Transcription of Episomal Papillomavirus DNA in Human Condylomata Acuminata and Buschke-Löwenstein Tumours

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SUMMARY

Condylomata acuminata and Buschke-Löwenstein tumours were analysed for the presence of human papillomavirus (HPV) transcripts. HPV DNA and RNA sequences were present in all 13 samples investigated. Ten contained HPV6 and three harboured HPV11. The HPV genomes were found exclusively as extrachromosomal circular molecules. In six biopsy specimens, viral RNA transcripts were not detectable by Northern blot analysis but could be demonstrated in dot blots. From seven HPV6-containing samples it was possible to obtain sufficient amounts of undegraded mRNA. We have found consistently one major species (1.4 kb). Less prominent species of 1.7, 1.85, 2.7 and 3.2 kb, respectively, were also detected. The 3' ends of the HPV6 mRNAs were located between nucleotides 3917 and 4441 in the putative early region and between nucleotides 7232 and 7696 in the putative late region. The arrangement of the 3' termini and the adjacent coding areas within the HPV6 genome show that the RNA species are transcribed from one DNA strand.

INTRODUCTION

Human genital warts (condylomata acuminata) are induced by human papillomaviruses (for review, see zur Hausen, 1977). The DNA of the papillomavirus types 6 (HPV6) and 11 (HPV11) has been detected by Southern blot analysis of DNA extracted from condylomata acuminata, and also from Buschke-Löwenstein tumours which are invasively growing non-metastasizing giant condylomata (Gissmann & zur Hausen, 1980; Gissmann et al., 1982a, 1983). The viral DNAs have been molecularly cloned (de Villiers et al., 1981; Gissmann et al., 1982b), and the sequence of HPV6b has recently been published (Schwarz et al., 1983).

Studies on the molecular biology of HPVs are particularly difficult owing to the lack of suitable cell culture systems. Therefore, biological data on HPV can only be obtained at present from human tumour material. This is the first report describing papillomavirus RNA species in human biopsy tumour material. The HPV6 transcripts were compared with the well-characterized transcripts of bovine papillomavirus type 1 (BPV-1) (Amtmann & Sauer, 1982; Engel et al., 1983). Additionally, the direction of transcription and the location of the 3' ends of the mRNA species were determined.

METHODS

Extraction of DNA and RNA. Total DNA plus RNA were extracted from solid tumour specimen according to the method of Krieg et al. (1983).

DNA and RNA blot hybridization. Purified DNA was fractionated by electrophoresis on 1% or 1.4% agarose gels (10 μg/tracker), transferred to nitrocellulose filter paper (Southern, 1975) and hybridized to 1 x 106 c.p.m./ml 32P-labelled nick-translated (Rigby et al., 1977) cloned HPV6b DNA (de Villiers et al., 1981) as described by Krieg et al. (1981). DNA blot hybridization was performed according to Amtmann & Sauer (1982). Sequences with homologies to HPV6 were visualized by autoradiography using XAR-5 X-ray films (Kodak) with intensifying screens.

Synthesis of cDNA. The procedure for cDNA synthesis was a modification of the protocol of Friedman & Rosbash (1977). Ten μg of total RNA was incubated in a 30 μl volume, containing 100 μg/ml actinomycin D, 200
µg/ml oligo(dT), 50 mM-Tris-HCl pH 8.3, 6 mM-MgCl₂, 140 mM-KCl, 20 mM-dithiothreitol, 30 µM-³²P-labelled deoxynucleoside triphosphates (Amersham, 400 Ci/mmol) and 18 units of avian myeloblastosis virus reverse transcriptase (Life Sciences). After 60 min incubation, SDS was added to a final concentration of 0.1% followed by digestion with proteinase K (0.5 µg/ml) for 30 min at 37 °C. The RNA was hydrolysed by NaOH treatment (final concentration 0.3 M). Purification of cDNA was carried out by chromatography on Sephadex G-75. Hybridization with the labelled cDNA (0.5 × 10⁶ c.p.m./ml) was performed in a 50 ml volume of 6 × saline sodium citrate (SSC) with Denhardt's solution (Denhardt, 1966) for 16 h at 68 °C with gentle shaking of the reaction vessel.

