Epstein-Barr Virus DNA

IX. Variation Among Viral DNAs from Producer and Nonproducer Infected Cells

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A comparative analysis of three Epstein-Barr virus DNAs from American patients with infectious mononucleosis (B95-8, Cherry, and Lamont) and four Epstein-Barr virus DNAs from African patients with Burkitt lymphoma (AG876, W91, Raji, and P3HR-1) indicated that the usual format of Epstein-Barr virus DNA includes a variable number of direct repeats of a 0.35×10^6 -dalton sequence (T_R) at both ends of the DNA, a 9×10^6 -dalton sequence of largely unique DNA (U_S), a variable number of repeats of a 2×10^6 -dalton sequence (I_R), and a 89×10^6 -dalton sequence of largely unique DNA (U_L). Within U_L there was homology between DNA at 26×10^6 to 28×10^6 daltons and DNA at 93×10^6 to 95×10^6 daltons. The relative sequence order (T_R , U_S , I_R , U_L , T_R) did not vary among "standard" Epstein-Barr virus DNA molecules of each isolate. B95-8 DNA had an unusual deletion extending from 91×10^6 to 100×10^6 daltons, and P3HR-1 DNA had an unusual deletion extending from 23.5×10^6 to 26×10^6 daltons. There was sufficient variability among the *EcoRI* and *BamHI* fragments of the DNAs to identify each isolate specifically. However, we discerned no distinguishing features for the two geographic or pathogenic origins of the seven isolates. Three intracellular DNAs (Raji, Lamont, and Cherry) and one virion DNA (P3HR-1) were heterogenous in molecular organization and had subpopulations of rearranged or defective molecules. Some regions, particularly 59×10^6 to 63×10^6 daltons and sequences around T_R , frequently participated in rearrangements. Restriction endonuclease maps of the standard and rearranged DNAs of the seven isolates are presented.

The host range of Epstein-Barr virus (EBV) *in vitro* is limited to primate B lymphocytes (18). B lymphocytes are also important targets of natural infection (8). EBV-infected lymphocytes proliferate in culture but are either nonpermissive or only partially permissive of virus replication. Viral DNA can be obtained in small amounts from purified virus produced by partially permissive B-lymphocyte cultures, such as B95-8 (28), P3HR-1 (19), AG876 (30), and W91 (27). Viral DNA can also be partially purified from infected, nonpermissive cell cultures. The latter approach involves several cycles of isopycnic banding of infected cellular DNA to enrich progressively for viral DNA, which is more dense (1.718 g/cm^3) than cellular DNA (1.695 g/cm^3).

The DNA of B95-8, an infectious mononucleosis isolate (28), consists of tandem direct repeats of a 0.35×10^6 -dalton sequence (T_R) at both ends, a short unique sequence of 9×10^6 daltons (U_S), a long unique sequence of 80×10^6 daltons (U_L), and tandem direct repeats of a 2×10^6 -dalton sequence (I_R), which separate U_S

and U_L (3, 11-15, 21, 23, 38). Recombinant DNA technology has been used to further purify and amplify viral DNA and to obtain precise restriction endonuclease maps of B95-8 viral DNA and partial maps of AG876 and W91 viral DNAs (3, 11-15, 21, 35). The overall sizes of B95-8, AG876, and W91 DNAs are 115×10^6 daltons (3, 5, 11, 32, 35). The DNAs of AG876 and W91, two Burkitt tumor isolates, differ from B95-8 DNA by the addition of a 9×10^6 -dalton sequence at a site in *BamHI* fragment I of B95-8 U_L (11, 21, 35). This 9×10^6 -dalton DNA sequence contains two *BamHI* cleavage sites, so that AG876 and W91 DNAs have three new contiguous *BamHI* fragments (fragments B1, W1 and I1; left to right in the map shown in Fig. 5 [35]). The B95-8 deletion begins in *BamHI*-B1, includes all of *BamHI*-W1, and ends in *BamHI*-I1 (35). Sequences which map at 93×10^6 to 95×10^6 daltons in the "additional" DNAs of AG876 and W91 have homology to sequences which map at 26×10^6 to 28×10^6 daltons (35). There is no homology between the additional DNA and cel-

lular DNA (35). At least 4.5×10^6 daltons and probably all of the additional DNA of AG876 and W91 is present in P3HR-1 viral DNA and in the DNA of cells infected with American strains of virus, indicating that B95-8 is an unusual deletion derivative (21, 35, 36).

The objective of our experiments was to determine whether there were other differences among B95-8, AG876, and W91 DNAs and to examine the extent of variation among other EBV DNAs. The other EBV DNAs analyzed were from the following sources: P3HR-1, a Burkitt tumor-derived viral DNA which is known to consist of at least two populations, one similar in organization to other EBV DNAs but with a deletion at 23.5×10^6 to 26×10^6 daltons and a second more highly defective population (5, 10, 14, 21, 36); Raji, a nonpermissively infected Burkitt tumor-derived cell line (34); and Lamont and Cherry, two nonpermissively infected cell lines established from the peripheral blood of patients convalescing from infectious mononucleosis (35). The sequences in each of the *EcoRI* and *BamHI* fragments of the virion (producer) and intracellular (nonproducer) DNAs were identified by Southern blot hybridizations, using individual labeled cloned fragments of EBV DNA which spanned the entire genome, including the region deleted from B95-8 DNA (3, 35). Previous attempts to compare the restriction endonuclease fragments of EBV DNAs from various sources have not extensively employed probes specific for different regions of EBV DNA (14, 39, 41). Therefore, it was not possible in previous studies to identify and map specific regions of variability in the EBV genome.

