Human papillomavirus type 6a DNA in the lung carcinoma of a patient with recurrent laryngeal papillomatosis is characterized by a partial duplication

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Transcriptionally active human papillomavirus type 6a (HPV-6a) DNA was detected in a lung carcinoma of a patient with recurrent laryngeal papillomatosis. The carcinoma contained episomal HPV-6a genomes that had a duplication of the upstream regulatory region, the late region and a portion of the early region. HPV-6a genomes found in benign laryngeal papillomas from the same patient did not contain this duplication. A role for the mutant molecules in the pathogenesis of the malignancy is suggested.

Human papillomavirus type 6 (HPV-6) causes benign lesions of the larynx, upper respiratory tract and genital tract (de Villiers et al., 1981; Mounts & Kashima, 1984). Unlike HPVs 16 and 18, HPV-6 does not immortalize or transform cells in culture (Watts et al., 1987; Pirisi et al., 1987; Kaur & McDougall, 1988), and it has been identified in malignant lesions only rarely (Boshart & zur Hausen, 1986; Kasher & Roman, 1988; Rando et al., 1986a; Zarod et al., 1988). In the few instances when HPV-6 genomes in malignant lesions were extensively analysed, they were found to have undergone mutations, insertions, and/or duplications in regulatory sequences, suggesting that such molecular changes are necessary to confer malignant potential (Boshart & zur Hausen, 1986; Rando et al., 1986a). Similarly, a duplication in HPV-11 genomes found in a lung cancer metastasis has been reported (Byrne et al., 1987).

The patient studied here was a 26-year-old non-smoking male with a 24-year history of recurrent respiratory papillomatosis who developed a poorly to moderately well-differentiated squamous cell carcinoma of the lung. Benign lesions from this patient were known to contain HPV-6 DNA. DNA and RNA hybridization analyses were performed in order to assess the possible role of a variant HPV-6 in the development of the malignancy and to determine whether molecular changes noted by others are consistently found in HPV-6 genomes associated with malignant lesions.

Tissue biopsies were frozen in liquid nitrogen, disrupted by vibration using a Braun Micro-Dismembrator II, and DNA was extracted with phenol and chloroform as described previously (Krieg et al., 1983). DNA was digested with restriction enzymes, subjected to electrophoresis through 0.8% agarose gels and blotted onto GeneScreen Plus nylon membranes (NEN) by a slight modification of the method of Southern (1975) as described previously (Steinberg et al., 1987). Unless otherwise specified, blots were probed at Tm - 24 °C with cloned HPV-6b DNA (de Villiers et al., 1981) purified from vector sequences and labelled with [α-32P]dCTP by random priming (Feinberg & Vogelstein, 1983). Blots were washed in 0.3 M-sodium chloride, 0.03 M-sodium citrate, 1% SDS at 68 °C (Tm - 22 °C).

Total cellular DNA isolated from a benign laryngeal papilloma from the patient was digested with PstI and analysed for HPV sequences by Southern blot hybridization. The cleavage pattern characteristic of HPV-6a was observed. Fig. 1 (a) shows a partial restriction map of HPV-6a arbitrarily linearized at one of the four PstI sites. The positions of the HPV open reading frames (ORFs) and the upstream regulatory region (URR) are also indicated (Schwarz et al., 1983). HPV-6a has not been completely sequenced, differs from the HPV-6b isolate sequenced by Schwarz et al. (1983). HPV-6a has an additional PstI site, which gives rise to the PstI-A and PstI-B fragments indicated in Fig. 1 (a), and HPV-6a DNA is approximately 250 bp longer than HPV-6b.

Restriction enzyme digestion and stringent hybridization were used to determine whether HPV was present in this patient's lung carcinoma (Fig. 2). Cleavage of the carcinoma DNA with enzymes that do not cut HPV-6a but which cleave frequently within the human genome (SacI, BglII, BclI) generated identical banding patterns (Fig. 2 a, lanes 2 to 4), which revealed the presence of HPV DNA and strongly suggested that the HPV DNA was extrachromosomal. However, the pattern was


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Fig. 1. (a) Genomic organization and partial restriction map of HPV-6a. E1 to E7 represent the ORFs of the early region, L1 and L2 represent the ORFs of the late region, URR indicates the location of the upstream regulatory region, and A to D denote the four PstI fragments. Approximate nt positions are indicated. (b) Structure and partial restriction map of the lung carcinoma HPV molecule containing a head-to-tail duplication. The open bars indicate the two URRs and the solid bars represent a complete or partial PstI-A fragment. Restriction enzyme cleavage sites are indicated: B, BamHI; E, EcoRI; H, HindIII; P, PstI; T, TaqI.

