DNA of Epstein-Barr Virus

II. Comparison of the Molecular Weights of Restriction Endonuclease Fragments of the DNA of Epstein-Barr Virus Strains and Identification of End Fragments of the B95-8 Strain

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Incubation of the DNA of the B95-8 strain of Epstein-Barr virus (EBV (B95-8) DNA) with *EcoRI*, *Hsu I*, *Sal I*, or *Kpn I* restriction endonuclease yielded 8 to 15 fragments separable on 0.4% agarose gels and ranging in molecular weight from less than 1×10^6 to more than 30×10^6 . *Bam I* and *Bgl II* yielded fragments smaller than 11×10^6 . Preincubation of EBV (B95-8) DNA with lambda exonuclease resulted in a decrease in the *Hsu I* A and *Sal I* A and D fragments, indicating that these fragments are positioned near termini. The electrophoretic profiles of the fragments produced by cleavage of the DNA of the B95-8, HR-1, and Jijoye strains of EBV were each distinctive. The molecular weights of some *EcoRI*, *Hsu I*, and *Sal I* fragments from the DNA of the HR-1 strain of EBV (EBV (HR-1) DNA) and of *EcoRI* fragments of the DNA of the Jijoye strain of EBV were identical to that of fragments produced by cleavage of EBV (B95-8) DNA with the same enzyme, whereas others were unique to each strain. Some *Hsu I*, *EcoRI*, and *Sal I* fragments of EBV (HR-1) DNA and *Kpn I* fragments of EBV (B95-8) DNA were present in half-molar abundance relative to the majority of the fragments. In these instances, the sum of the molecular weights of the fragments was in excess of 10^8 , the known molecular weight of EBV (HR-1) and (B95-8) DNA. The simplest interpretation of this finding is that each EBV (HR-1), and possibly also (B95-8), DNA preparation contains two populations of DNA molecules that differ in the arrangement of DNA sequences about a single point, such as has been described for herpes simplex virus DNA. Minor fragments could also be observed if there were more than one difference in primary structure of the DNAs. The data do not exclude more extensive heterogeneity in primary structure of the DNA of the HR-1 strain. However, the observation that the relative molar abundance of major and minor fragments of EBV (HR-1) DNA did not vary between preparations from cultures that had been maintained separately for several years favors the former hypothesis over the latter.

Current knowledge of the DNAs of human Epstein-Barr viruses (EBV) can be summarized as follows. (i) Virion DNA purified from HR-1 (11) and B95-8 (18) continuous lymphoblastoid cell cultures [designated EBV (HR-1) DNA and EBV (B95-8) DNA, respectively] is a linear double-stranded DNA of 100×10^6 daltons (23). Most DNA strands have a size less than 50×10^6 daltons in alkaline sucrose gradients (23). (ii) The buoyant density of EBV (HR-1) and (B95-8) DNAs is 1.718 g/cm^3 in neutral cesium chloride, suggesting a base composition of 57 to 58 guanine-plus-cytosine moles percent (12, 19, 23, 24, 30, 31). Fragments of EBV DNAs produced by mechanical shear have a distribution of buoyant density in neutral cesium chloride ranging from 1.716 to 1.721 g/cm^3 , suggesting

a narrow range of variation in proportional content of guanine and cytosine versus adenine and thymidine (23). EBV (B95-8) DNA is relatively enriched for sequences with a slightly higher than average buoyant density (23). (iii) Kinetic and absorptive reciprocal hybridizations with purified viral DNAs indicated that EBV (B95-8) and (HR-1) DNAs are approximately 85 to 92% homologous (23, 27) and that EBV (B95-8) DNA lacks approximately 8 to 15% of the sequences of EBV (HR-1) DNA (23). (iv) Covalently closed circular EBV DNA has been demonstrated within some infected cells (15). From this observation, it may be inferred that sequences near both termini of linear molecules possess homology.

