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Cleavage Map of BK Virus DNA with Restriction Endonucleases *Mbo*I and *Hae*III

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Specific cleavage of BK virus (MM) DNA with restriction endonuclease *Mbo*I gives rise to 10 fragments. Two techniques were used to determine the location of these fragments on the viral genome with respect to the three known sites for *Hin*dIII cleavage. In the first method, reciprocal digestion, individual *Mbo*I fragments were digested with *Hin*dIII and individual *Hin*dIII fragments were digested with *Mbo*I. In the second method, single-end ^{32}P-labeled *Hin*dIII subfragments were partially digested with *Mbo*I, and then the sizes of the radioactive partial products were used to deduce the nearest neighboring fragment. Information from these two methods is more than adequate to map all the *Mbo*I enzyme sites. Cleavage of BK virus (MM) DNA with restriction enzyme *Hae*III produces 21 fragments. With the aid of the same two methods, these fragments have also been ordered with respect to the known map locations of the *Hin*dIII and *Mbo*I sites.

The papovavirus BK virus (BKV) was initially discovered in England by Gardner et al. (5) in 1971 from the urine of an immunosuppressed renal allograft recipient. Since then, isolates similar to the original BKV have been obtained from the urine of other patients elsewhere in the world (1, 3, 10, 11, 14). A variant of BKV, BKV(MM), was later isolated from the urine and the brain tumor of a patient with Wiskott-Aldrich syndrome, a genetic disease characterized by defects in both cellular and humoral immunity (19). BKV(MM) is very similar to the prototype BKV except that the DNA of the former contains three *Hin*dIII sites instead of four (8). Direct evidence that BKV is a causative agent in human diseases is lacking (4, 21). However, the oncogenic potential of this virus has been demonstrated in hamsters both in vitro and in vivo (12, 15, 17, 20).

A detailed physical map is needed for determining the chemical and biological nature of BKV(MM) DNA, a covalently closed circular DNA consisting of about 5,200 base pairs. This communication describes the specific cleavage and physical mapping of BKV(MM) DNA with the use of restriction enzymes *Mbo*I and *Hae*III.

MATERIALS AND METHODS

Restriction enzyme abbreviations. *Bam*HI, *Eco*RI, *Hae*III, *Hha*I, *Hinc*II, *Hin*dIII, *Hpa*II, *Kpn*I, *Mbo*I, *Mbo*II, *Pst*I, and *Xba*I are specific restriction enzymes (16) from *Bacillus amyloliquefaciens* H, *Escherichia coli* RY13, *Haemophilus aegyptius, Haemophilus haemolyticus, Haemophilus influenzae* R_c, *Haemophilus influenzae* R_d, *Haemophilus parainfluenzae, Klebsiella pneumoniae* OK8, *Moraxella bovis, Providencia stuartii* 164, and *Xanthomonas badrii*, respectively. Each restriction DNA fragment produced by a complete digestion with a single enzyme is assigned a capital letter (2) in an order of decreasing size (A, B, C ..., where A is the largest) with an italicized prefix designating the enzyme used to produce the fragment (e.g., *Mbo*I-A). Each DNA fragment generated by simultaneous digestion of two enzymes is designated by the names of the enzymes used and followed by an arabic number, also in an order of decreasing size (e.g., *Hin*dIII-*Mbo*I 1, 2, 3 ..., where 1 is the largest [23]).

Cells. Human embryonic kidney cells were purchased from Microbiological Associates (Long Island, N.Y.). The cells were propagated in Dulbecco modified Eagle medium supplemented with 2 g of glucose per liter and 10% fetal calf serum in 105-mm-diameter plastic dishes. African green monkey kidney cells CV-1 and TC-7 were obtained and grown as previously described (9).

Viruses. The plaque-purified BKV(MM) strain was provided by P. M. Howley and K. K. Takemoto. Human embryonic kidney cells were infected with BKV(MM) at 0.001 to 0.01 PFU/cell. Virus stock was collected 10 to 18 days after infection at a time when extensive cytopathic effect was observed and about 80% of the cells were floating. The infected cells and the medium were frozen and then thawed twice in the original flasks before centrifugation at 1,500 rpm for 10 min. The supernatant solution was frozen in 20-ml portions, whereas the pellet was resuspended in 2 ml of phosphate-buffered solution per original flask. This suspension was subjected to two freeze-thaw cycles in a dry ice-EtOH bath. After the second thawing, the mixture was poured into a Dounce homogenizer, where

it was homogenized 12 times. Receptor-destroying enzyme (Microbiological Associates) was added (100 U to 1 ml of mixture), and the mixture was incubated overnight in a CO_2 incubator at 37°C. The mixture was then centrifuged, and the resulting pellet was homogenized twice in a Dounce homogenizer as described above. The pellet was resuspended in phosphate-buffered solution and centrifuged twice more. The supernatant solutions from each centrifugation were saved, pooled, and then added to the original supernatant solution. Small-plaque simian virus 40 was provided by D. Nathans and grown as described (9).

Viral DNA. For preparation of viral DNA, human embryonic kidney cells were infected with approximately 0.01 to 0.1 PFU of BKV(MM) per cell. Five to 8 days after infection, when about 15% of the cells showed cytopathic effect and about 25% cells were floating, the DNA was extracted from the cells according to the Hirt procedure (7). Pure superhelical viral DNA (form I) was obtained after isopycnic centrifugation in cesium chloride-ethidium bromide (22). Uniformly ^{32}P-labeled BKV(MM) DNA was obtained by using P_i-free Eagle medium and by adding 0.5 mCi of $^{32}P_i$ per plate 4 days after infection. The preparation of simian virus 40 DNA was as described (9).

Restriction enzyme cleavage of BKV(MM) DNA and simian virus 40 DNA. Restriction enzymes (16) *Eco*RI, *Hae*III, *Hha*I, *Hin*dIII, *Mbo*II, and *Pst*I were purchased from Bio-Lab. *Mbo*I was a generous gift of R. Roberts.

The standard cofactor mixture for most of these enzymes contained 10 mM Tris-hydrochloride (pH 7.5), 7 mM 2-mercaptoethanol, and 7 mM $MgCl_2$ (23). The cofactor mixture for *Hin*dIII and *Eco*RI also contained 50 mM NaCl. A typical reaction for complete digestion contains 0.3 U of enzyme per μg of DNA. Incubation was at 37°C for 3 to 15 h. The completeness of digestion was monitored by electrophoresis of a small sample in 1% agarose gel. For partial digestion, smaller amounts of terminally ^{32}P-labeled BKV(MM) DNA and enzyme were used. The incubation times were 2, 5, 10, 20 and 40 min. Samples were pooled before electrophoresis.

In vitro labeling of DNA. Labeling of DNA fragments at the 5'-terminus with γ-[^{32}P]ATP and T4 polynucleotide kinase was carried out according to the published procedure (12). The single-end, ^{32}P-labeled *Hin*dIII subfragments of BKV(MM) DNA were obtained by cleavage of the 5'-end, ^{32}P-labeled *Hin*dIII fragments with the single-cut enzymes *Eco*RI, *Pst*I, and *Hha*I.

Agarose gel electrophoresis. A slab gel (0.3 by 20 by 20 cm) of 1% agarose (SeaKem) containing 0.5 μg of ethidium bromide per ml was used. Electrophoresis was at 75 mA for 3 to 4 h.

