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Papillomavirus genomes in human cervical tumors: Analysis of their transcriptional activity

(human papillomaviruses/genital cancer/blot hybridization)

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ABSTRACT **Four of six human cervical carcinoma biopsies were shown to contain the DNA of the human papillomavirus type 16 (HPV-16) covalently linked with the tumor cell DNA. HPV-16-specific mRNA species were observed in only one of the four tumors. No such sequences were found in the three other specimens in conditions that permitted the detection of less than one mRNA molecule per cell. It is concluded that maintenance of the malignant nature of these cervical tumors does not depend on the continuous transcriptional activity of HPV-16.**

Papillomavirus infections of the human anogenital region are very common (1). Genital warts (condylomata acuminata and giant condylomata) consistently contain human genital papillomavirus (HPV) type 6 or type 11 (2, 3). The viral genomes persist exclusively as extrachromosomal circular molecules (4, 5), and they are transcriptionally active (5). Their biological activity in flat condylomata of the cervix, where both types are also occasionally seen (6), is not known. Integrated HPV-18 DNA has been detected in some cervical tumor biopsies and in HeLa cells (7). In two condylomata acuminata, however, HPV-18 DNA has been found in an extrachromosomal and in a transcriptionally active state (ref. 5; unpublished data).

HPV-16 appears to be rather widespread in genital lesions. HPV-16 DNA was detected in a small number of condylomata acuminata (8) and Buschke–Löwenstein tumors (unpublished data), in 80% of bowenoid lesions (9), and in atypical flat condylomata of the cervix displaying abnormal mitotic figures (6). The latter are regarded as possible precursers of cervical carcinomas (6, 10, 11). Neoplastic changes of the cervix are known to arise in close topological proximity to the atypical flat condylomata (12–14). It is suspected that papillomaviruses cause the appearance of the atypical condylomata (15); thus, HPV-16-containing lesions involve a relatively high risk (6, 15). Moreover, the fact that HPV-16 genomes were detected in as many as 60% of human cervical tumor biopsies (8) warrants a detailed investigation of their possible etiological role in human genital cancer. Therefore, we have analyzed both the physical state and the transcriptional activity of HPV-16 genomes in human cervical tumor biopsies.

MATERIALS AND METHODS

Biopsy Material. Biopsy specimens from human cervical tumors were provided by the Universitätsfrauenklinik Heidelberg. The invasive carcinomas included the clinical stages IB (CC328, CC527) and IVB (CC357, CC511).

Extraction of Nucleic Acids from Human Tumor Biopsies. High molecular weight DNA and RNA were prepared from human cervical tumor biopsies according to the method of Krieg *et al.* (16).

DNA and RNA Blot Hybridization. Tumor DNAs were digested by using various restriction enzymes, separated on agarose gels (10 μg of DNA per track), transferred to nitrocellulose filters, and hybridized under stringent conditions to ^{32}P-labeled nick-translated cloned HPV DNAs (1×10^6 cpm/ml) as described (17). RNA blot hybridization using methylmercury hydroxide gels was performed as described (18). Filters were then washed, dried, and exposed for 3–21 days to XAR-5 x-ray films (Kodak) with intensifying screens.

Synthesis of cDNA. The synthesis of ^{32}P-labeled oligo(dT)-primed cDNA was as described (5).

Plasmids. *Pst* I subfragments of HPV-16 were cloned into the *Pst* I site of pBR322 according to standard procedures. Cloned DNAs from HPV-6, -11, -16, and -18 were a gift from L. Gissmann (German Cancer Research Center). As myc-specific probe, the 1.4-kilobase (kb) *Cla* I/*Eco*RI fragment was used containing the third exon of the human c-*myc* gene (19), and as mos-specific probe, the plasmid pHM2A was used containing a 2.75-kb *Eco*RI fragment of the human c-*mos* protooncogene (20).

RESULTS

The Physical State of HPV-16 DNA in Cervical Carcinomas. A total of six tumor biopsies were screened with radiolabeled cloned DNA probes for the presence of HPV genomes. Four specimens contained HPV-16 DNA. DNA sequences of HPV-6, -11, or -18 were not detectable as shown by Southern blot analysis under stringent hybridization conditions, excluding cross-hybridization with other HPV sequences. Patients with tumors CC511 and CC527 (both of which contained HPV-16 DNA) had normal biopsy material taken from cervix and uterus, which was analyzed for the presence of HPV DNA sequences; in both cases, however, we failed to find HPV DNA (data not shown).