Two analyses of the fragments produced by

cleavage of EBV DNA with restriction enzymes have been reported. Some of the fragments produced by cleavage of EBV (B95-8) and (HR-1) DNAs were found to have similar molecular weights, whereas others were found to differ (10, 27). In one of these previous reports (27), avian myeloblastosis virus reverse transcriptase was used to label EBV DNA in vitro. The amount of radioactivity incorporated into fragments of DNA was variable and was not proportional to the molecular weight of the DNA fragment. High-molecular-weight fragments were underrepresented, even assuming that the labeling of fragments would be irrespective of size. In the other report (10) the DNA was labeled in vivo, but the quantitation of the label in each fragment was not presented.

In this paper, we report the results of an analysis of the fragments produced by cleavage of EBV (B95-8) DNA with six restriction enzymes, the comparative analysis of the fragments of EBV (HR-1) DNA, and preliminary results of identification of the end fragments of EBV (B95-8) DNA using lambda 5'-exonuclease. The long-term goal of these studies is to correlate biological function with base sequences and their organization in EBV DNA. The organization of viral DNA is likely to be important in control of transcription and may be related to the differential processing of specific viral RNA species in restringently infected cells (9, 21).

MATERIALS AND METHODS

Cell cultures. Cultures of HR-1 (11) (obtained from G. Klein, Karolinska Institute, Stockholm, Sweden, and from R. Glaser, Pennsylvania State University College of Medicine, Hershey), B95-8 (18) (obtained from G. Miller, Yale University, New Haven, Conn.), and Jijoye (obtained from P. Gerber, Division of Biological Standards, National Institutes of Health, Bethesda, Md.) cells were grown at 35°C in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum (both from GIBCO, Grand Island, N.Y.). Cultures were grown for 3-month intervals in media containing tylocine (60 µg/ml) (GIBCO) and spectinomycin (200 µg/ml) (The Upjohn Co., Kalamazoo, Mich.) or in media without antibiotics.

Restriction enzymes and marker DNAs. Restriction endonuclease *Sal* I (from *Streptomyces albus*), *Kpn* I (from *Klebsiella pneumoniae*), *Bam* I (from *Bacillus amyloliquefaciens*), and *Bgl* II (from *Bacillus globiggi*) and ³²P-labeled herpes simplex type 1 (MP strain) [HSV-1 (MP)] DNA were obtained from G. Hayward in the laboratory of B. Roizman, University of Chicago. *Eco*RI endonuclease was purchased from New England Biolabs, Beverly, Mass. *Hsu* I endonuclease was prepared from *Haemophilus suis* (*Hsu* I and *Hin* III are isoschizomers) by a procedure devised by R. Roberts and G. Hayward. All steps were carried out at 4°C. Briefly, 10 g of *H.*

suis cells was suspended in a buffer consisting of 50 mM NaCl, 10 mM 2-mercaptoethanol, and 20 mM Tris-hydrochloride (pH 7.6), sonically treated 25 times for 30 s at 300 W (Artek Corp., Farmingdale, N.Y.), and centrifuged at 100,000 × *g* for 90 min. One milliliter of 10% streptomycin sulfate was added for each 1,500 units of absorbancy at 260 nm, and the precipitate was removed by centrifugation at 10,000 × *g* for 30 min. A 50 to 70% saturated ammonium sulfate fraction of the supernatant was resuspended in a buffer consisting of 1 M NaCl, 10 mM 2-mercaptoethanol, and 20 mM Tris-hydrochloride (pH 7.6), dialyzed against the same buffer, and passed through a column (2.5 by 50 cm) of Bio-Gel A. The fractions containing the cleanest activity against T5 DNA were pooled, dialyzed against PC buffer (10% glycerol, 50 mM KCl, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 10 mM potassium phosphate, pH 7.9), and loaded onto a phosphocellulose (P. H. Whatman Corp.) column (25 by 1.6 cm). Fractions were eluted with a linear 0.05 to 1 M KCl gradient and tested for activity against T5 DNA. Fractions containing the desired activity were pooled, dialyzed against 50% glycerol in 0.3 M NaCl and 20 mM Tris-hydrochloride (pH 7.6), and stored at -20°C.

T5b1 phage was grown and its DNA was purified as described previously (7).

