Characterization of a New Type of Human Papillomavirus That Causes Skin Warts

GÉRARD ORTH,^{1*} MICHEL FAVRE,¹ AND ODILE CROISSANT²

Unité de Recherches sur l'Étiologie Virale des Cancers Humains, Laboratoire 147 associé au Centre National de la Recherche Scientifique, Unité 140 de l'Institut National de la Santé et de la Recherche Médicale, Institut Gustave-Roussy, 94800 Villejuif, France,¹ and Unité d'Oncologie Virale, Département de Virologie, Institut Pasteur, 75015 Paris, France²

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A human papillomavirus (HPV) was isolated from the lesions of a patient (ML) bearing numerous hand common warts. This virus was compared with the well-characterized HPV found in typical plantar warts (plantar HPV). ML and plantar HPV DNAs have similar molecular weights (5.26×10^6 and 5.23×10^6 , respectively) but were shown to be different by restriction enzyme analysis. When the cleavage products of both DNAs by endonuclease *EcoRI*, *BamI*, *HpaI*, or *Hind* were analyzed by electron microscopy, one, two, one, and four fragments were detected for ML HPV DNA instead of the two, one, two, and six fragments, respectively, detected for plantar HPV DNA. In contrast to plantar HPV DNA, a high proportion of ML HPV DNA molecules were resistant to these restriction enzymes. Most, if not all, of the molecules were either resistant to *BamI* and sensitive to *EcoRI* or sensitive to *BamI* and resistant to *EcoRI*. After denaturation and renaturation of the cleavage products of ML HPV DNA by a mixture of the two enzymes, the circular "heteroduplexes" formed showed one to three heterology loops corresponding to about 4 to 8% of the genome length. No sequence homology was detected between ML and plantar HPV DNAs by cRNA-DNA filter hybridization, by measuring the reassociation kinetics of an iodinated plantar HPV DNA in the presence of a 25-fold excess of ML HPV DNA, or by the heteroduplex technique. The two viruses had distinct electrophoretic polypeptide patterns and showed no antigenic cross-reaction by immunodiffusion or immunofluorescence techniques. Preliminary cRNA-DNA hybridization experiments, using viral DNAs from single or pooled plantar or hand warts, suggest that hand common warts are associated with viruses similar or related to ML HPV. The existence of at least two distinct types of HPVs that cause skin warts was demonstrated; they were provisionally called HPV type 1 and HPV type 2, with plantar HPV and ML HPV as prototypical viruses, respectively.

Papillomaviruses belong to the *Papovaviridae* family; they are distinct from polyomaviruses by virtue of the diameter of their naked icosahedral capsid (55 nm) and the size of their genome, a supercoiled, covalently closed circular double-stranded DNA molecule with a molecular weight of about 5×10^6 (6, 15).

In humans, a papillomavirus has been found associated with several benign epithelial tumors differing with respect to their localization and morphological and histological features: skin warts (the main clinical types being plantar warts, common warts, and flat warts), anal and genital condylomata, and oral and laryngeal papillomas (31). Biochemical and immunological characterization of viruses in these different lesions has been impeded by the lack of a cell system allowing their replication in vitro

(4) and by the widely variable viral content of warts and papillomas according to their clinical type (3, 5, 26). On the basis of transfer experiments, it has been long assumed that these lesions were all due to the same virus, the human papillomavirus (HPV) (31). The well-documented properties of the virus present in plantar warts were thus assumed to be those of HPV (6, 11, 13, 18, 21).

However, the incidence, mode of transmission, age distribution, and clinical evolution of various HPV-induced lesions may differ widely, suggesting that some clinical types may constitute distinct diseases induced by specific viruses (for a review, see G. Orth, F. Breitburd, M. Favre, and O. Croissant, *Cold Spring Harbor Conferences on Cell Proliferation*, vol. 4B, in press). This is further supported by molecu-

lar hybridization data, which failed to detect homologous sequences of skin wart virus DNA in DNA extracted from anogenital condylomata and laryngeal papillomas (9, 37). In addition, skin wart and condyloma viruses appeared to be antigenically distinct (2, 16).

In this paper, we report the biochemical and immunological characterization of a new type of HPV isolated from hand common warts. The DNA of this virus differs from that of the virus previously characterized in plantar warts by restriction enzyme analysis (13, 14, 18), and no sequence homology was observed between the two DNAs by using molecular hybridization techniques. Furthermore, the two viruses were shown to have different polypeptide patterns and distinct antigenic properties.

MATERIALS AND METHODS

Virus purification. Virus was purified from several independent samples of warts: two pools of hand warts (6 and 10 g, respectively) collected from the same patient (ML), a pool of hand wart parings obtained from six patients, pools of typical simple plantar warts from different patients (about 5 g/pool), and single plantar warts. Warts were kept at -20°C . Virus was extracted under conditions previously described (11), with the trypsin treatment omitted, and further purified by one equilibrium centrifugation in a CsCl gradient. The virus extracted from pooled plantar warts was further purified by a second centrifugation in a CsCl gradient and sedimentation in a sucrose gradient, as previously described (11). Protein concentration was determined by the method of Lowry et al. (23), with bovine serum albumin as a standard. Viral preparations were kept at -20°C .