Polyacrylamide gel electrophoresis. A slab gel (0.15 or 0.3 by 20 cm or 34 by 40 cm) of 3 to 6% polyacrylamide (acrylamide-bisacrylamide, 20:1) was run at 120 to 150 V until bromophenol blue migrated at the desired positions. The buffer was 40 mM Tris-hydrochloride (pH 7.9)-20 mM NaAc-2 mM EDTA. Analytical gels were dried under vacuum onto a sheet of Whatman 3 MM paper (23) before exposing the gels to Kodak XR-5 films and intensifying screen. The position of the bromophenol blue dye marker is shown (Fig. 2). The size of each DNA fragment was calculated from its electrophoretic mobility, using simian virus 40 *Hin*dII + -III and *Hae*III DNA fragments of known size and mobility as standards (23). Size estimation of the uniformly ^{32}P-labeled DNA fragments was also performed by the distribution of radioactivity in each specific enzyme digest (23).

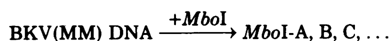
Elution of DNA fragments from gel. The gel strips containing radioactive DNA fragments were excised, ground, and soaked with 0.1 M triethylamine-bicarbonate (pH 8.5 to 9.0) overnight at 0°C in 10-ml siliconized glass vials. The gel debris was spun down and resuspended in the same buffer for another 30 min. The supernatant solutions thus collected were passed through a small DE-52 column (50- to 100-μl bed volume) packed in a 1-ml-capacity Eppendorf plastic pipette tip. The absorbed DNA was eluted with 0.2 ml of 2 M NaCl in a 1.5-ml Eppendorf plastic centrifugation tube and desalted by ethanol precipitation (22). The latter was carried out by freezing at −70°C for 5 to 10 min and centrifuging in an Eppendorf centrifuge (model 5412) for 15 min. In this way at least 95% of the counts in the DNA fragments of less than 1,000 base pairs were recovered from gels and columns. The yield of the DNA fragments larger than that size is generally lower.

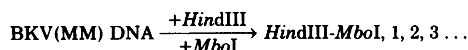
RESULTS

Rationale. In locating the unknown cleavage sites of *Mbo*I and *Hae*III on the circular map of BKV(MM) DNA, we made use of the known positions of the cleavage sites of *Bam*HI, *Eco*RI, *Hha*I, *Hin*dIII, *Hpa*II, *Kpn*I, *Pst*I, and *Xba*I. Two methods were used for mapping. The procedure of reciprocal digestion (2) is illustrated schematically in Fig. 1. First, the fragments obtained from digestion of uniformly ^{32}P-labeled BKV(MM) DNA with each enzyme are designated A, B, C ... (where A is the largest; the sizes of all fragments are shown in Table 1). The digestion products derived from simultaneous digestion of BKV(MM) DNA with *Hin*dIII and *Mbo*I are designated *Hin*dIII-*Mbo*I 1, 2, 3 ... (also in decreasing order, where 1 is the largest). Second (Fig. 1b), each of the isolated *Hin*dIII fragments was incubated with *Mbo*I. For example, cleavage of *Hin*dIII-A with *Mbo*I gave *Hin*dIII-*Mbo*I 3, 4, 5, 8, 9, 10, and 12. Finally, overlap analysis was carried out to position the *Mbo*I fragments which received *Hin*dIII cleavage with respect to the known sites of the *Hin*dIII fragments. For example (Fig. 1c), *Mbo*I-A spans from a part of *Hind*-A, over the entire *Hind*-C to a part of *Hind*-B. Knowing the order of *Hin*dIII fragments B, C, and A and the size of the *Hin*dIII-*Mbo*I subfragments 1, 3, and 6 (Table 2), the order of these subfragments can be arranged.

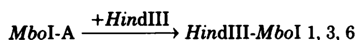
Another powerful method for mapping restriction enzyme fragments is based on the principle

(a) Production of DNA fragments by restriction enzymes





(b) Reciprocal cleavage of each isolated fragment



(c) Overlap analysis based on the known *Hind*III sites

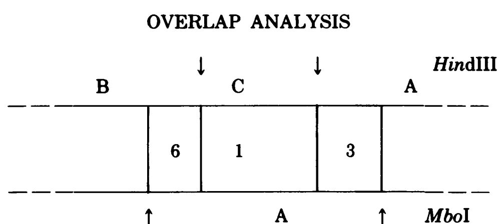


FIG. 1. *Scheme for reciprocal digestion of uniformly* ^{32}*P-labeled DNA and overlap analysis.*

of partial digestion of single-end-labeled DNA with the enzyme to identify the nearest neighboring fragments. This method has been reported previously (9, 18), and the details can be found in reference 18. Briefly, a single-end, ^{32}P-labeled DNA fragment is partially digested with a restriction enzyme whose cleavage sites are under determination. The partial digest is fractionated by electrophoresis on a polyacrylamide slab gel. After electrophoresis, the size of each partial product is estimated from their mobility with the use of suitable DNA markers. The size difference between two adjacent bands is then compared with the sizes of all the fragments resulting from the simultaneous digestion of DNA with enzymes (Table 2). Since all partial products share the same ^{32}P-labeled end, their order can be arranged in a unique way. The results obtained from this technique should be complementary to those from the first method.

Cleavage patterns of BKV(MM) DNA with restriction enzymes. In Fig. 2, incubation of uniformly ^{32}P-labeled BKV(MM) DNA with an excess of *Hind*III gave three fragments, *Hind*-A, -B, and -C (8), whereas no fragments were produced when incubated with *Hind*II (or *Hinc*II). Thus, all three *Hind* fragments, as previously reported, are generated by *Hind*III alone. We found that *Hind*-C actually consisted of three bands, designated C_a, C_b, and C_c (Fig. 2). Assay of radioactivity distribution (23) in each band revealed approximately equal proportions of these three fragments (C_a = 30%, C_b = 35%, C_c = 35%). The molar ratio of *Hin*-A, *Hin*-B, and *Hin*-C_a + C_b + C_c is 1:1:1 (data not shown), and their estimated sizes are shown in Table 1. Thus, three closely related species of DNA exist in this virus stock. The largest fragment (C_a) came from the parent BKV(MM), since it contains one *Hpa*II site. The two shorter fragments (C_b and C_c) came from deletion mutants of BKV(M), since the *Hpa*II site is lost (unpublished observation). These two deletion mutants are different from the wild-type BKV

TABLE 1. *Restriction fragments obtained by complete digestion of uniformly* ^{32}*P-labeled BKV(MM) DNA with HindIII, MboI, or HaeIII*[a]

Restriction fragment	Genome length (%)	Base pairs
*Hind*III		
A	46.35	2,410
B	40.80	2,122
C_a	13.10	681
C_b	12.70	660
C_c	12.60	655
*Mbo*I		
A	31.90	1,659
B	13.10	681
C	10.80	562
D	9.80	510
E	9.00	468
F	8.70	452
G	5.90	307
H	4.40	229
I	4.25	221
J	2.40	125
*Hae*III		
A	28.14	1,463
B	12.40	645
C	8.70	452
D	7.40	385
E_a	6.90	359
E_b	6.50	338
E_c	6.40	333
F	6.90	359
G	6.20	322
H	4.80	250
I	3.25	169
J	3.25	169
K	2.85	148
L	1.90	99
M	1.90	99
N	1.10	57
O	1.00	52
P	0.98	51
Q	0.91	47
R	0.84	44
S	0.55	29
T	0.54	26
U	0.10	5

[a] Genome length as a percentage of the total BKV(MM) DNA molecule was calculated on the basis of electrophoretic mobility. The number of base pairs was estimated assuming a total of 5,200 for BKV(MM) DNA.

