Rearrangements of the upstream regulatory region of human papillomavirus type 6 can be found in both Buschke-Löwenstein tumours and in condylomata acuminata

Albert Rübben,1* Sylvie Beaudenon,2 Michel Favre,2 Wolfgang Schmitz,1 Bettina Spelten1 and Elke-Ingrid Grussendorf-Conen1

1Hautklinik (Department of Dermatology) der RWTH-Aachen, Pauwelsstrasse 30, 5100 Aachen, Germany and 2Unité des Papillomavirus, Unité de l’Institut National de la Santé et de la Recherche Médicale 190, Institut Pasteur, 75015 Paris, France

Clinically malignant Buschke-Löwenstein tumours and benign condylomata acuminata are caused by human papillomaviruses (HPVs), predominantly HPV-6 and -11. In some cases, the HPV-6 genomes found in Buschke-Löwenstein tumours and in verru-ccous carcinomas differ from HPV-6b isolated from a benign genital wart, by rearrangements of the upstream regulatory region (URR). To evaluate the frequency and role of mutations of the URR of HPV-6 we analysed 42 condylomata acuminata and four Buschke-Löwenstein tumours by the polymerase chain reaction and restriction enzyme cleavage. Using only four different restriction enzymes we could demonstrate four distinct restriction patterns, indicating that naturally occurring HPV-6 isolates display a high degree of DNA polymorphism within the URR. One Buschke-Löwenstein tumour and two condylomata acuminata yielded rearranged URRs with DNA duplications. All three lesions harboured multiple HPV-6 variants, suggesting that cellular or environmental factors facilitate the development of rearrangements. Therefore, rearrangements of the URR may represent only secondary events in condylomata acuminata and Buschke-Löwenstein tumours which do not necessarily confer a higher malignant potential to the infected cell.

Introduction

Human papillomaviruses (HPVs), of which over 66 types have been identified, are associated with a wide range of epithelial tumours in man. Clinically, HPV types differ mainly in tissue specificity and oncogenic potential. Of the HPV types predominantly infecting mucosa, HPV-6 and -11 are associated mainly with benign condylomata acuminata and laryngeal papillomas (Gissmann & zur Hausen, 1980; Gissmann et al., 1982, 1983), whereas HPV-16 and -18 are strongly associated with cervical intraepithelial neoplasias and cervical cancer (Boshart et al., 1984; Dürst et al., 1983). Therefore HPV-6 and -11 are considered to be low risk viruses for malignant transformation of infected tissue (zur Hausen & Schneider, 1987). Nevertheless, in several cases it has been demonstrated that carcinomas may arise within condylomata acuminata (Grussendorf & Gahlen, 1974, Grussendorf & Bär, 1977), and HPV-6 and -11 have been detected in clinically malignant Buschke-Löwenstein tumours (Gissmann et al., 1983; Boshart & zur Hausen, 1986), low grade carcinomas of the vagina and larynx (Okagaki et al., 1984; Rando et al., 1986a, Sutton et al., 1987), and primary carcinoma of the urethra (Grussen-dorf-Conen et al., 1987).

All HPV types share a similar genomic structure consisting of a late region encoding structural proteins and an early region encoding proteins involved in replication and in the regulation of transcription of viral genes, and in immortalization and transformation of infected cells. An upstream regulatory region (URR) [synonyms: non-coding region (NCR) or long control region] of 500 to 1000 bp is situated between the late and early genes. The URR contains various enhancer and promoter elements which play an important role in the regulation of viral gene expression and virus-host cell interaction (Pfister, 1990).

Analysis of the URR in HPV-6-induced tumours has led to the identification of HPV-6 variants with rearranged URRs. Duplications within the 3' portion of the URR have been found in a benign and in an atypical condyloma acuminatum (Kulke et al., 1989; Wu &
described by Saiki (1988), and in a Buschke-Löwenstein tumour (Boshart & zur Hausen, 1986). A large head-to-tail duplication of the URR, the late region and a portion of the early region has recently been identified in a squamous lung carcinoma (DiLorenzo et al., 1992). It has been demonstrated by chloramphenicol acetyltransferase assay that duplicated sequences within the URR could produce enhancer functions (Wu & Mounts, 1988).

