Biochemical Characterization of Two Types of Human Papillomaviruses Associated with Epidermodysplasia Verruciformis

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The DNAs of the human papillomaviruses (HPVs) associated with the benign lesions of two patients suffering from epidermodysplasia verruciformis (patients JD and JK) were analyzed by using 12 restriction endonucleases. None of the restriction endonucleases were one-cut enzymes for the HPV DNA obtained from patient JD, referred to as the prototypical HPV-5, whereas five of them were one-cut enzymes for the DNA of the major virus found in patient JK, referred to as HPV-9. The molecular cloning of the two fragments resulting from the cleavage of HPV-5 DNA by endonuclease *Hind*III and of the single fragment obtained after treatment of HPV-9 DNA with endonuclease *Bam*HI was performed in *Escherichia coli* after the fragments were inserted in plasmid pBR322. A cleavage map of the two cloned genomes was constructed. Little sequence homology (4 to 5%) was detected between HPV-5 and HPV-9 DNAs by DNA-DNA hybridization experiments in liquid phase at saturation; this homology was reproducibly higher than that (2 to 3%) detected under the same conditions between these DNAs and HPV-1a DNA. In addition, blot hybridization experiments performed under stringent conditions showed no or little sequence homology between the DNAs of HPV-5 and HPV-9 and those of HPV prototypes of types 1, 2, 3, 4, and 7 associated with skin warts. These results confirm that HPV-5 and HPV-9 are two distinct HPV types.

The papillomaviruses, members of the Papovaviridae family, are characterized by a circular, superhelical DNA genome of about 8,000 nucleotide pairs (5, 10). In humans, papillomaviruses induce benign, cutaneous or mucous, epithelial tumors (skin warts of various types, oral or laryngeal papillomas, or anogenital condylomas). They are also associated with lesions showing no papillomatous structure, such as the flat lesions of the uterine cervix and the cutaneous macules which characterize epidermodysplasia verruciformis (EV) (for review, see references 24, 26, 27, 36, and 44). The lesions associated with a human papillomavirus (HPV) usually regress spontaneously or after treatment; however, in rare instances, a malignant transformation is observed (26, 44). This is the case of EV, a rare, life-long disease which is characterized by disseminated cutaneous lesions, usually resembling flat warts or reddish or pityriasis versicolor-like macules, and by an early development of multiple skin cancers in about 30% of the patients studied (19, 22, 32). EV constitutes the best model for studying the role of a papillomavirus in the genesis of human

cancer, together with genetic, immunological, and actinic factors (19, 22, 32).

Recent studies have shown the remarkable plurality of HPVs. At least 10 HPV types have been demonstrated so far (4, 8, 9, 12, 14, 15, 26-35; M. Favre, S. Jablonska, O. Croissant, S. Obalek, and G. Orth, manuscript in preparation; D. Kremsdorf, S. Jablonska, M. Favre, and G. Orth, manuscript in preparation), and the analysis of numerous isolates, with the aid of restriction enzymes and molecular hybridization experiments, has permitted the identification of subtypes in most types (12, 27, 31; Favre et al., in preparation). Some patients, suffering from EV, but showing only flat wartlike lesions, have been found to be infected with type 3 viruses, which are also associated with flat warts in the general population. No cancerous lesions were observed in such patients (27, 32). Distinct HPVs were identified in patients showing both flat wart-like and macular lesions, all of them having or having had carcinomas (27, 30, 32). Among these cases are patients JD, JK, and DD (Table 1), referred to as patients 1, 2, and 3 in this paper. The viruses found were initially

TABLE 1. Nomenclature of HPVs identified during previous studies of Polish patients suffering from EV

Proposed name (HPV type)	Previous name (HPV type)	First case found associated with the viruses ^a		Incidence in other patients ^b
		Patient	Characteristics of isolates ^b	
3a ^c	3 ^d	JD	Almost homogeneous in warts	Major virus in warts of patient 3; trace amounts in warts of patient 2
5 ^b	5b ^d	JD	Almost homogeneous in macules; found in two carcinomas	Minor component in macules and warts of patients 2 and 3; found in a carcinoma from patient 2
8 ^e	5c ^d	DD	Major virus in macules	Minor component in lesions of patient 2
9 ^b	5a ^d	JK	Major virus in warts and macules	Trace amounts in warts of patient 3

^a Patients JD, JK, and DD previously studied (32) are referred to as patients 1, 2, and 3 in this paper. The viruses have been identified by molecular hybridization experiments (30, 32) or restriction enzyme analysis (27) (or both) of DNA preparations obtained from pooled scrapings of flat wart-like lesions or reddish and pityriasis versicolor-like macular lesions.

^b From data to be published. In addition to the HPVs mentioned, the restriction enzyme analysis of DNA preparations obtained from pooled scrapings of lesions indicates the existence of at least two and three additional HPVs in patients 3 and 2, respectively (Orth et al., in preparation).

^c Favre et al., in preparation.

^d From reference 27.

