DNA of Epstein-Barr Virus

IV. Linkage Map of Restriction Enzyme Fragments of the B95-8 and W91 Strains of Epstein-Barr Virus

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The arrangement of EcoRI, Hsu I, and Sal I restriction enzyme sites in the DNA of the B95-8 and W91 isolates of Epstein-Barr virus (EBV) has been determined from the size of the single-enzyme-cleaved fragments and from blot hybridizations that identify which fragments cut from the DNA with one enzyme contain nucleotide sequences in common with fragments cut from the DNA with a second enzyme. The DNA of the B95-8 isolate was the prototype for this study. The data indicate that (i) approximately $95 \times 10^6$ to $100 \times 10^6$ daltons of EBV (B95-8) DNA is in a consistent and unique sequence arrangement. (ii) Both termini are variable in length. One end of the molecule after Hsu I endonuclease cleavage consists of approximately 3,000 base pairs, with as many as 10 additional 500-base pair segments. The opposite end of the molecule after Sal I endonuclease cleavage consists of approximately 1,500 base pairs, with as many as 10 additional 500-base pair segments. (iii) The opposite ends of the molecule contain homologous sequences. The high degree of homology between the opposite ends of the molecule and the similarity in size of the "additional" 500-base pair segments suggests that there are identical repeating units at both ends of the DNA. The arrangement of restriction endonuclease fragments of the DNA of the W91 isolate of EBV is similar to that of the B95-8 isolate and differs from the latter in the presence of approximately $7 \times 10^6$ daltons of "extra" DNA at a single site. Thus, the size of almost all EcoRI, Hsu I, and Sal I fragments of EBV (W91) DNA is identical to that of fragments of EBV (B95-8) DNA. A single EcoRI fragment, C, of EBV (W91) DNA is approximately $7 \times 10^6$ daltons larger than the corresponding EcoRI fragment of EBV (B95-8) DNA. Digestion of EBV (W91) DNA with Hsu I or Sal I restriction endonucleases produces two fragments (Hsu I D1 and D2 or Sal I G2 and G3) which differ in total size by approximately $7 \times 10^6$ daltons from the fragments of EBV (B95-8) DNA. Furthermore, the EcoRI, Hsu I, and Sal I fragments of EBV (W91) and (B95-8) DNAs, which are of similar molecular weight, have homologous nucleotide sequences. Moreover, the W91 fragments contain only sequences from a single region of the B95-8 genome. Two lines of evidence indicate that the "extra" sequences present in W91 EcoRI fragment C are viral DNA and not cellular. (i) The molecular weight of the "enlarged" EcoRI C fragment of EBV (W91) DNA is identical to that of the EcoRI C fragment of another isolate of EBV (Jijoie). (ii) The HR-1 clone of Jijoie has previously been shown to contain DNA which is not present in the B95-8 strain but is present in the EcoRI C and Hsu I D2 and D1 fragments of EBV (W91) DNA (N. Raab-Traub, R. Pritchett, and E. Kieff, J. Virol. 27:388-398, 1978).

Knowledge of the organization and structure of Epstein-Barr virus (EBV) DNA is necessary to an understanding of the biochemistry of cell transformation and virus replication but has been impeded by the relatively small quantities of virus which can be grown in the laboratory. The host range of EBV in vitro is restricted to primate B lymphocytes (14, 33). Infection of lymphocytes (7, 11, 29, 34) results in expression of a new intranuclear antigen (37), conversion of the cells to the potential for indefinite growth in vitro, and replication of virus in a variable small fraction of the infected cells. Both cell type and virus strain determine to a limited extent the frequency of permissive virus infection (6, 24, 30, 31). Marmoset lymphocytes seem to be more
permissive of virus replication than human lymphocytes and have therefore been used to reproduce virus in vitro (24, 26).

The DNAs of two isolates of EBV which have been grown in marmoset cells and of a third isolate, HR-1, have been partially characterized (10, 35, 36; E. Kieff, N. Raab-Traub, D. Given, W. King, A. T. Powell, R. Pritchett, and T. Dambaugh, in F. Rapp and G. de-The [ed.], *Oncogenesis and Herpesviruses*, in press). The B95-8 isolate was originally obtained from a lymphocyte culture from a patient with infectious mononucleosis (26), and W91 was isolated from the culture of a Burkitt tumor biopsy (23). The HR-1 virus is produced by a human lymphoblast cell line derived from a culture (Jijoye) of a Burkitt tumor biopsy (12).

Previous studies of the DNAs of purified virus have indicated the following. (i) The DNA of virus purified from HR-1 and B95-8 cultures is a linear, double-stranded DNA of approximately 100 × 10^6 daltons (35). Most DNA strands have a size less than 50 × 10^6 daltons in alkaline sucrose gradients (35). (ii) The buoyant density of EBV (strain HR-1 or B95-8) DNA is 1.718 g/cm^3 in neutral cesium chloride, suggesting a base composition of 57 to 58 mol% guanine plus cytosine (13, 20, 35, 39, 46, 47). (iii) Incubation of the DNA of the B95-8 strain of EBV with EcoRI, Hsu I, or Sal I restriction endonuclease yields 8 to 15 fragments ranging in molecular weight from less than 10^6 to more than 30 × 10^6 (10). All fragments are present in equimolar abundance, and the sum of the molecular weight of the fragments is approximately 105 × 10^6 (10). Treatment of EBV (B95-8) DNA with lambda exonuclease before cleavage with Sal I or Hsu I results in the disappearance of the Sal I A and D and Hsu I A fragments, indicating that these fragments are near the termini (10). Treatment of EBV (B95-8) DNA with Kpn I restriction endonuclease yields 15 fragments, some of which are present in submolar amounts (10). The total molecular weight of all fragments is approximately 130 × 10^6 (10). (iv) The molecular weights of some EcoRI, Hsu I, and Sal I fragments of the DNA of the EBV HR-1 strain are identical to those of fragments produced by cleavage of EBV (B95-8) DNA with the same enzyme, whereas the molecular weights of other fragments are unique to each strain (10). Limited amounts of virus could be purified from cultures of Jijoye cells, the cell line from which HR-1 was derived (12). The size of the EcoRI A fragment of EBV (Jijoye) DNA was not precisely determined (10). The size of all other fragments of EBV (Jijoye) DNA matches that of an EcoRI fragment of EBV (B95-8) and (HR-1) DNA, except that the EcoRI C fragment of EBV (Jijoye) DNA is approximately 7 × 10^6 daltons larger than the EcoRI C fragment of EBV (B95-8) DNA (10). Some Hsu I, EcoRI, and Sal I fragments of EBV (HR-1) DNA are present in half-molar abundance relative to the majority of the fragments (10). The sum of the molecular weight of the fragments produced by cleaving EBV (HR-1) DNA with Hsu I, Sal I, or EcoRI restriction endonuclease is in excess of 1.3 × 10^6 (10). (v) Kinetic and adsorptive hybridization experiments with purified viral DNA indicate that the DNA of the B95-8 strain [EBV (B95-8) DNA] shares approximately 85 to 92% of the DNA sequences of the DNA of the HR-1 strain [EBV (HR-1) DNA] (35, 43), and that EBV (B95-8) DNA lacks approximately 8 to 15% of the sequences of EBV (HR-1) DNA (35). The sequences missing in EBV (B95-8) DNA are contained in the EcoRI C and D and Hsu I E and N fragments of the HR-1 strain and in the EcoRI C and Hsu I D and E fragments are termed D1 and D2, respectively, in this study) fragments of the DNA of the W91 strain (36). (vi) Kinetic and adsorptive hybridization experiments indicate that the HR-1 virus contains more than 95% of the DNA sequences of the B95-8 strain (35). By removing from labeled EBV (B95-8) DNA all of the sequences homologous to HR-1, it is possible to demonstrate that the HR-1 virus lacks a small piece of DNA which is contained in the EcoRI A and J-K and Hsu I B fragments of EBV (B95-8) DNA and in the EcoRI A and Hsu B fragments of EBV (W91) DNA (36). (vii) Kinetic and adsorptive hybridization experiments with EBV (W91) DNA suggest that this DNA may contain all of the sequences present in both the B95-8 and HR-1 strains (36; Kieff et al., in press).

