Structure of Marek's Disease Virus DNA: Detailed Restriction Enzyme Map

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Purified virion DNA (120×10^6 molecular weight [MW]) of Marek's disease virus strain GA was cleaved with BamHI restriction endonuclease, and 27 out of the 29 fragments were cloned into bacterial plasmids. Restriction maps for BamHI, BglI, and SmaI endonucleases were constructed. The genomic structure of Marek's disease virus DNA was found to be similar to that of herpes simplex virus types 1 and 2. A long unique region (75×10^6 MW, located at 10×10^6 to 85×10^6 MW [10–85] from the left end of the genome), which was subdivided into segment 1 (22×10^6 MW, located at 10–32) and segment 2 (51×10^6 MW, located at 34–85) by direct repeats (32–34), was flanked by a long terminal region (10×10^6 MW, located at 0–10) and a long inverted region (10×10^6 MW, located at 85–95). A short unique region (8×10^6 MW, located at 103–111) was flanked by a short terminal region (8×10^6 MW, located at 111–119) and a short inverted region (8×10^6 MW, located at 95–103). The direct repeat fragments (0.9×10^6) could be isolated by cleavage with SmaI. The right terminal end was found to be heterogenous.

Marek's disease virus (MDV) is a member of the herpesvirus family which induces lymphoproliferative disease in chickens (1, 18). The induction of the disease can be prevented by vaccination with turkey herpesvirus (19). This is the first model system in which tumor induction is prevented by vaccination. The vaccination of chickens with turkey herpesvirus is now commonly practiced in the poultry industry. MDV DNA is a linear double-stranded DNA with a molecular weight (MW) of ca. 120×10^6 (13). Cebrian et al. (3) reported that the structures of MDV DNA and turkey herpesvirus DNA were similar to that of herpes simplex virus types 1 and 2, in which inverted repeat segments were detected at terminal and internal regions of the molecule by electron microscopy studies (21). A partial restriction map of MDV DNA has been reported by our laboratory (14). Because the availability of MDV DNA was poor, the ambiguity of the map remained a problem. Furthermore, not all the restriction fragments could be isolated, due to a lack of sufficient MDV DNA available for the study. In this report, we cloned 27 BamHI restriction fragments of MDV DNA in various plasmids, and the restriction map of MDV DNA was nearly completed.

MATERIALS AND METHODS

Virus propagation and DNA extraction. Eleven-day-old chicken embryo fibroblast cells were infected with cell-associated MDV strain GA in minimal essential medium with 2% fetal calf serum. When 80% of cells showed cytopathic effect, nucleocapsids were extracted from the infected cells by Nonidet P-40 and Triton X-100 treatment, as described previously (14). Viral DNA was isolated by centrifugation through a 10 to 30% continuous glycerol gradient after the purified nucleocapsids were treated with sodium dodecyl sulfate-proteinase K (14).

Ligation of plasmids and viral DNA. All enzymes used for DNA cloning, except calf intestine alkaline phosphatase, were purchased from Bethesda Research Laboratories. Specifications of the manufacturer were followed for the reaction of various enzymes. After digestion of pACYC184 or pBR322 with 5 U of restriction endonuclease BamHI per

μg of DNA in digestion buffer (50 mM NaCl, 10 mM Tris-hydrochloride [pH 7.5], 10 mM MgCl$_2$, and 1 mM dithiothreitol) at 37°C for 1 h, recircularization of the plasmid was inhibited by treatment with bacterial alkaline phosphatase in 10 mM Tris-hydrochloride (pH 8.0) at 65°C for 1 h. After extraction five times with buffer-saturated phenol and once with chloroform, the plasmid was ethanol precipitated (2). The plasmid DNA was dissolved in 10 mM Tris-hydrochloride (pH 7.5) containing 0.1 mM EDTA. Viral DNA was digested with BamHI and heated at 65°C for 10 min. Approximately 0.5 μg of phosphatase-treated plasmid was combined with 1 μg of BamHI-digested viral DNA, and 0.1 U of T4 ligase was added to the DNA mixture. This was then adjusted to 66 mM Tris-hydrochloride (pH 7.5)–6.6 mM MgCl$_2$–10 mM dithiothreitol (ligation buffer) in a total volume of 10 μl. The ligation mixture was incubated at 14°C for 12 h and then incubated at 65°C for 10 min to inactivate the ligase (2).

Transformation and identification of recombinant plasmid. The bacterial host used for transformation was *Escherichia coli* HB101. DNA preparations for transformations were prepared in 100 μl of 0.33× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.4) and 6.7 mM CaCl$_2$. Bacteria were grown to 2×10^8 cells per ml in L broth, pelleted by centrifugation, and washed with cold 100 mM MgCl$_2$. The cells were resuspended in cold 100 mM CaCl$_2$ and incubated for 20 min on ice. The cells were then pelleted and resuspended in 0.1 of the original volume of cold 100 mM CaCl$_2$. A 200-μl portion of cell suspension was added to each 100 μl of DNA preparation and incubated on ice for 30 min (17). The cell-DNA mixture was cultured in 2.7 ml of L broth for 1 h at 37°C and then plated onto LB agar containing antibiotic (20 μg of ampicillin per ml for pBR322 or 20 μg of chloramphenicol per ml for pACYC184) (4). The bacteria containing recombinant plasmids were further screened by LB agar plates containing 20 μg of tetracycline per ml. Each recombinant plasmid DNA in Ap^{r}Tc^s bacteria was identified by the boiling method of Holms and Quingly (11).

Colonies containing viral DNA fragments were grown in L broth with appropriate antibiotics, plasmid DNA was extracted by lysozyme-sodium dodecyl sulfate (1%)-NaCl (1 M) treatment, and the plasmid DNA in the supernatant was

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precipitated with polyethylene glycol 8000 (15). Closed circular plasmid DNA was purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients.