MATERIALS AND METHODS

Cell culture. The procedures and materials used for cell culture and purification of EBV from the culture fluids of producer cell lines have been described previously (7). AG876 (30), W91 (27), and P3HR-1 (19) are virus-producing cell lines that were derived independently by culture of African Burkitt tumor tissue or, in the case of W91, by infection of marmoset cells with tumor-derived virus. Raji is an EBV-infected African Burkitt tumor cell line which is not permissive of EBV replication (34). Cherry and Lamont are EBV-infected cell lines that were established by culture of lymphocytes of patients with infectious mononucleosis and are not permissive of virus replication (35; W. Henle and G. Henle, personal communication).

Preparation of viral DNA. DNAs from the partially permissive AG876, W91, and P3HR-1 cultures were extracted from virus purified from the culture media. DNA to be labeled *in vitro* by nick translation was purified further by equilibrium sedimentation in neutral CsCl. The DNA solution was adjusted to 25 mM Tris-hydrochloride (pH 7.6), 2.5 mM EDTA, 0.2%

(wt/vol) Sarkosyl, and 1.715 g of CsCl per cm^3 (Eastern Chemical, Hauppauge, N.Y.). The solution was centrifuged in a VTi50 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 40,000 rpm for 18 h at 20°C. Fractions with densities between 1.71 and 1.73 g/cm^3 were pooled and dialyzed against 1-mM Tris-hydrochloride (pH 7.6)–0.1 mM EDTA.

DNAs from nonpermissive Raji, Lamont, and Cherry cultures were enriched for EBV DNA by three cycles of isopycnic centrifugation of cellular DNA and repeated selection of higher-density DNA. The cells from 8 liters of a culture (about 5×10^9 cells) were collected by low-speed centrifugation, washed in cold phosphate-buffered saline, and suspended in cold 50 mM Tris-hydrochloride (pH 7.6)–10 mM EDTA containing 100 μg of proteinase K (EM Laboratories, Inc., Elmsford, N.Y.) per ml; 1% (wt/vol) sodium dodecyl sulfate was added while the cell suspension was swirled in a 45°C water bath. The viscous lysate was incubated at 39°C for 15 min. The lysate was homogenized in a Dounce apparatus by 10 slow strokes with a loose-fitting pestle, and this treatment yielded a homogeneous, viscous solution of high-molecular-weight DNA. The solution was adjusted to contain 2% (wt/vol) Sarkosyl and 1.715 g of CsCl per cm^3 . The lysate was diluted to 275 ml with a solution containing 25 mM Tris-hydrochloride (pH 7.6), 2.5 mM EDTA, and 1.715 g of CsCl per cm^3 . The mixture was centrifuged in a VTi50 rotor at 40,000 rpm for 18 h at 20°C. Fractions with a density lower than 1.730 g/cm^3 were pooled until significant viscosity was encountered due to the large amounts of high-molecular-weight cellular DNA present in the gradients. The remaining viscous fractions were pooled separately and centrifuged as described above to recover additional viral DNA, which was trapped in the viscous cellular DNA region. Fractions between 1.730 g/cm^3 and the viscous cellular DNA from this scavenger isopycnic banding were combined with the initial pool enriched for viral DNA and centrifuged through two additional cycles of isopycnic banding in neutral CsCl. Fractions with densities between 1.730 and 1.707 g/cm^3 were selected from these gradients and dialyzed against 1 mM Tris-hydrochloride (pH 7.6)–0.1 mM EDTA. This density-selected DNA had a size of about 100×10^6 daltons, as shown by comigration with intact herpes simplex virus type 2 DNA in a 0.2% agarose gel; 15 μg of a possible 50 μg (assuming that 60 EBV viral genome equivalents per cell are present in Raji [20, 33]) was recovered in 150 μg of total DNA. Therefore, a 500-fold enrichment of intracellular EBV DNA relative to cellular DNA could be obtained with this method.

Covalently closed circular recombinant plasmid DNAs containing B95-8 or AG876 *BamHI* fragments cloned in pBR322 (3, 35) or recombinant cosmid DNAs containing W91 *EcoRI* fragments (35) were purified by isopycnic banding in neutral CsCl gradients containing ethidium bromide. The *EcoRI*-D-1, Jhet fused end fragment from circular episomal B95-8 DNA cloned in Charon 4A (3) was a gift from Nancy Raab-Traub and Chris Beisel. AG876 *BamHI* fragment D1 (35) was cloned in pBR322 under P2 safety precautions in accordance with a Memorandum of Understanding and Agreement approved by the Institutional Biohazard Committee, as described previously (3). Am-

picillin-resistant tetracycline-sensitive colonies were screened by in situ hybridization (2) to identify colonies which contained DNA homologous to labeled cloned B95-8 *Bam*HI fragments H and X (3). DNA fragment *Bg*III-A (0 to 11×10^6 daltons) or the 3.4×10^6 - and 4.6×10^6 -dalton *Eco*RI components of cloned *Bam*HI-A (102×10^6 to 110×10^6 daltons) were separated by agarose gel electrophoresis, eluted from gel slices by electrophoresis, extracted with butanol to remove ethidium bromide, and concentrated by vacuum dialysis against 1 mM Tris-hydrochloride (pH 7.6)–0.1 mM EDTA.