Virus DNA preparation. Plantar and ML HPV DNAs were extracted from virions by incubation with Sarkosyl, and circular supercoiled (form I) molecules were separated from relaxed circular (form II) and linear (form III) molecules by centrifugation in a CsCl gradient in the presence of ethidium bromide (27). Viral DNA was also selectively extracted from single hand and plantar warts by a modification of the Hirt procedure (19). Warts were rinsed in 0.15 M NaCl and minced, and the fragments were incubated in 10 ml of 0.01 M Tris-0.01 M NaCl-0.01 M EDTA-0.5% sodium dodecyl sulfate (SDS) containing 50 μg of proteinase K (Merck) per ml. After a 6-h incubation at 37°C , the NaCl concentration was adjusted to 1 M, and the mixtures were maintained overnight at 4°C . After a 45-min centrifugation at 8,000 rpm, supernatants were extracted twice with phenol saturated with 0.1 M Tris (pH 8.0) and mixed with 2 volumes of ethanol at -20°C . Pellets were collected and dissolved in 0.01 M Tris-0.001 M EDTA (pH 7.9). Form I molecules were separated as previously described (27). Plantar HPV DNA (15 to 50 $\mu\text{g}/\text{ml}$) and ML HPV DNA (5 to 10 $\mu\text{g}/\text{ml}$) were kept at -20°C in the preceding buffer.

Electron microscopy. Viral particles were observed with a Siemens Elmiskop 101 electron micro-

scope after negative staining with 2% sodium phosphotungstate solution, pH 7.2. DNA samples and endonuclease DNA fragments were mounted on collodion-coated grids by the formamide technique of Davis et al. (8) and further Pt-Pd shadowed. Contour lengths were measured on electron micrographs by using a coordinatometer connected to a PDP 8 digital computer, taking the replicative form of ΦX174 phage ($\Phi\text{X174 RF}$) DNA molecules mounted on the same grid as an internal length standard.

Restriction enzyme analysis. Restriction endonucleases from *Escherichia coli* (*EcoRI*), *Haemophilus influenzae* (*Hind*, a mixture of *HindII* and *HindIII*), and *H. parainfluenzae* (*HpaI*), prepared as previously described (13), were a kind gift of M. Yaniv (Institut Pasteur, Paris, France). Endonuclease from *Bacillus amyloliquefaciens* (*BamI*) was a gift of T. Bickle (Biozentrum der Universität, Basel, Switzerland). The digestion conditions of plantar and ML HPV DNAs (0.1 to 0.2 μg of each) by *EcoRI*, *BamI*, *Hind*, *HpaI*, or both *EcoRI* and *BamI* have been already described (13, 14). Cleavage products were analyzed either by electron microscopy, as described above, or by vertical 2% agarose slab gel electrophoresis under the conditions described by Riou and Yot (Biochemistry, in press). After 14 h of electrophoresis at 20 mA, gels were stained with ethidium bromide (5 $\mu\text{g}/\text{ml}$), and the bands were localized and photographed under UV light.

Hybridization experiments. RNA-DNA filter hybridizations under paraffin were performed, as described by Kourilsky et al. (22), with cRNA's transcribed in vitro. *E. coli* RNA polymerase containing sigma factor was a gift of B. Lescure (Institut Pasteur, Paris, France). The transcription mixture (100 μl) contained 1 μg of both RNA polymerase and ML or plantar HPV DNA, 0.4 μmol of ATP, GTP, and CTP, and 0.25 mCi of tritiated uridine (Radiochemical Centre, Amersham, England; 50 Ci/mmol) in a solution of 0.04 M Tris (pH 7.9), 0.15 M KCl, 0.01 M MgCl_2 , and 0.001 M dithiothreitol. RNAs were extracted and purified under the conditions previously described (27). cRNA's (specific activity, 4×10^7 cpm/ μg) were kept at -20°C in $6 \times \text{SSC}$ ($1 \times \text{SSC}$ contains 0.15 M NaCl and 0.015 M sodium citrate). Four microliters of cRNA in $2 \times \text{SSC}$ -50% formamide (3,000 cpm) was spotted on nitrocellulose filters (2.5-mm diameter) carrying about 50 ng of heat-denatured viral DNA or heat-denatured human fetal thymus DNA prepared by the method of Sharp et al. (33) and on blank filters. Paraffin was immediately added, and filters were incubated for 15 h at 37°C . Filters were then processed as described previously (22) and counted in an SL 30 scintillation spectrometer (Intertechnique, Plaisir, France).

DNA-DNA reassociation kinetics were studied by using iodinated fragmented plantar HPV DNA (specific activity, 1.8×10^7 cpm/ μg) and unlabeled fragmented ML and plantar HPV DNAs prepared under the conditions described by Delap et al. (10). ^{125}I -labeled plantar HPV DNA (3.5 ng/100 μl) was denatured alone or in the presence of an excess of unlabeled fragmented ML or plantar HPV DNA, in 0.04 M sodium phosphate buffer (pH 6.8), by a 10-min

incubation at 100°C. DNA solutions (1 ml) in 0.086 M sodium phosphate buffer (pH 6.8)–0.08% SDS–1 M NaCl were allowed to reassociate at 68°C. Samples (100 μ l) were removed at various times and analyzed for single- and double-stranded DNA by hydroxyapatite (Bio-Rad Laboratories, Richmond, Calif.; DNA grade) chromatography as described by Delap et al. (10). Fractions were counted in an Intertech CG 30 gamma spectrometer.

