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DNA of Epstein-Barr Virus

VI. Mapping of the Internal Tandem Reiteration

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Epstein-Barr virus (B95-8) DNA consists of short (10×10^6) and long (87×10^6) unique DNA sequences joined by 10 tandem reiterations of a 1.85×10^6 DNA segment. The reiterated sequence contains *Bam*I and *Bgl*II sites separated by 4×10^5. The 4.5×10^5 and 14.0×10^5 segments generated by cleavage of the reiterated DNA with *Bam*I and *Bgl*II contain sequences which hybridize to each other, suggesting that the internal tandemly reiterated sequence has a direct or inverted repeat within it. The opposite ends of the linear, nicked, double-stranded DNA molecule (R. F. Pritchett, S. D. Hayward, and E. D. Kieff, J. Virol. **15**:556-569, 1975) consist of from 1 to 12 direct repeats of another 3×10^5 sequence (D. Given and E. Kieff, J. Virol. **28**:524-542, 1978; D. Given, D. Yee, K. Griem, and E. Kieff, J. Virol. **30**:852-862, 1979). There is no homology between the internal reiterated sequence and either terminus. However, part of the internal reiteration (less than 5×10^5) is reiterated at two separate locations in the long unique region. The internal reiterations are a source of variation within EBV (B95-8) DNA preparations. Thus, although the majority of molecules contain 10 tandem reiterations, some molecules have 9, 8, 7, 6, 5, 4, or fewer tandem reiterations. A consequence of this variability is that the *Kpn*I A fragment and the *Eco*RI/*Hsu*I A fragment consist of a family of seven or more fragments differing in the number of tandem internal reiterations. The *Eco*RI/*Hsu*I A fragment of EBV (W91) DNA is approximately 6×10^6 smaller than the largest and dominant *Eco*RI/*Hsu*I A fragment of EBV (B95-8) DNA. EBV (W91) DNA also differs from EBV (B95-8) DNA by an additional 7×10^6 to 8×10^6 of DNA in the long unique DNA region (D. Given and E. Kieff, J. Virol. **28**:524-542, 1978; N. Raab-Traub, R. Pritchett, and E. Kieff, J. Virol. **27**:388-398, 1978). These data suggest the possibility that the smaller number of internal reiterations in EBV (W91) DNA may be a consequence of the additional unique DNA and a restriction in the overall size of EBV DNA.

Epstein-Barr virus (EBV) DNA is a linear, nicked, double-stranded molecule of 105×10^6 (15). The buoyant density (11, 13, 15, 18, 24, 25) and melting temperature (11, 15) of the DNA are compatible with a guanine-plus-cytosine content of 57 to 58%. The DNA of the B95-8 isolate of EBV has been a prototype for studies of the organization of EBV DNA (for review, see reference 10). Both ends of the DNA consist of a variable number of direct repeats of the same sequence of 400 to 500 base pairs (6, 7). The size (8) and order (6) of the *Hsu*I, *Sal*I, and *Eco*RI restriction endonuclease fragments of EBV (B95-8) DNA are shown in Fig. 1. These data and the analysis of partially denatured molecules (4) indicate that, with the exception of the variability in the number of copies of the reiteration at each end of the DNA, the remainder of the DNA is organized in a single arrangement. The results of treatment of EBV DNA with *Kpn*I restriction endonuclease are discordant (8) as *Kpn*I produces a 20×10^6 fragment in submolar amounts, and the sum of the molecular weight of all fragments, including the submolar fragment, is approximately 20×10^6 more than the molecular weight of EBV DNA.

Analysis of the fragments produced by cleavage of the DNA with *Bam*I and *Bgl*II reveals the presence of a 2×10^6 fragment in excessive amounts (8, 17). Complementary RNA made from the 2×10^6 *Bam*I fragment, which is present in a 10-fold molar excess, hybridizes to the *Hsu*I A and *Eco*RI A fragments, indicating that there are approximately 10 tandem reiterations of the 2×10^6 sequence in the *Hsu*I A and *Eco*RI A fragments (17). Part of this reiterated DNA is transcribed in Burkitt tumor tissue (3) and in restringently infected Burkitt tumor cells grown

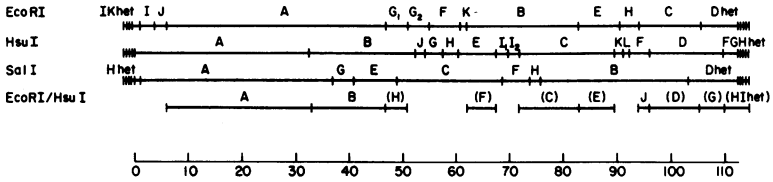

FIG. 1. *EcoRI (6), SalI (6), HsuI (6), and EcoRI/HsuI restriction endonuclease cleavage sites in EBV (B95-8) DNA. Fragments are designated by capital letters (6). A capital letter enclosed by parentheses above the EcoRI/HsuI fragments indicates tentative assignment of map location based on correspondence between the sizes of the fragments (Fig. 3) and the sizes of the EcoRI and HsuI overlap regions. The positions of the EcoRI/HsuI A, B, and J fragments were determined by hybridization of blots of agarose gels containing EcoRI/HsuI digest of EBV (B95-8) DNA to labeled HsuI A or B or EcoRI C fragment of EBV (B95-8) DNA.*

in culture (14). The objective of these experiments was to determine the location of the 2×10^6 reiteration within the *Eco*RI A and *Hsu*I A fragments. In the course of this work it became apparent that the discordant finding with *Kpn*I is due to the presence of a minor population of molecules which contain fewer copies of the internal reiteration but are otherwise identical to the dominant population.

MATERIALS AND METHODS

Cell culture, virus purification, and preparation of viral DNA. Viral DNA was obtained by sodium dodecyl sulfate-phenol extraction (12) of virus purified from the extracellular fluid of B95-8 and W91 cell cultures. Initial cultures of B95-8 and W91 cells were obtained from G. Miller, Yale University, New Haven, Conn. The procedures used to maintain cultures for virus purification and the purification of virus have been described previously (5).

