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Molecular Cloning and Characterization of the Genomes of Nine Newly Recognized Human Papillomavirus Types Associated with Epidermodysplasia Verruciformis

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The genomes of 11 human papillomaviruses (HPVs) found in benign lesions of eight patients suffering from epidermodysplasia verruciformis were cloned in *Escherichia coli* after insertion into plasmid pBR322. The study of the sensitivity of the cloned HPV DNAs to 14 restriction endonucleases permitted the construction of physical maps. DNA-DNA hybridization experiments, performed under stringent conditions, showed that these viruses represent nine new types, HPVs 14 (with subtypes a and b), 15, 17 (with subtypes a and b), 19, 20, 21, 22, 23, and 24. These HPVs were divided into three groups based on an absent or very weak cross-hybridization among the genomes of the viruses belonging to different groups.

Epidermodysplasia verruciformis (EV) is a rare disease characterized by disseminated, flat warts, macular lesions, or both, as well as by the early development of skin carcinomas in about one-third of EV patients (8, 12, 15). Early studies had stressed the role of genetic factors and actinic radiations in the pathogenesis of EV (8, 12). However, more recent studies have revealed the importance of immune factors (9) and the major role of specific human papillomavirus (HPV) types (10, 11, 15, 17–20, 22, 26). The study of a small number of EV cases has led to the characterization of six HPV types, after molecular cloning of their genomes (10, 11). These HPVs have been divided into three groups based on an absence of homology or a very weak homology among the genomes belonging to different groups (11). The first group includes HPVs 3a and 10, which are associated with flat warts found in some EV patients and in the general population; DNA sequences related to those of HPV 3a have been found in an EV carcinoma (6). The second group includes HPVs 5, 8, and 12 (11), the genomes of HPVs 5 and 8 having been detected in carcinomas of EV patients (15, 18, 20; G. Orth, S. Jablonska, L. A. Rueda, M. A. Lutzner, C. Blanchet-Bardon, S. Obalek, M. Favre, D. Kremsdorf, and O. Croissant, manuscript in preparation). The third group consists, so far, of only one virus, HPV 9. With the exception of an immunosuppressed renal-allograft recipient who was found to be infected by HPV 5 (13), the viruses of the two latter groups have been detected only in EV patients, most of them being infected by several viruses (4, 10, 11, 15, 17, 19, 20). It should be noticed that, among the 15 HPV types presently reported in the literature (1–3, 5–7, 10, 11, 15, 16, 18, 20–22), 4 have been found to be specifically associated with EV, a rare disease (11). However, a much higher degree of genetic heterogeneity has been revealed by a study of 60 EV cases carried out by our group, which permitted the identification of some 20 HPVs in benign lesions, including HPVs 3a, 5, 8, 9, 10, and 12 (10, 11), based on distinct restriction endonuclease cleavage patterns and on molecular hybridization experiments (Orth et al., in preparation). In this paper we report the molecular cloning of 11 HPV genomes from some of these patients, which has led to the characterization of nine new types.

Viral DNA preparations were selectively extracted (15) from scrapings of benign lesions of six European EV patients and of two South American EV patients (Table 1). The HPV DNAs were purified by equilibrium centrifugation in cesium chloride gradients, sedimentation in sucrose gradients in the presence of ethidium bromide, or both, by previously described procedures (10, 11, 15). The DNA preparations were treated with restriction endonucleases, and the digestion products were separated by electrophoresis in agarose gels (10, 11). Complex cleavage patterns were observed in most DNA preparations, suggesting that the patients were infected with more than one HPV type. In addition to HPVs 5, 8, and 12 (10, 11), and to HPV 2 (15) found in the common warts of one of the patients, 11 major DNA cleavage patterns, different from those of the previously characterized types, were identified (Table 1; Fig. 1). These viruses are designated (Table 1) according to a nomenclature established after characterization of the cloned genomes. The new HPV types were given a number, and the subtypes of a type were given the same number followed by a letter, based on the chronological order of their identification (2). The genomes of the 11 HPVs were cloned in *Escherichia coli* K-12 strain C600 (11). The DNAs were inserted as unit-length molecules, with the exception of the two HPV 24 DNA fragments generated by endonuclease *Bam*HI. They were inserted either in plasmid pBR322 (25), by using the unique cleavage sites of *Ava*I, *Bam*HI, and *Hin*dIII, or in a recombinant plasmid having integrated *Hin*dIII fragment B of HPV 5 DNA (10), which contains a unique *Sac*I site (Table 1; unpublished data).