^e From reference 35.

grouped within type 5 on the basis of experiments performed with antisera or specific probes of a prototype isolate obtained from lesions of patient 2 (27, 30, 32). Restriction enzyme analyses of DNA preparations obtained from different patients have shown the existence of distinct cleavage patterns, leading to the definition of subtypes of type 5 (HPV-5, subtypes a through d) (27). Furthermore, most of the patients were found to be infected by several viruses, in variable proportions, depending on the localization of the lesions (G. Orth, S. Jablonska, M. Favre, S. Obalek, and O. Croissant, manuscript in preparation). This is the case of patient 2, found to be infected by a major virus (first called HPV-5a [27]) and by several minor viruses, in particular, the virus detected in macular lesions of patient 1 and the major virus found in macular lesions of patient 3 (initially named HPV-5b and HPV-5c, respectively) (Table 1) (27). Molecular hybridization experiments, performed with a DNA preparation containing a high proportion of HPV-5a and with a homogeneous preparation of HPV-5b DNA, show that HPV-5a and HPV-5b present little sequence homology and that they are two distinct HPV types (Orth et al., in preparation). The detection of the sole HPV-5b genome in eight carcinomas of six patients, including patients 1 and 2, suggested that this virus has a higher oncogenic potential (M. Lutzner, C. Blanchet-Bardon, and G. Orth, submitted for publication); thus, HPV-5b has been considered as the prototype of type 5 (Table 1) (Orth et al., in preparation). Recently, an HPV DNA isolate showing a restriction cleavage pattern similar to that of the HPV-5c DNA found in patient 3 (27) and in a French patient (21) has been character-

ized in an Upper Volta patient and named HPV-8 by Pfister et al. (35) (Table 1). Finally, DNA-DNA hybridization experiments performed with the cloned HPV-8 DNA obtained from patient 3 show that this virus is an HPV type distinct from the former HPV-5a and HPV-5b (Kremsdorf et al., in preparation). We propose, therefore, that the former subtypes a, b, and c of type 5 be named HPV-9, HPV-5, and HPV-8, respectively (Table 1).

The heterogeneity of the viral DNA preparations obtained from mixtures of lesions from a single patient and the small quantity of DNA that can be extracted from them made the characterization of EV HPVs quite difficult. In the absence of a cellular system permitting an in vitro HPV replication, the cloning of the viral genomes in *Escherichia coli*, after insertion in plasmid pBR322, was a way to circumvent the difficulties (6, 15).

In this paper we report an analysis of the sensitivities of HPV-5 and HPV-9 DNAs to 12 restriction endonucleases. We also report the molecular cloning of the two fragments obtained after cleavage of HPV-5 DNA by *Hind*III endonuclease and that of the single fragment obtained after cleavage of HPV-9 DNA by *Bam*HI endonuclease. The cloned DNAs permitted the construction of restriction maps of these two genomes and the analyses of nucleotide sequence homologies between HPV-5 and HPV-9 genomes and between these genomes and those of the prototypes of HPV types associated with skin warts.

MATERIALS AND METHODS

DNA preparation. Viral DNAs were selectively extracted, using a modification of the method of Hirt (16,

26), from a deep plantar wart (HPV-1a) (8, 9), pooled hand common warts from a single individual (HPV-2, HPV-7) (27, 28, 31), palmar warts (HPV-4) (12, 27, 34), flat warts from a patient suffering from EV (HPV-3a) (27, 32), and scrapings of the macular lesions of patients 1 and 2, who were suffering from EV (27, 30, 32). Form I DNAs were purified by equilibrium centrifugation in cesium chloride gradients in the presence of ethidium bromide and by sedimentation in sucrose gradients in the presence of ethidium bromide (27, 31). DNA concentrations (10 to 20 µg/ml) were determined by electron microscopy, using the replicative form of bacteriophage ϕ X174 as a standard (31). The DNAs were kept at -20°C in buffer containing 10 mM Tris-hydrochloride and 1 mM EDTA (pH 7.9).

Restriction enzyme analysis. Endonucleases *Bgl*II, *Bgl*III, *Eco*RI, *Hind*II, and *Hpa*I (Boehringer Mannheim Corp.); *Ava*I, *Bam*HI, *Hind*III, *Pst*II, and *Sma*I (Bethesda Research Laboratories); and *Pvu*I and *Pvu*II (New England Biolabs) were used according to the instructions of the manufacturer. The unrestricted HPV DNAs obtained from lesions of patients 1 and 2 (1 µg) were separated by electrophoresis through agarose (1%) in vertical slab gels, as previously described (27). The HPV and recombinant DNAs (0.2 to 0.5 µg) were digested with one or a mixture of endonucleases, and the digestion products were separated by electrophoresis through agarose (1%) in horizontal slab gels, as previously described (6). The sizes of the fragments were determined by their electrophoretic mobility, using the *Hind*III digestion products of bacteriophage λ and simian virus 40 DNAs as standards (11, 25).