Restriction endonucleases and gel electrophoresis. Restriction endonucleases *Eco*RI, *Bam*HI, and *Hin*FI were obtained from Bethesda Research Laboratories, Rockville, Md. Enzyme digestions were performed by using the conditions specified by Bethesda Research Laboratories.

Separation of DNA fragments by gel electrophoresis was done in 0.4% (*Eco*RI digests), 0.8% (*Bam*HI digests), or 1% (*Hin*FI digests) agarose (Marine Colloids, Rockland, Maine) containing ethidium bromide.

Radioactive labeling of DNA and hybridization to nitrocellulose filters. Viral DNA fragments were immobilized on nitrocellulose filters (Millipore Corp., Bedford, Mass.) by the transfer technique of Southern (40). Identical filter strips of each restriction endonuclease digest were made by slicing preparative Southern blots into strips 2 mm wide.

³²P-labeled DNA fragment probes were made by nick translation of 50 to 200 ng of each cloned *Bam*HI or *Eco*RI EBV DNA fragment or of *Bg*III fragment A (37). The EBV DNA fragments were not separated from the pBR322 and Charon 4A cloning vectors. ³²P-labeled EBV total genome probe was made by nick translation of AG876 DNA.

For hybridization, nitrocellulose filters were preincubated for 1 h at 72°C in 6× SSC (pH 7.4; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll, 1 mM EDTA, 50 μg of salmon sperm DNA per ml, and 0.2% sodium dodecyl sulfate (6, 16). DNA probes were heat denatured. Each probe solution was made 6× SSC containing 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll, 1 mM EDTA, 50 μg of salmon sperm DNA per ml, and 0.2% sodium dodecyl sulfate. Hybridization was carried out at 72 to 74°C with shaking for 8 to 20 h. After hybridization, the nitrocellulose strips were washed with sequential changes of 2× SSC containing 0.2% sodium dodecyl sulfate, 0.5× SSC containing 0.2% sodium dodecyl sulfate, and 0.2× SSC containing 0.2% sodium dodecyl sulfate for 2 to 4 h at 72°C. The filters were dried and exposed to X-ray film (Kodak Corp., Rochester, N.Y.) for autoradiography or fluorography.

RESULTS

The *Eco*RI and *Bam*HI fragments of three virion and three intracellular EBV DNAs were compared with the fragments of virion and intracellular B95-8 DNAs. The virion DNAs were derived from cultures of AG876, W91, and P3HR-1 cells. The intracellular DNAs were de-

rived from cultures of Raji, Lamont, and Cherry cells. The intracellular DNAs were enriched at least 100-fold for viral DNA by three cycles of isopycnic centrifugation, as described above.

Comparison of the sizes and relative amounts of restriction enzyme fragments of EBV DNAs. The sizes of the *Eco*RI and *Bam*HI fragments of the virion and intracellular DNAs (Fig. 1) were determined from their electrophoretic mobilities in agarose gels compared with the mobilities of fragments of B95-8 DNA. The sizes of the B95-8 *Eco*RI and *Bam*HI fragments have been determined previously by comparison with intact and restriction endonuclease fragments of T5, lambda, φX174, and pBR322 DNAs (3, 11). The molecular weight of B95-8 DNA *Eco*RI fragment A has been estimated to be 40×10^6 , since it consists of 16×10^6 daltons of unique DNA and 12 tandem reiterations of the 2×10^6 -dalton I_R sequence (3, 12, 15, 38). Figure 1 shows an autoradiogram of the *Eco*RI and *Bam*HI fragments of the virion and intracellular DNAs, in which the viral DNA fragments were identified by hybridization to labeled virion AG876 DNA. Many of the *Eco*RI and *Bam*HI fragments of the virion and intracellular DNAs were similar in size to the fragments of virion and intracellular B95-8 DNAs. There were enough differences in the restriction fragments of each of the DNAs to distinguish each isolate (Fig. 1).

There was also heterogeneity in the relative amounts of several *Eco*RI and *Bam*HI fragments of P3HR-1, Raji, Cherry, and Lamont DNAs (Fig. 1), indicating that each of these DNAs was heterogeneous in molecular structure. The number of higher-molecular-weight *Eco*RI fragments (Fig. 1A), in particular, suggested that some fragments shared identical DNA sequences. Otherwise, the cumulative molecular weight of these fragments would have exceeded the size (115×10^6 daltons) of the EBV genome (3, 5, 11, 14, 32, 35).

