New Human Papilloma Virus Isolated from Epidermodysplasia Verruciformis Lesions

M. Yutsudo, T. Tanigaki, T. Tsumori, S. Watanabe, and A. Hakura

Department of Tumor Virology, Research Institute for Microbial Diseases, Osaka University, Yamadaoka, Suita 565 [M. Y., T. Ts., A. H.]; Department of Dermatology, Osaka University Medical School, Fukushima-ku, Osaka 553 [T. Ta.]; and Department of Medical Laboratory Technology, College of Bio-Medical Technology, Osaka University, Machikaneyama, Toyonaka 560 [S. W.], Japan

ABSTRACT

Human papilloma virus (HPV) was isolated from red plaques of a patient (N. F.) with epidermodysplasia verruciformis. Electron microscopic examination showed characteristic particles of papilloma virus as icosahedrons about 45 nm in diameter. DNA was extracted from these particles, and closed-circular DNA (Form I) was purified by centrifugation in CsCl containing ethidium bromide. The molecular weight of the DNA was about 5.0 x 10^6. A physical map of the HPV DNA was constructed using several restriction enzymes. The restriction endonuclease cleavage pattern of the HPV DNA was different from those of other types of HPV reported thus far, suggesting that the isolate was a new, as yet unclassified, HPV.

INTRODUCTION

Several types of HPV have been isolated from various skin warts and classified on the basis of their DNA sequence homology and immunological properties. The various types of HPV seem to be associated preferentially with particular types of warts: HPV-1 and HPV-4 are associated with plantar warts (2-4); HPV-2 and HPV-7 are associated with common hand warts (6, 11); HPV-3 is associated with flat warts, including verruca vulgaris; HPV-5 is associated with common warts; HPV-6 is associated with genital warts (condylomata acuminata); HPV-7 is associated with condylomata acuminata. Recently, HPV-8 was isolated from EV lesions (14, 15). EV is a lifelong skin disease first reported by Lewandowsky and Lutz (8) and is characterized by disseminated flat warts, red plaques, or pityriasis versicolor-like lesions of EV patients (12, 13). The investigations, including histological and immunological tests, that showed that this patient has EV will be reported elsewhere.

For isolation of viral particles, the red plaques on the skin were scraped off and lysed. The virus was then purified by isopyknic centrifugation in CsCl containing ethidium bromide. The virions were then incubated at 37°C for 2 hr in the presence of 0.5% sodium dodecyl sulfate and proteinase K (200 μg/ml). DNA was extracted twice with phenol and once with chloroform:isoamyl alcohol (24:1) and precipitated with ethanol. Form I DNA was then purified by centrifugation at 40,000 rpm (SW 50.1) for 48 hr in CsCl (p = 1.57) containing ethidium bromide.

MATERIALS AND METHODS

Purification of HPV DNA. Red plaques were scraped off from the back and chest of an EV patient (N. F.). The pooled materials were homogenized and sonicated, and virus particles were purified by low (5,000 rpm; 30 min) and high (30,000 rpm; 1 hr)-speed centrifugation. The virions were then incubated at 37°C for 2 hr in the presence of 0.5% sodium dodecyl sulfate and proteinase K (200 μg/ml). DNA was extracted twice with phenol and once with chloroform:isoamyl alcohol (24:1) and precipitated with ethanol. Form I DNA was then purified by centrifugation at 40,000 rpm (SW 50.1) for 48 hr in CsCl (p = 1.57) containing ethidium bromide.

Digestion with Restriction Endonucleases and Agarose Gel Electrophoresis. Restriction endonucleases EcoRI, BamHI, and HindIII were purchased from Takara Shuzo Co., Ltd., Japan; BglI, HindIII, Hhal, and XbaI were purchased from Bethesda Research Laboratories, Inc., and Hpal and HpaII were purchased from Boehringer Mannheim, Mannheim, West Germany. Digestion was carried out at 37°C for 3 hr in high-salt buffer (100 mM NaCl:50 mM Tris, pH 7.4:10 mM MgSO4) with EcoRI and XbaI, in medium-salt buffer (50 mM NaCl:10 mM Tris, pH 7.4:10 mM MgSO4:1 mM dithiothreitol) with BglI, BamHI, HindIII, Hhal, and HpaII, and in low-salt buffer (10 mM Tris, pH 7.4:10 mM MgSO4:1 mM dithiothreitol) with Hpal and HpaII. The resulting DNA fragments were subjected to 0.8% agarose gel electrophoresis at 20 V for about 15 hr in 40 mM Tris:20 mM sodium acetate:18 mM NaCl:2 mM EDTA (pH 8.05) (7), and bands were located either by staining with ethidium bromide or by the method of Southern (15). A 32P-labeled probe of HPV DNA was prepared with a nick-translation kit (Radiochemical Centre, Amersham, England).

