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Cloning and Characterization of the DNA of a New Human Papillomavirus from a Woman with Dysplasia of the Uterine Cervix

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A previous analysis of 121 female genital tract lesions from the United States and South America had revealed that a large number contained DNA sequences that were weakly homologous to a panel of human papillomavirus (HPV) probes. The DNA sequences of one of these viruses have been molecularly cloned and shown to be a new type of HPV which is called HPV 31. Among the cloned HPV genomes, HPV 31 is most closely related to HPV 16. Although absent from all genital condylomas studied, HPV 31 was present in approximately 20% of the mild and moderate dysplasias and in 6% of the invasive cervical cancers.

Papillomaviruses have been identified in humans and in numerous animal species, and substantial evidence suggests that some of these viruses may be oncogenic (12, 23, 29). The human papillomaviruses (HPV), with more than 30 types, compose the largest group. Several members of this group of viruses, especially HPV types 16 (HPV 16) and 18, are commonly detected in the 85% or more of cervical carcinomas that contain HPV DNA (2, 10, 12, 29). In the remaining 10 to 15% of carcinomas, HPV-like DNA sequences have not been detected.

During a recent survey of over 100 anogenital biopsy specimens, we detected a new type of HPV, designated HPV 31. We report here the cloning and characterization of HPV 31 DNA from a cervical dysplasia and its frequency in female genital tract neoplasias.

Cellular DNA suitable for restriction enzyme digestion and subsequent Southern blot hybridization studies (Fig. 1) was prepared by proteinase K digestion and phenol extraction as described previously (21). The nucleic acid extract (1 to 10 μg) was digested with restriction endonuclease *Pst*I or *Bam*HI (Bethesda Research Laboratories, Inc.). Digested DNA samples were electrophoresed in 1% agarose gels, depurinated in situ, and transferred to nitrocellulose (34).

Hybridization and washes were modifications of a previously described procedure (21). The filters were baked under a vacuum for 30 min at 80°C and prehybridized in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (34)–0.5 mM EDTA–10× Denhardt solution (8)–50 μg of tRNA per ml–20 mM sodium phosphate buffer (pH 7.4). Low-stringency hybridization (T_m −37°C) with ^{32}P-labeled DNA prepared by nick translation (31) was performed in 1.0 M NaCl–28% formamide–50 mM TES [*N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid]–10× Denhardt solution–0.5 mM EDTA–20 mM sodium phosphate (pH 7.4) at 41°C. Low- and high-stringency washes were done in 1.1× SSC at 52°C and 0.03× SSC at 65°C, respectively. The wash solutions also contained 0.1 mM EDTA and 10 mM sodium phosphate (pH 7.4).

Our results demonstrated that cervical dysplasias and carcinomas contained a greater diversity of HPV types than previously reported. In contrast to earlier reports (5, 12, 35), HPV 6 and HPV 11 were detected relatively infrequently in cervical dysplasias, whereas several new types of HPV were present. The *Pst*I-digested DNAs from several biopsies displayed similar patterns of fragment sizes that suggested the presence of a common new type of HPV.

DNA extracted from a mild dysplasia that demonstrated this pattern was digested with several restriction enzymes useful for cloning in λ L47 (24). Undigested samples and samples digested with enzymes that did not cut the putative new HPV DNA (see Fig. 3) exhibited bands which migrated at the positions of supercoiled and open circular DNA. *Eco*RI digestion produced a single band at 8 kilobases, indicating that it cut the circular DNA at a single site. Consequently, *Eco*RI was used to digest 2 μg of the cellular DNA and 1 μg of the phage DNA for molecular cloning as previously described (1, 25). By screening 2×10^5 plaques, 20 independent positive clones were obtained. Several were analyzed and found to contain the same DNA fragment; one of these was chosen and recloned into pBR322 for further analysis. The cloned DNA of the new HPV, which was called HPV 31, was labeled with ^{32}P by nick translation and hybridized at high stringency to Southern blots containing *Pst*I-digested total DNA from the above sample and 120 other anogenital lesions. Some of the latter samples exhibited a *Pst*I pattern similar to that of HPV 31, and these also gave positive hybridization signals. This demonstrated, first, that the correct DNA had been cloned, and second, that it was present in several independent biopsies.