Preparation of 5′-labeled probe and Southern blot hybridization. A 2-μg portion of cloned DNA was digested with 5 U of *Taq*I restriction endonuclease in 20 μl of digestion buffer (10 mM Tris-hydrochloride [pH 8.4], 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 100 mM NaCl) at 65°C for 1 h. After being heated at 80°C for 10 min, the digested fragments were mixed with 100 μCi of [γ-^{32}P]ATP and 10 U of T4 polynucleotide kinase in 50 μl of T4 kinase buffer (5 mM Tris-hydrochloride [pH 7.5], 10 mM $MgCl_2$, 10 mM dithiothreitol, and 5 μM ADP) and incubated at 37°C for 30 min (16). The reaction mixture was treated with phenol-chloroform, and the aqueous phase was loaded onto a G-50 column along with 100 μg of carrier DNA to separate the 5′-labeled DNA from unincorporated [γ-^{32}P]ATP. The fraction of labeled DNA was precipitated with ethanol. Viral DNAs cleaved with restriction endonuclease *Bam*HI, *Bgl*I, or *Sma*I were separated by 0.6% agarose gel electrophoresis and transferred to nitrocellulose paper by the method of Southern (20). Hybridization was carried out in 50% formamide in 50× Denhardt solution (8) (1× Denhardt is 0.02% Ficoll, 0.02% polyvinylpyrollidone, and 0.02% bovine serum albumin) and in 5 × SSC with 1 × 10^7 to 2 × 10^7 cpm of ^{32}P-probe per ml at 41°C for 24 h (7). The filter was washed once with 2× SSC–0.01% sodium dodecyl sulfate for 1 h with shaking at room temperature and once with 0.1× SSC for 1 h at room temperature. The filter was air-dried and exposed to X-ray film at −70°C with intensifying screens for 2 to 3 days. Since most of the restriction fragments were stainable by ethidium bromide before blotting, identification of the hybridized fragments was made mainly by comparison with the original picture taken after ethidium bromide staining. For some ambiguous fragments, the hybridized paper was rehybridized with viral DNA probe 5′-labeled with ^{32}P, as described above.

Cloning with cosmid DNA. A 1-μg sample of viral DNA was partially digested with 0.5 U of *Bam*HI in digestion buffer for 10 min, and terminal phosphate was removed by 0.02 U of calf intestine alkaline phosphatase (Boehringer Mannheim) treatment in 50 mM Tris-hydrochloride (pH 8.0)–0.1 mM EDTA. *Sal*I-*Bam*HI and *Pvu*II-*Bam*HI fragments, both containing the *cos* region, were prepared from pHC79 vector DNA, by the method of Ish-Horowicz and Burke (12). Calf intestine alkaline phosphatase-treated fragments and pHC79 fragments were ligated with 2 U of T4 ligase in ligation buffer in a total volume of 10 μl at 14°C for 12 h and then heat inactivated at 65°C for 10 min. The ligated fragments were packaged into λ bacteriophage heads, and transduction of *E. coli* was carried out as described previously (5, 6, 10, 11).

Location of fragments on the map. The location on the map is expressed in MW (× 10^6) from the left end of the genome.

RESULTS

Cloning of *Bam*HI restriction fragments of viral DNA in bacterial plasmid vectors. The digestion of viral DNA with *Bam*HI restriction endonuclease produced 29 fragments, ranging in MW from 0.5 × 10^6 to 15.5 × 10^6. Most of these fragments were successfully cloned into pBR322 and pACYC184 by shotgun insertion of the fragments into these
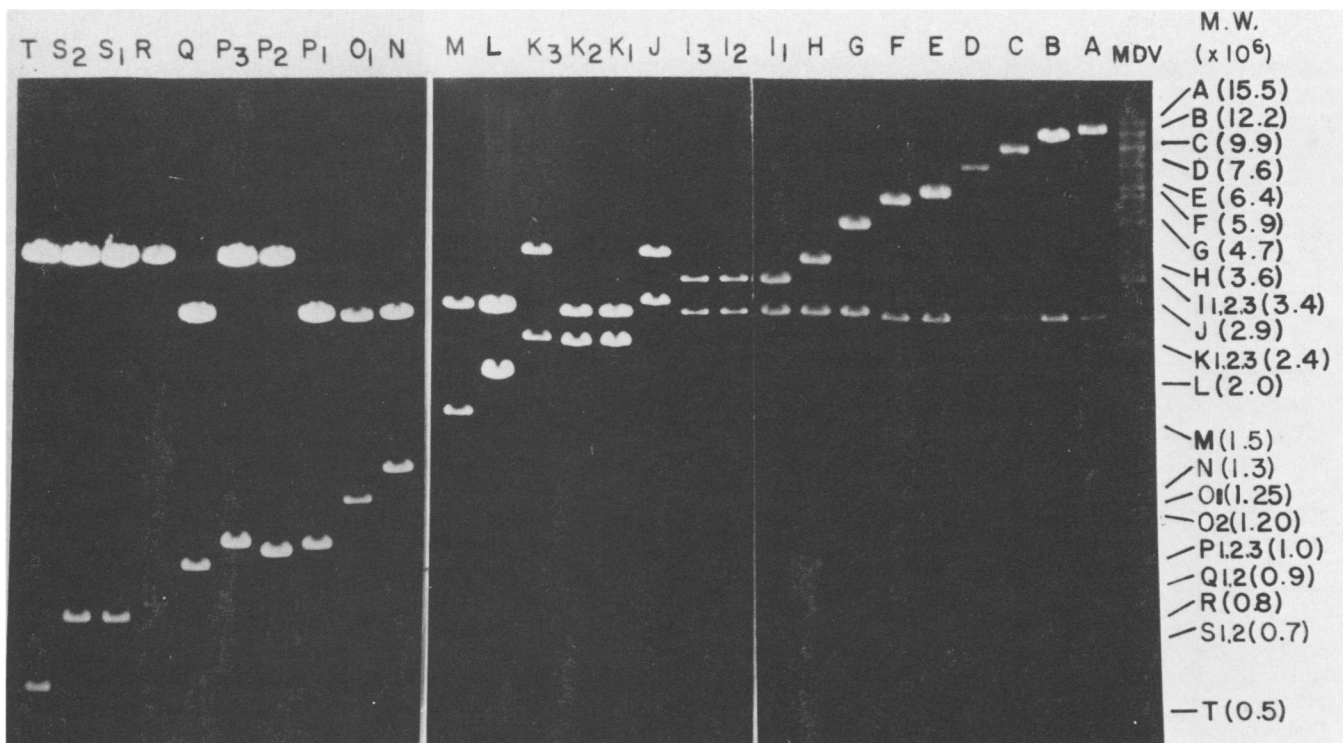

