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Anogenital Warts Contain Several Distinct Species of Human Papillomavirus

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Anogenital warts from 26 patients were examined for the presence of human papillomavirus (HPV). Although no whole, intact virus could be identified, varying amounts of nonintegrated HPV DNA were detected in 18 tissue specimens (70%) by employing both an agarose gel-ethidium bromide staining method and the Southern blot hybridization procedure. When hybridization analysis was performed under stringent conditions, six anogenital warts were observed to contain HPV genomic sequences related to either of the cutaneous viruses HPV type 1 (HPV-1) or HPV-2. In 12 tissue samples lacking sequence homology to either HPV-1 or HPV-2 under stringent conditions, HPV-related sequences were detected when the hybridization was performed under less stringent conditions, indicating that an HPV distinct from both HPV-1 and HPV-2 is also associated with these lesions. This anogenital HPV also appeared to be distinct from the other characterized types of HPV. These data indicate that at least three HPVs are associated with anogenital wart disease.

Anogenital warts (condylomata acuminata) are benign tumors afflicting the genitalia and perianal region in humans and are presumed to be caused by human papillomaviruses (HPVs) (12, 27). The spread of anogenital warts occurs during sexual contact (19) and is increasing at an alarming rate, establishing this syndrome as one of the major veneral diseases. There have been several clinical reports indicating that condylomata may develop marked cellular atypia and invade underlying tissues (3, 9, 26). Transmission of virus from genital warts at obstetrical delivery has been suggested as the cause of laryngeal papillomas in children developing symptoms in their first 6 months of life (2). Because of the clinical importance of infection and the possible transition of these benign tumors to malignancy, we have initiated studies to characterize the viruses involved in this disease.

Studies over the past several years have demonstrated a remarkable plurality of papillomaviruses, particularly those afflicting humans. Although it was previously thought that all HPV-induced lesions were due to a single virus type, it is now clear that multiple species of HPV exist (1). For example, seven distinct HPVs, which share little or no genetic homology, have been identified in various warts of humans. HPV types 1 (HPV-1), -2, and -4 are generally associated with plantar and common warts, whereas HPV-3 and -5 are associated with flat warts and lesions of epidermodysplasia verruciformis (7, 8, 10, 11, 21, 24). HPV-6 was identified in genital wart tissues (L. Gissmann, personal communication), and HPV-7 was isolated from common warts of meat-handlers (R. Ostrow, personal communication). To date, intact virus has only occasionally been identified in anogenital wart tissue (6, 20, 22). Hybridization studies initially failed to identify HPV-related DNA sequences in anogenital warts (5, 29), but more recent data detected a virus which shares some genetic homology to HPV-5 (previously designated HPV-4) (23). How this latter anogenital HPV relates to the HPV-6 isolate has not as yet been determined.

In this communication we conclusively demonstrate the presence of HPV DNA in 18 of 26 (70%) anogenital warts examined and report that at least three types of HPV, two closely related to common and plantar wart viruses and one apparently novel virus, are associated with these lesions.

MATERIALS AND METHODS

Purification of HPV and extraction of viral DNA. Plantar and common warts and flat wart-like lesions obtained from a patient with epidermodysplasia verruciformis served as sources of HPV-1, -2, and -5, respectively. The wart tissues were stored at −20°C until use and at that time were minced and then ground in a mortar with silicon carbide powder in a solution containing 50 mM sodium phosphate (pH 7.0) and 50 mM NaCl. Debris was removed by centrifugation at 10,000 × *g* for 30 min, and the supernatants

were centrifuged at 80,000 × g for 90 min to pellet virus. In some cases virus was further purified by equilibrium density gradient centrifugation in CsCl. Viral pellets were suspended in 50 mM sodium phosphate (pH 7.0)–50 mM NaCl–1 mM EDTA, CsCl was added to a density of 1.34 g/ml, and the mixture was centrifuged in an SW50.1 rotor at 37,000 rpm for 40 h at 20°C. The viral band at 1.34 g/cm^3 was collected and dialyzed against 10 mM Tris (pH 8.0) containing 1 mM EDTA. To extract viral DNA, the viral suspension was adjusted to 0.5% sodium dodecyl sulfate (SDS) plus 500 μg of self-digested pronase per ml, incubated at 37°C for 30 min, extracted three times with phenol-chloroform (1:1) saturated with 100 mM Tris-hydrochloride (pH 8.0) and 10 mM EDTA, and subsequently concentrated by ethanol precipitation.

