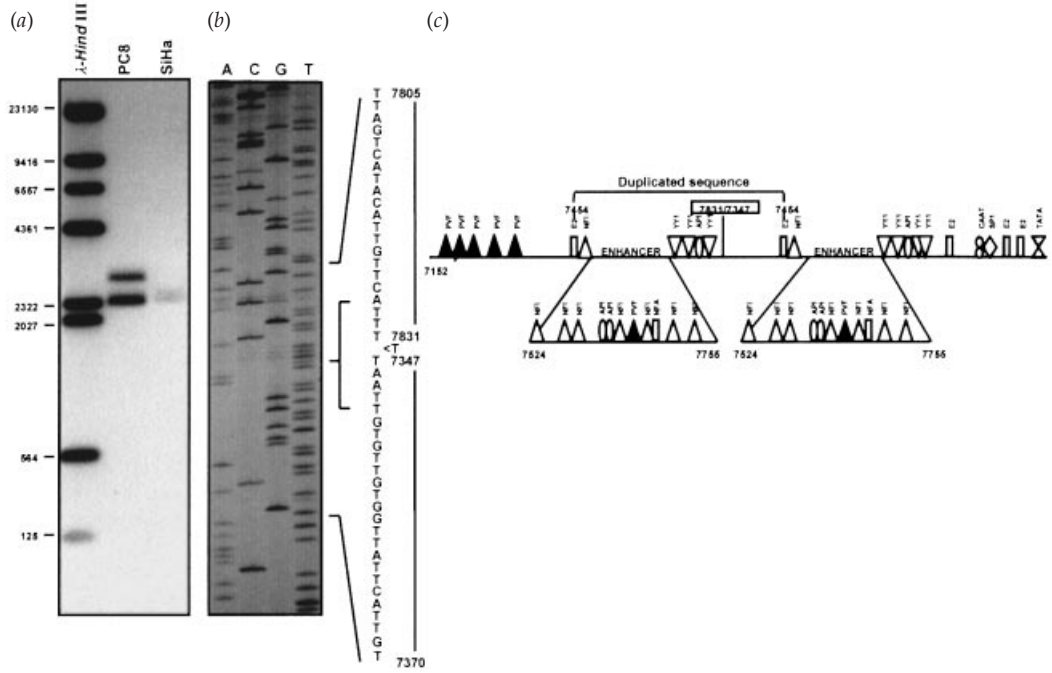

Fig. 2. (*a*) Southern blot analysis of DNA fragments isolated from penile carcinoma sample PC8 and the SiHa cervical cell line digested with *Bam*HI and *Taq*I and hybridized with a HPV-16 LCR-specific probe. (*b*) DNA sequence gel identifying the duplication in the lower strand of the HPV-16 LCR from the PC8 sample. Nucleotide positions refer to the published sequence of Seedorf *et al*. (1985). (*c*) Schematic representation of the rearranged HPV-16 LCR cloned from the PC8 sample showing the enhancer region and binding sites affected by the duplication event.