The purpose of the experiments described in this report is to determine the arrangement of restriction enzyme sites within EBV DNA. In these experiments, the linkage of restriction enzyme fragments within EBV DNA is estimated by demonstrating which fragments contain sequences that can be cleaved from EBV DNA in a single fragment by another restriction enzyme. Although it was known to contain a deletion, the DNA of virus produced by B95-8 cultures was analyzed first because of the relatively larger amounts of virus produced by B95-8 cultures. A comparative analysis of the DNA of the W91 strain was then undertaken.

**MATERIALS AND METHODS**

**Cell culture and virus purification.** Initial cultures of B95-8 and W91 cells were obtained from G. Miller, Yale University, New Haven, Conn. Cells were grown at a density of 5 × 10^6 viable cells per ml in media consisting of RPMI 1640 supplemented with...
10% fetal calf serum (both obtained from Grand Island Biological Co. [GIBCO], Grand Island, N.Y.). Cultures were maintained at 35°C and refed every 7 days by the addition of 1/3 volume of medium. At 3-month intervals cultures were switched between media containing tycloine (60 µg/ml; GIBCO) and spectinomycin (200 µg/ml; The Upjohn Co., Kalamazoo, Mich.) and media containing gentamicin (40 µg/ml; Schering Corp., Bloomfield, N.J.).

The procedures used in purifying virus from the supernatant media of cell cultures have been described (4). Briefly, 7 days after feeding, supernatant medium was harvested by aspiration from cultures which had been left unperturbed for at least 24 h. All subsequent steps were carried out at 4°C. The medium, containing virus, debris, and some cells, was centrifuged for 90 min at 9,000 rpm in a GS-3 rotor (Sorvall Corp., Newtown, Conn.). The pellet from 8 liters was suspended in 4 ml of 0.5 mM sodium phosphate, pH 7.4, homogenized with 25 strokes of a Dounce homogenizer, clarified by centrifugation for 10 min at 4,000 rpm in an SS34 rotor (Sorvall Corp.), layered onto a 5 to 30% (wt/vol) dextran gradient (dextran T10, Pharmacor Corp., Uppsala, Sweden), and centrifuged at 20,000 rpm for 1 h in an SW27 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). The single light-scattering band that formed midway between the top and bottom of the tube was removed, diluted with 0.5 mM sodium phosphate, pH 7.4, and centrifuged in an SW27 rotor for 90 min at 35,000 rpm. The virus pellet was resuspended in 0.1 M NaCl, 0.01 M EDTA, and 0.05 M Tris-hydrochloride, pH 7.4. Sodium laurel sulfate (BDH Laboratories, Poole, England) was added to a final concentration of 1% (wt/vol), and the mixture was incubated at 60°C for 2 min. The DNA solution was extracted twice with equal volumes of phenol and chloroform containing 2% (vol/vol) isoomyl alcohol (21) and was exhaustively dialyzed against 0.3 M NaCl, 0.01 M EDTA, 0.05 M Tris-hydrochloride, pH 7.6.

Restriction endonuclease treatment and separation of fragments in agarose gels. Lambda DNA (Bethesda Research Laboratories, Bethesda, Md.) was used to assay restriction enzyme activity. One unit of restriction enzyme activity was defined as that which would fully digest 1 µg of lambda DNA in 1 h in 100 µl of buffer. DNA extracted from purified EBV was incubated at 37°C at a concentration of 10 µg/ml in a solution containing 20 mM MgCl2, 20 mM NaCl, 20 mM Tris-hydrochloride, pH 7.4, and 100 U of Hsu I restriction endonuclease per ml (10); or 20 mM MgCl2, 50 mM NaCl, 100 mM Tris-hydrochloride, pH 7.4, and 100 U of EcoRI restriction endonuclease per ml (Bethesda Research Laboratories); or 20 mM MgCl2, 20 mM NaCl, 20 mM Tris-hydrochloride, pH 7.4, and 50 U of Sal I restriction endonuclease per ml (Bethesda Research Laboratories); or 10 mM MgCl2, 20 mM NaCl, 20 mM Tris-hydrochloride, pH 7.4, and 100 U of Xba I restriction endonuclease per ml (New England Biolabs, Beverly, Mass.); or 1 mM dithiothreitol, 10 mM MgCl2, 10 mM NaCl, 20 mM Tris-hydrochloride, pH 7.4, and 100 U of Xba I restriction endonuclease per ml (Bethesda Research Laboratories); or 1 mM dithiothreitol, 20 mM MgCl2, 20 mM NaCl, 20 mM Tris-hydrochloride, pH 7.4, and 100 U of Hpa I per ml (gift of Bernhard Roizman, University of Chicago). After 4 h, the digestion was terminated by the addition of 0.1 volume of a solution consisting of 60% sucrose, 0.01% (wt/vol) bromophenol blue, and 0.2 M EDTA. Up to 400 µl of reaction mixture was subjected to electrophoresis on cylindrical columns (1 by 28 cm) of 0.4% agarose (Seakem HGT, Marine Colloids, Rockland, Maine) at 1.0 V/cm for 18 h at 4°C in 0.5 µg of ethidium bromide per ml, 36 mM Tris, 1 mM EDTA, 30 mM NaH2PO4, pH 7.4 (9). The position of DNA fragments in the gels was determined by visualization under UV light. Gels were photographed with an MP4 camera with type 57 film (Polaroid Corp., Oak Brook, Ill.) and a Tiffin no. 21 filter.