Nick translation of HPV DNA. Viral DNA was radiolabeled in vitro by nick translation as follows: 0.5 to 1.0 μg of viral DNA in 15 μl of solution containing 10 mM Tris-hydrochloride (pH 7.4), 5 mM $MgCl_2$, and 50 mM KCl was incubated with 1 ng of DNase I at 37°C for 8 min. The nuclease was then inactivated at 68°C for 10 min, and the volume of the reaction mixture was increased to 100 μl by the addition of: 250 to 500 μCi of [α-^{32}P]dGTP (1,000 to 2,000 Ci/mmol); 2.7 nmol each of dATP, dCTP, and dTTP; 5 μmol of Tris-hydrochloride (pH 7.8); 5 μg of bovine serum albumin; 1 μmol of β-mercaptoethanol; and 0.55 μmol of $MgCl_2$. Two units of DNA polymerase I was then added, and the solution was incubated at 15°C for 1 h. The reaction was stopped by the addition of EDTA to 10 mM and then passed through a Sephadex G-50 column. The radiolabeled viral DNA was then subjected to SDS-pronase treatment and phenol-chloroform extraction as described above, concentrated by ethanol precipitation, suspended in 3 mM EDTA (pH 7.4), heated for 3 min at 100°C, and quenched on ice. The final product exhibited a specific radioactivity of approximately 3×10^7 to 7×10^7 cpm/μg of DNA and was 90 to 93% sensitive to S1 nuclease.

Extraction of DNA from anogenital warts. Anogenital warts afflicting the perianal, vulvar, and penile regions were excised from patients ranging in age from 18 to 55 years and were stored at −20°C. All tissues studied were small, papular warts meauring 2 to 5 mm in diameter. No giant condylomata acuminata (3) were included in these studies, and none of the patients from whom anogenital warts were obtained suffered from disseminated wart disease. For extraction of DNA, anogenital warts were thawed, minced, and then digested in a solution containing 10 mM Tris-hydrochloride (pH 8.0), 10 mM NaCl, 10 mM EDTA, 0.5% SDS, and 1 mg of pronase per ml for 2 to 8 h at 37°C. Total nucleic acids were then extracted three to four times with phenol-chloroform (1:1) saturated with 100 mM Tris-hydrochloride (pH 8.0) and 10 mM EDTA, and concentrated by ethanol precipitation. In some instances, before phenol-chloroform extraction, high-molecular-weight chromosomal DNA was separated from low-molecular-weight DNA, including HPV DNA, by the Hirt procedure (14). RNA was removed from the preparations by digestion in a solution containing 10 mM Tris-hydrochloride (pH 7.4), 10 mM EDTA, and 100 μg of pancreatic RNase per ml for 1 h at 37°C. The DNA was then extracted three times with phenol-chloroform and concentrated by ethanol precipitation.

CsCl-PI_2 equilibrium centrifugation of DNA from anogenital warts. The low-molecular-weight Hirt supernatant DNA from anogenital warts was subjected to cesium chloride-propidium diiodide (PI_2) equilibrium centrifugation as previously described (13). Briefly, 75 μl containing approximately 300 μg of DNA was added to 6.5 ml of solution consisting of 20 mM Tris-hydrochloride (pH 7.4), 6 mM EDTA, and 2.4 mg of PI_2. CsCl was added to give a final density of 1.532 g/ml, and the mixture was centrifuged for 60 to 70 h in a type 40 rotor (Beckman) at 33,000 rpm at 20°C. Form I DNA was pooled from regions of the CsCl-PI_2 gradient exhibiting densities between 1.543 and 1.563 g/cm^3, extracted four or five times with CsCl-saturated isopropanol, dialyzed against several changes of 10 mM Tris-hydrochloride (pH 8.0) containing 1 mM EDTA for 36 h at 4°C, and concentrated by ethanol precipitation.

Restriction endonuclease analysis of HPV DNAs. Restriction endonuclease analysis was performed on viral DNAs extracted from purified papillomavirus isolated from common and plantar warts and on HPV form I DNA prepared from an anogenital wart by CsCl-PI_2 equilibrium density centrifugation. Reaction mixtures (25 μl) containing 10 mM Tris-hydrochloride (pH 7.0), 7 mM $MgCl_2$, 60 mM NaCl, 1 mM dithiothreitol, 2 μg of bovine serum albumin, 100 to 400 ng of viral DNA, and 3 U of *Hin*dII restriction endonuclease (New England Biolabs) were incubated at 37°C for 3 h. The reactions were terminated by the addition of EDTA to 10 mM and SDS to 1.4%, and incubation was continued at 65°C for 10 min. The *Hin*dII fragments were separated on 1% agarose gels and visualized by ethidium bromide staining as described below.