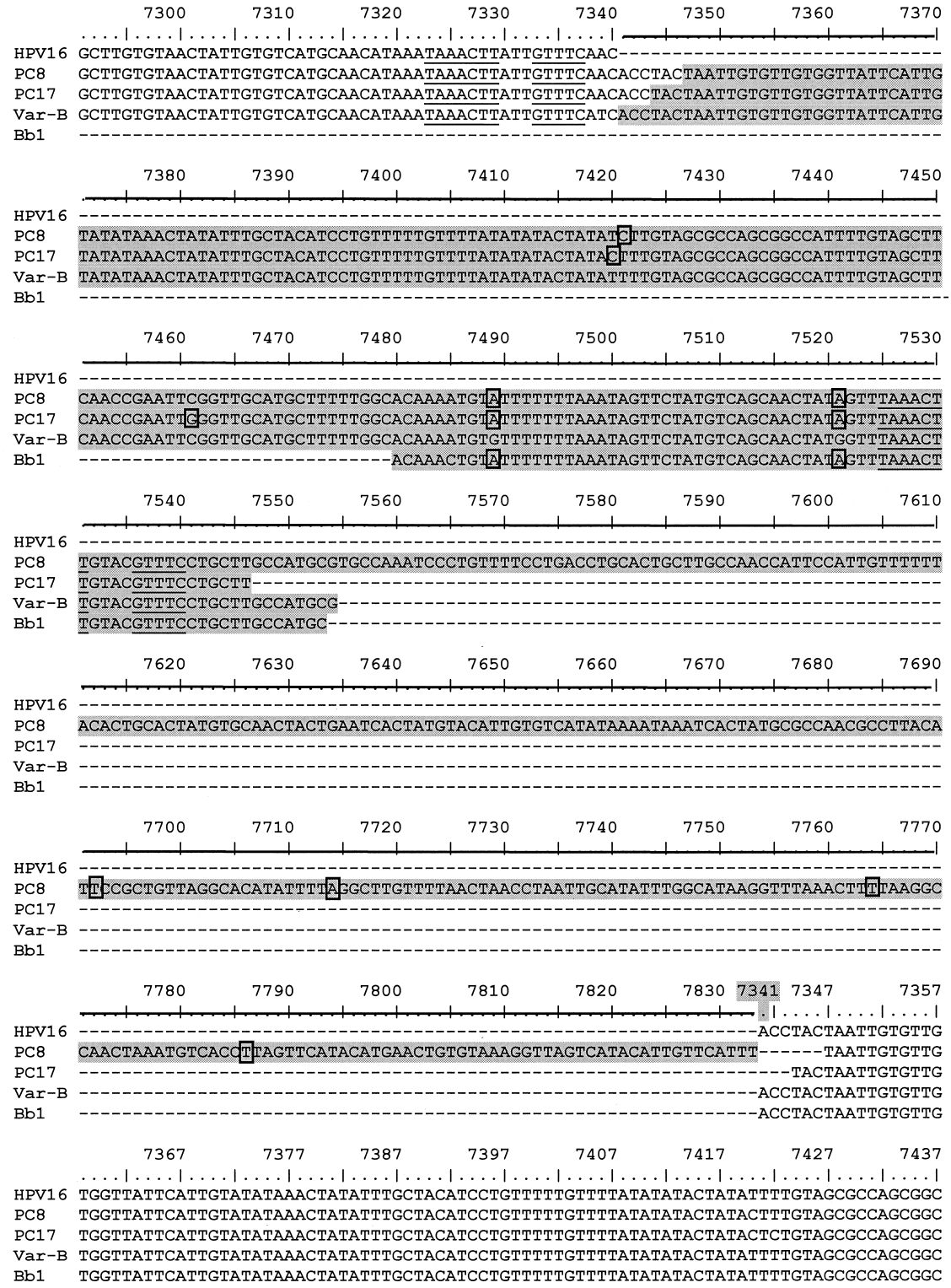

Fig. 3. Nucleotide sequences of rearranged and prototype HPV-16 LCRs. Duplicated regions are shaded in grey and are marked with a bold line above the nucleotide sequence. Nucleotide changes are boxed with a dark line. Nucleotide positions refer to the revised sequence of HPV-16 (HPV16R in the HPV Sequence Database).

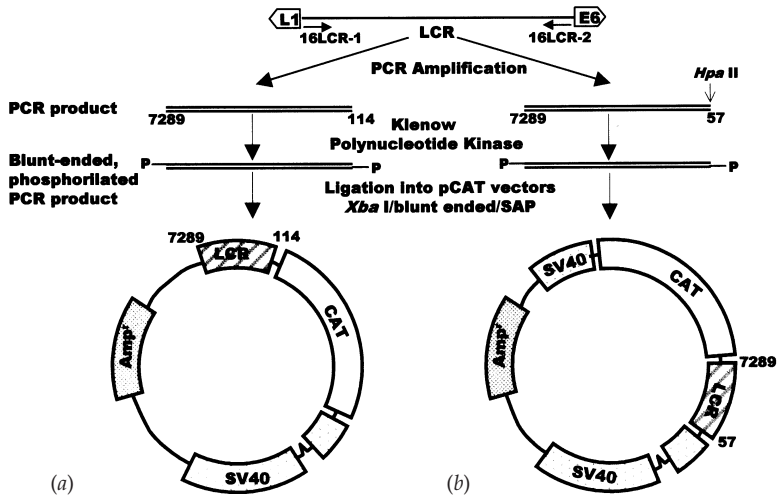

Fig. 4. Strategy for the isolation of HPV-16 LCR from genomic DNA of penile carcinomas. Each amplified PCR was cloned both into pCAT-Basic vector (*a*), which lacks eukaryotic promoter and enhancer sequences and, after the removal of P97 promoter, into the enhancer-less pCAT-Promoter vector (*b*) containing the SV40 promoter upstream from the CAT gene.