TABLE 2. *Restriction fragments obtained by complete digestion of uniformly ^{32}P-labeled BKV(MM) DNA with a combination of two restriction enzymes*[a]

Restriction fragment	Genome length (%)	Base pairs
*Hin*dIII-*Mbo*I		
1_a	13.10	681
1_b	12.70	660
1_c	12.60	655
2	10.80	562
3	10.00	520
4	9.80	510
5	9.00	468
6	8.00	416
7	5.90	307
8	5.70	296
9	4.40	229
10	4.25	221
11	3.00	156
12	2.40	125
*Hin*dIII-*Hae*III		
1	28.14	1,463
2	12.40	645
3	8.20	426
4_a	6.90	359
4_b	6.50	338
4_c	6.40	333
5	6.50	338
6	6.20	322
7	3.80	198
8	3.25	169
9	2.85	148
10	1.90	99
11	1.10	57
12	1.00	52
13	0.98	51
14	0.91	47
15	0.84	44
16	0.76	40
17	0.55	29
18	0.10	5
*Mbo*I-*Hae*III		
1	13.10	681
2	8.90	463
3	8.70	452
4_a	6.90	359
4_b	6.50	338
4_c	6.40	333
5	5.90	307
6	4.90	255
7	4.80	250
8	4.25	221
9	4.05	211
10	3.40	177
11	3.30	172
12	2.45	127
13	2.25	117
14	1.95	101
15	1.10	57
16	1.00	52
17	0.98	51
18	0.90	47
19	0.84	44
20	0.55	29
21	0.50	26
22	0.10	5

[a] Genome length and the number of base pairs were calculated as in Table 1.

because the latter contains more DNA than BKV(MM).

Complete digestion of BKV(MM) DNA with *Mbo*I resulted in 10 fragments (A through J), varying in length from 1,659 to 125 base pairs (Fig. 2, Table 1), which were fractionated into nine discrete bands in a 3.5% polyacrylamide gel.

Incubation of BKV(MM) DNA with *Hae*III produced 21 fragments (A through U), which migrated as 16 major bands in a 3.5% gel (Fig. 5 and 6). The relative molecular weight of the *Hae*III fragments (Table 1) was estimated by direct comparison to the known sizes of simian virus 40 DNA-*Hae*III fragments (23) fractionated in the same gel. We found the number and the molecular sizes of the *Hae*III fragments of these two viral DNAs to be rather similar and the total size of the genome to be approximately the same, namely, 5,200 nucleotide base pairs.

BKV(MM) DNA produced many more fragments when treated with a combination of any two enzymes (e.g., *Mbo*I and *Hin*dIII) (Fig. 2, Table 2). In almost every electrophoretic pattern we observed three closely migrated fragments, which confirm the presence of the two variants of the BKV(MM) genome. The difference in length between these two variant DNA molecules and the parent, as judged by their electrophoretic mobility, is approximately 21 base paris between bands a and b and 26 base pairs between bands a and c (Table 1).

Mapping of *Mbo*I cleavage sites. The known sites of the three *Hin*dIII fragments on the circular genome of BKV(MM) (Fig. 3b) have been used in locating the ten *Mbo*I fragments. Upon digestion of individual *Hin*dIII fragments with *Mbo*I, *Hind*-A gave seven subfragments as mentioned above (see also Fig. 4); *Hind*-B gave subfragments *Hin*dIII-*Mbo*I 1, 2, 6, 7, and 11; *Hind*-C (including C_a, C_b, and C_c) gave *Hin*dIII-*Mbo*I 1_a, 1_b, and 1_c, indicating that *Hind*-C received no cleavage from *Mbo*I (see Table 3).

In reciprocal digestion, each *Mbo*I fragment is incubated with *Hin*dIII. Either a uniformly labeled or a 5′-end-labeled fragment can be used, the two kinds of labeled DNA yielding complementary information (9). Figure 4b summarizes the results of digesting end-labeled *Mbo*I fragments by *Hin*dIII. Thus, digestion of *Mbo*I-A gave end-labeled subfragments *Hin*dIII-*Mbo*I 3 and 6 (Fig. 4b). Since these two fragments add up to only 18% genome length (Table 2) and *Mbo*I-A is 31.9% genome length (Table 1), there must be an unlabeled DNA fragment (e.g., *Hin*dIII-C, which is about 13% genome length; Table 1) between the labeled terminal fragments 3 and 6. This was confirmed by using the uniformly ^{32}P-labeled *Mbo*-A fragment for digestion

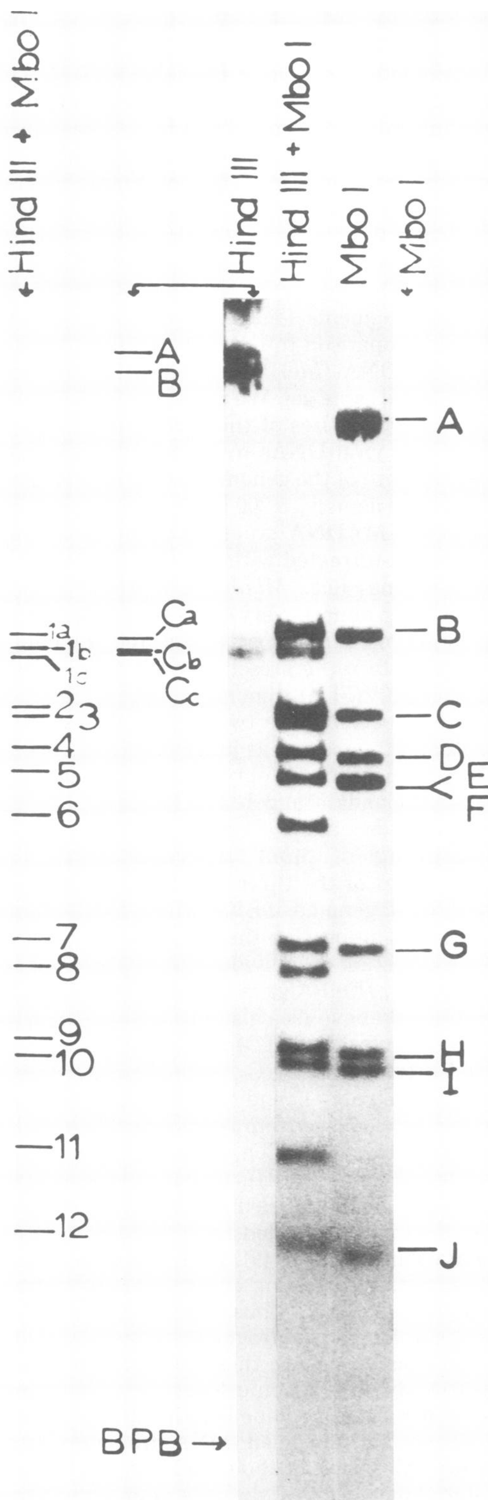


FIG. 2. *Polyacrylamide gel electrophoretic patterns of restriction fragments obtained by complete digestion of BKV(MM) DNA with restriction enzymes. Uniformly ^{32}P-labeled BKV(MM) DNA (0.1 μg, 1.5 × 10^5 cpm/μg) was incubated for 3 h at 37°C with excess restriction enzymes (0.2 to 3 U). The digests were fractionated by electrophoresis on a 3.5% polyacrylamide gel. The specific digests are indicated at the top of the gel. The gel pattern is also schematically depicted since resolution of some bands was lost during reproduction. The size estimation of each fragment has been performed according to procedures described previously (23). The results are summarized in Tables 1 and 2.*

with *Hin*dIII. *Mbo*I-E was cleaved into *Hin*dIII-*Mbo*I 8 and 11 by *Hin*dIII (Fig. 4b). The other *Mbo*I fragments were not cleaved by *Hin*dIII enzyme. By overlap analysis, several of the *Mbo*I sites on the genome were located using the known positions of the *Hin*dIII sites. A preliminary order of *Mbo*I fragments can be drawn as follows: -A-(J, I, D, H, F)-E-(C, B, G)-.