To determine whether the HPV-16 DNA was integrated into the host-cell genomes, we compared uncleaved tumor DNA samples (Fig. 1, lanes a, d, g, and k) with preparations that had been treated with the "no-cut" restriction endonuclease *Sst* I (lanes b, e, h, and l). In the undigested DNA preparations obtained from the four tumor specimens, the HPV-16 sequences migrated together with the high molecular weight fractions of DNA. After digestion with *Sst* I the HPV-16-specific signals were still larger than unit-length linear HPV-16 DNA (*Fo* III). Treatment of the DNA samples with the "single-cut" enzyme *Bam*HI, on the other hand, resulted in the appearance of two signals (lanes c, f, i, and m). These data document integration of the HPV-16 DNA sequences into the host-cell genome. We have not obtained any evidence for the existence of extrachromosomal HPV-16 DNA sequences in the four tumors.

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Abbreviations: HPV, human papillomavirus; BPV, bovine papillomavirus; kb, kilobase(s); bp, base pair(s).

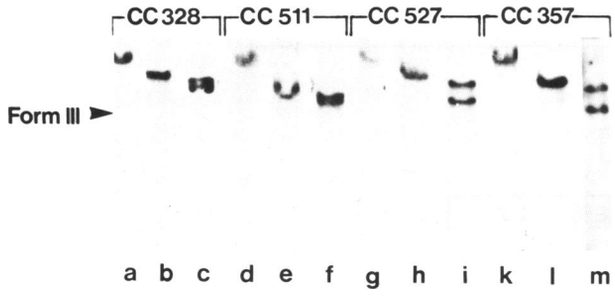

FIG. 1. Detection of HPV-16 DNA sequences in human cervical tumor biopsy specimens. The untreated or restriction enzyme-digested DNA samples (10 μg per track) were separated on a 1% agarose gel, transferred to nitrocellulose filter, and hybridized under stringent conditions to ^{32}P-labeled nick-translated cloned HPV-16 DNA (1×10^6 cpm/ml; $2–3 \times 10^8$ cpm/μg). Lanes contained the following: a–c, DNA of tumor CC328; d–f, DNA of tumor CC511; g–i, DNA of tumor CC527; k–m, DNA of tumor CC357. The untreated tumor DNAs were loaded in lanes a, d, g, and k, while in lanes b, e, h, and l the tumor DNAs were digested with *Sst* I, an enzyme that cuts outside the HPV-16 genome (K. Seedorf and W. Roewekamp, personal communication). Lanes c, f, i, and m contained the tumor DNAs after cleavage with *Bam*HI, cleaving the HPV-16 genome once within the late region (see Fig. 3). Sequences with homologies to HPV-16 were visualized in autoradiographs that had been exposed for 2 weeks.

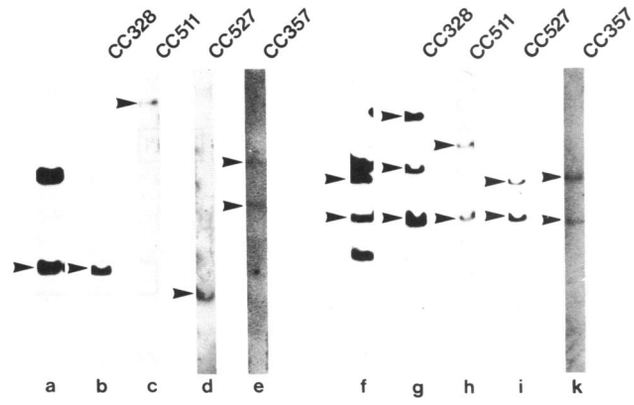

FIG. 2. Mapping of the integration areas on the HPV-16 genome. *Pst* I-cleaved DNA samples of tumors CC328, CC511, CC527, and CC357 were fractionated on a 1.4% agarose gel, transferred to nitrocellulose, and hybridized to a 1050-bp HPV-16 subfragment (lanes b–e) or to a hybrid plasmid containing two HPV-16 subfragments of 1750 and 2800 bp (lanes g–k). Lanes a and f contained the authentic HPV-16 fragments (1050 bp in lane a, 1700 and 2800 bp in lane f, indicated by arrows) that were used for the hybridization (additional bands in lanes a and f are derived from pBR322 DNA sequences). All fragments within the tumor DNAs hybridizing to the ^{32}P-labeled HPV-16 subfragments are indicated by arrows (lanes b–e and g–k).