The distribution of HPV 31 in various types of biopsy samples obtained in the United States and South America is shown in Table 1. HPV 31 was detected in approximately 20% of mild and moderate dysplasias, in 6% of invasive cervical carcinomas, and in 0% of the condylomata acuminata examined.

The extent of homology between the new isolate and a number of other HPV was determined by Southern blot analysis at various stringencies (Fig. 2). For this experiment, equal amounts of cloned DNA of HPV commonly present in genital lesions (HPV 6, 11, 16, and 18) in addition to HPV 1, 2, 4, and 10 and a 100-fold lower amount of HPV 31 were immobilized on nitrocellulose membranes and probed with HPV 31. At low stringency, these DNA fragments were detected by HPV 31 to various degrees. The least homology was evident with HPV 1, 2, and 4. After probing at moderately high stringency, only HPV 16 still demonstrated signif-

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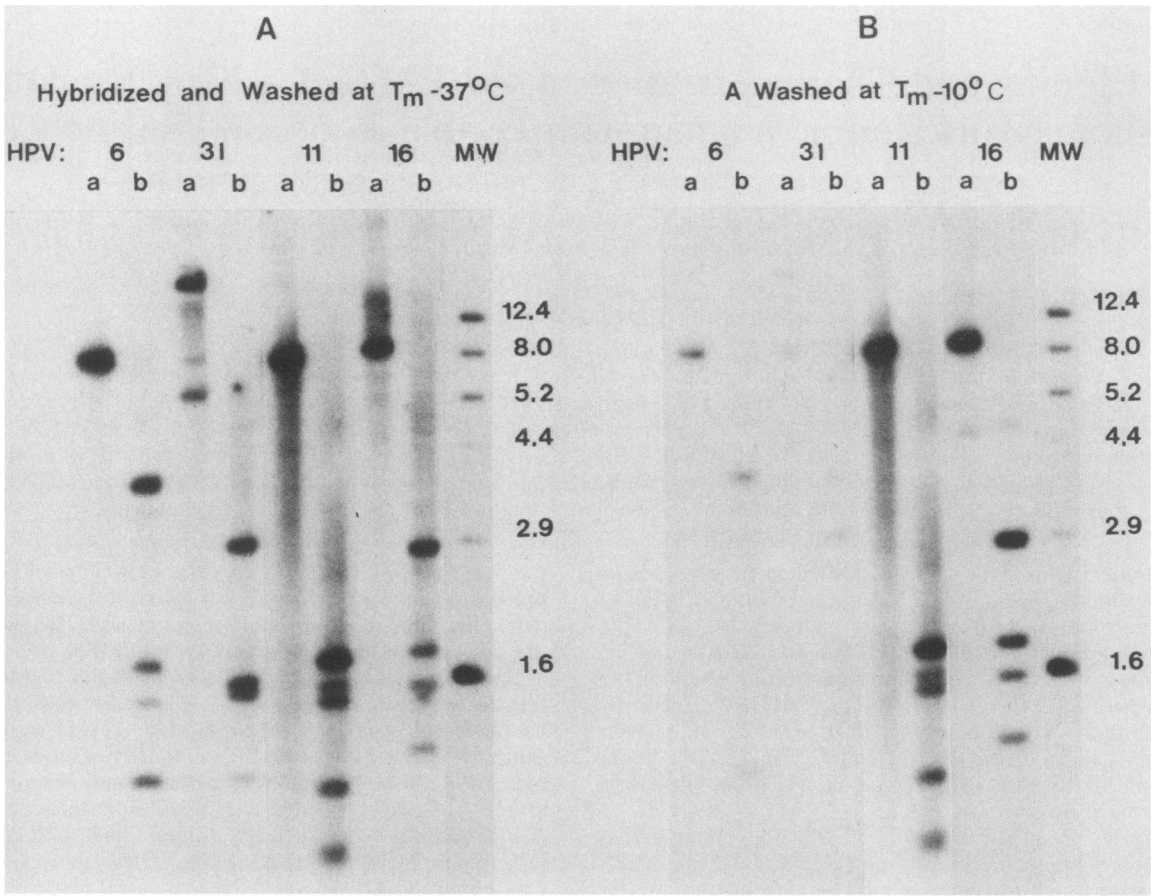