FIG. 1. Cloned *Bam*HI DNA after agarose gel electrophoresis. Viral DNA and recombinant plasmid DNAs were digested by the restriction endonuclease *Bam*HI and electrophoresed through 0.6% agarose gels. Gels were then stained with ethidium bromide (0.5 μg/ml) and photographed under UV illumination. Letters designate the various *Bam*HI restriction fragments of viral DNA which were inserted into pBR322 (2.7 × 10^6 MW), pACYC184 (2.6 × 10^6 MW), or pHC79 (3.7 × 10^6 MW) cloning vectors. *Bam*HI fragments A, B, C, D, E, F, I$_2$, and I$_3$ were inserted into pACYC184; G, H, I$_1$, K$_1$, K$_2$, L, M, N, O$_1$, P$_1$, and Q$_1$ were inserted into pBR322; J, K$_3$, P$_2$, P$_3$, R, S$_1$, S$_2$, and T were inserted into pHC79. *Bam*HI-O$_2$ and *Bam*HI-Q$_2$ have not been cloned.

vectors. Some of the small fragments were first cloned into pHC79 by using BamHI-partially digested viral DNA, followed by recloning of the desired small fragments by redigestion of the cloned fragments with BamHI, and then ligating and transforming E. coli host cells. This assured obtaining smaller fragments that were normally found by digestion of 1 to 2 μg of viral DNA with BamHI. Figure 1 illustrates all the cloned restriction fragments after digestion with BamHI and electrophoresis through a 0.6% agarose gel. The cloned BamHI A fragment was slightly smaller than the fragment obtained by BamHI digestion of viral DNA (Fig. 1). This is due to the possible deletion of the cloned BamHI A fragment. BamHI I fragments (3.4×10^6 MW) of molar ratio 4 were resolved into three species by Southern blot hybridization, due to the presence of BamHI-I_2 in the long inverted region (IR_L), K fragments (2.4×10^6 MW) of molar ratio 3 into three species, P fragments (1.0×10^6 MW) of molar ratio 3 into three species, and S fragments (0.7×10^6 MW) of molar ratio 2 were resolved into two species (Fig. 2).

To confirm the previously reported partial linkage map and to place cloned small fragments within the linkage map, BamHI, BglI, and SmaI digests of viral DNA were hybridized with ^{32}P-labeled individually cloned BamHI fragments. Every cloned BamHI fragment was hybridized to each corresponding fragment of BamHI digest of viral DNA. In addition, BamHI-D and BamHI-H were hybridized to each other (Fig. 3a); BamHI-A was hybridized to the smear region as well (Fig. 4a). (See Fig. 6 for a summary of the results of the hybridizations.) The locations of BamHI-I and BamHI-K fragments were not clear in the previous study, because the hybridization had been conducted with a combination of three different I and K species. By using individual clones of

I's and K's for the hybridizations, the locations of these fragments were successfully determined, and consequently the linkage of BamHI-K_1 to BamHI-H was moved from the region at 35–41, (i.e., MW of 35×10^6 to 41×10^6 from the left end of the genome) to the new location at 81–87. The regions BamHI-L to BamHI-K_3 (2–44) and BamHI-P_2 to BamHI-M (54–83) were further confirmed by cloning of BamHI partially digested viral DNA into cosmid pHC79 (data not shown). The order of the small fragments (M, N, P_2, P_3, R, S_1, S_2, and T) also was determined by partial digestion of cloned cosmid DNA spanning from BamHI-P_2 to BamHI-M and recloning into cosmid pHC79. The size of the BamHI A fragment shown on the map is the original size of the BamHI A fragment obtained from viral DNA.

Inverted repeat regions. Cebrian et al. (3) reported the presence of inverted repeat regions in MDV DNA and turkey herpesvirus DNA, which is also evident in herpes simplex virus types 1 and 2 DNA by electron microscopy studies (9). Figure 3a shows the homology between BamHI H and BamHI D fragments by Southern blot hybridization. Thus, BamHI-L, BamHI-I_2, and the left segment of BamHI-D, and BamHI-L, BamHI-I_2, and the right segment of BamHI-H, are obviously arranged as inverted repeats and should be considered as the long terminal region (TR_L) and the long inverted region (IR_L), respectively. Therefore, we aligned inverted repeat regions precisely according to the electron microscopy measurements of Cebrian et al. (3), in which TR_L and IR_L were found to have MWs of 10.0×10^6 and the short terminal region (TR_S) and the short inverted region (IR_S) had MWs of 8.0×10^6. This indicated that a portion of BamHI-A should be within the region encompassed by IR_S. Therefore, a radioactive ^{32}P-probe was made
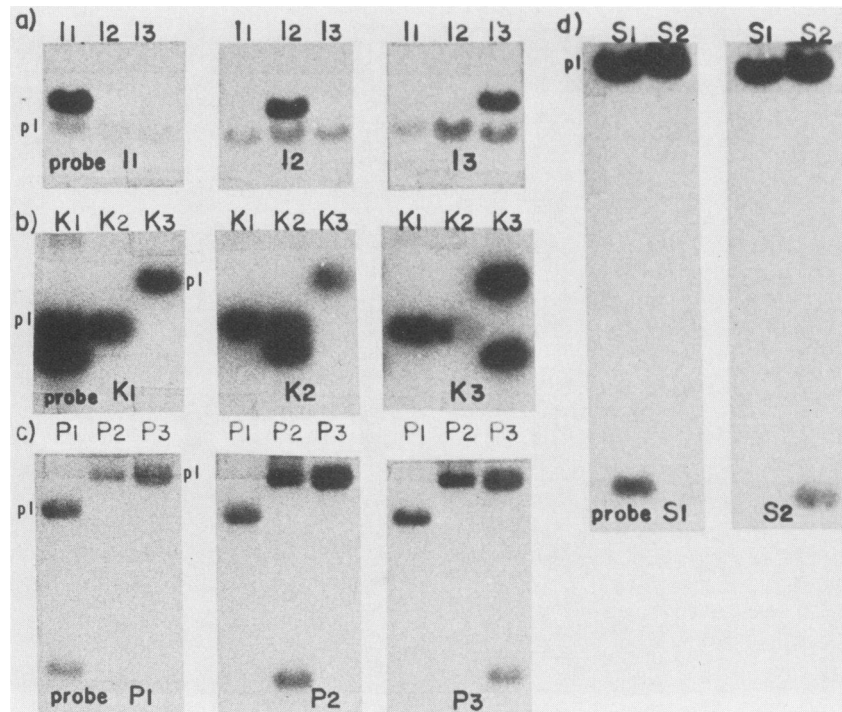