The terminal heterogeneous fragments *Eco*RI-Dhet and -L₁Jhet and *Bam*HI-Jhet and -Nhet were missing from the intracellular DNA of nonpermissively infected cells (Fig. 1), since the terminal fragments of the linear form of EBV DNA are joined in circular episomal molecules within cells (1, 3, 21, 23, 25, 35). Consistent with this observation, *Bam*HI-Jhet and -Nhet were less abundant in intracellular B95-8 DNA (Fig. 1B), which presumably represented a mixture of DNA from nonpermissively and permissively infected cells.

***Eco*RI and *Bam*HI restriction endonuclease maps of virion and intracellular EBV DNAs.** Labeled recombinant DNAs represent-

FIG. 2. Mapping of producer isolate AG876 DNA. Identical strips from preparative nitrocellulose filters containing the BamHI (A) and EcoRI (B) fragments of AG876 DNA were hybridized with labeled AG876 DNA (lane TP), cloned B95-8 BamHI fragments B1, W1, and I1, or cloned EcoRI-D-I, J. The fused end fragment as described in the text. The probe fragment is indicated above the hybridization result for each blot strip, and the strips are arranged from left to right in the order of the probe fragments in EBV DNA. A map of the B95-8 and AG876 BamHI fragments used as labeled probes is shown in Fig. 5 which defines the prototype FRV genome.

FIG. 3. Mapping of producer isolate W91 DNA. Identical strips from preparative Southern blots containing the BamHI (A) and EcoRI (B) fragments of W91 DNA were hybridized with labeled probes as described in the legend to Fig. 2.

FIG. 4. Mapping of producer isolate P3HR-1 DNA. Identical strips from preparative Southern blots containing the BamHI (A) and EcoRI (B) fragments of P3HR-1 DNA were hybridized with labeled probes as described in the legend to Fig. 2.

Linkage of EBV (AG876) DNA Fragments

Linkage of EBV (W91) DNA Fragments

Linkage of EBV (P3HR-1) DNA Fragments

A. Fragments similar in arrangement to fragments of EBV (B95-8 or A6876) DNA

B. Fragments different in arrangement from fragments of EBV (B95-8 or A6876) DNA

FIG. 5. Maps of the DNAs of producer isolates AG876, W91, and P3HR-1. The hybridization data shown in Fig. 2 through 4 are summarized. Maps of the cloned B95-8 and AG876 BamHI fragments used as probes are shown on the lines labeled **EBV DNA fragments**. Maps of the BamHI and EcoRI restriction enzyme fragments of each isolate are shown aligned above and below the prototype probe fragment map. The hybridization data are shown by arrows from each probe fragment to the homologous BamHI or EcoRI fragment(s). (A) Standard P3HR-1 DNA map. (B) Unusual or rearranged P3HR-1 DNA fragments. The deletion of intervening DNA is indicated by a zigzag line. The general structure of EBV DNA is shown at the bottom. The internal reiteration (I_R) region contains a variable, nonintegral number of copies of a 2×10^6 -dalton sequence. Since the size of the internal reiteration varied among the EBV DNAs, the map units for prototype EBV DNA are shown with a discontinuity across this region at 11×10^6 and 23×10^6 daltons. A modal number of six complete copies of the internal reiteration was assigned to prototype EBV DNA (12×10^6 daltons). D_L and D_R indicate sequence homologies or duplications between regions at the left and right of U_L in EBV DNA.

FIG. 6. Mapping of nonproducer isolate Raji DNA. Identical strips from preparative Southern blots containing the BamHI (A) and EcoRI (B) fragments of Raji cellular DNA enriched for viral sequences by CsCl density selection were hybridized with labeled probes as described in the legend to Fig. 2. The BglIII-A fragment of B95-8 virion DNA (0 to 11×10^6 daltons), B95-8 EcoRI-C cloned in Charon 4A (85×10^6 to 91×10^6 and 100×10^6 to 105×10^6 daltons; B95-8 DNA has a deletion of the intervening 9×10^6 daltons), and the plasmid vector pBR322 were also labeled and hybridized to blots of Raji DNA. (Several of the pBR322-EcoRI fragments hybridized weakly to identical 2.8×10^6 - and 1.2×10^6 -dalton fragments on Raji BamHI blots. Labeled pBR322 hybridized to the same fragments, but did not hybridize to Raji EcoRI blots. A possible explanation for these observations is that the BamHI enzyme contained a trace amount of pBR322-related DNA).

Fig. 7. Mapping of nonproducer isolate Chery DNA. Identical strips from preparative Southern blots containing the BamHI (A) and EcoRI (B) fragments of Chery cellular DNA enriched for viral sequences by CsCl density selection were hybridized with labeled probes as described in the legend to Fig. 2.

FIG. 8. Mapping of nonproducer isolate Lamont DNA. Identical strips from preparative Southern blots containing the BamHI (A) and EcoRI (B) fragments of Lamont cellular DNA enriched for viral sequences by CsCl density selection were hybridized with labeled probes as described in the legend to Fig. 2.

