JOURNAL OF VIROLOGY, Mar. 1977, p. 1210-1214

Vol. 21, No. 3
Printed in U.S.A.

Human Papillomavirus DNA: Physical Mapping of the Cleavage Sites of *Bacillus amyloliquefaciens* (*Bam*I) and *Haemophilus parainfluenzae* (*Hpa*II) Endonucleases and Evidence for Partial Heterogeneity

MICHEL FAVRE, GÉRARD ORTH,* ODILE CROISSANT, AND MOSHE YANIV

Unité de Biochimie Enzymologie, Laboratoire de Pharmacologie Moléculaire, no. 147 Associé au Centre National de la Recherche Scientifique, Institut Gustave-Roussy, 94800 Villejuif, and Département de Virologie and Département de Biologie Moléculaire, Institut Pasteur, 75015 Paris, France*

Received for publication 6 October 1976

The DNA of human papillomavirus (HPV) obtained from a pool of plantar warts is cleaved by *Bacillus amyloliquefaciens* (*Bam*I) and *Haemophilus parainfluenzae* (*Hpa*II) restriction endonucleases at one and four specific sites, respectively. These sites were localized on the previously established cleavage map of HPV DNA, using the *Hin*d, *Hin*dIII, *Hpa*I, and *Eco*RI endonuclease restriction sites as reference. The four *Hpa*II sites were mapped, clockwise, at 1.4, 41.1, 44.3, and 52.8% of the genome length from the unique *Bam*I cleavage site taken as point zero. The *Hpa*II site mapped at 1.4% of the genome length was absent in 40 to 50% of the molecules, thus showing a genetic heterogeneity of HPV DNA.

A human papovavirus belonging to the papillomavirus subgroup (11), the replication of which has not been obtained in vitro (3), is found in great amount in plantar warts (4, 9). Recent serological (2) and nucleic acid hybridization (13) data suggest the existence of differences between papillomaviruses inducing different clinical types of human benign tumors (plantar, common, or genital warts and juvenile laryngeal papillomas). The physical mapping of the viral DNA obtained from these tumors should allow precise determination of the genetic heterogeneity among human papillomaviruses (HPV).

In a previous paper, we showed that the DNA of the HPV is cleaved by the endonucleases *Eco*RI, *Hin*dII, *Hin*dIII, and *Hpa*I in two, three, three, and two fragments, respectively, and we further established their physical location (5). In this note, we report the location of the cleavage sites of HPV DNA by *Bacillus amyloliquefaciens* (*Bam*I) (12) and *Haemophilus parainfluenzae* (*Hpa*II) (10) endonucleases, which yield one fragment and three or four fragments, respectively, as already described (5).

Purification of HPV DNA from the virions and preparation of *Eco*RI, *Hin*d (a mixture of *Hin*dII and *Hin*dIII), *Hin*dIII, and *Hpa*I endonucleases have been reported previously (5). *Bam*I endonuclease (12) was a kind gift from T. Bickle (Biozentrum der Universität, Basel, Switzerland). *Hpa*II endonuclease was obtained by the procedure of Sharp et al. (10). Digestion conditions of HPV DNA by *Eco*RI, *Hin*d, *Hin*dIII, and *Hpa*I were those already described (5). Buffers used for digestion by *Bam*I and *Hpa*II were those described for *Hin*d and *Hpa*I, respectively. HPV DNA form I (2.5 μg) in 0.25 ml of buffer was incubated at 37°C for 1 h with *Bam*I (2 μl) and for 6 h with *Hpa*II (5 μl). When HPV DNA was digested successively by *H. influenzae* or *H. parainfluenzae* endonucleases and *Bam*I or *Eco*RI, *Bam*I or *Eco*RI enzymes were added 1 h before the end of the 6-h incubation. Cleavage products of HPV DNA were analyzed by electrophoresis on an acrylamide gradient (2.5 to 10%) under the conditions described by Jeppesen (8). The molecular weights of the fragments were determined either by electrophoresis, using the λ DNA *Hin*d fragments of known molecular weight (0.06×10^6 to 2.0×10^6) as standards (1, 5), or by electron microscopy as described previously (5).