To identify the recombinant plasmids, the electrophoretic mobilities of the digestion products of the recombinant DNAs and of the uncloned HPV DNAs were compared after treatment with a mixture of two restriction endonucleases which included the endonuclease used for insertion of the

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TABLE 1. Origin of the cloned HPV DNAs

Patient[a]	Nationality	Source[b]	Cloned HPV DNA type	Cloning enzyme[c]	Other HPV types found in patients[d]
1	Polish	Warts, knees	14a	*Hin*dIII	5
			15	*Bam*HI	
2	French	Warts, hands	14b	*Bam*HI	
3	Colombian	Macules, trunk	17a	*Bam*HI	5
4	Italian	Macules, chest	17b	*Sac*I	5
			22	*Sac*I	
5	Dutch	Macules, back	19	*Bam*HI	5, 8, 17a
		Macules, breast	24	*Bam*HI	
6	Colombian	Warts, hands	20	*Ava*I	5, 8, 24
7	Polish	Warts, knees	21	*Bam*HI	2, 12, 17a, 20
8	Polish	Macules, forearms	23	*Bam*HI	5, 8, 20

[a] Detailed case reports have been described for patients 2 (patient 1 in reference 12a) (14), 5 (24), 6 (patient 2 in reference 23), and 8 (patient SM in references 15 and 17).

[b] Viral DNA preparations were obtained from pooled scrapings of flat wartlike lesions or of reddish or pityriasis versicolor-like macular lesions.

[c] DNAs of HPVs 14b, 15, 17a, 19, 20, 21, and 23 were inserted as unit-length DNA molecules in plasmid pBR322 (25), after treatment with an enzyme (*Ava*I, *Bam*HI, or *Hin*dIII) cleaving both DNAs only once. HPVs 17b and 22 were inserted as unit length DNA molecules after cleavage with an enzyme (*Sac*I) which cleaves HPV DNA and the pBR322 recombinant plasmid containing *Hin*dIII fragment B of HPV 5 DNA (10; unpublished data) only once. HPV 14a DNA was inserted in plasmid pBR322 as a unit length DNA molecule, after incomplete digestion of the viral DNA preparation with *Hin*dIII, an enzyme which generates two fragments of 96.1 and 3.9% of the genome length. The HPV 24 *Bam*HI fragments A and B (83.1 and 16.9% genome length, respectively) were inserted separately in plasmid pBR322.

[d] HPV DNAs of preparations obtained from lesions of different localizations were identified by restriction enzyme analysis, blot hybridization experiments, or both with cloned, labeled probes (G. Orth et al., in preparation).

viral sequences into the plasmid (Fig. 1). The number and size of the fragments indicated that, in each case, the whole viral genomes were integrated. In the case of three patients (patients 1, 4, and 5 [Table 1]), two distinct HPV DNAs were cloned. The bands present in the uncloned DNA preparations, which do not appear as bands in the recombinant plasmids, were shown to correspond either to previously recognized HPV types (10, 11) or to HPV types characterized in the present study, by blot hybridization experiments (Table 1). A heterogeneity in DNA sizes was observed when the DNAs of the HPVs, uncloned or excised from the plasmid sequences, were analyzed by agarose gel electrophoresis (data not shown). The DNAs of HPVs 14b, 19, 20, and 21 have sizes similar to those of HPVs 3a, 5, 8, and 12 (ca. 7,700 nucleotide pairs) (10, 11), whereas the DNAs of HPVs 15, 17a, 17b, 22, and 23 have smaller sizes, similar to that of HPV 9 (ca. 7,200 nucleotide pairs) (10, 15).