RESULTS

Isolation of HPV from EV Lesions. An EV patient (N. F.), a 24-year-old man, has many red plaques but no pityriasis versicolor-like lesions (Fig. 1). Although the frequency of malignant conversion from EV is high (9, 10, 13), carcinoma has not yet been recognized in this patient. The investigations, including histological and immunological tests, that showed that this patient has EV will be reported elsewhere.

For isolation of viral particles, the red plaques on the back and chest of this patient were scraped off and pooled. The tissue was homogenized and sonicated, and the virus was collected by centrifugation. The suspension of the pellet was subjected to isopyknic centrifugation in CsCl. Virus particles that formed a band at a density of about 1.34 g/ml were observed by electron microscopy (Fig. 2). They were icosahedron-like particles about 45 nm in diameter. As described below, the molecular weight of the virion DNA was about 5.0 x 10^6. These features are characteristic of papilloma viruses.

Restriction Endonuclease Digestion of the HPV DNA. DNA was extracted from the virus particles and fractionated by CsCl:ethidium bromide equilibrium density centrifugation. DNA which banded at a density of 1.57 g/ml was identified as Form I HPV DNA.
The Form I DNA was digested with several restriction endonucleases and subjected to agarose gel electrophoresis (Fig. 3). When the DNA was cut with EcoRI or BglII, a linear molecule \( (M_c \sim 5.0 \times 10^6) \) was detected on the gel. It was a full genome-sized molecule generated by single-site digestion, judging by comparison of its mobility on gel with that of Form III DNA in the stock solution (Fig. 5). This conclusion was confirmed by the fact that the sum of the molecular weights of the fragments obtained by digestions with various restriction endonucleases was about \( 5 \times 10^6 \) (Table 1). HindIII gave a \( M_c = 4.7 \times 10^6 \) fragment. Therefore, HindIII might cut the molecule at 2 (or more) sites. Since we could not obtain much Form I DNA and used only a small amount for gel electrophoresis, we could not detect fragments with molecular weights less than \( 5 \times 10^5 \).

HindIII cut the molecule at 3 sites and generated 3 fragments, \( M_c = 2.45, 1.65, \) and \( 0.84 \times 10^6 \), respectively (Fig. 5). Other restriction endonucleases (XbaI, Hpal, HpalI, HhaI, and HindII) were also used to analyze the HPV DNA. These results were summarized in Table 1. No HPV that shows a cleavage pattern similar to that of DNA has been reported previously.

A restriction map of the HPV DNA was constructed. As shown in Figs. 4 and 5, the DNA was digested with 2 kinds of restriction endonuclease, and the products were analyzed by agarose gel electrophoresis. The molecular weights of the fragments are listed in Table 2. A preliminary physical map deduced from these data is shown in Chart 1. These results suggested that our isolate was a new HPV.

**DISCUSSION**

In this paper, we propose a physical map of the HPV DNA isolated from red plaques of an EV patient. No HPV DNA that gives the same restriction endonuclease cleavage pattern as our HPV DNA has been reported previously, suggesting that our isolate is a new HPV. Various viruses have variants in which several cleavage sites for restriction enzymes appeared newly or disappeared. However, as shown in Table 3, it seems unlikely that our HPV is a variant derived from other types of HPV by a minor base change. This conclusion would be strengthened by measuring the molecular weights of digested fragments in detail.

Different types of HPV are classified essentially on the basis of the extents of their DNA sequence homology (1). However, it was not possible to measure the extent of homology between