The electrophoretic pattern and the molecular weight of HPV DNA fragments obtained after cleavage by *Bam*I and *Hpa*II are shown in Fig. 1a and b and Table 1. *Bam*I endonuclease cleaves HPV DNA in a one-genome-length fragment, in agreement with the recent results of Gissmann and Zur Hausen (7). *Hpa*II endonuclease yields five fragments, the total molecular weight of which (8.95×10^6) greatly exceeds the molecular weight of the viral DNA

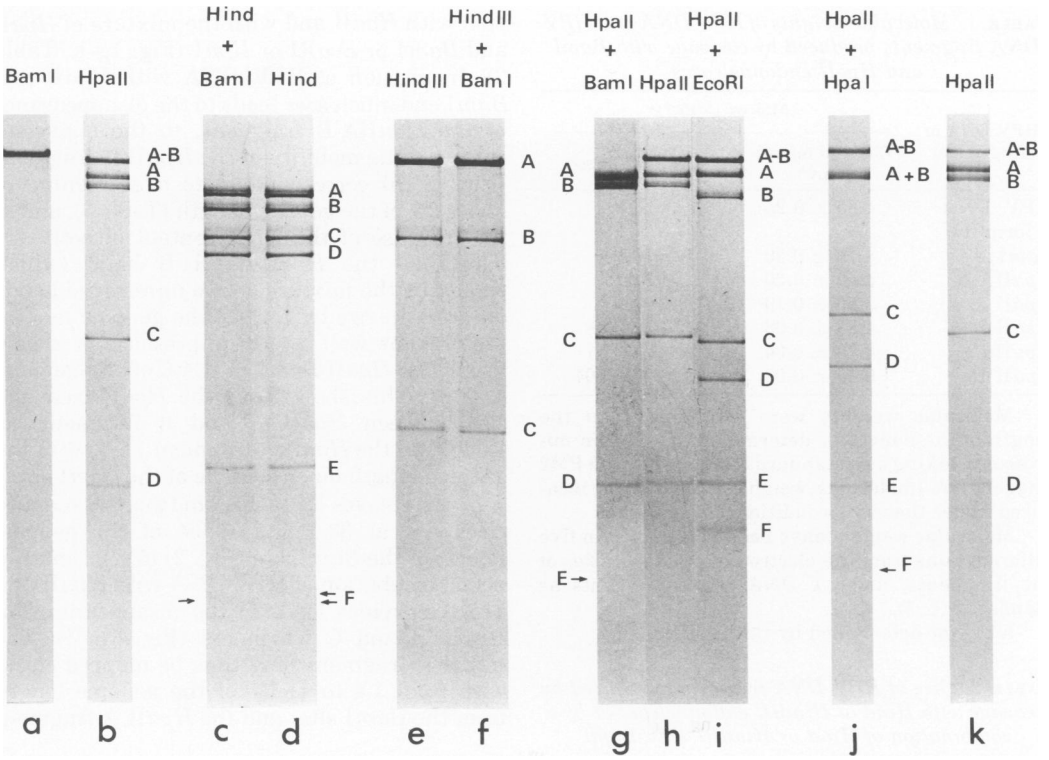

FIG. 1. *Polyacrylamide gel electrophoresis of restriction endonuclease cleavage products of HPV DNA. HPV DNA samples (2.5 μg) were incubated with restriction enzymes: (a) BamI, (b) HpaII, (c) Hind + BamI, (d) Hind, (e) HindIII, (f) HindIII + BamI, (g) HpaII + BamI, (h) HpaII, (i) HpaII + EcoRI, (j) HpaII + HpaI, (k) HpaII. Polyacrylamide gradient slab gels (2.5 to 10%) were run at 5 V/cm in 40 mM Tris-acetate, 20 mM sodium acetate (pH 7.9) (8) at room temperature for 8.5 h (a-i) or for 9.5 h (j and k). Bromophenol blue was used as a tracking dye. Gels were stained in 0.02% methylene blue for 2 h, and excess dye was removed with distilled water.*