All tumors contained the HPV-16 DNA as single copies. This is concluded from the results obtained after digestion of the DNA with the single-cut enzyme *Bam*HI revealing only two off-sized signals in each case (lanes c, f, i, and m).

DNA was isolated from different sections of specimens taken from three of the tumors. The restriction enzyme cleavage pattern in each section was determined and was found to be identical in all sections from any one tumor, although the pattern varied from tumor to tumor. This observation provides compelling evidence for the monoclonal origin of the three cervical carcinomas (unpublished data).

To identify the areas in which the circular viral genomes had been opened before integration, cloned *Pst* I HPV-16 DNA fragments were used as probes for hybridization with the *Pst* I-digested tumor DNA samples (Fig. 2). Tumor CC328 contained the authentic 1050-base-pair (bp) fragment (lane b) and the 1750-bp fragment, but lacked the 2800-bp fragment (lane g). Instead of the latter, two new DNA fragments, >2800 bp long, appeared, corresponding to the off-sized bands in lane g. This confines the site of opening to the 2800-bp fragment. This site could be mapped more precisely by using the multicut enzymes *Ava* II and *Hae* III (data not shown). Tumors CC527 and CC357 displayed homologies with the 2800-bp and 1750-bp fragments (lanes i and k), but lacked the 1050-bp fragment (lanes d and e). In the case of tumor CC357, two off-sized fragments appeared (lane e), but the analysis of the DNA from tumor CC527 revealed only one off-sized band (lane d). The lack of a discernible second signal may result from the small size (100–150 bp) of the HPV-16 sequences in the putative fragment. Southern blots with other restriction enzymes positioned the site of opening of HPV-16 in tumor CC527 within the 1050-bp *Pst* I fragment (data not shown). By double-digestion with *Apa* I/*Pst* I, the integration site on the HPV-16 genome in tumors CC357 and CC527 could be mapped more precisely within the 1050-bp fragment (data not shown). In tumor CC511, the 1050-bp and 2800-bp fragments, which are known to be adjacent to each other on the HPV-16 genome (K. Seedorf and M. Roewekamp, personal communication), were not present (lanes c and h). In each case, only one off-sized signal was detectable (lanes c and h), indicating the loss of one *Pst* I site. The recognition sequence of the single-cut enzyme *Hin*cII, 500 bp away from the missing *Pst* I site, was also absent, and further analysis (data not shown) revealed a deletion of at least 1900 bp of the HPV-16 genome in the tumor CC511. It should be added that the lack of authentic fragments provides further evidence for the absence of tandemly integrated full-length HPV-16 genomes.

A synopsis of the areas within which the HPV-16 genomes were opened and the positions of the open reading frames (K. Seedorf and W. Roewekamp, personal communication) is depicted in Fig. 3. It may be seen that a large deletion in tumor CC511 of at least 1900 bp affects the open reading frames E1, E2, E4, and E5. The latter were shown to be covered by the main bodies of the early mRNA species in the case of the related HPV-6b in genital warts (5). In tumor CC328, the viral genome was opened within the early region, near the 3′ end of the open reading frame E1, while in tumor CC527 the HPV-16 DNA was interrupted at the 3′ end of the early region. Tumor CC357, in contrast, possesses a complete early region of HPV-16. The site of opening was located beyond the putative early polyadenylylation site (K. Seedorf and W. Roewekamp, personal communication), at the beginning of the putative late region.

Expression of the Integrated HPV-16 Genomes. The RNA preparations from the HPV-16 DNA-containing tumors were examined for the presence of HPV-16-specific RNA sequences by the RNA blot procedure. In tumor CC328 only, three mRNA species of 1.4, 1.6, and 1.8 kb were identified, albeit in very low quantities (Fig. 4*A*). Tumors CC511, CC527, and CC357 did not contain detectable RNA with homology to HPV-16 DNA (Fig. 4*A*). Despite the fact that the analyses were carried out repeatedly, with different sections of the tumors used for the RNA preparations, we consistently failed to detect HPV-16-specific RNA sequences. The preparations from tumors CC357 and CC511 permitted enrichment for polyadenylylated RNA sequences. However, even after assaying 5 μg of poly(A)$^+$ RNA per lane, again no homology to HPV-16 was found (data not shown).

