Anatomy of Bovine Mammillitis DNA

I. Restriction Endonuclease Maps of Four Populations of Molecules That Differ in the Relative Orientation of Their Long and Short Components

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In this paper, we report that the DNA of bovine mammillitis virus (BMV) consists of two covalently linked components that are 71.5×10^6 and 15.7×10^6 in molecular weight and designated L and S, respectively. We further report that the BMV DNA consists of four equimolar populations differing only in the orientation of the L and S components relative to each other. This conclusion is based on the following: (i) The sum molecular weight of fragments generated by digestion of BMV DNA with *Hsu* I, *Hpa* I, *Bgl* II, or *Xba* I significantly exceeds the established molecular weight of the intact DNA. (ii) In each digest, the fragments form three groups differing in molar concentration. In reference to the molar concentration of intact DNA, each enzyme digest contained a set of four fragments 0.25 M in concentration, a set of four fragments 0.5 M in concentration, and a variable size set, unique for each enzyme digest, 1.0 M in concentration. (iii) Experiments involving digestion of intact DNA by lambda exonuclease followed by restriction endonuclease digestion established that each of four 0.5 M fragments were positioned at the termini of the BMV DNA. (iv) Complete maps for the fragments generated by each enzyme established that the 0.25 M fragments arise by fusion of the sequences of the terminal fragments when these are juxtaposed as a consequence of the inversion of L and S components. The maps also established the dimensions of the L and S components. We conclude that the structure of BMV DNA is similar to that of HSV DNA previously shown to consist of two unequal size components that invert relative to each other.

The genome of bovine mammillitis virus (bovid herpesvirus 2) (BMV) has been shown to be a linear DNA molecule 86×10^6 to 89×10^6 (T. G. Buchman, R. J. Jacob, and B. Roizman, submitted for publication) in molecular weight and to contain 64 guanine plus cytosine (G+C) mol % (6, 11).

Interest in BMV DNA stems from the observation that it is one of the few nonhuman herpesviruses whose DNA bears partial sequence homology (14%) to herpes simplex virus type 1 (HSV-1) DNA as determined by DNA-DNA hybridization (11); in similar types of experiments, HSV-1 was shown to share only 50% of its sequences with herpes simplex virus type 2 (HSV-2) (5).

It was of interest, therefore, to determine whether the sequence arrangements of BMV DNA and of HSV DNA are related.

Pertinent to this paper are several features of HSV DNA structure and sequence arrange-

ments. Specifically: (i) HSV-1 DNA consists of two covalently linked components, L and S, each consisting of unique sequences U_L and U_S bracketed by inverted repeats (10). Measurements in our laboratory showed that the L and S components comprise 82 and 18% of the DNA, respectively, and that the inverted repeats bracketing the L component, designated ab and $b'a'$, each contain 6% of total DNA, whereas the inverted repeats of the S component, designated ac and $c'a'$, each contain 4.3% of total DNA (7-9, 12). The sequence of regions identified in these measurements is $ab U_L b'a'a'c' U_S ca$. These measurements have since been confirmed (2). Restriction endonuclease analyses of HSV-2 DNA suggest that it has a similar structure (G. S. Hayward, T. Buchman, and B. Roizman, manuscript in preparation).

(ii) On the basis of the observation that the terminal regions of the L and S components are repeated internally as inversions, Sheldrick and

Berthelot (10) suggested that L and S components could, through legitimate recombination, invert relative to each other. Data reported from this laboratory and based on partial denaturation profiles, identification of the termini of the DNA, and restriction endonuclease analyses indicated that HSV-1 DNA consists of equimolar concentrations of four populations of DNA molecules differing solely in the relative orientation of L and S components (4, 8, 9). These data have since been confirmed (1, 2, 13, 14). In our studies, the four populations have been designated as P (prototype), I_L (inversion of L component), I_S (inversion of S component), and I_{SL} (inversion of both S and L components). One consequence of this arrangement of HSV DNA, pertinent to this study, is that restriction endonucleases that do not cleave within the reiterated regions generate three classes of fragments. The first class, corresponding in concentration to the molarity of intact DNA, comprises fragments mapping between the first and last cleavage within the U_L and U_S regions, respectively. The second class comprises the four terminal fragments present in 0.5 M concentration relative to the molarity of the intact DNA. The third class comprises 0.25 M fragments that arise through the joining of the 0.5 M L and S terminal fragments across the $b'a'a'c'$ junction in consequence of inversions of L and S components.

MATERIALS AND METHODS

Cells and virus. Maintenance of Vero cells and production of BMV stock were described previously (T. G. Buchman, R. J. Jacob, and B. Roizman, submitted for publication).

Preparation of ^{32}P -labeled DNA from purified virions and from whole cell extracts. ^{32}P -labeled viral DNA was isolated from purified virions as described previously (Buchman, Jacob, and Roizman, submitted for publication). Similar labeling, infection, and incubation procedures were used for preparation of BMV DNA from infected cells. In this instance, infected cells were suspended in the medium in which they were maintained and centrifuged for 1 h at 4°C and 24,000 rpm in a Beckman SW27 rotor. The pellets were suspended in TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5) containing nuclease-free Pronase at a final concentration of 1 mg/ml. Addition of sodium dodecyl sulfate to 0.6% solubilized the preparation, which was then incubated for 4 h at 39°C and subsequently dialyzed against many changes of TE buffer.