Preparation of labeled DNA. Eight liters of HR-1 or B95-8 cultures or 12 liters of Jijoye cultures was concentrated fivefold by aspiration of the supernatant media and incubated for 2 days with [³H]thymidine (60 Ci/mmol; New England Nuclear Corp., Boston, Mass.) or [³H]cytosine (50 Ci/mmol; New England Nuclear Corp., Boston, Mass.) at a final concentration of 3 µCi/ml. For those experiments in which DNA was labeled with [³²P]orthophosphate, cells were concentrated 10-fold, reincubated in media consisting of 50% old and 50% new phosphate-free RPMI 1640 (obtained from GIBCO) for 24 h, and then resuspended for 24 h in 50 µCi of [³²P]orthophosphate per ml in phosphate-free RPMI 1640 supplemented with 10% dialyzed calf serum and 0.05 M HEPES buffer (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid), pH 7.4. Virus was purified from the culture medium as previously described (2), and the radiolabeled viral DNA was isolated by gentle phenol extraction (23). Sedimentation of the labeled EBV (B95-8) and (HR-1) DNA preparations in sucrose velocity gradients using T4 DNA as a marker (23) demonstrated that more than 95% of the labeled EBV (HR-1) and (B95-8) DNA preparations sedimented with the velocity expected for molecules of 100 × 10⁶ daltons.

Exonuclease digestion. To identify those fragments located at the ends of EBV DNA molecules, a brief lambda exonuclease digestion was performed before incubation with restriction enzymes. The amount of lambda exonuclease (gift of G. Hayward in the laboratory of B. Roizman) required to eliminate terminal fragments from the gel profiles was determined by using T5b1 DNA. Each reaction mixture contained 3.5 µg of EBV DNA per ml, 67 mM glycine phosphate (pH 9.6), 50 µg of bovine serum albumin per ml, 1 mM 2-mercaptoethanol, and 3 mM MgCl₂ (28). The reaction mixture was incubated

at 25°C for 5 min, quenched in ice with the addition of 1/10 volume of 0.1 M EDTA, extracted with phenol, and dialyzed extensively against 0.15 M NaCl, 20 mM Tris (pH 7.6), and 1 mM EDTA, followed by 20 mM NaCl, 20 mM Tris (pH 7.6), and 1 mM EDTA.

Restriction enzyme analyses. EBV DNA (0.5 to 2 μ g) was incubated for 2.5 h at 37°C in 0.3 ml of a solution containing 20 mM $MgCl_2$, 20 mM NaCl, and 20 mM Tris-hydrochloride (pH 7.6) for digestion with *Hsu* I, *Sal* I, *Kpn* I, *Bam* I, and *Bgl* II or in a solution containing 20 mM $MgCl_2$, 50 mM NaCl, and 150 mM Tris-hydrochloride (pH 7.6) for digestion with *Eco*RI. The volume of restriction enzyme used in each reaction mixture was two- to fivefold greater than the volume needed to completely digest 3 μ g of T5b1 DNA. Two to 3 μ g of unlabeled T5b1 DNA was included in each incubation mixture to ascertain that the enzyme activity was adequate to yield limit digests (4). The enzyme reaction was terminated by the addition of 20 mM EDTA. In those experiments used to measure the size of the fragments of EBV DNA, fragments of T5b1 and HSV-1 (MP) DNA were used as internal markers (6). An *Hsu* I digest (6) of ^{32}P -labeled HSV-1 (MP) DNA was added to the sample before electrophoresis.