Heteroduplexes were prepared and analyzed under an electron microscope by the method of Davis et al. (8). Cleavage products of plantar HPV DNA by *Eco*RI or *Bam*I endonuclease and of ML HPV DNA by *Eco*RI or a mixture of *Bam*I and *Eco*RI endonucleases were obtained as described above. The denaturation mixture (22.5 μ l) contained 80 ng of cleaved HPV DNA (40 ng of each when two distinct preparations were mixed), 0.5 mM Na₂EDTA, and 2.5 mM NaOH. After a 15-min incubation at 25°C, the alkaline solution was neutralized with 2.5 μ l of a 1.8 M Tris-Hydrochloride–0.2 M Tris-hydroxide solution, and 25 μ l of redistilled formamide was added. The mixture was incubated for 30 min at 37°C or for 120 min at 25°C to allow for renaturation of denatured strands. Electron microscope analysis of renaturation products was performed with different concentrations of formamide in the spreading solution and in the hypophase: 30% onto 10% or 50% onto 17% (8). Duplex molecules were analyzed by examining electron micrographs.

Polyacrylamide gel electrophoresis. Plantar and ML HPV virions were SDS dissociated, and the viral polypeptides were analyzed by acrylamide gradient (7.5 to 20%) slab gel electrophoresis as previously described (12).

Antisera. Guinea pigs were immunized by intramuscular inoculation of full particles of ML HPV (G 206) (20 μ g of viral proteins per inoculation) or of plantar HPV purified either from pooled warts (G 121, G 122) or single plantar warts (G 254, G 260) (50 μ g of viral proteins per inoculation). The immunization cycle included three inoculations in complete Freund adjuvant at weekly intervals and was repeated after 2 weeks, except for guinea pigs immunized with virus purified from a single plantar wart. Animals were bled 2 weeks after the last inoculation. Antisera against guinea pig immunoglobulin G (IgG) were obtained from rabbits after two cycles of immunization (G. Orth, F. Breitburd, and M. Favre, in preparation), using electrophoretically pure guinea pig IgG.

Immunodiffusion tests. Immunodiffusion tests were performed on microscope slides, using 0.5% agarose gel in 0.05 M sodium phosphate buffer–0.15 M NaCl, pH 7.4. Circular wells (3.5-mm diameter) were spaced 6 mm from the center well (from edge to edge). Virus (10 μ l of a 1-mg/ml suspension) was placed in the center well, and serum dilutions were placed in peripheral wells. Diffusion was allowed to proceed for 48 h in a moist chamber.

Immunofluorescence tests. Electrophoretically pure IgG immunoglobulins obtained from anti-guinea pig IgG rabbit antiserum and from anti-ML (G 206) or anti-plantar (G 121, G 122) guinea pig antiserum were conjugated with fluorescein isothiocyanate. Conjugated globulins with a low fluores-

cein protein molar ratio (ranging from 1.5 to 2.5) were separated by DEAE-cellulose chromatography (G. Orth, F. Breitburd, and M. Favre, in preparation) and used at the lowest concentration (0.5 mg/ml) giving maximal fluorescence intensity. Viral capsid antigens were detected by a direct immunofluorescence test, using 7- μ m-thick frozen sections fixed for 10 min at –20°C in acetone. Antiviral antibodies were assayed on wart sections by an indirect immunofluorescence test, using fluorescein-labeled anti-guinea pig IgG rabbit globulins. Preparations were observed and photographed with a Zeiss Photomicroscope II.

RESULTS

Virus morphology. The virus characterized in this study was purified from the warts collected from a 20-year-old patient (ML) bearing a large number of common warts located exclusively on both hands (Fig. 1). This virus was provisionally named ML HPV; the virus purified from plantar warts was provisionally named plantar HPV. ML HPV purification included one differential centrifugation and equilibrium sedimentation in a CsCl gradient. The yield in full particles attained 60 and 70 μ g of viral protein per g of tissue for two different preparations. In negatively stained preparations, full particles appear as naked particles with a diameter ca. 53 nm and show the characteristic morphology of a papillomavirus (Fig. 2) (21).

Structure and molecular weight of ML HPV DNA. DNA molecules extracted from ML and plantar HPV virions were fractionated into supercoiled (form I) molecules and relaxed circular (form II) or linear (form III) molecules after centrifugation in a cesium chloride–ethidium bromide gradient. Contour lengths of the molecules were measured on electron micrographs, taking the length of Φ X174 RF DNA present on the same grid as a standard. The lengths of both ML and plantar HPV DNA molecules had similar and homogeneous distributions (Fig. 3), as expressed in Φ X174 RF DNA units. The length of ML HPV DNA determined for 246 molecules was 1.484 ± 0.025 that of Φ X174 RF DNA (58 molecules). It was not significantly different from the length of plantar HPV DNA measured for 218 molecules, which was 1.474 ± 0.010 that of the standard (78 molecules). Since Φ X174 RF DNA was shown to contain 5,375 base pairs (32), the molecular weights of ML HPV DNA and plantar HPV DNA were evaluated to 5.26×10^6 (7,977 base pairs) and 5.23×10^6 (7,922 base pairs), respectively.

Comparative sensitivity of ML and plantar HPV DNAs to restriction enzymes. ML and plantar HPV DNAs were cleaved by *Eco*RI,

FIG. 1. Multiple common warts of patient ML (left hand).

FIG. 2. Electron micrograph of ML HPV particles purified as described in the text and observed after staining with a 2% sodium phosphotungstate solution. Bar, 0.1 μ m.

FIG. 3. Histograms of the lengths of ML and planar HPV DNA molecules. ML HPV DNA (A) or planar HPV DNA (B) molecules were mixed with Φ X174 RF DNA molecules. Their contour lengths were measured on electron micrographs at a final magnification of $\times 40,000$ and expressed as fractional lengths of Φ X174 RF DNA.

