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Molecular Cloning and Characterization of the DNAs of Human Papillomaviruses 19, 20, and 25 from a Patient with Epidermodysplasia Verruciformis

ANTOINETTE GASSENMAIER,[1]† MATTHIAS LAMMEL,[2] AND HERBERT PFISTER[2]*

Institut für Virologie, Zentrum für Hygiene, D-7800 Freiburg,[1] and Institut für Klinische Virologie, D-8520 Erlangen,[2] Federal Republic of Germany

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Five human papillomavirus (HPV) DNAs from lesions of an epidermodysplasia verruciformis patient were cloned in lambda L 47: DNA of HPV 5, which predominated in the carcinoma; DNA of a variant type of HPV 8, which was not detected in the carcinoma DNA by Southern blot hybridization but only by cloning; and DNAs of three papillomaviruses that were isolated from warts. Southern blot and liquid phase DNA-DNA hybridization under stringent conditions showed that the three viruses from warts were new types, which we named HPVs 19, 20, and 25. These viruses cross-hybridized between 3 and 29% among themselves and with HPVs 5 and 8. After physical mapping with several restriction enzymes, the colinear genomes were aligned with HPV 8 DNA to define early and late regions. HPVs 8, 19, and 25 shared homology in different parts of their genomes.

Of human papillomaviruses (HPVs), 16 types have been described that differ in their nucleotide sequences, their antigenicity, and their biological properties (10). In epidermodysplasia verruciformis (EV), which represents a rare disease with disseminated, persistent warts, there is a tremendous heterogeneity of HPV. Types 3, 5, 8, 9, 10, 12, 14, and 15 have been detected in warts of EV patients (3–5, 8, 12, 16), and additional types are on the horizon (11). Of EV patients, ca. 30% develop skin cancers, mainly on sun-exposed areas (6), and it is important that only a few virus types (HPVs 5 and 8 and, once, HPV 3) persist in these carcinomas (7, 9, 11; G. Orth, personal communication). In the long run, these few virus types may provide a clue to understanding the role of the viruses in conversion to malignancy.

Some EV patients carry only one virus type (12), but usually they are infected by a number of different papillomaviruses, and even wart scrapings from small skin areas may contain up to six types (11). These viruses cannot be analyzed directly since they do not multiply in cell culture systems. Even in vivo, they are produced in small quantities which do not permit biochemical analysis. Molecular cloning of the individual HPV DNAs allows for arbitrary amplification and represents the only way to characterize the various types and to determine their relationship.

In this paper we report on cloning and partial characterization of five HPV DNAs from a EV patient (11). Cleavage, with the restriction enzymes *Bam*HI and *Eco*RI, of DNA from warts and from a carcinoma of this patient led to a number of fragments which hybridized to HPV 8 DNA to different degrees. The fragments were all derived from 7.7-kilobase papillomavirus DNAs because uncleaved samples revealed only full-length viral DNA of forms I, II, and III. The corresponding *Bam*HI and *Eco*RI fragments of different virus types could be identified by comparing qualitative and quantitative differences in the DNA cleavage patterns of individual wart scrapings. By Southern blot hybridization (14) under stringent conditions, we detected only a rather weak homology between HPV 8 and three of these viral DNAs, which made them interesting candidates for cloning. The fourth DNA that we cloned represented HPV 5 from the carcinoma of the patient. The fifth DNA was picked up by chance when we were cloning HPV 5 and had not previously been detected by blot hybridization.

HPV DNAs were cloned into lambda L 47 by using *Bam*HI or *Eco*RI cleavage sites according to a published protocol (2). To this end, we started with DNA from biopsies, which revealed only unequivocally defined virus-specific *Bam*HI or *Eco*RI fragments, i.e., the material could contain several HPV types giving rise to characteristic subgenomic fragments but only one type being linearized by the respective enzyme (Table 1). HPV DNA-containing phages were identified by plaque hybridization to ^{32}P-labeled HPV 8 DNA; positive plaques were amplified, and after DNA extraction and restriction enzyme cleavage, the cloned

TABLE 1. Origin and nomenclature of cloned HPV DNAs

Previous preliminary HPV designation (11)	Restriction enzyme fragment	Mol wt (10^6)	DNA source for cloning[a]	Clone designation
B	*Bam*HI	4.90	WS 4	L 19
C	*Bam*HI-A	4.40		L 25-1
	*Bam*HI-B	0.40	WS 6	L 25-2
	*Eco*RI	4.90	WS 2/1	L 25
D[b]	*Bam*HI-A	2.90		L 5-1
	*Bam*HI-B	2.10	WS 2/2	L 5-2
	*Eco*RI A + B	3.70		L 5
		1.20		
F	*Bam*HI-A	3.40		L 20-1
	*Bam*HI-B	1.20	WS 6	L 20-2
NI[c]	*Eco*RI	4.90	CC	L 8

[a] WS, DNA from wart scraping; individual numbers from reference 11. CC, DNA from the center of the carcinoma (11).
[b] HPV 5.
[c] NI, Not previously identified.