Molecular cloning of HPV-5 and HPV-9 DNAs in *E. coli*. Cloning of HPV-5 and HPV-9 DNAs in *E. coli* was performed after insertion into the bacterial plasmid pBR322, with the agreement of the French commission for the control of in vitro genetic recombination. *Hind*III fragments A and B of HPV-5 DNA and the HPV-9 genome, cleaved with *Bam*HI endonuclease, were inserted into the plasmid pBR322. These two enzymes cleave plasmid pBR322 once within the gene coding for tetracycline resistance (39). The binding of the viral and plasmid sequences and the transformation of *E. coli* K-12 strain DP50 (2) were performed by the method of Tanaka and Weisblum (41), with slight modifications. Briefly, the cleaved HPV and plasmid pBR322 DNAs were mixed in a 3:1 proportion (0.3 µg of HPV DNA to 0.1 µg of plasmid pBR322 DNA) in a reaction mixture (20 µl) containing 10 mM Tris-hydrochloride (pH 7.5), 10 mM MgCl_2 , 0.1 mM ATP, 2 mM dithiothreitol, and 1 U of T4 DNA ligase (Boehringer Mannheim Corp.). After an incubation period of 16 h at 4°C , the mixture was diluted to a volume of 0.3 ml by adding 100 mM Tris-hydrochloride (pH 7.4) and used to transfect *E. coli* K-12 DP50 bacteria treated with calcium chloride (41). Colonies containing recombinant plasmids were selected by their phenotype—resistance to ampicillin and sensitivity to tetracycline. The recombinant plasmids were amplified with chloramphenicol (150 µg/ml) (3) and selectively extracted by the clear lysate technique of Katz et al. (20), either with a small volume of culture (2 ml) to identify recombinant DNAs or with a great volume of culture (100 ml) for molecular studies of viral genomes. In the latter case, the recombinant DNAs were purified by equilibrium centrifugation in cesium chloride gradients

in the presence of ethidium bromide (27). The DNAs of HPV-5 and HPV-9 were separated from the plasmid DNA by cleaving the recombinant plasmids with the same endonuclease used for insertion and were purified by sedimentation in 5 to 21% sucrose gradients with a Beckman SW28 rotor at 25,000 rpm and 10°C for 29 h.

DNA blot hybridization. Form I DNA of HPV-1a, the equimolar mixture of the cloned *Hind*III-A and *Hind*III-B of HPV-5 DNA, and the cloned HPV-9 DNA (the single fragment produced by *Bam*HI) were labeled by nick translation in the presence of [α - ^{32}P]dCTP (2,000 to 3,000 Ci/mmol; Radiochemical Centre, Amersham, England) and of *E. coli* DNA polymerase I (grade I; Boehringer Mannheim Corp.), by the method of Wold et al. (43). The DNA sequences of HPV-5 and HPV-9, which were isolated from recombinant plasmids, and the uncloned DNAs of other HPVs (HPV-1a, HPV-2, HPV-3a, HPV-4, and HPV-7) were cleaved using one or a mixture of endonucleases, and the digestion products were separated by electrophoresis through agarose (1%) in horizontal slab gels. The DNA fragments were depurinated and denatured in situ and then transferred and immobilized on diazobenzoyloxymethyl paper (DBM paper) (1). The DNAs immobilized on DBM paper were incubated, before hybridization, in Denhardt solution (7) for 4 h at 60°C . The viral DNA, labeled with ^{32}P (specific activity, 7.4×10^7 to 9×10^7 cpm/µg; 2×10^4 cpm/cm 2), was hybridized with the DNA immobilized on DBM paper for 18 h at 37°C in a reaction medium containing 1 M NaCl, 10 mM Tris-hydrochloride (pH 7.4), 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 10 µg of denatured and sonicated salmon DNA per ml, and 50% formamide (17). The effective temperature for hybridization was determined from the melting temperature (T_m) of the HPV-1 genome, the only HPV whose percentage of guanine plus cytosine (G+C) is known (41%) (5, 13), using the equation T_m (degrees Celsius) = $81.5 + 16.6 (\log M) + 0.41 (\text{percent G+C}) - 0.72 (\text{percent formamide})$, where M is the molarity of the monovalent salt (15, 23, 37). The sheets of DBM paper were extensively washed in $4 \times \text{SSC}$ ($\text{SSC} = 0.15 \text{ M NaCl}$ and $0.015 \text{ M sodium citrate}$) (17). The hybrid molecules were detected by autoradiography, using RP Royal X-Omat X-ray films (Kodak) and Dupont Lightning Plus screens.

Liquid-phase reassociation of DNA. The HPV DNAs labeled with ^{32}P were fractionated by sedimentation in alkaline sucrose gradients (5 to 20%) in 300 mM NaOH–100 mM NaCl–1 mM EDTA–0.015% Sarkosyl (43). The fractions corresponding to fragments of 400 to 600 nucleotides (27) were mixed and dialyzed against 10 mM Tris-hydrochloride–1 mM EDTA (pH 7.9). After a 10-min incubation at 100°C , the single-stranded DNA was purified by batchwise chromatography on hydroxylapatite. The unlabeled viral DNAs (10 µg/ml) mixed with calf thymus DNA and calf thymus DNA alone (1 mg/ml) were sonicated under conditions permitting single-stranded DNA fragments of 4S to 6S (27) to be obtained. The DNAs were denatured for 5 min at 100°C , and the reassociation of the labeled probes (2,000 cpm), in the presence of calf thymus (25 µg) alone or mixed with unlabeled viral DNA (0.25 µg), was performed in sealed capillary tubes in 0.48 M NaCl–1 mM EDTA (pH 6.8) for 16 h at

FIG. 1. Restriction endonuclease analysis of HPV sequences integrated in recombinant DNAs. Unrestricted viral DNAs, obtained from macules of patients 1 (lane a) and 2 (lane b), were electrophoresed at 35 V for 14 h in 1% agarose vertical slab gels, as described in the text. Unclassified HPV-5 (lane c), plasmid pBR322/HPV-5 (pHPV-5) *HindIII*-A (clone 11) (lane d), pHPV-5 *HindIII*-B (clone 1) (lane e), pBR322 (lane f), pHPV-9 *Bam*HI (clone 6) (lane g), and unclassified HPV-9 (lane h) DNAs were cleaved with a mixture of *Bam*HI and *Hind*III endonucleases. The electrophoretic separation of the fragments was carried out at 150 V for 3 h in 1% agarose horizontal slab gels, as described in the text. The fragments are designated by capital letters for HPV DNA fragments and by small letters for pBR322 DNA fragments, according to their increasing electrophoretic mobilities. The *Bam*HI-*Hind*III-D fragment of pHPV-5 *Hind*III-A (clone 11) DNA ran off the gel.