Molecular weight of fragments of EBV DNA. The molecular weight of Kpn I, Xba I, and Hpa I fragments of EBV (B95-8) DNA and of Hsu I, Sal I, and EcoRI fragments of EBV (W91) DNA was determined by electrophoresis of the fragments in 0.35% agarose gels, using lambda DNA, EcoRI fragments of lambda DNA (44), and Hsu I, EcoRI, and Sal I fragments of EBV (B95-8) DNA (10) as markers. In each experiment 1 µg of intact and 2 µg of EcoRI fragments of lambda DNA were mixed with EBV DNA before electrophoresis. The relative position of lambda and EBV DNA fragments was measured in photographs of the gels and plotted against the logarithm of molecular weight of the lambda DNA fragment. The electrophoretic mobility of EcoRI, Hsu I, and Sal I fragments of EBV (W91) DNA was compared to that of EcoRI, Hsu I, and Sal I fragments of EBV (B95-8) DNA by electrophoresis of the DNA in alternate wells of a 7-mm-thick slab (22 by 28 cm) of 0.35% agarose.

Radioactive labeling of fragments of EBV DNA. Under direct visualization, slices containing individual fragments were cut from the gel. The agarose was dissolved in 5 volumes of 5 M NaClO4, 0.1 M Tris, pH 7.4 (48), at 45°C. The solution was loaded at 25°C onto a 0.25-mg column of hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories, Richmond, Calif.) which had been boiled in and pre-equilibrated with 0.04 M sodium phosphate, pH 6.8. The column was washed with 5 ml each of 0.04 and 0.18 M sodium phosphate, pH 6.8. The DNA was eluted with 1 ml of 0.5 M sodium phosphate, pH 6.8, dialyzed at 25°C against Dowex 50 (H form) in 100 ml of 1 M NaCl, 0.05 M Tris, 0.01 M EDTA to remove residual ethidium bromide, dialyzed against 5 mM Tris-hydrochloride, pH 7.4, at 4°C, and frozen at −20°C.

For radioactive labeling (16), 0.05 to 0.5 µg of DNA in 95 µl of a solution consisting of 10−10 g of activated DNAse (38), 1 mM dithiothreitol, 10 mM MgCl2, and 50 mM Tris-hydrochloride, pH 7.4, was incubated at 37°C for 5 min and then at 80°C for 10 min. Ten micromolar concentrations of each of three deoxynucleotides (P-L Laboratories, Milwaukee, Wis.), 200 pmol of 32P-labeled dCTP or dGTP (250 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and 5 U of Escherichia coli DNA polymerase I (Boehringer-Mannheim Corp., Indianapolis, Ind.) were added, and the reaction mixture was incubated at 16°C for 4 to 6 h. After the addition of 5 volumes of column buffer, labeled DNA was separated from unincorporated nucleotide on a column (1 by 30 cm) of Sephadex G50 in 0.01 M Tris, 0.001 M EDTA, 0.1% (wt/vol) Sarkosyl, pH 7.4. The fractions containing DNA were combined.
with 1 mg of calf thymus DNA (Sigma Corp., St. Louis, Mo.), adjusted to 0.5% sodium dodecyl sulfate and 0.1 M NaCl, and extracted with phenol and chloroform containing 2% (vol/vol) isooctyl alcohol, and the DNA was precipitated at -20°C by the addition of 2 volumes of ethanol. The product of the polymerase reaction had a specific activity which varied between 1 x 10^5 and 2 x 10^6 cpm/μg and sedimented at 4 to 6S in alkaline sucrose gradients (17).

Preparation of filters containing separated fragments (41) of EBV DNA and hybridization with labeled DNA in solution. Agarose gels containing separated restriction enzyme fragments of 0.5 to 1.5 μg of EBV DNA were immersed in a solution containing 0.5 N NaOH and 1.5 M NaCl for 30 min and then in a solution containing 3 M NaCl and 0.5 M Tris, pH 7.0, for 15 min. The gel was laid onto filters soaked with 20× SSC (SSC consisted of 0.15 M NaCl and 15 mM sodium citrate) and covered with a sheet of nitrocellulose (HAWP, Millipore Corp., Bedford, Mass.) and a polyethylene mask exposing only the area of the nitrocellulose in contact with the gel. Dry cellulose filters were layered over the mask to continuously remove fluid from the upper surface of the nitrocellulose. After 4 h the gel was peeled from the nitrocellulose filter. The filter was cut in half lengthwise, rinsed in 2× SSC, and dried at 70°C for 2 h. The elution of DNA from the gel was routinely monitored by UV illumination after restaining the gel in 0.5 μg of ethidium bromide per ml.

For hybridization, 10^4 to 10^6 cpm of labeled EBV DNA was mixed with 1 mg of calf thymus DNA. The DNAs were denatured in 0.2 M NaOH, neutralized with 0.2 M HCl, and incubated at 68°C in 1 ml of a solution consisting of 5 mM EDTA, 1.25 M NaCl, and 50 mM Tris-hydrochloride, pH 7.4, in a sealed 10-ml pipette containing a filter to which separated fragments of EBV DNA had been fixed. After 18 h, the filter was removed, washed for 4 h in 4× SSC at 55°C, and dried at 60°C for 1 h. The filter was then exposed to X-ray film (SB5, Kodak Corp., Rochester, N.Y.) at room temperature or to X-ray film with intensifying screen (Cronex lightning plus, E. I. du Pont de Nemours & Co., Wilmington, Del.) at -70°C.