(4.8×10^6). A fourfold increase in the amount of enzyme does not alter the cleavage pattern, whereas when an equimolar mixture of HPV and simian virus 40 DNAs is incubated under the same conditions, the totality of simian virus 40 DNA is cleaved to give full-length linear molecules (data not shown). Furthermore, similar cleavage patterns have been obtained with three different preparations of HPV DNA. Table 1 shows that the molecular weight of the genome may be obtained either by adding the molecular weights of the largest fragment and the two smallest fragments (4.77×10^6) or by adding the molecular weights of the four smaller fragments, A, B, C, and D (4.73×10^6). These results show that there are four *Hpa*II cleavage sites on HPV DNA, yielding four fragments named by letters according to their increasing electrophoretic mobility, and that one site is missing in a fraction of the DNA molecules, thus giving rise to the large fragment named *Hpa*IIA-B. The fraction of partially resistant molecules may be determined to be about 40 to 50% from the values of the area ratio of *Hpa*IIA-B to *Hpa*II A and B fragments (1.1, 0.7, 0.7), as determined by measuring the area under the peaks obtained in a densitometric recording of the negatives of the photographs of three different DNA preparations.

The location of the single *Bam*I cleavage site has been inferred from the electrophoretic pattern (Fig. 1c-f) and from the size of the fragments (Table 2) produced by *Hin*d or *Hin*dIII endonucleases, or by a mixture of *Hin*d or *Hin*dIII endonucleases with *Bam*I. *Hin*d (a mixture of *Hin*dII and *Hin*dIII endonucleases) and *Hin*dIII cleave HPV DNA into six and three fragments, respectively (5, 7). After successive treatment of HPV DNA by *Hin*d and *Bam*I or *Hin*dIII and *Bam*I endonucleases, the electrophoretic mobility of *Hin*d F and *Hin*dIII C fragments (Fig. 1c–f) is slightly increased, corresponding to a shortening of 0.2 to 0.3% of the genome length. This localizes the *Bam*I site unambiguously on the *Hin*d F fragment at 0.3% of the genome length of the *Hin*dIII site be-

TABLE 1. *Molecular weights of HPV DNA and HPV DNA fragments produced by cleavage with BamI and HpaII endonucleases*

HPV DNA or fragments	Mol wt ($\times 10^{-6}$)	
	Electron microscopy[a]	Electrophoresis[b]
HPV DNA form II	4.80 ± 0.25	ND[c]
*Bam*I A	4.78 ± 0.20	ND
*Hpa*IIA-B	4.22 ± 0.20	ND
*Hpa*II A	2.30 ± 0.10	ND
*Hpa*II B	1.88 ± 0.08	2.0 ± 0.08
*Hpa*II C	0.40 ± 0.06	0.44 ± 0.01
*Hpa*II D	0.15 ± 0.03	0.14 ± 0.004

[a] Molecular weights were estimated from the length of the molecules determined by electron microscopy, taking as a standard the length of the PM2 phage DNA (molecular weight, 6.4×10^6; 6) measured under the same conditions.

[b] Molecular weights have been determined in five different runs from the electrophoretic mobilities of the fragments, using λ DNA *Hin*d fragments as standards (1, 5).

[c] ND, Not determined by this method.