DNA samples were layered onto 0.4% (wt/vol) cylindrical agarose gels (1 by 28 cm) and electrophoresed at 40 V for 36 h at 4°C (5). Fragments of T5b1 DNA and EBV DNA were visualized and photographed under UV light after staining for 2 to 4 h in a solution containing 0.5 μ g of ethidium bromide per ml. The relative position and absorbance of EBV and T5b1 DNA fragments were determined by scanning transparency photographs of the ethidium bromide-stained gels in an Acta II spectrophotometer equipped with gel scanner. The position of fragments of 3H -labeled EBV and ^{32}P -labeled HSV-1 DNAs was determined by cutting the gels transversely into 1-mm slices and measuring the radioactivity present in each slice (6). Relative molar ratios were calculated by dividing the amount of radioactivity in each EBV DNA fragment by the molecular weight of the fragment as determined from its electrophoretic migration relative to T5b1 and HSV-1 (MP) markers. Between 85 and 90% of the radioactively labeled EBV (B95-8) or (HR-1) DNA applied to the gel was routinely recovered in specific fragments. The relative molar ratios listed in Table 2 represent the results of analyses of three 3H -thymidine-labeled DNA preparations. Average relative molar ratios of between 0.4 and 0.6 were considered to be 0.5; those between 0.8 and 1.2 were considered to be 1. To obtain molar ratios of low-molecular-weight fragments, gels were run for shorter intervals than those shown in Fig. 1 and 2.

RESULTS

Comparison of the DNAs of the B95-8 and HR-1 strains of EBV. Between 4 and 8 μ g of DNA was regularly recovered from virus purified from 8 liters of B95-8 and HR-1 cultures. The specific activity of the purified DNA varied between 2×10^4 and 10^5 3H cpm/ μ g when cultures were incubated in 3H -thymidine or

3H -cytosine and was approximately 10^4 cpm/ μ g when cultures were incubated with ^{32}P -orthophosphate.

Initially, six restriction endonucleases (*Hsu* I, *Sal* I, *Eco*RI, *Kpn* I, *Bgl* II, and *Bam* I) were tested to determine which would yield a small number of fragments of EBV (B95-8) DNA that could be resolved on agarose gels (Fig. 1). The molecular weights of EBV DNA fragments were estimated by comparison of their electrophoretic mobility with that of fragments of T5b1 and HSV (MP) DNA. The relative molar ratios were calculated as described in Materials and Methods. The results were as follows: (i) *Eco*RI, *Hsu* I, and *Sal* I each yielded up to 15 fragments, ranging in molecular weight from 0.6×10^6 to 32×10^6 , whereas *Bam* I and *Bgl* II each gave more than 20 fragments smaller than 11×10^6 (Fig. 1, Table 1). (ii) Almost all fragments produced by digestion of EBV (B95-8) DNA with *Eco*RI, *Sal* I, and *Hsu* I contained an amount of 3H -thymidine (Fig. 1, Table 1) proportional to their molecular weight. One *Eco*RI fragment (fragment D) contained between one-quarter and one-half of the expected 3H -thymidine label (Fig. 1 and Table 1). Two minor fragments (Fig. 1, *Hsu* I fractions 168 to 171 and *Eco*RI fractions 149 to 151) were consistently observed, but were not assigned letter designations because they were present in molar ratios of less than 0.25. Two other fragments, the G fragment of *Eco*RI and I fragment of *Hsu* I, contained more label than would be expected if these bands contained single fragments of their estimated molecular weight (Fig. 1 and Table 1). Analysis of the *Sal* I, *Hsu* I, and *Eco*RI fragments of EBV (B95-8) DNA labeled with ^{32}P -orthophosphate and of *Sal* I and *Hsu* I fragments labeled with 3H -cytosine confirmed the relative molar ratios obtained with 3H -thymidine-labeled DNA. The only discrepancy observed was that the *Hsu* I C fragment contained slightly more 3H -thymidine label and the B fragment slightly less than was expected for fragments of their size (Fig. 1), whereas the reverse result was obtained when the DNA was labeled with 3H -cytosine (data not shown). (iii) The sums of the molecular weights of the fragments produced by *Eco*RI, *Sal* I, and *Hsu* I digestion (Table 1) were close to the reported molecular weight for EBV (B95-8) DNA (23). (iv) Treatment of EBV (B95-8) DNA with *Kpn* I yielded at least 15 separable fragments. The *Kpn* I C and E fragments probably consisted of two components, since these fragments contained approximately twice as much label as expected for fragments with their estimated molecular weight (Table 1). In addition, three *Kpn* I fragments (B, L, and M) were present in approximately half-molar amounts. The sum of