RESULTS

The strategy used in these studies is to link restriction enzyme fragments of EBV DNA by determining their homology to a single, separated, labeled fragment of viral DNA. Two points are important in evaluating the data. (i) The linkage is unambiguous if the labeled fragment possesses homology to only a single region of the DNA compatible with the size of the fragment. In most instances, this was tested by determining whether the labeled fragment hybridized only to itself and not to another fragment of the same enzyme digest. (ii) The blotting technique is useful for determining which unlabeled DNA fragments possess homology to a specific labeled fragment if all fragments are transferred from gel to filter. In most of the experiments reported here, the fragments on the blot which possessed homology to a specific labeled fragment were identified by comparing the radiofluorogram with a radiofluorogram of the same blot made after rehybridization of the blot to labeled viral DNA. The representation of the fragments in the second autoradiogram was similar to the appearance of fragments in ethidium bromide-stained gels from which the blot was prepared, indicating that all fragments, including those in excess of 10^4, were transferred efficiently. The efficient transfer of large fragments is probably due to the relatively short single-strand length of the DNA. The DNA used in these studies was extracted from virus which was purified from the extracellular fluid of cultures, where it had accumulated over a 7- to 10-day period before harvest. Under these conditions the DNA is highly nicked and sediments in alkaline sucrose gradients (17) at 8-10S, suggesting a single-strand length of approximately 1 to 3 kilobases.

Linkage of fragments of EBV (B95-8) DNA. EcoRI fragments A through K of EBV (B95-8) DNA were individually cut from a 0.4% agarose gel, eluted from the gel, labeled in vitro with DNA polymerase I, denatured, and incubated with blots of EcoRI, Hsu I, or Sal I fragments of EBV (B95-8) DNA. The results are indicated in Table 1 and are as follows. (i) Labeled fragments F, G, I, and J specifically hybridized to unlabeled EcoRI fragments F, G, I, and J, respectively (Fig. 1A). Labeled fragments A, B, and K hybridized to fragments A, B, and K, respectively, but to a lesser extent to other fragments (Fig. 1A). (ii) On Hsu I blots (Fig. 1B) it was apparent that labeled EcoRI fragment A hybridized specifically to Hsu I fragments A and B; B fragment hybridized specifically to Hsu I fragments C, E, and I; C fragment to Hsu I fragments D and F; the D-E fragments to C and D and to multiple discrete bands extending from I to the region between E and F in a stepwise pattern with approximately 10 increments (terned Hsu I het); the F fragment to G and H; the 2 molar G fragment hybridized to B, G, and J; H fragment to F, K, and L; the I fragment to A; the J fragment to A; and the labeled EcoRI K fragment to Hsu I fragment E. (iii) On Sal I blots (Fig. 1C) labeled EcoRI fragment A hybridized specifically to Sal I fragments A, E, and G; B fragment to Sal I fragments B, C, H, and F; C fragment to fragments B and D; D-E fragments to fragments B, D, and to multiple discrete bands extending from past H to E in approximately 10 stepwise increments (terned Sal I het); F fragment to C; G fragment to C and E; H fragment to B; I fragment to A and to Sal I het; J fragment to A; and K fragment to C. From these data (summarized in Table 1), the known
TABLE 1. Hybridization of labeled restriction enzyme fragments of EBV (B95-8) DNA to blots of fragments of EBV DNA

<table>
<thead>
<tr>
<th>³²P-labeled fragments of EBV (B95-8) DNA</th>
<th>EBV (B95-8) DNA on blot</th>
<th>EBV (W91) DNA on blot</th>
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<td>C</td>
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Sal

| B                                     | A | A, I, J | A |     |     |     |
| B                                     | B | C, E, B, H | C, D, F |     |     |     |
| C                                     | C | B, F | E, G, H, I, J |     |     |     |
| D                                     | D, het | C, D, het | D, het | C, D | D, het | D |
| E                                     | E | A, G | B |     |     |     |
| F                                     | F |     | C, I |     |     |     |
| G                                     |     | G |     |     |     |     |
| H                                     | H, D, het | B, het, D | C, het |     |     |     |

Eco

| B                                     | A | A, E, G | A | A, B | A |     |
| C                                     | B, D | D, F | C | D₁, D₂, F | B, D, G₂ |     |
| D, E                                 | B, D, het | C, D, het | D, E |     |     |     |
| F                                     | F |     | G, H | F |     |     |
| G                                     | C, E | G | B, J, G | G |     |     |
| H                                     | B | H | F, K, L | H |     |     |
| I                                     | A, het | I | A | I | A | A |
| J                                     | A | J | A |     |     |     |
| K                                     | C | K | E |     |     |     |

Xba-A


Hpa-B


size of the fragments (10), and the previous results of lambda exonuclease degradation, which indicated that Hsu I-A and Sal I-A and -D are near termini (10) and that EcoRI fragment I precedes J at the terminus (D. Hayward and E. Kieff, unpublished observations), a tentative linkage map could be constructed (Fig. 4A).