TABLE 2. *Size of HPV DNA fragments produced by cleavage with Hind or HindIII endonuclease or by a combination of Hind or HindIII with BamI endonuclease*[a]

DNA fragment	Size (%)			
	*Hin*d	*Hin*d + *Bam*I	*Hin*dIII	*Hin*dIII + *Bam*I
A	33.5	33.6	77.6	77.7
B	27.4	27.6	17.5	17.6
C	18.0	18.0	4.9	4.7
D	16.0	16.0		
E	3.9	3.9		
F	1.2	0.9		

[a] The sizes of the fragments represent their molecular weights expressed as percentages of the total molecular weights of the fragments (*Hin*d, 4.78×10^6; *Hin*d + *Bam*I, 4.72×10^6; *Hin*dIII, 4.71×10^6; *Hin*dIII + *Bam*I, 4.70×10^6). Molecular weights were estimated by electron microscopy with a standard deviation of about 4% or by electrophoresis (a mean of three different runs) for *Hin*d F, *Hin*d-*Bam*I F, *Hin*dIII C, and *Hin*dIII-*Bam*I C, using the λ *Hin*d fragments as standards.

tween *Hin*d C and F fragments, in agreement with Gissmann and Zur Hausen (7). *Bam*I endonuclease being so far the only one of the studied enzymes to yield a single cleavage site on HPV DNA, the *Bam*I site is thus taken as point zero on the cleavage map (Fig. 2).

The *Hpa*II cleavage sites have been located by comparing the electrophoretic pattern and the size of the fragments obtained after incubation with *Hpa*II and with the mixture of *Hpa*II and *Bam*I or *Eco*RI or *Hpa*I (Fig. 1g-k, Table 3). Incubation of HPV DNA with *Hpa*II and *Bam*I endonucleases leads to the disappearance of the *Hpa*IIA-B fragment, to the slight increase of the mobility of the *Hpa*II A fragment (Fig. 1g-h) corresponding to a shortening of about 2% of the genome length (Table 3), and to the increase of the DNA content of both this band and the *Hpa*II-*Bam*I B band (which should be the mixture of two unresolved bands differing in size by 1.4% of the genome length; Fig. 1g), as well as to the presence of a new fragment, *Hpa*II-*Bam*I E (1.4% of the genome length). This shows that the *Hpa*II cleavage site between *Hpa*II A and B fragments is located on the *Hpa*II A fragment at 1.4% of the genome length on either side of the *Bam*I site.

*Eco*RI cleaves HPV DNA in two sites mapped clockwise at 33.3 and 46.3% of the genome length of the *Bam*I site (Fig. 2) (5, 7). Simultaneous incubation of HPV DNA with *Hpa*II and *Eco*RI enzymes leads to the disappearance of *Hpa*II B and C fragments (Fig. 1h, i). The *Hpa*II B fragment may thus be mapped clockwise from 1.4 to 41.1% of the genome length from the *Bam*I site, and the *Hpa*II A fragment

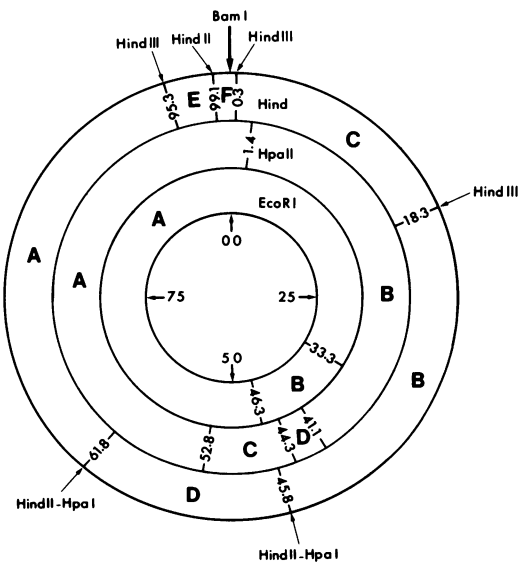

FIG. 2. *Cleavage map of HPV DNA. The map was constructed taking BamI cleavage site as point zero. The outer ring shows the HindII, HindIII, and HpaI cleavage sites and the order of the fragments obtained with the mixture of HindII and HindIII (5); the median ring shows the HpaII cleavage sites; and the inner ring shows the EcoRI cleavage sites (5). The distances from point zero are given as percentages of the HPV genome length.*

TABLE 3. *Size of HPV DNA fragments obtained by cleavage with HpaII endonuclease or by a combination of BamI, EcoRI, or HpaI endonuclease with HpaII*[a]