FIG. 2. Identification of individual clones of multimolar subfragments of viral DNA. BamHI digestion of viral DNA yielded multimolar subfragments of I, K, P, and S fragments. The multimolar subfragments were individually cloned. To prove that cloned DNA fragments are of different species, BamHI-digested BamHI-I, BamHI-K, BamHI-P, and BamHI-S clones were electrophoresed and blot hybridized to different ^{32}P-labeled clones of BamHI-I, BamHI-K, BamHI-P, and BamHI-S, respectively. Detection of (a) three different I subfragments, (b) three K subfragments, (c) three P subfragments, and (d) two S subfragments. pl, Cloning vectors pACYC184, pBR322, and pHC79.
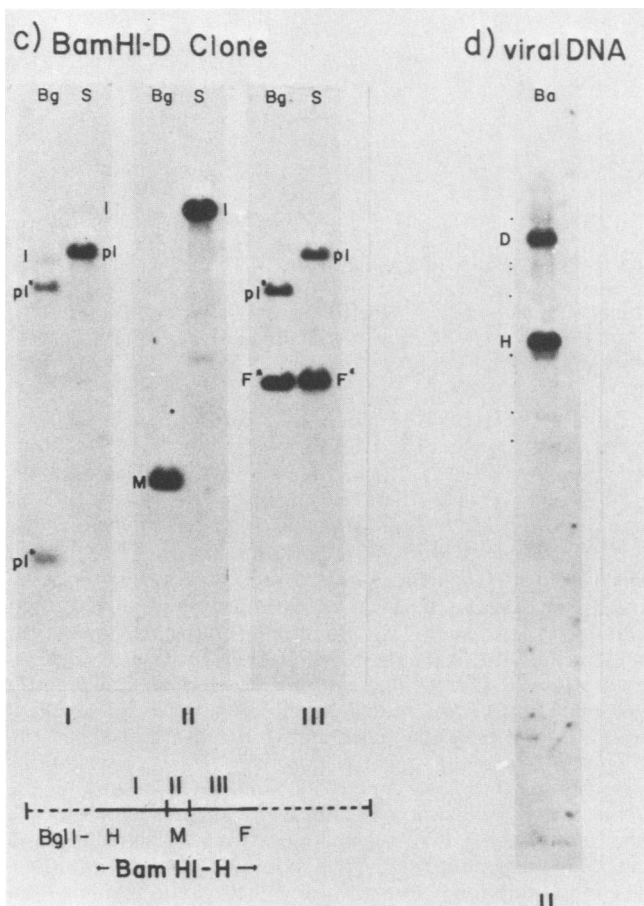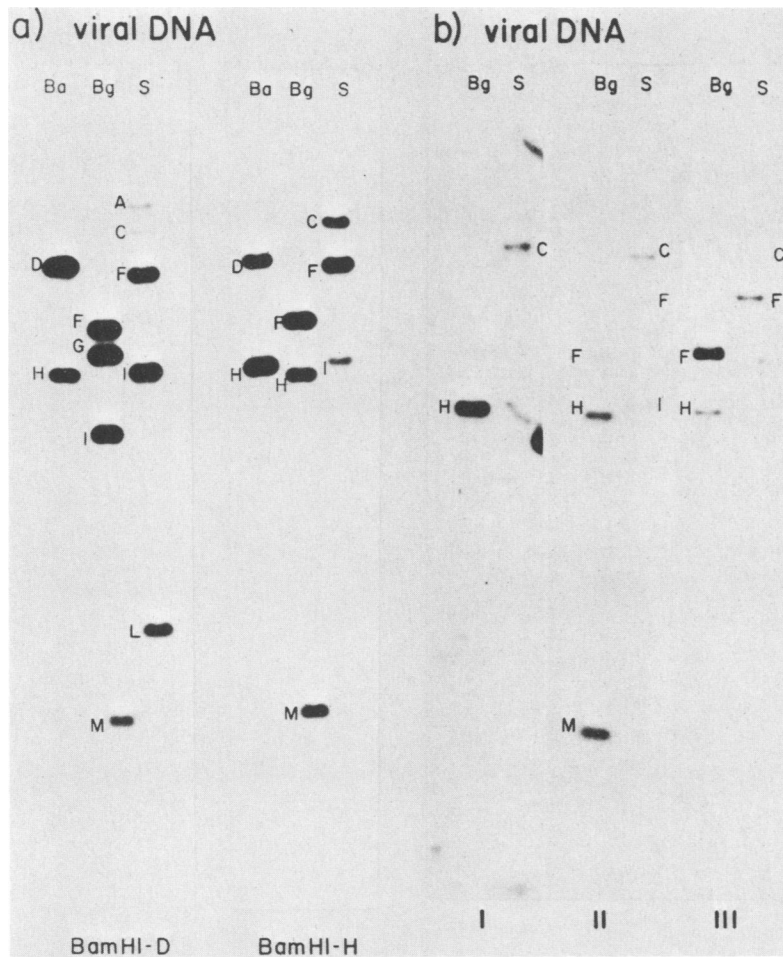
FIG. 3. Region of homology between *Bam*HI-D and *Bam*HI-H. Hybridization probes were 5'-^{32}P-labeled and hybridized to Southern blots of restriction endonuclease digests of viral DNA or to cloned DNA fragments. (a) Hybridizations of ^{32}P-labeled cloned *Bam*HI-D or *Bam*HI-H DNA to *Bam*HI-, *Bgl*I-, or *Sma*I-digested viral DNA; (b) hybridizations of the ^{32}P-labeled *Bgl*I I, *Bgl*I II, or *Bgl*I III subfragment