Purification of ^{32}P -labeled viral DNA. Whole cell extract from a 750-cm^2 infected cell culture was brought to a density of 1.712 g/cm^3 with CsCl and banded by centrifugation. Viral DNA-containing fractions from several tubes were pooled and recentrifuged to concentrate the viral DNA and to remove residual contaminating RNA and host DNA. CsCl was removed by dialysis against TE buffer. Restriction en-

donuclease digestion patterns of viral DNA purified from whole cell extracts could not be differentiated from those of virion- or nucleocapsid-purified DNA.

Restriction endonucleases. *Bgl* II, *Xba* I, *Hpa* I, *Hsu* I, and *Eco*RI restriction endonucleases were prepared from appropriate bacterial cell pastes by using standard procedures: streptomycin sulfate precipitation of bacterial nucleic acids, ammonium sulfate fractionation of proteins, molecular-sieve chromatography through agarose beads, and phosphocellulose ion-exchange chromatography. All restriction endonuclease preparations were shown to be free of detectable contaminating endo- or exonuclease activities before use. All incubations were carried out at 37°C for 2.5 h with demonstrated excess enzyme to achieve limit digestion. The digestions were done in 20 mM Tris (pH 7.6), 20 mM MgCl_2 , and 20 mM NaCl for all endonucleases except *Eco*RI, which required 50 mM NaCl. Reactions were quenched by the addition of EDTA to 20 mM.

Lambda 5'-exonuclease. Lambda 5'-exonuclease was purified by the method of Radding (7) from *Escherichia coli* 1100 (AT11) cells induced with an empirically determined amount of mitomycin C ($5\text{ }\mu\text{g/ml}$). Purification was carried through the phosphocellulose step, and the preparation was found to be free of contaminating endonucleases by assay against supercoiled plasmid DNA. Reaction mixtures contained glycine titrated with KOH to pH 9.6 (67 mM), 1 mM mercaptoethanol, and 50 μg of bovine serum albumin per ml. After addition of DNA and exonuclease, the mixture was brought to 20°C . The addition of MgCl_2 to 10 mM started the reaction: portions were removed at successive time points, added directly to 10 mM EDTA (pH 8), and immediately extracted with phenol. The digested DNA was then dialyzed against several changes of TE buffer.

Agarose gels: electrophoresis, slicing, and autoradiography. Quenched restriction endonuclease digests containing 6% sucrose and 0.001% bromophenol blue were loaded onto 0.4 or 0.45% agarose gels (30 cm) that had been prepared in Loening electrophoresis buffer (36 mM Tris, 30 mM NaH_2PO_4 , and 1 mM Na_2EDTA , pH 7.7). After electrophoresis (40 h, 50 V, and 25-cm dye migration for all gels except those represented in Fig. 6a to i, in which electrophoresis was accomplished for 16 h, 65 V, and 16-cm dye migration), one of three procedures was followed. For preparation of autoradiograms, the gels were sliced longitudinally and air-dried onto Whatman no. 1 filter paper. The paper strips were cut out, mounted on a solid support, and permitted to expose Cronex 2DC medical X-ray film. For determination of molar ratios and molecular weights, the gels were sliced into 1-mm-thick disks by using a parallel razor blade device. The individual slices were autoclaved in 1 ml of water at 110°C for 15 min directly in scintillation vials. Strips of glass fiber paper (2.5 by 4 cm) were added, and the vials were dried overnight at 80°C . Radioactivity was measured in toluene-based scintillation fluid. Preparative isolation of restriction endonuclease fragments also involved disk slicing as described above, but the disks were suspended in 1 ml of TE buffer and ^{32}P radioactivity was measured directly by using the Cer-

enkov procedure. In this instance, appropriate disks were recovered from the scintillation vials, pooled, and dissolved by gentle agitation in 5 volumes of KI-saturated TE buffer at 37°C. After dialysis against TE buffer (during which a weak gel matrix reformed), the agarose was pelleted at $20,000 \times g$ for 20 min at 4°C; the fragment-containing supernatant was stored at 4°C for not more than 48 h.

Determination of fragment molecular weights. Digested BMV DNA labeled with ^{32}P was subjected to co-electrophoresis with ^3H -labeled lambda DNA previously digested with *EcoRI* or with ^3H -labeled HSV-1 (MP) DNA previously digested with *Bgl* II. Gels were processed as described above, and radioactivity was measured in a toluene-based scintillation fluid. Calibration curves of known standard fragment molecular weight were constructed (data not shown), and BMV fragment molecular weights were read directly from the calibration curves.

RESULTS

Electrophoretic profiles and quantitation of BMV DNA fragments generated by limit digestion with four restriction endonucleases. ^{32}P -labeled purified BMV DNA was digested with either *Bgl* II, *Xba* I, *Hpa* I, or *Hsu* I restriction endonucleases, and the limit digests were subjected to electrophoresis in agarose gels as described above. The selection of the enzymes was based on the observation that BMV DNA is rich in G+C residues, and, therefore, to minimize the number of fragments, we selected enzymes that are known to have A+T-rich cleavage sites.