The sensitivity of the cloned viral genomes to 14 restriction endonucleases was analyzed, and physical maps were established (Fig. 2). Between 22 and 33 cleavage sites were localized by previously described methods (11). No obvious analogy was detected among these maps, with the exception of that between HPVs 14a and 14b on one hand and HPVs 17a and 17b on the other hand (Fig. 2). Among the 21 and 31 sites localized on the DNAs of HPVs 14a and 14b, respectively, 15 were found to be common when one of the two *Bam*HI cleavage sites of the HPV 14a DNA was aligned with the unique *Bam*HI cleavage site of the HPV 14b DNA. Similarly, 21 of the 29 cleavage sites situated on the DNA of HPV 17a were also found on the DNA of HPV 17b (with 26 sites), when the unique *Bam*HI cleavage sites were aligned. No obvious analogy was detected between these maps and those previously established for the HPVs associated with EV (HPVs 3a, 5, 8, 9, 10, and 12) (10, 11, 18, 20, 22), with cutaneous warts (HPVs 1, 2, and 4) (7; M. Favre, S. Jablonska, S.Obalek, O. Croissant, and G. Orth, manuscript in preparation), and with mucocutaneous or mucous membrane lesions (HPVs 6b, 11a, 13, and 16) (1, 3, 5, 21), with the exception of the HPV 14a map, which is closely related to the map of an HPV isolated from a Japanese EV patient (26). The latter isolate differs from HPV 14a by an additional *Bam*HI site and an additional *Hin*dII site, whereas the localizations of the *Ava*I, *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dII, and *Hin*dIII sites are similar in the two viruses. Cross-hybridization experiments have confirmed that these two viruses are very closely related (M. Yutsudo, M. Favre, and G. Orth, unpublished data).

The existence of sequence homologies among the DNAs of newly characterized HPV DNAs, as well as between the latter and the DNAs of previously characterized EV HPVs (HPVs 3a, 5, 8, 9, 10, and 12), HPVs associated with cutaneous warts (HPVs 1, 2, 4, and 7), and HPVs associated with lesions of the mucous membranes (HPVs 6b, 11a, 13, and 16), was investigated. Experiments with blot hybridization and DNA-DNA hybridization in liquid phase at saturation followed by nuclease S1 digestion were carried out under stringent conditions, as previously described (10, 11). Blot experiments, performed with 200 ng of immobilized HPV DNAs, have shown no cross-hybridization or almost no cross-hybridization between the genomes of HPVs 1, 2, 4, 6b, 7, and 11a and ^{32}P-labeled, newly cloned EV HPV DNAs (data not shown) or between the unlabeled EV HPV DNAs and probes specific for HPVs 13, 16 (data not shown), and 18 (L. Gissmann and H. zur Hausen, personal communication). Similarly, no cross-hybridization or almost no cross-hybridization was detected between the DNAs of HPVs 14a, 14b, 15, 17a, 17b, 19, 20, 21, 22, 23, and 24 and the DNAs of HPVs 1a and 11a by reassociation at saturation (Table 2). The newly cloned HPV DNAs presented no cross-hybridization, almost no cross-hybridization, or less than 50% cross-hybridization among themselves and with the genomes of the other EV-associated HPVs (HPVs 3a, 5, 8, 9, 10, and 12), with the exception of HPVs 14a and 14b on one hand and of HPVs 17a and 17b on the other hand, which showed a high cross-hybridization (Table 2). These observations justify the classification of the new viruses into nine new types (HPVs 14, 15, 17, 19, 20, 21, 22, 23, and 24) plus two subtypes within types 14 (HPVs 14a and 14b) and 17 (HPVs 17a and 17b), HPVs 16 and 18 having, meanwhile, been isolated from carcinomas of the uterine cervix (1, 3). The DNAs of HPVs 14, 19, 20, 21, 22, and 23 show cross-hybridization levels varying from 6 to 38% among themselves, and these DNAs cross-hybridize significantly (4 to 13%) only with the DNAs of HPVs 5, 8, and 12. These viruses are thus members of the