Agarose gel electrophoresis and filter hybridization. DNA samples were electrophoresed in either 0.7% or 1.0% horizontal agarose (Seakem) gels in buffer containing 50 mM Tris-acetate (pH 8.05), 2 mM EDTA, and 18 mM NaCl for 10 to 16 h. DNA was visualized by staining with ethidium bromide and then transferred to nitrocellulose (Schleicher and Schuell, BA85) by the method of Southern (28). The filters were dried at 80°C for 2 h under vacuum and then were preincubated for 24 h at 37°C in hybridization solution containing 500 μg of depurinated calf thymus DNA per ml; 50 μg of yeast tRNA per ml; 0.06% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin; 0.1% SDS; 1 M sodium chloride; 20 mM sodium phosphate (pH 6.8); and either 20% or 50% formamide, depending upon whether hybridization was to be performed under less stringent or stringent conditions, respectively (15, 17). After preincubation, fresh hybridization solution (4 to 8 ml) containing ^{32}P-labeled viral DNA (1×10^6 to 2×10^6 cpm/ml) and 10% dextran sulfate was added to the filters and incubated for 24 h at either 37°C for less stringent hybridization (T_m − 48°C) or 41°C for stringent hybridization (T_m − 22°C). The filters were washed successively with several changes of 2× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate)–0.1% SDS and 0.2× SSC–0.1% SDS at room temperature. The less stringent and stringent hybridization filters were then washed for 3

to 6 h at 50°C in solutions containing 0.1% sodium pyrophosphate and either 6× SSC–0.1% SDS or 0.4× SSC–0.1% SDS, respectively. After air drying, the filters were covered with plastic wrap and exposed to Kodak RP-Royal X-Omat X-ray film with an intensifying screen at −70°C. Using this procedure, as little as 15 to 30 pg of viral DNA could be detected after 4 to 5 days.

Electron microscopy of HPV form I DNA isolated from an anogenital wart. HPV form I DNA from an anogenital wart was isolated by CsCl-PI_2 equilibrium density centrifugation and then subjected to agarose gel electrophoresis. The HPV form I DNA band was visualized by ethidium bromide staining and subsequently eluted from agarose by incubating the crushed gel slice overnight at 4°C in a solution containing 10 mM Tris-hydrochloride (pH 8.0) and 10 mM EDTA. After being concentrated by ethanol precipitation, the DNA was spread for electron microscopy by the basic protein monolayer method of Davis et al. (4). The electron microscope was calibrated from micrographs of a carbon replica (54,000 lines per in.).

RESULTS

Examination of anogenital warts for the presence of HPV virions. Anogenital warts were disrupted by conventional procedures consisting of grinding with silicon carbide powder in a mortar (25). After cellular debris was removed by low-speed centrifugation, supernatant fractions were further centrifuged to pellet any free viral particles. The putative viral pellets were then subjected to equilibrium density gradient centrifugation in CsCl to band HPV-like particles if they were indeed present. Several anogenital warts excised from the vulvar and perianal regions were analyzed by this procedure, and none of the samples contained sufficient virus particles to produce a visible band in the CsCl equilibrium density gradients (Fig. 1B). In contrast, an equivalent amount of plantar wart tissue prepared in the same manner yielded a distinct viral band at 1.34 g/cm^3 (Fig. 1A). Furthermore, electron microscopic analysis of putative viral pellets before CsCl equilibrium density gradient centrifugation was also unsuccessful in revealing HPV-like particles from anogenital wart specimens (data not shown).

Detection of HPV-like DNA in anogenital warts. Since whole, intact HPV-like viral particles could not be identified in anogenital warts by the above procedure, an attempt was made to determine whether DNA molecules corresponding to either form I, II, or III of the HPV genome could be detected. In these studies, the wart tissues were minced and disrupted by treatment with SDS-pronase, and the resultant cell lysates were subjected to the Hirt fractionation procedure (14) to separate high-molecular-weight chromosomal DNA from any free forms of HPV-like DNA that might be present. The

