JOURNAL OF VIROLOGY, Dec. 1981, p. 977-982
0022-538X/81/120977-06$02.00/0

Vol. 40, No. 3

Cloning Overlapping DNA Fragments from the B95-8 Strain of Epstein-Barr Virus Reveals a Site of Homology to the Internal Repetition

G. N. BUELL,[1]† D. REISMAN,[1] C. KINTNER,[1]‡ G. CROUSE,[2]§ AND B. SUGDEN[1]*

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706,[1] and Department of Biological Sciences, Stanford University, Stanford, California 94305[2]

Received 22 May 1981/Accepted 30 July 1981

Overlapping, sheared DNA fragments from the B95-8 strain of Epstein-Barr virus were cloned in Charon 4A. Eleven recombinant phages plus one recombinant plasmid contained all of the sequences found in B95-8 virion DNA. Analysis of recombinant DNA molecules revealed a previously undetected site of homology to the internal repetition found in Epstein-Barr virus DNA. This site was adjacent to or at a site which was unstable when the recombinant DNA was propagated as phage DNA in procaryotic hosts.

Epstein-Barr virus (EBV) is a human herpesvirus which in vitro infects human B-lymphocytes and transforms them into lymphoblasts capable of being propagated indefinitely. No cell in culture has yet been identified which supports lytic growth of EBV (13). The primary sources of virus are producer cell lines: transformed cell lines which contain a small percentage of cells that release virus at any given time. As a consequence of this inefficient release, large amounts of EBV are not available, and only its virion DNA has been purified and studied in detail. To generate large amounts of pure fragments of EBV DNA, both to construct a restriction map and to aid in future functional mapping studies, we cloned randomly sheared fragments of DNA from the B95-8 strain of EBV (10) into the λ vector Charon 4A (2). We screened these recombinants and identified 11 overlapping members which together with one recombinant plasmid apparently represent a complete copy of EBV DNA. In analyzing these recombinant DNAs, we identified a previously unrecognized site of homology to the large internal region of repetitious sequences. A region near or at this site of homology is strikingly unstable when propagated in procaryotic hosts.

Recombinant phages were constructed by ligating sheared fragments of B95-8 EBV DNA containing synthetic termini to the 19.8-kilobase (kb) and 10.9-kb *Eco*RI fragments of Charon 4A DNA (9). Approximately 600 recombinant phages were screened using the Benton-Davis plaque assay (1) by hybridization with labeled B95-8 viral DNA or DNA from the recombinant plasmid pBR1 (described below). Positive plaques were picked, purified, and grown as plate stocks. Phage DNAs were analyzed by digestion with *Eco*RI.

The EBV DNA sequences in recombinant phages were aligned with a restriction map of B95-8 EBV DNA by Southern transfers (7, 12). Recombinant phage DNA was labeled in vitro and hybridized to nitrocellulose transfers containing *Eco*RI, *Hin*dIII, *Sal*I, and *Bam*HI digests of virion DNA (strip blots). Eleven recombinant phages appeared to contain overlapping sequences which spanned the restriction map of B95-8 EBV DNA. The strip blots for several phage hybridization probes are shown in Fig. 1. EB90-99 (Fig. 1B) mapped to the right end of the viral DNA molecule (*Eco*RI fragment D and *Sal*I fragment D). This phage contained homology with both the right and left terminal repeats as seen by the ladder of bands in each strip blot of Fig. 1B (7). Both EB0-8 and EB26-36 probes (Fig. 1D and E) hybridized to the 3.2-kb *Bam*HI internal repetition. EB26-36 also hybridized to unique sequence DNA which mapped to the right of the internal repetition, whereas EB0-8 hybridized to the unique sequences which mapped to the left of the internal repetition and the left terminus of virion DNA. Neither EB0-8 nor EB26-36 contained a complete copy of the unit of the repeat in the internal repetitious DNA mapping approximately between 6 and 25% of the genome. We therefore cleaved virion DNA with *Bam*HI and cloned one copy of this repeat into the *Bam*HI site of pBR322 (14). This

† Biogen S. A., Geneva, Switzerland.
‡ Division of Biology, California Institute of Technology, Pasadena, CA 91125.
§ Cancer Biology Program, Frederick Cancer Research Center, Frederick, MD 21701.