PC17, an insertion of 202 bp homologous to the LCR region from nt 7344–7545 was present at nt 7544. This insertion affects the 5′ end of the enhancer region and the binding sites for NF-IL6. Fig. 3 shows nucleotide alignment of PC8 and PC17 LCRs along with the prototype European (E) HPV-16 as well as Bb1 and Var-B sequences described by Chan *et al.* (1992) and Schwartzman Fang *et al.* (1993).

Most of the nucleotide changes observed in all five samples are typical of the African lineage and allow their classification as members of the Af-1 group (Tornesello *et al.*, 1997). Sequencing analysis of the five Ugandan HPV-16 LCRs (nt 7289–93) showed seven point mutations: a common mutation at nt 7521 (G → A) reported in 80% of the isolates sampled throughout the world and mutations at nt 7489 (G → A), 7764 (C → T) and 7786 (C → T) that are distinctive to the Ax group (Af-1, Af-2 and Asian lineages). Four out of five isolates contain mutations at nt 7833 (G → T), specific to Af-1 and Af-2, and at nt 7714 (T → A), observed only in Af-1. In addition, the C → G transversion was observed in four out of five samples at nt 7461, which falls within an E2-binding site. The E6 gene of all five samples, as previously described (Tornesello *et al.*, 1997), contains Af-1-specific non-synonymous changes at nt 132 (G → C), 143 (C → G) and 145 (G → T). Only sample PC8, also containing mutations at nt 286 (T → A), 289 (A → G) and 335 (C → T, H → Y), shows all six nucleotide changes consistently detected by Yamada *et al.* (1997) in the E6 gene of Af-1 isolates. Nucleotide sequencing analysis of the E7 gene consistently shows two Af-1 synonymous point mutations at nt 789 (T → C) and 795 (T → G) in all five PC samples (Tornesello *et al.*, 1997).

Functional activity of mutated HPV-16 LCRs

The expression of HPV early genes, including the E6 and E7 genes, at the transcriptional level is mainly regulated through the LCR region upstream of the E6 gene. The DNA fragment covering the whole LCR region of HPV-16 isolates from samples PC4, PC8, PC17 and the HPV-16 prototype were cloned either (i) upstream of the CAT reporter gene into the pCAT-Basic vector, under expression control of their own P97 promoter or (ii) digested with *Hpa*II restriction enzyme in order to remove the HPV-16 promoter (nt 58–114) and cloned downstream of the CAT expressing gene into the pCAT-Promoter plasmid, which contains the SV40 promoter (Fig. 4). The pCAT-Control plasmid, which contains the supposedly ubiquitously active SV40 enhancer and the SV40 promoter, was used as positive control.

Transfection experiments were performed in four cell lines: SiHa and HeLa cells, which are derived from cervical carcinomas and contain, recombined into their chromosomes, transcriptionally active papillomavirus genomes types 16 and 18, respectively; and the human cervical carcinoma HT3 and the teratocarcinoma NTERA-2 cell lines, which are both free of endogenous papillomavirus genomes.

Data from the outcome of these experiments are summarized in Table 1 and Fig. 5. All three constructs (pCAT-Basic, pCAT-Promoter and pCAT-Control) have been tested in all four cell lines to evaluate, under our experimental conditions, the expression activity of the SV40 promoter/enhancer, which has been described as cell type-dependent. The promoter/enhancer-less pCAT-Basic shows a low basal CAT activity in all tested cell lines; the enhancer-less pCAT-Promoter shows a higher CAT activity (5–10-fold increment) in all four cell lines, with maximum activity in the HT3 cell line. pCAT-Control shows CAT activity in all cervical carcinoma-derived cell lines with maximum activity in the NTERA-2 cell line.

Recombinant plasmids containing the HPV promoter/enhancer were 10–30-fold less active when compared to HPV enhancer/SV40 promoter constructs, confirming that the SV40 promoter has a stronger activity than the HPV-16 P97 promoter. The recombinant constructs pPC4/114, pPC8/114,

Table 1. CAT activity of HPV-16 LCR variants in epithelial and non-epithelial cell lines

The constructs described in the tables contain the CAT reporter gene downstream of LCR variants from the HPV-16 field isolates PC4, PC8 and PC17 identified in the corresponding samples. The constructs labelled 114 (*a*) contain the whole HPV-16 LCR with the P97 autologous promoter driving the CAT gene; the constructs labelled 57 (*b*) contain the heterologous SV40 promoter upstream and the HPV-16 LCR, depleted of the P97 promoter, downstream of the CAT gene. The constructs were transfected into both epithelial and non-epithelial cell lines. The enzymatic activity is expressed as pmol/min/mg proteins; the expression activity of each construct has been reported as folds of activations above the respective basal constructs pCAT-Basic and pCAT-Promoter (Folds C/basal) as well as above the prototype HPV-16 LCR (Folds M/Prot).