Cloned HPV6 DNA. Cloned HPV6b DNA was a gift of L. Gissmann, German Cancer Research Center. The plasmids were prepared as described by Ish-Horowicz & Burke (1981). Plasmids pAmp2 and pAmp21 contain the 3.5 × 10⁶ and 1.6 × 10⁶ kb BamHI/EcoRI restriction fragments of HPV6b, respectively (de Villiers et al., 1981).

Dot blot analysis. Purified DNA (10 µg) and RNA (20 µg) in 10 x SSC were heat-denatured (10 min at 100 °C) and dropped, using a sterile pipette, onto a nitrocellulose sheet. The filter was dried, baked at 80 °C for 2 h and then hybridized with ³²P-labelled HPV6b DNA.

(a) (b) (c) (d) (e) (f) (g) (h) (i) (k) (l) (m) (n) (o)

Fig. 1. Detection of HPV6 DNA sequences in two genital warts. Total tumour DNA (10 µg/track) untreated or cleaved with several restriction enzymes was fractionated on a 1-4% agarose gel and hybridized to 1 × 10⁶ c.p.m./ml of a mixture of ³²P-labelled pAmp2 and pAmp21 (10⁸ to 2 × 10⁸ c.p.m./µg). Lanes (a) to (g) and (h) to (o) contained the two tumour DNAs, respectively. (b, k) Untreated DNA; (a, i) HpaI-cleaved DNA; (c, l) HpaI-cleaved DNA; (d, m) EcoRI-cleaved DNA; (e, n) BamHI-cleaved DNA; (f, o) HindIII-cleaved DNA; (g, h) PstI-cleaved DNA.
RESULTS

HPV DNA analysis in human biopsy material

HPV DNA sequences were revealed by the Southern blotting technique. The data obtained from two out of 13 biopsy specimens are shown in Fig. 1. Both undigested and restriction enzyme-digested DNA samples were run on a 1.4% agarose gel and hybridized with $^{32}$P-labelled cloned HPV6b DNA. Lanes (b) and (k) contained untreated DNA, lanes (d) and (m) DNA digested with a no-cut enzyme, lanes (e), (f), (l), (n) and (o) DNA treated with single-cut enzymes. The data show unequivocally the plasmid state of the HPV DNA, as digestion with single-cut enzymes generated only form III (FoIII) unit-length DNA and digestion with no-cut enzymes failed to alter the migration properties of the viral DNA in relation to the undigested samples. The remaining 11 genital warts also contained exclusively extrachromosomal HPV DNA sequences (data not shown) in agreement with the results of Gissmann et al. (1982a). Digestion of the DNAs with multi-cut enzymes (lanes a, c, g, h and i) permitted the identification of the virus types or subtypes in the lesions (Table 1). Six of the tumours contained HPV6a, one HPV6b, and three biopsy specimens harboured a still unidentified subtype of HPV6, as concluded from the $PstI$ and $HpaII$ restriction patterns. The HPV11 types in three samples were not analysed in greater detail with regard to possible minor alterations.

Detection of HPV mRNA species

The isolation of intact mRNA from solid genital warts became feasible after the establishment of a new technique which permits the simultaneous extraction of DNA and RNA (Krieg et al., 1983). Aliquots from eight specimens were either pretreated with DNase-free RNase (Fig. 2a), with RNase-free DNase I (b), and with RNase (c). The samples were immobilized on nitrocellulose filters and hybridized with $^{32}$P-labelled HPV6 DNA sequences. All eight samples contained both HPV DNA (a) and RNA (b). No distinction between HPV6 and HPV11 can, however, be made, owing to the extensive homologies between the viral DNAs (Gissmann et al., 1983). Pretreatment of the RNA with DNase-free RNase (Fig. 3c) abolished the signals, thus providing evidence of the presence of HPV transcripts in all of the tested human biopsy specimens.