68°C. The percentage of reassociation was determined either by digestion with nuclease S1 (40) or by batch-wise chromatography on hydroxylapatite (43). After precipitation with trichloroacetic acid, the radioactivity was measured with a scintillation spectrometer.

RESULTS

Molecular cloning of HPV-5 and HPV-9 DNAs and characterization of recombinant DNAs. The HPV DNAs were extracted and purified from scrapings of reddish macular lesions or pityriasis versicolor-like lesions which were localized on the back of patient 1 and at different locations on the trunk of patient 2 (27, 32). The HPV-5 DNA preparation obtained from the lesions of patient 1 was homogeneous, as shown by electrophoretic analysis in an agarose gel of uncleaved viral DNA (Fig. 1, lane a) or DNA treated with a mixture of *Bam*HI and *Hind*III (Fig. 1, lane c) which produced four fragments of molecular weights 2.84×10^6 , 1.77×10^6 , 0.33×10^6 , and 0.2×10^6 (the latter fragment is not visible in Fig. 1). In contrast, the heterogeneity of the HPV DNA preparation obtained from patient 2 was shown by the presence of two classes of form I and II molecules (Fig. 1, lane b), one of which (minor) had an electrophoretic mobility analogous to that of HPV-5 DNA, the other of which (of higher mobility) corresponded to the DNA of the major virus, HPV-9. Similarly, the

cleavage of the HPV DNA preparation from patient 2 by a mixture of endonucleases *Bam*HI and *Hind*III (Fig. 1, lane h) resulted in four major fragments (molecular weights, 1.95×10^6 , 1.70×10^6 , 0.74×10^6 , and 0.43×10^6) and in numerous minor fragments corresponding to additional species of HPV DNA (Table 1).

Table 2 summarizes the sensitivity of HPV-5 and HPV-9 DNAs to 12 restriction endonucleases. Among these, none cleaved the HPV-5 DNA only once, whereas five enzymes cleaved the viral genome twice (*Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, and *Pvu*II). With the exception of *Bgl*II, these enzymes cleaved the DNA of plasmid pBR322 once, and two of them, *Bam*HI and *Hind*III, interrupt the gene coding for resistance to tetracycline (39). *Hind*III-A and *Hind*III-B of HPV-5 DNA (93.4 and 6.6% of the genome, respectively) were chosen for insertion in plasmid pBR322. Among the five endonucleases (*Bam*HI, *Bgl*II, *Eco*RI, *Pst*I, and *Pvu*II) which cleaved the HPV-9 genome only once (Table 2), the *Bam*HI restriction site was used to insert the viral genome in plasmid pBR322.

To characterize the recombinant plasmids, the electrophoretic mobilities of the digestion products of recombinant DNAs, HPV DNAs obtained from patients 1 and 2, and DNA of plasmid pBR322, treated with a mixture of endonucleases *Bam*HI and *Hind*III, were compared

TABLE 2. Sensitivity of HPV-5 and HPV-9 DNAs to several restriction endonucleases^a

DNA fragment	Size of fragments obtained after cleavage of:			
	HPV-5 DNA		HPV-9 DNA	
	Mol wt ($\times 10^6$) ^b	% of genome ^c	Mol wt ($\times 10^6$) ^b	% of genome ^c
<i>Ava</i> I				
A ^d	2.40	47.5	3.40	73.1
B	1.55	30.7	1.25	26.9
C	1.10	21.8		
<i>Bam</i> HI				
A	3.0	57.7	4.75	100
B	2.20	42.3		
<i>Bgl</i> II				
A	3.0	58.8	4.80	100
B	2.0	39.2		
C	0.10	2.0		
<i>Bgl</i> III				
A	2.95	57.8	2.30	47.9
B	2.15	42.2	1.60	33.3
C			0.90	18.8
<i>Eco</i> RI				
A	3.80	75.3	4.70	100
B	1.25	24.7		
<i>Hind</i> III				
A	1.80	37.6	1.90	39.4
B	0.58	12.1	1.60	33.2
C	0.58	12.1	0.82	17.0
D	0.44	9.2	0.50	10.4
E	0.40	8.4		
F	0.38	7.9		
G	0.31	6.5		
H	0.30	6.2		
<i>Hind</i> III				
A	4.70	93.4	2.40	49.2
B	0.33	6.6	1.70	34.8
C			0.78	16.0
<i>Hpa</i> I				
A	2.95	58.6	2.70	56.2
B	0.90	17.9	1.55	32.6
C	0.90	17.9	0.50	11.2
D	0.28	5.6		
<i>Pst</i> I				
A	3.10	60.5	4.70	100
B	1.25	24.4		
C	0.48	9.4		
D	0.29	5.7		
<i>Pvu</i> II				
A	2.80	55.4	4.75	100
B	2.25	44.6		

^a In addition to the listed endonucleases, HPV-5 and HPV-9 DNAs were found to be uncleaved by *Pvu*I and *Sma*I endonucleases.

