Sequence analysis of the long control region of human papillomavirus type 16 variants and functional consequences for P97 promoter activity

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Genital human papillomaviruses (HPV) are considered to be one of the main risk factors for the development of cervical cancer. The P97 promoter at the E6-proximal end of the long control region (LCR) regulates the transcription of viral genes, especially the oncogenes E6 and E7. The LCR contains binding sites of several viral and cellular transcription factors, which either activate or repress the P97 promoter. Intratype variants of HPV-16 belong to six geographically clustered phylogenetic groups distributed all over the world. These variants exhibit differences in E6 protein activities and in tumour progression in vivo. Seven HPV-16 variants were investigated by sequencing the entire LCR (nt 7060–124) and by comparing the transcriptional activities of their P97 promoters. Previously unknown nucleotide variations were identified in all LCRs investigated. In luciferase assays, 3–3- and 2–8-fold increases in P97 promoter activity were detected in the Asian American c and North American 1 variants when compared with the European reference clone. The African variants 1a and 2a exhibited P97 promoter activities comparable to the European reference clone. After recombining different LCR fragments, the region responsible for enhanced transcription in the Asian American c and North American 1 variants could be attributed to the E6-proximal end of the LCR (nt 7619–124).

Introduction

Human papillomaviruses (HPV) infect epithelial cells of the skin and mucosa and induce benign lesions that can develop into malignant neoplasias. The genital HPVs, especially HPV types 16 and 18, are the main risk factors for the development of cervical intraepithelial lesions and cervical cancer. HPV DNA can be found in more than 90% of all cervical carcinomas and, in nearly 50% of these cases, HPV-16 DNA is detected (Bosch et al., 1995; Das et al., 1992; Londesborough et al., 1996). The region responsible for the transformation of cells can be mapped to the viral genes E6 and E7. The encoded proteins interact with a number of cellular factors, e.g. p53 and pRB, and are sufficient for the immortalization of human keratinocytes (Dyson et al., 1989; Münger et al., 1989; Scheffner et al., 1990). The long control region (LCR) is important for replication of the viral DNA and contains the P97 promoter at its E6-proximal end, which is responsible for the transcription of E6 and E7. It harbours an epithelial cell-specific enhancer and several additional binding sites for cellular and viral transcription factors. The viral E2 protein binds as a dimer to its palindromic recognition sites in the LCR and can repress or activate the expression of E6 and E7, depending on the E2 concentration (Doslatni et al., 1991; Steger & Corbach, 1997; Tan et al., 1994). Cellular factors like AP1, GRE, NF1, NF-IL6, Oct-1, SP1, TEF-1, TEF-2 and YY1 have also been shown either to stimulate or to inhibit P97 promoter activity (Bauknecht et al., 1992, 1996; Chan et al., 1990; Chong et al., 1990, 1991; Hoppe-Seyler et al., 1991; Ishiji et al., 1992; Kyo et al., 1993, 1995; Mittal et al., 1993; O’Connor & Bernard, 1995; O’Connor et al., 1996; Tan et al., 1994).

HPV types are defined by sequence variation of more than 10% between themselves, whereas intratype variants vary by up to only 2% in conserved regions of the genome, like E1 or L1, and by up to 5% in the LCR. The nucleotide sequences in just one region of the genome can be used to define HPV types and intratype variants. HPV-16 variants can be divided into six geographically clustered phylogenetic groups, the European (E) group, two African (Af) groups and the Asian (As), Asian
American (AA) and North American (NA) variants (Wheeler et al., 1997; Yamada et al., 1995, 1997). There are certain indications that the HPV-16 prototype and HPV-16 variants exhibit differences in their biological and biochemical properties. Non-European HPV-16 variants can be detected more often in neoplasias of higher grade than the prototype HPV-16 (Londesborough et al., 1996; Xi et al., 1997, 1998; Zehbe et al., 1998), and AA variants of HPV-16 show differences in the degradation of p53 due to their E6 proteins (Stöppler et al., 1996). An Asian American variant also exhibits increased P97 promoter activity compared with the prototype HPV-16 (Veress et al., 1999).

