Characterization of a New Type of Human Papillomavirus Found in a Lesion of Bowen's Disease of the Skin

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The genome of a human papillomavirus (HPV) found in a patient with Bowen's disease of the skin was molecularly cloned. Blot hybridization experiments, performed under stringent conditions, revealed no cross-hybridization between this HPV DNA and the other known HPV DNAs, showing that this HPV represents a new type, tentatively named HPV34. In relaxed hybridization conditions, the highest cross-hybridization was observed with HPV16 DNA. The physical map of HPV34 DNA was aligned with the genetic map of HPV16 DNA by heteroduplex mapping. HPV34 was not detected in 12 additional patients with Bowen's disease of the skin, but a closely related HPV DNA was found in one patient with penile Bowenoid papulosis.

The first evidence for the role of human papillomaviruses (HPVs) in the development of human cancers was obtained from the study of epidermodysplasia verruciformis, since the genomes of specific HPV types, mainly HPV5 and less frequently HPV8, HPV14, and HPV17, have been found in skin carcinomas observed in epidermodysplasia verruciformis patients (15, 19, 21, 23, 29). This was further supported by the detection of HPV5 DNA in the skin cancers of an immunosuppressed renal allograft recipient (16). HPV DNA sequences have also been detected in a high proportion of genital intraepithelial neoplasias and invasive carcinomas, and the genomes of two specific HPV types, HPV16 and, less frequently, HPV18, have been shown to be associated with the majority of these tumors, especially with carcinomas of the uterine cervix (1, 3, 6, 10, 26). In contrast, the association of HPVs with premalignant and malignant skin tumors in the general population has been poorly substantiated. Only a limited number of cases of Bowen's disease of the skin, an *in situ* squamous cell carcinoma with characteristic histological features (14), have been investigated. HPV2 DNA sequences have been detected in one case (24) and HPV16 genomes were detected in two of five cases (10).

To better define the etiological role of HPVs in the cutaneous tumors observed in the general population, we initiated a study aimed at the detection of HPV DNA sequences in various types of neoplasia of the skin. We first studied patients with Bowen's disease of the skin, and we report here the molecular cloning and characterization of a new HPV type found in one of these patients.

Biopsies of Bowen's disease were taken from 13 Polish patients (9 women and 4 men) with ages ranging from 58 to 82 years. The lesions had been present from 6 months to 15 years and were located on the face in three patients, on the trunk in two patients, on the hands and fingers in three patients, and on the legs and feet in five patients. The specimens were kept in Eagle medium before freezing at -80°C.

The total cell DNA was extracted from the biopsies (19). The DNA preparations were treated with *Pst*I restriction enzyme, and the digestion products were analyzed by blot

hybridization experiments as previously described (12, 13). The hybridizations were carried out in either stringent ($T_m - 20^\circ\text{C}$) or relaxed ($T_m - 40^\circ\text{C}$) conditions with various ³²P-labeled HPV DNA probes (9). The DNA probes were specific for HPVs associated with cutaneous warts (a mixture of HPV1, -2, -4, and -7 DNAs or a mixture of HPV3, -10, and -28 DNAs) and with epidermodysplasia verruciformis (a mixture of HPV5, -8, and -14 DNAs or a mixture of HPV17, -20, -23, and -24 DNAs) or specific for HPVs associated with mucocutaneous and mucous membrane lesions (HPV16 DNA, HPV18 DNA, or a mixture of HPV13 and -32 DNAs). HPV DNA sequences were only detected in the DNA preparations obtained from three biopsies of a single lesion after hybridization under relaxed conditions with a mixture of HPV3, 10, and 28 DNA probes or with an HPV16 DNA probe (Fig. 1, lane a). The pattern observed, that is, four bands with molecular weights corresponding to 3.0, 2.0, 1.3, and 0.61 kilobase pairs (kbp), respectively, differed from any of the known *Pst*I HPV DNA cleavage patterns. This newly recognized HPV will be referred to as HPV34 in this paper.

