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Analysis of the Physical State of Different Human Papillomavirus DNAs in Intraepithelial and Invasive Cervical Neoplasm

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The integration of human papillomavirus (HPV) DNA into the human genome has been generally accepted as a characteristic of malignant lesions. To gain a better understanding of this phenomenon, genomic DNA from 181 cervical biopsy specimens was isolated and analyzed for HPV type and physical state of the HPV genome. These specimens represented the full spectrum of cervical disease, from condyloma to invasive carcinoma. Discrimination between integrated and episomal HPV DNA was accomplished by the detection of HPV-human DNA junction fragments on Southern blots. In most cases in which ambiguous Southern blot results were obtained, the specimens were reanalyzed by two-dimensional gel electrophoresis. Of the 100 biopsy specimens of cervical intraepithelial neoplasia analyzed, only 3 showed integrated HPV DNA, in contrast to 56 (81%) of 69 cervical carcinomas ($P < 0.001$) showing integrated HPV DNA. Of the 40 carcinomas containing HPV 16 DNA, 29 (72%) had integrated HPV DNA, of which 8 (20%) also had episomal HPV DNA. In 11 (27%) cancers, only episomal HPV 16 DNA was detected. All 23 HPV 18-containing carcinomas had integrated HPV DNA, and 1 also had episomal HPV 18 DNA. The difference between HPV types 16 and 18 with respect to frequency of integration was statistically significant ($P < 0.01$). The results of this study indicate that detectable integration of HPV DNA, regardless of type, occurs infrequently in cervical intraepithelial neoplasia. The absence of HPV 16 DNA integration in some carcinomas implies that integration is not always required for malignant progression. In contrast, the consistent integration of HPV 18 DNA in all cervical cancers examined may be related to its greater transforming efficiency in vitro and its reported clinical association with more aggressive cervical cancers.

Cervical cancer is one of the most common malignant diseases in women worldwide. A factor that appears to play an important role in the development of this cancer is infection with certain types of human papillomavirus (HPV), including types 16, 18, 31, 33, 35, and 45 (4, 11, 20, 21, 23, 25). In contrast, other HPVs which infect the anogenital tract, such as types 6, 11, 42, 43, and 44, are not associated with cervical cancer (5, 19, 21). The ready accessibility of cervical tissues and the relative ease with which keratinocytes can be immortalized by specific HPV DNAs has led to considerable in vivo and in vitro investigation of the role that these viruses play in the pathogenesis of cervical neoplasms. There is clear evidence that HPV DNA is maintained as an episome in benign productive infections (12, 17). In contrast, integrated HPV DNA has often been identified in biopsy specimens of cervical cancers (10, 12, 16), in cell lines isolated from cervical neoplasms (1, 31), and in immortalized human keratinocytes (2). Integration usually disrupts the E1 or E2 open reading frames, potentially leading to a deregulation of viral gene expression (1, 7, 8, 26). These observations fostered the attractive hypothesis that disruption of the E2 repressor allows overexpression of the E6 and E7 oncoproteins, which might promote the development of neoplasia (8, 26). Recent studies, however, failed to detect evidence of HPV integration in at least 30% of the analyzed cancers (13, 22), thus questioning the absolute dependence of malignant progression on HPV integration.

Two groups (10, 17) have reported the detection of integrated HPV genomic sequences in over 50% of cervical intraepithelial neoplasm (CIN), suggesting that this phenomenon might have clinical value as a prognostic indicator of the subset of preinvasive lesions likely to develop into cancer. Furthermore, articles to date have focused primarily upon HPV 16-containing tumors (8, 10, 12, 13, 17, 22), thereby projecting a limited perspective. This study was undertaken to determine the frequency of HPV integration in a spectrum of premalignant and malignant cervical lesions and to study the integration profiles of different cancer-associated HPV types.

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MATERIALS AND METHODS

Tissue specimens. Samples of CIN and cervical cancer were obtained from women with well-developed lesions within the lower genital tract who came to gynecologic facilities in five geographic regions: Detroit, Mich.; Atlanta, Ga.; Washington, D.C.; Hamilton, Ontario, Canada; and Seattle, Wash. Samples of cervical tissue were also taken under colposcopic control from areas with no apparent disease. One hundred and eighty-one specimens containing HPV DNAs of interest were selected from a larger set of specimens, whose general demographic and clinical characteristics are described elsewhere (15, 17a). Biopsy specimens were bisected, and one portion was submitted for standard histopathologic diagnosis, while the other portion was frozen at −70°C for subsequent molecular analysis. Specimens which did not contain HPV DNA and those

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TABLE 1. Restriction endonucleases used in analyzing the physical state of HPV DNA in clinical specimens