* Corresponding author.
† Present address: Dermatologische Klinik mit Poliklinik, D-8520 Erlangen, Federal Republic of Germany.

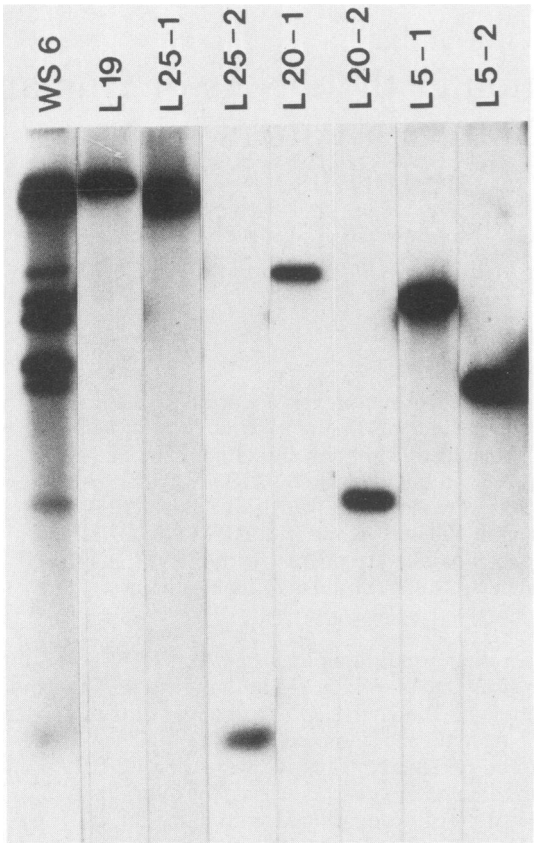

FIG. 1. Comparative analysis of HPV DNA from wart scraping (WS) 6 (11) and from lambda L 47 clones L 19, L 25-1, L 25-2, L 20-1, L 20-2, L 5-1, and L 5-2. HPV DNAs were cloned from tumor DNA (see Table 1) in agreement with the German Committee for Biological Safety, as described previously (2). The DNAs were cleaved with *Bam*HI, and virus-specific DNA was visualized by Southern blot hybridization (14) with ^{32}P-labeled HPV 8 DNA under stringent conditions.

fragments were identified by comigration with authentic fragments within wart DNA (Fig. 1). The cloned DNAs were assigned to individual HPVs by the method of Pfister et al. (11) as summarized in Table 1. The sum of the molecular weights of *Bam*HI fragments A and B from isolate F did not correspond to that of a full-length genome, which might imply that we are still missing a small *Bam*HI fragment of isolate F (HPV 20 [3]).

The cloned DNAs were further characterized by cleavage with a number of restriction enzymes, and cleavage sites were physically mapped by standard procedures. The physical map of HPV 5 was published previously (11) and is not discussed further in this paper.

The second HPV type from the carcinoma turned out to be a variant strain of HPV 8, based on a number of characteristic restriction enzyme cleavage products. HPV 8a$_2$, as it was called, differed from HPV 8a$_1$ (12) by one additional cleavage site each for *Bam*HI, *Hin*dII, *Hin*dIII, and *Pst*I. The *Eco*RI and *Hpa*I fragments were the same as those of HPV 8a$_1$ (Fig. 2). The *Bam*HI fragments of HPV 8a$_2$ (2.5×10^6 each) differed in size from those of another variant, HPV 8a$_3$ (2.7×10^6 and 2.2×10^6), which was present in the warts of the Turkish patient (11). Physical maps of HPV DNAs from L 19, L 20, and L 25 are shown in Fig. 2. They show no similarities with physical maps of the HPV types published so far (HPVs 1 to 13).