There are several possible explanations for the apparent absence of HPV-16 transcripts from the three tumors. One trivial explanation might be that the RNA had been degraded either before or during the extraction procedure. This,

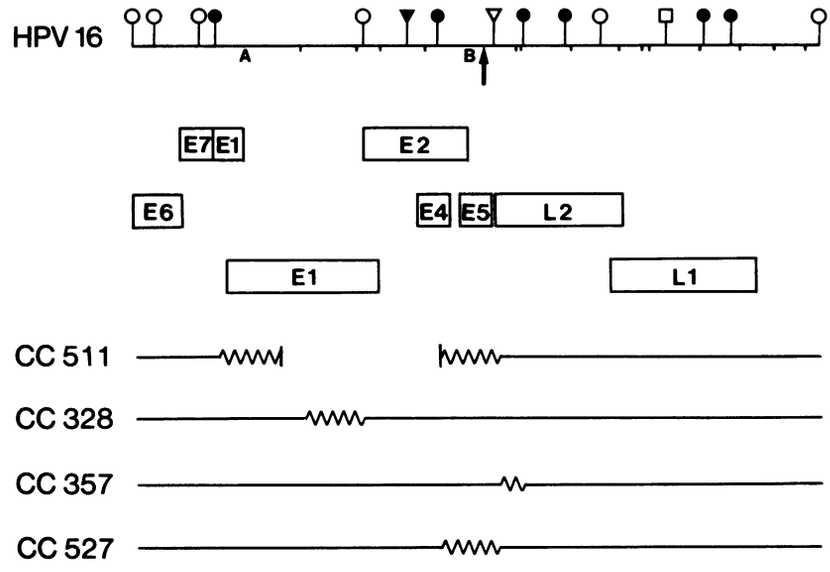

FIG. 3. Organization of HPV-16 genomes in cervical carcinomas. (*Upper*) Linear map of HPV-16 (7900 bp) with the cleavage sites of the single-cut enzymes *Apa* I (▽), *Bam*HI (□), *Hin*cII (▼) and the multicut enzymes *Pst* I (●), *Ava* II (○). Arrow indicates the putative early polyadenylylation signal (K. Seedorf and W. Roewekamp, personal communication). (*Middle*) Putative open reading frames (K. Seedorf and W. Roewekamp, personal communication). (*Lower*) Organization of the HPV-16 DNA sequences in the four cervical tumors CC511, CC328, CC357, and CC527 is indicated. Horizontal lines indicate the HPV-16 sequences present in the tumors; wavy lines span the region where the HPV-16 genome was opened before integration. The interrupted region in tumor CC511 symbolizes the minimum region of the HPV-16 DNA, which is deleted within this tumor.

however, was ruled out by the demonstration of the two c-*myc* transcripts of 2.2 and 2.4 kb (21) in the HPV-16 DNA-containing tumors after hybridization of the tumor RNA preparations in RNA blots with the cloned human c-*myc* oncogene DNA (Fig. 4*B*). The presence of c-myc mRNA which shows an extreme instability ($t_{1/2}$, 10 min) in normal and transformed human cells (21), renders possible the detection of very unstable messengers. In addition, in