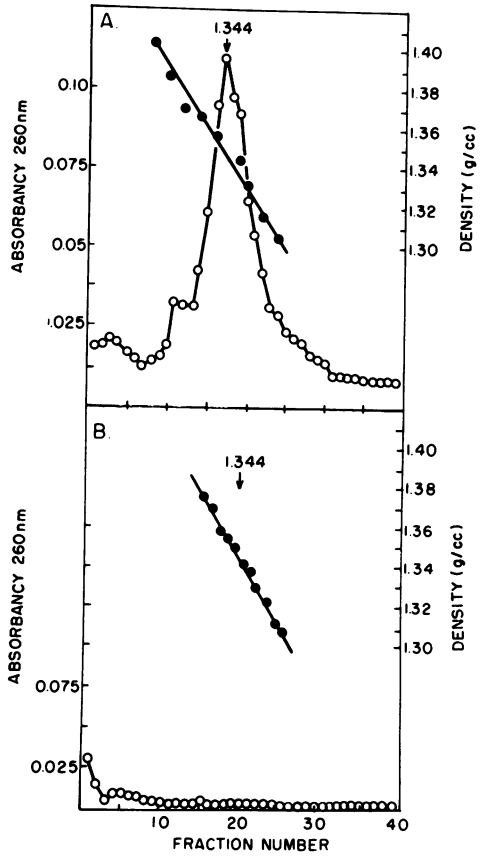

FIG. 1. *CsCl equilibrium density gradient centrifugation of plantar and anogenital wart tissue extracts. Approximately 1 g of plantar warts and anogenital wart tissue was minced and then disrupted by grinding in a mortar with silicon carbide powder in a solution containing 50 mM sodium phosphate (pH 7.0) and 50 mM NaCl. Debris was removed by centrifugation at 10,000 × g for 30 min, the supernatants were further centrifuged at 80,000 × g for 90 min, and the resultant pellets were suspended in buffer consisting of 0.05 M $NaPO_4$ (pH 7.0), 0.05 M NaCl, and 0.01 M EDTA. CsCl was then added to a density of 1.34 g/ml, and the mixtures were centrifuged in an SW50.0 rotor at 37,000 rpm for 40 h at 20°C. Fractions were collected, the density was determined, and the absorbancy at 260 nm was measured. (A) Plantar warts; (B) anogenital wart tissue. Symbols: ○, absorbancy at 260 nm; ●, density of CsCl (grams per milliliters).*

Hirt supernatant fractions were then analyzed by electrophoresis in agarose gels, which were subsequently stained with ethidium bromide to visualize the fractionated DNA molecules. By employing this method of analysis, DNA molecules comigrating with the various forms of HPV DNA could be identified in 6 of 26 anogenital warts examined. Three anogenital wart DNA preparations containing HPV-like DNA are

shown in Fig. 2A (slots 2, 4, and 6). All three forms of HPV DNA were present in one of these samples, whereas in the other two, only HPV-like form I DNA molecules could be visualized.

The ability to visualize HPV-like DNA in the agarose gels by ethidium bromide staining was dependent upon the extent of background from heterogeneous cellular DNA present in the Hirt supernatant fraction. For example, although the presence of HPV-like form I DNAs was easily identified in some of the anogenital wart DNA preparations (e.g., Fig. 2A, slot 6) and less evident in others (e.g., Fig. 2A, slots 2 and 4), in the majority of cases the presence of cellular DNA in the preparations (e.g., Fig. 2A, slots 7 and 8) precluded the identification of HPV-like DNA. Form I HPV-like DNA could readily be observed in the samples shown in Fig. 2A (slots 2 and 4) if the cellular DNA background was removed by subjecting the DNA preparations to equilibrium density centrifugation in CsCl-PI_2 before analysis by agarose gel electrophoresis (Fig. 2B, slots 2 and 4). When several anogenital wart DNA preparations similar to those shown in Fig. 2A (slots 7 and 8) were analyzed by this procedure, sufficient amounts of HPV-like form I DNA were not present to allow visualization by ethidium bromide staining (data not shown). However, HPV-like DNA could be demonstrated in the majority of these specimens, if they were first transferred to nitrocellulose paper and then analyzed by the more sensitive procedure of filter hybridization (see below).

The superhelical structure of the HPV-like DNA isolated by the combined CsCl-PI_2–agarose gel procedure was confirmed by electron microscopy. The anogenital wart DNA band comigrating with HPV form I DNA in the agarose gel (Fig. 2B, slot 2) contained circular DNA molecules in a superhelical configuration (Fig. 3b and c). Also, some form II molecules were observed (Fig. 3a, d, and e) which were presumably generated during the preparation of form I DNA for electron microscopy. The size of these molecules averaged 5×10^6 daltons, which is in the range of the papillomavirus genome and half the size of the only species of superhelical DNA molecules present in normal human tissue, mitochondria DNA (ca. 10×10^6 to 12×10^6 daltons) (16, 29).