Linkage of EBV (Raji) DNA Fragments

A. Fragments similar in arrangement to fragments of EBV(B95-8 or AG876) DNA

B. Fragments different in arrangement from fragments of EBV (B95-8 or AG876) DNA

Linkage of EBV (Cherry) DNA Fragments

A. Fragments similar in arrangement to fragments of EBV(B95-8 or AG876) DNA

B. Fragments different in arrangement from fragments of EBV(B95-8 or AG876) DNA

Linkage of EBV (Lamont) DNA Fragments

FIG. 9. Maps of the DNAs of nonproducer isolates Raji, Cherry, and Lamont. The hybridization data shown in Fig. 6 through 8 are summarized and presented as described in the legend to Fig. 5. The EBV nonproducer viral DNA maps are presented in a linear format. Nonproducer EBV genomes actually exist as circular episomes in which the ends of each linear molecule are joined.

and W91. The *EcoRI*-C of each virion and intracellular DNA was identified by labeled *Bam*HI fragments W, V, b, B1, W1, I1, I, and A (Fig. 2 through 4 and 6 through 8), and was 9×10^6 daltons larger than the equivalent fragment of B95-8 DNA. Thus, AG876, W91, Raji, Cherry, and Lamont DNAs each had 9×10^6 daltons of additional DNA. Hybridization of the labeled *Bam*HI fragments to *Bam*HI fragments of each virion and intracellular DNA (Fig. 2 through 4 and 6 through 8) confirmed that the additional DNA mapped at 91×10^6 to 100×10^6 daltons in the U_L component of these DNAs. In four of these DNAs, W91 (Fig. 4), Raji (Fig. 6), Cherry (Fig. 7), and Lamont (Fig. 8), the additional DNA had two *Bam*HI sites, which yielded *Bam*HI fragments identical in size to AG876 *Bam*HI fragments W1 and I1 (35). Two types of variability were present in this region. First, P3HR-1 DNA had only a single *Bam*HI site in its additional DNA. AG876 *Bam*HI fragments W1 and I1 both hybridized to a P3HR-1 DNA fragment (fragment W1I1 [Fig. 5]) equal in size to AG876 *Bam*HI fragments W1 and I1, suggesting that P3HR-1-W1I1 contained all of the sequences of AG876 *Bam*HI fragments W1 and I1. Second, the molecular weight of *Bam*HI-B1 varied extensively among the DNAs; it was 7.2×10^6 in AG876 DNA, 6.6×10^6 in W91 DNA, and 6.4×10^6 in P3HR-1, Raji, Cherry, and Lamont DNAs (Fig. 2 through 4 and 6 through 8).

Homology was previously demonstrated between sequences mapping in U_L of AG876 or W91 DNA at 93×10^6 to 95×10^6 daltons in the additional DNA of *Bam*HI-B1 and at 26×10^6 to 28×10^6 daltons in *Bam*HI-H (35). Labeled *Bam*HI-H and -B1 hybridized to fragments from both map locations in AG876, W91, and P3HR-1 virion DNAs (Fig. 2 through 5) and Raji, Cherry, and Lamont intracellular DNAs (Fig. 6 through 9). Thus, there was homology between DNAs at two map locations in the U_L component of each of the EBV DNAs except B95-8, which has a deletion of DNA at 93×10^6 to 95×10^6 daltons (35).

Differences among standard EBV DNAs. A complete or nearly complete set of *EcoRI* or *Bam*HI fragments identical in size and sequence content to B95-8 or W91 DNA fragments was identified in the analysis of each virion and intracellular DNA. The finding of a complete set of similar or identical fragments indicated that at least some of the molecules in each DNA had common restriction endonuclease maps. This set of EBV restriction endonuclease fragments which was in common among the EBV DNAs was either the dominant population of fragments

or a major population and is referred to as the standard type of EBV DNA.

There were several types of differences among the standard EBV DNAs in addition to those described above.

First, in several instances adjacent *EcoRI* fragments were missing (e.g., *EcoRI*-I and -I₁Jhet from AG876 [Fig. 2 and 5], Cherry [Fig. 7], and Lamont [Fig. 8 and 9] DNAs), and the sequences usually identified with these fragments were present in a larger *EcoRI* fragment equal in size to the sum of the sizes of the missing fragments, whereas the colinear *Bam*HI fragments were not altered. Similarly, some adjacent *Bam*HI fragments were missing (e.g., *Bam*HI-X and -H from AG876 DNA [Fig. 2 and 5], *Bam*HI-B and -G or *Bam*HI-W1 and -I1 from P3HR-1 DNA [Fig. 4 and 5], and *Bam*HI-A and -Nhet or *Bam*HI-E and -Y from Raji DNA [Fig. 6 and 9]), and the sequences usually identified with these probe fragments were in a larger *Bam*HI fragment equal in size to the sum of the sizes of the missing fragments, whereas the colinear *EcoRI* fragments were not altered. These variations were probably due to mutations at *EcoRI* and *Bam*HI sites.

Second, in several instances the *Bam*HI fragment probes identified new, smaller *EcoRI* fragments (e.g., *EcoRI*-J1 of W91 [Fig. 3 and 5] and Lamont [Fig. 8 and 9] DNAs and *EcoRI*-G2a and -G2b of P3HR-1 DNA [Fig. 4 and 5]), whereas the colinear *Bam*HI fragments were not altered in size. These new fragments were probably consequences of mutations which generated new *EcoRI* sites.