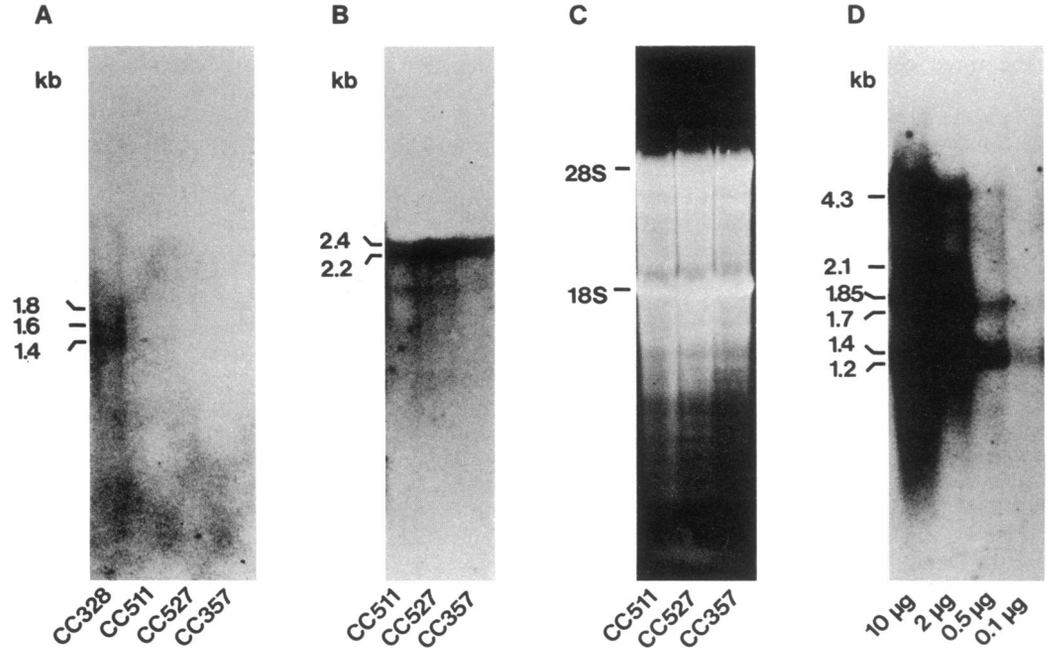

FIG. 4. RNA blot analysis of RNA from cervical carcinomas and determination of the level of detection in RNA blotting. (*A*) Samples of total RNA from cervical tumors (20 µg per track) were fractionated on a 1.4% methylmercury hydroxide gel and blotted as described (18). The filter was hybridized to ^{32}P-labeled cloned HPV-16 DNA (1.5×10^6 cpm/ml; 3×10^8 cpm/µg). Sequences with homologies to HPV-16 were visualized in autoradiographs that had been exposed for 3 weeks. (*B*) Tumor RNAs (10 µg per track) from three tumors were separated as described in *A*. The filter was hybridized to the cloned 3′ exon of human c-*myc* DNA (19). Autoradiograms were exposed for 3 days. (*C*) Tumor RNAs from *B* were visualized by staining the gels with ethidium bromide before blotting. (*D*) Various amounts of total RNA from ID13 cells were separated as in *A*. Lanes contained 10 µg, 2 µg, 0.5 µg, and 0.1 µg of ID13 RNA. In each case, the total amount of 10 µg of RNA per lane was established by the addition of 3T3 cellular RNA to the ID13 RNA. BPV-1-specific RNA species were visualized after exposure of the autoradiographs for 10 days.

ethidium bromide-stained gels, all three RNA preparations displayed the presence of the undegraded 28S and 18S ribosomal RNA (Fig. 4*C*). It might also be assumed that the CC511, CC527, and CC357 tumors did contain HPV-16-specific transcripts, but in quantities that had escaped our detection. With this in mind, we determined the level of detection of specific mRNA species in RNA blot analysis by using the bovine papillomavirus type 1 (BPV-1) transcripts in the BPV-1-transformed ID13 mouse cell line. In this system, the number of viral transcripts was shown to comprise 15–30 molecules per cell (22). Serial dilutions of total RNA from ID13 cells were therefore subjected to RNA blot analysis. We were able to detect the BPV-1-specific signals, even in samples that had been diluted to a ratio of 1:100 (Fig. 4*D*). This means that even if transcripts are present in only one of two or three cells, they can still be detected by our method.

Similar experiments using the simian virus 40 (SV40)-specific transcripts from the well-studied SV40-transformed 3T3 cells for calibration confirmed this level of detection (data not shown). Thus, if the carcinomas had contained one or more HPV-16-specific transcripts per cell, these would not have been overlooked.