This has led to the hypothesis that duplications of the URR might raise the otherwise low oncogenic potential of HPV-6 by enhancing transcription of transforming genes E6 and E7. Rearrangements within the 5' portion of the URR of HPV-6 have been found in two squamous carcinomas of the vulva (Kasher & Roman, 1988; Rando et al., 1986a). In one case (Rando et al., 1986b) the rearranged URR seemed to constitute a stronger enhancer than the analogous region from HPV-6b, but this finding could not be reproduced in the second case (Farr et al., 1991). Duplications within the URR have also been detected in laryngeal carcinomas associated with HPV-11 (Byrne et al., 1987), and in anogenital carcinomas containing HPV-16 (Di Luca et al., 1989, Scheurlen et al., 1986) and HPV-33 (Cole & Streeck, 1989).

Despite the interesting implications of these findings, no epidemiological data have been published on the frequency of mutations and rearrangements within the URR of HPV-6 in condylomata acuminata and Buschke-Löwenstein tumours. Rearranged HPV-6 genomes should predominate in histologically or clinically more malignant lesions if they confer a stronger malignant potential on HPV-6. Epidemiological data would also indicate whether rearranged HPV-6 variants represent naturally occurring viruses which might constitute an important risk factor for the infected patient. The aim of this study was to evaluate the frequency and type of mutations within the URR of HPV-6 in condylomata acuminata and Buschke-Löwenstein tumours in the general population.

**Methods**

**Patients and specimens.** Tissue specimens were obtained from patients attending the Department of Dermatology of the RWTH-Aachen: 47 patients had anogenital condylomata acuminata and four had Buschke-Löwenstein tumours. After surgical removal, one part of each specimen was stored frozen at −20 °C and one portion was fixed in formalin and embedded in paraffin for histological examination. Large specimens were divided into equal smaller portions which were analysed separately. DNA was extracted from biopsies as described (Orth et al., 1980).

**Polymerase chain reaction (PCR).** Amplifications were performed as described by Saiki et al. (1985). The following two primer pairs were used to amplify the URR of HPV-6, URR1/2 [nucleotides (nt) 6487 to 320 of the published HPV-6b sequence (Schwarz et al., 1983); sense strand: 5' GGCTGCAGACCCATATGGTG 3', antisense strand 5' TTTTCCATGAATTTCTAGGCCAGC 3'; URR3/4 (nt 7446 to 284): sense strand 5' GCTGTTGCACCGGTGTTTGTG 3', antisense strand 5' ATATGGATAGCCGCCTCGAA 3'.

Amplification with primer pair URR1/2 was performed with 20 cycles of 1 min denaturation at 92 °C, 1 min annealing at 55 °C and 2 min extension at 72 °C, followed by 15 cycles of 1 min denaturation at 92 °C, 1 min annealing at 55 °C and 2.5 min extension at 72 °C. Elongation time during the last 15 cycles was prolonged to favour the amplification of long DNA sequences. Amplification with primers URR3/4 was performed with 35 cycles of 1 min denaturation at 92 °C, 1 min annealing at 55 °C and 1.5 min extension at 72 °C. Both programmes terminated with an extension step of 7 min duration. PCR products were separated on 10% agarose gels in Tris–borate–EDTA and stained with ethidium bromide.

**Restriction enzyme cleavage experiments.** Analysis of PCR products by restriction endonuclease digestion was performed to compare the resulting restriction patterns with the published sequence of HPV-6b (Schwarz et al., 1983). Rsal (Pharmacia), Ddel (Pharmacia), Psrl (Boehringer Mannheim), HpaII (Pharmacia), and HaeII (Pharmacia) (1 unit of each) were used for cleavage after ethanol precipitation of PCR products and resuspension in water. Double digestions were performed to determine restriction maps. DNA fragments were separated on 1% 5% agarose gels.