of *Bam*HI-H to *Bgl*I- or *Sma*I-digested viral DNA; (c) hybridizations of the ^{32}P-labeled *Bgl*I I, *Bgl*I II, or *Bgl*I III subfragment of *Bam*HI-H to *Bam*HI and *Bgl*I or *Bam*HI and *Sma*I doubly digested *Bam*HI-D that has been cloned into the *Bam*HI site of pACYC184; and (d) hybridization of ^{32}P-labeled *Bgl*I-M DNA (the *Bgl*I II subfragment of *Bam*HI-H) to *Bam*HI-digested viral DNA. Ba, *Bam*HI; Bg, *Bgl*I; S, *Sma*I.
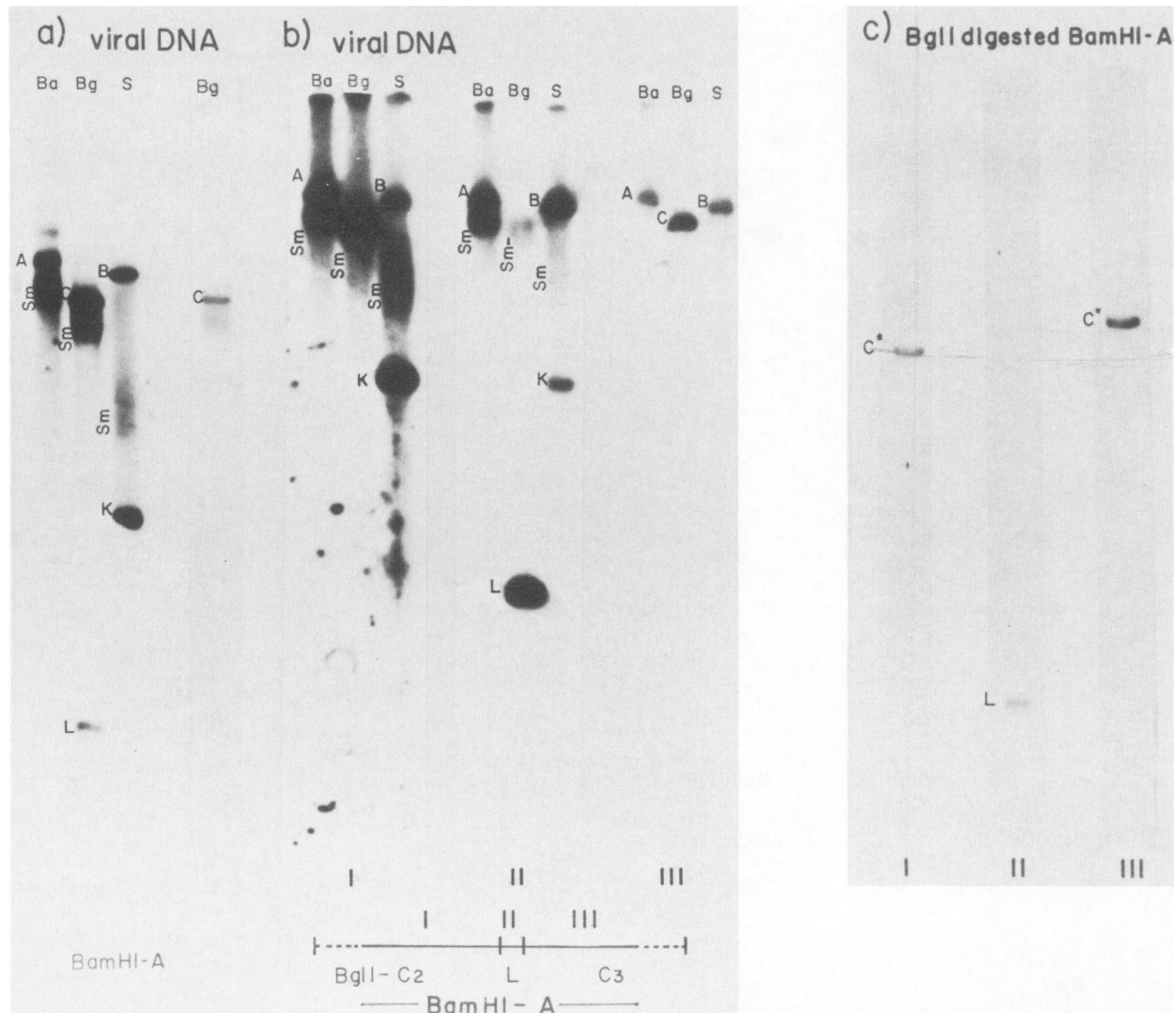
FIG. 4. The region of homology between *Bam*HI-A and the right terminal region. Hybridization probes were 5'-^{32}P-labeled and hybridized to Southern blots of restriction endonuclease digests of viral DNA or to cloned *Bam*HI-A. (a) ^{32}P-labeled *Bam*HI-A DNA hybridized to *Bam*HI-, *Bgl*I-, or *Sma*I-digested viral DNA. Column 4 (Bg) is a short exposure of column 2 (Bg); (b) the ^{32}P-labeled *Bgl*I 1, *Bgl*I II, or *Bgl*I III subfragment of *Bam*HI-A hybridized to *Bam*HI-, *Bgl*I-, or *Sma*I-digested viral DNA; and (c) the ^{32}P-labeled *Bgl*I I, *Bgl*I II, or *Bgl*I III probes hybridized to *Bgl*I-digested *Bam*HI-A DNA cloned into the *Bam*HI site of pACYC184.