Another, even more sensitive, approach was used for detection of HPV-16-specific polyadenylylated RNA species. Tumor RNA samples served as templates for oligo(dT)-primed reverse transcription of polyadenylylated RNAs, as described recently (5). The ^{32}P-labeled cDNAs were then hybridized in dot blots to cloned HPV-16 and cellular DNA fragments. The cDNA probes from all four tumors hybridized to well-defined cellular genes, such as human c-*myc* and actin (Fig. 5). With this method, which in our hands is 2–3 times more sensitive than the RNA blot analysis, we failed to detect HPV-16-specific polyadenylylated RNA sequences in tumors CC357, CC511, and CC527, whereas the cDNA prepared from tumor CC328 gave a strong hybridization signal (Fig. 5). This method, however, does not exclude the presence of HPV-16 mRNA missing viral poly(A) sequences, such as hybrid mRNAs with cellular sequences at the 3′ end.

Additional RNA blots were done by using ^{32}P-labeled cRNAs as hybridization probes (23) transcribed from two plasmids with HPV-16 inserts covering the E6/E7 and the E2/E4/E5 region, respectively. By this procedure, which significantly increases the sensitivity of the RNA blot analysis by at least a factor of 10 (23), we were able to detect HPV-16-related transcripts only in tumor CC328, while tumors CC357, CC511, and CC527 lacked any detectable HPV-16-specific RNA (data not shown).

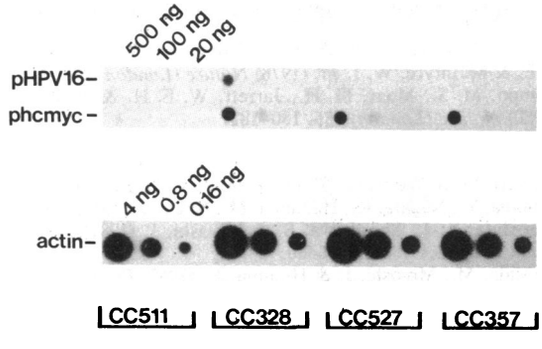

FIG. 5. Search for HPV-16 RNA in human cervical tumors by reverse transcription of polyadenylylated tumor RNAs. Five micrograms of total tumor RNA was used as template for the synthesis of oligo(dT)-primed ^{32}P-labeled cDNA by reverse transcription. The labeled cDNAs (0.2×10^6 cpm/ml) from the tumors CC511, CC328, CC527, and CC357 were hybridized in dot blots to decreasing amounts of pHPV-16 plasmid DNA (500 ng, 100 ng, 20 ng, respectively), cloned human c-myc DNA (500 ng, 100 ng, 20 ng, respectively), and cloned actin DNA from rat (4 ng, 0.8 ng, 0.16 ng, respectively).

DISCUSSION

The purpose of the work presented here was the analysis of the functional state of HPV-16 genomes in human cervical carcinomas. Six tumor specimens were available for our study, four of which contained HPV-16 DNA as a single copy exclusively in an integrated state. Despite the presence of the HPV-16 DNA, it was possible in only one of the four tumor specimens to reveal a transcriptional activity of the HPV-16 DNA.

The integration pattern of the HPV-16 genomes was found to be different in all four tumors, suggesting random integration events. The identification of the organization of the HPV-16 genomes should provide clues relevant to their function in the integrated state. We have therefore attempted to map the region within which they had been opened prior to their integration. In tumor CC328, the arrangement of the integrated HPV-16 DNA was such that the intact E2/E4/E5 region was found to be separated from the putative promoter region. Nevertheless, this tumor turned out to be the only case in which we detected HPV-16-specific transcripts. They were shown to utilize the polyadenylylation signal at the 3′ end of the "early" region, and must have been initiated, therefore, by a cellular promoter located within the flanking cellular DNA. Whether these messengers that were present only in low amounts were translated into biologically active proteins is unknown. The significance of the presence of HPV RNA in cervical carcinoma specimens deserves a critical evaluation because of the close proximity of neoplastic tissue to the flat condylomata (12–14). Contaminations of carcinoma specimens with condyloma material have recently been reported, with squamous carcinoma biopsies containing small areas of condyloma-like tissue (24) and the HPV-specific signals in the *in situ* hybridization experiments resulting from condyloma cells.