Type of digest[a]	Endonuclease used with HPV type:										
	6	11	16	18	31	33	35	45	51	52	56
Noncut A	*Bgl*II	*Bgl*II	*Bgl*II	*Bgl*II	*Bgl*II	*Bam*HI	*Nco*I	*Bgl*II	*Bgl*II	*Hin*dIII	*Hin*dIII
Noncut B	*Kpn*I	*Nco*I	*Eco*RV	*Hin*dIII	*Bam*HI	*Eco*RI	*Hin*dIII	*Eco*RI	*Eco*RI	*Sph*I	*Eco*RI
Cut A	*Bam*HI	*Bam*HI	*Bam*HI	*Eco*RV	*Eco*RV	*Bgl*II	*Bgl*II	*Bam*HI	*Hin*dIII	*Eco*RV	*Bam*HI
Cut B	*Nco*I	*Hin*dIII	*Nco*I	*Nco*I	*Hin*dIII	*Eco*RV	*Eco*RV	*Hin*dIII	*Bam*HI[b]	*Eco*RI	*Sph*I
Cut C	*Eco*RV[b]	*Eco*RV[b]	*Sph*I	*Bam*HI[b]	*Nco*I[b]	*Nco*I[b]	*Bam*HI[b]	—[c]	*Nco*[b]	*Bgl*II[b]	*Eco*RV[b]

[a] Each specimen was subjected to digestion with five different enzymes which depended on the type of HPV DNA.
[b] Enzymes which cleaved the HPV genomes twice; all others indicated in the cut A, cut B, and cut C rows cleaved within the HPV genome only once.
[c] —, An additional enzyme which would cleave HPV 45 once or twice was not mapped.

which had HPV other than types 6, 11, 16, 18, 31, 33, 35, 45, 51, 52, and 56 were excluded from the study because of insufficient numbers of specimens or lack of association with cervical cancer.

DNA extraction, Southern blotting, and HPV typing. DNA was extracted from biopsy specimens or tissue culture cell lines (HTB 31 and SiHa, obtained from the American Type Culture Collection, Rockville, Md.) by proteinase K digestion and phenol extraction. Genomic DNA (2.5 to 10 μg) was digested with *Pst*I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), electrophoresed in 1% agarose gels, and transferred to Hybond N (Amersham) filters by the method of Southern (28). To assess probe performance, vector contamination, and hybridization stringency, we included known amounts of gel-purified HPV DNA fragments and vector DNA on each gel. Selected size fragments from HPV types 11, 16, 18, and 31 (at a concentration of approximately 30 pg per band) were also included as a molecular size standard. Virus typing was accomplished by hybridizing the Southern blots with probes specific for HPV types 6, 11, 16, 18, 31, 33, 35, 45, 51, 52, and 56 (4, 11, 18, 20, 21, 23, 24, 27). Some specimens were typed by a dot-blot method (Vira-Type; Life Technologies, Inc.) and subsequently verified by Southern blotting. Probes were prepared by radiolabeling gel-purified HPV DNA fragments with ^{32}P by using a random primer DNA labeling kit (Bethesda Research Laboratories). Southern blots were prehybridized for 2 h in hybridization solution without the probe and then hybridized overnight at 55°C (high stringency, T_m −25°C) in hybridization buffer containing 30% formamide, 5% sodium dodecyl sulfate (SDS), 1 M NaCl, 50 mM $NaPO_4$ (pH 7.4), 1 mM EDTA, 1% gelatin, 50 μg of tRNA per ml, and approximately 1×10^7 dpm of ^{32}P-labeled probe per ml. Three 20-min washes were performed at 65°C (high stringency, T_m −10°C) in 0.03× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5 mM $NaPO_4$ (pH 7.4)–1 mM EDTA–0.05% SDS. The moist filters were sealed in plastic pouches and subjected to autoradiography with intensifying screens at −70°C. Probe was removed prior to rehybridization by placing the filters in boiling 0.1% SDS–1 mM EDTA for 2 min, which was followed by rapid cooling in the same solution at approximately 20°C.

Integration analysis. A panel of five restriction endonucleases was used for each virus type, such that the first two enzymes would not cleave the corresponding HPV genome and the last three would cut the HPV DNA once or twice (Table 1). Biopsy specimens were first analyzed undigested and also after digestion with the enzymes appropriate to that HPV type, using conditions of high stringency in each instance.