**Southern blot hybridization.** The sample DNAs (5 to 10 μg) were digested with Psrl, electrophoretically separated in 1% agarose slab gels (Agarose NA; Pharmacia), denatured and transferred to nitrocel-
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Fig. 2. PCR-mediated amplification with primer pair URR3/4 of HPV-6 DNA obtained from specimens lb, lc, 9 and 13.

Results

PCR and Southern blot analysis of the URR

The PCR with primer pair URR1/2 detected HPV-6 DNA sequences in all Buschke-Löwenstein tumours (specimens la to 4) and in biopsies from 42 patients of 47 with condylomata acuminata (specimens 5 to 46). PCR with primer pair URR1/2 amplified part of L1, the entire URR of HPV-6 and part of E6. Sequence data from HPV-6b predicted a PCR product of 1735 bp (Schwarz et al., 1983).

Electrophoresis and ethidium bromide staining of amplified products obtained from specimen DNA showed a predominant band of approx. 1850 bp (Fig. 1). One large Buschke-Löwenstein tumour was divided into three portions (lb to lc). PCR products from two portions (lb and lc) showed additional bands of greater molecular size (approx. 2070 and 2450 bp). Specimen 9 yielded two additional bands of approx. 1870 and 2000 bp, and specimen 13 showed an enlarged amplification product of approx. 1960 bp.

For further characterization of the enlarged and additional HPV-6 DNA species in specimens lb, lc, 9 and 13, primer pair URR3/4 was designed to amplify the 3' portion of the URR and part of E6 (Fig. 2). Blotting of the PCR products and hybridization with a subgenomic HPV-6 probe (nt 7438 to 7863) confirmed the amplification of HPV-6 DNA sequences (data not shown). Sequence data from HPV-6b predicted a PCR product of 740 bp. Analysis of the Buschke-Löwenstein tumour of patient 1 showed the presence of additional bands of approximately 980 and 1360 bp in specimens lb and lc (Fig. 2). The difference in length from the amplified sequence from specimen la (760 bp) was 220 and 600 bp, respectively, and was equivalent to the results obtained with primer pair URR1/2. A Southern blot with the extracted total cell DNA obtained from specimens la, lb and lc confirmed that the Buschke-Löwenstein tumour of patient 1 contained three HPV-6 DNA species differing in the length of the URR, and that these DNAs were unevenly distributed in the tumour (Fig. 3). Specimen la (lane 1) showed fragments corresponding to HPV-6a DNA (approx. 3-7, 1-7, 1-5, 1-1 kbp). The HPV-6 DNA in specimen la was named AC1A. Specimen lb contained an additional band of approx. 1-9 kb (lane 2). The difference in size from the 1-7 kb fragment containing the URR corresponded to the size difference of approx. 220 bp demonstrated by PCR. The enlarged HPV-6 DNA present in specimen lb was named AC1B. Specimen lc (lane 3) showed an additional fragment of 0-6 kb, confirming the size difference found by PCR. The enlarged DNA species was named AC1C.

PCR-mediated DNA amplification of DNA from specimen 9 with primer pair URR3/4 revealed three products of approx. 740, 790 and 920 bp (Fig. 2). The difference in length between the PCR products was equivalent to the difference found by amplification with primer pair URR1/2. The HPV-6 DNA in the condyloma acuminatum of patient 9 yielding a URR of normal size was named AC9A. The HPV-6 DNA with a URR
Restriction enzyme cleavage of PCR product obtained using URR1/2 with RsaI (a), DdeI (b) and HaeII + HaeIII (c). Lanes 1, ACV1; lanes 2, ACV2; lanes 3, ACV3; lanes 4, ACV4; lanes 5, specimen 1a; lanes 6, specimen 1c; lanes 7, specimen 1b; lanes 8, specimen 9; lanes 9, specimen 13; lanes 10, specimen 27. Additional or enlarged bands are indicated by arrowheads. a, double bands.

Fig. 4. Restriction enzyme cleavage of PCR product obtained using URR1/2 with RsaI (a), DdeI (b) and HaeII + HaeIII (c). Lanes 1, ACV1; lanes 2, ACV2; lanes 3, ACV3; lanes 4, ACV4; lanes 5, specimen 1a; lanes 6, specimen 1c; lanes 7, specimen 1b; lanes 8, specimen 9; lanes 9, specimen 13; lanes 10, specimen 27. Additional or enlarged bands are indicated by arrowheads. a, double bands.