FIG. 1. Determination of types of HPV present in anogenital lesions. Total DNA extracted from four different lesions was characterized by Southern analysis at low and high stringency. The types of HPV DNAs detected are indicated at the top of each pair of lanes: HPV 6 and 11 (from two different condylomata acuminata), HPV 31 (from a mild dysplasia), and HPV 16 (from an invasive cervical carcinoma). Each sample was digested with *Bam*HI (lane a) and *Pst*I (lane b). Lane MW contains selected fragments of various HPV and pBR322 as molecular weight markers. (A) Autoradiograph of a Southern blot hybridization at low stringency (T_m −37°C) to a probe mixture of HPV 10 (19), 11 (14), and 16 (10) which was washed at T_m −37°C. (B) The same filter as shown in panel A after a wash at high stringency (T_m −10°C). It demonstrates, as expected, a significantly greater loss of signal in lanes containing HPV 6 or 31 than in lanes with HPV 11 or 16, which are homologous to the probes. The probes used in this experiment had greater than 2×10^8 dpm/μg of DNA.

icant hybridization; subsequent washing of this filter at T_m −10°C removed all signals except that to the HPV 31 control DNA.

In another experiment, HPV 31 was hybridized to Southern blots containing equal amounts (50 ng) of various other HPV DNAs (provided by L. Gissmann and G. Orth) which included HPV 3, 5, 7, 8, 9, 12, 13, 15, 17, 19, 20, 21, 22, 23, 24, 28, and 29 as well as the previously tested HPV 1, 2, 4, 6, 10, 11, 16, and 18. A probe of HPV 31 detected all of these viral DNAs to various degrees under low-stringency conditions (T_m −37°C); however, HPV 16, again, gave the strongest signal. In contrast, at high stringency (T_m −10°C), all of these DNAs failed to hybridize detectably even though the film was exposed for a longer period. The results were normalized with 5 ng of HPV 31 DNA spotted on one corner of the filters (data not shown).

Saturation hybridization analysis with hydroxylapatite chromatography also was performed, as previously described (22). Under standard conditions of stringency (T_m −25°C), HPV 31 and HPV 16 were found to share 35 to 40% sequence homology (data not shown). Based on previously established criteria (4), this result establishes HPV 31 as a new type of HPV.

A restriction endonuclease map was derived for HPV 31

TABLE 1. Distribution of HPV 31 in various clinical samples from the United States and South America

Source of sample	No. of isolates with HPV 31/no. of isolates tested (%) for samples from:					
	Normal cervix	Condylomata acuminata	Mild dysplasia	Moderate dysplasia	Severe dysplasia + CIS[a]	Invasive cervical carcinoma
United States	0/9 (0)	0/5 (0)	3/14 (21)	2/10 (20)	0/2 (0)	2/39 (5)
Brazil		0/1 (0)	2/8 (25)	1/5 (20)	1/5 (20)	0/6 (0)
Peru						2/17 (12)

[a] CIS, Carcinoma in situ.