The autoradiograms of the electrophoretically separated fragments are shown in Fig. 1. In addition, an identical set of gels was sliced, and

FIG. 1. Autoradiograms of the ^{32}P -labeled BMV DNA restriction endonuclease fragments electrophoretically separated in agarose gels. The bands are designated on the left by a letter in descending order according to the molecular weight ($\times 10^6$) on the right. The symbol $\circ$ (preceding the letter) identifies terminal fragments present in 0.5 M concentration as indicated in the text. The pairs of letters on the right of each profile identify 0.25 M fragments; these names indicate the terminal fragments whose juxtaposition at the junction at the L and S region has resulted in fusion, giving to the particular 0.25 M fragment.

the radioactivity in each disk was measured as described above. The ^{32}P radioactivity profiles are shown in Fig. 2. The procedure used for determination of the molecular weights of the DNA fragments in each band is described above.

The salient features of the data presented in Fig. 1 and 2 are as follows: First, the sum of the molecular weights of the fragments generated by each enzyme far exceeded the previously established molecular weight for BMV DNA. Thus, the sums for *Hsu* I, *Bgl* II, *Xba* I, and *Hpa* I fragments were ($\times 10^6$) 155.8, 140.3, 175.7, and 190.9, respectively. In addition, examination of the radioactivity profiles (Fig. 2) indicated the presence in each digest of three groups of

fragments differing in molar concentration. Thus, on the basis of the amount of radioactivity and the molecular weight of the DNA in each *Hsu* I band, it was readily calculated that fragments *Hsu* I A, B, C, and D were present in concentrations 0.25 of those of *Hsu* I E, I, J, L, M, N, O, and P, whereas fragments *Hsu* I F, G, H, and K were present at 0.5 the concentration of fragments *Hsu* I E, I, J, L, M, N, O, and P. Similar deductions can be made for each of the remaining digests, provided it is assumed that some of the bands contained more than one fragment.

These observations are similar to those originally made with HSV-1 DNA (6-8) and permit us to construct a model concerning the possible structure of BMV DNA. Specifically, by analogy with the structure of HSV DNA, we could envision that BMV DNA consists of two covalently linked components of unequal size that could also be designated as L and S. We could also envision (Fig. 3) that L and S invert relative to each other in such a fashion that BMV DNA consists of four equimolar populations differing in the orientations of L and S. Thus, if one population is arbitrarily designated as P (prototype), the other populations become I_L , I_S , and I_{SL} (inversion of L, S, or both L and S). If this were the case, restriction endonuclease digests of the four molecular arrangements of BMV DNA should contain three groups of fragments: (i) four terminal fragments, each present twice among the four forms and thus present in 0.5 M concentration relative to intact DNA; (ii) four fragments spanning the junction of the L and S components, each present in only one of the four forms and thus present in 0.25 M concentration relative to intact DNA; (iii) remaining fragments located between the 0.25 and 0.5 M fragments present in each form and therefore present in 1.0 M concentration relative to intact DNA. In this model, the 0.25 M fragments would arise from fusion of terminal fragment sequences in that arrangement of the DNA in which these terminal sequences are juxtaposed as the junction of the L and S components. For example, if the 0.5 M *Hsu* I H and K are the terminal fragments of the P arrangement, the sequences contained in *Hsu* I H will be juxtaposed to the sequences of *Hsu* I K at the junction of L and S in I_{SL} arrangement of the DNA. Because there is no cleavage site at the junction, the *Hsu* I digestion of the I_{SL} arrangement would generate a 0.25 M (with respect to total BMV DNA) fragment containing the sequences of *Hsu* I H and K. A cursory examination of the 0.25 and 0.5 M fragments indicates that the data are consistent with the model.

FIG. 2. Radioactivity profiles of ^{32}P -labeled BMV DNA limit digested with *Bgl* II, *Xba* I, *Hpa* I, and *Hsu* I restriction endonucleases and subjected to electrophoresis through agarose gels. The gels were identical to those used to prepare the autoradiograms displayed in Fig. 1. Profiles were aligned according to the common internal standard (^3H -labeled lambda DNA digested with *Eco*RI). Fragments are identified according to the nomenclature shown in Fig. 1; 0.25 M fragments are identified by their double-letter designations.

FIG. 3. Model of BMV DNA structure showing the arrangement of 0.5 and 0.25 M fragments generated by limit digestion of intact BMV DNA with Hsu I. The model shows four molecules differing in the relative orientation of two components shown here as unequal in size, L-large and S-small components. The 0.25 M fragments are designated both by single letters reflecting the size of the fragment relative to others and by the double letter designation identifying the sequences in the terminal fragments, which, in that particular arrangement of the DNA, are juxtaposed to form one fragment. Thus, fragment A (21.2×10^6) is composed of sequences from juxtaposed fragments F (12.0×10^6) and G (9.2×10^6), etc., which otherwise are terminal fragments in the I_{SL} arrangement of the BMV DNA.