Separation and determination of the molecular weight of restriction endonuclease fragments of EBV. EBV DNA was incubated with a two- to fivefold excess of *Kpn*I (New England Biolabs, Beverly, Mass.), *Eco*RI, *Hsu*I (8) (or its isoschizomer, *Hin*dIII), *Sal*I, *Xba*I, *Bam*I, or *Bgl*II (BRL, Bethesda, Md.) for 2.5 h at 37°C (6). For double digestion with *Eco*RI, *Hsu*I, *Bam*I, or *Bgl*II, 2 to 4 μg of the DNA was incubated at 37°C with a fivefold excess of enzyme in a solution consisting of 50 mM NaCl, 7 mM $MgCl_2$, 2 mM β-mercaptoethanol, and 20 mM Tris-hydrochloride, pH 7.4. *Eco*RI, *Hsu*I, *Sal*I, *Xba*I, *Kpn*I, *Eco*RI/*Hsu*I, *Bam*I, and *Bgl*II DNA fragments were separated by electrophoresis at 4°C in 0.3 to 0.4% (wt/vol) agarose gels in cylinders (1 by 28 cm) or in slabs (0.5 by 23 by 27 cm) (6, 8). *Bam*I, *Bgl*II, *Bam*I/*Eco*RI, *Bam*I/*Hsu*I, *Bgl*II/*Hsu*I, and *Bam*I/*Bgl*II fragments were also separated in 0.8% (wt/vol) agarose gels. DNA fragments were stained with ethidium bromide and photographed (type 57 film, Polaroid Corp., Oak Brook, Ill.) under UV illumination (6, 8). DNA fragments to be cleaved with a second enzyme or to be labeled in vitro were cut from the gel under direct visualization. The agarose was dissolved in 5 volumes of 5 M sodium perchlorate at 45°C, the DNA was separated by chromatography on hydroxylapatite (HTP, Bio-Rad Laboratories, Richmond, Calif.), and the ethidium bromide was removed by dialysis against Dowex 50 resin in the H form (6). The molecular weights of the *Eco*RI/*Hsu*I fragments were determined from a log linear plot of the electrophoretic mobility of the fragments in a 0.3% (wt/vol) agarose slab gel relative to lambda DNA (BRL) (22), *Eco*RI fragments of lambda DNA (22), *Hsu*I fragments of EBV (B95-8) and (HR-1) DNAs (8), and *Eco*RI fragments of EBV (B95-8) DNA (8) as reference DNAs in adjacent wells on the same slab. The molecular weights of *Bam*I or *Bgl*II fragments were similarly determined from the comparative electrophoretic mobility of the fragments in 0.3% (wt/vol) agarose gels with the *Eco*RI fragments of EBV (HR-1) DNA (8) as described above or in 0.8% (wt/vol) agarose slab gels relative to *Eco*RI fragments of lambda DNA (22). The sizes of the *Bam*I or *Bgl*II fragments of the separated *Hsu*I A or *Eco*RI A fragments and of the *Bam*I/*Hsu*I, *Bam*I/*Eco*RI, *Bgl*II/*Hsu*I, *Bgl*II/*Eco*RI, or *Bam*I/*Bgl*II fragments were determined by electrophoresis in 0.8 to 1% agarose gels relative to the *Eco*RI fragments of lambda DNA (22). The molecular weight of the smallest *Eco*RI fragment of lambda DNA is 2.1×10^6 (22). Therefore, determination of the molecular weight of fragments smaller than 10^6 from their electrophoretic mobility relative to the *Eco*RI fragments of lambda DNA is subject to error, even in 1% agarose gels.

Labeling of DNA in vitro. EBV DNA or fragments of EBV DNA were labeled in vitro by nick translation (6, 9) using DNA polymerase I of *Escherichia coli* (Boehringer Mannheim Corp., New York) and α-^{32}P-labeled dCTP (300 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). The specific activity of the labeled DNA was 0.5×10^8 to 1×10^8 cpm/μg.

Hybridization of labeled DNA to blots of fragments of EBV DNA. Blots (20) of separated fragments of EBV DNA were incubated at 68°C in 1 ml of a solution consisting of 10^4 to 10^5 cpm of denatured, labeled EBV DNA, 1 mg of denatured calf thymus DNA, 0.01 M EDTA, 1.25 M NaCl, and 0.05 M Tris-hydrochloride, pH 7.4. After 18 h the filter was washed for 4 h in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) 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.), with an intensifying screen (Cronex Lightning Plus, Du Pont Co., Wilmington, Del.) at −70°C.

RESULTS

***Kpn*I fragments of EBV (B95-8) DNA.** The order of arrangement of the *Kpn*I fragments in

EBV (B95-8) DNA was determined by hybridization of blots of *Kpn*I fragments of EBV DNA to labeled *Eco*RI, *Hsu*I, *Sal*I, or *Bam*I fragments whose map positions have been previously determined (6). The results (Fig. 2) indicate that each of the *Kpn*I fragments of EBV DNA, with the exception of the minor fragments slightly smaller than A and the B fragment, mapped to a specific and unique location. The B (previously C [8]) fragment was 2 M relative to