Fig. 2. Southern blot analysis of DNA from the lung carcinoma (Ca) and a laryngeal papilloma (P) from the same patient. The entire HPV-6b genome was used as the probe for all lanes except (c) 4 and 5, in which the probe used was the PstI-A fragment of the cloned HPV-6a. (a) Digestion with enzymes which do not cut HPV-6a. Lane 1, P digested with BgII; lanes 2, 3 and 4, Ca digested with SacI, BglII, or BclI, respectively. (b) Digestion with enzymes that cleave HPV-6a once. Lane 1, P digested with BamHI; lane 2, Ca digested with HindIII. (c) BamHI-PstI digestions. Lane 1, the EMBL3 clone of the larger BamHI fragment from the carcinoma; lane 2, Ca genomic DNA; lane 3, Ca DNA enriched for the smaller BamHI fragment; lanes 4 and 5, lanes 2 and 3 reprobed with the PstI-A fragment of HPV-6a. Lane 1 is from a separate gel, but dotted lines indicate bands of equal size. (d) Ca digested with BamHI-TaqI.
clearly not that of monomeric HPV. Bands which comigrated with the supercoiled (form I) and nicked circular (form II) HPV bands in the benign laryngeal papilloma (Fig. 2a, lane 1) were seen, as well as a faint band between these and a higher Mr band. Further restriction enzyme analysis was done in order to determine the identity of the novel bands.

Cleavage of DNA from the benign papilloma with BamHI (Fig. 2b, lane 1) or with EcoRI or HindIII (not shown) gave a single band of 8.1 kbp. Cleavage of the carcinoma DNA with BamHI generated two fragments, the expected 8.1 kbp linear HPV-6a band and an additional smaller band of 5.4 kbp (Fig. 2b, lane 2). Cleavage with two additional single-cutters for HPV-6a, EcoRI (not shown) and HindIII (Fig. 2b, lane 3), gave the expected 8.1 kbp band but also an additional band of higher Mr, (approximately 14 kbp).

The 8.1 kbp BamHI fragment was cloned from the carcinoma DNA into EMBL3, and the phage clone was doubly digested with BamHI and PstI (Fig. 2c, lane 1). Five fragments were observed, having sizes of 3.8, 1.8, 1.1, 0.8 and 0.7 kbp, although the two smallest fragments were only faintly visible on the original autoradiogram. This cleavage pattern was identical to that expected for HPV-6a and was also seen with the total carcinoma DNA (Fig. 2c, lane 2). (Note that lanes 1 and 2 were from different gels, but dotted lines connect bands of equal Mr.) This cloned DNA was analysed using several other restriction enzymes, and in all cases the cleavage pattern was as expected for HPV-6a (not shown).

Attempts to clone the 14 kbp EcoRI fragment and the 5.4 kbp BamHI fragment have been unsuccessful to date. The 5.4 kbp BamHI fragment was therefore eluted from a gel to enrich for these sequences for further analysis. Digestion of this fragment with PstI gave a banding pattern identical to that expected for HPV-6a when probed with the full-length HPV-6b genome, except that the 3.8 kbp PstI-A fragment was missing (Fig. 2c, lane 3). Only a very faint band was seen, which we believe came from trace contamination by the larger BamHI fragment. When the blot in Fig. 2c, lanes 2 and 3 was reprobed with the 3.8 kbp PstI-A fragment from the cloned HPV-6a, it hybridized to the expected 3.8 kbp band and to a band at 1.1 kbp (Fig. 2c, lanes 4 and 5). This indicated that the 5.4 kbp BamHI fragment represented a partial HPV-6a genome which did not include 2.7 kbp of the 3.8 kbp PstI-A fragment. Failure to detect the new 1.1 kbp PstI fragment when the total carcinoma DNA digest was probed with full-length HPV-6 was due to the presence of a normal PstI fragment of this size (PstI-D; see Fig. 1a). The 5’ end of the PstI-A deletion was mapped between the TaqI sites present at nucleotides (nt) 266 and 724 as digestion of the carcinoma DNA with BamHI and TaqI generated a novel band of 1.7 kbp which was due to the absence of the TaqI site at nt 724 (Fig. 2d).