*Bam*I, *Hpa*I, and *Hind*II+III endonucleases, and the digestion products were analyzed by electron microscopy. ML HPV DNA differs from planar HPV DNA by both the number and the size of the fragments generated and by the presence of entirely or partially resistant molecules (Fig. 4). The molecular weights of the fragments are reported in Table 1. A single fragment, of one genome unit length, was detected after cleavage of ML HPV DNA by

*Eco*RI or *Hpa*I endonuclease. Two or four fragments, which are named by letters according to their decreasing sizes, were detected after cleavage by endonuclease *Bam*I or *Hind*II+III, respectively; their total molecular weights (5.15×10^6 and 5.37×10^6 , respectively) nearly equal that of ML HPV DNA. The number of the fragments obtained after cleavage of planar HPV DNA by *Eco*RI, *Bam*I, or *Hpa*I (2, 1, 2, respectively) and their molecular weights are in agreement with the results previously reported (13, 14, 18). However, of the six fragments generated by *Hind*II + III (13), *Hind*-C and *Hind*-D cannot be resolved (Fig. 4), and *Hind*-F (molecular weight, 60,000) is too small to be seen in the electron microscope.

As indicated by electron microscopy (Fig. 4) and confirmed by agarose gel electrophoresis for *Eco*RI and *Bam*I endonucleases (Fig. 5a, b, d), a high proportion of ML HPV DNA molecules were found resistant to the enzymes. A fourfold increase in the amount of enzymes did not alter the cleavage pattern; furthermore, when a mixture of equal amounts of ML HPV and simian virus 40 DNAs were incubated in the presence of *Eco*RI (Fig. 5c) or *Bam*I endonuclease, all of the simian virus 40 DNA was cleaved into linear genome-length molecules. The presence of molecules resistant to the enzymes was observed for the three ML HPV DNA preparations tested, one of them being obtained from a single wart, in contrast to planar HPV DNA, which is usually fully sensitive (13, 14). However, the proportion of resistant molecules varied from one preparation to another for a given enzyme (Table 2). Most of the

FIG. 4. Histograms of the lengths of the digestion products of ML and plantar HPV DNAs by restriction endonucleases. The lengths of the fragments and resistant molecules were measured on electron micrographs at a final magnification of $\times 40,000$ and expressed as fractional lengths of $\Phi X174$ RF DNA molecules measured on the same micrographs. Black histograms represent measurable resistant circular molecules; diagonally striped histograms represent partially resistant molecules.

molecules resistant to *Bam*I endonuclease showed neither of the two sites; conversely, only molecules partially resistant to *Hind*II+III were detected, as shown by the existence of a fragment whose molecular weight (4.89×10^6) nearly equals the sum of the molecular weights of *Hind*-A, -B, and -C fragments (Fig. 4). No doubly resistant molecules were detected by electron microscopy (data not shown) or by agarose gel electrophoresis (Fig. 5e) when ML HPV DNA was cleaved by a mix-

ture of *Eco*RI and *Bam*I endonucleases. Three bands migrating like linear unit molecules and *Bam*I-A and -B fragments were detected (Fig. 5e). For the two preparations of ML HPV DNA, the percentage of linear unit molecules generated was nearly equal to that of molecules resistant to *Bam*I endonuclease and hardly lower than that of the molecules sensitive to *Eco*RI enzyme (Table 2). Thus, the ML HPV DNA preparations essentially contained *Eco*RI sensitive *Bam*I-resistant (*Eco*RI S-*Bam*I R) mole-

cles and *EcoRI*-resistant *BamI*-sensitive (*EcoRI* R-*BamI* S) molecules in varying proportions. If they contained a small proportion of *EcoRI*-sensitive *BamI*-sensitive (*EcoRI* S-

TABLE 1. Sensitivity to restriction enzymes of ML and plantar HPV DNAs

Cleavage products	Mol wt ($\times 10^6$) ^a	
	ML HPV DNA	Plantar HPV DNA
<i>EcoRI</i> -A	5.29	4.68
B		0.67
<i>BamI</i> -A	3.94	5.18
B	1.21	
<i>Hind</i> -A	2.49	1.77
B	1.70	1.38
C	0.91	ND ^b
D	0.28	ND
E		0.25
F		ND
<i>HpaI</i> -A	5.36	4.44
B		0.82

^a Molecular weights were estimated from the lengths of the fragments determined by electron microscopy, taking as a standard the length of Φ X174 RF DNA molecules present on the same grids; the molecular weight of Φ X174 RF DNA was assumed to be 3.55×10^6 (32).

^b ND, Not determined by this method.

FIG. 5. Agarose slab gel electrophoresis of cleavage products of ML HPV DNA by *EcoRI*, *BamI*, or a mixture of *EcoRI* and *BamI* endonucleases. Samples (0.2 μ g) of ML HPV DNA (a), cleavage products of ML HPV DNA by *EcoRI* (b), *BamI* (d), or a mixture of *BamI* and *EcoRI* (e), as well as a mixture of equal amounts (0.2 μ g) of ML HPV DNA and simian virus 40 DNA cleavage products by *EcoRI* (c), were subjected to electrophoresis as described in the text. The genome-unit-length molecules of simian virus 40 DNA migrate like ML HPV DNA form I molecules.

BamI S) molecules, the cleavage products were not detected either by electron microscopy or by agarose gel electrophoresis (Fig. 5e).