The patient was a 69-year-old woman who had undergone hysterectomy for cervical carcinoma 10 years earlier. She had had for 3 years one lesion of Bowen's disease on the periungual site of the left middle finger (Fig. 2A). Histological examinations of three biopsies revealed the characteristic features of an *in situ* squamous cell carcinoma of Bowen's type (Fig. 2B) (14). The patient had also had for 6 years two common warts on the right palm and index finger, respectively. After blot hybridization with an HPV4 DNA probe (Fig. 1, lane b), the DNA preparation extracted from one common wart yielded a *Pst*I cleavage pattern typical for HPV4 DNA.

To better characterize the HPV DNA sequences detected in the Bowen's disease lesion, the tumor DNA preparations were pooled and treated with 20 different restriction enzymes and analyzed by blot hybridization experiments performed in relaxed conditions with an HPV16 DNA probe. Surprisingly, no cleavage was observed after treatment with 14 enzymes, as illustrated for *Hind*III and *Sac*I (Fig. 1, lanes d and e), and this was further confirmed after molecular cloning of the HPV DNA sequences (see the legend to Fig. 3). The HPV34 DNA was cleaved once by *Bgl*II (Fig. 1, lane

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FIG. 1. Blot hybridization analysis of HPV DNA sequences found in a patient with Bowen's disease of the skin. The total cell DNA was extracted (19) from three biopsies of the lesion illustrated in Fig. 2A (lanes a and c to g), from a palmar common wart (lane b) found in the same patient, and from a lesion of penile Bowenoid papulosis of another patient (lane i). HPV34 DNA was inserted in plasmid pSP64 at the *Bgl*II cleavage site and cloned in *E. coli* K-12 C600. The cloned HPV DNA sequences were excised and purified from the plasmid sequences (lane h) as previously described (12). The DNA preparations were either untreated (lane c) or treated with *Pst*I (lanes a, b, and h), *Hind*III (lane d), *Sac*I (lane e), *Bgl*II (lanes f and i), or a mixture of *Bgl*II and *Pst*I (lane g). The DNAs were electrophoresed in vertical 1.2% agarose slab gels (lanes a to f) or in horizontal 1.0% agarose slab gels (lanes g to i) (19), depurinated, denatured, and transferred to Genescreen-plus hybridization membranes (New England Nuclear Corp., Boston, Mass.) as previously described (12). Hybridizations were carried out in 1 M NaCl at 42°C in the presence of 50% formamide (lanes b and g to i) or 20% formamide (lanes a and c to f), with ³²P-labeled cloned HPV16 DNA (lanes a and c to f), HPV4 DNA (lane b), and HPV34 DNA (lanes g to i) probes. The specific activities of the probes varied between 8.0×10^7 and 1.5×10^8 cpm/μg. The hybrids were detected by autoradiography as previously described (12). The sizes of the fragments, indicated in kilobase pairs, were deduced from the sizes of λ *Hind*III DNA fragments taken as standards (17). The migration of free monomeric HPV DNA molecule forms I, II, and III are indicated. Arrowheads point to oligomeric DNA molecules.

f), *Bgl*II, and *Hpa*I and twice by *Hinc*II, *Hpa*II, and *Xba*I (data not shown). In untreated tumor DNA, bands migrating like free monomeric HPV DNA molecule forms I, II, and III as well as two slow-migrating species were detected (Fig. 1, lane c). The unmodified electrophoretic mobilities of the slow-migrating molecules after treatment with no-cut enzymes for HPV34 DNA (Fig. 1, lanes d and e) indicated that they corresponded to oligomeric molecules.