FIG. 1. Distribution of tritium label in agarose gel electropherograms of fragments of EBV (B95-8) DNA produced by treatment with *Hsu* I, *Sal* I, *Bgl* II, *Eco*RI, *Kpn* I, and *Bam* I restriction enzymes. The procedures used in labeling and purifying EBV DNA, in treating the DNA with restriction enzymes, in separating fragments of the DNA in 0.4% agarose gels, and in determining the distribution of radioactivity in the gels are described in Materials and Methods. The direction of electrophoresis is from left to right.

the molecular weights of the *Kpn* I fragments of EBV (B95-8) DNA was approximately 1.3×10^8 (Table 1). Intact T5b1 DNA mixed with the EBV (B95-8) DNA before incubation with *Kpn* I enzyme was fully digested.

The results of analyses on agarose gels of the fragments produced by digestion of EBV (HR-1) DNA with *Eco*RI, *Hsu* I, or *Sal* I (Fig. 2) indicated the following. (i) In each instance, some of the fragments were identical in molecular weight to a fragment produced by treating EBV (B95-8) DNA with the same enzyme (Fig. 4). Other fragments in each EBV DNA preparation were unique (Fig. 4). (ii) Some fragments of EBV (HR-1) DNA produced by *Eco*RI, *Hsu* I, and *Sal* I contained half as much label as expected for a fragment of their size (Fig. 2 and Table 1). Each enzyme produced at least two such fragments present in half-molar amounts

(Fig. 2 and Table 1). The finding of half-molar fragments was confirmed by analyses in which the amount of DNA in each fragment was determined from the relative optical density of the fragment in ethidium bromide-stained gels. Three minor components (*Hsu* I fractions 93 to 98, 136 to 149, and 157 to 159) were consistently present, but were not assigned letter designations because they were present in molar ratios of less than 0.25. (iii) The sums of the molecular weights of the *Eco*RI, *Hsu* I, and *Sal* I fragments were 132×10^6 , 160×10^6 , and 130×10^6 , respectively (Table 1). (iv) Identical results were obtained using EBV purified from HR-1 cultures originally obtained from G. Klein and passaged for 3 years in our laboratory or HR-1 cultures more recently obtained from R. Glaser.

The HR-1 cell line was originally derived (11) from a continuous culture of lymphoblasts (Ji-

TABLE 1. *EBV restriction enzyme fragments*

Frag- ment	B-95								HR-1							
	<i>Eco</i> RI		<i>Hsu</i> I		<i>Sal</i> I		<i>Kpn</i> I		<i>Eco</i> RI		<i>Hsu</i> I		<i>Sal</i> I			
	Size ^a	Molarity	Size	Molarity	Size	Molarity	Size	Molarity	Size	Molarity	Size	Molarity	Size	Molarity	Size	Molarity
A	32.5	1	28 ^b	1	32 ^b	1	29	1	26	1	35	0.5-1	27.5	0.5		
B	21	1	20	1	27.5	1	22	0.5	21	1	27	0.5	22.5	0.5		
C	11.61	1	17.6	1	19.5	1	11	2	20	1	17.6	1	12.8	1		
D	8.2	0.25-0.5	13.8	1	11.0 ^b	1	8.4	1	14.5	1	16.0	0.5	11.0	2		
E	7.5	1	7.0	1	8.3	1	6.9	2	12.5	0.25	11.3	2	9.2	1		
F	5.4	1	4.0	1	5.0	1	6.6	1	10	0.5	9.6	1	8.3	1		
G	4.1	1.5-2	3.5	1	4.0	1	6.2	1	8.0	1	8.0	1	7.5	1		
H	3.6	1	3.1	1	2.0	ND ^c	4.1	1	5.4	1	4.4	1	4.8	1		
I	2.8	1	2.2	2			4.0	1	4.2	0.5	4.0	1	4.0	0.5		
J	2.2	1	2.1	1			2.6	1	3.5	0.5	3.5	1	3.4	0.5		
K	1.5	1	1.7	1			2.5	1	2.8	1-2	3.1	1	2.5	2		
L	1.0	1	1.3	1			2.4	0.5	2.1	ND ^c	2.2	ND ^c	2.0	2		
M	0.6	ND ^c	1.0	ND ^c			2.1	0.5	1.5	ND ^c	2.1	ND ^c	1.3	3		
							1.8	ND ^c			1.6	ND ^c				
							1.5	ND ^c			1.5	ND ^c				
Total mol wt ^d	105 × 10 ⁶		105 × 10 ⁶		109 × 10 ⁶		129 × 10 ⁶		132 × 10 ⁶		158 × 10 ⁶		130 × 10 ⁶			