(*a*)

Cell line	Sample	Enzymatic activity pmol/min/mg (±SD)*	Expression activity	
			Folds C/basal	Folds M/Prot16
SiHa	pCAT-Basic	1·93 (±0·33)	1·00	–
	pProt16/114	5·54 (±1·57)	2·87	1·00
	pPC4/114	7·45 (±1·54)	3·86	1·34
	pPC8/114	13·84 (±2·09)	7·16	2·50
	pPC17/114	14·05 (±3·11)	7·27	2·53
HeLa	pCAT-Basic	1·48 (±0·45)	1·00	–
	pProt16/114	2·90 (±0·87)	1·96	1·00
	pPC4/114	3·52 (±1·02)	2·38	1·22
	pPC8/114	5·23 (±1·43)	3·54	1·81
	pPC17/114	1·80 (±0·89)	1·21	0·62
HT3	pCAT-Basic	3·47 (±0·77)	1·00	–
	pProt16/114	3·43 (±1·75)	0·99	1·00
	pPC4/114	6·00 (±1·41)	1·73	1·75
	pPC8/114	14·48 (±2·84)	4·17	4·23
	pPC17/114	8·73 (±3·11)	2·51	2·54
NTERA-2	pCAT-Basic	2·46 (±0·09)	1·00	–
	pProt16/114	3·06 (±0·86)	1·24	1·00
	pPC4/114	3·19 (±0·90)	1·30	1·04
	pPC8/114	3·41 (±0·24)	1·38	1·11
	pPC17/114	4·26 (±2·09)	1·73	1·39

(*b*)

Cell line	Sample	Enzymatic activity pmol/min/mg (±SD)*	Expression activity	
			Folds C/basal	Folds M/Prot
SiHa	pCAT-Promoter	8·31 (±2·12)	1·00	–
	pProt16/57	89·28 (±12·27)	10·74	1·00
	pPC4/57	94·21 (±18·59)	11·34	1·06
	pPC8/57	143·11 (±32·59)	17·22	1·60
	pPC17/57	130·34 (±29·03)	15·68	1·46
HeLa	pCAT-Promoter	4·15 (±0·89)	1·00	–
	pProt16/57	33·22 (±9·40)	8·01	1·00
	pPC4/57	41·96 (±12·19)	10·11	1·26
	pPC8/57	61·09 (±10·85)	14·72	1·84
	pPC17/57	15·61 (±2·00)	3·76	0·47
HT3	pCAT-Promoter	48·26 (±12·52)	1·00	–
	pProt16/57	91·12 (±12·38)	1·89	1·00
	pPC4/57	99·71 (±10·90)	2·07	1·09
	pPC8/57	347·87 (±72·02)	7·21	3·82
	pPC17/57	100·14 (±15·59)	2·07	1·10
NTERA-2	pCAT-Promoter	7·11 (±0·53)	1·00	–
	pProt16/57	4·60 (±0·12)	0·65	1·00
	pPC4/57	6·39 (±0·60)	0·89	1·36
	pPC8/57	7·04 (±2·00)	0·99	1·53
	pPC17/57	8·41 (±21·00)	1·18	1·82

* All results represent the average values of CAT assays, normalized by β-galactosidase assay, from at least four independent transfections, each performed in duplicate using two different DNA preparations. Standard deviations among corresponding samples from independent transfections were lower than 30%.