For example, the 0.5 M Hsu I fragments were F, G, H, and K with molecular weights ($\times 10^6$) of 12.0, 9.2, 9.0, and 6.5, respectively. The 0.25 M fragments A, B, C, and D had molecular weights ($\times 10^6$) of 21.2, 18.5, 18.2, and 15.5, respectively. Simple arithmetic shows that the 0.25 M fragment Hsu I A (21.2×10^6) could have arisen as a consequence of fusion of Hsu I F (12.0×10^6) and G (9.2×10^6) in that arrangement of the DNA in which fragments H and K were terminal. Similarly, the 0.5 M Hsu I fragment B (18.5×10^6) could have arisen from fusion of Hsu I F (12.0×10^6) and K (6.5×10^6) when Hsu I G and H were terminal. In the same fashion, the 0.25 M fragment C (18.2×10^6) and D (15.5×10^6) could have arisen from fusion of Hsu I G (9.2×10^6) and H (9.0×10^6) and of K (6.5×10^6) and H (9.0×10^6), respectively.

The model also predicts that the fusion products of Hsu I G and K and of Hsu I F and H should not exist: in fact, 0.25 M fragments with molecular weights corresponding to the sums of the fragments were not observed. Analysis of the 0.25 and 0.5 M fragments generated by other enzymes is consistent with this model. Proof that the structure of BMV DNA is as described by the model requires evidence that the arrange-

ment of the restriction endonuclease fragments in the physical map of BMV DNA is as predicted by the model.

Demonstration that the 0.5 M fragments are located at the termini of the BMV DNA molecules. The procedure used in this experiment was similar to that described in the analyses of HSV DNA (4). In preliminary experiments, purified lambda 5'-exonuclease free of endonucleolytic activity was titrated by digestion of a fixed amount of DNA for a fixed time interval. The concentration of enzyme was then increased until the rate of release of mononucleotides could no longer be increased by further addition of the enzyme. BMV DNA was then digested for 2, 5, and 8 min with the saturating concentration of the 5'-exonuclease. The quenched digests were redigested with restriction endonucleases and subjected to electrophoresis on agarose gels. The autoradiogram of one set of electrophoretically separated fragments (Hsu I) is shown in Fig. 4. In each instance, the results showed that the 0.5 M fragments were the first to disappear from the profiles, indicating that they were located at the termini of the BMV molecules. As previously described for HSV, extended digestion resulted in the loss of additional fragments.

Restriction endonuclease maps of BMV DNA. The restriction endonuclease maps of BMV DNA were constructed by analysis of fragments generated by redigestion singly or in groups of all fragments generated by one enzyme with a second enzyme. Three points should be made concerning the presentation of the data. First, for the sake of simplicity, it is convenient to present the mapping process in a series of nine steps. Each step is schematically presented in a panel (Fig. 5), which contains only information that has been already established. All of the experimental data are contained in the autoradiograms of the electrophoretically separated fragments shown in Fig. 6. Second, again for the sake of simplicity, the diagrams show only one, the arbitrarily selected P, arrangement of the BMV DNA. In this arrangement, Hsu I H and K are the terminal fragments, and, consequently, if our model is correct, the 0.25 M junction-spanning fragment must necessarily be Hsu I A. By selecting the terminal fragments of the Hsu I restriction endonuclease fragment maps, we necessarily assign the terminal fragments to all other putative arrangements of BMV DNA. Thirdly, it is also convenient to designate the 0.25 M fragments with two letters, each identifying the fragments whose sequences are juxtaposed as a consequence of the inversion of the putative components. Thus, Hsu I A

FIG. 4. Autoradiograms of electrophoretically separated fragments generated by ^{32}P -labeled BMV DNA digested by lambda 5'-exonuclease followed by Hsu I. ^{32}P -labeled BMV DNA was digested with a saturating concentration of lambda 5'-exonuclease for 0, 2, 5, and 8 min as described in the text. These preparations were then limit digested with Hsu I and subjected to electrophoresis through agarose gels. Note the rapid disappearance of the four 0.5 M fragments, F, G, H, and K, from the gels.

becomes Hsu I FG. Whereas Hsu I FG appears as a 0.25 M junction-spanning fragment in the P arrangement shown in Fig. 5a, Hsu I F and G also appear individually in the digest and are the terminal fragments in the I_{SL} arrangement, which places Hsu I H and K in juxtaposition at the junction of the two components.

The purpose of the first step in the mapping procedure (Fig. 5a and 6a) was to identify the Bgl II terminal fragments corresponding to the terminal Hsu I fragments by digesting the Hsu I terminal fragments with Bgl II. Bgl II fragments E, K, L, and M are 0.5 M and terminal, as determined by lambda 5'-exonuclease digestion. Digestion of Hsu I K (6.5×10^6) with Bgl II yielded a fragment identical in size to Bgl II

M (4.2×10^6) and a smaller fragment (2.3×10^6). The 2.3×10^6 fragment cannot be a Bgl II terminal fragment because it corresponds in size to none of the 0.5 M Bgl II terminal fragments. The 4.2×10^6 fragment, however, does correspond to the terminal Bgl II M. Similarly, digestion of Hsu I H (9.0×10^6) with Bgl II yielded fragments 4.8×10^6 and 4.2×10^6 in molecular weight; the 4.2×10^6 fragment cannot be a Bgl II terminal fragment because the 4.2×10^6 Bgl II terminus has already been identified as being contained in Hsu I K. Therefore, the 4.8×10^6 must be the Bgl II terminus, and, in fact, it corresponds to the 0.5 M Bgl II L. Hsu I G (9.2×10^6) was not cleaved by Bgl II, and, therefore, must be contained in its entirety within the larger Bgl II terminal fragment E (11.8×10^6). Finally, digestion of Hsu I F (12.0×10^6) with Bgl II yielded two fragments, one of which (5.6×10^6) corresponds in size to the remaining 0.5 M terminal fragment Bgl II K.