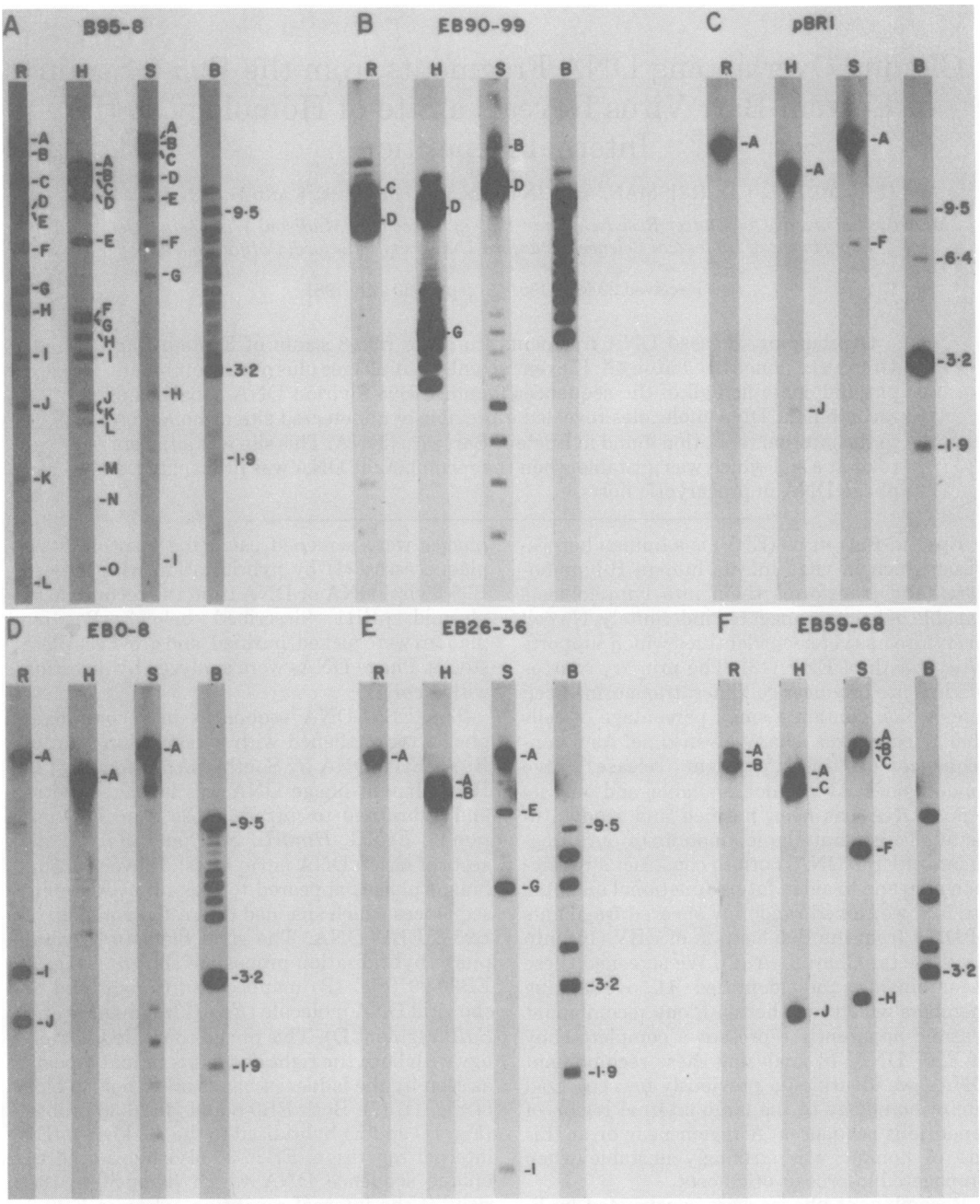

FIG. 1. *Hybridization of recombinant DNAs to digests of B95-8 virion DNA. B95-8 virion DNA (20 to 30 μg) was digested with EcoRI (R), HindIII (H), SalI (S), or BamHI (B). Each digest was placed into a 12-cm-wide slot of a 20-cm-long agarose gel and electrophoresed. The DNA in each gel was transferred to a separate piece of nitrocellulose, which was then cut into 0.8-cm-wide strips. (A) Autoradiograms were obtained after a set of four strips of nitrocellulose, one derived from each digest, was hybridized with labeled B95-8 DNA to detect all EBV restriction fragments. (B, C, D, E, and F) Autoradiograms obtained after hybridization of separate recombinant DNAs labeled in vitro to sets of the four strips to detect homology between recombinant DNAs and restriction fragments of B95-8 virion DNA.*