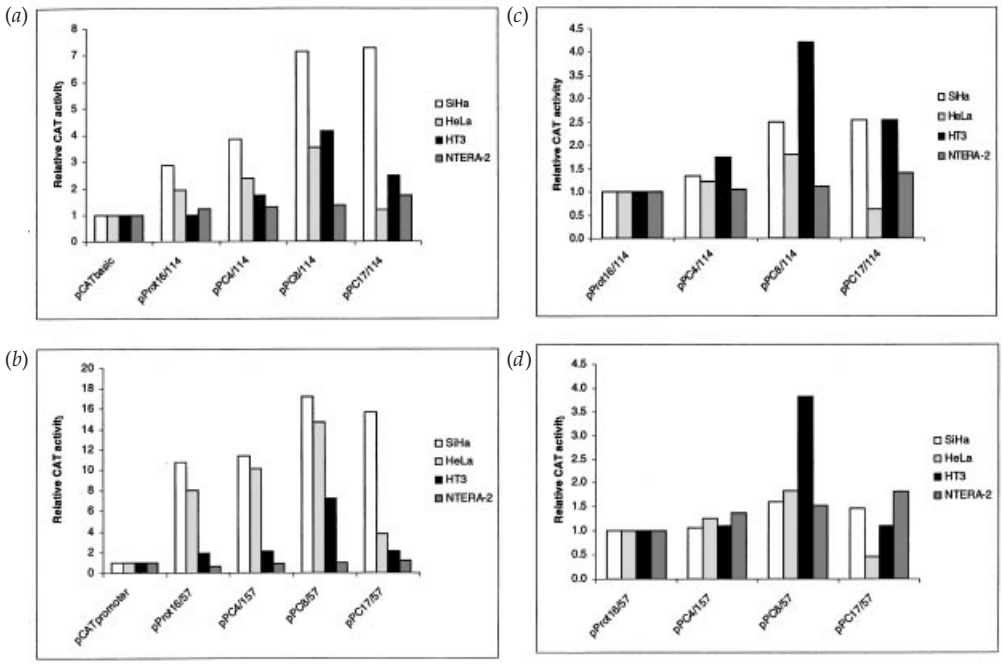

Fig. 5. Transcriptional activation of different HPV-16 LCR variants. The LCR regions from different HPV-16 isolates were cloned into the enhancer- and promoter-less pCAT-Basic reporter vector (*a* and *c*) and into the enhancer-less pCAT-Promoter reporter vector (*b* and *d*). pProt16/57 and pProt16/114 reporter vectors contain the LCR enhancer of the HPV-16 prototype with homologous (P97) and the heterologous (SV40) promoters, respectively. The relative CAT activity of pCAT-Basic (*a*), pCAT-Promoter (*b*), pProt16/114 (*c*) and pProt16/57 (*d*) has been chosen as the reference unit (value 1·0) in each histogram and the activities of the other constructs have been expressed as folds of activity above them.

pPC17/114 cloned from PC4, PC8 and PC17 samples, respectively, expressed in the SiHa cell line showed a 1·34-, 2·50- and 2·53-fold increase in comparison to the CAT activity of the pProt16/114 construct containing prototype sequences. All HPV constructs were active in cervical carcinoma cell lines but were not functional in NTERA-2 cells, suggesting that the genetic alterations present in these isolates do not affect the ability of their enhancers to retain their functional activity in epithelial cells. Furthermore, as shown in Table 1, rearrangements within the LCR region significantly increase its promoter activity. The rearranged enhancer is, in fact, more active in all cell lines in comparison to the prototype sequence whether the CAT expression is driven by the HPV homologous promoter P97 (1·81–4·23-fold increase) or by the SV40 heterologous promoter (1·60–3·82-fold increase). However, the expression levels of the PC17 rearranged LCR constructs are lower than the pProt16 construct in the HeLa cell line (0·62-fold activity with the homologue and 0·47-fold activity with the SV40 heterologous promoter). Such decreased activity could be due either to an intrinsic weak activity of this enhancer in HeLa cells or to the interference of the HPV-18 sequences present in the HeLa cells.

In contrast, the HPV-16 Af-1 class point mutations present in the HPV-16 PC4 isolate do not significantly increase the expression efficacy of the HPV-16 LCR, which shows a maximum 1·75-fold increase of CAT activity, in comparison with the prototype sequence in HT3 cells.

Biological properties of rearranged LCRs

The whole E6/E7 region of HPV-16 isolated from PC8 and PC17, under expression control of their own LCR regulatory region, has been cloned into the pRc/CMV vector, which is depleted of the CMV early promoter (Fig. 6) and carries neomycin resistance. The PC8 E6/E7 and PC17 E6/E7 transforming activity has been examined in NIH3T3 cells by DNA-mediated gene transfer technology. Morphologically transformed foci were detected 4–6 weeks after transfection. No foci were observed in control cultures transfected with pRc/CMV DNA vector alone. The pRc/Prot16 construct induced 21 foci/μg DNA; the pRc/PC8Δ, pRc/PC8 and pRc/PC17 constructs induced an average of 31·3, 64·6 and 51·6 foci/μg DNA, respectively. Thus the pRc/PC8Δ, pRc/PC8 and pRc/PC17 transformation efficiency of 156·5 ($P < 0{\cdot}005$), 323 ($P < 0{\cdot}001$) and 258 ($P < 0{\cdot}005$) foci per 1×10^5 treated cells was 1·49-, 3·07- and 2·45-fold higher than the number of foci (105 per 1×10^5 treated cells) induced by pRc/R16 DNA (Table 2). To estimate the transfection efficiency, transfected NIH3T3 cells were selected with G418