The controls that were included in all nucleic acid hybridizations (and which are not shown here) consisted of DNA and RNA isolated from human foreskin fibroblasts, human embryonic lung cells and HeLa cells. All control hybridizations failed to reveal specific signals with HPV6b $^{32}$P-labelled DNA.

![Fig. 2. Dot blot analysis of DNA and RNA from genital warts. Total DNA (10 µg) and total RNA (20 µg), immobilized on a nitrocellulose paper, were hybridized to 1.5 x 10$^6$ c.p.m./ml of a mixture of $^{32}$P-labelled pAmp2 and pAmp21 (10$^9$ to 2 x 10$^9$ c.p.m./µg). (a) DNA, (b) DNase I-treated RNA, (c) RNase-treated RNA from eight tumours.](image)

Table 1. HPV DNA detected in genital biopsy material

<table>
<thead>
<tr>
<th></th>
<th>Condyloma acuminatum (n = 9)</th>
<th>Buschke-Löwenstein tumour (n = 4)</th>
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<tbody>
<tr>
<td>HPV6a</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>HPV6b</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>HPV6c</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>HPV6</td>
<td>2*</td>
<td>1*</td>
</tr>
<tr>
<td>HPV11</td>
<td>1</td>
<td>2</td>
</tr>
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* New subtype of HPV6.
In seven of the 13 tumours, it was possible to discern discrete RNA species. The remaining six tumours contained either too little virus-specific nucleic acids, or the RNA may have been partially degraded in the tumour samples prior to the extraction procedure. The RNA from six condylomata acuminata and one Buschke-Löwenstein tumour, all containing HPV6 DNA, was suitable for Northern blot analysis. The number and size of the HPV6-specific transcripts were determined by electrophoresis through methylmercuric hydroxide gels, followed by transfer of the RNA to nitrocellulose filters and hybridization with $^{32}$P-labelled cloned HPV6b DNA. Five RNA species were identified, and, by using two cloned HPV6b DNA fragments as probes, these were partially mapped. Fig. 3(a) shows the analysis of the RNA of one condyloma acuminatum (lanes 2) and one Buschke-Löwenstein tumour (lanes 3) using the complete HPV6b DNA as $^{32}$P-labelled probe (lanes 1, size marker). Three mRNA species of 1.4, 1.85 and 2.7 kb were detectable (Fig. 3b) using as a probe the coding region for the putative main bodies of the early mRNAs (pAmp21, see Fig. 5). It is possible, however, that the most abundant 1.4 kb species may consist of two different species. The abundant 'early' transcripts of the closely related BPV-1 in tissue culture cells and in bovine warts, for example, are represented by two major species of 1.1 and 1.3 kb (Amtmann & Sauer, 1982). In the hamster system (tumours and tissue culture cells), however, the two bands could not be distinguished after gel electrophoresis (Amtmann & Sauer, 1982). Likewise, it is not clear whether the 1.4 kb signal described here represents one or two species.