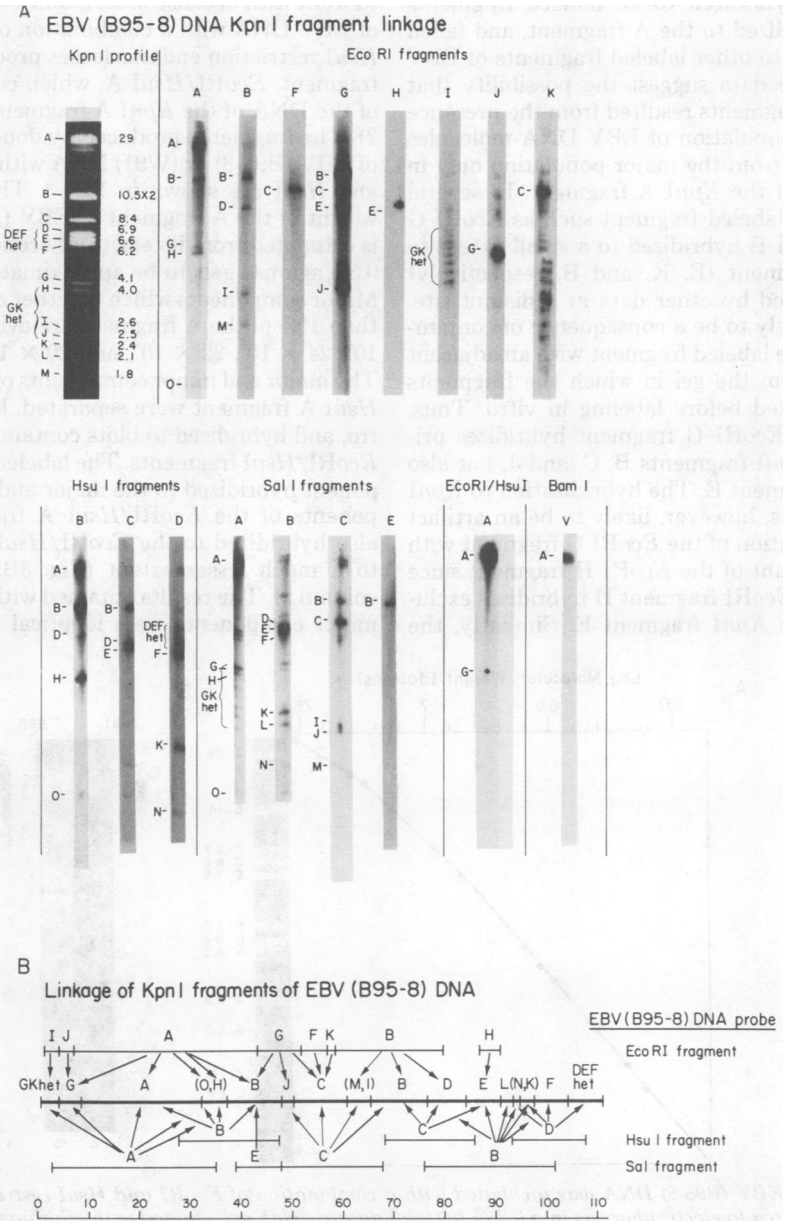

FIG. 2. *Mapping of KpnI fragments of EBV (B95-8) DNA by hybridization to labeled fragments of known map position (Fig. 1). (A) Photograph of an ethidium bromide-stained agarose gel of an electrophoretically separated KpnI digest of EBV (B95-8) DNA is shown in the upper left. The sizes of the fragments were determined from their electrophoretic mobility in 0.3% (wt/vol) and 0.4% (wt/vol) agarose gels (6, 8). Radiofluorograms of blots of KpnI restriction endonuclease fragments of EBV (B95-8) DNA which had been hybridized to the separated ^{32}P-labeled EcoRI, HsuI, SalI, EcoRI/HsuI, or BamI fragments of EBV (B95-8) DNA. (B) Summary of linkage data for KpnI fragments. The arrows drawn indicate hybridization of the labeled EcoRI, HsuI, or SalI fragment to KpnI fragments on blots. The order of the KpnI O and H, M and I, and N and K fragments within the map distances indicated by parentheses has not been determined.*

other fragments (8), could be resolved into two discrete bands in 0.3% agarose gels, and mapped to two distinct locations, 37×10^6 to 48×10^6 and 66×10^6 to 77×10^6. The minor fragments smaller than A ranged in size from 16×10^6 to 27×10^6, hybridized to all labeled fragments which hybridized to the A fragment, and failed to hybridize to other labeled fragments of EBV DNA. These data suggest the possibility that the minor fragments resulted from the presence of a minor population of EBV DNA molecules which differ from the major population only in the length of the *Kpn*I A fragment. In several instances, a labeled fragment such as *Eco*RI G or K or *Hsu*I B hybridized to a small extent to a *Kpn*I fragment (E, K, and B, respectively) which mapped by other data at a distant site. This was likely to be a consequence of contamination of the labeled fragment with an adjacent fragment from the gel in which the fragments were separated before labeling in vitro. Thus, the labeled *Eco*RI G fragment hybridizes primarily to *Kpn*I fragments B, C, and J, but also to *Kpn*I fragment E. The hybridization to *Kpn*I fragment E is, however, likely to be an artifact of contamination of the *Eco*RI G fragment with a small amount of the *Eco*RI H fragment since the labeled *Eco*RI fragment H hybridizes exclusively to the *Kpn*I fragment E. Similarly, the *Eco*RI fragment K is contaminated with a small amount of *Eco*RI het, and the *Hsu*I fragment B is contaminated with the *Hsu*I fragment C.

_Eco_RI/_Hsu_I A fragments of EBV (B95-8) and (W91) DNA. The *Kpn*I enzyme is difficult to work with because of its lability (6). Digestion of EBV DNA with a combination of *Eco*RI and *Hsu*I restriction endonucleases produces a large fragment, *Eco*RI/*Hsu*I A, which contains most of the DNA of the *Kpn*I A fragment (Fig. 1 and 2). The fragments produced by double digestion of EBV (B95-8) or (W91) DNA with both *Eco*RI and *Hsu*I are shown in Fig. 3. The molecular weight of the A fragment of EBV (B95-8) DNA is estimated from its electrophoretic mobility in 0.3% agarose gels to be approximately 28×10^6. Minor components which together comprise less than 10% of the A fragment are evident at 26×10^6, 24×10^6, 22×10^6, and 20×10^6 (Fig. 3A). The major and minor components of the *Eco*RI/*Hsu*I A fragment were separated, labeled in vitro, and hybridized to blots containing all of the *Eco*RI/*Hsu*I fragments. The labeled major component hybridized to the major and minor components of the *Eco*RI/*Hsu*I A fragments and also hybridized to the *Eco*RI/*Hsu*I J fragment to a much lesser extent (Fig. 3B; see Fig. 8, column 5). The results obtained with the labeled minor components were identical to those ob-