Analysis of the sequence heterology in ML HPV DNA by heteroduplex mapping. Sequence heterogeneity observed was further studied by analysis of the duplexes formed after denaturation and renaturation of the digestion products of ML HPV DNA by a mixture of *EcoRI* and *BamI* endonucleases. From the experimental values reported in Table 2, it can be inferred that the preparation studied (ML₂ HPV DNA) contains about 22% *EcoRI* S-*BamI* R molecules and 67% *EcoRI* R-*BamI* S molecules. After denaturation and renaturation of the cleavage products, both circular and linear duplexes may be expected. Circular duplexes should be formed from *EcoRI* S-*BamI* R molecules and *EcoRI* R-*BamI* S fragments in a proportion of about 29%, whereas linear duplexes should correspond mainly to unit-length *EcoRI* S-*BamI* R duplexes and *BamI*-A and *BamI*-B fragments generated from *EcoRI* R-*BamI* S molecules. The data (Table 3) show that the observed proportion of circles among all of the duplexes scored (circles, linear unit molecules, and *BamI*-A fragments) is about 23%, near the expected value. Among the 98 circular duplexes analyzed, 84 molecules bore at least one heterology loop (Fig. 6) corresponding to about 2 to 4% of the genome length. Thirty-nine duplexes showed a single loop, 39 duplexes showed two loops, and 6 duplexes showed three loops. Among these 84 duplexes, 25 contained a single-strand segment of about 15 to 25% genome length (Fig. 6). In contrast, among the 323 linear duplexes observed, only 5 of one unit length showed one or two heterology loops. Furthermore, after denaturation and renaturation of the cleavage products of ML HPV DNA by *EcoRI* (Table 3), no heterologous sequences were observed in the linear duplexes supposed to be formed from *EcoRI* S-*BamI* R molecules.

Thirty-nine circular duplexes appeared suitable for the mapping of the heterology loops and single-strand segment. Figure 7 shows that, if one end of one of the heterology loops is taken as the origin, the second heterology region is located at about 0.6 and 0.8 genome fractional length. Thus, by selecting the heteroduplexes formed between molecules differing in their sensitivity to the enzymes *EcoRI* and *BamI*, two classes of molecules are observed with a sequence heterology corresponding to about 4 to 8% of the genome length. Finally, Fig. 7 shows that the single-stranded region observed on some of the duplexes has a defined position from one of the loops taken as origin (from 0.1 to 0.3 fractional length); the size of the

TABLE 2. Percentage of ML and plantar HPV DNA molecules resistant to restriction enzymes

HPV DNA prepn ^a	% of molecules resistant to the enzyme ^b :				% of linear unit mol- ecules after treat- ment with <i>EcoRI</i> + <i>BamI</i> ^c
	<i>EcoRI</i>	<i>BamI</i>	<i>HpaI</i>	<i>HindIII</i> + <i>III</i>	
ML ₁	20 (297) ^d	71 (412)	ND ^e	ND	67 (274)
ML ₂	67 (162)	24 (203)	29 (232)	28 (166)	22 (158)
P ₁	2 (173)	2 (158)	2 (122)	0 (153)	1 (150)

^a ML₁ and ML₂ HPV DNA preparations were obtained from virions purified from two different pools of ML patient warts, and the P₁ HPV DNA preparation was obtained from virions purified from pooled plantar warts from different patients.

^b Circular molecules (*EcoRI*, *HpaI*), circular and linear unit molecules (*BamI*), and fragments corresponding to the sum of *Hind*-A, -B, and -C fragments (*HindIII* + *III*) were considered to be resistant molecules. Linear unit molecules obtained after treatment with *BamI* endonuclease were observed only in the ML₁ HPV DNA preparation and represented 2% of the molecules. Linear unit molecules (*EcoRI*, *HpaI*) and *BamI*-A and *Hind*-A fragments were taken as sensitive molecules. Circular and linear unit-length molecules were scored on electron micrographs, and the number of fragments was deduced from the length histograms obtained from the same micrographs.

^c The percentage of linear unit molecules, obtained after treatment with a mixture of *EcoRI* and *BamI* endonucleases, was evaluated from the number of linear unit molecules and *BamI*-A fragments obtained from the length histograms of the cleavage products. No fragments corresponding to molecules sensitive to both enzymes were detected on the length histograms.

^d Total number of molecules or fragments scored.

^e ND, Not determined.

TABLE 3. Evidence for sequence heterology in ML HPV DNA by heteroduplex technique

Digestion products denatured and rena- tured ^a	No. of duplexes scored ^b	% of circular duplexes		% of linear duplexes	
		With loop	Without loop	With loop	Without loop
ML ₂ HPV DNA (<i>EcoRI</i> + <i>BamI</i>)	421	20	3	1	76
ML ₂ HPV DNA (<i>EcoRI</i>)	214	0	26	0	74
P ₁ HPV DNA (<i>EcoRI</i>) + P ₁ HPV DNA (<i>BamI</i>)	383	0	42	0	58
P ₁ HPV DNA (<i>EcoRI</i>)	200	0	1	0	99
P ₁ HPV DNA (<i>BamI</i>)	320	0	0	0	100

^a ML₂ HPV and plantar P₁ HPV DNA preparations are described in Table 2, footnote a. ML₂ HPV DNA cleaved by a mixture of *EcoRI* and *BamI* or by *EcoRI* alone, a mixture of equal amounts of P₁ HPV DNA cleaved by *EcoRI* or *BamI*, and P₁ HPV DNA cleaved by *EcoRI* or *BamI* were denatured and then renatured for 120 min at 25°C as described in the text. Renaturation products were spread on 50% formamide, and duplexes were analyzed on electron micrographs.

^b The percentage of circular and linear duplexes was obtained from the number, on electron micrographs, of circular molecules, linear unit molecules, and duplexes of the size of the ML HPV DNA *BamI*-A fragment or of the P₁ HPV DNA *EcoRI*-A fragment.

segment suggests that it may correspond to the *BamI*-B fragment.