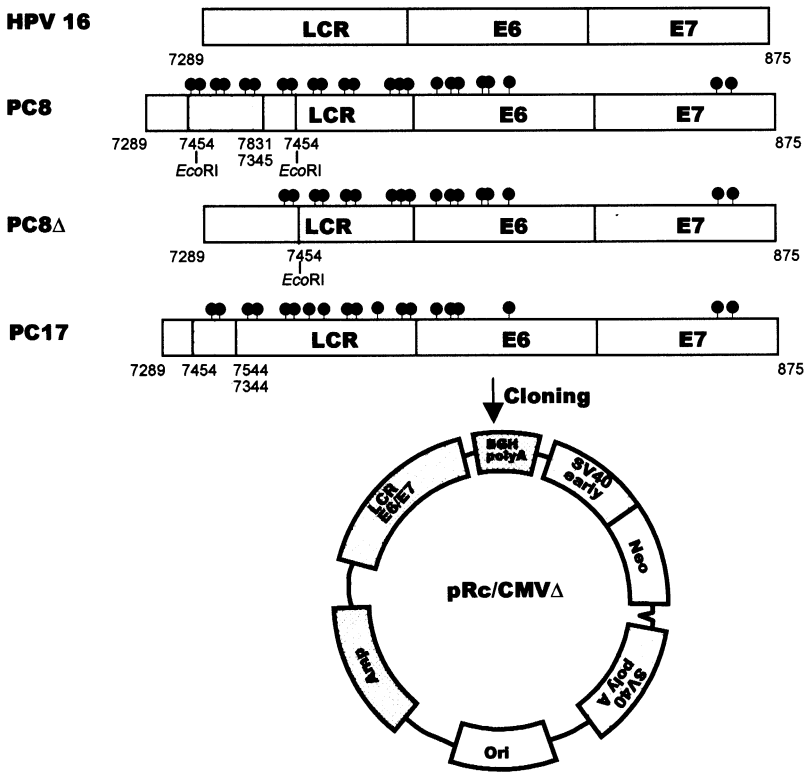

Fig. 6. Schematic representation for cloning HPV-16 LCR/E6/E7 regions from the HPV-16 prototype clone and PC8 and PC17 isolates into pRc/CMVΔ vector. Point mutations (nucleotide positions described in the text) are shown by black dots above LCR regions.

Table 2. The *in vitro* transforming activity of E6/E7 genes driven by prototype (pRc/Prot16), typical Af-1 (pRc/PC8Δ) and rearranged (pRc/PC8 and pRc/PC17) LCRs

Sample	No. foci per 1.0×10^5 treated cells	Transformation induction	
		Folds over pRc/Prot16	Folds over pRc/PC8Δ
NIH3T3	0	…	…
pRc/CMV	0	…	…
pRc/Prot16	105 (±9·41)	1·00	…
pRc/PC8Δ	156 (±7·58)	1·49	1·00
pRc/PC8	323 (±12·39)	3·07	2·06
pRc/PC17	258 (±10·20)	2·45	1·65

(400 μg/ml) and observed for 21 days for focus formation. The average number of G418-resistant colonies was 552 ± 12·28 colonies/μg pRc/CMV-based constructs, indicating that the transformation efficiency ranged from 11·9 to 3·8% of the cells expressing genes transfected with Af-1 typical, rearranged or prototype HPV-16 LCR, respectively. The 3-fold increase in transformation efficiency seems compatible with the enhancer/promoter strength of each construct presented in the CAT assay.

The results of these experiments strongly suggest that rearrangements, namely those resulting in duplications of regulatory regions of the viral genome are associated with an increased transformation activity of the virus.

Discussion

Although many studies have shown that the E6 and E7 genes of high-risk HPVs are expressed in cervical carcinoma and play a relevant role in both the immortalization process of primary human keratinocytes (Barbosa & Schlegel, 1989; Barbosa *et al.*, 1991) or rodent cells (Phelps *et al.*, 1988; Peacock *et al.*, 1990) and the transformation of immortalized murine fibroblast NIH3T3 cells (Yasumoto *et al.*, 1986; Bedell *et al.*, 1989), little is known about the specific natural changes in the host cell environment and/or in the viral DNA that might increase the oncoprotein expression during the course of an HPV infection.