Detection of HPV-like DNA in anogenital warts by filter hybridization. Although superhelical HPV-like DNA could be detected in some anogenital warts by direct staining of agarose gels with ethidium bromide, the number of warts that contained the necessary amount of HPV DNA (>10 ng) required for detection with this technique was only a fraction of the total number of warts analyzed (ca. 23%). We therefore further analyzed anogenital warts for the presence of HPV-related DNA by the Southern blot hybridization procedure (28), which pro-

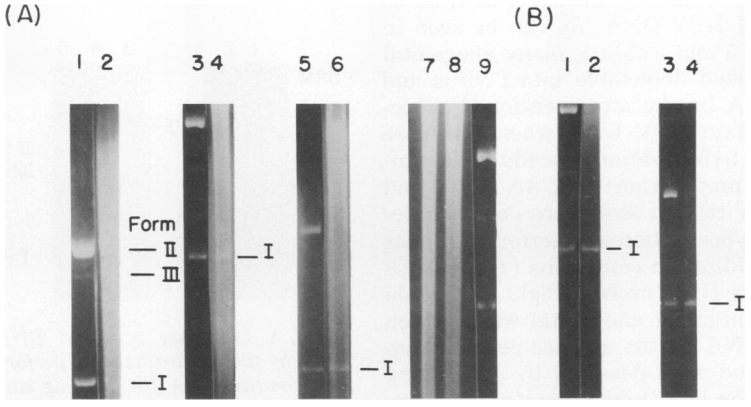

FIG. 2. *Detection of HPV-like DNA in anogenital warts. (A) A low-molecular-weight DNA Hirt supernatant fraction was prepared from several anogenital wart specimens, and approximately 10 to 20 μg of cellular DNA was subjected to electrophoresis either in a 0.7% or a 1% horizontal agarose slab gel. The presence of HPV-like DNA was detected subsequent to staining the gels with ethidium bromide (1 μg/ml) by use of a UV light box. The positions of DNA molecules present in the anogenital preparations which comigrated with forms I, II, and III of HPV DNA are indicated by the lines. Slots 2, 4, 6, 7, and 8, Anogenital wart DNA from patients 1, 26, 24, 15, and 12, respectively; slots 1, 3, 5, and 9, HPV DNA extracted from purified plantar wart virions. (B) A portion of the Hirt supernatant DNA from the anogenital wart analyzed in (A), slots 2 and 4, was subjected to CsCl-PI_2 equilibrium centrifugation as described in the text and then analyzed by agarose gel electrophoresis. The position of DNA molecules present in the anogenital wart preparations which comigrated with HPV form I DNA is indicated by the lines. Slots 2 and 4, Anogenital wart DNA samples; slots 1 and 3, HPV DNA extracted from purified plantar wart virions.*

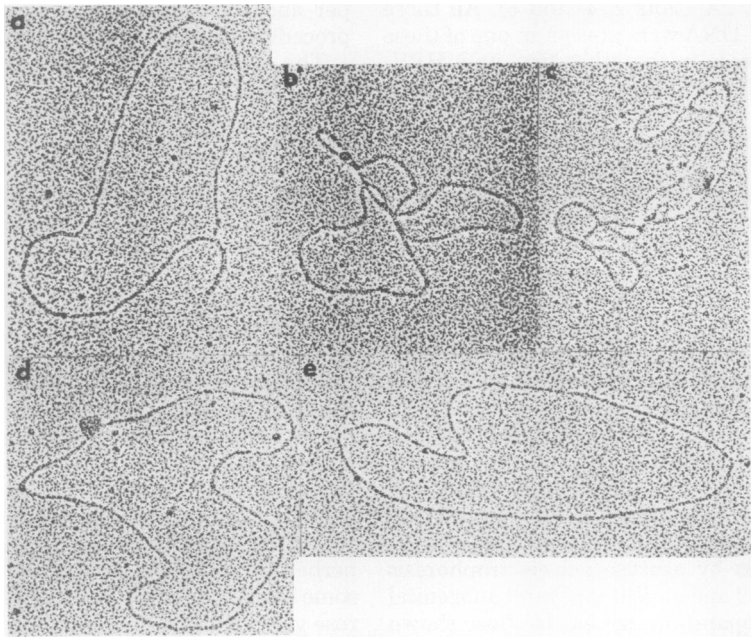

FIG. 3. *Electron microscopy of HPV-like form I DNA isolated from an anogenital wart. HPV-like form I DNA from an anogenital wart was prepared by CsCl-PI_2 equilibrium density centrifugation and then subjected to agarose gel electrophoresis. The HPV-like form I DNA band was visualized by ethidium bromide staining and subsequently eluted from agarose by incubating the crushed gel slice overnight at 4°C in a solution containing 10 mM Tris-hydrochloride (pH 8.0) and 10 mM EDTA. After being concentrated by ethanol precipitation, the DNA was spread for electron microscopy by the basic protein monolayer method of Davis et al. (4). The electron microscope was calibrated from micrographs of a carbon replica (54,000 lines per in.). × 100,500. (b, c) Form I DNA molecules; (a, d, e) form II DNA molecules.*