For specimens which produced blots that were not easily

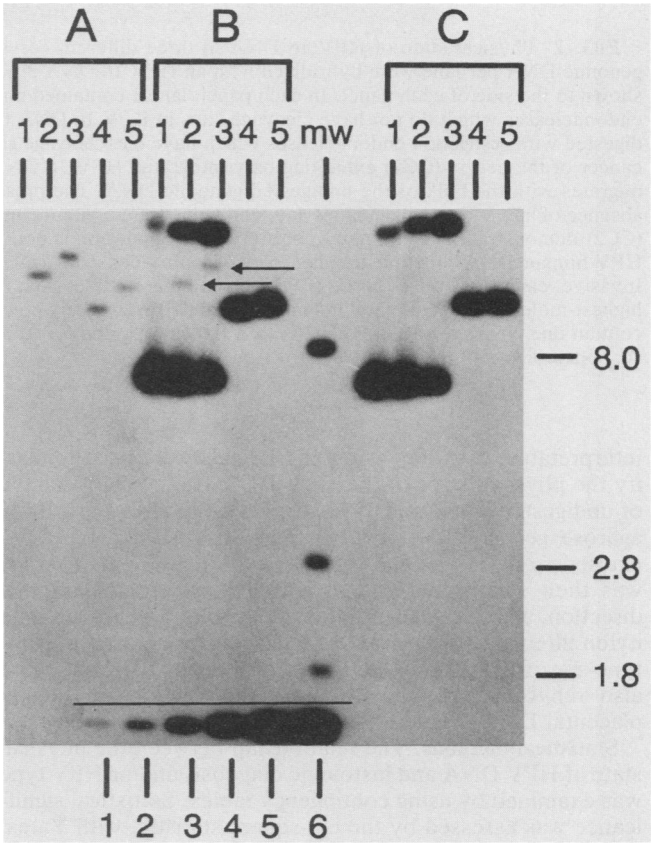

FIG. 1. Detection of integrated HPV 16 DNA in a model system. A Southern blot of DNA from human carcinoma cell lines combined with HPV 16 plasmid DNA (10.8 kb in size) was hybridized with an HPV 16 DNA probe. The sizes, in kilobases, of the molecular size standards (lane mw) are shown to the right. In each panel, lane 1 contained undigested DNA. The DNAs in lanes 2 and 3 were digested with restriction endonucleases which have no cleavage sites in HPV 16 DNA (*Bgl*II and *Eco*RV, respectively), and the DNAs in lanes 4 and 5 were digested with restriction endonucleases which have one cleavage site in HPV 16 DNA (*Bam*HI and *Stu*I, respectively). (A) 2.5 μg of SiHa DNA. (B) 2.5 μg of SiHa DNA plus 250 pg of HPV 16 plasmid DNA. Arrows indicate HPV-human DNA junction fragments not obscured by plasmid DNA. (C) 2.5 μg of HTB 31 DNA (a cervical carcinoma cell line with no detectable HPV DNA) plus 250 pg of HPV 16 plasmid DNA. Lanes 1 through 6 at the bottom contained full-length gel-purified HPV 16 DNA of 3, 6, 27, 81, 243, and 729 pg, respectively. Vector DNA (10 pg) (not visible) was used to verify that the probe was free of contaminating vector sequences.