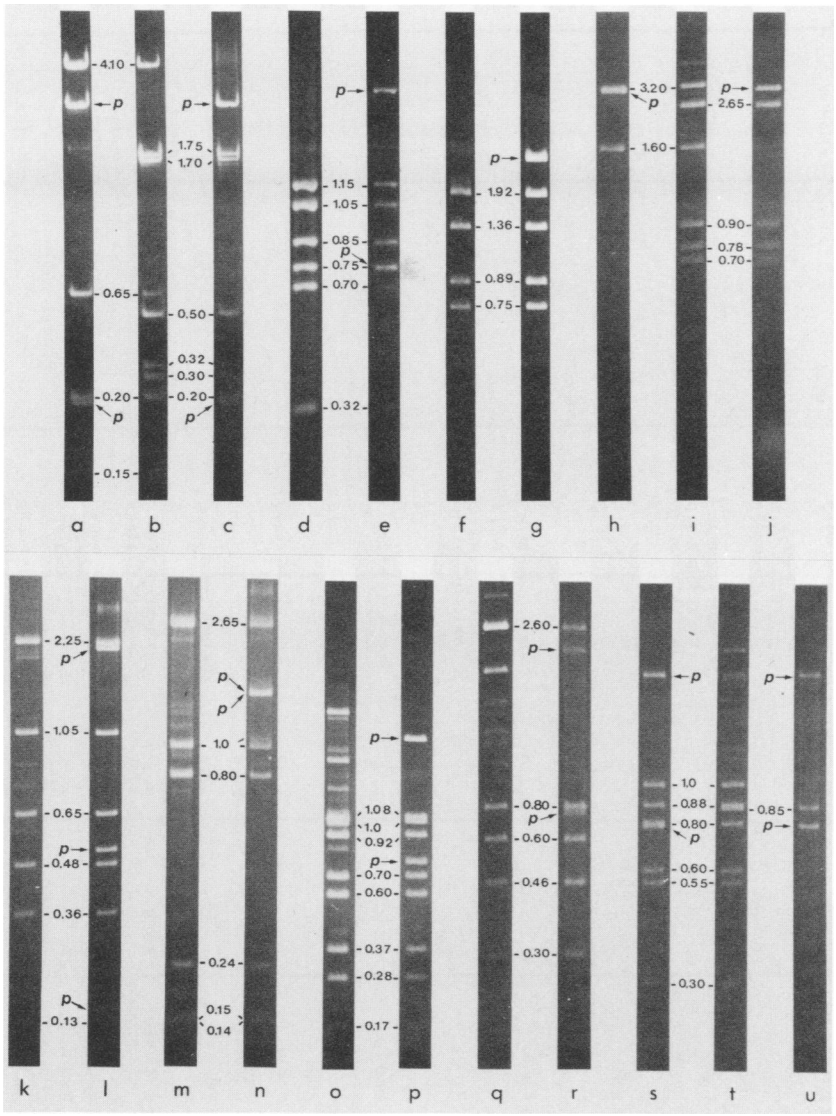

FIG. 1. Characterization of HPV DNAs inserted in recombinant plasmids with the aid of restriction endonucleases. The viral DNAs were selectively extracted by a modified Hirt method (15), purified, inserted in plasmid pBR322 (25), and then cloned in *E. coli* K-12 strain C600 (11). The recombinant plasmids were extracted as previously described (10, 11). Cloning of the viral genomes was performed with the agreement of the French commission for the control of in vitro genetic recombination. The following DNAs were run in the different slots: pBR322/HPV 14a (pHPV14a) (lane a), uncloned HPVs 14a and 15 (lane b), pHPV15 (lane c), uncloned HPV 14b (lane d), pHPV14b (lane e), uncloned HPV 17a (lane f), pHPV17a (lane g), pHPV17b (lane h), uncloned HPVs 17b and 22 (lane i), pHPV22 (lane j), uncloned HPV 19 (lane k), pHPV19 (lane l), uncloned HPV 20 (lane m), pHPV20 (lane n), uncloned HPV 21 (lane o), pHPV21 (lane p), uncloned HPV 23 (lane q), pHPV23 (lane r), pHPV24 *Bam*HI fragment A (lane s), uncloned HPV 24 (lane t), and pHPV24 *Bam*HI fragment B (lane u). The DNAs were cleaved by a mixture of *Bam*HI and *Hin*dIII (lanes a, b, and c), *Bam*HI and *Pst*I (lanes d, e, o–t, and u), *Bam*HI and *Bgl*II (lanes f and g), *Sac*I and *Bgl*II (lanes h, i, and j), *Bam*HI and *Hin*dII (lanes k and l), and *Ava*I and *Pst*I (lanes m and n). The DNA fragments were separated by electrophoresis in agarose slab gels, as previously described (10, 11). The fragments observed after ethidium bromide staining of the gels are shown. The molecular weights ($\times 10^6$) of the fragments corresponding to the genomes of the major virus or viruses are indicated. *p*, pBR322 DNA fragments.

second group of EV HPVs previously defined (11). Similarly, the DNAs of HPVs 15 and 17, which show ca. 20% cross-hybridization between themselves and ca. 6% cross-hybridization with the HPV 9 DNA, belong to the third group of EV HPVs (11). Finally, HPV 24, which shows almost no homology with the genomes of the other HPVs, has been considered the first member of a fourth EV HPV group.

These results were confirmed by blot hybridization experiments, in which each newly characterized HPV DNA was digested with a mixture of endonucleases which generated 4 to 10 fragments and was hybridized with at least six ^{32}P-labeled EV HPV DNA probes (data not shown). When members of the same group were compared by twos, a variable extent of cross-hybridization was always observed, and at least two fragments were revealed in most cases by heterologous probes (in 31 of the 35 combinations analyzed). When the DNAs of viruses belonging to different groups were compared by twos, a weak cross-hybridization was detected in 24 of the 33 combinations analyzed, the homologous sequences being in most cases (21 combinations) local-