vides sufficient sensitivity to detect as little as 15 to 30 pg of HPV-DNA. As can be seen in Figures 4 and 5 and Table 1, many anogenital warts that lacked detectable intact virus and HPV-like DNA by the aforementioned procedures did contain HPV DNA when the more sensitive filter hybridization procedure was employed. In six preparations (Fig. 4A and B and Table 1), HPV-related sequences could be detected when hybridization was performed under stringent hybridization conditions (T_m − 23°C) with cutaneous HPV probes. Eight additional HPV DNA-containing anogenital warts which lacked viral DNA by the agarose gel-ethidium bromide method were detected by performing the hybridization assay under less stringent conditions (T_m − 48°C) (Fig. 5 and Table 1). In our hands, these conditions of hybridization allowed the detection of conserved nucleotide sequences between several distinct HPV groups (Fig. 6B) which were not detected by stringent hybridization conditions (Fig. 6A). Furthermore, as demonstrated by Law et al. (17), these less stringent conditions also permit the detection of conserved nucleotide sequences between HPV and distantly related animal papillomaviruses (Fig. 6B). Thus, although both HPV-1- and HPV-2-related nucleotide sequences could be detected

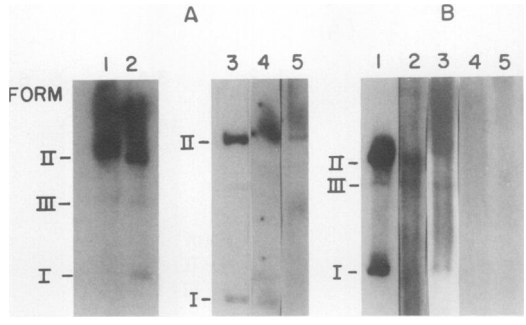

FIG. 4. *Detection of HPV DNA in anogenital warts by filter hybridization performed under stringent conditions. A 10- to 20-μg sample of DNA extracted from an anogenital wart was subjected to electrophoresis in a 1% horizontal agarose gel. After transfer of DNA to nitrocellulose, the filters were hybridized with a ^{32}P-labeled viral DNA probe under stringent conditions as described in the text. (A) Hybridizations performed with HPV-1 [^{32}P]DNA. Slots 1 and 3, HPV-1 DNA; slots 2, 4, and 5, anogenital wart DNA prepared from patients 1, 16, and 17, respectively. (B) Hybridizations performed with HPV-2 [^{32}P]DNA. Slot 1, HPV-2 DNA; slots 2 and 3, anogenital wart DNA prepared from patients 3 and 39, respectively; slots 4 and 5, negative control DNAs prepared from human lymphocytes and spleen, respectively.*

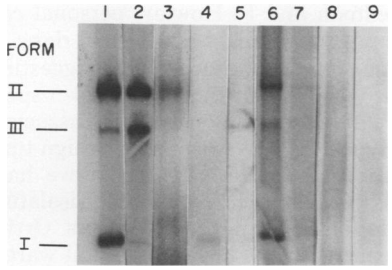

FIG. 5. *Detection of HPV DNA in anogenital warts by filter hybridization performed under less stringent conditions. 10 to 20 μg of DNA extracted from anogenital warts was analyzed as described in Fig. 4 except that hybridization with HPV-2 [^{32}P]-DNA was performed under less stringent conditions, as described in the text. Slot 1, HPV-2 DNA; slots 2 to 7, anogenital wart DNA prepared from patients 21, 23, 18, 29, 27, and 25, respectively; slots 8 and 9, negative control DNAs prepared from human lymphocytes and spleen, respectively.*

TABLE 1. *HPV DNA in anogenital warts*[a]