Partial digestion of single-end-labeled *Hin*dIII subfragments with *Mbo*I was carried out for further ordering of *Mbo*I fragments. 5'-^{32}P-labeled *Hin*dIII fragments A and B were digested with *Eco*RI and *Pst*I to generate single-end-labeled *Hin*dIII -A_1, -A_2, -B_1, and -B_2 (Fig. 3b). Each of these fragments was subjected to partial cleavage with *Mbo*I to produce a series of incompletely digested fragments. The DNA bands (sharing the same labeled end) resulting from partial digestion were then analyzed for size. The size difference between two adjacent bands was calculated and compared to the known size of DNA fragments produced by *Hin*dIII-*Mbo*I simultaneous digestion (Table 2). The partial *Mbo*I cleavage of *Hin*dIII-A_1 produces subfragments a, b, c, d, and e (in an order of decreasing length) (Fig. 3, Table 4). Fragment d (the neighboring band larger than e) is longer than fragment e (corresponding to *Hin*dIII-*Mbo*I 3) by 2.4% genome length of the BKV(MM) DNA (Table 4), which is equivalent in size to *Hin*dIII-*Mbo*I 12 shown in Table 2. Thus, fragment 12 is immediately adjacent to fragment 3. Similarly, fragment c is larger than d by 4.30% genome length, which corresponds to *Hin*dIII-*Mbo*I 10, establishing the order of fragments as 3-12-10. By extending this analysis, the order of the component *Mbo*I fragments included in *Hin*dIII-A_1 can be deduced as *Hin*dIII-*Mbo*I 3-12-10-5-4B (Table 4, Fig. 4a and b) or *Mbo*I-A-J-I-F-D- (Fig. 4c). In a similar manner, partial digestion of *Hind*-A_2 by *Mbo*I defines the order of *Hin*dIII-*Mbo*I 8-9-4A (or *Mbo*I E-H-D), and *Hind*-B_1 corresponds to *Hin*dIII-*Mbo*I 6-7-1A (or *Mbo*I A-G-B), etc. (Table 4, Fig. 4b).

The data obtained from *Hin*dIII-*Mbo*I reciprocal digestion using uniformly ^{32}P-labeled DNA fragments and partial cleavage of single-end-la-

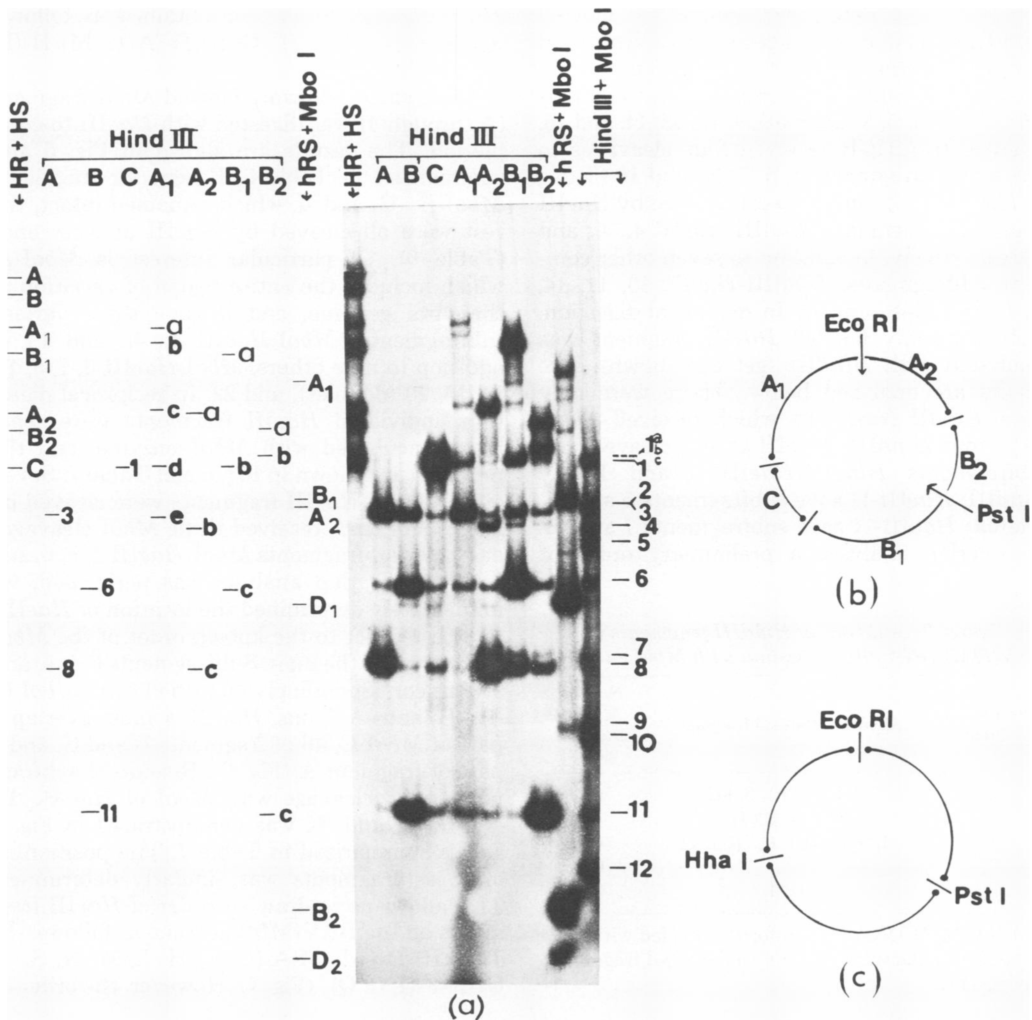


FIG. 3. *(a) Partial cleavage patterns of the single-end, ^{32}P-labeled HindIII fragments digested with MboI. The single-end, ^{32}P-labeled EcoRI HindIII-A_1 and -A_2 as well as PstI HindIII-B_1 and -B_2 were obtained by treating the 5'-^{32}P-labeled HindIII fragments A and B with EcoRI and PstI, respectively. Each single-end-labeled fragment was then partially digested with MboI (lanes A_1, A_2, B_1, and B_2). Electrophoresis of the partial digests (1.5 × 10^4 cpm) was carried out on a 4% polyacrylamide gel. Lanes A, B, and C: Complete digestion of 5'-^{32}P-labeled HindIII-A, -B and -C fragments, respectively, with MboI resulted in single-end-labeled subfragments HindIII-MboI 3 and 8 for HindIII-A, 6 and 11 for HindIII-B, and 1 for HindIII-C (indicating no MboI site on this fragment). Lane HR + HS: Mixture of two double digests, HindIII-EcoRI (HR) and HindIII-PstI (HS) digests of BKV(MM) DNA, was used as a reference for HindIII-A, -B, and -C as well as for HindIII-A_1, -A_2, -B_1, and -B_2 fragments. Lane hRS + MboI [also see (c)]: 5'-^{32}P-labeled DNA from an HhaI-EcoRI-PstI digest was incubated with MboI to give six single-end-labeled bands: MboI-A_1 (21.3%), MboI-A_2 (10.6%), MboI-B_1 (11.8%), MboI-B_2 (2.4%), MboI-D_1 (7.6%), and MboI-D_2 (2.05%). (b) A circular HindIII cleavage map of BKV(MM) DNA. The 5'-^{32}P-labeled ends are marked with dots; the cleavage sites of EcoRI and PstI are marked with arrows. (c) Circular map of BKV(MM) DNA with the sites of three single-cut restriction enzymes. BKV(MM) DNA was cleaved simultaneously by HhaI, EcoRI, and PstI and then labeled at the 5'-ends.*

beled *Hin*dIII fragments with *Mbo*I are complementary. Taken together, these data completely define the physical map for *Mbo*I (Fig. 4c).