The very low amount of plantar HPV DNA molecules resistant to *EcoRI* or *BamI* endonuclease does not allow similar experiments. However, after denaturation and renaturation of the cleavage products of plantar HPV DNA by *EcoRI*, *BamI*, or the equimolar mixture of these cleavage products, no heterologous sequences were found in the duplexes observed (Table 3). This confirms the homogeneity of plantar HPV DNA already shown by restriction enzyme analysis (13, 14).

Evaluation of the extent of homology between ML and plantar HPV DNAs, using molecular hybridization techniques. ML HPV

DNA and plantar HPV DNA obtained from virions purified either from a pool of 20 plantar warts from different patients or from a single wart were transcribed in vitro, and cRNA's were used as probes in cRNA-DNA filter hybridization experiments with HPV DNAs extracted from single or pooled hand and plantar warts. Only a little, if any, sequence homology was detected between ML and plantar HPV DNAs (Table 4). Furthermore, viral DNA preparations obtained either from single hand warts of three patients bearing numerous hand warts or from a pool of hand wart parings annealed with ML cRNA, whereas no annealing was observed with plantar cRNA's. A lower extent of annealing with ML HPV cRNA was observed

FIG. 6. Sequence heterology in ML HPV DNA. Circular heteroduplexes showing two heterology loops were obtained after denaturation and renaturation of the cleavage products of ML HPV DNA by a mixture of *EcoRI* and *BamI* endonucleases. A single-strand region (asterisks) is indicated on one of the duplexes. Bar, 0.1 μ m.

for two hand HPV DNA preparations as compared with ML HPV DNA preparations. This cannot be explained by the 2.5-fold-lower amount of DNA bound to the filters, since, in our experimental conditions, a 10-fold-lower amount of homologous DNA resulted in about a 2-fold-lower extent of annealing. These results may thus indicate that the DNAs are only partially related to ML HPV DNA. Regardless, the data reported show that plantar warts are essentially induced by a single type of HPV and suggest that hand common warts are preferentially caused by the HPV characterized in ML patient or by related viruses.

The sequence homology between ML HPV and plantar HPV genomes was further studied by DNA-DNA hybridization techniques. The effect of cold ML HPV DNA on the kinetics of reassociation of an iodinated plantar HPV DNA was analyzed. The reassociation kinetics of 125 I-labeled plantar HPV DNA alone or in the presence of an excess of cold plantar or ML HPV DNA is shown in Fig. 8. Data are expressed by the equation $1/fss = \frac{K C_0 t}{2} + 1$,

where C_0 is the total concentration of plantar HPV DNA, t is the time of incubation under reannealing conditions, fss is the fraction of plantar HPV DNA that remains single stranded at time t , and K is the second-order reassociation rate constant (33). Figure 8 shows that the kinetics follow a second-order reaction and that in the presence of a 1.5- and a 4-fold excess of cold plantar HPV DNA, the reassociation rate is increased 2.6- and 4.8-fold, respectively, which are near the expected values (2.5 and 5, respectively). Conversely, no detectable effect on the reassociation rate of 125 I-labeled plantar HPV DNA was observed in the presence of a 25-fold excess of ML HPV DNA. This confirms that ML and plantar HPV DNAs

FIG. 7. Schematic diagram of the circular duplex molecules obtained after denaturation and renaturation of the cleavage products of ML HPV DNA by a mixture of *EcoRI* and *BamI* endonucleases. The end of one of the heterologous regions was taken as the origin to map the other heterologous region (—■—) and the single-strand segment (---). Histograms of the lower part of the diagram show the frequency of occurrence of heterologous sequences (black) and of single-strand regions (white).

TABLE 4. Extent of sequence homology between HPV DNAs of hand and plantar warts as detected by cRNA-DNA hybridization^a

Viral DNA on filters ^b	Radioactivity bound (%) on filters after hybridization with different ³ H-labeled cRNA's ^c		
	ML ₂ HPV cRNA	P ₁ HPV cRNA	P ₃ HPV cRNA
Hand HPV			
ML ₂	59.1	0.2	3.4
ML ₃	47.0	-0.5	4.0
H ₄	11.0	1.0	ND ^d
H ₅	55.3	0.1	-0.3
H ₆	17.5	0.4	0.2
Plantar HPV			
P ₁	2.5	49.4	42.1
P ₂	0.2	ND	75.7
P ₃	0.4	ND	55.6
P ₄	0.3	ND	55.9
P ₅	0.3	ND	72.2
P ₆	0.6	ND	69.3
P ₇	0.2	ND	50.0
P ₈	0.8	44.4	ND
P ₉	0.2	46.9	73.7
Human fetal thymus	0.3	0.2	0.1

^a Filter hybridization in paraffin (22) was performed at 37°C for 17 h in 2× SSC-50% formamide with about 50 ng of DNA (or 20 ng for H₄ and H₆ HPV DNAs) and 3,000 cpm of tritiated cRNA per filter.

^b Hand HPV DNAs were purified from virions obtained from pooled warts of patient ML (ML₂), from a single ML wart (ML₃), and from pooled hand wart parings of several patients (H₄), or selectively extracted from single warts of two different patients (H₅ and H₆). Plantar HPV DNAs were either purified from virions obtained from different pools of warts of several patients (P₁ and P₂) and from five single warts from different patients (P₃ to P₇), or selectively extracted from single warts of two different patients (P₈ and P₉).

^c Tritiated cRNA's were obtained by in vitro transcription with *E. coli* RNA polymerase of purified form I DNA molecules extracted from virions obtained from pooled ML warts (ML₂ HPV), pooled plantar warts (P₁ HPV), and a single plantar wart (P₃ HPV).