Based on the Southern blot hybridization analysis, we believe that the lung carcinoma contained extrachromosomal HPV-6a existing in two forms: normal 8.1 kbp monomers (present in 10-fold excess) and 14 kbp molecules with a 5.4 kbp head-to-tail duplication of the URR, the late region and a portion of the early region. Fig. 1(b) shows a partial restriction map of the larger species. The 14 kbp band seen upon HindIII or EcoRI digestion represented the linearized head-to-tail duplication. As the partial duplication did not include the HindIII or EcoRI site, there was only a single cleavage site for these enzymes. The duplicated region did include the BamHI site, and the presence of the second BamHI site accounts for the additional 5.4 kbp BamHI fragment. The uppermost band seen with the non-cutting enzymes represented form II molecules of the larger species. The faint extra band could be form I of this species. The large molecules can be thought of as head-to-tail dimers in which one of the monomers has a 2.7 kbp deletion in the PstI-A fragment.

To determine which ORFs were present in the partially duplicated early region, DNA from the carcinoma was digested with EcoRI, and a polymerase chain reaction (PCR; Saiki et al., 1988) was performed using primers which flanked the PstI-A fragment (nt 37 to 53 and nt 4060 to 4076 of the prototype HPV-6). Digestion with EcoRI prior to PCR prevented amplification of the full-sized PstI-A fragments and only allowed amplification of the PstI-A fragments having the deletion. The resulting PCR product was trimmed at both ends with PstI, cloned into a bacterial plasmid, and a subfragment of the cloned PCR product was sequenced using the chemical cleavage method of Maxam & Gilbert (1980). Nucleotides 437 to 3089 were found to be deleted. The partial early region therefore contained the 5’ end of the E6 ORF, the 3’ end of the E2 ORF, and intact E4 and E5 ORFs. At the deletion site, nt 430 and 3096 were joined by one copy of the sequence 5’ GCTGTG 3’. This 6 bp sequence is normally present at both nt 431 to 53 and nt 4060 to 4076 of the prototype HPV-6). Digestion with EcoRI prior to PCR prevented amplification of the full-sized PstI-A fragments and only allowed amplification of the PstI-A fragments having the deletion. The resulting PCR product was trimmed at both ends with PstI, cloned into a bacterial plasmid, and a subfragment of the cloned PCR product was sequenced using the chemical cleavage method of Maxam & Gilbert (1980). Nucleotides 437 to 3089 were found to be deleted. The partial early region therefore contained the 5’ end of the E6 ORF, the 3’ end of the E2 ORF, and intact E4 and E5 ORFs. At the deletion site, nt 430 and 3096 were joined by one copy of the sequence 5’ GCTGTG 3’. This 6 bp sequence is normally present at both nt 431 to 436 and nt 3090 to 3095 of the prototype HPV-6 (Schwarz et al., 1983) and may have facilitated a recombination event which generated the 14 kbp HPV molecules having a head-to-tail duplication.

The duplication described here included the HPV URR, a segment of the viral genome which contains the origin of DNA replication and transcriptional regulatory elements (Rando et al., 1986b; Wu & Mounts, 1988). Duplication of the URR could therefore affect both viral DNA replication and the expression of viral genes. HPVs 6 and 11 have very low transforming ability, but Rosen & Auborn (1991) have shown that cloned HPV-11 DNA containing a duplication of the URR acquires the
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ability to cooperate with ras in transforming baby rat kidney cells, whereas the prototype HPV-11 cannot.