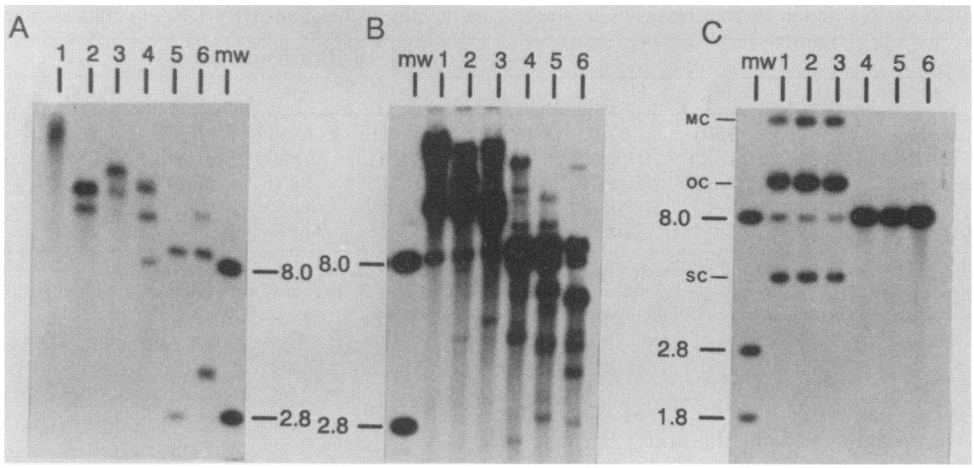

FIG. 2. Physical state of HPV 16 DNA in three different cervical cancer specimens. Southern blots containing approximately 5 μg of genomic DNA per lane were hybridized with an HPV 16 DNA probe. The sizes, in kilobases, of the molecular size standards (lane mw) are shown to the side of each panel. In each panel, lane 1 contained undigested DNA. The DNAs in lanes 2 and 3 were digested with restriction endonucleases which do not have cleavage sites in HPV 16 DNA (*Bgl*II and *Eco*RV, respectively), and the DNAs in lanes 4, 5, and 6 were digested with restriction endonucleases which have one cleavage site in HPV 16 DNA (*Bam*HI, *Nco*I, and *Sph*I, respectively). (A) Invasive cancer of the cervix (CC1) exhibiting only integrated HPV 16 DNA. The smear in lane 1 is characteristic of integrated HPV DNA, which migrates with the bulk of the undigested genomic DNA. The presence of more than two HPV-human junction fragments per lane and the absence of any 8-kb bands in lanes 4, 5, and 6 illustrate nontandem multicopy integration of the HPV DNA. (B) Invasive cancer of the cervix (CC2) demonstrating a complex mixture of episomal monomers, episomal multimers, and integrated HPV 16 DNA. The many different HPV-human DNA junction fragments evident in lanes 2 through 6 indicate the presence of multicopy integration of HPV 16 DNA. (C) Invasive cancer of the cervix (CC3) containing only episomal forms of HPV 16. The multimeric episomal DNA (visible as the highest-molecular-weight band in lanes 1, 2, and 3) was totally converted to an 8-kb linear molecular form upon digestion with enzymes that contain one cleavage site in HPV 16 DNA (lanes 4, 5, and 6). Abbreviations used in panel C: mc, multimeric circles; oc, open circles; and sc, supercoils.

interpretable, two-dimensional (2-D) gels were used to clarify the physical state of the HPV DNA (14). A 5-μg sample of undigested genomic DNA was electrophoresed in a 0.4% agarose gel at 25 V for 14 h, and the resulting lane was excised from the gel and recast within a 0.8% gel. This gel was then electrophoresed at a right angle to the original direction, using 60 V for 5 to 6 h. A 2-D blot was prepared on nylon filters and hybridized at high stringency with an HPV type-specific probe. As a further aid, these 2-D blots were also rehybridized with a human genomic probe (human placental DNA; Sigma).

Statistical methods. The relationship between the physical state of HPV DNA and histologic diagnosis and/or HPV type was examined by using contingency tables. Statistical significance was assessed by the chi-square statistic, with Yates' continuity correction as appropriate, using the BMDP package, release 89 (BMDP Inc., Los Angeles, Calif.).

RESULTS

Detecting integrated HPV 16 DNA in a model system. The ability of the Southern blot analysis to detect integrated HPV DNA against a strong background of episomal HPV DNA is demonstrated in Fig. 1. The supercoiled, open circular, and linear forms of the episomal HPV DNA (Fig. 1C) form a predictable pattern which is easily distinguished from the random pattern produced by HPV DNA from the human cervical carcinoma cell line SiHa, which contains one to two copies of integrated HPV 16 DNA per cell (1) (Fig. 1A). Despite an approximately 100-fold excess of plasmid DNA, integrated HPV sequences remained easily detectable in Fig. 1B. The episomal HPV DNA in this model system did not contain high-molecular-weight multimers; therefore, interpretation of Southern blots was relatively clear-cut.

Physical status of HPV DNA at different points in the pathologic spectrum. Southern blot hybridization allowed confident distinction between episomal, integrated, or episomal-plus-integrated HPV DNA patterns in 163 clinical specimens (Fig. 2), but confirmatory 2-D gel electrophoresis was needed in 18 instances (Fig. 3). Of these 18, 5 did not contain enough DNA for further analysis, and 1 specimen remained ambiguous even after 2-D analysis. The 12 latent HPV infections (normal tissue containing HPV DNA) and the 39 CIN 1 specimens displayed only episomal HPV DNA (Table 2). Both episomal and integrated HPV DNAs were detected in 3 of 61 CIN 2-CIN 3 specimens; all remaining CIN 2-CIN 3 biopsy specimens contained exclusively episomal HPV sequences (Table 2). In contrast, integration was documented in 56 (81%) of the 69 cancers, 47 (68%) showing exclusively integrated sequences and 9 (13%) having both episomal and integrated HPV DNA. Overall, these differences in the physical state of HPV DNA at various points in the pathologic spectrum were highly significant (Yates-corrected χ^2, $P < 0.001$). Comparisons of the various subsets within Table 2 showed statistically significant differences between invasive cancer and either normal-CIN 1 or CIN 2-CIN 3. Differences between normal-CIN 1 and CIN 2-CIN 3 were not significant.