In order to correlate sequence variations in the HPV-16 LCR and transcriptional activities of the P97 promoter, we investigated different HPV-16 variants by sequencing the complete LCRs and determining the P97 promoter activity.

**Methods**

**Plasmid constructs.** PCR-amplified LCR regions from nt 7009 to 124 of the HPV-16 variants Af1a, Af2a, AAc and NA1 were ligated into the Invitrogen PCR II vector. The LCR fragments were cut out of these vectors by using the *HindIII* and *BamHI* restriction sites, which were included in the primers used in the PCR (Dong et al., 1994). The purified fragments were ligated into the precut vector pALuc (Kyo et al., 1993) so that the P97 promoters of the different LCRs were placed in front of the luciferase reporter gene (Fig. 1a). To generate recombinant LCR constructs, the 1024 bp LCR fragments were purified and cleaved with DralI at nt 7619 (Fig. 1a), thereby creating a 610 bp 5'- and a 414 bp 3'-LCR fragment. These fragments were religated to create four different chimeric LCR fragments, consisting of the Af1a 5'-region and the AAc or NA1 3'-region or the Af1a 3'-region and the AAc or NA1 5'-region. These recombinant LCR fragments were introduced back into the precut pALuc vector by using the *HindIII* and *BamHI* restriction sites. Before use in luciferase assays, the different pALuc clones were sequenced for confirmation.

**Cell culture and functional assays.** The human cervical carcinoma cell line C33A (ATCC HTB-31) was grown in DMEM supplemented with 10% (v/v) FCS. Twenty-four h prior to transfection, 6 × 10⁶ cells were plated into 6 cm² culture dishes. At 2 h prior to transfection, the cells were washed once with PBS and 2 ml fresh DMEM containing 10% (v/v) FCS was added. Within each experiment, each test DNA was transfected onto three different plates. In all assays, a total amount of 5 µg DNA per culture dish was used, comprising 4 µg test DNA and 1 µg RSV-β-Gal plasmid as an internal control for transfection efficiencies. Water was added to a volume of 153 µl and 175 µl of HEPES-buffered saline (pH 7.05) and 22.5 µl 2 × HEPES-buffered saline (pH 7.05) and 22.5 µl 2 × HEPES-buffered saline (pH 7.05) were added, quickly mixed on a vortexer and, after 20 min incubation at room

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**Fig. 1.** Schematic representation of the HPV-16 LCR. (a) The complete LCR fragments (nt 7009–124) of the different HPV-16 variants were cloned into the pALuc vector in front of the luciferase gene (Luc) by using *HindIII* and *BamHI* restriction sites. The recombined LCR fragments (Af1a–AAC, Af1a–NA1, AAC–Af1a and NA1–Af1a) were constructed by restricting the original fragments at the DralI restriction site (nt 7619), thereby creating fragments of 610 and 414 bp. These fragments were religated so that the 5' region was from Af1a and the 3' region was from AAc or NA1. Reciprocal exchanges were also made. The four different recombined fragments were then again placed in front of the luciferase gene of the pALuc vector by using the *HindIII* and *BamHI* restriction sites. (b) Some of the main features of the HPV-16 LCR fragment are depicted, including the enhancer region and the P97 promoter start site. The CAAT region, the TATA box and binding sites of several cellular or viral factors are shown. Binding site positions are given for selected factors.
temperature, the DNA precipitates were added to the cells. Following incubation overnight at 37 °C, the cells were washed again with PBS and new medium was added. The cells were harvested 48 h after transfection and lysed by four 2 min freeze–thaw cycles. Luciferase activities were quantified as described before (de Wet et al., 1987) and the P97 promoter activities were determined by calculating the luciferase/β-galactosidase activity ratios.