The tumor DNA was cleaved with *Bgl*II. The digestion products were fractionated in a 5 to 40% preformed sucrose gradient, and the fractions were analyzed by electrophoresis in an agarose gel with linear HPV16 DNA as an 8-kbp standard (17). DNA fragments of about 8 kbp were inserted into the polylinker region of plasmid pSP64 (18), using the unique *Bam*HI site, since *Bgl*II and *Bam*HI recognize the same tetranucleotide sequence within their hexanucleotide recognition sequences (17). After ligation, the recombinant plasmids were transfected into *Escherichia coli* K-12 C600 (17), with the agreement of the French commission for the control of in vitro genetic recombination. Colonies containing recombinant plasmids were selected by in situ hybridiza-

tion (17), using an HPV16 DNA probe under relaxed annealing conditions. Of 3,000 colonies, 2 yielded intense signals, and the recombinant plasmids were amplified and extracted from the 2 colonies as previously described (12). Since the cloning site was destroyed during insertion, the size of the cloned DNA sequences was estimated by treatment of recombinant plasmids with a mixture of *Hind*III and *Sac*I, two no-cut enzymes for HPV34 DNA, whose cleavage sites are located on either side of the insert in the polylinker region of pSP64 (18). Only one of the two recombinant plasmids yielded the expected 8-kbp DNA band and was further characterized.

The 8-kbp insert was excised by treatment with a mixture of *Hind*III and *Sac*I and separated from the plasmid sequences as previously described (12). The electrophoretic mobilities of the digestion products of the uncloned DNA treated with a mixture of *Bgl*II and *Pst*I and of the purified cloned DNA treated with *Pst*I were compared by blot hybridization with the cloned DNA as a probe. Seven bands (with sizes corresponding to 3.0, 1.5, 1.3, 0.61, 0.56, 0.46, and 0.23 kbp, respectively) were observed in the two DNA preparations (Fig. 1, lanes g and h). The sum of the sizes of

FIG. 2. Clinical and histological features of the Bowen's disease lesion containing HPV34 DNA sequences. (A) Morphology of the lesion. (B) Histology of the lesion showing the typical features of an in situ squamous cell carcinoma of Bowen's type, namely, a loss of normal polarity, atypical keratinocytes with large hyperchromatic nuclei extending throughout the acanthotic epidermis, multinucleated cells, abnormal mitotic figures, individually keratinizing cells, and hyperkeratosis with parakeratosis (14).

these bands (7.66 kbp) suggested that the whole HPV34 genome was integrated. Two of these bands (1.5 and 0.46 kbp) corresponded to the *Bgl*II cleavage products of the 2-kbp *Pst*I fragment (Fig. 1, lane a). The two bands of 0.56 and 0.23 kbp were not detectable with the HPV16 DNA probe in relaxed hybridization conditions (Fig. 1, lane a). A physical map of the cloned HPV34 DNA (Fig. 3) was constructed as previously described (13). No obvious similarities were observed with any previously published HPV DNA maps (1, 5, 7-9, 11-13, 22, 25).

The DNA sequence homology between HPV34 DNA and HPV1 to HPV26 DNAs (1, 5-9, 11-13, 20, 22, 25) was analyzed, as well as that between HPV34 DNA and HPV DNAs recently isolated from cutaneous warts (HPV27, R. S. Ostrow, personal communication; HPV28 and -29, M. Favre, S. Jablonska, S. Obalek, O. Croissant, and G. Orth, manuscript in preparation), from a laryngeal carcinoma (HPV30, T. Kahn and L. Gissmann, personal communication), from a cervical intraepithelial neoplasia (HPV31, A. Lorincz and G. Temple, personal communication), from a focal epithelial hyperplasia of the oral mucosa (HPV32, S. Beaudenon, F. Praetorius-Clausen, D. Kremsdorf, M. A. Lutzner, N. Worsaae, and G. Orth, submitted for publication), and from a cervical carcinoma (HPV33, S. Beaudenon, D. Kremsdorf, S. Jablonska, O. Croissant, and G. Orth, manuscript in preparation). Blot hybridization experiments, performed under stringent conditions ($T_m = 20^\circ\text{C}$), revealed no cross-hybridization between the HPV34 DNA probe and HPV1 to -29, -32, and -33 DNAs (Fig. 4A). Similarly, no cross-hybridization was observed with blotted HPV30 and -31 DNAs (kindly provided to us by T. Kahn and L. Gissmann and by A. Lorincz and G. Temple, respectively). It thus seems justified to consider HPV34 as a new HPV type.