^b The molecular weights of the DNA fragments were evaluated from their electrophoretic mobilities,

FIG. 2. Electrophoretic analysis of restriction endonuclease digests of pHPV-5 *Hind*III-A and pHPV-5 *Hind*III-B recombinant plasmids. pHPV-5 *Hind*III-A clone 11 (lanes a and e through g) and clone 4 (lane b) DNAs and pHPV-5 *Hind*III-B clone 1 (lane c) and clone 5 (lane d) DNAs were cleaved with *Eco*RI (lanes a through d), *Bam*HI (lane e), *Pvu*II (lane f), and a mixture of *Bgl*II and *Pvu*I (lane g) endonucleases. The electrophoretic separation of the fragments was carried out at 150 V for 3 h in 1% agarose horizontal slab gels, as described in the text. The fragments are designated by their molecular weights for HPV DNA fragments. Underlined molecular weights indicate pBR322/HPV DNA fragments; the molecular weights of the HPV-5 DNA sequences contained in mixed fragments were obtained by subtracting the molecular weights of the pBR322 DNA sequences.

(Fig. 1, lanes c through h). Several recombinant DNAs, which had integrated *Hind*III-A or *Hind*III-B of HPV-5 DNA (Fig. 1, lanes d and e) and the genome of HPV-9 (Fig. 1, lane g), were identified. The orientation of the viral sequences in the recombinant plasmids was determined by cleaving with *Eco*RI the recombinant plasmids which had integrated *Hind*III-A or *Hind*III-B of HPV-5 DNA or HPV-9 DNA. Two fragments, whose molecular weights differed according to the orientation of the viral sequences, were obtained for each type of recombinant plasmid (Fig. 2, lanes a through d; Fig. 4, lanes a and b).

The sizes of *Hind*III-A of HPV-5 DNA and of the linear HPV-9 DNA inserted in the recombinant plasmids, used for the construction of cleavage maps, were determined by measuring in the electron microscope the digestion prod-

using the *Hind*III fragments of λ bacteriophage and simian virus 40 DNAs as standards (11, 25).

^c The molecular weights of the genomes were obtained from the sum of the molecular weights of the fragments.

^d The fragments are designated by capital letters according to their increasing electrophoretic mobility.

FIG. 3. Cleavage map of HPV-5 DNA. One of the two *Hind*III cleavage sites was taken as point zero. The distances from the origin are given in fractional length of the HPV-5 genome.

ucts of the recombinant DNAs treated with the same endonuclease used for insertion of the viral sequences. The lengths of *Hind*III-A of HPV-5 DNA and of the linear HPV-9 DNA, measured on 50 molecules, represented 1.608 ± 0.11 and 1.648 ± 0.09 , the length of the pBR322 DNA, respectively (the latter was determined by measuring 50 molecules). Since the pBR322 DNA contains 4,362 base pairs (39), the molecular weights of *Hind*III-A of HPV-5 DNA and of HPV-9 DNA were estimated to be 4.63×10^6 (7,014 base pairs) and 4.74×10^6 (7,188 base pairs), respectively. Assuming that *Hind*III-A of HPV-5 represented 93.4% of the genome length (Table 2), the molecular weight of HPV-5 DNA was estimated to be 4.96×10^6 (7,510 base pairs).

Construction of a cleavage map of HPV-5 DNA. A cleavage map of the HPV-5 genome was built by using the recombinant plasmids containing *Hind*III-A or *Hind*III-B of HPV-5 DNA.

The 10 cleavage sites, corresponding to five enzymes cleaving the HPV-5 genome twice (*Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, and *Pvu*II) (Table 2) were localized. With the exception of one *Eco*RI cleavage site, all of the other sites were found on *Hind*III-A; this could be deduced from

the number of fragments produced by recombinant plasmids (Fig. 2). The distances between the *Bam*HI, *Bgl*II, *Eco*RI, and *Pvu*II cleavage sites and the *Hind*III insertion sites were deduced from the sizes of the viral sequences in mixed fragments produced after cleavage of recombinant plasmids, which had integrated viral DNA in the two orientations, by endonucleases *Bam*HI, *Eco*RI, and *Pvu*II and by a mixture of endonucleases *Bgl*II (which does not cleave the pBR322 DNA) and *Pvu*I (which cleaves the pBR322 DNA once and does not cleave the HPV-5 DNA) (Fig. 2). The sizes of the fragments produced after cleavage of the uncloned HPV-5 DNA by endonuclease *Eco*RI (Table 2) permitted the unambiguous orientation of *Hind*III-A and *Hind*III-B in relation to each other. A cleavage map of the prototypical HPV-5 genome was built, using one of the *Hind*III sites as the origin (Fig. 3).