^d ND, Not determined.

have little, if any, sequence homology.

Finally, plantar and ML HPV DNAs were analyzed by the heteroduplex technique. When a mixture of equal amounts of ML HPV DNA cleaved by *Eco*RI and plantar HPV DNA cleaved by *Bam*I was denatured, reannealed for 120 min at 25°C, and spread on 50% formamide, no heteroduplex structures were observed on electron micrographs. Similar results were obtained when the cleavage products of ML and plantar HPV DNAs by *Eco*RI were mixed, de-

natured, reannealed for 30 min at 37°C, and spread on 30% formamide.

All of these results show clearly that ML HPV and plantar HPV are two widely distinct types of HPV.

Polypeptide composition of ML HPV virions. The polypeptide composition of ML HPV virions was analyzed by SDS-acrylamide gradient slab gel electrophoresis and compared with that of plantar HPV. The two types of HPV give completely different polypeptide patterns (Fig. 9a and c), as further demonstrated by co-electrophoresis of the two SDS-dissociated viruses (Fig. 9b). The main components of plantar HPV are the major polypeptide of molecular weight 54,000, a polypeptide with a molecular weight of 76,000, and three low-molecular-weight polypeptides that were shown previously to be cellular histones associated with the viral DNA (12). In contrast, the main components of ML HPV are a major polypeptide with a molecular weight of 67,000, a polypeptide with a molecular weight of 53,000, and four low-molecular-weight polypeptides, one of them migrating like one of the histone-like components of plantar HPV. In addition, numerous minor bands were detected in the electropherogram of SDS-dissociated ML HPV virions, which probably represent cellular contaminants since the purification of these virions included only a

FIG. 8. Kinetics of reassociation of denatured and fragmented iodinated plantar HPV DNA alone or in the presence of an excess of cold plantar or ML HPV DNA. Iodinated fragmented plantar HPV DNA (3.5×10^{-3} μ g/ml; 1.8×10^7 cpm/ μ g) was allowed to reassociate alone (□) or in the presence of a 1.5-fold (○) or 4-fold (■) excess of cold plantar HPV DNA fragments or of a 25-fold excess of ML HPV DNA fragments (●), in 0.08 M sodium phosphate buffer (pH 6.8)–0.08% SDS, for various times of incubation at 68°C. The fraction of labeled DNA remaining single stranded was determined by hydroxyapatite chromatography as described by Delap et al. (10).

FIG. 9. Viral polypeptide patterns of ML and plantar HPV virions. Prior to electrophoresis, ML and plantar HPV virions were dissociated in 4 M urea-5% 2-mercaptoethanol-3% SDS for 4 min at 100°C. ML HPV virions (40 μ g of proteins) (a), a mixture of ML and plantar HPV virions (20 μ g of each) (b), and plantar HPV virions (60 μ g of proteins) (c) were subjected to electrophoresis for 15 h at 35 V in an SDS-acrylamide gradient slab gel, as described in the text.

single equilibrium centrifugation in a CsCl gradient.

Serological studies. ML serum was not suitable for testing the antigenic relationships between ML HPV and plantar HPV, since no antibodies to these two viruses could be detected by immunodiffusion tests. Serological relationships between the two viruses were checked, using guinea pig antisera prepared against purified ML HPV and plantar HPV isolated from single plantar warts and showing high titers against the homologous viruses (Table 5).

No cross-reaction could be detected between the two viruses, either by immunodiffusion or by the more sensitive indirect immunofluorescence test (Table 5). The lack of antigenic relationship between ML HPV and plantar HPV is

illustrated in Fig. 10. Adjacent sections of either ML or plantar warts were stained with fluorescein-labeled IgG obtained from ML HPV antiserum or from a plantar HPV antiserum prepared against virions purified from pooled plantar warts of several patients. In hand and plantar wart sections stained with homologous conjugates, viral antigen was detected in the nuclei of keratinizing cells and in keratinized cells (Fig. 10a and d), as expected (35). In contrast, no fluorescence was obtained with heterologous conjugates (Fig. 10b and c). The amounts of antigen varied widely in the 10 ML warts studied. The higher amount of viral antigen detected was lower than that usually found in plantar warts.

DISCUSSION

The results reported in this paper show clearly that two distinct types of papillomavirus may be isolated from human skin warts. HPV found in hand common warts of patient ML was different from HPV found in single or pooled, simple plantar warts. The DNAs of the

TABLE 5. Serological comparison of ML HPV and plantar HPV by immunodiffusion and immunofluorescence techniques^a

Guinea pig anti-serum ^b	Antibody titers using:			
	ML HPV antigens		Plantar HPV antigens	
	ID	FA	ID	FA
Anti-ML HPV (G 206)	160	2,560	Neg ^c	<10
Anti-plantar HPV (G 254)	ND ^d	<10	160	640
Anti-plantar HPV (G 260)	ND	<10	160	640

^a The immunodiffusion (ID) test was performed as described in the text. The fluorescent antibody (FA) test was performed by the indirect immunofluorescence procedure. Frozen sections of ML hand warts and plantar warts were successively incubated for 30 min at 37°C in the presence of serial dilutions of the sera and, after being washed, in the presence of fluorescein-labeled anti-guinea pig IgG rabbit globulins. Titers were expressed as the reciprocal of the highest dilution of the serum giving optimal fluorescence for the detection of the viral capsid antigen.