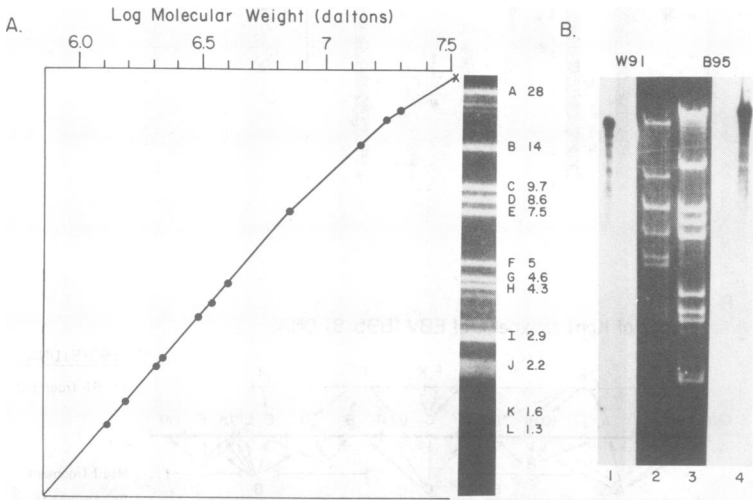

FIG. 3. *(A) EBV (B95-8) DNA was incubated with a combination of EcoRI and HsuI restriction endonucleases, subjected to electrophoresis in a 0.35% (wt/vol) agarose slab gel, stained with ethidium bromide, and photographed under UV illumination. The sizes of the EcoRI/HsuI fragments were determined from a log linear plot of the electrophoretic mobility of the EcoRI/HsuI fragments relative to EcoRI or HsuI fragments of EBV (B95-8) DNA or HsuI fragments of EBV (HR-1) DNA (8). The calibration curve shown above was constructed using HsuI (B through L) fragments of EBV (B95-8) DNA and intact lambda DNA. (B) EcoRI/HsuI fragments of EBV (W91 or B95-8) DNA were separated by electrophoresis in adjacent wells of a 0.35% agarose slab gel. Photograph of the ethidium bromide-stained gel with the fragments of W91 (column 2) and B95-8 (column 3) DNA. Autoradiograms of blots of the EcoRI/HsuI fragments of EBV (W91 or B95-8) DNA which were hybridized to the ^{32}P-labeled EcoRI/HsuI A fragment are shown to the left (column 1) and to the right (column 4), respectively.*

tained with the major component. As many as 10 minor components differing in molecular weight by approximately 2×10^6 could be distinguished in fluorograms of blots of EBV (B95-8) DNA hybridized to the labeled *Eco*RI/*Hsu*I A fragment (Fig. 3B). These data suggested the possibility that the difference in sizes of the *Eco*RI/*Hsu*I A fragments was due to variation in the number of copies of the 2×10^6 reiteration (6, 17) and that there was homology between the *Eco*RI/*Hsu*I A fragment and another region of the EBV genome (*Eco*RI/*Hsu*I J fragment; see below and Fig. 8, column 5). The size of the *Eco*RI/Hsu I A fragment of EBV (W91) DNA is 22×10^6. Minor components which together constituted approximately 10% of the total DNA were visible at 20×10^6, 18×10^6, 16×10^6, 14×10^6, 12×10^6, and 10×10^6 in fluorograms of blots of EBV (W91) DNA hybridized to the labeled *Eco*RI/*Hsu*I A fragment (Fig. 3B).

Mapping within the *Eco*RI/*Hsu*I A fragment. *Bam*I and *Bgl*II are known to cleave within the tandem reiteration in the *Eco*RI/*Hsu*I A fragment (6, 17). Therefore, a series of experiments was undertaken using *Bam*I and *Bgl*II to establish the arrangement of sequences within the *Eco*RI/*Hsu*I A fragment. One objective of this approach was to demonstrate directly that the difference in the sizes of the major and minor *Eco*RI/*Hsu*I A fragments was due to a difference in the number of copies of the reiterated DNA and not to a difference in the sizes of the unique sequence components of the *Eco*RI/*Hsu*I A fragment. A log linear plot of the size and electrophoretic mobility of the *Bam*I and *Bgl*II fragments of EBV (B95-8) DNA in 0.8% (wt/vol) agarose gels is shown in Fig. 4. The molecular weight of the reiterated DNA fragment, *Bam*I V, previously termed *Bam*I S (17) or *Bgl*II R, was determined to be 1.85×10^6 relative to the molecular weight of *Eco*RI fragments of lambda DNA.

The *Bgl*II A fragment electrophoresed in 0.8% gels as a broad band and in 0.3% gels as multiple discrete bands differing by approximately 3×10^5 in molecular weight, indicating that the *Bgl*II A fragment extends to include one end of the DNA (6, 7). Three approaches were used to map the *Bam*I and *Bgl*II cut sites within the *Eco*RI/*Hsu*I A fragment. In the first approach, the *Bam*I and *Bgl*II fragments which contain sequences homologous to the *Eco*RI/*Hsu*I A fragment were identified by hybridization of the labeled *Eco*RI/*Hsu*I A fragment DNA to blots of separated *Bam*I and *Bgl*II fragments of EBV DNA (Fig. 5A and B). The second approach was to tentatively identify the *Bam*I and *Bgl*II fragments which contain *Eco*RI or *Hsu*I sites by hybridization of the labeled *Eco*RI/*Hsu*I A fragment to blots of EBV DNA doubly cut with *Bgl*II and *Hsu*I (Fig. 5A), with *Bam*I and *Eco*RI (Fig. 5B), or with *Bam*I and *Hsu*I (Fig. 5B). The third approach was to recut the isolated *Eco*RI A or *Hsu*I A fragment with *Bam*I or *Bgl*II and to determine the size of the resultant fragments (Fig. 5C). The results are summarized in Fig. 6 and were as follows.

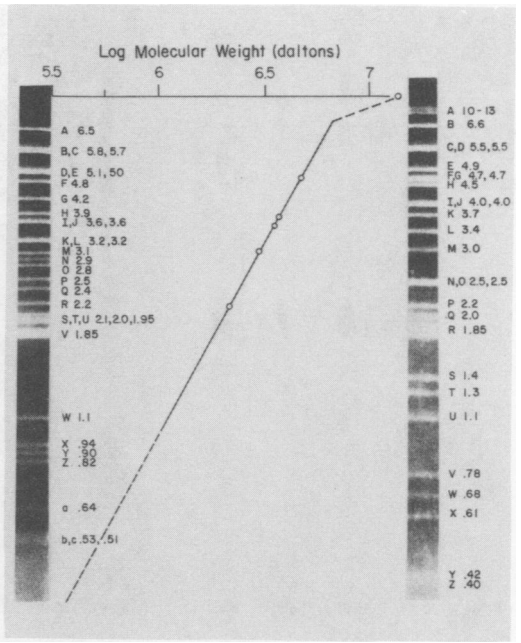

FIG. 4. *Log linear plot of size and electrophoretic mobility of BamI (photograph of ethidium bromide-stained gel on the left) and BglII (photograph of ethidium bromide-stained gel on the right) fragments of EBV (B95-8) DNA in 0.8% agarose gels relative to EcoRI fragments of lambda DNA (22). The molecular weights of the two largest fragments (BamI and BglII) were determined in 0.4% agarose gels relative to HsuI (MP) DNA (8). The broken line indicates regions of the log linear plot drawn by extrapolation. The estimate of the molecular weights of BamI and BglII fragments from these regions is therefore subject to greater error.*

(i) The *Eco*RI/*Hsu*I A fragment hybridizes primarily to the *Bgl*II A, C, or D and R fragments (Fig. 5A, column 2) and to the *Bam*I B or C, G, V, and X fragments (Fig. 5B, column 2).