Patient no.	Sex	Location of lesions	Method of detection			
			Agarose gel-ethidium bromide staining	Southern blot procedure		
				Stringent		Less stringent (HPV-1 or HPV-2)
				HPV-1	HPV-2	
1	F	Perianal and vulvar	+	+	−	+
2	F	Vulvar	+	−	−	ND
3	F	Vulvar	−	−	+	+
6	F	Vulvar	−	−	−	−
7	F	Vulvar	−	−	−	−
8	F	Vulvar	−	−	−	+
12	M	Perianal	−	−	−	+
13	F	Vulvar	+	+	−	+
14	F	Vulvar	−	−	−	−
15	M	Perianal	−	−	−	+
16	M	Perianal	−	+	−	+
17	F	Vulvar	−	+	−	ND
18	F	Perianal	−	−	−	+
20	F	Perianal	−	−	−	−
21	M	Perianal	+	−	−	+
23	M	Penile	−	−	−	+
24	F	Perianal	+	−	−	+
25	M	Penile	−	−	−	+
26	F	Perianal	+	−	−	+
27	M	Perianal	−	−	−	+
28	M	Perianal	−	−	−	−
29	F	Perianal	−	−	−	+
32	M	Penile	−	ND	ND	−
33	M	Penile	−	ND	ND	−
39	F	Vulvar	−	−	+	+
42	F	Vulvar	−	ND	ND	−

[a] Cellular DNA extracted from anogenital warts was analyzed for HPV DNA by the agarose gel-ethidium bromide staining method and the Southern blot procedure performed with either a ^{32}P-labeled HPV-1 or HPV-2 DNA probe under stringent or less stringent hybridization conditions as described in the text. +, HPV DNA detected; −, HPV DNA not detected; ND, not done.

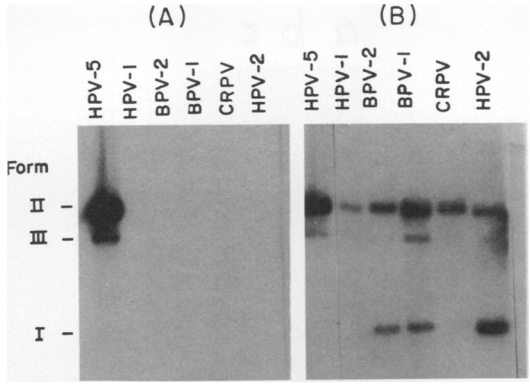

FIG. 6. *Detection of genetic homology between papillomavirus DNAs. Approximately 25 to 100 ng of papillomavirus DNA extracted from CsCl-purified HPV-1, HPV, HPV-5, bovine papillomaviruses BPV-1 and BPV-2, and the cottontail rabbit papillomavirus (CRPV) were subjected to electrophoresis, transferred to nitrocellulose, and then hybridized with ^{32}P-labeled HPV-5 DNA either under stringent (A) or less stringent (B) hybridization conditions as described in the text. In both cases, the homologous hybridization was detected only after a 2- to 4-h exposure to X-ray film, whereas the heterologous hybridization under less stringent conditions required 1 to 2 days of exposure. No hybridization was detected with heterologous viral DNA under stringent conditions even after exposure for 12 days.*

by stringent hybridization conditions in some of the anogenital warts analyzed (Fig. 4A and B), 12 of these tumors contained nucleotide sequences more distantly related to the cutaneous HPV-1 and -2 (Fig. 5 and Table 1). These data indicate that nonintegrated forms of HPV DNA are present in the majority of anogenital warts (70%) analyzed and that different types of HPVs, one of which is totally distinct from either HPV-1 or HPV-2, are associated with these lesions. One anogenital HPV DNA that did not hybridize to either HPV-1 or HPV-2 under stringent conditions was analyzed by *Hin*dII restriction endonuclease mapping. The pattern of the HPV isolate was compared with those of HPV-1 and a mixture of two subtypes of HPV-2 (Fig. 7). Although one of the restriction fragments of the anogenital HPV comigrated with that of HPV-1, the other two showed a migration distinct from that of the other two HPV-1 and all the HPV-2 restriction fragments.

DISCUSSION

By employing a sensitive filter hybridization assay, we have been able to identify HPV DNA in the majority (70%) of anogenital warts examined. When hybridization analysis was performed under stringent conditions, six of the anogenital warts were observed to contain HPV

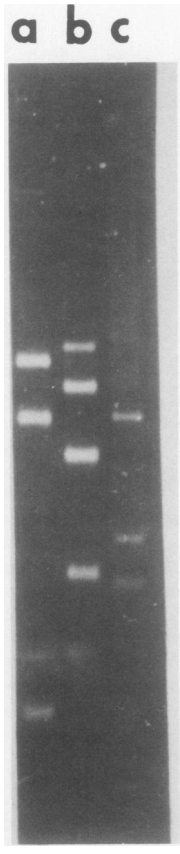

FIG. 7. *Agarose gel electrophoresis of HindII fragments of HPV DNA from plantar (HPV-1), common (HPV-2), and anogenital warts. HindII restriction endonuclease analysis was performed either on viral DNA extracted from purified papillomavirus isolated from common (HPV-2) and plantar warts (HPV-1) or on HPV-like form I DNA prepared from an anogenital wart by CsCl-PI_2 equilibrium density gradient centrifugation as described in the text. The HindII fragments were separated by electrophoresis in a 1% agarose gel, and the DNA fragments were visualized by ethidium bromide staining. Slots: a, HPV-1 DNA; b, HPV-2 DNA; c, HPV form I DNA from an anogenital wart excised from patient 2.*