^b Anti-ML HPV antiserum was obtained by immunization of a guinea pig (G 206) with a preparation obtained from a pool of ML hand warts. Anti-plantar HPV antisera were obtained by immunization of two guinea pigs (G 254 and G 260) with two different plantar HPV preparations purified from single plantar warts.

^c Neg, Negative when tested undiluted.

^d ND, Not determined, since a sufficient amount of ML HPV was not available.

FIG. 10. Lack of antigenic relationships between ML HPV and plantar HPV, as shown by the direct immunofluorescence technique. Adjacent frozen sections of a patient ML hand wart (a, b) and of a plantar wart (c, d) were incubated either with anti-ML HPV fluorescein-labeled IgG (a, c) or anti-plantar HPV fluorescein-labeled IgG (b, d); both labeled globulins were used at a concentration of 0.5 mg/ml. Magnification, $\times 400$.

two viruses differed by their sensitivity to the restriction enzymes *EcoRI*, *BamI*, *HpaI*, and *HindII*+*III* and showed no sequence homology by cRNA-DNA and DNA-DNA hybridization techniques. The two viruses had distinct polypeptide patterns and showed no antigenic cross-reaction by the immunodiffusion test or by the more sensitive immunofluorescence technique. Thus, the differences between ML and plantar HPV are greater than those between human papovaviruses belonging to the polyomavirus genus, JC virus and BK virus, and between these viruses and simian virus 40 (28). It is proposed that, provisionally, plantar HPV, the DNA of which is well characterized (13, 14, 18), be called HPV type 1 and that ML HPV and the related viruses found associated with hand common warts in this study should constitute a new type of HPV called HPV type 2. Evidence for the existence of two distinct types of HPV causing skin warts was also recently obtained by Gissmann et al. (17). On the basis of the sensitivity of viral DNA to *H. aegyptius* (*HaeIII*) endonuclease, cRNA-DNA hybridizations, and electrophoretic mobilities and antigenic properties of viral polypeptides, these authors have characterized three viral isolates (HPV types 1 to 3) closely related to HPV type 1 and an entirely different type of virus, HPV type 4. However, no conclusion can be drawn about the relationships between this virus and ML HPV, since different restriction enzymes were used, and the clinical type and anatomical location of the warts associated with this virus were not clearly defined.

Restriction enzyme analysis of HPV type 1 DNA molecules had shown only minor genetic heterogeneity (13, 14, 17, 18); when this was checked by heteroduplex technique, using a preparation of HPV type 1 DNA purified from pooled warts, where one *HpaII* cleavage site was missing in about 40% of the molecules (data not shown) (14), no deletion or heterology loops could be detected. In contrast, the greater genetic heterogeneity of ML HPV DNA was shown by the high proportion of resistant molecules to restriction enzymes and by the detection of up to 8% sequence heterology by the heteroduplex technique, although deleted molecules as reported for face and hand wart HPV DNA, referred to as human wart virus DNA (36), were not observed. Furthermore, two of three additional preparations of hand HPV DNAs annealed to a lower extent with ML HPV cRNA as compared with the control, suggesting that these DNAs shared only a partial sequence homology with ML HPV DNA. More studies are required before concluding that the genetic variability is a general characteristic of

the HPVs belonging to type 2 and that it may play a role in their pathogenicity.

HPV type 1 causes most of the typical plantar warts and has been found to be associated with some warts of low virus content restricted to the anal region (Orth et al., in press). HPV type 2 has been isolated from multiple hand common warts, but its specificity for this clinical type of wart has to be further confirmed. Much work remains to be done to determine the role of the two viruses, or of additional types of HPV, in the production of other clinical types of skin warts (common warts at different locations, mosaic plantar warts, flat warts, and filiform warts). Indeed, the existence of a third type of HPV has recently been suggested by immunological studies on virus isolated from the lesions of patients suffering from epidermodysplasia verruciformis. Epidermodysplasia verruciformis is a rare disease characterized by a lifelong generalized eruption of flat wartlike skin lesions and by the transformation of some of the lesions into carcinomas in about 25% of the patients (20). Papillomavirus particles isolated from two epidermodysplasia verruciformis patients were shown to be immunologically distinct from virions obtained from a pool of either plantar or hand warts, as assayed by immune electron microscopy or complement fixation tests (F. Pass, M. Reissig, K. Shah, M. Eisinger, and G. Orth, J. Natl. Cancer Inst., in press). Furthermore, no antigenic relationships could be shown between HPV types 1 and 2 and the virus present in the lesions of five epidermodysplasia verruciformis patients by immunofluorescence studies (G. Orth and S. Jablonska, in preparation). As far as the virus of genital condylomata is concerned, serological (2, 16), molecular hybridization (9, 17, 37), and epidemiological (25) data suggest that it could constitute a type of virus distinct from skin wart viruses.

No clear-cut conclusion on the pathogenicity of the different viruses can be drawn from the serological studies on human wart virus infection. Viral antigens obtained from pools of plantar warts were found suitable for serological studies in patients bearing warts at different locations (7, 24). Total identity between preparations of purified virus from pools of simple plantar warts, mosaic plantar warts, or hand warts has been reported (24). These results may be explained either by a single clinical type of wart corresponding to several types of viruses or by a single virus inducing several types of warts. Furthermore, antiviral antibodies were detected, by different serological techniques, in 10 to 50% of the patients bearing active warts and in 20 to 100% of the patients at

the time of wart regression (1, 16, 29, 30, 34). In most of these studies, the sources of antigen and/or sera were poorly defined. The discrepancy of the results may be explained by the differences in the antigen used. This further stresses the need for a better characterization of HPVs and their pathogenicity.

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