(ii) The *Bgl*II A fragment extends to the end of the DNA and is 10×10^6 to 13×10^6. This fragment must therefore contain the short, unique region. The *Bgl*II CD fragment must lie to the right of the reiterated *Bgl*II R fragment and include the first *Hsu*I cut site, since it is reduced in size from 5.8×10^6 to 4.0×10^6 when the DNA is cut with both *Hsu*I and *Bgl*II (Fig. 5A, column 4) and contains sequences homologous to the *Eco*RI/*Hsu*I N B fragment (Fig. 5A,

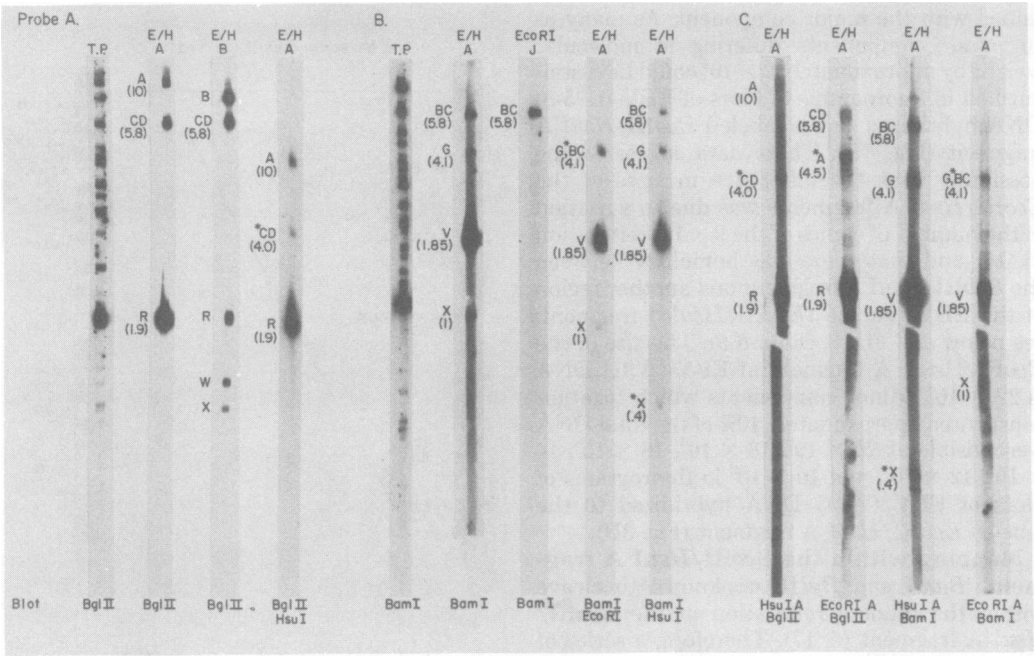

FIG. 5. *Radiofluorograms of blots of BglII or BglII/HsuI fragments (A) or BamI, BamI/HsuI, or BamI/EcoRI fragments (B) of EBV (B95-8) DNA or of blots of isolated EcoRI A or HsuI A fragment recut with BamI or BglII (C), which had been hybridized to labeled EBV (B95-8) DNA (designated T.P.) or to labeled restriction enzyme fragments of EBV (B95-8) DNA (designated by the letters at the top of each blot). The molecular size of each fragment was determined by comparison of electrophoretic mobility to EcoRI fragments of phage lambda DNA coelectrophoresed in adjacent wells.*

FIG. 6. *Map of the BamI sites from 4.2 × 10⁶ to 33 × 10⁶ and of the BglII cut sites from 0 to 34.3 × 10⁶ in EBV (B95-8) DNA.*

column 3). The arrangement of the *Bgl*II fragments and of the *Eco*RI and *Hsu*I cleavage sites (6) from 0 to 36 × 10^6 is shown in Fig. 6. The arrangement was confirmed by the results of recleavage of the purified *Hsu*I A or *Eco*RI A fragment with *Bgl*II (Fig. 5C). As expected, cleavage of the *Hsu*I A fragment with *Bgl*II resulted in three fragments: the *Bgl*II A fragment, the reiterated *Bgl*II R fragment, and a third fragment, *Bgl*II CD, which is reduced in size because it extends beyond the *Hsu*I cleavage site (Fig. 5C, column 1, and Fig. 6). Cleavage of the isolated *Eco*RI A fragment with *Bgl*II resulted in full-size *Bgl*II R and CD fragments and a *Bgl*II A fragment reduced in size because of cleavage by *Eco*RI at the *Eco*RI sites located at 1 × 10^6, 3.8 × 10^6, and 6 × 10^6 from the left end of the *Bgl*II A fragment (Fig. 5C, column 2, and Fig. 6).

(iii) Similarly, the *Bam*I BC fragment contains sequences homologous to the *Eco*RI J fragment (Fig. 5B, column 3) and is reduced in size from 5.8 × 10^6 to 4.1 × 10^6 when the DNA is cut with *Eco*RI and *Bam*I (Fig. 5B and Fig. 5C, column 4), indicating that this fragment must lie to the left of the reiteration. The *Bam*I X fragment must cross the *Hsu*I cut site since it is reduced from 1 × 10^6 (Fig. 5B, columns 2 and 4) to 0.4 × 10^6 (Fig. 5B, column 5, and Fig. 5C, column 3) when the DNA is cleaved with *Bam*I and *Hsu*I. Furthermore, as expected, the labeled *Eco*RI/*Hsu*I B fragment hybridizes to the *Bam*I X fragment (data not shown). The *Bam*I G fragment must extend from the reiteration to the *Bam*I X fragment since it is homologous to the *Eco*RI/*Hsu*I A fragment and is not decreased in size by cleavage with *Hsu*I (Fig. 5B, column 5) or *Eco*RI (Fig. 5B, column 4). The map of the *Bam*I sites from 4.2 × 10^6 to 33 × 10^6 is shown in Fig. 6. These results were confirmed by analysis of the fragments produced when the purified *Hsu*I A or *Eco*RI A fragment was cut with *Bam*I and identified on blots with the labeled *Eco*RI/*Hsu*I A fragment (Fig. 5C, columns 3 and 4). As expected, the *Bam*I BC, G, and V fragments were cleaved from the *Hsu*I A fragment and were identical in size to the fragments cut from intact EBV (B95-8) DNA, whereas the

X fragment was reduced to approximately 4×10^5, confirming that the size of the component of the X fragment to the left of the first *Hsu*I cut site is approximately 4×10^5. Furthermore, the *Bam*I BC fragment is smaller when cleaved from the *Eco*RI A fragment since the *Eco*RI site at 6×10^6 is in the *Bam*I BC fragment (Fig. 5C, column 4, and Fig. 6).