The purpose of the second step in the mapping procedure was to identify the Xba I terminal fragments corresponding to the terminal fragments of Bgl II and Hsu I previously identified. The procedure involved digestion of the Xba I 0.5 M fragments and of the 0.25 M fragments formed by fusion of the corresponding 0.5 M fragments (Fig. 5a and 6b and c). The rationale underlying these experiments is the prediction that digestion of a terminal (0.5 M) fragment with a second enzyme should generate a set of fragments, one of which is the terminal fragment generated also by digestion of the intact DNA with the second enzyme. However, digestion of the 0.25 M fragment containing the 0.5 M fragment with the same second enzyme should yield the same set of fragments except that the sequences within the new terminal fragment would be contained within a larger fragment spanning the junction between the two components. For example, digestion of Xba I E with Hsu I yielded two fragments, 12.0×10^6 and 4.8×10^6 in molecular weight. The 4.8×10^6 fragment also appeared in the Hsu I digest of the 0.25 M Xba I EL and EM, both of which contain the sequences of Xba I E. Therefore, the 4.8×10^6 fragment should be nonterminal, i.e., it should arise from sequences located distal to the junction of the two components. The 12.0×10^6 fragment corresponded in size to fragment Hsu I F, and its sequences must be contained within the 16.5×10^6 fragment appearing in the digest of Xba I EL and EM. It should be noted here that the sum of the molecular weights of the two fragments generated by Hsu I digestion of Xba I E (12.0×10^6 and 4.8×10^6) was smaller than that of the parent fragment molecular weight (19×10^6). As discussed later in the text, Xba I E

probably contains, within a small region, numerous *Hsu* I cleavage sites, and the resulting minute fragments would not be detected in these gels. The conclusion that fragment *Xba* I E contains the terminal *Hsu* I F fragment emerges also from analysis of the fragments generated by digestion of *Xba* I E with *Bgl* II (Fig. 6c). In this instance, the digest contained two fragments with molecular weights of 13.4×10^6 and 5.6×10^6 . The 13.4×10^6 fragment was present in the digest of the 0.25 M fragments *Xba* I EL and EM with *Bgl* II and, therefore, is located distal to the terminus. The 5.6×10^6 fragment in the digest corresponded in size to the terminal fragment *Bgl* II K and, as expected, was absent from the *Bgl* II digest of *Xba* I EL and EM. We have previously shown that *Bgl* II K was contained within *Hsu* I F. This set of digests

The fourth step in the procedure involved estimation of the minimal dimensions of the two

FIG. 6. Autoradiograms of electrophoretically separated fragments generated by digestion of isolated BMV DNA fragments with a second restriction endonuclease. Numbers appearing below fragments are molecular weights ($\times 10^{-6}$) estimated by the electrophoretic mobilities of external standards. Occasional bands representing either undigested material or partial cleavage products are not identified with respect to their apparent molecular weights.

FIG. 6 g-i.

components. The minimal size of the left component based on the sum of the *Hpa* I fragments E and F is 38.5×10^6 (Fig. 5b). The minimal size of the right component based on the *Bgl* II or *Hsu* I digests is approximately 16×10^6 . If the second component were the smaller component and if its size were 16×10^6 , then 1.0 M fragments totaling 6.8×10^6 should fit between *Xba* I fragments L and M, whereas 1.0 M fragments totaling 4.8×10^6 should fit between *Hpa* I J and K.

Digestion of *Bgl* II E with *Xba* I yielded two fragments, 4.3×10^6 and 6.8×10^6 . The smaller fragment (4.3×10^6) corresponded to the contained *Xba* I terminus, whereas the larger fragment (6.8×10^6), which filled precisely the predicted size of the missing sequence, corresponded in size and relative concentration (1.0 M) to either *Xba* I I or J. Discrimination between the two possible *Xba* I fragments rested on two observations. First, digestion of *Xba* I I with *Hpa* I (Fig. 6d) yielded a 3.5×10^6 fragment corresponding in size to *Hpa* I L, whereas *Xba* I J did not yield fragments corresponding to any of the *Hpa* I 1.0 M fragments. The second observation concerns peculiar digestion properties of fragments spanning the center of the S component with *Hpa* I; specifically, it was observed that *Bgl* II E and *Hsu* I G upon digestion with *Hpa* I yielded fragments whose summed molecular weights were less than those of the original fragments. One possible explanation is that the central region of the S component has closely spaced *Hpa* I cleavage sites generating fragments too small to be detected in these gels. This phenomenon was also observed in *Hpa* I digests of *Xba* I I, but not in *Hpa* I digests of *Xba* I J. We conclude, therefore, that *Xba* I I fits between fragments L and M, whereas *Hpa* I L fits between fragments K and J.