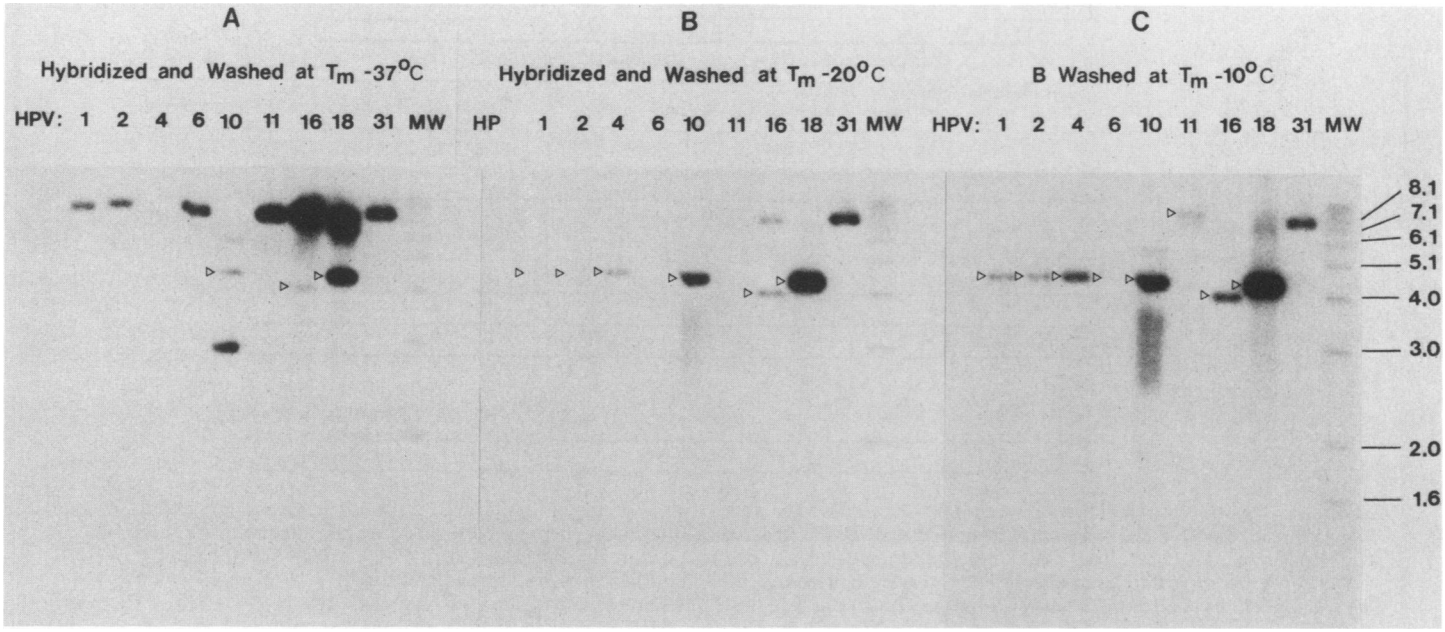

FIG. 2. DNA sequence homology between HPV 31 and several other types of HPV. ^{32}P-labeled HPV 31 was hybridized to HPV 1 (6, 18), 2 (18), 4 (18), 6 (9), 10 (two fragments of 3 and 5 kilobases), 11, 16, 18 (2), and 31 and molecular weight markers (MW). HPV 31 (100 pg) and the other HPV clones (10 ng each) were linearized with the appropriate restriction enzymes, partially purified by gel electrophoresis, and run on a 1% agarose gel. Two nitrocellulose blots were prepared. (A) The filter was probed with HPV 31 at low stringency (T_m −37°C) and washed at T_m −37°C. (B) The other filter was probed at moderately high stringency (T_m −20°C) and washed at T_m −20°C. (C) After autoradiography, panel B was rewashed at high stringency (T_m −10°C) and autoradiographed for a period of time equivalent to two times that used in panels A and B. Sequences homologous to pBR322 (▷) were identified by rehybridization of the filter shown in panel A with pBR322 probe devoid of HPV sequences (data not shown). Although the HPV 31 probe was gel purified, vector sequences are apparent due to the trace contamination of pBR322 in the probe and the relatively large quantities of HPV DNAs that were needed to visualize weak cross-hybridization.