Mapping of *Hae*III cleavage sites. Localization of the 10 *Mbo*I sites on the BKV(MM) genome in addition to the 11 sites mentioned earlier has facilitated the subsequent mapping of other enzymes which produce a larger number of fragments on BKV(MM) DNA. Ordering of the 21 fragments produced by *Hae*III is pre-

sented below. First, each uniformly labeled *Hin*dIII fragment was digested with *Hae*III to completion. *Hin*dIII-A received 10 cleavages by *Hae*III to produce 11 subfragments, *Hin*dIII-*Hae*III 2, 3, 4, 5, 8 (doublet), 9, 12, 13, 14, and 15, whereas *Hind*III-B received four cleavages to produce subfragments 1, 6, 7, 10, and 16 (Table 5). *Hind*-C_a, -C_b, and -C_c were cleaved by *Hae*III to give subfragments *Hin*dIII-*Hae*III 4_a, 4_b, and 4_c, respectively, in addition to seven other common subfragments, *Hin*dIII-*Hae*III 10, 11, 14, 16, 17 (doublet), and 18. In reciprocal digestion, each uniformly labeled *Hae*III fragment was incubated with *Hin*dIII (gel not shown). The results are analyzed below. There were only three *Hae*III fragments which received cleavages from *Hin*dIII. *Hae*III-C was cleaved into subfragments *Hin*dIII-*Hae*III 3 and 16 by *Hin*dIII; *Hae*III-D gave subfragments 5 and 16, whereas *Hae*III-H gave subfragments 7 and 14. By overlap analysis, a preliminary order of

TABLE 3. *Analysis of HindIII fragments of BKV(MM) DNA after digestion with MboI enzyme*[a]

*Hin*dIII fragment	*Hin*dIII-*Mbo*I subfragment[b]	No. of *Mbo*I cleavage sites
A	3, 4(D), 5(F), 8, 9(H), 10(I), 12(J)	6
B	1_a(B), 2(C), 6, 7(G), 11	4
C_a	1_a	0
$C_{b,c}$	1_b, 1_c	0

[a] BKV(MM) DNA was uniformly labeled with ^{32}P.
[b] Letters in parentheses refer to the *Mbo*I fragments (see Fig. 4b).

*Hae*III fragments can be obtained as follows: -C-(B, F, I, J, K, O, P, Q, R)-D-(A, G, M)-H-(E, L, N, S, T, U)-.

Next, each uniformly labeled *Mbo*I fragment (A through J) was digested with *Hae*III to completion. The results are shown in Fig. 5 and summarized in Table 6. Except for fragments *Mbo*I-B, -G, and -I, which remained intact, the rest were all cleaved by *Hae*III at least once (Table 6). Of particular interest is *Mbo*I-A, which includes the entire region of variation in the viral genome, and it gave three variant subfragments (*Mbo*I-*Hae*III 4_a, 4_b, and 4_c) in addition to nine others, *Mbo*I-*Hae*III 3, 7, 9, 12, 14, 15, 20 (doublet), and 22. In reciprocal digestion, individual *Hae*III fragments were separately incubated with *Mbo*I enzyme, and the products are shown in Fig. 6 and Table 7. Seven out of the 21 *Hae*III fragments were cleaved by *Mbo*I. *Hae*III-A received three *Mbo*I cleavages and gave subfragments *Mbo*I-*Hae*III 1, 5, 6, and 9. When overlap analysis was performed, we immediately determined the location of *Hae*III-A with respect to the known order of the *Mbo*I fragments on the map. Subfragments 6, 1, 5, and 9 were correspondingly identified from *Mbo*I-C, -B, -G, and -A. Thus, *Hae*III-A must overlap a part of *Mbo*I-C, all of fragments B and G, and a part of fragment A (Fig. 7). Release of subfragment upon cleavage with *Mbo*I of *Hae*-B, -D, -F, -G, -J, and -K was demonstrated in Fig. 6 and is summarized in Table 7. The positioning of these fragments was similarly determined. This allows us to draw an order of *Hae*III fragments on the BKV(MM) genome as follows: -B-J-(P, R)-D-G-L/M-A-(C, E, H, L/M, N, S, T, U)-K-F-(I, O, Q)- (Fig. 7). However, the order of

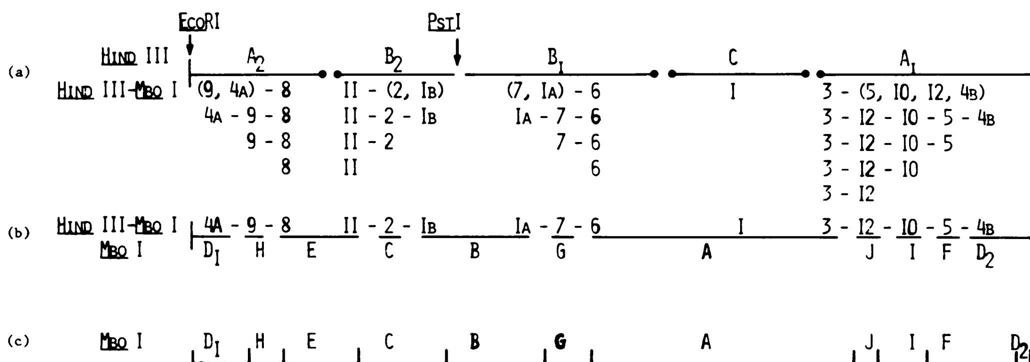


FIG. 4. *Ordering of the MboI fragments on the BKV(MM) genome. (a) Summary of an analysis of the single-end-labeled HindIII fragments by partial digestion with MboI. The dotted end indicates the 5'-*^{32}P*-labeled terminus. The resolved orders of the HindIII-MboI subfragments are shown under each single-end,* ^{32}P*-labeled HindIII fragment. (b) Summary of overlap analysis of the MboI fragments cut by HindIII. Each isolated 5'-end-labeled MboI fragment was completely digested with HindIII (gel not shown). (c) Complete physical map of MboI on the BKV(MM) genome is deduced from the results of (a) and (b).*

TABLE 4. *Analysis of single-end-labeled HindIII fragments after partial digestion with MboI*[a]

Single-end-labeled *Hin*dIII fragments	Partial *Mbo*I cleavage products	Genome length (%)		Specific *Hin*dIII-*Mbo*I subfragment identified[b]	Order of subfragments deduced
		Estimated size	Size difference		
A_1	a	28.55			a = 3-12-10-5-4B
			2.05	4B (D_2)	
A_1	b	26.50			b = 3-12-10-5
			9.00	5 (F)	
A_1	c	17.50			c = 3-12-10
			4.30	10 (I)	
A_1	d	13.20			d = 3-12
			2.40	12 (J)	
A_1	e[c]	10.80		3 (A_2)[d]	e = 3
A_2	1	17.8			a = 8-9-4A
			7.6	4A (D_1)	
A_2	b	10.2			b = 8-9
			4.5	9 (H)	
A_2	c*	5.7		8 (E_1)	c = 8
B_1	a	25.4			a = 6-7-1A
			11.8	1A (B_1)	
B_1	b	13.6			b = 6-7
			5.8	7 (G)	
B_1	c*	7.8		6 (A_3)[d]	c = 6
B_2	a	15.4			a = 11-2-1B
			1.4	1B (B_2)	
B_2	b	14.0			b = 11-2
			10.8	2 (C)	
B_2	c[c]	3.2		11 (E_2)	c = 11

[a] Single-end, ^{32}P-labeled *Hin*dIII-A_1, -A_2, -B_1, and -B_2 fragments were obtained as described in the legend to Fig. 3. They were partially digested with *Mbo*I to produce the partial cleavage products a, b, c, etc.
[b] The corresponding *Mbo*I fragment is given in parentheses.
[c] This is the end fragment in the single-end, ^{32}P-labeled *Hin*dIII fragment.
[d] Considering *Hin*dIII-*Mbo*I subfragment as *Mbo*I-A.