(iv) An unexpected finding when blots of *Bam*I fragments were hybridized to the labeled *Bgl*II R or *Bam*I V fragment was that the radiofluorogram appeared identical to that shown in Fig. 5B, column 2. All four *Bam*I fragments, including X, hybridized to the labeled *Bgl*II R or *Bam*I V fragment. These data indicated that the *Bam*I X fragment which is separated from the tandem reiterations by the length of the *Bam*I G fragment, contains sequences homologous to *Bgl*II R and *Bam*I V fragments. The sequences homologous to *Bam*I V and *Bgl*II R fragments must lie to the left of the *Hsu*I cut site in the *Bam*I X fragment shown in Fig. 6, since the labeled *Eco*RI/*Hsu*I A fragment hybridized to the 0.4×10^6 fragment of X and not to the 0.6×10^6 fragment of *Bam*I X (Fig. 5B, column 5). The homology between the internal reiteration and the *Bam*I X fragment is further defined by studies with labeled components of the reiterated DNA below.

To confirm that the major and minor components of the *Eco*RI/*Hsu*I A fragment do not differ in the content of unique sequences, a minor band was separated from a gel, incubated with *Bam*I, and re-electrophoresed. The sizes of the fragments produced by cleavage of the major and minor *Eco*RI/*Hsu*I A fragment components with *Bam*I were identical (data not shown).

Arrangement of *Bam*I and *Bgl*II sites within the internal tandem reiterations. The sizes of the components of *Bgl*II A and *Bam*I BC to the right of the *Eco*RI J-A site are 4.5×10^6 (Fig. 5C, column 2) and 4.1×10^6 (Fig. 5C, column 4), respectively. The sizes of the components of *Bgl*II CD and *Bam*I G and X to the left of the *Hsu*I A-B site are 4×10^6 (Fig. 5A, column 4) and 4.1×10^6 and 0.4×10^6 (Fig. 5B, column 5), respectively. As summarized in Fig. 6, these observations, that the distance from the *Eco*RI J-A cut site to the *Bgl*II site within the first internal reiteration is 4×10^5 longer than the distance to the first *Bam*I site and that the distance from the *Bgl*II site in the last reiteration to the *Hsu*I A-B site is 5×10^5 shorter than the corresponding *Bam*I distance, both suggest that the *Bam*I site is 4×10^5 to 5×10^5 to the left of the *Bgl*II site in the reiteration. To confirm the distance between the *Bam*I and *Bgl*II sites within the reiterated DNA, the sizes of the *Bam*I/*Bgl*II double-digest fragments were determined by electrophoresis in a 1% agarose gel with *Eco*RI fragments of lambda DNA in adjacent wells. As expected, the reiterated DNA is cleaved into two components (Fig. 7, column 2). The electrophoretic mobility of the large component of the reiterated DNA, *Bgl*II R_l, is slightly faster than the smallest *Eco*RI fragment of lambda, indicating a size of 1.4×10^6. The rapid mobility of the smaller component, *Bgl*II R_s, places it in the nonlinear part of the gel but suggests a size of approximately 4×10^5 to 5×10^5.

The data, which are summarized in Fig. 6, indicate that the *Bam*I site in the internal reiteration is approximately 4×10^5 to the left of the *Bgl*II site and therefore in the leftward 1.4×10^6 of the reiterated DNA. If the *Bam*I site is near the left end of the reiteration, *Bam*I BC would contain only the left end of the reiterated sequence and *Bam*I G would contain most of one complete reiteration, i.e., all of the DNA between the *Bam*I and *Bgl*II sites, *Bgl*II R_s and most of the rest of the reiteration, *Bgl*II R_l. If there are no reiterations within the reiterated 1.85×10^6 sequence, the extent of hybridization of labeled *Bgl*II R_l to *Bam*I BC as opposed to *Bam*I G should be proportional to the amount

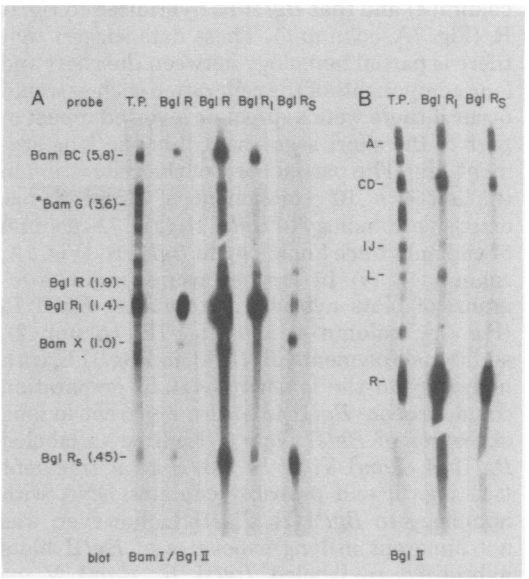

FIG. 7. *Radiofluorograms of blots of fragments of EBV (B95-8) DNA produced by cleavage with a combination of BamI and BglII (BamI/BglII) (A) or by cleavage with BglII (B) which were hybridized to the ^{32}P-labeled EBV (B95-8) DNA (T.P., column 1) or to labeled BglII R fragment or to the large (Bgl R_l) or small (Bgl-R_s) fragment obtained when BglII R is cut into two fragments by BamI. The fragments which hybridize are identified by name, where known, and by size on the left.*