and is shown in Fig. 3. Nucleotide sequence analysis of several papillomavirus DNAs, bovine papillomavirus type 1 (3), cottontail rabbit (Shope) papillomavirus (11), deer papillomavirus (17), HPV 1 (7), HPV 6 (32), and HPV 16 (33), has revealed a common organization of papillomavirus early and late open reading frames. Using the restriction map for HPV 31 and by determining which fragments share sequence homology, we compared the sequence arrangement of this clone to that of HPV 6 and 16. These two HPV are also commonly present in anogenital lesions, and their entire nucleotide sequence and genetic organization are known (32, 33). Gel-purified and nick-translated *Pst*I-*Eco*RI-*Bam*HI-digested fragments of HPV 6b were hybridized to a Southern blot of *Pvu*II-*Sal*I-*Eco*RI and *Ava*II-*Nco*I-*Eco*RI digests of HPV 31 as previously described (2). Each fragment of HPV 6b hybridized with only a discrete region of HPV 31, and contiguous segments of HPV 6b reacted only with the corresponding contiguous segments of HPV 31, allowing us to construct the map shown in Fig. 4. An analogous experiment with HPV 16 indicated a similar alignment with HPV

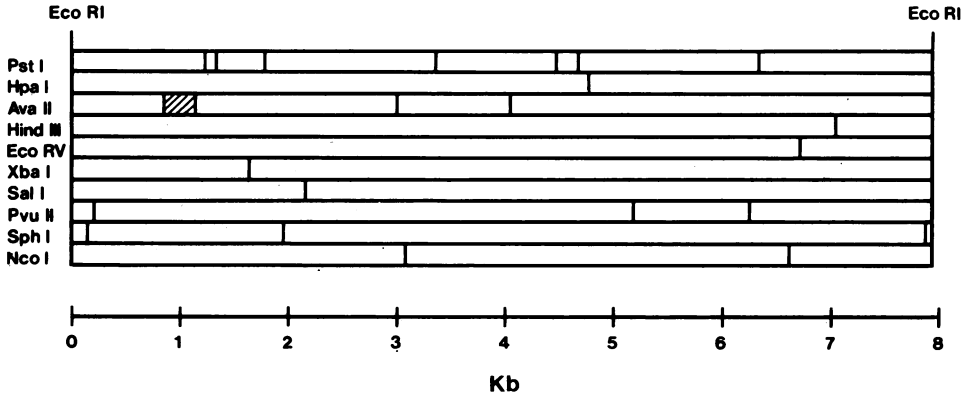

FIG. 3. Restriction endonuclease map of HPV 31. The expected direction of transcription is from left to right, based on the data for HPV 6b (32). The hatched region contains an unknown number of *Ava*II restriction sites. The following enzymes did not cut HPV 31 DNA: *Xho*I, *Pvu*I, *Cla*I, *Bam*HI, *Ava*I, *Nru*I, *Bal*I. Kb, Kilobases.