TABLE 5. *Analysis of uniformly labeled HindIII fragments after digestion with HaeIII enzyme*

*Hin*dIII fragment	*Hin*dIII-*Hae*III subfragment	No. of *Hae*III cleavage sites
A	2, 3, 4, 5, 8 (doublet), 9, 12, 13, 14, 15	10
B	1, 6, 7, 10, 16	4
C_a	4_a, 10, 11, 14, 16, 17 (doublet), 18	7
$C_{b,c}$	4_b, 4_c, 10, 11, 14, 16, 17 (doublet), 18	7

the fragments in parentheses has not yet been determined.

Partial digestion (see above) of certain single-end, ^{32}P-labeled *Hin*dIII fragments provided this information (see Fig. 8 for a typical gel) and completed the order: -B-J-P-R-D-G-M-A-H-L-N-U-T-E-S-C-K-F-O-I-Q-. When the three 5'-terminally ^{32}P-labeled *Hin*dIII-C fragments (including C_a, C_b, and C_c) were cleaved by *Mbo*II [the *Mbo*II cleavage map of BKV(MM) DNA will be reported elsewhere] to completion, three single-end-labeled *Mbo*II-*Hin*dIII-C_1 fragments ($C_{a\text{-}1}$, $C_{b\text{-}1}$, and $C_{c\text{-}1}$) were obtained in addition to a small single-end-labeled DNA piece of about 15 nucleotides in length. The partial cleavage patterns of each of these three *Mbo*II-*Hin*dIII-C_1 fragments with *Hae*III are shown and illustrated schematically with the detailed linear map in Fig. 8. Each partial digest could be resolved in seven bands, designated a through g. The size of the fragment in each band was estimated, and the component *Hae*III fragments in each radioactive partial product were deduced as described earlier (see also the legend to Fig. 8). Of special interest is that *Hae*III-E is the only fragment having three sizes of DNA, whereas each of the rest has its unique size. The result obtained from the partial digestion of another single-end-labeled fragment, *Hha*I-*Hin*dIII-C_1 (the 5'-^{32}P-labeled end was at the *Hin*dIII site), with *Hae*III confirmed the above finding that *Hae*III-E, at map positions 0.64 to 0.71, is the only region where variation takes place in the entire viral genome.

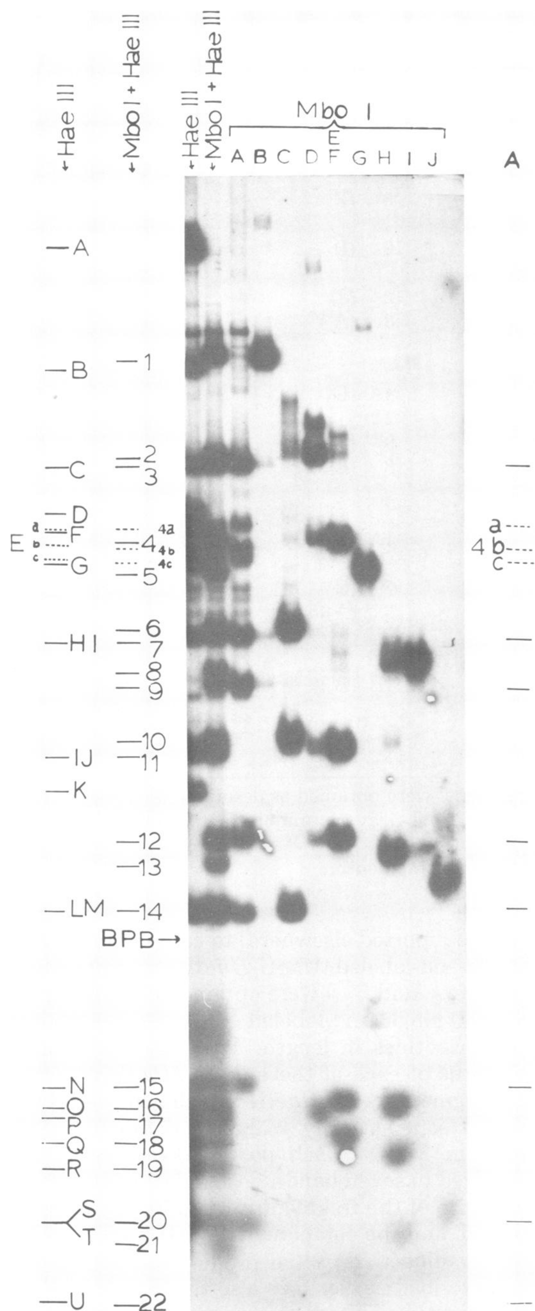


FIG. 5. *Cleavage of individual MboI fragments with HaeIII. Each isolated MboI fragment of BKV(MM) DNA was digested with HaeIII to completion. The digests were fractionated on a 3.5% polyacrylamide slab gel with both digests of HaeIII and MboI-HaeIII as direct markers. The banding patterns of the markers are drawn out at the left-hand side of the autoradiogram. The HaeIII cleavage pattern of MboI-A fragment is also depicted, which includes the three species of the MboI-HaeIII 4 subfragment (4_a, 4_b, and 4_c), corresponding to HaeIII-E_a, -E_b, and -E_c. The MboI fragments are indicated at the top.*

TABLE 6. *Analysis of uniformly labeled MboI fragments after digestion with HaeIII*

*Mbo*I fragment	*Mbo*I-*Hae*III subfragment	No. of *Hae*III cleavage sites
A	3, 4_a, 4_b, 4_c, 7, 9, 12, 14, 15, 20 (doublet), 22	9
B	1	0
C	6, 10, 14	2
D	2, 17	1
E	4, 12	1
F	11 (doublet), 16, 18, 21	4
G	5	0
H	13, 17, 19, 21	3
I	8	0
J	14, 20	1

TABLE 7. *Analysis of uniformly labeled HaeIII fragments after digestion with MboI*

*Hae*III fragment	*Hae*III-*Mbo*I subfragment	No. of *Mbo*I cleavage sites
A	1, 5, 6, 9	3
B	2, 11	1
C	3	0
D	4, 21	1
E	4_a, 4_b, 4_c	0
F	8, 14, 21	2
G	10, 12	1
H	7	0
I	11	0
J	13, 17	1
K	12, 20	1
L/M	14	0
M/L	14	0
N	15	0
O	16	0
P	17	0
Q	18	0
R	19	0
S	20	0
T	20	0
U (not done)	22	0

A linear cleavage map of BKV(MM) DNA consisting of 41 specific sites in relative position has been constructed (Fig. 9).