### Site-directed mutagenesis

Site-directed mutagenesis was performed with the AAc and NA1 variant clones by using the QuickChange site-directed mutagenesis kit (Stratagene) in order to change the nucleotide at position 7729 from a cytosine to an adenosine residue, as found in the European reference clone. The primers used comprised 34 base-pairs from bp 7715 to 7748 and contained the intended nucleotide exchange. After obtaining positive clones, the whole LCRs were sequenced to confirm the mutated nucleotide in the context of the former LCR sequences.

### Results

#### Different HPV-16 LCR clones exhibit new sequence variations

The HPV-16 LCR fragments analysed here were collected in a study on the worldwide distribution of HPV-16 variants (Yamada et al., 1997). Seven variant LCRs were sequenced from nt 7009 to 124 and compared with the HPV-16 European reference (HPV16R sequence in the HPV database). Due to the sequence alterations found between nt 7485 and 7842 (Table 1), the seven HPV-16 LCR clones were identified as being African 1a (Af1a), African 2a (Af2a), Asian American c (AAc) and North American 1 (NA1) variants, according to the results of Yamada et al. (1997).

The two Af1a variant clones (B2, D1) differed by just one nucleotide each from the sequence published by Yamada et al. (1997). Af1a B2 had a T/C transition at nt 7775 and Af1a D1 had a deletion of a T at nt 7645. In the regions abutting the 5’ and 3’ ends of that report by Yamada et al. (1997), both Af1a variants revealed nine nucleotide exchanges each compared with the reference sequence. Three deviations occurred in only Af1a B2 (nt 7293, 7314 and 7853) or Af1a D1 (nt 7087, 7200 and 7210) (Table 1); the other six nucleotide exchanges (nt 7060, 7193, 7232, 7876, 31 and 83) were found in both Af1a B2 and Af1a D1. The Af2a variant clones E1 and F1 exhibited three changes compared with the sequence of Yamada et al. (1997), whereas the Af2a F3 clone did not show any nucleotide exchanges. Af2a E1 had a transversion at nt 7578 (C/G) and an A/G transition at nt 7742, whereas the Af2a F1 variant had only one transition (G/A), at nt 7522. As in the case of the Af1a variants, there were several nucleotide changes in the neighbouring regions of the LCRs of the Af2a variants (Table 1). There were five nucleotide exchanges found in all three Af2a variants (nt 7060, 7193, 7233, 7387 and 7435) and some differences distinctive for each clone. Af2a E1 had two T/C transitions, at positions 7334 and 31, Af2a F1 had an A/T transversion at nt 7458, Af2a F3 had another T/C transition at 7293 and both Af2a F1 and Af2a F3 contained a C/G transversion at nt 31. The sequence of the LCR of the AAc variant showed a nucleotide exchange at nt 7827 (T/C transition) that was not found in the former published sequences from Yamada et al. (1997). In the abutting regions, seven more nucleotide differences were noted between the AAc variant and the European reference (nt 7060, 7193, 7233, 7339, 7394, 7866 and 7894). Comparing the nucleotide sequence of our AAc variant with the one published by Veress et al. (1999), five sequence alterations could be identified. The NA1 variant had no nucleotide changes compared with the region published by Yamada et al. (1997). There were six more nucleotide changes (nt 7060, 7193, 7227, 7233, 7283 and 7394) in the region from nt 7060 to 7458 when compared with the European reference clone (Table 1).

At six positions (7060, 7193, 7489, 7521, 7764 and 7786), the same nucleotide was found in all HPV-16 variants except for the European prototype variant. The T at nt 7193 and the A at nt 7521 can also be identified in some European isolates (Yamada et al., 1997; Veress et al., 1999; unpublished observations). At the other four positions, more than 10 independent European isolates showed the same nucleotide as the European reference clone (unpublished results).

With this more extensive sequencing of the HPV-16 LCRs of variants Af1a, Af2a, NA1 and AAc, we identified new sequence alterations in each one compared with the European reference clone and with former published sequences (Table 1).