**Table 1**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>EcoRI</th>
<th>BglII</th>
<th>HindIII</th>
<th>BamHI</th>
<th>XbaI</th>
<th>Hpal</th>
<th>HpalI</th>
<th>HhaI</th>
<th>HindII</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5.0</td>
<td>5.0</td>
<td>4.7</td>
<td>2.45</td>
<td>2.5</td>
<td>3.8</td>
<td>1.88</td>
<td>2.85</td>
<td>2.65</td>
</tr>
<tr>
<td>B</td>
<td>1.65</td>
<td>2.35</td>
<td>0.87</td>
<td>1.39</td>
<td>1.05</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.84</td>
<td></td>
<td>0.92</td>
<td>0.52</td>
<td>0.59</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.48</td>
<td></td>
<td>0.46</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5.0</td>
<td>5.0</td>
<td>4.7</td>
<td>4.94</td>
<td>4.85</td>
<td>4.67</td>
<td>4.77</td>
<td>4.88</td>
<td>3.94</td>
</tr>
</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>HindIII</th>
<th>EcoRI</th>
<th>HindIII</th>
<th>EcoRI</th>
<th>BglII</th>
<th>HindIII</th>
<th>BamHI</th>
<th>HindIII</th>
<th>BamHI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5.0</td>
<td>5.0</td>
<td>2.35</td>
<td>2.5</td>
<td>2.35</td>
<td>2.5</td>
<td>2.5</td>
<td>2.35</td>
<td>2.5</td>
</tr>
<tr>
<td>B</td>
<td>3.9</td>
<td>3.9</td>
<td>3.35</td>
<td>3.35</td>
<td>3.35</td>
<td>2.0</td>
<td>1.65</td>
<td>1.65</td>
<td>1.65</td>
</tr>
<tr>
<td>C</td>
<td>2.65</td>
<td>2.65</td>
<td>2.25</td>
<td>2.25</td>
<td>2.25</td>
<td>1.2</td>
<td>0.58</td>
<td>0.58</td>
<td>0.58</td>
</tr>
<tr>
<td>D</td>
<td>0.75</td>
<td>0.75</td>
<td>1.35</td>
<td>1.35</td>
<td>1.35</td>
<td>0.42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.7</td>
<td>4.7</td>
<td>5.0</td>
<td>5.0</td>
<td>4.70</td>
<td>3.55</td>
<td>4.85</td>
<td>4.85</td>
<td>4.85</td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>EcoRI</th>
<th>BamHI</th>
<th>HindIII</th>
<th>HindIII</th>
<th>HindIII</th>
<th>HindIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>1</td>
<td></td>
<td>2</td>
<td>4</td>
<td>&gt;5</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
the new isolate and other defined types of HPV by hybridization because it was almost impossible to collect or exchange all types of HPV, mainly owing to the absence of an in vitro system for efficient replication of HPV. To determine whether our isolate belongs to a new type or a subtype of HPV such as HPV-5 (10), we plan to isolate a cloned DNA of our HPV and to submit it to the hybridization test.

Although the patient described does not yet have a recognizable carcinoma (possibly because he is still quite young), the cloned DNA obtained might be useful in studies on the mechanism of skin carcinogenesis. HPV-related sequences in tumor tissues of other EV patients must be examined and compared with HPV DNAs from EV patients bearing skin carcinomas. We must also try the transformation assay of cultured human cells and mouse NIH3T3 cells with the cloned DNA.

ACKNOWLEDGMENTS
The authors are grateful to Dr. Y. Hosaka for carrying out electron microscopic studies on HPV and to Dr. K. Toyoshima for his interest in this work.

REFERENCES
Fig. 1. Red plaques of the EV patient (N. F.). Many red plaques were seen on the chest, back, and face. Red plaques on the chest and back were scraped off for virus purification.

Fig. 2. Electron microscopic appearance of the virions. The virions purified by CsCl density gradient centrifugation were dialyzed against phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 1.16 g Na2HPO4, 0.2 g KH2PO4 per liter) and were stained negatively with uranyl acetate. The diameter of the virions is about 45 nm. Bar, 100 nm.

Fig. 3. Agarose gel electrophoresis of the HPV DNA digested with several restriction endonucleases. The DNA was applied to 0.8% agarose gel electrophoresis and was stained with ethidium bromide. Lanes 1 and 7, markers for estimation of molecular weight (DNA digested with HindIII; the sizes of each fragment were 14.63, 6.13, 4.07, 2.83, 145, and 1.26 x 10^6, respectively); Lane 2, undigested DNA; Lane 3, DNA digested with HindIII; Lane 4, DNA digested with BglII; Lane 5, DNA digested with EcoRI; Lane 6, DNA digested with XbaI; Lane 8, DNA digested with HpaI; Lane 9, DNA digested with HpaII; Lane 10, DNA digested with HhaI.

Fig. 4. Agarose gel electrophoresis of the HPV DNA digested with 2 kinds of restriction enzyme. The DNA was subjected to 0.8% agarose gel electrophoresis and stained with ethidium bromide. Lane 1, DNA digested with HindIII; Lane 2, DNA digested with EcoRI and HindIII; Lane 3, DNA digested with EcoRI; Lane 4, DNA digested with EcoRI and BglII; Lane 5, DNA digested with BglII; Lane 6, DNA digested with XbaI and BglII; Lane 7, DNA digested with XbaI; Lane 8, DNA digested with XbaI and HindIII; Lane 10, DNA digested with XbaI and EcoRI.

Fig. 5. Agarose gel electrophoresis of the HPV DNA digested with 2 kinds of restriction enzyme. The DNA was applied to 0.8% agarose gel electrophoresis and analyzed by the blotting method of Southern (15). 32P-HPV DNA (5.0 x 10^6 cpm) was used for hybridization. Molecular weight markers (see Fig. 3) were located by staining with ethidium bromide. Lane 1, undigested DNA; Lane 2, DNA digested with BglII; Lane 3, DNA digested with BglII and HindIII; Lane 4, DNA digested with HindIII; Lane 5, DNA digested with HindIII and BamHI; Lane 6, DNA digested with BamHI; Lane 7, DNA digested with BamHI and BglII.
New Human Papilloma Virus Isolated from Epidermodysplasia Verruciformis Lesions

M. Yutsudo, T. Tanigaki, T. Tsumori, et al.