with *Bam*HI-A and then blot hybridized with *Bam*HI, *Bgl*I, and *Sma*I digests of viral DNA (Fig. 4a). *Bam*HI-A probe hybridized to the smear region with an MW of 10.0×10^6, as well as to the *Bam*HI A fragment. In the *Bgl*I digest, *Bgl*I-C, the smear region, and *Bgl*I-L were hybridized with the probe, as it also hybridized to *Sma*I-B and *Sma*I-K as well as to the smear region between *Sma*I-B and *Sma*I-K. The smear region of the *Bgl*I digest was designated as the terminal region in the previous study (14), and therefore the smear region found in the *Bam*HI digest should be considered to be of the same nature. The results indicated that *Bam*HI-A hybridized to the smear terminal region located at 111–123, which should include the TR$_S$ region, and also that the terminal end of the molecule is heterogeneous.

To identify the junctions between IR$_S$ and the short unique region (U$_S$) and between U$_S$ and TR$_S$, the cloned *Bam*HI A fragment was divided into three *Bgl*I subfragments: I (part of *Bgl*I-C$_2$), II (*Bgl*I-L), and III (part of *Bgl*I-C$_3$). Since *Bgl*I cleaves the fragment close to the center, subfragments I and

III had to be separated along with pACYC184 DNA fragments. Subfragments I, II, and III did not share sequence homology with each other (Fig. 4c). When the same ^{32}P-probes of I, II, and III were hybridized with *Bam*HI, *Bgl*I, and *Sma*I digests of viral DNA, subfragment I hybridized to the smear regions of *Bam*HI, *Bgl*I, and *Sma*I digests of viral DNA, and subfragment II hybridized to the smear region of the *Bam*HI digest observed as subfragment I hybridization, whereas subfragment III did not hybridize to the smear region (Fig. 4b). It was noted that hybridization of subfragment I to *Bgl*I-C was not clearly observed (Fig. 4b), suggesting that *Bgl*I-C$_2$ may also be heterogeneous. The slight hybridization between subfragment II and *Sma*I-K or subfragment II and the smear region of *Bgl*I and *Sma*I digests may be due to the presence of partial sequence homologies. When the hybridization results were aligned with the IR$_S$ and TR$_S$ regions, it was concluded that subfragment I and part of subfragment II (*Bgl*I-L) should be placed within IR$_S$. The other junction between U$_S$ and TR$_S$ should reside within
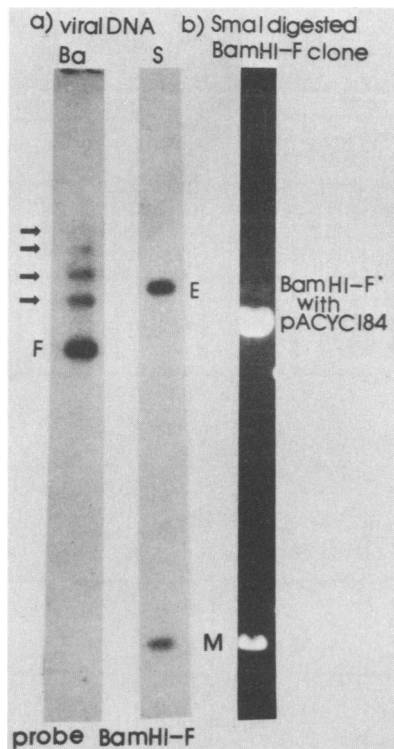
FIG. 5. Tandem repeats within *Bam*HI F fragment. Variable numbers of tandem repeats (*Sma*I M fragments) within the *Bam*HI F fragment were detected by Southern blot hybridization analysis. (a) ^{32}P-labeled *Bam*HI-F DNA hybridized to *Bam*HI- and *Sma*I-digested viral DNA; (b) ethidium bromide stain of *Sma*I-digested *Bam*HI-F DNA cloned into the *Bam*HI site of pACYC184.

*Bgl*I-C_3 left of *Bam*HI-P_1, since *Bam*HI-P_1 did not hybridize to the smear region, and the terminal IR_S should contain one *Bgl*I site near the junction.

To identify the junction between TR_L and U_{L1} and also between U_{L2} and IR_L (U_{L1} and U_{L2} are subdivisions of the long unique region $[U_L]$ and are located at 10–32 and 34–85, respectively), three of the *Bgl*I subfragments cleaved from *Bam*HI-H, I (part of *Bgl*I-H), II (*Bgl*I-M), and III (part of *Bgl*I-F), were made radioactive by 5'-labeling with $[\gamma\text{-}^{32}P]ATP$ and then hybridized with *Bam*HI-D that had been digested with *Bgl*I and *Sma*I (Fig. 3c). Subfragment I hybridized only faintly to *Bgl*I-I and *Sma*I-I in the *Bam*HI-D digest, whereas subfragment II (*Bgl*I-M) hybridized strongly to *Bgl*I-M and *Sma*I-I in the *Bam*HI-D digests. Subfragment III hybridized only to the portions of *Bgl*I-F and *Sma*I-F fragments derived from the *Bam*HI-D digest. When the same probes were hybridized with viral DNA digests by using *Bgl*I and *Sma*I (Fig. 3b), major hybridizations were observed with *Bgl*I-H and *Sma*I-C for subfragment I. The center probe, subfragment II (*Bgl*I-M), hybridized to *Bgl*I-H and to a lesser extent to *Bgl*I-F, as well as to *Bgl*I-M and *Sma*I-C, *Sma*I-F, and *Sma*I-I. This indicates that a homologous sequence is present between *Bgl*I-M and the adjacent fragment, *Bgl*I-H, and slightly present in *Bgl*I-F. Subfragment III hybridized to *Bgl*I-F and *Bgl*I-H, as well as to *Sma*I-F and *Sma*I-C. This also indicates the presence of homology between *Bgl*I-F and *Bgl*I-H segments. Subfragment I did not hybridize to *Bgl*I-M (subfragment II) or *Bgl*I-F, whereas subfragments II (*Bgl*I-M) and III hybridized to *Bgl*I-H. Thus the homologies