^a Molecular weight estimated from position of marker DNAs as described in Materials and Methods.

^b End fragment.

^c ND, Molarities not calculated. The total number of counts per minute in these fragments was less than two times the total background.

^d In those instances where the relative molar ratio was clearly in excess of 1, the fragment was considered to have two components of equal molecular weight.

joye) established from a Burkitt tumor biopsy. Comparison of the DNA of EBV from Jijoye and HR-1 cultures would therefore be of interest. However, the production of EBV from Jijoye cultures is smaller and less constant than from the HR-1 cell line (4). To obtain sufficient EBV (Jijoye) DNA for analysis, it was necessary to take a broad region of the dextran gradient. This DNA was not run on sucrose gradients before treatment with restriction enzyme. The summary data of fragments of EBV (Jijoye) DNA (Fig. 4) were derived from analyses of electropherograms of [³H]thymidine-labeled and unlabeled EBV (Jijoye) DNA (Fig. 3) run in parallel with electropherograms of fragments of EBV (HR-1) DNA. The data indicate that the *Eco*RI A fragment of EBV (Jijoye) DNA is larger than the corresponding fragment of HR-1 DNA and that there is no Jijoye fragment corresponding to the D fragment of EBV (HR-1) DNA. The differences between fragments of EBV (Jijoye) DNA and EBV (HR-1) DNA were confined to EBV (HR-1) DNA fragments that were not present in EBV (B95-8) DNA. However, the molecular weights of the EBV (Jijoye) DNA fragments which differed from those of EBV (HR-1) DNA did not correspond to the molecular weights of fragments of EBV (B95-8) DNA. The background in the region of lower-molecular-weight fragments of EBV (Jijoye) DNA was much higher than in the comparable analyses of EBV (HR-1) or

(B95-8) DNA, likely as a result of contamination and random degradation of the EBV (Jijoye) DNA. Therefore, the relative molar abundance of fragments of EBV (Jijoye) DNA could not be determined from these data.

Identification of the end fragments of EBV (B95-8) DNA. The distribution of [³H]thymidine label in *Sal* I or *Hsu* I restriction endonuclease fragments of [³H]thymidine-labeled EBV (B95-8) DNA was compared with that of DNA which had been preincubated with lambda exonuclease in order to identify the end fragments. Lambda exonuclease is a processive enzyme that attacks 5'-phosphoryl termini of native DNA preferentially (1), although not exclusively (26). After lambda exonuclease treatment and cleavage with restriction enzyme, fragments positioned at ends of native EBV DNA would be expected to consist of native DNA with a single-strand 3'-hydroxyl tail. The migration of DNA that contains single- and double-stranded segments is aberrant on agarose gels (8). In control experiments undertaken to determine the proper condition for lambda exonuclease treatment, terminal fragments of T5b1 DNA of 7.7×10^6 and 8.6×10^6 daltons were more than 90% reduced without any decrease in the penultimate fragments. The results of lambda exonuclease pretreatment of EBV (B95-8) DNA illustrated in Fig. 5 and 6 indicate the following. (i) The *Hsu* I A fragment was removed by lambda exonuclease

FIG. 2. Distribution of tritium label in agarose gel electropherograms of fragments of [^3H]thymidine-labeled EBV (HR-1) DNA produced by treatment with *Hsu* I, *Sal* I, and *Eco*RI restriction enzymes. The procedures used are described in Materials and Methods. The direction of electrophoresis is from left to right.

digestion (Fig. 5) and is therefore located near (within 8×10^6 daltons) an end of the EBV (B95-8) DNA molecule. No change was apparent in the amount of fragments B through J, and presumably the other end is a fragment smaller than J. (ii) *Sal* I A and D fragments were significantly decreased by lambda exonuclease pretreatment (Fig. 6) and were similarly identified as terminal fragments. The *Sal* I D fragment differed from other *Sal* I fragments in that it consisted of multiple discrete, but closely packed, bands, all of which decreased after

lambda exonuclease treatment. The simplest interpretation of this finding is that there exists slight length variability at the D-fragment terminus of the EBV (B95-8) DNA molecule.