#### Analysis of the P97 promoter activity of the different HPV-16 LCR clones

Some of the nucleotide exchanges identified in the seven different HPV-16 LCR clones were located near to or within known binding sites for cellular or viral proteins (Fig. 1 b). Therefore, the binding of cellular transcription factors to their recognition sites might be hindered or stimulated. There is also the possibility that new protein-binding sites were generated due to some of the nucleotide changes identified.

In order to determine whether sequence alterations can influence P97 promoter activity, the complete HPV-16 LCR fragments (nt 7009–124) of the different variants were cloned into the pALuc vector in front of the luciferase gene (Fig. 1 a). Cells of the C33A line were transfected and luciferase activities were analysed 48 h later. A significantly enhanced transcriptional activity of the P97 promoter was noted for the variants AAc and NA1. Here, the activity was stimulated 3-3- and 2.8-fold, respectively, when compared with the European reference clone, which was set at 1.0 in all assays (Fig. 2). The variants Af1a B2 and Af1a D1, as well as Af2a E1, Af2a F1 and Af2a F3, exhibited more or less the same transcriptional activity as the reference clone.

#### Enhancement of P97 promoter activity can be attributed to the 3’ region of the LCR

In order to determine which part of the HPV-16 LCR of the variants AAc and NA1 was responsible for the stimulation of
Table 1. Sequence alterations identified in the LCRs of the HPV-16 variants

With the help of the consensus sequences at nucleotides 7485–7842 (Yamada et al., 1997), the seven LCR clones were identified as belonging to the Af1a, Af2a, AAc and NA1 variants of HPV-16. The nucleotide position and the nucleotide of the European reference sequence are noted. The nucleotide exchanges identified in the different HPV-16 variants are listed to the right. The line between nt 7578 and 7645 depicts the region where some of the LCRs were recombined with each other (Fig. 1a). –, No nucleotide exchange compared with the European reference sequence; o, deletion of the nucleotide at this position.

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P97 promoter activity, recombinants of the LCR fragments of the two more-active variants AAc and NA1 and the less-active variant Af1a (B2) were constructed. The LCR fragments were cut and religated by using the DraIII restriction site at nt 7619 (Fig. 1a). The pALuc vectors contained the 5′ region of the less-active Af1a LCR fragment fused to the 3′ region of the more-active variants AAc and NA1 (Af1a–AAc, Af1a–NA1) or the 5′ region of the LCR of the variants AAc and NA1 fused...
Comparing all sequence variations in the E6-proximal part of the LCR among the more-active AAc and NA1 variants and the less-active ones, we identified a single nucleotide substitution at position 7729 that was restricted to AAc and NA1 isolates. With the help of site-directed mutagenesis, the A/C transition was reversed in the AAc and NA1 variants (Mut-AAc, Mut-NA1), leaving all other sequence variations unchanged. By using these mutated clones in luciferase expression experiments, we could demonstrate that the overall activity of Mut-AAc and Mut-NA1 was reduced compared with the original clones AAc and NA1, with expression levels comparable to the European reference clone (Fig. 3).

**Discussion**

In this study, we have analysed in detail seven different HPV-16 LCR variants by sequencing and by defining broad regions responsible for enhanced promoter activity of some of the variants investigated. In our more extensive sequencing, comprising 1024 bp (nt 7009–124), we identified additional sequence alterations in the Af1a, Af2a, AAc and NA1 variants compared with the results of Yamada et al. (1997) (Table 1). These sequence differences are probably due to the use of cloned PCR fragments in the present study, compared with direct sequencing of pooled PCRs reported by Yamada et al. (1997). Although we sequenced a larger region of the LCR, the phylogenetic relationship between the different variants did not vary from the results published previously. Af1a, Af2a, AA and E each represented a discrete branch on the phylogenetic tree of HPV-16 variants, whereas NA1 and AA were distinct, but still belonged to the same major branch.