To determine whether the HPV DNA present in the carcinoma was transcriptionally active and to characterize the transcripts, tissue was disrupted as described above and total cellular RNA was isolated using the guanidinium–caesium chloride method of Chirgwin et al. (1979). Northern blot analysis of 20 μg total RNA clearly revealed the presence of four HPV-6 transcripts when the entire HPV-6b cloned genome was used as a probe (Fig. 3a, lane 1). In addition, a fifth transcript of low abundance, having a size of 4.2 kb, was detectable on the original autoradiogram. When this same blot was hybridized with a subgenomic fragment of HPV-6b (nt 3212 to 3610) coding for the E4 ORF, an identical pattern was observed (data not shown). This result was expected, because the E4 segment of the genome is included in all of the early HPV-6 transcripts which have been described by Chow et al. (1987). The abundance and size of the predominant transcript of 1.3 kb is consistent with this transcript being the E1/E4 transcript described previously in benign lesions containing HPV-6 or HPV-11 (Chow et al., 1987).

To determine whether the malignant lesion contained HPV transcripts not present in a benign papilloma, the HPV transcript pattern of the carcinoma (Fig. 3a, lane 1) was compared to that of a laryngeal papilloma from the same patient (Figure 3b, lane 1). Twenty μg total RNA was loaded in each lane; however, the films in Fig. 3 (a) were exposed approximately ten times longer than those in Fig. 3 (b). This indicates that HPV transcripts were more abundant in the laryngeal papilloma than in the lung carcinoma, probably reflecting virion production in the papilloma. Although the transcript patterns were similar, they differed in that there was a 7.3 kb transcript present in the carcinoma but absent from the papilloma, even on a very long exposure. Using oligo(dT) priming of RNA from the carcinoma, an HPV-specific cDNA of this same size was generated (T. Crook, personal communication). For this reason, we believe that the 7.3 kb band represents a mature messenger RNA, not a nuclear precursor.

Hybridization using a subgenomic HPV-6b fragment (nt 7815 to 7902 and 1 to 617) coding for the E6/E7 region of the genome showed that the novel 7.3 kb transcript contained E6/E7 sequences (Fig. 3a, lane 2). A second and possibly critical difference between the transcript patterns of the papilloma and carcinoma was the increased amount of the 1.9 kb E6/E7 transcript, relative to the other HPV transcripts, in the carcinoma. As described by Chow et al. (1987), the most abundant transcripts in HPV-6 and HPV-11 lesions contain a short leader sequence from the E7 region of the genome. However, the E6/E7 probe used for our analysis does not hybridize to this leader sequence. Bands detected with the E6/E7 probe therefore represent transcripts from the E6/E7 ORFs. In human carcinomas and carcinoma-derived cell lines containing integrated HPV-16 or HPV-18, both the E6 and E7 genes are preferentially retained and transcribed (Androphy et al., 1987; Choo et al., 1987; Schneider-Gadicke & Schwarz, 1986; Schwarz et al., 1985; Seedorf et al., 1987; Smotkin & Wettstein, 1986). Moreover, the genes for E6 and E7 are sufficient for immortalization and transformation by HPVs 16 and 18 (Bedell et al., 1989; Crook et al., 1989; Watanabe et al., 1989; Yutsudo et al., 1988).

There are several possible explanations for the differences seen when the HPV transcription patterns of the carcinoma and the benign papilloma are compared.
Altered transcription from the partially duplicated HPV-6 molecules may have occurred due to duplication of regulatory sequences as discussed above. Altered RNA processing may have occurred as a result of deletion of splice donor and splice acceptor sites in the reiterated region of these genomes. Deletion of E1 and E2 coding regions from the reiterated region may also have contributed to the altered transcription pattern. However, it is unlikely that a gene dosage effect played a significant role, because 90% of the HPV-6 genomes in the carcinoma were full-length monomers. Finally, differences in the relative abundance of the various HPV transcripts may have been seen, at least in part, because the benign papilloma was a productive lesion and the carcinoma was not.

In this report, we have demonstrated the presence in a lung carcinoma of HPV-6a genomes having a duplication of the URR, late region and part of the early region. HPV-6a genomes in benign lesions from the same patient did not have this duplication. This finding supports the hypothesis that sequence changes are necessary to confer malignant potential on HPV-6.

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**References**


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