Employing as a probe the coding region for the putative late region and the 5' part of the early region (pAmp2; this HPV6 DNA fragment covers the area between the BamHI site and the EcoRI site; for details, see Fig. 5), two weak signals were obtained corresponding to two mRNA species of 1.7 kb and 3.2 kb (Fig. 3c).
Fig. 4. Mapping of the 3' ends of HPV6 mRNA in condylomata acuminata. 32P-labelled cDNA (0.5 x 10^6 c.p.m./ml), synthesized from total RNA isolated from genital warts, was hybridized to pAmp21 and pAmp2 DNA restriction fragments. The restriction enzymes in the linear maps are indicated by the following symbols: •, ApaI; □, AvaII; ▼, HaeII; ▲, HpaII; ○, PstI. Open bars indicate restriction fragments hybridizing with 32P-labelled cDNA. The dotted lines indicate the outer limits within which the 3' ends were confined to the physical map of HPV6b. (a) Top, HPV6b EcoRI/BamHI fragment (1.6 x 10^6 kb) without the pBR322 moiety with the recognition sites of the restriction endonucleases ApaI, AvaII, HpaII, PstI. Lanes a, pAmp21 DNA digested with HpaII; b, pAmp21 DNA digested with BamHI/PstI; c, pAmp21 DNA digested with HpaII/PstI; d, pAmp21 DNA digested with ApaI/BamHI; e, pAmp21 DNA digested with ApaI/PstI. a to e show ethidium-bromide stained gels; a' to e' show autoradiographs of nitrocellulose filters hybridized to 32P-labelled cDNA. (b) Top, HPV6b BamHI/EcoRI fragment (3.5 x 10^6 kb) without the pBR322 moiety with the recognition sites of the restriction enzymes ApaI, AvaII, HaeII, HpaII, PstI. Lane f shows pAmp2 DNA digested with EcoRI/PstI; g, pAmp2 DNA digested with HpaII/PstI; h, pAmp2 DNA digested with HpaII/PstI; i, pAmp2 DNA digested with AvaII; k, pAmp2 DNA digested with HaeII; l, pAmp2 DNA digested with MboII (MboII recognition sites are not indicated; the two sites of interest are in positions 7028 and 381, respectively). e to l show ethidium-bromide stained gels; e' to l' show autoradiographs of nitrocellulose filters hybridized to 32P-labelled cDNA. The minor bands in lane f' result from partial digestion products, seen in lane f.

Another observation deserves comment, namely the absence of the weak signals (seen in Fig. 3b, c) when a mixture of labelled pAmp21 and pAmp2 was used as probe (Fig. 3a). This could be accounted for by the reduced relative content, within the labelled probe, of the early and the late DNA sequences. Hence, the minor bands were probably too faint to become visible.

**Mapping of the 3' ends and direction of transcription**

Polyadenylated 3' ends of the HPV mRNAs were approximately mapped as described previously (Amtmann & Sauer, 1982). Total RNA served as template for reverse transcription using oligodeoxythymidylic acid as primer and 32P-labelled nucleoside triphosphates as precursors. The cDNA sequences were hybridized to HPV6b DNA restriction fragments to identify the 3' ends of the early and late messenger species in the tumours.

Fig. 4 shows the ethidium bromide-stained gels containing HPV6b DNA restriction fragments (lanes a to l). The same gels were employed in Southern blots to reveal, after hybridization with 32P-labelled cDNA those fragments which display homologies to the 3' ends of the polyadenylated HPV6-specific mRNAs (lanes a' to l'). The approximate position of the 3' ends of the putative early RNA species could be assigned to the region between nucleotides 3917 and 4441 as indicated in Fig. 4(a) by the dotted vertical lines. The 3' ends of the putative late mRNA species were confined to the region between nucleotides 7232 and 7696 (Fig. 4b).
In some cases, depending on the sizes of the labelled probe, the restriction fragment adjacent to the fragment containing the 3' ends also hybridized to the cDNA probe (Fig. 4a, b, lanes e', i', k'), thus permitting assessment of the direction of transcription. The HPV6 RNAs are located in a head-to-tail arrangement (Fig. 5) which indicates that all of the HPV RNAs are transcribed from the same DNA strand, as described previously for BPV-1 (Amtmann & Sauer, 1982; Engel et al., 1983).

**DISCUSSION**

The genomes of the human papillomaviruses types 1a and 6b display striking similarities with the bovine papillomaviruses types 1 and 2 (for review, see Pfiester, 1984). The open reading frames of all these viruses can be readily superimposed, revealing a very similar genome organization.