HPV DNAs from L 19, L 20-1 + -2, and L 25 were compared with reference DNAs for HPV types 1 to 6, 8 to 11, 13, and 16 by Southern blot hybridization under stringent conditions. The DNAs of HPVs 1, 4, 6, 8, 9, and 16 were cloned in pBR322; the DNAs of HPVs 2, 3, 10, 11, and 13 were cloned in lambda L 47 (summarized in reference 10). L 19, L 20, and L 25 cross-hybridized only with one another and with HPVs 5, 8, and 9. The exact degree of homology between the new isolates and HPVs 5, 8, and 9 was determined by kinetics of liquid phase hybridization. Reannealing of 25 ng of labeled DNA was measured in the presence of 100 to 400 ng of unlabeled homologous and heterologous DNAs. Calf thymus DNA served as a negative control. Evaluation according to Sharp et al. (13) revealed less than 30% homology between HPVs 5, 8, and 9 and L 19, L 20, and L 25 (Table 2).

L 19, L 20, and L 25 were also compared with recent HPV isolates from EV patients (HPVs 12 [see reference 5], 14, 15, 17, 19 to 24 [see reference 3]) and with an HPV DNA isolate from a cervical carcinoma (HPV 18 [1]). L 19 and L 20 DNA turned out to be identical to isolates 19 and 20, respectively, based on complete cross-reactivity in Southern blot hybridization and the identical positions of most restriction enzyme cleavage sites. A radioactive probe of L 25 disclosed only some DNA fragments of isolates 12, 14, 20, and 24 and to a limited extent all *Bam*HI/*Hin*dII or *Bam*HI/*Pst*I fragments of isolates 19 and 21. An extremely weak cross-hybridization was observed with isolates 17 and 22, whereas isolate 23 showed almost no hybridization. No similarities were detected between the physical maps of L 25 and the other EV isolates (3). These data permitted a final classification of isolates B, C, and F. Isolates B and F were referred to as HPVs 19 and 20, respectively (3). Isolate C showed no relationship or a limited relationship to all other HPV types and was called HPV 25. This latter virus could not be directly compared

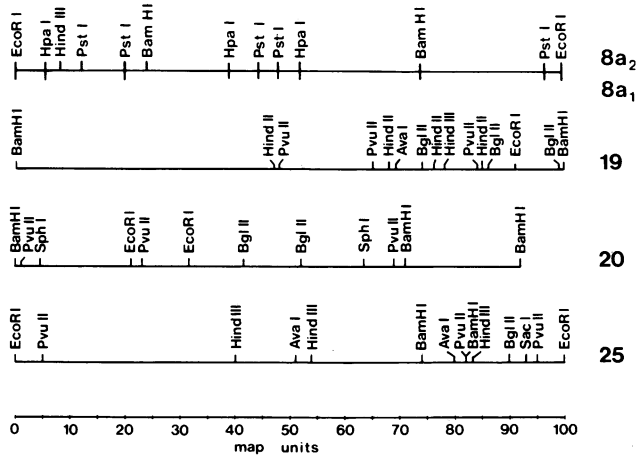

FIG. 2. Restriction endonuclease cleavage maps of L 8, L 19, L 20, and L 25 viral inserts. L 20-1 and L 20-2 are joined. L 8 virus is referred to as 8a$_2$, and the positions of cleavage sites are directly compared with those of the previously published variant HPV 8a$_1$ (12). Restriction enzymes were purchased from Bethesda Research Laboratories, Gaithersburgh, Md., and used by the recommendations of the manufacturer.