genomic sequences related to either HPV-1 or HPV-2, thus indicating a strong possibility that HPV-1 and -2 are associated with anogenital wart disease. Moreover, when 12 anogenital warts which showed no hybridization to either of the cutaneous viruses under stringent conditions were hybridized under less stringent conditions with a cutaneous HPV probe, an additional anogenital HPV was identified. The *Hin*dII restriction map of this HPV not only differed from those of HPV-1 and HPV-2 but also from those of HPV-4, HPV-5, and HPV-7 (C. Heilman and P. Howley, personal communication; R. Krzyzek, unpublished data; R. Ostrow, personal communication), suggesting that this virus is distinct from the known species of HPV. These data demonstrate the association of three types of HPV with these benign tumors.

In addition to the HPVs that we have described, two other HPVs have been isolated from these lesions by other investigators. Orth et al. (23) reported HPV in seven genital wart specimens which was shown by complementary RNA-hybridization to be unrelated to either HPV-1, -2, or -3. A low level of nucleotide sequence homology (3.5 to 8.8%) to HPV-5 was observed, but the exact extent of homology was not determined. We analyzed several anogenital warts containing the novel HPV by Southern blot hybridization with a HPV-5 DNA probe under stringent conditions, and no detectable sequence homology was observed (data not shown). This suggests either that our novel anogenital HPV is distinct from the isolate of Orth et al. (23) or that the low levels of hybridization detected by these authors were not reproducible in our hands. Gissmann has obtained an HPV isolate from a single anogenital wart which has only been partially characterized but appears to be distinct from the known types of HPV because of its *Hin*dII restriction map (personal communication). The *Hin*dII restriction map of our novel HPV, which shares no genetic homology with either HPV-1 or HPV-2, is different from that of the Gissmann isolate (personal communication). The relatedness of our novel HPV to that of Gissmann and of Orth et al. (23) will undoubtedly require further study. Presently, these data collectively indicate that possibly five types of HPV are associated with anogenital wart disease. It is conceivable that there are additional anogenital HPVs, since morphologically distinct anogenital warts have been described by Oriel (19) and Meisels et al. (18). The anogenital warts studied by us all had a similar papular morphology, and thus no correlation between wart morphology and the type of HPV associated with the lesion could be made.

Our results also indicate that there is great variation in the amount of HPV DNA present in anogenital warts which appears to be related to neither the sex of the patient, the location of the lesion, nor the type of HPV identified (Table 1). In some cases, viral DNA can be readily visualized by the agarose gel-ethidium bromide staining method, but in most cases it can only be detected by the more sensitive hybridization procedure. The inability to identify HPV DNA in all the anogenital warts examined may reflect quantities of viral DNA below the sensitivity of

our hybridization method. In addition, our data, with the possible exception of the finding that the novel HPV was identified in the majority of anogenital warts, do not indicate any significant correlation between the type of anogenital wart and the species of HPV associated with this lesion (Table 1). This relationship may indeed exist but might only become evident in analyses performed on greater numbers of tissue samples than examined in this study.

Clearly, in some instances relatively large amounts of free, unintegrated DNA can be identified in anogenital wart tissue despite the lack of intact virus. These findings suggest that the involved tissues are nonpermissive for HPV replication and that HPV DNA accumulates with little or none being packaged into mature virions. The reason for nonpermissiveness is not clear. It has been suggested that this is a function of the unique nature of the virus involved, but the likelihood that HPV-1 and -2 are involved, and likewise fail to assemble, tends to negate this hypothesis. Lack of assembly may reflect changes in local tissues (e.g., temperature or pH) which in turn create a nonpermissive situation.

The study of anogenital warts has been limited by the amounts of tissue available (specimens typically contain 0.1 to 2.0 g of tissue, largely connective tissue stroma) and the lack of intact virions. The techniques applied in this study begin to circumvent these problems, but are still lacking material for repetitive hybridizations and restriction endonuclease analysis. We have directed further experimental efforts toward amplifying the genomes through molecular cloning procedures involving bacterial plasmids.

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