Third, labeled B95-8 *Bam*HI-X and -H hybridized to a single P3HR-1 *Bam*HI fragment, *Bam*HI-XH (Fig. 4 and 5), which was 2.5×10^6 daltons smaller than the sum of the sizes of the probe fragments. This was consistent with previous data which indicated that the sequences at the right of *Bam*HI-X and at the left of *Bam*HI-H are deleted in P3HR-1 DNA (21, 22, 36). The difference between the sizes of *Bam*HI-X and -H and the size of P3HR-1 *Bam*HI-XH defined the size of the P3HR-1 deletion. The P3HR-1 strain is unique among EBV isolates because it is not able to initiate growth transformation of primary lymphocytes (26, 29). Some of the DNA in the *Bam*HI-X-*Bam*HI-H region which is deleted from P3HR-1 (22) encodes polyadenylated polyribosomal RNA in nonpermissively infected cell lines growth transformed by EBV (22).

Fourth, the labeled 3.9×10^6 -dalton B95-8 *Bam*HI-H fragment identified similar-sized *Bam*HI fragments in Raji (Fig. 6), Cherry (Fig. 7), and Lamont (Fig. 8) DNAs, but fragments

larger than B95-8 *Bam*HI-H in AG876 (Fig. 2) and W91 (Fig. 3) DNAs. The W91 *Bam*HI fragment H had a molecular weight of 4.5×10^6 (Fig. 3), and the homologous AG876 *Bam*HI fragment D1 had a molecular weight of 5.2×10^6 . AG876 *Bam*HI-D1 was also identified by the 1.2×10^6 -dalton B95-8 *Bam*HI-X fragment, which mapped just to the left of B95-8 *Bam*HI-H, suggesting that AG876 *Bam*HI-D1 was a fusion of *Bam*HI-H and -X resulting from the absence of the intervening *Bam*HI site. To compare these fragments further, we analyzed *Hin*FI fragments of recombinant plasmid DNAs containing B95-8 *Bam*HI-H or -X, AG876 *Bam*HI-D1, or W91 *Eco*RI-A. We found that the orders of *Hin*FI fragments (left to right) in B95-8 *Bam*HI fragments X and H were as follows: 0.08×10^6 , 0.3×10^6 , 0.5×10^6 , and 0.2×10^6 and 0.4×10^6 , 0.2×10^6 , 2.1×10^6 , 1.1×10^6 , and 0.08×10^6 daltons, respectively. Most of the *Hin*FI fragments of AG876 *Bam*HI-D1 and *Bam*HI-X and -H from B95-8 and W91 were identical in size. The molecular weight of the largest *Hin*FI fragment, an internal fragment of *Bam*HI-H, was different in the three DNAs (2.1×10^6 in B95-8, 2.35×10^6 in AG876, and 2.65×10^6 in W91 [data not shown]).

Fifth, the sizes of the *Eco*RI-A fragments which were identified with specific probes (Fig. 2 through 4 and 6 through 8) varied among the isolates and among molecules of each isolate. Fragments differing in molecular weight by increments of 2×10^6 were detected in each EBV virion and intracellular DNA (Fig. 2 through 4 and 6 through 8), presumably as a consequence of differences in numbers of copies of I_R . The numbers of repetitions of I_R shown in Fig. 5 and 9 are estimates based on the number of components of *Eco*RI-A identified in Fig. 2 through 4 and 6 through 8. Some of the forms of W91 *Eco*RI-A may have differed because of the deletion of a unique sequence of DNA, in addition to having fewer copies of I_R . The molecular weights of the smallest components of W91 *Eco*RI-A were 4×10^6 to 8×10^6 (Fig. 3B), whereas the molecular weights of the unique sequence components of *Eco*RI-A are at least 11×10^6 .

Rearranged sequences in EBV DNA. The labeled *Bam*HI fragments identified fragments with altered sequence arrangements in virion P3HR-1 DNA and intracellular Raji, Cherry, and Lamont DNAs (Fig. 4 and 6 through 8). EBV sequences which were not linked in the standard EBV genome recombined in new linkages. In particular, sequences from *Eco*RI-B were frequently in novel fragments which contained sequences not linked to *Eco*RI-B in

standard EBV DNA. Labeled *Bam*HI-Y and -R (fragments which mapped in *Eco*RI-B) and *Bam*HI-C, -V, and -X (fragments which mapped in *Eco*RI-A) hybridized to some of the same P3HR-1 (Fig. 4B) and Raji (Fig. 6B) *Eco*RI fragments. Therefore, sets of P3HR-1 and Raji *Eco*RI fragments (P3HR-1 *Eco*RI fragments A1 through A3 [Fig. 5] and Raji *Eco*RI fragments A1 and A2 [Fig. 9]) contained sequences which mapped in either *Eco*RI-A or -B in standard DNA.