In order to identify virus mutations involved in the transformation process we have analysed penile cancers from the Kyadondo County in Uganda, where this cancer represented the most frequently diagnosed cancer in men in the 1950s and 1960s (Kyalwazi, 1966; Dodge *et al.*, 1973). The annual crude rate of penile cancer has been declining from the 2·2 cases per 100 000 observed in the period 1964–1968, with a peak of 10·6 in the Nyoro tribe of the Bunyoro District (Schmauz & Jain, 1971), to 0·9 per 100 000; penile cancer in the Kyadondo County, however, still shows one of the highest

incidence rates in the world, with a 2·8 age standarized rate (World) per 100000 per year in comparison to < 1·0 age standarized rate (World) in Western countries (IARC, 1997). In this region Af-1 is the prevalent HPV-16 class in male (Tornesello *et al*., 1997) as well as female (Buonaguro *et al*., 2000) genital lesions; 22·7% of all female cancers are represented as genital lesions (IARC, 1997). However, there is no evidence on the different oncogenic activity of Af-1 HPV-16 in comparison to HPV-16 variants of the E class and on the type of genetic alterations present in such lesions. In particular, it would be relevant to discriminate between an increased Af-1 oncogenic activity (i.e. E6/E7 with higher binding affinity to oncosuppressors) and a spectrum of genetic mutations/rearrangements that would increase the expression of the HPV-16 oncogenes in a population which could be more susceptible to HPVs for socio-economic reasons.

HPV-16 DNA from PC biopsies of five Ugandan patients has been characterized by gene sequencing. Three of these samples were characterized by biological assays, targeted to evaluate the LCR promoter activity in a CAT expression system and the transforming activity of E6/E7 driven by their own LCRs in an NIH3T3 morphological transformation assay. Nucleotide sequence analysis of the LCR region, amplified by PCR, identified all HPVs as members of the Af-1 class (Tornesello *et al*., 1997). Further nucleotide mutations have not been observed. Only the PC8 and PC17 samples have shown an unusual LCR region, characterized by a duplication of 495 bp and 190 bp fragments, respectively, which starts in a co-linear region between 7340–7350, with the resultant doubling of several nuclear factor binding sites.

Functional analysis of the typical Af-1 LCR and rearranged Af-1 LCR has been performed in CAT assay experiments following cloning of the LCR regions upstream of the reporter gene. Both typical and rearranged Af-1 LCR regions showed enhancer activity higher than HPV-16 class E prototype in epithelial cells, irrespective of the presence of endogenous HPV viral genomes. In the HT3 cells, in particular, the typical Af-1 LCR shows a 1·75-fold increase in enhancing activity and the rearranged LCR shows a 4·23-fold higher activity than that seen with the HPV-16 E prototype. Thus the rearranged LCR regions present a significantly higher activity than the typical Af-1 LCR. The enhancer activity of HPV-16 and HPV-18 LCRs, containing point mutations upstream from and within the enhancer region, was reported to be an average 2-fold higher than the prototype LCR in HPV-positive oral cancer cell lines (Chen *et al*., 1997). All these results would suggest that point mutations in the LCR are able to induce a modest increment of enhancer activity, while rearrangements can be involved in major expression modifications.

The modulation of the oncogenic activity of HPV-16 variants by LCR modifications has been tested in NIH3T3 cells, which being immortalized can be used to analyse, in a single hit fashion, the transforming activity correlated to alterations of cell-cycle checkpoints of direct dominant oncogenes and/or indirect oncogenic genes that inhibit oncosuppressors. The *in vitro* transforming assay, cloning the whole LCR/E6/E7 region from prototype and mutated isolates in the pRc/CMVΔ expression vector, has been used to examine whether differences in the strength of enhancer/promoter activity of HPV-16 variants result in an increased E6/E7-dependent transforming activity. A significantly higher number of transformed foci were obtained with E6 and E7 genes expressed by rearranged LCRs. In particular, constructs of the Af-1 variant with duplications within the enhancer region showed a transforming efficiency 2·06- and 1·65-fold higher than the prototype LCR. Although the LCR CAT-driving activity in NIH3T3 is modest in the transient CAT assay on a pooled cell population, the expression of E6/E7 driven by the LCR enhancer–promoter has been detected in morphologically transformed foci (Yasumoto *et al*., 1986; Buonaguro *et al*., 1994). In our experimental conditions we cannot verify a significantly higher transforming activity of the Af-1 variant as compared to the E prototype; on the other hand the LCR-rearranged Af-1 isolate is associated with a significantly higher frequency of transformation events. This may be because the LCR-rearranged Af-1 isolate shows a higher probability of expressing sufficient levels of transforming genes due to an increased susceptibility to nuclear factors.