Thus, when attempting to identify hitherto unknown transcripts of HPV6 in human biopsy material, it was to be expected that RNA species resembling the previously described BPV-1 transcripts might be revealed. Six out of 13 condylomata acuminata and Buschke-Löwenstein tumours contained RNA which was, however, not amenable to detailed analysis, although specific HPV6 or HPV11 RNA sequences could be demonstrated by dot blot hybridization. The viral RNA species in the seven other tumours could be characterized in greater detail by Northern blot analysis. As we have shown in this report, one prominent species of 1.4 kb and four minor species of 1.7, 1.85, 2.7 and 3.2 kb were present in the human wart biopsies. Both the number and the sizes of the HPV6 transcripts were shown, indeed, to resemble those detected in the case of BPV-1 (Amtmann & Sauer, 1982; Engel et al., 1983).

Employing different fragments of HPV6b as a $^{32}$P-labelled probe (Fig. 3), the five RNA species could be distinguished by three 'early' transcripts (1.4, 1.85 and 2.7 kb) and two 'late' transcripts (1.7 and 3.2 kb). The small amounts of RNA precluded an enrichment of polyadenylated RNA; hence, the existence of additional viral mRNA species beyond the level of
Human papillomavirus transcription
detection cannot be ruled out. BPV-1-induced warts contain five ‘early’ and three ‘late’ species (Engel et al., 1983). The sizes of the RNAs in the human wart biopsy specimens correspond approximately to the size classes of BPV-1 mRNAs in bovine warts with the exception of the large species which were not detectable in the human material.

Densitometric measurements showed a 20- to 50-fold excess of ‘early’ mRNA species over ‘late’ mRNA (data not shown). Such varying amounts of either the ‘early’ or the ‘late’ transcripts can be readily accounted for by the structure of the warts from which they were obtained. The permissive tissue, namely the keratinized periphery, where virus particle production takes place, comprises only a few cell layers. Hence, putative ‘late’ transcripts were found in minor amounts. Additionally, the yield of mature virions in condylomata acuminata is very low (Grussendorf-Conen et al., 1983).

Mapping of the 3’ ends of the HPV6-specific transcripts revealed two polyadenylation sites, one of which was located between nucleotides 3917 and 4441 for the putative ‘early’ transcripts and the other between nucleotides 7232 and 7696 for the putative ‘late’ transcripts; the latter \(HaeII\) fragment contains a polyadenylation signal (Schwarz et al., 1983). A poly(A)-related sequence is located at position 4554; however, our data have shown that the ‘early’ polyadenylation site maps at least 100 base pairs upstream (Fig. 4a). This position agrees approximately with the corresponding poly(A) sites of HPV1 and BPV-1. The Southern blots enabled, in addition, determination of the direction of transcription. In common with the BPV-1 system (Amtmann & Sauer, 1982; Engel et al., 1983), all HPV6 mRNAs are transcribed from one DNA strand. Together with the characterization of the 3’ ends of the polyadenylated viral RNAs, the Northern blot data permitted a preliminary mapping of the main bodies of the RNA species to regions of the HPV6 genome (Fig. 5) without, however, providing information about the 5’ ends or splicing of the transcripts. The two ‘early’ species of 1.4 and 1.85 kb would suffice to code for the gene products of the open reading frames E4 and E2 (Fig. 5), while the large 2.7 kb species contains coding capacity for a part of the putative E1 protein. Similarly, the two ‘late’ species (1.7 kb, 3.2 kb RNA) could encode the putative gene products of the two large open reading frames L1 and L2 in the late region (Fig. 5).

After this paper was completed, HPV18 DNA was also identified in one condyloma acuminatum containing HPV6a. HPV18 has recently been found in some genital cancer biopsies (Boshart et al., 1984). Both forms I and II of HPV18 DNA were found (H. Lehn & G. Sauer, unpublished results) and the genome was expressed, as shown by reverse transcription of the ‘early’ and ‘late’ polyadenylated viral RNAs.

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REFERENCES


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