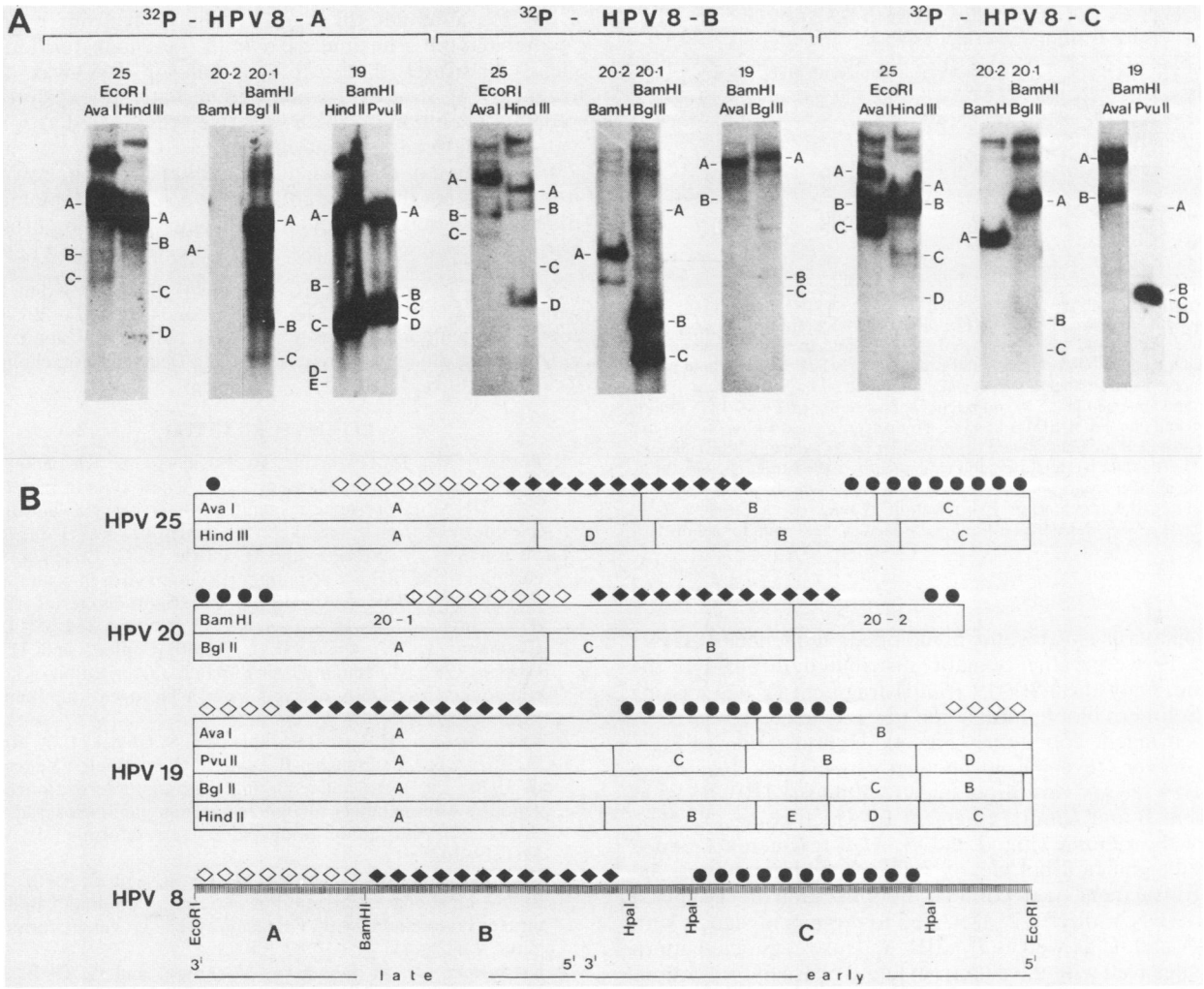

FIG. 3. Aligning of the genomes of HPVs 8, 19, 20, and 25 (B) based on Southern blot hybridization (A) with subgenomic probes under relaxed conditions (10% formamide–1 M NaCl at 37°C). Cloned DNAs of HPVs 19, 20, and 25 were excised from λ L 47 DNA by *Bam*HI or *Eco*RI. All DNAs except L 20-2 were digested with a second enzyme as indicated above the gels in (A) and hybridized with ^{32}P-labeled fragments A, B, and C of HPV 8 DNA (see the physical map of HPV 8 at the bottom). The data obtained for the different cleavage patterns were summarized, and homologous regions of HPVs 8, 19, 20, and 25 are indicated by symbols in common. Early and late regions of the HPV 8 genome and 5′ to 3′ polarity of protein reading frames as derived from a comparison with bovine papillomavirus 1 (data not shown) are given at the bottom.

with HPV 7, but HPV 7 was unrelated to HPV 5 and showed different cleavage patterns, so that it could not be the same virus type.

Southern blots of restriction enzyme digests of HPVs 19, 20, and 25 were hybridized to ^{32}P-labeled subgenomic fragments of HPV 8 to look for a possible alignment of the four genomes. As expected, the HPV 8 probes hybridized to distinct fragments of HPVs 19, 20, and 25. The results are schematically summarized in Fig. 3. The data are in line with a colinear arrangement of the DNA sequences of the four HPV types. Early and late parts of the HPV 8 genome were defined earlier by hybridization to HPV 1 and bovine papillomavirus 1 DNA fragments (1a) in which these regions were defined by sequence data and transcription analysis (Fig. 3) (for review, see reference 10).

The data presented in this paper further support the plurality of HPV among EV patients. The large number of rather closely related HPV types that lead to similar tumors is unique for EV and leads to speculations about the origin of this heterogeneity. It is probably a result of research intensity combined with the fact that EV patients select for a number of HPV types by their impaired cellular immunity (10). So far, there is no clear-cut evidence for an increased mutation or recombination rate in the course of the life-long infection of an individual patient. Independent isolates from different countries revealed nearly identical cleavage maps (HPVs 19 and 20 [3; this paper] and three isolates of HPV 5 [7, 9, 11]). This indicates that the HPV types are well preserved as defined entities, which argues against a high mutation rate.