Type-specific differences in integration patterns. The association of DNA integration with invasive cancers was seen regardless of HPV type (Fig. 4). Among the invasive cancers, however, differences were observed in the integration profiles of HPV 16 and HPV 18 DNA (Table 3 and Fig. 4). Of the 40 HPV 16-containing cancers, 21 (52%) revealed exclu-

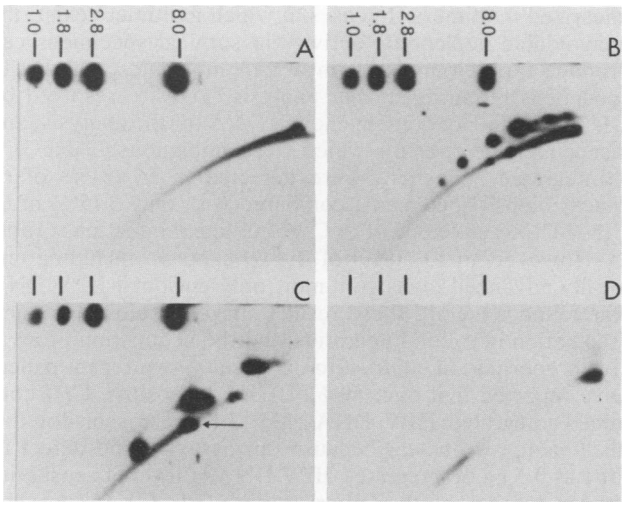

FIG. 3. 2-D gel electrophoresis of three different HPV 16-containing cervical cancers. Blots of 2-D gels containing approximately 5 μg of genomic DNA were hybridized with either labeled HPV 16 DNA (A, B, and C) or human DNA (D). Molecular size standards in kilobases (run in the first dimension) are shown above each panel. (A) Example of a specimen (CC1) containing exclusively integrated HPV 16 DNA. The only HPV 16 DNA recognized by hybridization in this specimen is in the lower arc (linear DNA), which spans the entire distribution of the human DNA (data not shown). (B) The HPV 16 DNA in this specimen (CC4) was present as both monomeric and multimeric episomal forms as well as integrated HPV DNA. The specimen shows hybridization of HPV 16 DNA in two arcs. The lower arc indicates linear DNA, whereas the upper arc indicates circular DNA forms. The hybridization signal in the lower arc encompasses the entire distribution of human DNA (data not shown); therefore, this specimen contained integrated HPV DNA as well as episomal HPV DNA. (C) This specimen (CC3) shows only episomal HPV 16 DNA. The distinct spots of hybridization in the upper arc indicate monomeric and multimeric episomal HPV DNA. Some linearized episomal DNA is visible in the lower arc (arrow), and the smear arcing down to the left of it represents some degradation of this DNA. (D) Membrane from panel C rehybridized with a probe of human placental DNA. This probe defines the boundaries of the human DNA and confirms that the hybridization signal from the HPV DNA probe (panel C) does not encompass all the linear human DNA present. Therefore, there is no integrated HPV DNA present in this specimen.

sively integrated HPV DNA, and another 8 (20%) displayed both integrated and episomal HPV DNAs. However, 11 (27%) specimens showed only episomal copies of the HPV genome. In contrast, 22 of 23 (96%) of the HPV 18-containing cancers revealed exclusively integrated HPV DNA and the remaining case had both integrated and episomal HPV DNA (Table 3). The difference in frequency of integration between the DNA of HPV types 16 and 18 is statistically significant (Yates-corrected χ^2, $P < 0.01$).

TABLE 2. Differences in physical state of HPV DNA, by histologic diagnosis[a]

Row	Histology	Episomal (%)	Episomal + integrated (%)	Integrated (%)
1	Normal-CIN 1	51 (100)	0 (0)	0 (0)
2	CIN 2-CIN 3	58 (95)	3 (5)	0 (0)
3	Invasive cancer	13 (19)	9 (13)	47 (68)

[a] Overall χ^2 for 3 × 3 table, 123.0; $P < 0.001$. Partial χ^2 for row 1 versus row 3, 77.6; $P < 0.001$. Partial χ^2 for row 2 versus row 3, 78.4; $P < 0.001$. Partial χ^2 for row 1 versus row 2 1.0; $P > 0.3$.

Among the six cancers containing DNA of less prevalent HPV types, integration profiles were as follows. Of two HPV 31 DNA-positive tumors, one contained integrated and one contained episomal HPV DNA. The single tumor with HPV 35 DNA displayed integrated HPV DNA. Of three HPV 45-associated tumors, two contained integrated and one contained episomal HPV DNA. The low frequency of these HPV types in cervical cancer specimens precluded statistical analysis; however, the findings of four cases with exclusively integrated HPV DNA and two cases with exclusively episomal HPV DNA broadly resemble integration profiles in HPV 16 cancers (Fig. 4).

Comparison of matched biopsy specimens from the same cancer. A second biopsy specimen (taken from a different part of the primary tumor) was available for analysis from 5 of the 69 cervical cancers. The initial biopsies from four of these five tumors (one containing HPV 16 DNA, two HPV 18 DNA, and one HPV 45 DNA) revealed exclusively integrated HPV DNA. In all four instances, identical integration patterns were seen in the second biopsy specimen, as illustrated for one pair of biopsy specimens in Fig. 5.