To link the EcoRI fragments and to triply cross check the results, Hsu I fragments A through E and K and L and Sal I fragments A through H of EBV (B95-8) DNA were individually cut from an agarose gel, eluted from the gel, labeled in vitro with DNA polymerase I, denatured, and incubated with blots of EcoRI, Hsu I, or Sal I fragments. The results are indicated in Table 1 and are as follows. (i) Labeled Hsu I fragment A hybridized specifically to Hsu I-A and to Hsu I-I; labeled Hsu I-B hybridized to B and, to a lesser extent, to A; labeled Hsu I-C hybridized almost exclusively to C, D to D, and E to E (Fig. 2A). (ii) Labeled Hsu I fragment A hybridized to EcoRI fragments A, I, D, and J and to multiple discrete bands located from near I to past K (termed EcoRI het, Fig. 2B); labeled Hsu I-B hybridized to EcoRI fragments A and G; to fragments B and E; D to C and D; E to B; K to H; and Hsu I fragment L hybridized preferentially to EcoRI fragments H and E (Fig. 2B). (iii) Labeled Hsu I fragment A hybrid-
ized specifically to Sal I fragments A, D, and Sal I het; labeled Hsd I fragment B to fragments A, E, G, and C to B, F, and H; D to B and D; and labeled Hsd I fragment E hybridized to Sal I fragment C (Fig. 2C). (iv) The Sal I A and Sal I B fragments of EBV (B95-8) DNA were separated by 3 mm in the gel from which the fragments were cut for labeling. Labeled Sal I fragment A hybridized predominantly to Sal I fragment A (Fig. 3A), to EcoRI fragment A (Fig. 3B), and to Hsd I fragment A (Fig. 3C). Many other fragments were visible in the autoradiograms to a lesser extent. Labeled Sal I fragment B hybridized to Sal I fragment B and, to a lesser extent, to Sal I fragment A (Fig. 3A); to EcoRI fragments B, C, E, and H (Fig. 3B); and to Hsd I fragments C, D, and F (Fig. 3C). The failure to observe hybridization of the labeled Sal I B fragment to Hsd I fragments K and L was due to the fact that these small fragments had moved past the end of the gel from which this blot was made. The hybridization of labeled Sal I-B to Sal I fragment A, Hsd I fragment A, and EcoRI fragment A was probably due to contamination of the Sal I fragment B with Sal I fragment A. Labeled Sal I fragment C hybridized specifically to Sal I fragment C (Fig. 3A); to EcoRI fragments B and F (Fig. 3B); and to Hsd I fragments E, G, H, I, and J (Fig. 3C). Sal I fragment D consisted of multiple discrete bands. However, two components of average mobility for the cluster were present in equal amounts and in excess of the other components. The Sal I fragments were subjected to electrophoresis on 0.3% agarose gels for 24 h to achieve separation between the two major components of Sal I-D. The two components were individually labeled and hybridized to blots of Sal I, Hsd I, or EcoRI fragments. Both components yielded identical results (data not shown). Labeled Sal I D fragment hybridized to Sal I fragment D and to Sal I het (Fig. 3A); to EcoRI fragments C and D and to multiple discrete bands from past J to H in approximately 10 stepwise increments (termed EcoRI het, Fig. 3B); to Hsd I-D and Hsd I het (Fig. 3C). Labeled Sal I fragment E hybridized specifically to Sal I fragment E (Fig. 3A); to EcoRI fragments A and G (Fig. 3B); and to Hsd I fragment B (Fig. 3C). Labeled Sal I fragment F hybridized specifically to Sal I-F (Fig. 3A) and to Hsd I fragments C and I (Fig. 3C). Labeled Sal I fragment G hybridized specifically to Sal I fragment G (Fig. 3A). Labeled Sal I fragment H was contaminated with Sal I het and hybridized to Sal I fragments H, D, and Sal I het (Fig. 3A); to EcoRI-B, -D, and EcoRI het (Fig. 3B); and to Hsd I-C and Hsd I het (Fig. 3C). The Hsd I and Sal I data are listed in Table 1 and diagrammatically represented in Fig. 4B and 4C, respectively. The composite EcoRI, Hsd I, and Sal I linkage data are summarized in Fig. 4D.

No large fragments provided linkage between Hsd I-B and -J or between EcoRI-G and -F, or between Sal I-E and -C. To verify this linkage, a search was made for a fragment of another enzyme which would cross this region. EBV (B95-8) DNA was incubated with Kpn I, Xba I, or Hpa I (Fig. 5), and the size of fragments was determined by electrophoresis in agarose gels, using lambda DNA and EcoRI fragments of lambda DNA as internal markers. Treatment of EBV (B95-8) DNA with Kpn I had previously been found to yield fragments present in molar amounts, as indicated in Fig. 5, and several fragments in submolar amounts. The largest of the submolar fragments was approximately 22 \times 10^6 and had been termed fragment B (10). The concentration of Kpn I enzyme used in the previous study was severalfold in excess of that required to digest T\sb{b}\sb{1} DNA, and T\sb{b}\sb{1} DNA included in the EBV DNA Kpn I digestion reaction was completely digested (10). In preparation for this series of experiments Kpn I was standardized using T\sb{b}\sb{1} and lambda DNA. The enzyme was found to deteriorate rapidly over several weeks of storage at -20°C in a buffer consisting of 0.2 M KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM Tris-hydrochloride, pH 7.4, and 0.2 mg of bovine serum albumin per ml. At a time when 2.5 \mu l of Kpn I completely digested 2 \mu g of lambda DNA in 100 \mu l, 30 \mu l of Kpn I incubated with 2 \mu g of EBV DNA in 300 \mu l gave the same result as that obtained previously. However, hybridization of labeled EcoRI, Sal I, or Hsd I fragments of EBV DNA to blots of separated Kpn I fragments indicates that the fragment previously termed B hybridizes to the same labeled fragments as Kpn I fragment A and therefore more likely arises from variation in the size of the A fragment than as an artifact of incomplete digestion. Moreover, no large Kpn I fragment extends from Hsd I fragment B through Hsd I fragment J (Kieff et al., in press; Given and Kieff, manuscript in preparation).

Fig. 2. Autoradiograms of blots of Hsd I (A), EcoRI (B), or Sal I (C) restriction endonuclease fragments of EBV (B95-8) DNA which had been incubated with \textsuperscript{32}P-labeled EBV (B95-8) DNA (designated "total") or with \textsuperscript{32}P-labeled separated Hsd I restriction endonuclease fragments of EBV (B95-8) DNA (designated by fragment letter at the top of each blot). The preparation of blots, separation of fragments of EBV DNA, conditions of hybridization, and procedures for radiofluorography are described in the text.
To determine which Xba I or Hpa I fragments would be useful for demonstrating linkage to the right of Hsu I fragment B, blots of Hpa I or Xba I digests were incubated with labeled Hsu I fragment B or with labeled EcoRI fragment A, which overlaps Hsu I-B and extends to the left (Fig. 4). Labeled Hsu I fragment B hybridized to Xba I fragment A and to Hpa I fragments B and F. Labeled EcoRI-A hybridized to Hpa I fragments A, B, and F. These data indicated that Xba I A fragment and Hpa I B fragment were likely to provide useful linkage data. Xba I A and Hpa I B fragments were therefore cut out of gels, labeled, denatured, and incubated with blots of Sal I, EcoRI, and Hsu I digests of EBV (B95-8) DNA. The results were as follows: Xba I fragment A (Fig. 6) specifically hybridized to EcoRI fragments I, J, A, G, and possibly EcoRI het, to Sal I fragments A, E, and G, and to Hsu I fragments A, B, and J. Labeled Hpa I fragment B (Fig. 6) hybridized specifically to EcoRI fragments A, G, and F, to Hsu I fragments A, B, G, and J, and to Sal I fragments A, C, E, and G.