When hybridization experiments were performed in relaxed conditions ($T_m = 40^\circ\text{C}$), the HPV34 DNA probe revealed conserved DNA sequences in most of the HPV DNAs, the highest cross-hybridization being observed with HPV16 DNA and, to a lesser extent, with HPV32 and -33 DNAs (Fig. 4B). The sequence homology between HPV34 and HPV16 DNAs was further studied by heteroduplex mapping. HPV16 DNA has previously been cloned from penile Bowenoid papulosis (S. Beaudenon, unpublished data). The HPV16 recombinant plasmid used in this study was obtained by inserting HPV16 DNA in the polylinker region of plasmid pSP62 (18), using the unique *Bam*HI site. The HPV16 DNA and the plasmid sequences were in the same $5' \rightarrow 3'$ orientation, according to their nucleotide

FIG. 3. Physical map of HPV34 DNA. The unique *Bgl*II site has been taken as the origin. The distances from the origin are given in percentages of the HPV34 genome. The seven *Pst*I fragments have not been mapped. *Bal*I, *Bam*HI, *Cl*aI, *Eco*RI, *Hind*III, *Nde*I, *Nru*I, *Pvu*I, *Pvu*II, *Sac*I, *Sal*I, *Sma*I, *Tth*1111, and *Xma*I do not cleave uncloned or cloned HPV34 DNA.

FIG. 4. DNA sequence homology between HPV34 and other HPV DNAs, as detected by blot hybridization experiments. Cloned HPV1, -3, -6, -10, -11, -13, -16, -18, -28, -29, -32, -33, and -34 DNAs were excised and purified from plasmid sequences as previously described (12). DNAs (5 ng) were electrophoresed and blotted on nitrocellulose filters (BA85; Schleicher & Schuell, Inc., Keene, N.H.). The filters were allowed to hybridize for 16 h at 42°C in $5\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)- $5\times$ Denhardt solution (17)-50 mM sodium phosphate buffer (pH 6.5)-250 μg of heat-denatured yeast tRNA per ml in the presence of 50% formamide ($T_m = 20^\circ\text{C}$) (A) or 20% formamide ($T_m = 40^\circ\text{C}$) (B), with ^{32}P -labeled HPV34 DNA (3×10^4 cpm/ μm^2 ; specific activity, 1.2×10^8 cpm/ μg). The filters were washed in $2\times$ SSC-0.1% (wt/vol) sodium dodecyl sulfate at 68°C ($T_m = 20^\circ\text{C}$) (A) or 48°C ($T_m = 40^\circ\text{C}$) (B) (10). The hybrids were detected by autoradiography after a 26-h exposure.

sequences (27, 28), as determined by restriction enzyme analysis (data not shown). Heteroduplex molecules were formed, either between excised cloned HPV DNAs or between one excised cloned HPV DNA and the linearized recombinant plasmid containing the other HPV DNA. The pSP64-HPV34 recombinant plasmid was linearized by using the unique *Sal*I cleavage site located in the polylinker region at 13 nucleotides upstream from the HPV34 DNA sequences inserted in the direction $0 \rightarrow 1$ (Fig. 3). The pSP62-HPV16 recombinant plasmid was linearized by using the unique *Sma*I cleavage site located in the polylinker region at six nucleotides upstream from the HPV16 DNA sequences oriented in the direction $0 \rightarrow 1$ of the HPV16 physical map (see Fig. 5C). Heteroduplex molecules were formed in 30% formamide and spread at room temperature (22 to 24°C) in 30% ($T_m = 41^\circ\text{C}$) or 50% ($T_m = 27^\circ\text{C}$) formamide (2, 4).

In 30% formamide, HPV34 and HPV16 DNAs were paired over about 65% of the genome length (Fig. 5A). In contrast, in 50% formamide, four to six small duplex segments (corresponding to about 8% of the genome length) were observed on rarely detected heteroduplex molecules (Fig. 5B). The regions of partial homology were mapped, and the relative orientation of the HPV34 and HPV16 DNA maps was determined (Fig. 5C). The regions yielding stable heteroduplexes at $T_m = 41^\circ\text{C}$ were distributed throughout the genomes. The less-conserved regions were located in the noncoding region, in the E5 open reading frame, and in segments of each other's open reading frames. The regions of strongest homology, detected in 50% formamide, were mapped in the E1, L2, and L1 open reading frames. The *Bam*HI site, taken as the origin of the physical map of HPV16 DNA, was mapped at 0.7 map unit on the HPV34 DNA map.