The remaining pair of biopsy specimens contained a complex mixture of monomeric and multimeric episomes of HPV 16 DNA (Fig. 6). Digestion with restriction endonucleases that linearize HPV 16 DNA converted the various multimeric forms into two discrete bands, both of which are greater than 8 kb (Fig. 6A, lanes 4 and 6). Fragments of this length indicate that the HPV DNA had been rearranged. In addition, digestion with *Nco*I did not produce the expected band pattern. Rather, *Nco*I appears to have cleaved the HPV 16 DNA twice, suggesting a duplication. Band patterns from the two biopsy specimens were similar, except that specimen B exhibited additional strong bands and a different distribution of high-molecular-weight multimers. Unfortunately, the presence of large numbers of high-molecular-weight episomes makes it impossible to determine whether the additional small bands in Fig. 6B, lanes 4, 5, and 6, were the result of integration events or of DNA rearrangement within the multimeric episomes.

High-molecular-weight and genomically rearranged episomes. Of the 22 cancers which contained episomal HPV DNA, 20 (91%) had high-molecular-weight forms of HPV DNA (episomes that were dimeric or longer). Of the 100 premalignant lesions with episomal HPV DNA, 72 (72%) had high-molecular-weight forms. However, high-molecular-weight episomes were also present in 10 (83%) of 12 latent HPV infections. The observed differences in the prevalence of high-molecular-weight episomes between the three pathologic categories were not statistically significant. In addition, episomal molecules with unit lengths exceeding 8 kb, indicative of genomic rearrangement, were seen in 3 (4%) of 69 cancers, 2 (2%) of 100 CIN, and 1 (8%) of the 12 latent HPV infections.

DISCUSSION

Accurately determining the physical state of HPV DNA in tumor biopsy specimens is not straightforward. The simultaneous existence of integrated and episomal HPV DNA in lesions poses the greatest challenge for detection, since concomitant episomal forms can mask the presence of relatively small amounts of integrated HPV DNA. The differentiation between rearranged multimeric HPV epi-

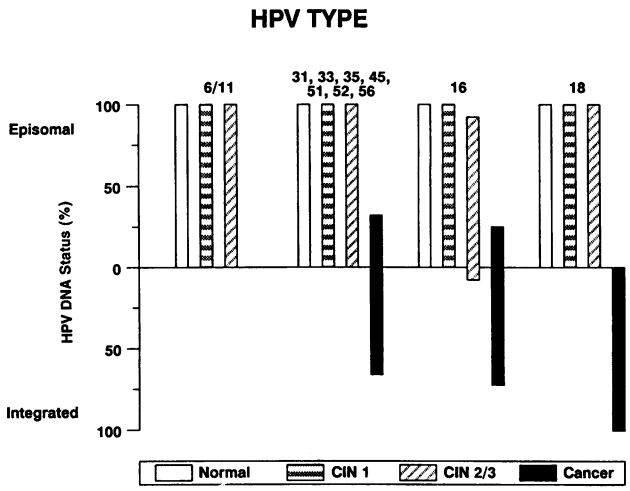

FIG. 4. Bar graph comparing episomal and integrated (includes concomitant episomal) HPV DNA, according to histologic diagnosis and HPV type.

somes and integrated HPV DNA is also problematic. HPV multimers containing deletions or duplications have been documented (6, 9) and might yield unpredictable fragment sizes in some assays, which could be easily mistaken for evidence of integration. To address these concerns, we used two distinct methods for detecting integrated HPV DNA in this study. The principal method used an HPV type-specific panel of restriction endonucleases and subsequent Southern blot analysis. The observed outcomes were predictable patterns of DNA fragments for episomal HPV and unpredictable patterns of DNA fragments in specimens containing integrated HPV molecules (Fig. 1 and 2). These unpredictable integration patterns were reproducible in different biopsy specimens from the same cancer (Fig. 5). The use of five different restriction endonucleases for each specimen reduced the chance of faint HPV-human junction fragments being obscured by episomal HPV DNA in the same specimen (Fig. 1). This analysis was able to define clearly the physical state of HPV DNA in 163 (90%) of the 181 specimens. The remaining 18 specimens yielded results which were difficult to interpret by this assay owing to the presence of both high-molecular-weight episomal forms of HPV DNA (multimers) and smaller HPV DNA fragments of unpredictable sizes. Therefore, these specimens were reanalyzed, when possible, by 2-D gels. 2-D gel electrophoresis was used only as a confirmatory technique since it cannot always distinguish between integrated HPV DNA and randomly linearized multimers. In cases in which multimers exist, the unavoidable nucleolytic activity in surgical specimens can produce large, randomly linearized molecules. Of the 18 specimens requiring further analysis, 11 were resolved by 2-D gels, 5 did not contain enough DNA for the analysis, and 1 specimen gave results which were ambiguous (Table 3).