DISCUSSION

By using *Hin*dII + -III, *Hpa*II, and *Eco*RI, Howley et al. (8) constructed the first physical map of BKV(MM) DNA. This simple map with five cleavage sites has enabled us to do further mapping of the viral genome with many other restriction enzymes.

*Mbo*I, which recognizes a tetranucleotide sequence, ↓GATC (6), was first used to cleave BKV(MM) DNA to completion. The resulting 10 *Mbo*I fragments were ordered and mapped on the genome (Fig. 4) by means of *Hin*dIII-

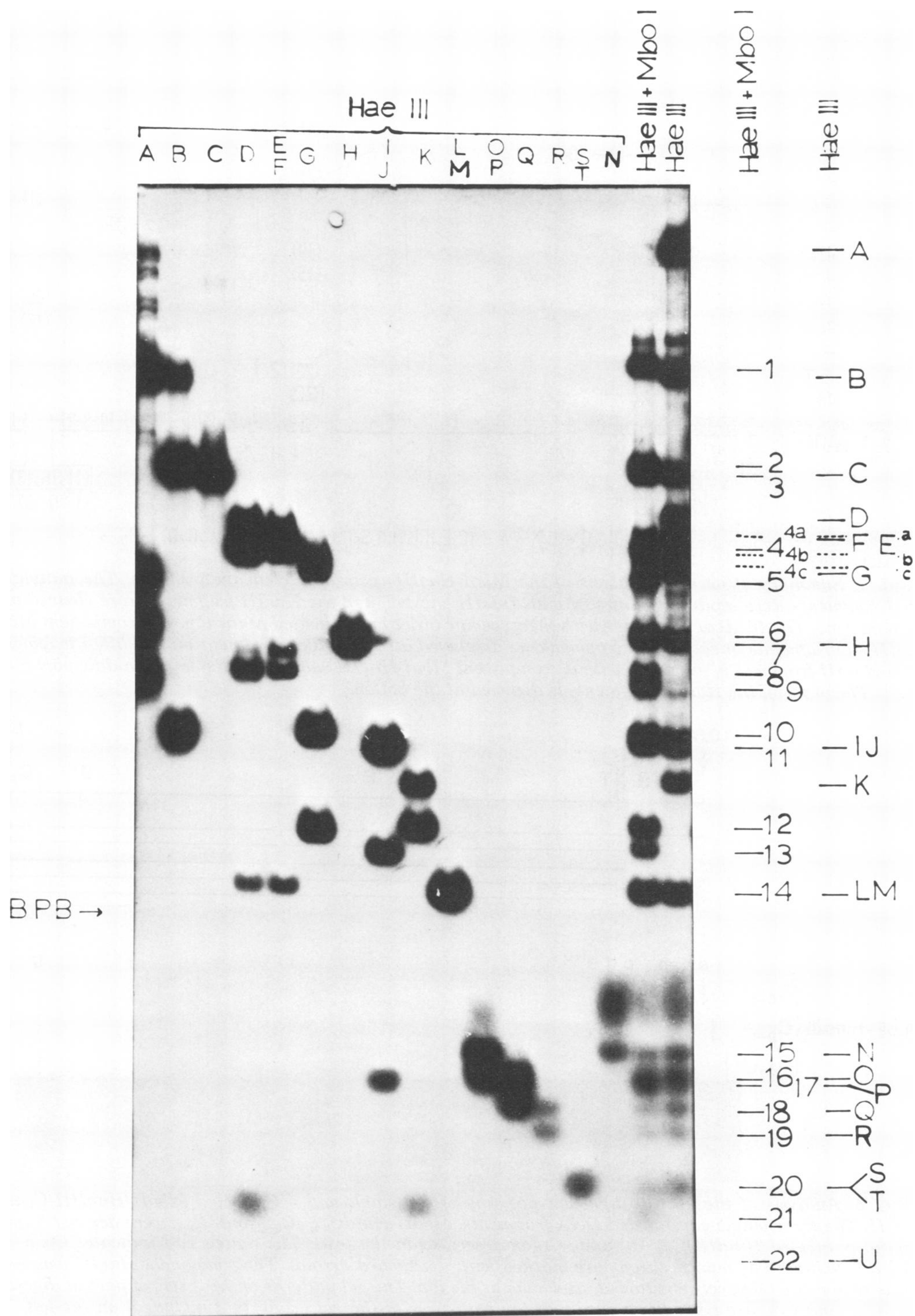


FIG. 6. *Cleavage of individual HaeIII fragments with MboI. Digestion of each isolated HaeIII fragment with MboI enzyme and fractionation of the resulting digests were as described (23). Each HaeIII fragment is marked at the top of the gel; specific markers are schematically drawn alongside for identification of MboI-HaeIII subfragments (see text for details). Nonspecific bands seen in some lanes are due to cross-contamination of the neighboring bands during isolation. For example, in lane R, the upper band was derived from the contaminant HaeIII-Q when HaeIII-R was isolated. BPB, Bromophenol blue.*

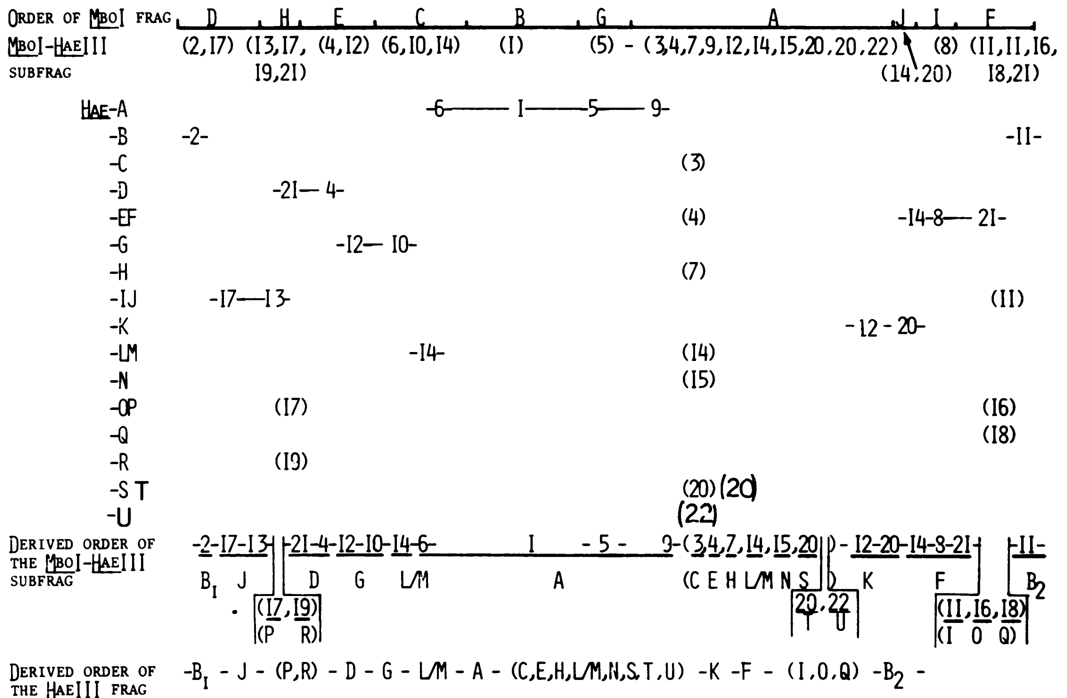


FIG. 7. *Summary of overlap analysis of the MboI-HaeIII reciprocal cleavage patterns. The individual MboI fragments were separately digested with HaeIII enzyme, and the HaeIII fragments were cleaved with MboI enzyme. The 10 MboI fragments with their specific order are indicated at the top. The component MboI-HaeIII subfragments in each MboI fragment are displayed directly under the linear map of MboI fragments. Each HaeIII fragment is indicated with its component MboI-HaeIII subfragment(s) in the middle part of the figure. The order of the HaeIII fragments is deduced at the bottom.*