of *Bgl*II R_l in *Bam*I BC as opposed to *Bam*I G and to the distance from the left end of the reiterated sequence to the *Bam*I site as opposed to the distance from the *Bgl*II site to the right end of the reiteration. Furthermore, *Bgl*II R_s should hybridize to *Bgl*II A and *Bam*I G, but not to *Bam*I BC (Fig. 6). To estimate the relative amount of *Bgl*II R_l in *Bam*I BC versus *Bam*I G and to further check the data shown in Fig. 6, *Bgl*II R_l, *Bgl*II R_s, *and Bgl*II R were isolated from gels, labeled in vitro, and hybridized to blots of *Bgl*II and *Bam*I/*Bgl*II fragments of EBV DNA. The results are as follows. (i) Labeled *Bgl*II R_l hybridizes extensively to the *Bam*I BC fragment (Fig. 7A, column 2) and hardly at all to the *Bam*I G fragment (Fig. 7A, column 2) from which *Bgl*II R_s had been removed by digestion with *Bgl*II and *Bam*I. An overlong exposure of the blot (Fig. 7A, column 3) demonstrated that the hyrbridization to *Bam*I G was barely above background. These data indicate that the *Bam*I site must be near the right end of the left 1.40×10^6 component of the reiterated sequence. (ii) As expected, *Bgl*II R_s hybridized extensively to *Bgl*II A (Fig. 7B, column 3) and hardly at all to *Bam*I BC (Fig. 7A, column 5). (iii) A surprising finding was that *Bgl*II R_l hybridized slightly to *Bgl*II R_s (Fig. 7A, column 4) and that *Bgl*II R_s hybridized to *Bgl*II R_l (Fig. 7A, column 5). These data suggest that there is partial homology between the short and long components of the reiteration such as would occur if there were a direct or inverted repeat of part of the short segment within the long segment. (iv) The partial copy of the reiteration in the left 4×10^5 component of *Bam*I X has extensive homology to *Bgl*II R_s (Fig. 7A, column 5) and only trace homology to *Bgl*II R_l (Fig. 7A, column 4). (v) In long exposure, radiofluorograms of blots hybridized to labeled *Bgl*II R_l (Fig. 7A, column 4, and Fig. 7B, column 2), additional fragments, *Bgl*II IJ and *Bgl*II L, with homology to the labeled *Bgl*II R_l preparation could be seen. *Bgl*II IJ is also apparent in long exposures of *Bgl*II blots hybridized to labeled *Bgl*II R, *Bam*I V, or *Eco*RI/*Hsu*I A fragment (see Fig. 8) and probably contains DNA with homology to *Bgl*II R_l. *Bgl*II L, however, was not apparent in long exposures of *Bgl*II blots hybridized to labeled *Bgl*II R, *Bam*I V, or *Eco*RI/*Hsu*I A and is probably identified in Fig. 7B, column 2, because of contamination of the labeled *Bgl*II R_l preparation with another fragment of similar size.

Homology between the internal reiteration and DNA in the long, unique region. Long-exposure radiofluorograms of blots of *Eco*RI, *Hsu*I, *Eco*RI/*Hsu*I, or *Bgl*II fragments which had been hybridized to labeled *Eco*RI/*Hsu*I A, *Bam*I V, or *Bgl*II R fragments revealed homology between the internal reiteration and the *Hsu*I F (Fig. 8, column 2), *Eco*RI C (Fig. 8, column 3), *Eco*RI/*Hsu*I J (Fig. 8, column 5), and *Bgl*II IJ (Fig. 8, column 6) fragments. The *Bgl*II IJ fragment is part of *Eco*RI C (Fig. 8, column 7). These results indicate that within the region of overlap of *Eco*RI C and *Hsu*I F, i.e., between 94×10^6 and 97×10^6 from the left end of EBV DNA (Fig. 1), there exists a sequence with homology to the internal reiteration. The extent of hybridization of the labeled *Eco*RI/*Hsu*I A or *Bam*I V fragment to *Eco*RI C, *Hsu*I F, *Bgl*II IJ, or *Eco*RI/*Hsu*I J fragment was determined from scans of autoradiograms to be less than 3% of the extent of hybridization to *Eco*RI A, *Hsu*I A, *Bgl*II R, or *Eco*RI/*Hsu*I A fragment, indicating that there is less than 1 complete copy of the reiteration in the *Eco*RI C, *Hsu*I F, *Bgl*II IJ, and *Eco*RI/*Hsu*I J fragments. The *Bgl*II IJ fragment has homology both to the large, *Bgl*II R_l (Fig. 7B, column 2), and small, *Bgl*II R_s (Fig. 7B, column 3), component of the reiteration.

DISCUSSION

The results of the mapping of the *Bam*I and *Bgl*II restriction enzyme cleavage sites near the internal reiteration which joins the short (10×10^6) and long (87×10^6) unique regions of EBV (B95-8) DNA are summarized in Fig. 6 and 9. The existence of short and long unique DNA sequences joined by reiterated DNA and bounded at the termini by direct repeats is sim-

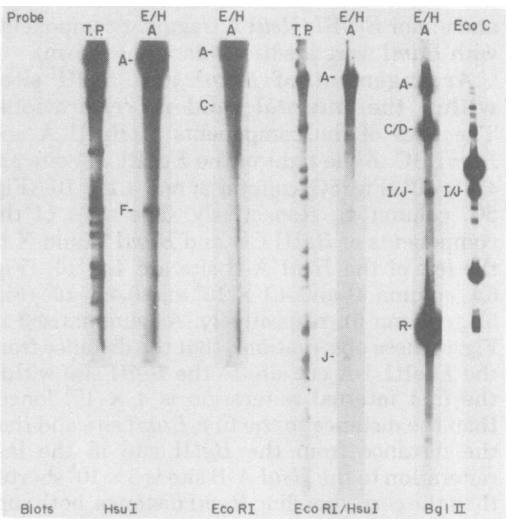

FIG. 8. *Radiofluorograms of blots of the HsuI, EcoRI, EcoRI/HsuI, or BglII fragment of EBV DNA which was hybridized to the labeled EcoRI/HsuI A fragment (E/H A), labeled EcoRI fragment C (EcoRI C), or labeled EBV DNA (T.P.).*

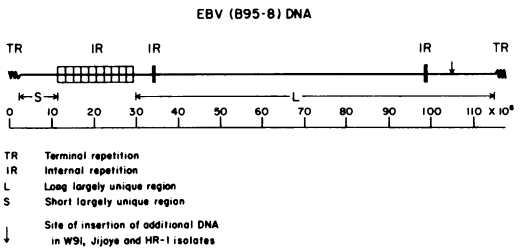

FIG. 9. *Diagram of organization of EBV (B95-8) DNA.*

ilar to the molecular organization of herpes simplex virus (19, 23), bovine mammillitis virus (2), and pseudorabies virus (21) DNAs. However, EBV DNA differs from these other herpes viral DNAs in that there are tandem direct internal reiterations in EBV DNA and there is no homology between the internal reiteration and either terminus. Moreover, the short and long unique regions of EBV DNA do not invert relative to each other during DNA replication as has been observed with herpes simplex (23), bovine mammillitis (2), and pseudorabies viruses (21).