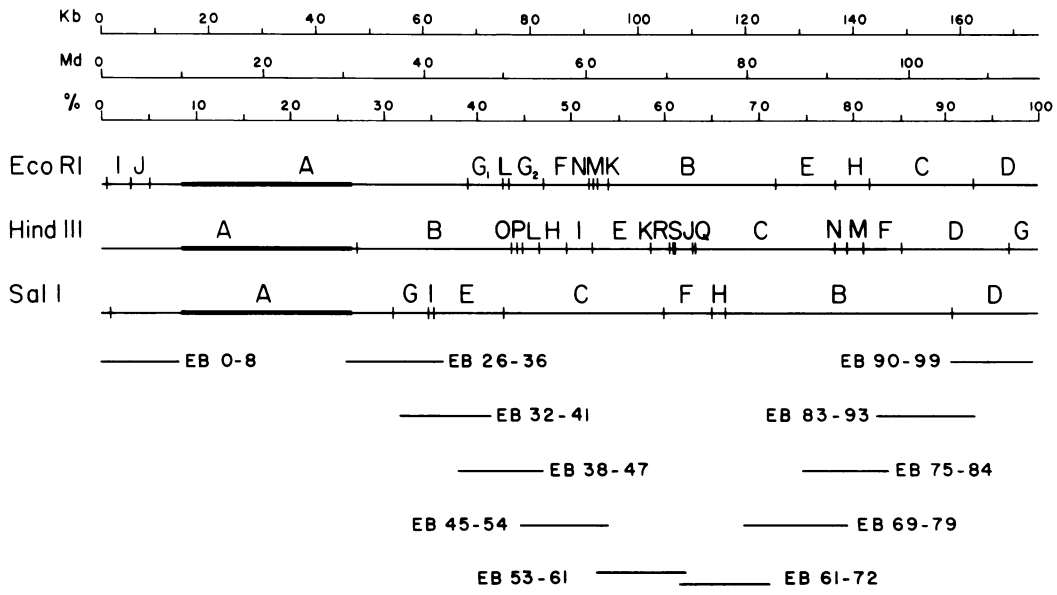

FIG. 2. *EcoRI, HindIII and SalI restriction maps for the B95-8 strain. The ordering of restriction sites is based on the map refined by Dambaugh et al. (4), on data obtained from double-digestion experiments (data not shown), and on data obtained from hybridizing recombinant DNAs to restriction fragments of virion DNA (examples in Fig. 1). The distances between restriction sites are based on double-digestion experiments and on analyses of digested, recombinant phage DNAs. The region containing the large, internal repetition is designated with a thickened bar. Given below the restriction maps are the physical map locations of the EBV DNA inserts contained within the library of lambda and plasmid EBV recombinants. λ recombinants are designated with EB and with the region of EBV DNA they contain in percent units.*

recombinant plasmid, pBR1, hybridized readily to the large internal repetition from which it was derived. In the strip blot containing virion DNA digested with *Bam*HI, pBR1 hybridized to a 9.5-kb fragment all but 200 base pairs (bp) of which was contained within EB0-8, a 1.9-kb fragment contained within EB26-36, a 3.2-kb fragment which was its homolog, and a 6.4-kb fragment which was a dimer of itself resistant to cleavage with a 10-fold excess of *Bam*HI (Fig. 1; unpublished data). Plasmid pBR1 also hybridized to a region mapping to 61% of the genome (discussed below). A summary of the data derived from the strip blots of all 11 recombinant phages is given in Table 1.

Several lines of evidence indicated that the 11 recombinant phages plus 1 recombinant plasmid, pBR1, contained all of the sequences which were present in B95-8 virion DNA. (i) We determined that all neighboring pairs of phages contained overlapping sequences. DNA from each phage was digested with *Eco*RI, *Hin*dIII plus *Eco*RI, or *Sal*I plus *Eco*RI. Products were electrophoresed in 0.6% agarose gels and transferred to nitrocellulose. For each phage insert, its shared homology with the overlapping phage insert was demonstrated by hybridization (data not shown). (ii) The lengths of the fragments produced by cleaving the recombinant phage DNAs with *Eco*RI, *Hin*dIII, or *Sal*I were measured (Table 1). When a recombinant phage spanned all of one or more fragments of cleaved virion DNA, the measured lengths for the phage and virion DNAs were identical with the single exception of *Hin*dIII fragment J (discussed below). (iii) For each recombinant phage, the lengths of all of the double-digestion products resulting from digestion with *Eco*RI, *Hin*dIII, and *Sal*I were determined. Those lengths agreed closely with the lengths of the double-digestion products of uncloned virion DNA. For example, the digestion of phage EB75-84 with *Eco*RI and *Hin*dIII indicated that *Hin*dIII fragment N was digested with *Eco*RI to yield 1.4- and 0.4-kb fragments. These two fragments had comigrating homologs in *Eco*RI plus *Hin*dIII digests of virion DNA. This finding differed from earlier findings (4) and was therefore confirmed by detecting the *Eco*RI cleavage products of *Hin*dIII fragment N in Southern transfers of EB75-84 and virion DNAs, using labeled *Hin*dIII fragment N as a hybridization probe. (iv) The number of sites in each phage insert recognized by a restriction endonuclease equaled the number of