To demonstrate that the heterogeneity in electrophoretic mobility of the terminal fragments was due to variability in the length of duplex DNA and not to single-strand tails, EBV DNA was treated with Sal I restriction endonuclease and then with activated pancreatic DNAse to make single-strand nicks at each 300 to 500 bases. The activity of the pancreatic DNAse was assayed, using 3H-labeled PMB9 as an internal marker (gift of H. Avni and A. Markovitz, University of Chicago). The extent of nicking produced by the DNase was determined by velocity sedimentation of an aliquot of the reaction mixture in alkaline sucrose gradients (17). The DNA was then subjected to electrophoresis in a 0.33% agarose gel transferred to a filter and incubated with 32P-labeled EBV (B95-8) DNA or 32P-labeled Sal I D fragment of EBV (B95-8) DNA. The results (Fig. 7) indicate that (i) there is no decrease in the abundance of the terminal heterogeneous bands after DNase treatment, and (ii) there is a slight decrease in the relative amount of high-molecular-weight Sal I fragment A. These data indicate that the heterogeneous fragments are not more susceptible to DNase than other fragments of EBV DNA and are therefore unlikely to contain single-strand regions as long as 300 to 500 bases.

**Linkage of fragments of EBV (W91) DNA.** The size of EcoRI, Sal I, and Hsu I fragments of EBV (W91) DNA (Fig. 8) was determined by electrophoresis in 0.4% agarose gels, using lambda DNA and EcoRI fragments of lambda DNA as internal markers and using EcoRI, Sal I, and Hsu I fragments of EBV (B95-8) DNA in adjacent wells of a 0.35% slab gel. The size of each of the fragments of DNA of the W91 strain was indistinguishable from that of the corresponding fragment of EBV (B95-8) DNA (10), with the following exceptions. (i) The EcoRI C fragment of EBV (W91) DNA is approximately 19 × 10^6, whereas the C fragment of B95-8 DNA is 12 × 10^6. (ii) Hsu I digestion of EBV (W91) DNA produces two fragments, D1 and D3, of approximately 11 × 10^6 and 10 × 10^6, respectively, instead of Hsu I fragment D of EBV (B95-8) DNA, which is 14 × 10^6. EBV (W91) DNA has two Sal I fragments, G2 and G3, of 3.4 and 3 megadaltons, which are not present in the Sal I digest of EBV (B95-8) DNA. In addition, the Sal I B fragment of EBV (W91) DNA has a slightly slower electrophoretic mobility than the Sal I B fragment of EBV (B95-8) DNA, suggesting that it is approximately 2 megadaltons larger.

These data suggested the possibility that the arrangement of sequences of EBV (W91) DNA could be similar to that of EBV (B95-8) DNA and that the major difference between the DNA of the two strains might be that the DNA of the W91 strain contains approximately 7 × 10^6 of "extra" DNA in the Hsu I-D–EcoRI-C overlap region of EBV (B95-8) DNA. To confirm this, blots containing separated unlabelled Hsu I, EcoRI, or Sal I fragments of EBV (W91) DNA were hybridized with labeled fragments of EBV (B95-8) DNA. The results are indicated in Table 1 and are as follows. (i) Labeled EBV (B95-8) DNA EcoRI fragments A–I hybridized specifically to the corresponding EcoRI fragment of EBV (W91) DNA (Fig. 9A). In several instances (Fig. 9A) the same blot was used for two or three sequential hybridizations and autoradiograms with different fragments. Therefore, hybridization of the second or third probe to fragments to which the first probe had hybridized would not have been recognized in these experiments. Nevertheless, the data clearly indicate a high degree of sequence homology between each EcoRI fragment of EBV (B95-8) DNA and the
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\[ \text{Summary of linkage} \]

\[ \text{32P-labeled EcoRI fragments} \]

\[ \text{32P-labeled HsuI fragments} \]

\[ \text{32P-labeled SalI fragments} \]

\[ \text{0} \quad 10 \quad 20 \quad 30 \quad 40 \quad 50 \quad 60 \quad 70 \quad 80 \quad 90 \quad 100 \quad \times 10^6 \text{ daltons} \]
corresponding EcoRI fragment of EBV (W91) DNA. (ii) To further confirm the homology between EBV (B95-8) and (W91) DNA restriction enzyme fragments of similar size and to demonstrate that each W91 fragment contained sequences from only a single region of the B95-8 genome, selected large fragments of EBV (B95-8) DNA which span restriction enzyme sites and which together cover the entire genome were hybridized to EcoRI, Sal I, or Hsu I blots of EBV (W91) DNA. Labeled EBV (B95-8) DNA EcoRI fragment C hybridized specifically to EBV (W91) DNA Hsu I fragments D, D, and F (Fig. 9B) and to Sal I fragments B, D, and G2 (Fig. 9C). Labeled EBV (B95-8) DNA EcoRI fragment I hybridized specifically to EBV (W91) Hsu I fragment A (Fig. 9B) and Sal I fragment A (Fig. 9C). Labeled EcoRI fragment B of EBV (B95-8) DNA hybridized to Sal I fragments B, C, F, and H of EBV (W91) DNA. Labeled Hsu I fragment A of EBV (B95-8) DNA hybridized only to the Hsu I A (Fig. 9B) and Sal I A fragments of EBV (W91) DNA (Fig. 9C). Labeled Hsu I fragment B of EBV (B95-8) DNA hybridized to Hsu I-B (Fig. 9B) and Sal I A, E, C, and G (Fig. 9C) fragments of EBV (W91) DNA. Labeled Sal I D fragment of EBV (B95-8) DNA hybridized primarily to Hsu I-D and Hsu I het (Fig. 9B) and to Sal I-D (Fig. 9C) of EBV (W91) DNA. Labeled Xba I A fragment of EBV (B95-8) DNA hybridized to the Hsu I A and B fragments of EBV (W91) DNA. Labeled Hpa I B fragment of EBV (B95-8) DNA hybridized to W91 Hsu I fragments A, B, G, and J. The hybridization data are summarized in Table 1 and Fig. 10 and indicate that EBV (W91) and (B95-8) DNAs have identical sequence arrangements and that W91 DNA contains additional DNA inserted at a single locus.

**DISCUSSION**

Two lines of evidence indicate that there is a consistent and unique sequence arrangement of the DNA of the B95-8 strain (Fig. 11). Thus, (i) treatment of EBV (B95-8) DNA with EcoRI, Sal I, or Hsu I restriction endonuclease yields fragments present in equal molar abundance, and the sum of the molecular weights of the fragments produced by cleavage with each enzyme is approximately 10^6 (10), the known molecular weight of EBV (B95-8) DNA (35). The previous data (10) included a discordant finding, i.e., that digestion of EBV DNA with Kpn I restriction endonuclease yields at least one fragment present in submolar amounts. Subsequent analysis of the fragments produced by cleavage of EBV (B95-8) DNA with Kpn I restriction endonuclease indicates that the fragment previously termed B maps in the same region of the DNA as the Kpn I A fragment and reflects variation in the size of the A fragment (Given and Kieff, manuscript in preparation). (ii) The analysis reported here of homology between fragments generated by treatment with different restriction enzymes indicates that there is a unique order of fragments with little homology between separate portions of the genome. An exception is the homology seen between fragments containing the opposite ends of the DNA.