P3HR-1 *Eco*RI-A1 through -A3 also included sequences from *Bam*HI-M and -S (Fig. 4 and 5). Raji *Eco*RI-A1 contained sequences from the right end of *Eco*RI-A and DNA which mapped in *Bam*HI-P and -O (Fig. 6 and 9). The *Eco*RI-A2 DNA rearrangement in Raji linked I_R sequences and *Bam*HI-H sequences with DNA from *Bam*HI-P and -O (Fig. 6 and 9). This rearranged linkage of DNA was confirmed by the demonstration of a new mosaic Raji *Bam*HI fragment, fragment O1, which was homologous to *Bam*HI-H and -P probes but did not have sequences from the intervening *Bam*HI probe fragments (Fig. 6A and 9).

Another class of unusual fragments which contained rearranged DNA was Raji and Cherry *Eco*RI-B1. Sequences from *Bam*HI-P and -O were linked with DNA from the standard genome *Eco*RI-B region (Fig. 6, 7, and 9).

Two other unusual *Eco*RI fragments were present in P3HR-1 virion DNA and the intracellular EBV DNAs. First, labeled *Bam*HI-B and -G identified a new *Eco*RI fragment, *Eco*RI fragment F2, in P3HR-1 DNA (Fig. 4 and 5) and Raji, Cherry, and Lamont DNAs (Fig. 6 through 9). The faint staining of *Eco*RI-F2 with labeled EBV genomic DNA (Fig. 1A, 4B, 6B, 7B, and 8B) indicated that *Eco*RI fragment F2 had a low relative abundance in these DNAs. Second, a class of novel fragments appeared to have a rearrangement or deletion in the terminal portion of the *Eco*RI-D-*Eco*RI-I, Jhet region. The P3HR-1 *Eco*RI-Dhet fragments (Fig. 4 and 5) were smaller than the unique sequence component of *Eco*RI-Dhet in standard DNA. The small sizes of Cherry *Eco*RI-D2 and -D3 and some of the small Lamont *Bam*HI and *Eco*RI T_R -containing fragments also suggested the deletion of terminal sequences (Fig. 7 through 9).

Raji *Eco*RI-D1 contained sequences which mapped in *Eco*RI-Dhet and *Bam*HI-G and -D in standard DNA (Fig. 6B and 9). This DNA rearrangement was confirmed by the presence of sequences from the standard DNA probe fragments in the novel Raji *Bam*HI-A1 fragment (Fig. 6A and 9). Fragment probes which mapped

between these recombined DNA sequences in standard DNA did not hybridize to the mosaic Raji *EcoRI*-D and *Bam*HI-A1 fragments. Raji *EcoRI* and *Bam*HI fragments which mapped in the intervening or deleted region (Raji *EcoRI*-C and -H and *Bam*HI-I1 and -T [Fig. 1 and 6], in particular) were underrepresented on blots of Raji DNA hybridized with labeled EBV (AG876) DNA compared with fragments which mapped in regions where rearrangements did not occur.

DISCUSSION

These data extend previous knowledge of the "wild-type" EBV genome by indicating that the linkage maps of the American EBV DNAs Cherry and Lamont are similar to those of the African Burkitt isolates AG876, W91, P3HR-1, and Raji, particularly in the region deleted from B95-8 DNA. Previous data (35) indicated that Cherry and Lamont DNAs contain sequences from this region but did not define the sizes of the fragments within this region or demonstrate the duplication of sequences at 26×10^6 to 28×10^6 and 93×10^6 to 95×10^6 daltons in *Bam*HI-H and -B1. The sizes of the additional DNAs of W91, AG876, P3HR-1, Raji, Lamont, and Cherry are nearly identical. Furthermore, the placement of the *Bam*HI sites within the additional DNA of each isolate results in *Bam*HI-W1 and -I1 fragments of identical size and similar-sized *Bam*HI-B1 fragments. The similarities in size and sequence homology between the AG876 fragments *Bam*HI-W1, -I1, and -B1 and the *Bam*HI fragments of W91, P3HR-1, Raji, Lamont, and Cherry clearly establish that these sequences are common to most and probably all EBV DNAs. Thus, the general structure of EBV DNA includes the features of I_R , T_R , U_L , and U_S described above and also a U_L sequence with a molecular weight of 89×10^6 . U_L contains a duplication of sequences which map at both 26×10^6 to 28×10^6 and 93×10^6 to 95×10^6 daltons. The DNAs of the herpesviruses papio and pan, which are homologous to 40% of EBV DNA, share these organizational features (16, 17, 24; Heller and Kieff, manuscript in preparation). Therefore, these features define this subgroup of herpesvirus DNAs.

The seven (including B95-8) EBV DNAs examined in this study exhibit a range of variability. Four of the seven (P3HR-1, Raji, Cherry, and Lamont) consist of at least two populations of molecules—a standard population, similar in organization to B95-8, and at least one population of defective molecules. There is sufficient variation in the fragments generated by the two restriction enzymes used in this study (*EcoRI*

and *Bam*HI) to distinguish among the standard viral DNA molecules of each isolate. Thus, differences among the DNAs confirm that these isolates are independent. The ability to distinguish isolates could be used for epidemiological analyses of virus infections in communities or laboratories.