Restriction fragment length polymorphism of the URR

PCR products of all specimens obtained with primer pair URR1/2 were examined by cleavage with RsaI or DdeI, and by double digestion with HaeII and HaeIII to compare the resulting restriction maps to that of the published HPV-6b DNA sequence. Analysis by agarose gel electrophoresis allowed estimation of the size of the fragments to approx. 10 bp accuracy. Five variants could be distinguished (Fig. 4 and 5, Table 1). Twenty-two condylomata acuminata and three Buschke-Löwenstein tumours showed the predominant variant ACV1 which differed from the homologous DNA sequence of HPV-6b by the absence of a DdeI site at nt 7565 of the HPV-6b DNA sequence, by an insertion of approx. 100 bp between the RsaI site at nt 7331 and the DdeI site at nt 7440, and by an insertion of approx. 20 bp between the HaeII site at nt 7696 and the DdeI site at nt 7843. Ten condylomata acuminata yielded variant ACV2 which differed from the DNA sequence of HPV-6b by an
Rearrangements of the URR of HPV-6

Table 1. Frequency of subtypes detected by restriction enzyme cleavage of PCR product NCR1/2

<table>
<thead>
<tr>
<th>Type</th>
<th>Condyloma acuminatum</th>
<th>Buschke-Löwenstein tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACV1</td>
<td>22</td>
<td>3</td>
</tr>
<tr>
<td>ACV2</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>ACV3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>ACV4</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>AC27</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>4</td>
</tr>
<tr>
<td>Duplication</td>
<td>2*</td>
<td>1†</td>
</tr>
</tbody>
</table>

* Both ACV2. † ACV1.

insertion of approx. 90 bp between the RsaI site at nt 7331 and the DdeI site at nt 7440. Variant ACV3 was detected in four condylomata acuminata and one Buschke-Löwenstein tumour. ACV3 differed from ACV1 by an additional RsaI site at approx. nt 7830. Five condylomata acuminata showed variant ACV4, which differed from ACV1 only by an additional HaelII site at approx. nt 6700. HPV-6 DNA from specimen 27, a condyloma acuminatum, showed a different pattern characterized by a missing DdeI site at nt 7565, by an insertion of approx. 80 bp between the RsaI site at nt 7331 and the DdeI site at nt 7440, and by an insertion of approx. 20 bp between the HaeII site at nt 7696 and the DdeI site at nt 7843 (AC27) (Fig. 4 and 5). All isolates yielding enough DNA demonstrated the PstI cleavage pattern of HPV-6a by Southern blot analysis.

Characterization of rearranged HPV-6 variants

Restriction fragment analysis of the amplified products obtained with primer pair URR1/2 demonstrated that the increases in size of DNAs AC1B, AC1C, AC9B, AC9C and AC13A were due to rearrangements in the 3' portion of the URR (Fig. 4). For further characterization of the enlarged HPV-6 DNAs, the amplified products obtained with primer pair URR3/4 were examined by cleavage with RsaI, DdeI, HaeII, HpaII and PstI. AC1B contained an insertion of approx. 220 bp between the HaeII site at nt 7696 and the PstI site at nt 224 (Fig. 6), and AC1C showed an insertion of approx. 600 bp between the primer-binding sites of URR3/4. Sequencing of the URR of AC1B and AC1C demonstrated exact tandem duplications of 218 bp (nt 7707 to 2) and 592 bp (nt 7586 to 255) (Fig. 6). The 20 bp insertion found in isolates AC1A, AC1B and AC1C could be identified as the 20 bp insert at nt 7720 already described by Kulke et al. (1989). AC9B contained an insertion of approx. 50 bp between the HaeII site at nt 7696 and the DdeI site at nt 7843 (Fig. 6). The larger DNA species, AC9C, from the same specimen showed an insertion of 180 bp between the RsaI site at nt 7633 and the DdeI site at nt 7863. AC13A demonstrated an insertion of 140 bp in the same region. In both cases the HaeII site at nt 7696 was duplicated, suggesting DNA duplications within the 3' portion of the URR.