Heterogeneity had been observed previously in the Sal I D and EcoRI D fragments of EBV (B95-8) DNA (10). The disappearance of the heterogeneous Sal I D fragment after limited lambda exonuclease digestion of EBV DNA before incubation with restriction enzyme indicates that the Sal I D fragment is at or near the terminus (10). The data obtained in this study confirm that Sal I-D is a terminal fragment and
indicate that (i) cleavage of EBV (B95-8) DNA with EcoRI, Hsu I, or Sal I results in each instance in two fragments with heterogeneous electrophoretic mobility, EcoRI-D and EcoRI het, Hsu I-A and Hsu I het, and Sal I-D and Sal I het. (ii) The heterogeneity is unlikely to be due to variable lengths of single-strand regions in the DNA since treatment of the DNA with DNase before digestion with restriction enzyme did not alter the distribution of the heterogeneous bands. (iii) Two series of observations suggest (but do not directly prove) that the heterogeneity in electrophoretic mobility of Hsu I-A, Sal I-D, EcoRI-D, Hsu I het, Sal I het, and EcoRI het is due to variation in the number of copies of a reiterated sequence. First, the difference in electrophoretic mobility of the components of the heterogeneous fragments appears to be constant. The heterogeneity is clearly resolved into 8 to 12 components when the terminal fragment is small (EcoRI het [Fig. 3B], Sal I het [Fig. 1C], or Hsu I het [Fig. 1B]), less clearly resolved in the case of Sal I-D (Fig. 2C) and EcoRI-D (Fig. 2B), and poorly resolved in blots of Hsu I-A (Fig. 2A). From the known size of fragments (10) which have electrophoretic mobility similar to that of the heterogeneous fragments, the variability in size between the fastest and slowest components of Sal I-D (10 × 10^6 to 13 × 10^6), EcoRI-D (6.5 × 10^6 to 9.5 × 10^6), Hsu I het (2 × 10^6 to 5 × 10^6), Sal I het (1 × 10^6 to 4 × 10^6), or EcoRI het (0.5 × 10^6 to 3.5 × 10^6) is estimated to be 3 megadaltons. From the difference in electrophoretic mobility of individual components of Sal I het, EcoRI het, and Hsu I het, the components are estimated to vary in size by similar increments of approximately 3 × 10^5. This estimate agrees well with an estimate of the average variation, 3 × 10^5, derived by dividing the overall variation of 3 × 10^6 by the number of components (8 to 12). Second, the heterogeneous fragments preferentially hybrid-
ize to labeled EBV (B95-8) DNA, as would be expected if they contained reiterated sequences. Thus, the heterogeneous fragments are more apparent relative to other fragments in radiofluorograms and autoradiograms of blots of fragments hybridized with 32P-labeled EBV (B95-8) DNA (e.g., Sal I het in Fig. 1C, 2C, and 3A) than in ethidium bromide-stained gels from which the blots were prepared or in autoradiograms of 32P-labeled fragments of EBV (B95-8) DNA. (iv) The Hsu I het and the heterogeneous EcoRI D fragment clearly map within the Sal I-D terminus (Fig. 1B, 2B, 3B and C and 4). From the hybridization data with labeled EcoRI fragment C and labeled Hsu I fragment D which place Hsu I-D internal to Hsu I het and from the estimated size of the terminal Hsu I het, EcoRI D, and Sal I D fragments, the order of restriction endonuclease sites at the Sal I-D terminus is Sal I site, approximately 5,000 base pairs; EcoRI site, approximately 6,500 base pairs; Hsu I site; and a terminus consisting of approximately 3,000 base pairs with between 1 and 10 additional units of 400 to 500 base pairs. The arrangement of restriction enzyme sites at the Hsu I-A end is less precisely known. From their variable size and homology to Hsu I-A and lack of homology to EcoRI-J and -A and Sal I-A, EcoRI het and Sal I het must lie very near the terminal end of Hsu I-A. The penultimate EcoRI-I hybridizes to Sal I-A and Sal I het (Fig. 1C) and therefore must extend from the Sal I A fragment to within 1,500 base pairs of the terminal variability in Sal I het. (v) The terminal fragments from both ends of the DNA possess homology. Thus, Sal I-D hybridizes to Sal I het (Fig. 3C) and Hsu I-A hybridizes to Hsu I het (Fig. 2B). The homology is in the terminal part of these fragments since the penultimate EcoRI I (Fig. 1), Sal I A (Fig 3), and Hsu I D (Fig. 2) fragments possess no homology to their opposing termini. From the extent of hybridization of Sal I-D to Sal I het (Fig. 3A) and from the lack of hybridization of Hsu I-D to Sal I het (Fig. 2C), it is likely that the homology between opposite ends of the molecule is in terminal, reiterated sequences.

Although the B95-8 strain of EBV is similar to most other isolates in its ability to transform uninfected B lymphocytes into lymphocytes capable of long-term growth in vitro and in its ability to replicate in a small fraction of transformed cells (22, 23, 25), kinetic and absorptive hybridization data (36) indicate that the B95-8 DNA is missing approximately 10% of the sequences of the HR-1 strain. The sequences deleted in EBV (B95-8) DNA are contained in the EcoRI C and D and Hsu I E and N fragments of DNA of Epstein-Barr Virus. IV.
A