HPV 25 showed a peculiar evolutionary position with a roughly equal distance to HPVs 8 and 19, which in turn were more distantly related (Table 2). We followed this line of evidence to the molecular level by cross-hybridization experiments with subgenomic fragments.

As observed with a number of other HPV and bovine

TABLE 2. Cross-hybridization between DNAs of different HPV isolates from epidermodysplasia verruciformis[a]

HPV type	% Cross-hybridization with HPV DNA:					
	5	8	9	L 19	L 20	L 25
5	100	27	2	11	6	29
8		100	4	10	3	29
9			100	2	1	4
L 19				100	6	25
L 20					100	8
L 25						100

[a] Cross-hybridization was determined from the kinetics of DNA reassociation in liquid phase. Labeling HPV DNAs by nick translation was by a published protocol (2). The unlabeled DNAs were cut to the same size by using nick-translation conditions and replacing [^{32}P]TTP with the cold analogon. Labeled and unlabeled DNAs were denatured by boiling (20 min), chilled on ice, and annealed in 0.5 M sodium phosphate buffer (pH 6.8)–0.1% sodium dodecyl sulfate– 2 mM EDTA at 68°C. To measure reassociation, single- and double-stranded molecules were separated by hydroxylapatite chromatography (15). The data were plotted and evaluated by the method of Sharp et al. (13). The numbers represent the percentage of cross-hybridization, with 100% representing the homologous hybridization. Values of calf thymus DNA hybridization were defined as 0% and were used as the 0 point for a 0 to 100% scale.

papillomavirus isolates, the homologous sequences of HPVs 8 and 19 were rather equally distributed throughout the genome. Only the HPV 19 *Hin*dII fragment D was missing after Southern blot hybridization to ^{32}P-labeled HPV 8 DNA under stringent conditions (Fig. 4). Hybridization to HPV 25, however, revealed an unequal distribution. Homology with HPV 8 was very pronounced within the HPV 25 *Ava*I fragment B and *Hin*dIII fragment B, which cover the same part of the genome (Fig. 2 and 4). *Ava*I fragments A and C showed significant but clearly reduced hybridization as did *Hin*dIII fragment A. In contrast, hybridization of ^{32}P-labeled HPV 19 DNA to HPV 25 DNA led to prominent signals with *Ava*I-A and -C as well as *Hin*dIII-A. This reciprocal pattern correlates well with the data from liquid phase hybridization. These data could be reconciled with a recombination between an HPV 8- and an HPV 19-related virus, giving rise to

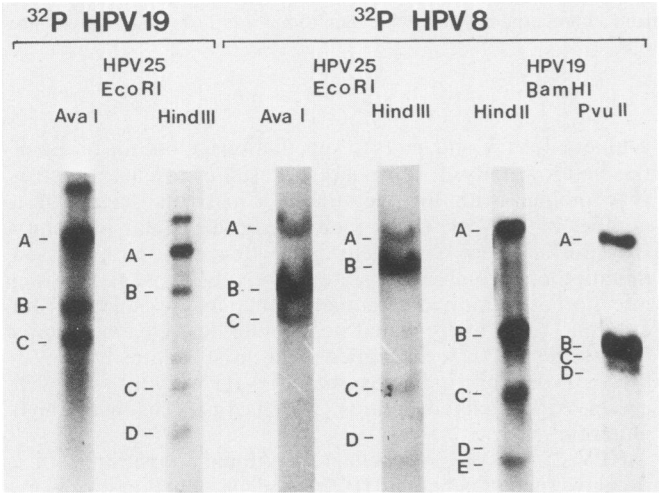

FIG. 4. Southern blot hybridization of ^{32}P-labeled HPV 19 and HPV 8 DNA to DNA fragments of HPV 25 and HPV 19. Note the reciprocal labeling of HPV 25 fragments with the HPV 19 and HPV 8 probes, respectively.

HPV 25, although they are certainly far from proof of recombination. The limited overall homology furthermore clearly indicates that recombination—if any—was not a recent event. The data could be alternatively explained by separate evolution in which selective pressure led to conservation of different genome regions.

Irrespective of the mechanism of diversification, however, it is interesting that different virus types share homology in different regions of the genome. This fact will be important in correlating biological properties with certain genotypes.

We thank G. Orth and L. Gissmann for kindly providing blots with DNA of HPV types 12, 14, 15, and 17 to 24 for direct comparison with our isolates by Southern blot hybridization.

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