The fifth step in the procedure was to place additional *Bgl* II fragments in the L component (Fig. 5c). Specifically, digestion of *Xba* I F with *Bgl* II yielded four fragments (Fig. 6c). From the number of generated fragments, we predict that *Xba* I F should yield one 0.5 M *Bgl* II terminus (4.8×10^6) and two 1.0 M *Bgl* II fragments (6.9×10^6 and 2.7×10^6) (Fig. 6c). These 1.0 M fragments can be identified as *Bgl* II J and N from the enzyme digest of intact BMV DNA (Fig. 1 and 2). The order of *Bgl* II J and N relative to *Bgl* II L was deduced from the profiles of fragments generated by digestion of *Hsu* I H with *Bgl* II (Fig. 6a). If *Bgl* II N were adjacent to *Bgl* II L, we would expect that digestion of *Hsu* I H with *Bgl* II would yield three fragments, two of which would have molecular weights of 4.8×10^6 and 2.7×10^6 . Since

the digest of *Hsu* I H with *Bgl* II yielded only two fragments with molecular weights of 4.8×10^6 and 4.2×10^6 , we conclude that the fragment order from left to right is *Bgl* II L, J, and N. Digestion of *Xba* I E with *Bgl* II (Fig. 6c) yielded two fragments, 5.6×10^6 and 13.4×10^6 . Since the 5.6×10^6 fragment was previously identified as the terminal *Bgl* II K fragment, the 13.4×10^6 fragment must be a digestion product of a larger *Bgl* II fragment lying to the left of the 0.25 M fragment, *Bgl* II EK. The identity of that larger *Bgl* II fragment emerges from examination of Fig. 6f. Specifically, *Bgl* II D is the only fragment that yields a 13.4×10^6 fragment when digested with *Xba* I. Confirmation emerges from the prediction that *Bgl* II D should not be cleaved by *Hpa* I. This is shown to be the case in Fig. 6g.

The purpose of the sixth step in the mapping procedure was to complete the *Bgl* II restriction endonuclease map (Fig. 5d). At the left side of the L region, three *Bgl* II fragments, totaling 14.4×10^6 in molecular weight, and one *Hpa* I fragment, 17.5×10^6 in molecular weight, have been positioned. The next *Bgl* II fragment must, therefore, yield one or more fragments totaling 3×10^6 upon digestion with *Hpa* I. Only three 1.0 M *Bgl* II fragments, i.e., B, G, and H, have not yet been positioned. Examination of the *Hpa* I digests of these *Bgl* II fragments (Fig. 6g) shows that *Bgl* II G is not cleaved by *Hpa* I, and, therefore, cannot lie to the right of *Bgl* II N. DNA of *Bgl* II B, EK, and EL were digested together with *Hpa* I because these fragments could not be resolved on our gels (Fig. 1). The digest of these fragments yields new fragments, 3.5×10^6 and 2.7×10^6 in molecular weight; however, these new fragments arise from the E component of *Bgl* II EK and EL, since *Bgl* II E digested with *Hpa* I was shown to generate identical 3.5×10^6 and 2.7×10^6 fragments. *Bgl* II B does not yield a fragment of approximately 3×10^6 upon digestion with *Hpa* I, and, therefore, cannot lie to the right of *Bgl* II N. By exclusion, *Bgl* II H must lie to the right of *Bgl* II N. Experimentally, *Bgl* II H yields not one but three fragments of approximately 3×10^6 upon digestion with *Hpa* I. A second of these three fragments must correspond, therefore, to a completely contained *Hpa* I molar fragment, which can only be *Hpa* I M. Thus, *Hpa* I M must lie to the right of *Hpa* I F. The third of the 3×10^6 fragments generated by *Hpa* I digestion of *Bgl* II H must represent part of an as yet unmapped *Hpa* I 1.0 M fragment. The unassigned 1.0 M *Hpa* I G, H, and I each have a molecular weight of approximately 10×10^6 . Cleavage of one of these generates a 3×10^6 fragment corresponding to the third of the *Bgl* II H fragments.

This implies that the fragment lying immediately to the right of *Bgl* II H must yield, upon digestion with *Hpa* I, a 7×10^6 fragment complementing the 3×10^6 fragment. *Bgl* II G is not cleaved by *Hpa* I, whereas *Bgl* II B yields a 6.9×10^6 fragment. *Bgl* II B, therefore, must be positioned just to the right of *Bgl* II H, whereas *Bgl* II G fits precisely between *Bgl* II B and *Bgl* II D. The *Bgl* II restriction endonuclease map of BMV is thus completed.

The purpose of the seventh step in the mapping procedure was to complete the *Xba* I restriction endonuclease map. We expected to find only three *Bgl* II cleavage sites among the unmapped *Xba* I molar fragments G, H, J, K, and N (Fig. 5d and e). Examination of *Bgl* II digests of these fragments (Fig. 6c) shows that the *Bgl* II cleavage sites occur in *Xba* I fragments G, H, and K. Digestion of *Xba* I G with *Bgl* II yields a fragment 4.9×10^6 in molecular weight. Examination of the digests of *Bgl* II fragments with *Xba* I (Fig. 6f) revealed that a corresponding 4.9×10^6 fragment was present in *Bgl* II B digest. We conclude that *Xba* I G contains a portion of *Bgl* II B and either a portion of *Bgl* II H to the left or a portion of *Bgl* II G to the right. Discrimination between these two possibilities was based on the following. If *Xba* I G were to extend to the left of *Bgl* II B, it would necessarily completely contain *Hpa* I M. However, *Hpa* I digest of *Xba* I G (Fig. 6d) did not contain a fragment corresponding in size to *Hpa* I M. Thus, *Xba* I G must extend from *Bgl* II B to the right into *Bgl* II G.