Construction of a cleavage map of HPV-9 DNA. Fifteen cleavage sites were localized, corresponding to enzymes which cleaved the HPV-9 DNA once (*Bam*HI, *Eco*RI, and *Pvu*II), twice (*Ava*I), three times (*Hind*III and *Hpa*I), or four times (*Hind*II) (Table 2). The distances between the *Ava*I, *Eco*RI, and *Pvu*II cleavage sites and

FIG. 4. Electrophoretic analysis of restriction endonuclease digests of HPV-9 recombinant plasmids. pHPV-9 clone 6 (lanes a and c through h) and clone 12 (b) DNAs were cleaved with *EcoRI* (a and b), *PvuII* (c), *AvaI* (d), *HindIII* (e), *HpaI* (f), *HindII* (g), and *HindII-HindIII* (h). The electrophoretic separation of the fragments was carried out at 150 V for 3 h in 1% agarose horizontal slab gels, as described in the text. The fragments are designated by their molecular weights for HPV DNA fragments. Underlined molecular weights indicate pBR322/HPV DNA fragments, and the unlabeled DNA fragments correspond to pBR322 DNA fragments. The molecular weights of HPV-9 DNA sequences contained in mixed fragments were obtained by subtracting the molecular weights of the pBR322 DNA sequences.

the *Bam*HI insertion site and the distances between the closest *HindII* and *HindIII* cleavage sites and the *Bam*HI insertion site were deduced from the sizes of the viral sequences in mixed fragments produced after cleavage of recombinant plasmids, which had integrated viral DNA in the two orientations, by endonucleases *AvaI*, *EcoRI*, *HindII*, *HindIII*, and *PvuII* (Fig. 4). The other *HindII* and *HindIII* cleavage sites were localized by comparing the sizes of the fragments produced after cleavage of recombinant plasmids by endonucleases *HindII* and *HindIII* and by a mixture of endonucleases *HindII* and *HindIII* (*HindII-HindIII*) (Fig. 4, lanes e, g, and h). The localization of the *HpaI* cleavage sites was obtained by comparing the sizes of the fragments produced after cleavage of recombinant plasmids by endonucleases *HindII* and *HpaI* (Fig. 4, lanes f and g). These data permitted the construction of a cleavage map of the HPV-9 genome, using the unique *Bam*HI cleavage site as the origin (Fig. 5).

Analysis of sequence homology between HPV DNAs. *HindIII*-A and *HindIII*-B of HPV-5 DNA (in an equimolar mixture) and the linear molecules of HPV-9 DNA (purified from recombinant plasmids) were used for studies of homolo-

gy of nucleotide sequences by hybridization in liquid medium at saturation (42, 43) or by blot hybridization (1, 38).

The experiments of reassociation in liquid medium at saturation were performed at 68°C in 0.48 M NaCl–1 mM EDTA (pH 6.8). This corresponded to an effective hybridization temperature lower by 25°C than the T_m of HPV-1 DNA, the only HPV whose percentage of G+C is known (41%) (5, 13), as deduced from the equation derived by Schildkraut and Lifson (37). The results reported in Table 3 show that only 4 to 5% of HPV-5 and HPV-9 DNAs hybridize with each other, whether the reassociation is evaluated by measuring the fraction resistant to nuclease S1 (40) or by batchwise chromatography on hydroxylapatite (43). A weaker hybridization level, 2 to 3%, was observed between the HPV-1a DNA and the HPV-5 or HPV-9 DNA. Similar results were obtained when HPV DNA probes were annealed in the presence of amounts of unlabeled HPV DNA ranging from 1 to 5 µg/ml (data not shown).

The nucleotide sequence homologies existing between these genomes and those of prototypes of other HPVs associated with cutaneous lesions (HPV-1, HPV-2, HPV-3, HPV-4, and HPV-7) were also analyzed by blot hybridization experi-

TABLE 3. DNA sequence homology between HPV-1a, HPV-5, and HPV-9 as determined by cross-hybridization in liquid phase^a

[³² P]DNA	Unlabeled DNA	Percentage of hybridization	
		S1 nuclease	Hydroxylapatite
HPV-1a	Calf thymus	9.0 (0)	5.1 (0)
	HPV-1a	82.6 (100)	83.3 (100)
	HPV-5	11.0 (2.7)	7.4 (2.9)
	HPV-9	11.4 (3.3)	6.3 (1.5)
HPV-5	Calf thymus	8.5 (0)	2.8 (0)
	HPV-1a	8.6 (0.1)	4.7 (1.7)
	HPV-5	77.3 (100)	69.9 (100)
	HPV-9	12.3 (5.5)	6.5 (4.1)
HPV-9	Calf thymus	9.6 (0)	11.7 (0)
	HPV-1a	11.3 (2.3)	12.3 (0.8)
	HPV-5	12.3 (3.7)	14.9 (4.3)
	HPV-9	82.0 (100)	88.6 (100)

^a ³²P-labeled HPV DNAs were annealed in the presence of calf thymus DNA or unlabeled HPV DNAs, as described in the text. The specific activities of HPV-1a, HPV-5, and HPV-9 DNA probes were 1.2×10^8 cpm/µg, 8.2×10^7 cpm/µg, and 6.8×10^7 cpm/µg, respectively. The percentage of hybridization was determined by measuring the nuclease S1-resistant fractions or by batchwise chromatography on hydroxylapatite. The numbers in parentheses represent values corrected for the self-annealing of the probes and normalized to 100% for the homologous hybridization.