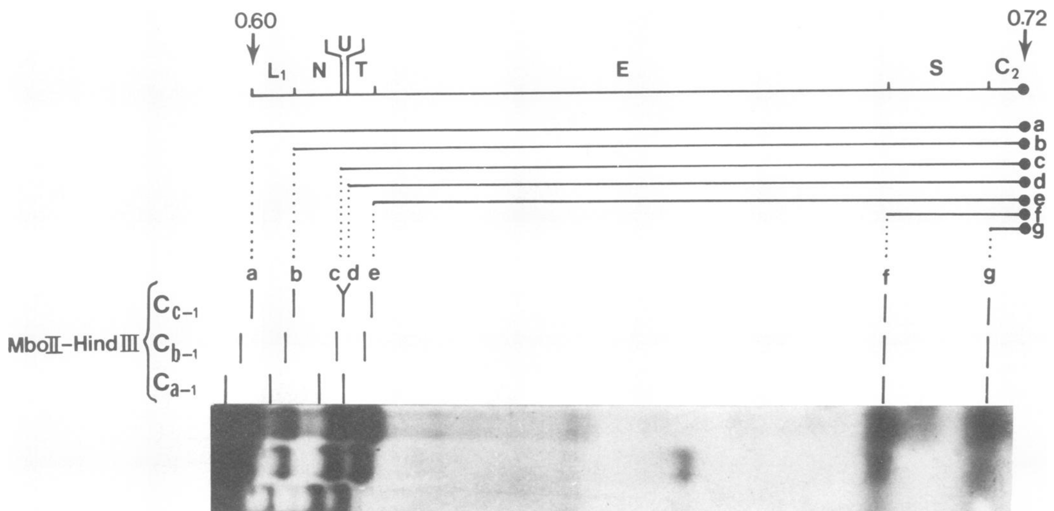


FIG. 8. *Analysis of the partial digestion patterns of the single-end, ^{32}P-labeled MboII-HindIII-C_1 with HaeIII. The three single-end-labeled DNA fragments, MboII-HindC$_{a\text{-}1}$, -C$_{b\text{-}1}$, and -C$_{c\text{-}1}$, were derived from 5′-terminally labeled HindIII-C$_a$, -C$_b$, and -C$_c$ (as described in the text). The dotted end indicates the 5′-^{32}P-labeled terminus. The map positions are marked with downward arrows. The component HaeIII fragments in this region are relatively positioned, as shown at the top. The gel patterns of three sets of partial digestion (C$_{a\text{-}1}$, C$_{b\text{-}1}$, and C$_{c\text{-}1}$) are lying horizontally at the bottom, with the gel origin (not included) on the left. The incompletely cleaved products sharing the same radioactive end were resolved in seven bands (a–g) and subjected to size estimation for HaeIII fragments. The deduced order of the component HaeIII fragments in each product is drawn as follows: g = C$_2$; f = S-C$_2$; e = E-S-C$_2$; d = T-E-S-C$_2$; c = U-T-E-S-C$_2$; b = N-U-T-E-S-C$_2$; A = L$_1$-N-U-T-E-S-C$_2$. Bands c and d were resolved close to each other. The size difference between them is about five nucleotides (corresponding to HaeIII-U in length). However, this has been confirmed with the results obtained from partial cleavage of another single-end-labeled (the 5′-^{32}P-terminus was on the HindIII site) fragment, HhaI-HindIII-C$_1$, with HaeIII. In lane C$_{b\text{-}1}$, a nonspecific band was also observed.*

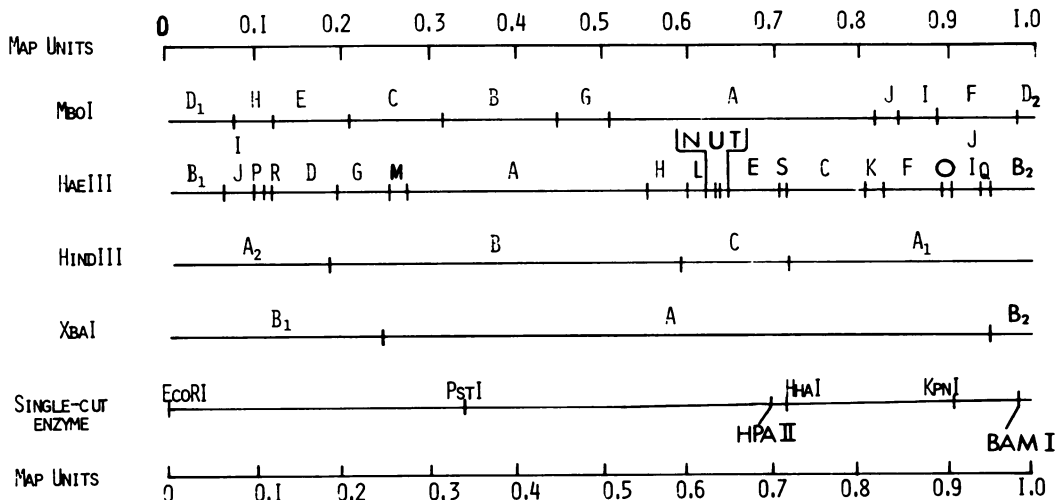


FIG. 9. *Physical map of BKV(MM) DNA. Circular DNA is represented as a linear structure, using the unique EcoRI site as the 0/1.0 site. The cleavage sites of MboI and HaeIII are located as described in the text; those of the HindIII, EcoRI, and HpaII are from Howley et al. (8), and those of XbaI and single-cut enzymes are from Yang and Wu (24).*

MboI reciprocal digestions of the uniformly ^{32}P-labeled DNA fragments, as well as *MboI* partial digestions of the single-end-labeled *HindIII* subfragments.

Determination of the *MboI* sites and the sites from eight other enzymes (see Fig. 9) of BKV(MM) DNA has allowed us to map the 21 sites of *HaeIII* by using similar techniques.

It is noteworthy that even though the BKV(MM) stock contained three species of related viruses, by using the two aforementioned powerful techniques we have been able to identify the location of the variant DNA and positioned the region of variation between map positions 0.64 to 0.71, corresponding to *HaeIII*-E. This region of variation among the genomes of the three species of BKV(MM) is most likely in the same region of the genome that the variation between BKV(MM) and prototype BKV maps. The DNA molecules of the two variants of BKV(MM) are smaller than those of the parent BKV(MM) by about 21 to 26 nucleotides in length, due partly to the loss of *HpaII* sites of the variants. They are viable deletion mutants of the parent BKV(MM), since no matter how low the multiplicity of infection, the proportion of the three virus species remained essentially unchanged.

The specific cleavage map of the 41 sites with their relative positions marked on the BKV(MM) genome has therefore been constructed. The size of the resultant fragments ranges from 6 (of *HaeIII*-U) to 614 (of *PstI*-*MboI* B_1) nucleotides in length. From our experience in DNA sequencing work, using the powerful technique of Maxam and Gilbert (13), DNA fragments of up to 300 nucleotides in length can be completely sequenced. Thus, at least 80% of the fragments obtainable from the present map are suitable for complete sequence analysis. We have already initiated a program for extensive sequence analysis.

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