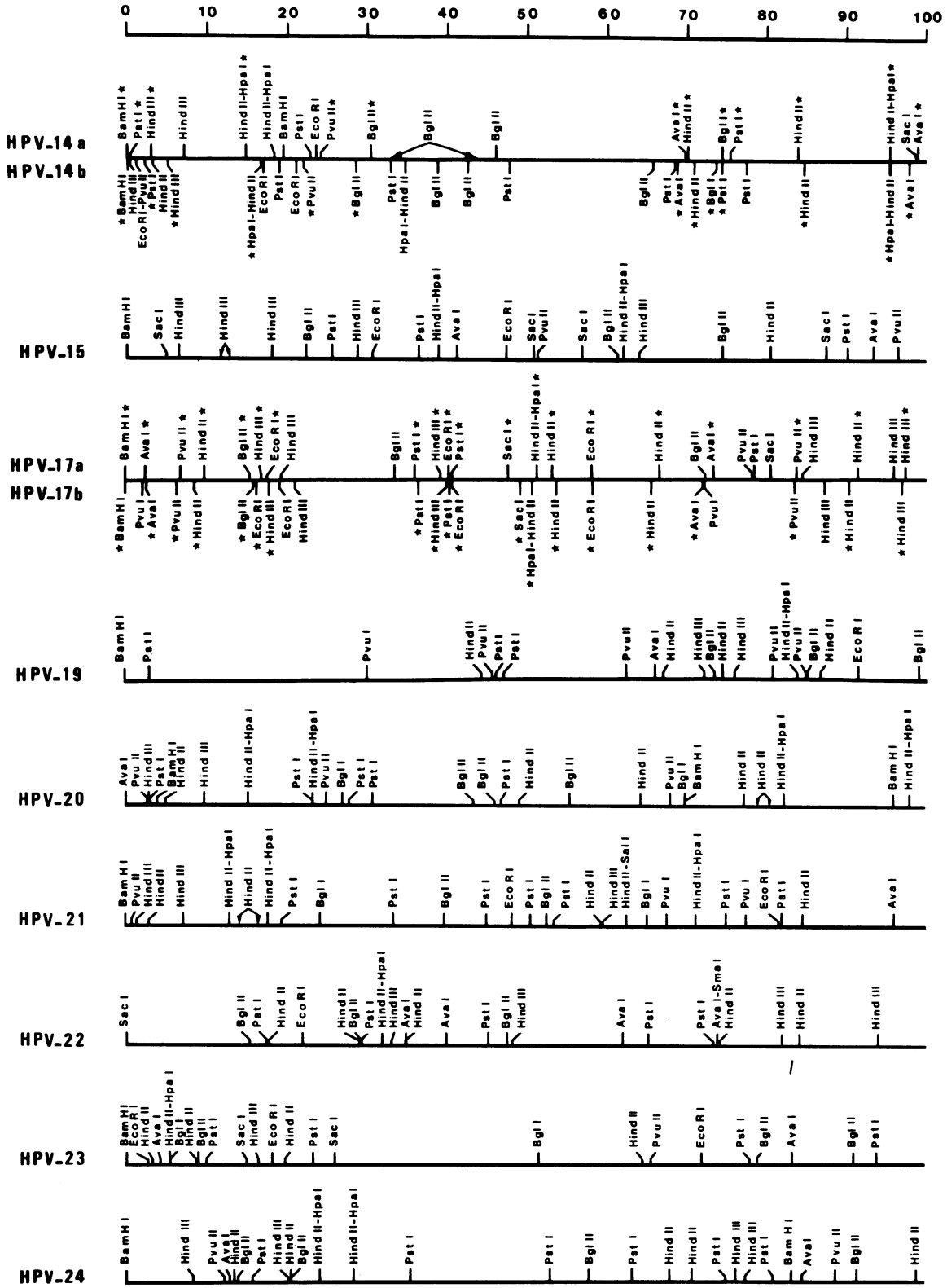

FIG. 2. Cleavage maps of EV HPV DNAs. Mapping of the sites indicated by arrows was not possible. The cleavage maps of HPVs 14a and 14b were aligned by taking as their origins one of the two *Bam*HI sites of the HPV 14a DNA and the unique *Bam*HI site of HPV 14b DNA. The cleavage maps of HPVs 17a and 17b were aligned by taking as their origins their unique *Bam*HI sites. Cleavage sites differing by less than 2% of the genome length in their localization were considered conserved (⋆). No-cut enzymes were *Pvu*I, *Sal*I, and *Sma*I for DNAs of HPVs 14a and 23; *Pvu*I, *Sac*I, *Sal*I, and *Sma*I for HPV 14b DNA; *Bgl*I, *Pvu*I, *Sal*I, and *Sma*I for DNAs of HPVs 15, 17a, and 17b; *Bgl*I, *Sac*I, *Sal*I, and *Sma*I for HPV 19 DNA; *Eco*RI, *Pvu*I, *Sac*I, and *Sma*I for HPV 20 DNA; *Sac*I and *Sma*I for HPV 21 DNA; *Bam*HI, *Bgl*I, *Pvu*I, *Pvu*II, and *Sal*I for HPV 22 DNA; and *Bgl*I, *Eco*RI, *Pvu*I, *Sac*I, *Sal*I, and *Sma*I for HPV 24 DNA.