Some new nucleotide changes identified in this study were specific for the individual Af1a and Af2a variant clones. The two Af1a clones B2 and D1 differed in nine nucleotide positions and the three Af2a clones E1, F1 and F3 exhibited seven, four and intratype variations, respectively. Comparing the intertype variations between the Af1a and Af2a LCR clones, they exhibited 16–19 differences. An intertype comparison of the Af1a and Af2a variants with the E, AAc and NA1 variants also revealed 16–20 sequence variations. This confirmed previous results, showing that the E, Af1a, Af2a and AAc/NA1 variants belong to different branches of the phylogenetic tree.

The variants AAc and NA1 exhibited several additional nucleotide changes relative to the reference sequence and some of these were distinct for the two variants. For instance, the nucleotide changes at positions 7339, 7507, 7827, 7886 and 7894 were only seen in the AAc variant and the nucleotide changes at positions 7227 and 7283 were specific to the NA1 variant. Whereas the sequence of the AAc variant studied here differed from that published by Veress et al. (1999) in five nucleotide positions, 11 nucleotide changes were identified between the AAc and NA1 variants. This indicates that the relationship between the AAc and NA1 variants is intermediate.
to those found with intra- and intervariant isolates and confirms a closer phylogenetic relationship of AAc and NA1 variants to each other than to the E, Af1a or Af2a group of variants (16–20 nucleotide exchanges).

The HPV-16 LCR variants exhibited differences in promoter activity when the complete LCRs were cloned in front of the reporter gene luciferase. The variants AAc and NA1 showed a roughly 3-fold increase in P97 promoter activity, whereas the Af1a and Af2a variants were more or less as active as the reference clone. A 1.7-fold increase in P97 promoter activity of an AAc variant has also been observed by Veress et al. (1999). It seems that enhanced P97 promoter activity can be attributed to the AAc variants in general, but minor sequence alterations affect P97 promoter activity. Investigations by Xi et al. (1997, 1998) demonstrated an increased tendency of progression in infections with non-prototype variants such as the AAc variant. Stöppler et al. (1996) showed stimulated induction of differentiation-resistant colonies of human foreskin keratinocytes when studying an AA isolate. The assumed enhanced oncogenic potential of the Asian American variants could therefore be due both to differences in the biological activities of the E6 protein and to an upregulation of the P97 promoter, which controls the expression of E6.

The NA1 LCR increased the P97 promoter activity 2.8-fold. This result indicates that the AA and NA1 variants share biological properties in line with their close sequence relationship. One might speculate that this also reflects a higher oncogenic potential of North American variants. The results of transient-transfection experiments with recombined LCR fragments demonstrated that the enhanced transcriptional activity could be attributed to the 3′ region comprising bp 7619–124 of the LCRs of the AAc and NA1 variants (Fig. 3). The activities of the recombined LCRs Af1a–AAc and Af1a–NA1 were even higher (3.9- and 3.4-fold) than those of the original LCR fragments. The increased P97 promoter activity could not be attributed to the enhancer region located in the 5′ region of the LCR of either variant. Similar results were also obtained by Veress et al. (1999), who showed that the enhanced transcriptional activity of the AAc variant could be ascribed to nucleotide changes in the 3′ region of the LCR. Contrary to their results, which showed no enhanced P97 promoter activity of the chimeric LCR compared with the original AAc LCR, we demonstrated an increase in P97 promoter activity when the 3′ region of our AAc variant was fused to the 5′ region of the Af1a variant. This indicates that the overall sequence context of the LCR may be important for promoter activity.

We identified a single nucleotide substitution at position 7729 (A/C transition) that was characteristic for the more-active variants AAc and NA1. Electrophoretic mobility shift assays with C33A nuclear extracts did not exhibit differences in protein binding to oligonucleotides with either A or C at this position (unpublished results). With the help of site-directed mutagenesis, the A/C transition was reversed in the AAc and NA1 variants, generating Mut-AAc and Mut-NA1, with all other sequence variations unchanged. Luciferase expression experiments demonstrated that this single nucleotide exchange reduced the P97 promoter activity of the variants AAc and NA1 almost to that of the European reference clone. Therefore, the enhanced transcriptional activity of AAc and NA1 can be attributed mainly to the A/C transition at nt 7729.

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