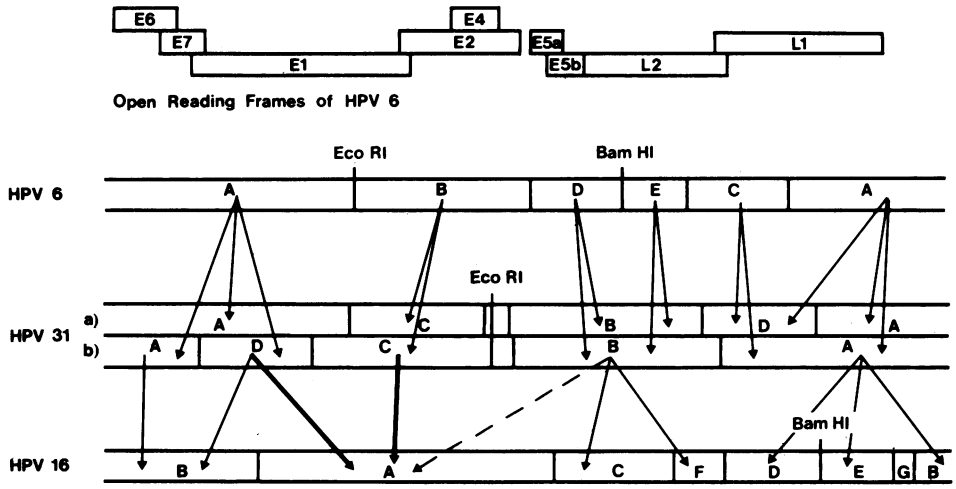

FIG. 4. Regions of homology between HPV 6b, HPV 16, and HPV 31 detected at T_m −37°C and T_m −20°C. Fragments of the *Bam*HI-linearized HPV 6b clone were generated with *Eco*RI and *Pst*I as indicated, then gel purified, nick translated with ^{32}P, and used to probe Southern blots containing: *Nco*I-*Sph*I restriction digests (a) or *Pvu*II-*Sal*I restriction digests (b) of the purified *Eco*RI fragment of HPV 31. Similarly, the indicated fragments of HPV 31 were labeled and used to probe a *Pst*I restriction digest of HPV 16 linearized with *Bam*HI. The solid thin lines connect regions which hybridized only at T_m −37°C. (The dotted line indicates a very weak signal at T_m −37°C.) The thick lines connect regions which hybridized also at T_m −20°C. Each of the maps is arranged so that the ends correspond to the relative position of the *Hpa*I site of HPV 6b. The positions of the open reading frames deduced for HPV 6b are shown above the homology map.

31. The region of highest homology between HPV 16 and HPV 31 was mapped to the large *Pst*I fragment of HPV 16 spanning the E1 and E2 regions. Preliminary sequence data for the L1 and E6 regions of HPV 31 (M. D. Goldsborough, unpublished data) verify the alignment shown.

Although numerous types of HPV have been found in mucosal biopsies (5, 10, 12, 15, 16, 20, 26–29, 35), most attention has focused on HPV 6, 11, 16, and 18 because of their frequent occurrence in a spectrum of benign to malignant anogenital lesions. For example, HPV 16 and HPV 18 DNA sequences are present more frequently in malignant tumors than in benign lesions, whereas HPV 6 and HPV 11 are the principal virus types found in benign condylomata acuminata and exophytic condylomas of the cervix (2, 10, 12, 15). Although rarely found in cervical carcinomas, HPV 6 is associated with the nonmetastasizing anogenital verrucous carcinomas (similar to Buschke-Lowenstein tumors) (12, 13, 29, 30, 36).

The evidence that HPV 31 is a member of the family of HPV is as follows. (i) The mild dysplasia from which it was cloned had the histological morphology characteristic of HPV infection, and papillomavirus common antigen was detectable by immunocytochemistry (A. B. Jenson, personal communication). (ii) The viral DNA of HPV 31 appeared predominantly in the form of supercoiled and relaxed covalently closed circles and migrated on gel electrophoresis as an 8-kilobase fragment after single-site cleavage, as expected for a typical papillomavirus. (iii) The DNA sequence arrangement of HPV 31 closely matches those of HPV 6 and HPV 16, which are similar in organization to other cloned papillomaviruses. (iv) The early regions of HPV 31 and HPV 16 hybridized under conditions of moderately high stringency and therefore share significant homology (Fig. 4).

Although the DNA sequence of HPV 31 is more closely related to that of HPV 16 than to any other known HPV, saturation hybridization analysis indicated that it is too distantly related to be classified as a subtype of HPV 16. Limited nucleotide sequence analysis of HPV 31 has been performed. A comparison of nucleotide sequences of HPV 31 with those of HPV 16 revealed an overall homology of 70% in the L1 region and 56% homology within a 200-base-pair segment of the E6 region (M. D. Goldsborough, unpublished data).

Similar to HPV 6 and HPV 11, HPV 31 is more prevalent in mild dysplasias than cancers. It was, however, found in a significantly higher percentage of anogenital carcinomas than either HPV 6 or HPV 11 and was absent in the condylomata acuminata that we have examined (A. Lorincz, et al., unpublished data). Its association with the various grades of anogenital neoplasias, therefore, is intermediate between HPV 6 and HPV 11 on one hand and HPV 16 and HPV 18 on the other. Determination of the entire DNA sequence of HPV 31 is under way and will permit a more detailed comparison with the other members of this interesting group of viruses.

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