TABLE 2. Extent of cross-hybridization between HPV DNAs as determined by hybridization in liquid phase[a]

Unlabeled HPV DNA	% Hybridization with ^{32}P-labeled HPV DNA type:													
	3a	5	14a	14b	19	20	21	22	23	9	15	17a	17b	24
1a	0.1	0.3	0.2	0.3	0	0.8	2.9	0	0	1.0	0.4	0.2	0	0
11a	1.6	1.0	1.3	0	0.3	0.1	0.7	3.7	0	0.1	0.1	0.6	3.3	0
3a	100	1.8	1.0	0	1.5	0.1	1.5	0	1.9	0.1	1.7	1.2	1.8	3.6
10	32.3	ND	ND	0.1	0	0.1	1.9	3.0	ND	0	1.6	0.1	2.0	ND
5	0.2	100	12.1	12.4	5.8	9.3	9.4	10.1	5.8	4.3	0.7	3.6	0	2.4
8	1.1	15.7	9.9	13.4	8.5	5.6	5.0	7.1	5.8	3.5	1.5	3.2	3.8	0.1
12	0.1	19.3	9.2	12.5	5.3	8.6	9.3	11.7	4.0	3.6	1.2	0.1	1.9	0
14a	0.2	13.2	100	88.8	14.6	32.4	32.9	10.1	24.6	3.0	2.2	2.4	4.1	ND
14b	ND	10.5	94.1	100	9.3	28.4	35.4	9.5	28.2	0	0	0	0	0
19	ND	7.2	21.4	20.6	100	7.6	8.8	15.5	27.7	0	0	0	2.2	1.0
20	ND	9.9	28.8	37.9	6.2	100	25.4	13.7	14.1	0	0	2.1	0	3.6
21	ND	10.5	38.7	40.5	6.4	37.5	100	9.8	18.6	0.1	0	2.5	0.3	0
22	ND	7.2	7.4	ND	17.3	7.2	10.8	100	17.9	0	0	0.1	0.1	0
23	ND	ND	ND	ND	ND	ND	ND	21.2	100	0	0.5	0	0	0.1
9	0.4	3.1	0.5	1.2	0	2.0	1.0	0	0	100	5.5	6.3	5.4	0
15	0.4	3.3	2.1	3.3	0	0.1	0.8	0	0	7.8	100	22.5	21.6	0
17a	0.7	1.2	1.4	2.8	0	0.1	0.1	0.8	1.4	7.6	19.5	100	92.7	0
17b	ND	ND	ND	1.4	0	0.3	3.4	ND	ND	ND	ND	86.3	100	ND
24	ND	ND	0.1	2.6	ND	ND	ND	0.8	0	0.2	0	1.1	1.1	100

[a] HPV DNAs were labeled by nick translation and fractionated by sedimentation in alkaline sucrose gradients (5 to 20%), as previously described (15). The labeled HPV DNAs (4,000 cpm) were annealed in 0.48 M NaCl–1 mM EDTA (pH 6.8) at 68°C, with either calf thymus (20 μg) or unlabeled HPV DNAs (0.20 μg), as previously described (10, 11). The specific activities of the HPV DNA probes varied between 5.3×10^7 and 2×10^8 cpm/μg. The percentage of hybridization was determined by measuring the nuclease S1-resistant fractions. The numbers represent the values corrected for self-annealing of the probes (4 to 15%) and normalized to 100% for the homologous hybridization (75 to 95%). ND, Not determined.

ized in a single fragment. These experiments have shown no clear-cut evidence that any HPV type could be derived from the genetic recombination of two other types. For example, the HPV 15 DNA fragments labeled by an HPV 9 DNA probe are also labeled by an HPV 17a DNA probe, and the HPV 17a DNA fragments labeled by an HPV 9 DNA probe are also labeled by an HPV 15 DNA probe (Fig. 3).