DISCUSSION

These studies were undertaken to begin to determine the arrangement of sequences in EBV DNA. *Eco*RI, *Hsu* I, *Sal* I, and *Kpn* I restriction enzymes cleave EBV DNA into fragments that are separable on 0.4% agarose gels, whereas *Bam* I and *Bgl* II yield fragments whose size is too close to be separable. On the basis of their susceptibility to degradation by lambda exonuclease, the *Hsu* I A and *Sal* I A and D fragments of EBV (B95-8) DNA have been identified as terminal fragments.

The analyses reported here of the size and relative molar abundance of restriction enzyme fragments of the DNA of EBV strains indicate that there are differences in base sequence between strains of EBV and between DNA molecules of the same strain of EBV. Several lines of evidence indicate that there is heterogeneity in the DNA molecules of at least one strain of EBV. Thus, some *Hsu* I and *Sal* I fragments of EBV (HR-1) DNA are present in equal molar abundance; other fragments are present in half-molar abundance; and the sum of the molecular weight of the fragments produced by each enzyme is in excess of the known molecular weight of EBV (HR-1) DNA (23). In evaluating the significance of these observations, the following points should be considered. (i) The presence of minor bands is unlikely to be an artifact of incomplete digestion of the DNA. T5b1 DNA was incubated with EBV DNA in each enzyme reaction mixture and was itself completely digested. Moreover, the ratio of minor to major bands was constant in repeated experiments and was not affected by increasing the concentration of restriction enzymes. (ii) The EBV (HR-1) DNA used in these analyses sedimented in sucrose velocity gradients as a single band of approximately 100×10^6 daltons. (iii) EBV (HR-1) DNA contains only EBV homologous sequences. Thus, DNA from cells infected with EBV drives denatured EBV (HR-1) DNA more than 90% into hybrid, whereas DNA from uninfected cells has no effect on the renaturation of EBV (HR-1) DNA (13, 14, 20, 22). (iv) Analysis on agarose gels of the restriction enzyme fragments of EBV DNA purified from HR-1 cultures that have been maintained separately for several years yielded identical results.

Taken together, these data suggest that EBV (HR-1) DNA contains at least two populations of molecules with a high degree of homology

FIG. 3. Electropherogram of fragments of [^3H]thymidine-labeled EBV (Jijoye) DNA produced by treatment with *EcoRI* restriction enzyme. The photograph of an agarose gel stained with ethidium bromide is positioned above a graph of the distribution of tritium label in that gel. The procedures used are described in Materials and Methods. The direction of electrophoresis is from left to right.

and with unique and common restriction enzyme fragments. The simplest explanation for these observations is that they may be due to alternative molecular arrangements of identical DNA sequences, possibly in association with inverted repeats in the DNA. Precedence for this hypothesis has been established from studies of the sequence of herpes simplex DNA (25, 29). Another possibility is that the HR-1 cultures contain a stable mixture of two closely related strains of EBV. This possibility has been suggested by studies that have found two distinct types of EBV-related nuclear antigen, EBNA, in lymphocytes infected with EBV (HR-1) (3). Comparative analysis of the DNA in other strains of EBV could help to distinguish between these alternatives. The analyses reported here of EBV (B95-8) DNA are ambiguous in this regard in that submolar fragments and a total molecular weight for all fragments in excess of 100×10^6 were clearly obtained in only one instance, i.e., digestion of EBV (B95-8) DNA with *Kpn* I restriction nuclease.