Integrated sequences were detected in 56 (81%) of 69 cancer biopsy specimens, compared with only 3 (5%) of 61 CIN 2-CIN 3 and none of 51 CIN 1 or latent infections (Table 2). However, in 13 (19%) of the 69 cancers, including one locally advanced invasive tumor, only episomal HPV DNA was found. Overall, these results suggest a close temporal association between integration and the acquisition of invasive properties. In contrast to our results, two recent papers have reported that over 50% of HPV 16-positive CIN contained integrated HPV DNA (10, 17). The reason for this discrepancy is unclear because our assays could detect as little as 0.5 pg of integrated HPV DNA, a level of sensitivity similar to that of the other studies (10, 17). A possible explanation for the differences in various studies could be the result of inaccurate histopathologic diagnosis. Because the specimens in this study were not reviewed by a single pathologist, some variation in grading could have occurred. Hence, we grouped CIN 2 and CIN 3 into a single category to reduce subjectivity. It is generally accepted that the discrimination between CIN and cancers is highly accurate. All the cancers in this study were well advanced and clinically overt, thus the chances of misclassification were very low. Misclassification between CIN 1 and CIN 2-CIN 3 would have a negligible impact on the overall conclusions of the study. Despite the use of a rigorous Southern blot analysis method and confirmatory 2-D gels, it is still possible that the presence of some integrated HPV DNA was missed. However, this was less likely in specimens of more advanced cancers which could not have been contaminated with adjacent CIN and in which there were low levels of episomal HPV DNA (Fig. 2C). Furthermore, our ability to detect integrated HPV DNA in every cancer specimen containing HPV 18 diminishes the chances that we missed many occurrences of integrated HPV 16 DNA as a result of suboptimal study performance.

The integration event frequently disrupts the E1 or E2 open reading frame of the HPV genome and in this way may deregulate the E6 and E7 transforming genes (7, 8, 26, 31), leading to carcinogenesis. While these events may be important steps in some cancers, the present study shows that they are not obligatory, since 19% of the cancers analyzed contained only episomal HPV DNA. Fuchs et al. (13) also detected predominantly episomal HPV 16 DNA in 36% of cervical cancers. It is possible that rearrangement or mutations within these viral episomes resulted in functional

TABLE 3. Physical state of HPV DNA by virus type and histopathologic diagnosis [a]

Histology	n	HPV 6 or 11			HPV 16			HPV 18			HPV 31, 33, 35, 45, 51, 52, 56		
		Epi only	Int plus Epi	Int only	Epi only	Int plus Epi	Int only	Epi only	Int plus Epi	Int only	Epi only	Int plus Epi	Int only
Normal	12	3	0	0	3	0	0	3	0	0	3	0	0
CIN 1	39	8	0	0	12	0	0	8	0	0	11	0	0
CIN 2-CIN 3	61	5	0	0	28	3[b]	0	8	0	0	17	0	0
Cancer	69	n/a[c]			11	8[d]	21	0	1[b]	22	2	0	4

[a] Partial χ^2 for HPV 18 versus HPV 16, 7.66; $P < 0.01$. Abbreviations: Epi, episomal; Int, integrated; *n*, total number of specimens; n/a, not applicable.
[b] In two specimens, junction fragments could be from rearranged multimers.
[c] No cancers contained HPV type 6 or 11 DNA.
[d] In three specimens, junction fragments could be from rearranged multimers.

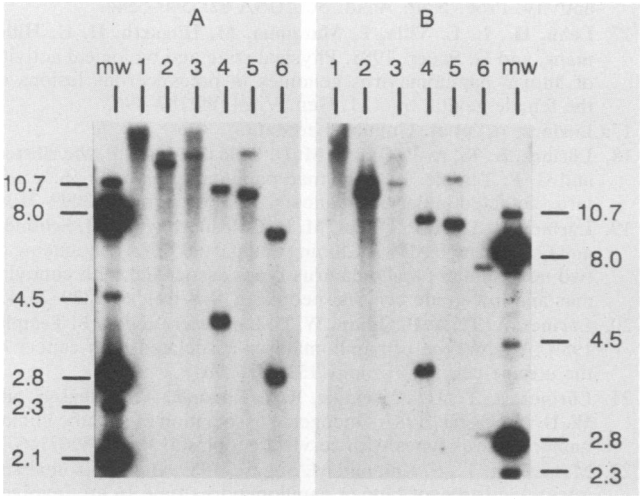

FIG. 5. Comparison of integrated HPV 16 DNA in separate biopsy specimens from one cervical cancer. A Southern blot containing approximately 5 μg of genomic DNA per lane was hybridized with an HPV 16 DNA probe. The sizes, in kilobases, of the molecular size standards (lanes mw) are shown. In each panel, lane 1 contained undigested DNA, DNAs in lanes 2 and 3 were digested with two separate restriction endonucleases which have no cleavage sites in HPV 16 DNA (*Bgl*II and *Eco*RV, respectively), and DNAs in lanes 4, 5, and 6 were digested with three separate restriction endonucleases which have one cleavage site in HPV 16 DNA (*Bam*HI, *Nco*I, and *Sph*I, respectively). (A) DNA isolated from the original biopsy specimen of a cervical cancer (CC5a). (B) DNA isolated from an independent biopsy specimen of a different region of the same cervical cancer lesion (CC5b).