Many of the differences observed among the standard viral genomes do not affect the map coordinates of DNA and appear to result from a loss or gain of a restriction endonuclease site without any detectable addition or deletion of DNA. Some *EcoRI* and *Bam*HI sites appear to vary more than others. The *EcoRI* site between *EcoRI*-I_Jhet and *EcoRI*-I is present in B95-8, Raji, W91, and P3HR-1 DNAs, but absent in AG876, Cherry, and Lamont DNAs. Most other *EcoRI* sites are rarely, if ever, altered. AG876 is an African Burkitt lymphoma isolate, whereas Cherry and Lamont are American infectious mononucleosis isolates. Therefore, the difference does not appear to have a geographic or pathogenic basis and probably resulted from mutation of one of the nucleotides at the enzyme recognition site. The changes could accumulate preferentially at certain sites as a consequence of increased mutational frequency or as a consequence of less stringent counterselection, which might occur in a noncoding region of DNA. Similarly, no geographic or pathogenic basis is suggested by the differences observed among the DNAs in *Bam*HI-H or *Bam*HI-B1 since these fragments are indistinguishable in Raji, Cherry, and Lamont DNAs.

Of the viral genomes defined in this study, P3HR-1 DNA is unusual in that the standard P3HR-1 DNA has a deletion of 2.5×10^6 daltons. Previous data defined the left and right boundaries of the P3HR-1 deletion, which extends from the left of the *Hind*III cut site in B95-8 *Bam*HI-X to within the 2.1×10^6 -dalton *Hin*FI fragment of B95-8 *Bam*HI-H (21, 22, 36). The significance of this deletion arises because of the unique inability of the P3HR-1 clone of Jijoye to transform cells, whereas Jijoye and all other isolates of EBV can transform cells (18, 26, 27, 29, 30). The P3HR-1 deletion includes DNA which encodes mRNA in restringently infected growth-transformed cells (21, 22). Recent data indicate that Jijoye has these DNA sequences (W. King and E. Kieff, unpublished data). The maps of P3HR-1 and Raji DNAs presented here will be useful in ongoing efforts to obtain transformation-positive recombinants between P3HR-1 and endogenous Raji DNA or other EBV genomes (9, 10, 43–45).

Nonstandard (or defective) viral genomes have been described in previous studies of P3HR-1 DNA which included partial denatura-

tion mapping (5) and attempts at identifying the sequences present in unusual restriction endonuclease fragments (21, 36; D. Given, Ph.D. thesis, University of Chicago, Chicago, Ill., 1979). The partial denaturation data suggested that the most common, nonstandard P3HR-1 DNA molecule consists of repeats of a sequence of 30×10^6 to 40×10^6 daltons (5). From the frequencies of the nonstandard *EcoRI*-A1, -A2, and -A3 fragments compared with the frequency of the standard *EcoRI* fragment A and from the frequencies of the *EcoRI*-C1 and -D1het fragments compared with the frequencies of the standard *EcoRI*-C and -Dhet fragments (Fig. 4 and 5), it is likely that the repeating unit of the most common nonstandard P3HR-1 DNA consists of the following components: I_R , *Bam*HI fragments M, S, Y, R, B1, W1, I1, and A, *EcoRI*-Dhet, and T_R . These data suggest that there are multiple recombinational events in the generation of the defective P3HR-1 DNAs.

Nonstandard DNAs similar in organization to P3HR-1 are also evident in the analyses of Raji, Cherry, and Lamont intracellular EBV DNAs. In each of these instances, altered forms which include the sequences of *Bam*HI fragments Y, R, K, and B are evident. The prevalent participation of these sequences in altered forms suggest that they might include an origin of DNA replication or DNA which recombines more easily with other regions of the EBV genome.

The coexistence of both standard and mosaic forms of EBV DNA within cells is of importance to ongoing studies of intracellular EBV DNAs. Attempts to discern linkage between cellular and viral DNA should take into consideration the observation reported here that altered forms of viral DNA occur as a consequence of recombination between distant parts of the viral genome. Studies of putative recombinants between superinfecting and intracellular EBV DNAs and mapping of viral RNAs must take into consideration the possibility that altered forms of intracellular EBV DNA may participate in these processes.

The finding of altered linkages between parts of the viral genome in each of the intracellular DNAs is a surprising outcome of this study and may correlate with the unusual properties of EBV. Multiple copies of EBV DNA persist in nonpermissively infected cells over many cellular generations (1, 20, 25, 33). The EBV genome differs from cellular DNA in its circular episomal structure and its higher guanine plus cytosine content (1, 25, 32), yet in nonpermissively infected cells the EBV genome is replicated by a cellular polymerase (42). Whether the frequency of mutation or recombination of viral DNA during replication by cellular enzymes differs from

the frequency of mutation or recombination of cellular DNA or of viral DNA replicated by viral polymerase is not known. Furthermore, the presence of multiple genomes within the same cell might permit functional complementation and limit selection against unfavorable errors or rearrangements in the three regions of the EBV genome which encode mRNA in nonpermissively infected cells (21, 22, 31).

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ADDENDUM IN PROOF

Soon after this paper was submitted for publication, the partial denaturation and restriction endonuclease patterns of P3HR-1, M-ABA, CC 34-5, QIMR-WIL, and B95-8 virion EBV DNAs were compared (G. Bornkamm, H. Delius, V. Zimmer, J. Hudewentz, and M. Epstein, *J. Virol.* 35:603-618, 1980).

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