FIG. 5. Cleavage map of HPV-9 DNA. The unique *Bam*HI cleavage site was taken as point zero. The distances from the origin are given in fractional length of the HPV-9 genome.

ments (Fig. 6). These experiments were performed under stringent conditions (in the presence of 50% formamide and at a molar concentration of monovalent ion at 37°C, which corresponds to an effective hybridization temperature lower by 25°C than the T_m of HPV-1 taken for a control) (17, 23, 37). Under these conditions, only hybrids with at least 82% homologous bases are stable (18). The HPV DNAs were cleaved by one endonuclease or by a mixture of endonucleases, producing four to six fragments (Table 4) whose maps have been reported for only prototypical HPV-1 (HPV-1a) (8, 9, 12), HPV-4 (15), HPV-5, and HPV-9 (Fig. 3 and 5) DNAs. As shown in Fig. 6, no or very little hybridization could be detected when the labeled DNAs of HPV-1a, HPV-5, and HPV-9 were hybridized with heterologous DNAs. The labeled HPV-9 DNA showed a weak or very weak hybridization level with, in order of decreasing label intensity, the HPV-5 DNA fragments produced by *Hind*III-*Pvu*II (6.6 to 34.4 map units [MU]), *Pvu*II-*Eco*RI (34.4 to 81.7 MU), and *Eco*RI-*Pvu*II (81.7 to 90.7 MU) (Fig. 3 and Table 4). Reciprocally, the labeled HPV-5

DNA showed hybridization with the *Bam*HI-*Eco*RI fragment (0 to 33.3 MU) of HPV-9 DNA and a very weak cross-hybridization with *Hind*III-C (40.3 to 55.5 MU) (Fig. 5). The labeled DNAs of HPV-5 and HPV-9 showed only a very weak degree of cross-hybridization with fragment *Hind*II-*Hind*III-A (61.8 to 95.3 MU) of HPV-1a DNA (9), whereas the labeled HPV-1a DNA showed a very weak degree of cross-hybridization with fragment *Hind*III-*Pvu*II (6.6 to 34.4 MU) of HPV-5 DNA (Fig. 3) and a stronger degree of hybridization with *Hind*III-B (55.5 to 91.2 MU) of HPV-9 DNA (Fig. 5). In addition, the labeled HPV-5 DNA did not form stable hybrids with the HPV-2 and HPV-7 DNAs and showed only a very weak degree of hybridization with two fragments of HPV-3a DNA, representing 24.0% of the genome length, and a weak degree of cross-hybridization with fragments *Hind*III-*Hpa*I (36.0 to 75.0 MU) and *Hind*III-B (3.6 to 36.0 MU) of HPV-4 DNA (15) (Fig. 6 and Table 4). The labeled HPV-9 DNA did not form stable hybrids with the HPV-2, HPV-3a, HPV-4, and HPV-7 DNAs (Fig. 6). Under the same conditions, the labeled HPV-1a

FIG. 6. DNA sequence homology between HPV genomes as detected by blot hybridization experiments. (Left upper panel) HPV DNAs were cleaved with *HindII-HindIII* endonucleases for HPV-1a (lane a) and HPV-3a (lane c) DNAs; *HindII* for HPV-2 DNA (lane b); *BamHI*, *HindIII*, and *HpaI* for HPV-4 DNA (lane d); *HpaI* for HPV-7 DNA (lane e); *EcoRI* and *PvuII* for an equimolar mixture of *HindIII*-A and *HindIII*-B/HPV-5 cloned DNA fragments (lane f); and *EcoRI* and *HindIII* for cloned *BamHI*/HPV-9 DNA (lane g). The cleavage products (approximately 0.2 μg of DNA per slot) were electrophoresed at 150 V, for 3 h, in 1% agarose horizontal slab gels. The DNA fragments were depurinated, denatured, transferred on DBM paper, and hybridized with ^{32}P -labeled HPV-1a DNA (specific activity, 6×10^7 cpm/ μg) (right upper panel), HPV-5 DNA (specific activity, 7.3×10^7 cpm/ μg) (left lower panel), or HPV-9 DNA (specific activity, 7.4×10^7 cpm/ μg) (right lower panel), as described in the text. The blots were exposed for 6 h, using RP Royal X-Omat X-ray film (Kodak).

DNA did not form stable hybrids with the HPV-2 DNA and showed a small degree of hybridization with fragment *HpaI-HindIII* (75.0 to 86.0 MU) of HPV-4 DNA (15), which was only detected at $T_m - 43^\circ\text{C}$ by Heilman et al. (15). A weak cross-hybridization was observed with three fragments of HPV-3a DNA, representing 31.0% of the genome length, and with *HpaI*-B of

HPV-7 DNA, representing 31.6% of the genome (Fig. 6 and Table 4).