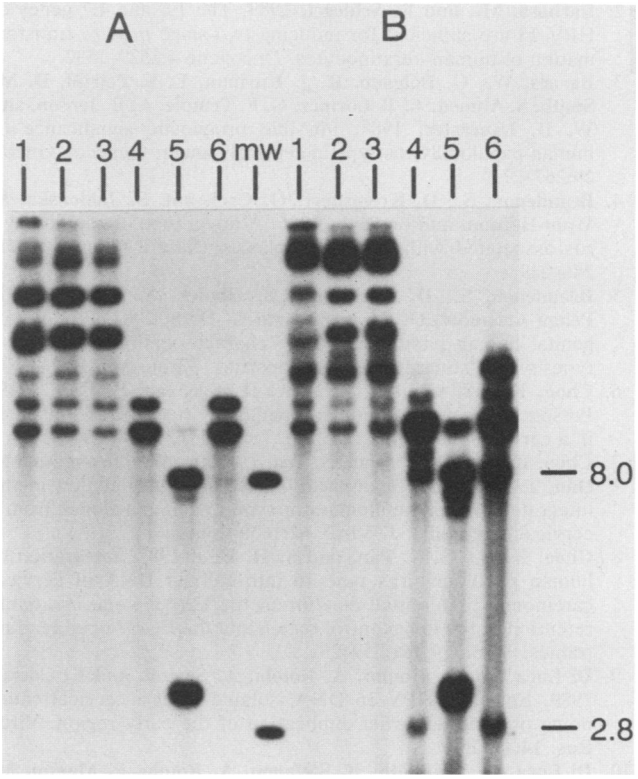

FIG. 6. Comparison of episomal HPV 16 DNA in separate biopsy specimens from one cervical cancer. Southern blots containing approximately 5 μg of genomic DNA were hybridized with an HPV 16 DNA probe. The sizes, in kilobases, of the molecular size standards (lane mw) are shown to the right. In each panel, lane 1 contained undigested DNA, lanes 2 and 3 contained DNA digested with two separate restriction endonucleases which do not have cleavage sites in HPV 16 DNA (*Bgl*II and *Eco*RV, respectively), and lanes 4, 5, and 6 contained DNA digested with three separate restriction endonucleases which have one cleavage site in HPV 16 DNA (*Bam*HI, *Nco*I, and *Sph*I, respectively). (A) DNA isolated from the original biopsy specimen of a cervical cancer (CC6a). (B) DNA isolated from an independent biopsy specimen of a different region of the same cervical cancer (CC6b).

alterations equivalent to integration. However, the presence of simple multimers or genomically rearranged HPV episomes was not predictive of malignancy, since these patterns were seen in many specimens of benign tissue.

The analysis of various HPV types in our study revealed a significant difference between HPV 16 and HPV 18 integration in cervical cancers ($P < 0.01$). Most previous reports investigating HPV integration directly (8, 10, 12, 13, 17, 22) focused only on HPV 16-containing lesions and did not present a detailed comparative analysis of the integration profiles of other HPV types. Different integration profiles of HPV 16 and 18 in cancers have also been suggested in a study which used the in situ hybridization technique (29). It was noted that most cancers containing HPV 18 consistently failed to express transcripts from the E2 region but continued to express E6, E7, and E1. In contrast, most HPV 16-containing cancers expressed the entire early region. The implication from these observations is that integration of HPV 18 in the E2 region coupled with a loss of all episomal molecules leads to the absence of E2 open reading frame expression. The observed consistent integration of HPV 18 DNA in cancers may relate to the more aggressive properties of this HPV type. HPV 18 was predicted to be clinically more aggressive than HPV 16 based on the relatively low prevalence of HPV 18 in CIN compared with cervical cancers (15, 21). In fact, recent studies have shown HPV 18-containing cancers to have a poorer prognosis than HPV 16-containing cancers (3, 30). HPV 18 DNA is also more efficient than HPV 16 at immortalizing and conferring serum resistance to human keratinocytes in vitro (2, 23a). It is possible that the more aggressive transforming capacity of HPV 18 results in a higher growth rate or level of genetic instability in these cells which favors HPV DNA integration. Alternatively, the DNA sequence of HPV 18 may in some manner enhance the integration event, resulting in an altered integrated HPV DNA which promotes rapid progression to aggressive malignancy. These two hypotheses may not be mutually exclusive.

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