between subfragments II (*Bgl*I-M), III, and *Bgl*I-H should exist at the left-end portion of *Bgl*I-H; that is, within the *Bam*HI K_1 fragment. Similarly, subfragment II (*Bgl*I-M) hybridized to *Bgl*I-F faintly, whereas subfragment III did not hybridize to *Bgl*I-M (subfragment II). Therefore, the homology between subfragment II (*Bgl*I-M) and *Bgl*I-F should reside in the right-end portion of *Bgl*I-F; that is, within *Bam*HI-I_2.

Further experiments showed that subfragment II (*Bgl*I-M) hybridized not only to *Bam*HI-D and *Bam*HI-H, but also to some other fragments (Fig. 3d). Thus, these experiments indicate that the junction of IR_L and U_{L2} should be placed at the right-end region of *Bgl*I-H, and that of TR_L and U_{L1} should be placed at the left-end region of *Bgl*I-I. By alignment of the linkage map with the electron microscopy measurements of Cebrian et al. (3), the junction between IR_S and IR_L should be present to the left of the *Bam*HI A segment or within the gap to which *Bam*HI-O_2 should be assigned, as mentioned below.

Presence of direct repeat units. The presence of direct repeat units was found within the *Bam*HI F fragment when the ^{32}P-labeled DNA fragment was hybridized to the *Bam*HI and *Sma*I digest of viral DNA (Fig. 5a). The probe detected an *Sma*I fragment (0.9×10^6 MW) of molar ratio 5. The molar ratio was determined with consideration of MW and densitometry tracing. When cloned *Bam*HI F fragment in pACYC184 was digested with *Sma*I endonuclease, fragments of 0.9×10^6 MW were also obtained but with a molar ratio of 2 (Fig. 5b). When ^{32}P-labeled, cloned *Bam*HI-F was hybridized to the *Bam*HI digest of viral DNA, the probe detected, in addition to the *Bam*HI F fragment, fragments of MW 9.5×10^6, 13.1×10^6, 16.7×10^6, and 18.5×10^6. This indicates that the majority of molecules contain 2 mol of *Sma*I-M (0.9×10^6 MW) within the *Bam*HI F fragment. Some contain 6, 10, 14, and 16 U of *Sma*I-M, assuming that the difference in the MW of these bands reflects the difference in the number of repeat units.