The positioning of *Xba* I K is based on the argument that it cannot extend from the position of *Bgl* II B to the left into *Bgl* II M. If this were the case, *Xba* I J would have to be entirely contained within *Bgl* II H. This cannot occur because *Bgl* II M, unlike *Xba* I J, is not cleaved by *Hsu* I (Fig. 6b and h). We conclude, therefore, that *Xba* I H extends from *Bgl* II B to the left into *Bgl* II H, whereas *Xba* I K extends from *Bgl* II G to the right into *Bgl* II D.

Xba I H yielded a fragment corresponding in size to *Hpa* I M upon redigestion with that enzyme (Fig. 6), resulting in the alignment of *Xba* I H and *Hpa* I M (Fig. 5e).

Xba I J and N are completely contained within *Bgl* II B, and the order of these fragments relative to *Xba* I H and *Xba* I G is ascertained by examination of the *Hpa* I digest of *Xba* I J (Fig. 6d). If *Xba* I J were to lay immediately to the left of *Xba* I G, cleavage of *Xba* I J with *Hpa* I should yield fragments of 5.4×10^6 and 1.3×10^6 in molecular weight. If, however, *Xba* I J were to lay to the right of *Xba* I H, the *Hpa* I digest of *Xba* I J would be predicted to contain two fragments, 4.2×10^6 and 2.5×10^6 . The

latter set of fragments was in fact observed experimentally.

The eighth step completed the *Hsu* I restriction endonuclease map. Digestion of *Xba* I F with *Hsu* I (Fig. 6b) yielded three fragments, one of which corresponded to the *Hsu* I terminus. Of the remaining two fragments, one must correspond to a completely contained 1.0 M *Hsu* I I fragment. Since no *Hsu* I 5.0×10^6 fragment exists, the contained fragment must be *Hsu* I O and is positioned to the right of *Hsu* I H. *Hsu* I E was positioned to the right of *Hsu* I O, since, upon redigestion with *Bgl* II (Fig. 6a), it yielded fragments corresponding to *Bgl* II N and H.

Hsu I L is readily placed because it is not cleaved by *Xba* I (Fig. 6i); rather it is completely contained within *Xba* I G. Similarly, *Hsu* I M is not cleaved by *Bgl* II because it is contained within *Bgl* II G (Fig. 6h). Digest of *Bgl* II G with *Hsu* I yielded, in addition to the 4.8×10^6 fragment corresponding to *Hsu* I M, a 3.0×10^6 fragment corresponding to part of adjacent *Hsu* I L (see digest of *Hsu* I L with *Bgl* II, Fig. 6a, for confirmation) and a 2.1×10^6 fragment. That 2.1×10^6 fragment must correspond to a portion of an unmapped 1.0 M *Hsu* I fragment. Both *Hsu* I I and J were excluded because upon digestion with *Bgl* II, neither yielded a corresponding 2.1×10^6 fragment (Fig. 6a). *Hsu* I N is completely contained within *Bgl* II B (Fig. 6a and h) and, therefore, is not the source of that 2.1×10^6 fragment. Thus, *Hsu* I P, the only remaining 1.0 M *Hsu* I fragment, must be the source of that fragment and is assigned a position to the right of *Hsu* I M.

The remaining 1.0 M fragments, *Hsu* I I and J, must fit between *Hsu* I E and N and between *Hsu* I P and F. *Xba* I digest of *Hsu* I J (Fig. 6i) yielded 5.0×10^6 and 2.0×10^6 fragments. This asymmetrical cleavage pattern places *Hsu* I J to the right of *Hsu* I P, whereas the more symmetrically cleaved *Hsu* I I is assigned immediately to the right of *Hsu* I E.

In the ninth and final step of the mapping procedure, we ordered *Hpa* I fragments G, H, and I, thus completing the *Hpa* I map of BMV. These three fragments migrate too close to each other to be separated in our agarose gel system (Fig. 1 and 2). We therefore divided the complex band into five fractions, where fraction 1 contained the most slowly migrating and therefore the largest DNA segments. Each fraction was digested with the other three restriction endonucleases and subjected to electrophoresis through agarose gels (Fig. 7).

On the basis of the *Bgl* II map, we predicted that only one of the three *Hpa* I fragments G, H, and I should contain a *Bgl* II cleavage site. The data show that DNA containing *Bgl* II

FIG. 7. Autoradiogram of electrophoretically separated fragments generated by digestion of fractions from the *Hpa* I "GHI complex" with a second restriction endonuclease. Numbers appearing below fragments are molecular weights ($\times 10^6$) estimated by the electrophoretic mobilities of external standards.

cleavage sites is present in fractions 1, 2, and 3. The data also show that fractions 4 and 5 contain two different *Hpa* I fragments, since digestion of the DNA in these fractions with *Xba* I yields markedly different patterns. Thus, fraction 2 best represents *Hpa* I G. Since this fragment contains a *Bgl* II cleavage site, it must extend from *Bgl* II H to the right into *Bgl* II B.