The role of rearrangements in HPV-transforming activity has been also suggested by several *in vitro* studies showing changes of the oncogenic potential following genetic alterations of the LCR region. Rosen *et al*. (1991) reported that 'low-risk' HPV-11 DNA, which does not normally transform cells *in vitro*, is able to transform baby rat kidney cells in a *ras*-dependent focus assay when two copies of the LCR were present (Rosen & Auborn, 1991). Furthermore, Romanczuk *et al*. (1991) reported that the major determinant of the differential immortalization of HPV-16 and HPV-18 lies within the LCR/E6/E7 region. In particular, the HPV-18 LCR/E6/E7 is more efficient in this immortalization function than the analogous region of the HPV-16 genome. The E6 and E7 genes of either HPV-16 or HPV-18, when regulated by the same heterologous promoter, immortalized primary human keratinocytes with the same efficiency, suggesting that the difference in immortalization activities was not due to the different E6 or E7 genes themselves but rather to a difference in the transcriptional regulatory regions upstream of these genes.

Analysing all HPV-16 LCR duplication regions identified to date in our laboratory and reported by other groups (Schwartzman Fang *et al*., 1993; Chan *et al*., 1992), it becomes evident that the regions of rearrangements are not random, particularly at the 5′ end where the region lying between nt 7340 and 7346 is constantly involved (Fig. 3). Furthermore, at the 5′ end as well as the 3′ end, TAAACTT and GTTTC direct repeats are present, suggesting that these motifs are involved in homologous recombination events and are eventually facilitating rearrangements of the intervening sequences.

The identification of rearrangements in two out of the five samples analysed suggests that such genetic alterations within the LCR seem not to be a rare event in PC, in constrast to the situation reported for cervical carcinoma. The frequency of rearrangements in HPV-16 isolated from female genital lesions, on the other hand, could have been underestimated, considering that the genetic patterns we have described fall in a nucleotide region previously not well characterized as shown by the modest number of sequences of that region (Human Papillomaviruses Compendium, 1996). The etiopathogenesis of these genetic alterations and the molecular mechanisms involved in the transformation events are not well understood. In particular, it would be relevant to determine whether the HPV LCR rearrangements represent a consequence of accumulating genetic alterations during the transformation process or whether they precede the frequently detected cellular genetic aberrations identified as a chromosomal imbalance in the E6/E7-dependent immortalization/transformation (Solinas-Toldo *et al*., 1997; Havre *et al*., 1995; White *et al*., 1994; Reznikoff *et al*., 1994). In the latter case, the genetic alterations of the LCR would increase the expression of E6 and E7, with the consequent decrease of p53 and pRb. E7 binding to pRb with the release of the transcription activator E2F, whose function is important for G_1–S transition, would be relevant for the immortalization of the HPV-infected cells. The E6-dependent reduction of p53 would have as a consequence the decrease of $p21^{cip1}$ (the major cyclin-dependent kinase inhibitor) and the resulting abrogation of the p53-dependent growth arrest following DNA damage with accumulation of chromosomal instability. The chromosomal imbalances observed in HPV-infected cells are prevalently represented by additional copies of chromosomes (3, 5, 19 and 20) or by the loss of chromosomes (1, 2, 4, 11, 14, 15 and 22). Numerous structural aberrations, however, consist of imbalances of only a portion of chromosome arms, particularly amplification of chromosome regions (Solinas-Toldo *et al*., 1997).

Moreover, rearrangements of the integrated HPV E6/E7 regulatory sequences would further activate the expression of the viral oncoproteins. Those cells characterized by such abnormalities would then have a growth advantage above normal and HPV-infected cells and could progress to malignant transformation.

In conclusion, although the Af-1 HPV-16 variants prevalently present in the Ugandan population in both men and women have been suggested to confer along with Ax variants a 6·5-fold higher risk of developing cervical intraepithelial neoplasia (CIN) 2–3 than the prototype-like HPV-16 variant (Xi *et al*., 1997), no clinical/epidemiological data are available on the oncogenic risk associated with rearranged LCR. This study represents one of the first reports on the biological efficacy and *in vitro* transforming activity of rearranged LCR of the HPV-16 Af-1 class.

Supported by grants from Ministero della Sanità (Ricerca Corrente 2000), Progetto AIDS-Istituto Superiore di Sanità (no. 30B.34) and the ICSC-World Laboratory (project MCD-2/7). We thank Miss Chris Hattie McBride for polishing the English form of the manuscript.

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Received 5 April 2000; Accepted 11 August 2000