B95-8 EcoRI Fragments on W91 EcoRI Blots

Total F B F B DE G A G A DE H I

B

B95-8 Fragments on W91 HsuI Blots

Total HsuA HsuB XbaA EcoC EcoI Sol D Sol D HpaB

C

B95-8 Fragments on W91 SalI Blots

Total HsuA HsuB EcoC EcoI EcoB Sol D
the HR-1 strain and in the EcoRI C and Hsu I D1 and D2 (previously termed E; 36) fragments of the W91 strain (36; Kieff et al., in press). The analysis of EBV (W91) DNA in this report indicates that this DNA is nearly identical in structure and organization to the B95-8 DNA and differs from the DNA of the B95-8 strain in the presence of this extra DNA in the EcoRI C, Hsu I D2 and D1, and Sal I G2 and G3 fragments (Fig. 11). Two lines of evidence indicate that the extra DNA in EBV (W91) EcoRI C and Hsu I D1 and D2 fragments is viral DNA and not cellular. Thus, (i) hybridization data with labeled EBV (HR-1) DNA from which sequences homologous to EBV (B95-8) DNA had been removed indicate that the extra DNA contained in EBV (W91) DNA Hsu I D2 and D1 fragments and in the overlapping EcoRI C fragment is homologous to EBV (HR-1) DNA (36). (ii) The HR-1 strain was derived from a lymphocyte clone of the Jijoye Burkitt tumor lymphoblast culture (12). There is a striking similarity between the size of the EcoRI fragments of EBV (W91) DNA and those of EBV (Jijoye) DNA (10). The EcoRI C fragment of EBV (Jijoye) DNA is approximately 7 to 8 megadaltons larger than that of EBV (B95-8) DNA (10) and identical in size to that of EcoRI C fragment of EBV (W91) DNA. The similarity in size of the EcoRI fragments of EBV (W91) and (Jijoye) (10) DNAs suggests that EBV (Jijoye) DNA is similar in structure and sequence arrangement to EBV (W91) DNA.

Several aspects of the data reported here require comment and further investigation. (i) The precision of the map of restriction enzyme sites in EBV DNA (Fig. 11) is dependent on the accuracy of determination of the size of fragments. The variation in determination of the size of fragments is as much as 10% for fragments in excess of 2 \times 10^7 and 20% for fragments larger than 3 \times 10^7. In regions of the genome with a low density of EcoRI, Sal I, and Hsu I restriction endonuclease sites, this may result in error in the map distance (Fig. 11) between restriction enzyme sites. Determination of the size of fragments produced by simultaneous or sequential cleavage of the DNA with two enzymes and the use of additional restriction enzymes which have cut sites in these regions of the DNA should improve the precision of the map. Thus, the size of the EcoRI A–Hsu I A overlap region has been determined to be 25 \times 10^6 by electrophoretic analysis of EBV (B95-8) DNA fragments produced by double digestion with EcoRI and Hsu I (Given and Kieff, manuscript in preparation). (ii) We cannot exclude the possibility that there might exist alternative arrangements of DNA within regions of the genome which do not have EcoRI, Sal I, or Hsu I restriction sites. (iii) It is not known if the homologous sequences at the ends of the DNA are on the same strand or on the opposite strands. Circular DNA molecules differing 10% from the size of viral DNA have been found in cells infected with the B95-8 strain of EBV (1). If the homology is between sequences on opposite strands and reiterated, the terminal homologous sequences could be a mechanism by which circular DNA molecules of different sizes could arise after infection. (iv) The function(s) of the DNA sequences missing in EBV (B95-8) DNA is not known. Although previous studies have indicated that the DNA of cells infected with virus from outside the Burkitt endemic region possess at least 90% of the DNA of the HR-1 strain (6, 15, 18, 32), it is possible that some of the extra DNA present in the W91 and Jijoye-HR-1 Burkitt tumor isolates may be unique to isolates from the Burkitt endemic region.

The structure and organization of the DNA of several herpesviruses have been investigated. Three types of structures have been described. Herpes simplex type 1 (8, 40, 45), herpes simplex type 2 (27), pseudorabies (42), and bovine mastitis (3) virus DNAs have similar molecular weights (10^8, 10^7, 9.2 \times 10^6, and 10^7, respectively) and consist of a long and a short segment which invert relative to each other during virus replication, producing a population of DNA molecules which varies in sequence arrangement. *Herpesvirus aeteles* (5) and *H. saimiri* (2) DNAs have molecular weights of 90 \times 10^6 and 100 \times 10^6, respectively. Both consist of a unique region of approximately 70 \times 10^6 of DNA with a low guanine-plus-cytosine content (36 to 38 mol%) bounded on both sides by repetitive DNA of high guanine-plus-cytosine content (72 to 75 mol%). Analysis of partially denatured molecules of human cytomegalovirus DNA (19) and of the sedimentation properties of murine cytomegalovirus DNA (28) suggests that cytomegaloviruses may differ from the other herpesviruses in that the full complexity of viral DNA may be contained in molecules of 1.3 \times 10^6 to 1.5 \times 10^6 daltons. Of these three types of structures, EBV seems to be similar to *H. aeteles* and *H. saimiri* in that both DNAs consist of long,

![Fig. 9. Radiofluorograms of blots of EcoRI (A), Hsu I (B), or Sal I (C) fragments of EBV (W91) DNA which had been incubated with ^32P-labeled EBV DNA (designated "total") or with separated ^32P-labeled EcoRI, Sal I, Hpa I, Hsu I, or Xba I fragments of EBV (B95-8) DNA.](http://jvi.asm.org/Downloaded from http://jvi.asm.org)
FIG. 10. Summary of homology between labeled EcoRI, Hsu I, and Sal I fragments of EBV (B95-8) DNA and unlabeled fragments of EBV (W91) DNA.
labeled unique regions of DNA bounded at each terminus by homologous reiterated sequences.

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ADDITIONAL

Treatment of EBV (B95-8) DNA with Bam I and Bgl II yields fragments of $2 \times 10^8$ daltons which are present in supramolar amounts (10). Rymo and Forsblum (Nucleic Acid Res. 5:1387-1402, 1978) have recently reported that the $2 \times 10^8$-dalton Bam I fragment, Bam I-S, is present in 11-fold excess of other fragments and that complementary RNA to Bam I-S hybridizes almost exclusively to the EcoRI A and Hsu I A fragments. We have confirmed these observations. Bam I S fragment, purified by electrophoresis and re-electrophoresis in 20-cm 0.8% agarose gels and labeled in vitro with dCTP and E. coli DNA polymerase I, hybridizes almost exclusively to the A fragments of EcoRI and Hsu I and to the Hsu I A/EcoRI C fragment of the Hsu I-F and EcoRI-C double digest. However, hybridization is also evident to the Hsu I F and EcoRI C fragments. These data suggest that Bam I-S consists of 8 to 10 copies of a $2 \times 10^8$-dalton sequence tandemly reiterated in the Hsu I A/EcoRI A fragment and one or two copies of a second $2 \times 10^8$-dalton sequence contained in the Hsu I-F and EcoRI-C overlap region. Furthermore, the $\alpha_{32P}$-labeled Bam I S fragment DNA hybridizes extensively to the supramolar $2 \times 10^8$-dalton Bgl II fragment (10).

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