With regard to the differences reported here

between the DNA of strains of EBV, the following points should be made. (i) EBV (HR-1) is produced from the HR-1 clone (11) of a continuous lymphoblast culture (Jijoye) established from a Burkitt tumor biopsy. EBV (B95-8) is produced from a clone of marmoset cells infected in vitro with EBV derived from a continuous culture of lymphoblasts established from a patient with infectious mononucleosis (18). EBV (B95-8) has the ability to transform but cannot induce early antigen on superinfection, whereas EBV (HR-1) can induce early antigen but lacks the ability to transform (16, 17). The enhanced ability of EBV (B95-8) to transform could result from the presence of DNA sequences required for transformation or from the absence of DNA sequences that might inhibit transformation. (ii) Some of the fragments produced by treating EBV (B95-8) or (HR-1) DNA with *EcoRI*, *Hsu* I, and *Sal* I restriction enzymes have identical molecular weights in agarose gels. Since EBV (B95-8) DNA contains approximately 85% of the sequences of EBV (HR-1) DNA (23, 27) and EBV (HR-1) DNA

FIG. 4. Comparison of the molecular weights of fragments of EBV (B95-8), (Jijoye), and (HR-1) DNAs treated with *EcoRI*, *Hsu I*, or *Sal I* restriction enzyme. Fragments present in molar ratios of less than 1 are indicated by narrow bands.

FIG. 5. Agarose gel electropherogram of *Hsu I* restriction enzyme fragments of [^3H] thymidine-labeled EBV (B95-8) DNA (upper panel) and of [^3H]thymidine-labeled EBV (B95-8) DNA which had been treated with lambda exonuclease (lower panel) to digest 5' ends of the DNA. The direction of electrophoresis is from left to right. The bottom end of the agarose gel (to the right of fragment G) shown in the lower panel contracted during the electrophoresis. The small band of labeled DNA migrating more rapidly than fragment G in the lower panel is likely, therefore, to be fragment H.

contains more than 95% of the sequences of EBV (B95-8) DNA (23), it is likely that the fragments which are of the same molecular weight have similar base sequences. Sequences unique to each strain are likely to be contained

FIG. 6. Agarose gel electropherogram of *Sal I* restriction enzyme fragments of [^3H]thymidine-labeled EBV (B95-8) DNA (upper panel) and of [^3H]thymidine-labeled EBV (B95-8) DNA which had been treated with lambda exonuclease to digest 5' ends of the DNA. The direction of electrophoresis is from left to right.

in one or more fragments whose molecular weight is distinctive. Hybridization of fragments with DNA will permit identification of sequences unique to EBV (HR-1) DNA and may permit the identification of sequences unique to EBV (B95-8) DNA. (iii) EBV (Jijoye) is not as amenable to biochemical analyses as its high-virus-producing HR-1 clone. Nevertheless, it is clear from these studies that the treatment of EBV (Jijoye) DNA with *EcoRI* restriction enzyme yields fragments that are identical and others that differ in molecular weight from fragments of EBV (HR-1) DNA. As might be anticipated from the common lineage of EBV (Jijoye) and (HR-1), there are fewer differences between their DNA fragments than between those of EBV (HR-1) and (B95-8) DNAs. That the size of many of the fragments common to EBV (Jijoye) and (HR-1) is also common to EBV (B95-8) DNA and that all of the fragments which differ also differ from those of EBV (B95-8) DNA suggest that there are sequences preferentially conserved in EBV DNA.

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ADDENDUM IN PROOF

Current studies using "blots" of fragments of viral DNA (N. Traub, R. Pritchett, and E. Kieff, manuscript submitted for publication) have identified those *EcoRI* and *Hsu I* fragments of the DNA of the HR-1 strain of EBV that contain DNA sequences missing from the DNA of the B95-8 strain. These sequences are components of the *EcoRI* C and D and the *Hsu I* E and N fragments of EBV (HR-1) DNA. The *EcoRI* K (and possibly A) and *Hsu I* B fragments of the DNA of the B95-8 strain of EBV contain sequences that are missing from EBV (HR-1) DNA.

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