DNA fragment	Size (%)			
	*Hpa*II	*Hpa*II + *Bam*I	*Hpa*II + *Eco*RI	*Hpa*II + *Hpa*I
A-B	88[b]		ND[c]	ND
A	48.6	46.6	48.4	38.6
B	39.7	40.4	32.6	38.6
C	8.5	8.7	8.0	11.7
D	3.2	2.9	6.1	6.8
E		1.4	3.0	2.8
F			1.9	1.5

[a] The sizes of the fragments represent their molecular weights expressed as percentages of the total molecular weights of the fragments (*Hpa*II, 4.73×10^6; *Hpa*II + *Bam*I, 4.83×10^6; *Hpa*II + *Eco*RI, 4.75×10^6; *Hpa*II + *Hpa*I, 4.75×10^6). Molecular weights were estimated by electrophoresis (a mean of two different runs) or by electron microscopy for *Hpa*II A, *Hpa*II-*Bam*I A, and *Hpa*II-*Eco*RI A fragments.

[b] The size of the *Hpa*IIA-B fragment represents its molecular weight determined by electron microscopy expressed as a percentage of the molecular weight of the HPV DNA.

[c] ND, Not determined.

may be mapped from 52.8 to 1.4% of the genome length from point zero (Fig. 2). From the size of the four new fragments produced by *Eco*RI (Table 3), it may be inferred that *Hpa*II B gives rise to *Hpa*II-*Eco*RI B and C fragments, as expected from the cleavage map (Fig. 2), and that *Hpa*II C gives rise to *Hpa*II-*Eco*RI D and F fragments. This localizes the ends of the *Hpa*II C fragment at 1.9 and 6.1% of the genome length on either side of the *Eco*RI site. The cleavage map (Fig. 2) shows that this *Eco*RI site is about at 5 and 6.5% of the genome length of the nearest ends of the *Hpa*II A and B fragments. The size of the fragments given in Table 3 proves that the *Hpa*II C fragment is close to *Hpa*II A and is separated from *Hpa*II B by the *Hpa*II D fragment (3.2% of the genome length), thus locating the fourth *Hpa*II site at about 44.3% of the genome length from point zero (Fig. 2). This is further confirmed by the analysis of the products obtained after sequential digestion of HPV DNA by *Hpa*II and *Hpa*I endonucleases.

*Hpa*I cleaves HPV DNA in two sites (5), the mapping of which may be deduced to be at 45.8 and 61.8% of the genome length from the *Bam*I site (Fig. 2). Successive treatment of HPV DNA with *Hpa*II and *Hpa*I endonucleases leads to the disappearance of *Hpa*II A and C fragments (Fig. 1j, k). *Hpa*II A generates the *Hpa*II-*Hpa*I A fragment, with the same migration as *Hpa*II B, and the *Hpa*II-*Hpa*I C fragment; the *Hpa*II C fragment yields *Hpa*II-*Hpa*I D and F fragments. The sizes of the four new fragments (Table 3) are in agreement with the values predicted from the cleavage map (Fig. 2).

The study of the sensitivity of the DNA of HPV isolated from plantar warts to restriction enzymes *Bam*I (7; this paper), *Eco*RI (5, 7), *Hin*dII and *Hin*dIII (5, 7), and *Hpa*I (5) and *Hpa*II (this paper) has allowed the physical mapping of 15 cleavage sites on this DNA. In one out of three viral DNA preparations, four *Hpa*II weak bands, at the detection limit, have been observed, corresponding to molecular weights of 1.55×10^6, 1.30×10^6, 1.15×10^6, and 0.86×10^6, respectively, the total molecular weight of which represents the viral genome molecular weight (data not shown). This suggests the existence of an HPV variant, which represents about 1 to 2% of the DNA molecules, as previously inferred from the cleavage pattern obtained with *Eco*RI (5). Furthermore, Gissmann and Zur Hausen have recently reported the existence of an additional *Bam*I site and *Hin*dIII site in one out of three DNA preparations obtained from three plantar wart samples, thus supporting a genetic heterogeneity of HPV DNA (7). These two additional sites were not evidenced in our study; however, we have observed the absence of the *Hpa*II site located at 1.4% of the genome length from the *Bam*I site in 40 to 50% of the DNA molecules purified from virions obtained from three different pools of plantar warts. Viral DNA preparations obtained from three different single warts gave a mixture of the four *Hpa*II fragments as well as the *Hpa*IIA-B fragment, whereas the *Hin*dII,III digest gave a molar yield of all six fragments. Thus, the DNA microheterogeneity is present in single warts.