DISCUSSION

In this paper we have shown that 27 out of 29 fragments of viral DNA were cloned into bacterial plasmids pBR322 and pACYC184 and into cosmid pHC79. The use of the cosmid vector helped this study because cloning partially *Bam*HI-digested DNA into it revealed small fragments which could not have been detected by conventional use of 1 to 2 μg of viral DNA. Digestion and ligation of partially digested cloned DNA resulted in isolation of small fragments individually. The ambiguity that was not resolved in the previous study in regards to the identity of multimolar subfragments was clarified by the cloning of individual fragments into plasmid vectors. Some of the fragments were misplaced in the previous study due to insufficient amounts of individual fragments available for hybridization, and also because individual subfragments having a multimolar ratio could not be used for hybridization. A typical example was the misplacement of *Bam*HI-K_1, which was previously located next to *Bam*HI-K_3; however, hybridization with the cloned K_1 fragment has resulted in placing the fragment in a different position. Similarly, the locations of some *Bam*HI I fragments were corrected in the present study. Two fragments, *Bam*HI-O_2 and *Bam*HI-Q_2, were not cloned. Alignment of each fragment on the map indicated three gaps (Fig. 6), all in the inverted repeat region to which these two fragments should fit by size. *Bam*HI-O_2, with a molar ratio of unity, should be placed in the gap between *Bam*HI-A and *Bam*HI-L. *Bam*HI-Q_2 should be placed next to *Bam*HI-I_2. Since the
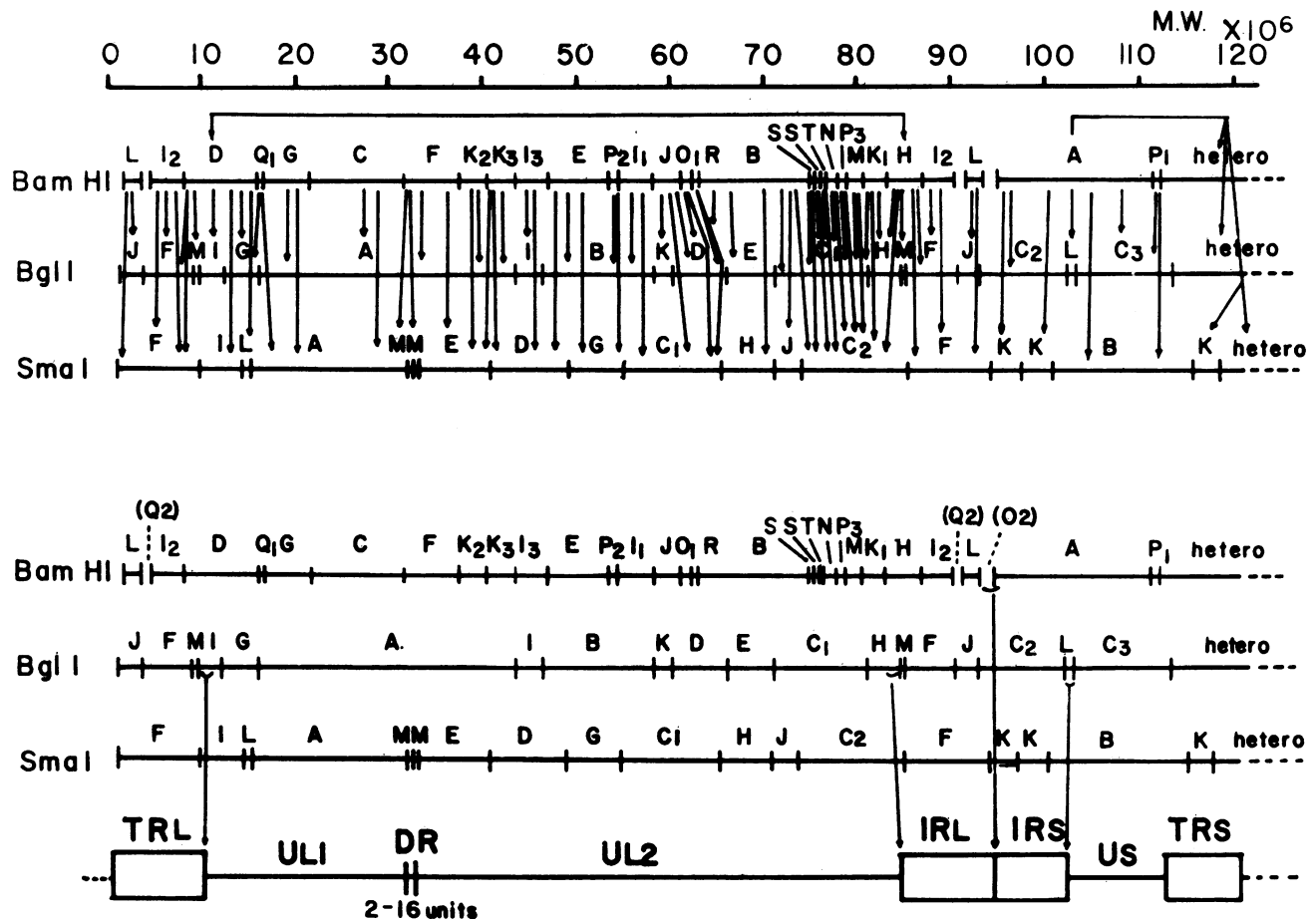
FIG. 6. *Bam*HI, *Bgl*I, and *Sma*I restriction endonuclease maps of MDV DNA. (a) Summary of hybridization of cloned *Bam*HI fragments to *Bam*HI, *Bgl*I, and *Sma*I digests of viral DNA. Arrows, Hybridization between fragments. (b) Physical map of MDV DNA. DR, Direct repeat; ⅄, junction region. Heterogeneity in the TR$_L$ region has not been detected in this study; however, Cebrian et al. (3) have reported the presence of heterogenous tails on both ends of viral DNA. The location on the map is expressed in MW ($\times$ 10^6) from the left end of the genome.

*Bam*HI-O$_2$ of unit molar ratio resides within the inverted regions, this fragment should cover the junction between IR$_S$ and IR$_L$. Most of the linkages, but not all, were confirmed by cloning *Bam*HI partially digested viral DNA into the cosmid vector.

This study revealed the presence of four unique features in the structure of MDV DNA: (i) inverted repeat regions; (ii) terminal heterogeneity, possibly in IR$_S$; (iii) simple repeat units in *Bam*HI-F; and (iv) a sequence homologous to *Bgl*I-M throughout viral DNA.

Neither inverted regions nor flip-flop structures of viral DNA were identified in the previous study. In the present study, inverted regions were identified by analysis of the linkage map of *Bam*HI fragments. The locations of *Bam*HI-L and *Bam*HI-I$_2$, together with the sequence homology with opposite orientation between *Bam*HI-H and *Bam*HI-D, clearly indicate the presence of inverted repeat regions located at 0–10 and 85–95. The other inverted repeat regions, IR$_S$ and TR$_S$, were detected by hybridization of *Bam*HI-A, which detected the presence of the homologous sequence at the terminal region, characterized by heterogeneity. *Bam*HI-A hybridized to the smear region of the *Bgl*I digest, which has already been shown to be a terminal region (14). Therefore, judging from the result of the hybridization of *Bam*HI, *Bgl*I, and *Sma*I digests of viral DNA with ^{32}P-

labeled *Bam*HI-A, together with the alignment of the linkage map to the measurement of inverted repeat regions by electron microscopy (3), IR$_S$ and TR$_S$ should be located at 95–103 and 111–119, respectively. The junction between TR$_L$ and U$_{L1}$, as well as that between U$_{L2}$ and IR$_L$, lies in proximity to the *Bgl*I M fragment. The junction between IR$_S$ and U$_S$ should be located within *Bgl*I-L, and that between U$_S$ and TR$_S$ should be located at the left end of *Bgl*I-C. The border between IR$_L$ and IR$_S$ should be located within *Bam*HI-O$_2$ as already discussed, because this fragment is of unit molar ratio and is located near the border if aligned to the electron microscopic data.

Left-terminal heterogeneity, which was observed by electron microscope (3), has not been detected in this study. This might be due to the unavailability of cloned *Bam*HI-O$_2$ for hybridization. Subfragment I, generated by digestion of *Bam*HI-A with *Bgl*I, did not seem to hybridize clearly to *Bgl*I-C but only to the smear region. Since *Bgl*I-C$_2$ should be located under *Bam*HI-A, it is possible that *Bgl*I-C$_2$ may also be heterogeneous. This must be confirmed by further study.

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