The previously reported results (10, 11, 18–20, 22) and those reported in this paper show the remarkable plurality of EV-associated HPVs. Of the 17 HPV DNAs isolated from benign lesions of 13 EV patients and cloned by our group, 15 were distinct types (HPVs 3a, 5, 8, 9, 10, 12, 14a, 15, 17a, 19, 20, 21, 22, 23, and 24) (10, 11). Evidence for an additional type (HPV 25) has been recently obtained (4). Moreover, a certain genetic heterogeneity was observed within some of these types. Incomplete but high cross-hybridizations associated with differences in the cleavage maps led to the recognition of subtypes of HPVs 14 and 17. Differences in the cleavage maps have been observed for different isolates of HPV 5 (10, 13, 18–20) and HPV 8 (11, 22) as well as for the isolates of HPVs 19 and 20 described in this paper or reported by others (4). With the exception of HPVs 3a and 10, the other EV HPVs have not been found, so far, in the general population. Conversely, the HPVs associated with skin warts or genital lesions have seldom (HPV 2) or never been identified in our series of 60 EV patients (Orth et al., in preparation). Hybridizations performed under the most stringent conditions, that is, hybridizations in liquid phase at saturation followed by nuclease S1 digestion, have permitted the division of the 13 HPV types specifically associated with EV into three groups, with almost no cross-hybridization between the members of different groups. Blot hybridization experiments, carried out under the usual stringent conditions (T_m − 20°C) (10, 11), have revealed a weak cross-hybridization between the HPV DNAs belonging to different groups, whereas cross-hybridization was only rarely observed between EV HPVs and HPVs associated with cutaneous warts or mucous membrane lesions (10, 11). The analysis of heteroduplex DNA molecules, formed under various conditions of stringency, has shown that the EV HPVs are much more related among themselves than with HPVs associated with skin warts in the general population (O. Croissant, G. Pehau-Arnaudet, N. Bonneaud, and G. Orth, manuscript in preparation). This suggests the existence of phylogenetic relationships among EV HPVs. EV seems, therefore, to be

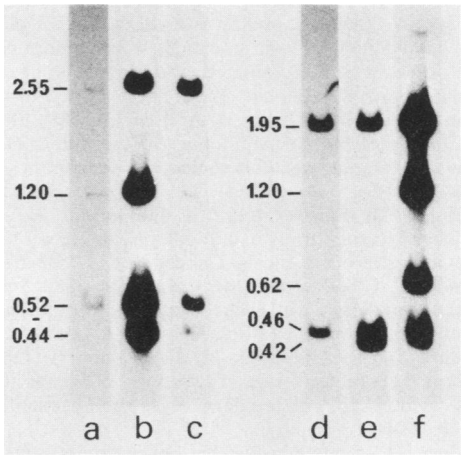

FIG. 3. DNA sequence homology among HPVs 9, 15, and 17a as indicated by blot hybridization experiments. HPV 15 (lanes a, b, and c) and HPV 17a (lanes d, e, and f) were cleaved with *Bam*HI and *Pst*I (HPV 15) or *Bam*HI and *Hin*dII (HPV 17a) endonucleases. The cleavage products (50 ng) were electrophoresed at 95 mA for 4 h in a vertical 1.2% agarose slab gel. The DNA fragments were denatured, transferred to Gene Screen hybridization transfer membrane, and hybridized with ^{32}P-labeled HPV 9 DNA (specific activity, 7.1×10^7 cpm/μg) (lanes a and d), HPV 15 DNA (specific activity, 1.7×10^8 cpm/μg) (lanes b and e), or HPV 17a DNA (specific activity, 8.1×10^7 cpm/μg) (lanes c and f); hybrids were detected by autoradiography as previously described (11).

characterized by an abnormal sensitivity to a related group of HPVs, which may depend on a specific, cell-mediated immunological deficiency. A puzzling question is whether the diversification of EV HPVs has been favored by one or more cellular gene defects, of unknown nature, likely to be involved in the pathogenicity of the disease (12).

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