Discussion

PCR analysis of the URR of HPV-6 in 42 condylomata acuminata and four Buschke-Löwenstein tumours demonstrated a high degree of DNA polymorphism within the URR. By comparison of restriction fragments generated with only four enzymes we could distinguish four variants of HPV-6 with differences within the URR, and one variant with an additional restriction site within L1.

Two regions within the URR of HPV-6 frequently differed from the HPV-6b sequence. All our specimens showed insertions of approx. 80 to 100 bp between the RsaI site at nt 7331 and the DdeI site at nt 7440. Boshart & zur Hausen (1986) have reported that the HPV-6b clone used for sequence analysis contains a deletion of approx. 120 bp in this region owing to a cloning artefact. The previously described variants HPV-6vc (Rando et al., 1986a), HPV-6e, HPV-6g (Wu & Mounts, 1988), HPV-6-T70 and HPV-6-W50 (Kasher & Roman, 1988) all show insertions in this region. Sequencing will show whether the 80 to 100 bp inserts found in the HPV-6 isolates are similar to these. It is worth stressing that...
duplications found in the 3' portion of the URR suggests rearrangements rather than naturally occurring viruses.

Our finding of two condylomata acuminata of normal appearance bearing putative DNA duplications indicates that rearrangements are not restricted to HPV-6-induced lesions expressing a more malignant phenotype, although these lesions may progress at later stages. Up to now, HPV-6 variants bearing duplications within the 3' portion of the URR have been found in four cases of condyloma acuminatum (HPV-6g, HPV-6ma, HPV-6-AC9B and HPV-6-AC13A) and only two cases of Buschke-Löwenstein tumour (HPV-6d and HPV-6-AC1B,C). This might be a further argument against duplication of the URR having a significant role in carcinogenesis. Moreover, eight of 10 Buschke-Löwenstein tumours analysed (Boshart & zur Hausen, 1986 and our data) provide no evidence for structural alterations in the URR of HPV-6 and HPV-11. Nevertheless, taking into account the rarity of Buschke-Löwenstein tumours in the general population, one might argue that the relative frequency of structural rearrangements within the 3' portion of the URR of HPV-6 is higher in Buschke-Löwenstein tumours than in condylomata acuminata.

Our study also demonstrates that all three lesions containing HPV-6 variants with a rearranged URR have HPV-6 DNA of lower molecular size and additional rearranged HPV-6 variants. This indicates that the HPV-6 variants initially infecting the tissue most probably did not contain rearrangements within the URR. These HPVs are represented by isolates AC1A and AC9A showing variants ACV1 and ACV2, which are frequent in the condylomata acuminata. Isolates AC1B and AC1C, and AC9B and AC9C differ from isolates AC1A and AC9A only by duplications. They are most probably the result of subsequent mutations during tumour growth. A similar coexistence of two HPV-6 DNA species in one lesion has recently been observed in a lung carcinoma (Di Lorenzo et al., 1992). The uneven distribution of isolates AC1B and AC1C in the Buschke-Löwenstein tumour of patient 1 can be explained by rearrangements occurring at different locations and times. The finding of multiple rearranged variants in all three lesions suggests that these lesions either facilitated the occurrence of rearrangements within the URR or the survival of rearranged HPV-6 DNA. The existence of cellular or environmental factors favouring viral rearrangements is speculative, but should nonetheless be considered.

The epidemiological data obtained could not demonstrate a clear correlation between genomic rearrangements and malignant potential of HPV-6. Host factors are probably responsible for most Buschke-Löwenstein tumours and rearrangements in the 3' portion of the URR may represent only secondary events in condylomata acuminata and Buschke-Löwenstein tumours which do not necessarily confer a higher malignant potential to the infected cell. Experiments to determine the enhancer activity and the transforming potential of
the rearranged HPV-6 variants found in the Buschke-Löwenstein tumour and the condylomata acuminata are in progress.

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References


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