DISCUSSION

These results describing the biochemical characterization of the cloned HPV-5 and HPV-9 DNAs confirm that these genomes are two distinct molecular species. Their DNAs have differ-

TABLE 4. Restriction endonuclease HPV DNA fragments used for sequence homology studies

DNA	Enzyme	No. of fragments	MW of DNA fragments ($\times 10^6$) ^a
HPV-1a	<i>HindII-HindIII</i>	6	1.80 ^b , 1.40, 0.94, 0.81, 0.19, 0.06
HPV-2	<i>HindII</i>	4	2.54 ^c , 1.65, 0.92, 0.10
HPV-3a	<i>HindII-HindIII</i>	6	1.62 ^d , 1.25, 0.74, 0.63, 0.50, 0.36
HPV-4	<i>BamHI + HindIII + HpaI</i>	5	1.98 ^e , 1.58, 0.70, 0.58, 0.18
HPV-7	<i>HpaI</i>	4	2.50, 1.78, 0.83, 0.25
<i>HindIII</i> fragments A + B/HPV-5	<i>EcoRI + PvuII</i>	6	2.45 ^f , 1.63, 0.49, 0.43, 0.32, 0.01
<i>BamHI</i> /HPV-9	<i>EcoRI + HindIII</i>	5	1.75 ^g , 1.60, 0.81, 0.44, 0.44

^a The molecular weights of the DNA fragments were evaluated from their electrophoretic mobilities, using the *HindIII* fragments of λ bacteriophage and simian virus 40 DNAs as standards (11, 25).

^b The six *HindII-HindIII* fragments of HPV-1a DNA are localized at 61.8 to 95.3 MU, 18.3 to 45.8 MU, 0.3 to 18.3 MU, 45.8 to 61.8 MU, 95.3 to 99.1 MU, and 99.1 to 0.3 MU, respectively, on the cleavage map of the genome (9).

^c The sizes of the four *HindII* fragments of HPV-2 DNA are in agreement with previous data (27, 28).

^d The sizes of the six *HindII-HindIII* fragments of HPV-3a DNA are in agreement with previous data (27).

^e The five *BamHI + HindIII + HpaI* fragments of HPV-4 DNA are localized on the cleavage map of the genome at 36.0 to 75.0 MU, 3.6 to 36.0 MU, 86.0 to 100 MU, 75.0 to 86.0 MU, and 0 to 3.6 MU, respectively (15).

^f The six *EcoRI + HindIII + PvuII* fragments of HPV-5 DNA are localized on the cleavage map of the genome at 34.4 to 81.7 MU, 6.6 to 34.4 MU, 81.7 to 90.7 MU, 90.7 to 100 MU, 0 to 6.4 MU, and 6.4 to 6.6 MU, respectively (Fig. 3).

^g The five *BamHI + EcoRI + HindIII* fragments of HPV-9 DNA are localized on the cleavage map of the genome at 55.5 to 91.2 MU, 0 to 33.3 MU, 40.3 to 55.5 MU, 91.2 to 100 MU, and 33.3 to 40.3 MU, respectively (Fig. 5).

ent molecular weights (about 7,500 and 7,200 nucleotide pairs, respectively). The cleavage maps of the two genomes showed no obvious analogy. Only a small sequence homology (4 to 5%) was detectable in reassociation experiments performed at saturation in liquid medium. This homology was reproducibly higher than that observed between these genomes and the HPV-

1a genome (2 to 3%). The sequence homology between HPV DNAs was further studied by blot hybridization experiments which were performed under stringent conditions, using restriction fragments of prototypical skin HPV DNAs and HPV-1a, HPV-5, and HPV-9 DNA probes. Blot hybridization experiments showed that the nucleotide sequences forming stable hybrids between HPV-5 and HPV-9 DNAs are mostly localized in the regions between 6.6 and 34.4 MU on the cleavage map of HPV-5 DNA (Fig. 3) and between 0 and 33.3 MU on the cleavage map of HPV-9 DNA (Fig. 5). Furthermore, these experiments revealed no sequence homology between the HPV-5 or HPV-9 DNAs and the genomes of other viruses associated with cutaneous lesions or a degree of homology lower than that existing between the DNAs of HPV-5 or HPV-9 and the DNA of HPV-1a.

A study of 10 Polish patients, including patients 1 and 2, suffering from a severe form of EV (that is, presenting flat wart-like and macular lesions and, in the case of seven patients, carcinomas) reveals the multiplicity of viruses specifically associated with this disease (27, 30, 32; Orth et al., in preparation). At least 10 viruses (including HPV-3 found in four patients) have been identified, each showing its own cleavage pattern when submitted to the action of different restriction endonucleases. In addition, most of the patients were infected by several HPVs, seven in the case of patient 2, in variable proportions depending on the localization of the lesions (Orth et al., in preparation). Blot hybridization experiments, performed under stringent conditions, revealed that the genomes of four of these viruses showed some sequence homology with HPV-5 DNA and that one genome showed some sequence homology with HPV-9 DNA, although to an extent of less than 50%. One of these viruses, present in a small proportion in patient 2, present as the major virus in patient 3 (27, 30, 32), and later found also in a French patient (21), was initially called HPV-5c (Table 1) (27). A closely related virus was found later in an Upper Volta patient and named HPV-8 by Pfister et al. (35). After being cloned in *E. coli*, the genome of HPV-5c was found to share about 17 and 2% sequence homology with HPV-5 and HPV-9 genomes, respectively, as shown by DNA reassociation experiments (Kremsdorf et al., in preparation). Thus, this virus is a distinct type, HPV-8, by the criteria adopted for the classification of papillomaviruses (4). It should be noticed that all of the EV HPV isolates from Polish patients which cross-hybridized with HPV-5 DNA also cross-hybridized with HPV-8 DNA, and the extent of homology was less than 50%. This suggests the existence of additional HPV types in EV patients.

The molecular cloning of these viral genomes and their characterization, which are at present being studied, will allow us to better define the relationships between the numerous viruses associated with EV.

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