The DNA in fraction 4 is predominantly *Hpa* I H; this DNA yielded two fragments, 7.2×10^6 and 2.6×10^6 , upon digestion with *Xba* I, whereas the DNA in fraction 5 (*Hpa* I I) yielded three fragments of molecular weight 4.5×10^6 , 2.6×10^6 , and 2.2×10^6 . On the basis of the *Xba* I map, we concluded that *Hpa* I H contains the single *Xba* I site separating *Xba* I G and K, whereas *Hpa* I I completely contained fragment *Xba* I N as well as the two cleavage sites that bound it.

These assignments are supported by the observation that *Hpa* I H yielded the completely contained *Hsu* I M (4.8×10^6) upon digestion with that enzyme, whereas *Hpa* I I yielded no fragment larger than *Hsu* I N (4.0×10^6) upon digestion.

Possible clustering of restriction endonuclease cleavage sites within BMV DNA. The *Hpa* I map contains a small region at map position 76 in Fig. 5f, for which no fragment is assigned; similarly, the *Hsu* I map harbors small gaps at positions 32, 37, and 58. Fragments of other enzymes spanning these regions, when redigested with *Hpa* I or *Hsu* I, as appropriate, invariably generate fragments whose summed molecular weight is less than the molecular weight of the starting fragment. We suspect that

these apparent gaps are loci containing clusters of restriction sites spaced less than 500,000 molecular weight units apart, since this is the lower limit of detection in the present gel system.

Sequence arrangement of BMV DNA. Central to the model presented in Fig. 3 is the concept that BMV DNA consists of two components that invert relative to each other and thereby generate four equimolar populations of DNA molecules. The maps of fragments generated by one restriction endonuclease (*Bgl* II) for all the arrangements of BMV DNA are shown in Fig. 8. Relevant to the model are the following findings: (i) We have shown that all four 0.5 M fragments are terminal: this is demonstrated in step 1 of the mapping procedure. (ii) We have also shown that the position of the 0.25 M fragments relative to the 1.0 M fragments is fixed, as predicted by the model: this is shown in steps 6, 7, and 8 of the mapping procedure. (iii) We have also shown that the sequences contained in the digests of the 0.25 M fragments are also present in appropriate terminal fragments and therefore that the 0.25 M fragments must arise by fusion of sequences of the terminal fragments when these are juxtaposed at the junction of the L and S components. This is shown in step 6 of the mapping procedure.

Similar maps of all four arrangements of the DNA can be constructed for all fragments generated by other restriction endonucleases.

Since the 0.25 M fragments consist of sequences also present in the terminal fragments, we can calculate the size of the L and S components. From the *Bgl* II and *Xba* I restriction endonuclease fragment maps, we calculated the molecular weights of the L and S components to be 71.5×10^6 and 15.7×10^6 , respectively.

DISCUSSION

Analysis of the restriction endonuclease fragments generated by four enzymes indicates that BMV DNA consists of two covalently linked, unequally sized components that invert relative to each other and that BMV DNA consists of

four populations of molecules differing in the orientation of these two components. By analogy with HSV DNA (10, 12), these components have been designated L and S. The sizes of the BMV L and S components have been calculated to be 71.5×10^6 and 15.7×10^6 , respectively, i.e., similar in relative size to those of HSV DNA (10, 12).

As indicated above, a characteristic of HSV DNA is that the L and S components consist of unique sequences bound by the inverted repeats *ab* (6.0×10^6) and *ca* (4.3×10^6), respectively. It is conceivable that the inverted repeats bracketing the two components are obligatory if the two components are to invert relative to each other. Inverted repeats analogous to *ab* and *ca* of HSV DNA have not been established as yet within BMV DNA; however, if they are present, they must be less than 4.8×10^6 and 4.2×10^6 in molecular weight for the L and S regions, respectively. Our reasoning is based upon the expectation that the presence of restriction enzyme cleavage sites within putative reiterated sequences in either L or S should yield two terminal fragments of identical size. This has not been observed with any of the restriction enzymes used in this study.

The size of BMV DNA as determined from this and preceding studies is $87.5 \pm 3 \times 10^6$. Bearing in mind the maximum size of the repeated regions, the minimum size of the unique sequences is 70×10^6 . This is consistent with the sum of the unique sequences in *Herpesvirus saimiri* DNA (3) and within the range of the unique sequences of HSV DNA (79×10^6) (12). If the size of the putative reiterated sequences is smaller than the maximal estimates, then it could be concluded that the decrease in size of the BMV genome with respect to the HSV genome is entirely at the expense of the reiterated sequences.

Selection of the BMV arrangement designated as P (prototype) was purely arbitrary. Recent studies on HSV-1 DNA have shown that not all of the four forms are capable of initiating infection. If a similar situation prevails for BMV DNA, the P form may not be among the arrangements capable of initiating infection.

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FIG. 8. *Bgl* II restriction endonuclease maps of all four arrangements of BMV DNA.

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