A rather severe alteration in the HPV-16 genome was detected in tumor CC511, where a deletion was encountered that encompassed at least 1900 bp of the E1/E2/E4/E5 region (Fig. 3). The E2/E4/E5 region was shown to be essential for the transformation of tissue culture cells by BPV-1 (25, 26). The main bodies of the early BPV-1 and HPV-6 mRNA species are transcribed from this area. In the case of BPV-1, these mRNA species were found in transformed tissue culture cells, in bovine warts, and in hamster tumors (18, 27), and the corresponding HPV-6 early mRNAs were detected in benign genital tumors (5). As the organization of the HPV-16 genome bears a close relationship with BPV-1 and HPV-6b (K. Seedorf and W. Roewekamp, personal communication), it is reasonable to assume that its E2/E4/E5 region may be endowed with similar biological properties. This, however, although not conclusive, makes it rather unlikely that the deleted HPV-16 genome was responsible, by virtue of its biological activity, for the maintenance of the malignant state of tumor CC511. This suggestion is corroborated by the absence of detectable HPV-16-specific RNA sequences. However, recent data on BPV-1 showed that the E6/E7 region alone can transform C127 mouse cells (28). The presence of putative HPV-16 RNA species transcribed from the E6/E7 region in tumors CC357, CC511, and CC527 can be ruled out by the RNA blot analysis (Fig. 4*A*) and by blot experiments with ^{32}P-labeled cRNA from the E6/E7 region (data not shown).

In the apparently undeleted HPV-16 genome in tumor CC527, the 3′ end of the E2/E4/E5 region was also affected by the integration event, and we again failed to detect HPV-16-specific transcripts. It is especially interesting that

tumor CC357 lacked detectable HPV-16 transcripts, despite the availability of a complete and uninterrupted early region.

Our data suggest that the integrated HPV-16 genomes are biologically inactive in three of the four tumors. On the other hand, malignant conversion of HPV-induced human diseases (genital warts, laryngeal papillomas, epidermodysplasia verruciformis) has frequently been reported in recent decades (29). Most women with cervical cancer had been infected by papillomaviruses; 93% of those patients had antibodies against a papillomavirus-specific antigen (30).

The contention that papillomaviruses and extrinsic factors interact synergistically in tumor development is generally accepted (29). BPV-4, for example, induces papillomas in the alimentary tract of cattle harboring episomal BPV-4 genomes (31, 32). The conversion of papillomas into carcinomas was demonstrated histologically within the same lesions, but it took place only in conjunction with the ingestion of bracken fern, which contains a carcinogen. These papilloma-derived malignant tumors, however, lacked BPV-4 DNA sequences (ref. 29; M. S. Campo, personal communication). Hence, it appears that BPV-4, while obviously not responsible for the maintenance of the carcinomas, nevertheless provides an essential prerequisite for their appearance by inducing the papillomas from which they arise. On the other hand, in the cottontail rabbit papillomavirus-induced carcinomas of domestic rabbits, viral DNA and mRNAs were present in the tumors (33, 34). A situation similar to carcinomas in cattle may exist in the HPV-16-containing cervical carcinomas, but with one difference: that the HPV-16 DNA is maintained in the tumor cells owing to its integrated state.

Human T-cell leukemia virus (HTLV-1) is thought to be the etiological agent of human adult T-cell leukemia (35, 36). As in the case of HPV-16 reported here, Gallo and co-workers failed to detect HTLV-1-specific transcripts in four of five specimens of primary leukemic cells from patients with adult T-cell leukemia despite the presence of integrated HTLV-1 proviral DNA (37).

These apparent contradictions—that is, the availability of tumor virus genomes (in the case of HPV-16 and HTLV-1) and the absence of viral transcripts in tumor cells bearing these sequences—can be reconciled in our view by the following assumptions:

It is conceivable that the HPV-16 genomes may be involved in a rather indirect fashion in the genesis of the tumors, in that through the formation of flat atypical condylomata for which they are considered to be responsible, they provide one of the most important prerequisites for the formation of the carcinomas. The monoclonality of the tumors, as implied by our own and by Gallo's data (37), clearly refers the factors accounting for the malignant conversion to an extremely rare event. Thus, we consider HPV-16 a potential hazard in the first steps of the multistage development of human cervical cancer. Continuous expression of the HPV-16 genomes is not required, however, for maintenance of the malignant state.

Note Added in Proof After this manuscript was submitted for publication, Schwarz and coworkers described the structure and transcription of HPV-18 in cervical carcinoma cell lines, and, in addition, the presence of HPV-16-transcripts in two of three HPV-16 DNA-containing cervical carcinoma biopsies (38).

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