HPV34 DNA sequences have been searched for, by blot hybridization experiments performed in stringent conditions, in the total cell DNA extracted from the other 12 patients with Bowen's disease of the skin, from 13 specimens of squamous cell carcinomas of the skin, from 12 biopsies of genital Bowen's disease, from 36 samples of Bowenoid papulosis of the external genitalia or of the anal region, and from scrapings of benign lesions of 17 epidermodysplasia verruciformis patients. No evidence for the presence of HPV34 DNA sequences was obtained in any

FIG. 5. Partial homology maps of HPV34 and HPV16 DNAs, as determined by heteroduplex analysis. (A) HPV34 and HPV16 DNAs excised from pSP64-HPV34 and pSP62-HPV16 recombinant plasmids, respectively, were annealed and spread in the presence of 30% formamide ($T_m = 41^\circ\text{C}$) (2, 4). (B) Heteroduplex molecules, formed in 30% formamide between *Sma*I-linearized pSP62-HPV16 recombinant plasmid and excised cloned HPV34 DNA, were spread in the presence of 50% formamide ($T_m = 27^\circ\text{C}$). The extremities of HPV16 or -34 and plasmid (P) DNA sequences, deduced from length measurements, are indicated by arrows. The bar represents 0.1 μm . (C) Partial homology maps were obtained by analysis of heteroduplex molecules formed between excised cloned HPV34 and *Sma*I-linearized pSP62-HPV16 recombinant plasmid DNAs and between excised cloned HPV16 and *Sma*I-linearized pSP64-HPV34 recombinant plasmid DNAs. The plasmid sequences made it possible to deduce the orientation of the heteroduplexes. The lengths of single-stranded and double-stranded regions of HPV34-HPV16 heteroduplex molecules were measured with a digital length calculator (Numonics) connected to a Nova 3D computer (Data General). The histograms represent the number of double-stranded 0.01-genome-length segments observed in heteroduplexes formed in 30% formamide and spread in 30% ($\square$; seven molecules) or 50% ($\blacksquare$; seven molecules) formamide, as a function of their position on the HPV34 physical map. The alignment of HPV34 and HPV16 DNA maps was deduced from the oriented partial homology maps. A

of these lesions, with the exception of one case of penile Bowenoid papulosis, in which an HPV DNA showing a high degree of cross-hybridization with HPV34 DNA was detected (Fig. 1, lane i). This HPV DNA differed, however, from HPV34 DNA by its sensitivity to *Bgl*II (two cleavage sites) (Fig. 1, lane i), *Bam*HI (one cleavage site), *Hinc*II (three cleavage sites), and *Hind*III (two cleavage sites), while neither HPV DNA was cleaved by *Eco*RI, *Sal*I, or *Sma*I (data not shown). Thus, HPV34 has been detected in only two cases of cutaneous or genital intraepithelial neoplasias showing features of Bowen's atypia. This suggests that HPV34 may have an oncogenic potential but that its prevalence is low.

Contrary to previous reports (10, 24), neither HPV2 nor HPV16 have been detected in any of the 13 specimens of extragenital Bowen's disease of the skin studied. Conversely, HPV16 has been found in a high proportion of genital Bowen's disease cases and in Bowenoid papulosis (10; G. Orth, S. Jablonska, S. Obalek, and S. Beaudenon, unpublished data). The results reported in this paper suggest that Bowen's disease of the skin and genital Bowen's disease usually have different causes. The association of Bowen's disease of the skin with HPVs is not as well substantiated as that of genital Bowen's disease. However, it is still possible that Bowen's disease of the skin involves an as yet unrecognized HPV(s) which may not have been detected with the available HPV DNA probes.

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