A survey of viral DNA preparations from different types of warts and papillomas now in progress should help to clarify the relation between the possibly different viruses and tissue specificity.

We gratefully acknowledge F. Breitburd for critical reading of the manuscript. We are greatly indebted to P. Agache for generously providing human plantar warts. We also thank C. Dauguet for technical collaboration, N. Jibard and D. Fortin for excellent assistance, and D. Cany for preparation of illustrations.

This investigation was supported by the Centre National de la Recherche Scientifique through the Laboratoire Associé no. 147 (Pharmacologie Moléculaire), and by a grant from the Institut National de la Santé et de la Recherche Médicale (A.T.P. no. 73.4. 432.18, "Enzymologie des Virus Oncogènes").

LITERATURE CITED

1. **Allet, B., and A. I. Bukhari.** 1975. Analysis of bacteriophage Mu and λ-Mu hybrid DNAs by specific endonucleases. J. Mol. Biol. 92:529-540.

2. **Almeida, J. D., J. D. Oriel, and L. M. Stannard.** 1969. Characterization of the virus found in human genital warts. Microbios 3:225–232.
3. **Butel, J. S.** 1972. Studies with human papillomavirus modeled after known papovavirus systems. J. Natl. Cancer Inst. 48:285–299.
4. **Favre, M., F. Breitburd, O. Croissant, and G. Orth.** 1975. Structural polypeptides of rabbit, bovine, and human papillomaviruses. J. Virol. 15:1239–1247.
5. **Favre, M., G. Orth, O. Croissant, and M. Yaniv.** 1975. Human papillomavirus DNA: physical map. Proc. Natl. Acad. Sci. U.S.A. 72:4810–4814.
6. **Franklin, R. M.** 1974. Structure and synthesis of bacteriophage PM2, with particular emphasis on the viral lipid bilayer. Curr. Top. Microbiol. Immunol. 68:107–159.
7. **Gissmann, L., and H. Zur Hausen.** 1976. Human papillomavirus DNA: physical mapping and genetic heterogeneity. Proc. Natl. Acad. Sci. U.S.A. 73:1310–1313.
8. **Jeppesen, P. G. N.** 1974. A method for separating DNA fragments by electrophoresis in polyacrylamide concentration gradient slab gels. Anal. Biochem. 58:195–207.
9. **Rowson, K. E. K., and B. W. J. Mahy.** 1967. Human papova (wart) virus. Bacteriol. Rev. 31:110–131.
10. **Sharp, P. A., B. Sugden, and J. Sambrook.** 1973. Detection of two restriction endonuclease activities in *H. parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. Biochemistry 12:3055–3063.
11. **Wildy, P.** 1971. Classification and nomenclature of viruses, p. 38–39. *In* J. L. Melnick (ed.), Monographs in virology, vol. 5. S. Karger, Basel.
12. **Wilson, G. A., and F. E. Young.** 1975. Isolation of a sequence-specific endonuclease (BamI) from *Bacillus amyloliquefaciens H*. J. Mol. Biol. 97:123–125.
13. **Zur Hausen, H., W. Meinhof, W. Schreiber, and G. Bornkamm.** 1974. Attempts to detect virus-specific DNA in human tumors. I. Nucleic acid hybridizations with complementary RNA of human wart virus. Int. J. Cancer 13:650–656.