The minor *Kpn*I (8) and *Eco*RI/*Hsu*I fragments arise through the presence in each EBV (B95-8) DNA preparation of molecules with a smaller number of internal reiterations in the DNA. Thus, although the majority of molecules of EBV (B95-8) DNA contain 10 to 11 tandem reiterations, some molecules have 9, 8, 7, 6, 5, or 4 tandem reiterations. We do not as yet know whether such variant molecules are always generated during the course of viral DNA replication. The findings that cells infected with the B95-8 isolate and the cell line from which the B95-8 virus was isolated contained circular DNA molecules 6×10^6 smaller than EBV (B95-8) DNA (1) support the hypothesis that shorter DNA molecules containing fewer copies of the internal reiteration may be infectious and may yield progeny with a larger or smaller number of internal tandem reiterations. Furthermore, the finding that the *Eco*RI/*Hsu*I A fragment of EBV (W91) and (AG876) (N. Raab-Traub and E. Kieff, manuscript in preparation) DNAs is smaller than the *Eco*RI/*Hsu*I A fragment of EBV (B95-8) DNA suggests that these Burkitt tumor isolates of EBV, which have 7×10^6 of additional DNA inserted near the distal end of the long, unique region (6, 16), may have a smaller number of internal tandem reiterations, possibly as a consequence of the additional unique DNA.

Previous estimates of the size of the *Hsu*I A and *Eco*RI A fragments were based on the electrophoretic mobility of these fragments in 0.4% agarose gels relative to the *Hsu*I fragments of herpes simplex virus (MP strain) DNA, the largest fragment of which is 27.5×10^6, or the *Hsu*I fragments of T_5b1 DNA, the largest fragment of which is 34×10^6 (8). The log linear relationship between molecular weight and electrophoretic mobility in 0.4% agarose gels is parabolic for fragments in excess of 23×10^6 to 25×10^6 (8). The *Hsu*I A and *Eco*RI A fragments are slightly larger than the largest standards, and previous estimates of the sizes of these fragments are therefore subject to error. Three lines of evidence indicate that the size of the *Eco*RI/*Hsu*I A fragment is 28×10^6 and, therefore, that the true sizes of the *Hsu*I A and *Eco*RI A fragments are 34×10^6 and 42×10^6 daltons, respectively. First, the electrophoretic mobility of the *Eco*RI/*Hsu*I A fragment relative to the *Hsu*I B fragment of EBV (B95-8) DNA (20×10^6 [8]), the *Hsu*I B fragment of EBV (HR-1) DNA (27×10^6 [8]), and intact lambda DNA (32×10^6 [22]) is compatible with a size of 28×10^6. Second, six minor bands, each differing by approximately 2×10^6 in molecular weight, are clearly distinguishable above the *Eco*RI/*Hsu*I B fragment in blots of the *Eco*RI/*Hsu*I fragments which were hybridized to the labeled *Eco*RI/*Hsu*I A fragment. The molecular weight of the *Eco*RI/*Hsu*I B fragment is 14×10^6. The size of the *Eco*RI/*Hsu*I A fragment, which is larger than the largest minor *Eco*RI/*Hsu*I A fragment, must therefore be 28×10^6. Third, the molecular weight of the unique-sequence component of the *Eco*RI/*Hsu*I A fragment is 8.5×10^6. The size of the reiterated component, *Bam*I V or *Bgl*II R fragment, is 1.85×10^6 to 1.9×10^6. The *Bam*I V fragment component is present in at least 10-fold excess of other *Bam*I fragments (17).

The revised estimates of the sizes of the *Hsu*I A and *Eco*RI A fragments suggested by these data bring the sum of the molecular weights of the *Eco*RI or *Hsu*I fragments of EBV (B95-8) DNA to 116×10^6, which is approximately 10% larger than estimates based on a measurement of the length of EBV DNA (1, 7, 15). Although the latter estimates of the molecular weight of EBV (B95-8) DNA are based ultimately on the length of ϕX174 DNA, a molecule of known molecular weight, there are several possible sources of error in the determination of the molecular weight of EBV DNA from its length, the most significant of which is the possibility that the higher guanine-plus-cytosine content of EBV DNA results in a higher mass per unit length than that of ϕX174 replicative-form II or PM2 DNA.

The significance of the internal tandem reiteration, of the homology between the components of the reiteration which is likely to be a consequence of a reiteration within the reitera-

tion, and of the homologous DNA sequences in the *Bam*I X and *Hsu*I F fragments of the long, unique region is uncertain. From the relative hybridization of labeled *Bam*I V or *Bgl*II R to the *Hsu*I F and *Hsu*I A fragments, the length of the region of homology between the reiterated DNA and *Hsu*I F fragment is estimated to be less than 5×10^5, whereas the component of the *Bam*I X fragment homologous to the internal reiteration is contained in the 4×10^5 region of *Bam*I X fragment to the left of the *Hsu*I A-B site. The *Hsu*I F sequences homologous to the internal reiteration have more homology to the *Bgl*II R_i component than to the *Bgl*II R_s component, whereas the reverse is true for *Bam*I X. It is not known whether the DNA in *Bam*I X and *Hsu*I F, which is homologous to the internal reiteration, is a continuous sequence or is separated by regions of nonhomology or whether the homologous sequences within the reiteration or in *Bam*I X and *Hsu*I F are direct or inverted repeats. Regions of homology in the DNA could play a role in DNA or RNA synthesis or could be a consequence of gene duplication. Pertinent to the latter is the observation that the internal reiteration encodes stable polyadenylated RNA in Burkitt tumor tissue and the most abundant RNA in restringently infected Burkitt tumor cells grown in culture (3, 14).

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