TABLE 1. *Data from strip blots of 13 recombinant phages*

Phage designation	Length of insert (kb[a])	Orientation of insert[b]	No. of sites within phage insert[c]			Hybridization of phage to digests of virion DNA detects these fragments readily[d]		
			*Eco*RI	*Hin*dIII	*Sal*I	*Eco*RI	*Hin*dIII	*Sal*I
EB0-8	14.5	−	3	0	1	A, I, J (left termini)	A	A (left termini)
EB26-36	18.2	−	0	1	3	A	A, B	A, E, G, I
EB32-41	17.0	−	1	0	2	A, G_1	B	E, G, I
EB38-47	15.7	+	3	4	1	A, G_1, G_2, L	B, H, L, O, P	E, C
EB45-54	16.0	+	4	4	0	F, G, K, M, N	E, H, I, L, P	C
EB53-61	15.0	+	2	4	1	B, K, M	E, J, K, Q, S	C, F
EB61-72	18.7	−	1	2	2	B, E	C, J, R	B, F, H
EB69-79	19.3	−	2	1	0	B, E, H	C, N	B
EB75-84	15.6	+	2	3	0	C, E, H	C, F, M, N	B
EB83-93	18.2	+	0	1	1	C	D, F	B, D
EB90-99	15.3	+	1	1	1	C, D	D, G	B, D
EB59-68	15.0	+	0	5	3	A, B	A, C, J, K, Q, R, S	A, B, C, F, H
EB57-65	14.0	−	0	6	2	B	C, D, J, K, Q, R, S	C, F, H

[a] The lengths of the EBV DNA inserts were measured in 0.6% agarose gels with restriction fragments of λ DNA as molecular weight standards.

[b] The orientation of the inserted DNA indicates the orientation of the EBV DNA relative to the λ vector DNA. The orientation was determined by comparison of fragments generated by digesting recombinant phage DNA with *Hin*dIII or *Sal*I to those generated by digesting with *Hin*dIII and *Eco*RI or *Sal*I and *Eco*RI. +, The 19.8-kb λ arm mapped to the left end of the recombinant phage when the EBV DNA had the orientation shown in Fig. 2.

[c] The number of restriction sites within the EBV DNA inserts was based on the number of fragments generated when the recombinant DNAs were digested with a restriction enzyme and electrophoresed in a 0.6% agarose or a 3.5% acrylamide gel. Restriction fragments less than 200 kb in length may not have been detected.

[d] Recombinant phage DNAs were hybridized to restriction fragments of virion DNA in experiments similar to those shown in Fig. 1. Only the results of readily detected hybridizations are shown. For example, hybridization between the DNA of EB53-61 and the *Eco*RI, *Hin*dIII, and *Sal*I A fragments of virion DNA was detected only after long exposures, and so this hybridization is not noted above. To be consistent, the nomenclature of Dambaugh et al. (4) was used where possible to assign letters to restriction fragments; therefore, the left terminal fragments of *Eco*RI and *Sal*I were not named. However, where we identified fragments previously unidentified, as in digestions with *Hin*dIII, we labeled them alphabetically in descending order of size.

fragments detected on strip blots containing digests of virion DNA for that phage probe minus one. Data indicating the number of restriction sites in each phage insert and the fragments detected when each phage was hybridized to strip blots are summarized in Table 1. A phage insert with, for instance, three *Hin*dIII sites hybridized to four *Hin*dIII fragments found on strip blots. Phage inserts EB38-47, EB45-54, and EB59-68 contained small *Hin*dIII fragments. Their lengths and positions were determined by electrophoresis in 3.5% acrylamide gels. *Hin*dIII fragments O, P, Q, R, and S were, respectively, 870, 670, 420, 420, and 270 bp. Lengths were measured relative to *Hin*fI fragments of pBR322 (14). These *Hin*dIII digestion products were detected in uncloned virion DNA when recombinant phage DNAs were hybridized to strip blots. (v) Complete copies of the termini of virion DNA were present in the recombinant phage. Digestions of EB0-8 showed a faint ladder of submolar *Eco*RI fragments which differed from one another by 500 bp and had lengths of 2.8, 2.3, 1.8, and 1.3 kb. Similar fragments produced upon digestion of virion DNA map at the left terminus (7). B95-8 virion DNA contains multiple copies of a 500-bp direct repeat at both termini (6, 7). Apparently EB0-8 resulted from linker addition to a terminal fragment of virion DNA which contained at least four copies of the terminal repeat. Some of these repeats were deleted during propagation of EB0-8 in *Escherichia coli*.

The restriction maps in Fig. 2 are based on the data derived from characterizing recombinant phage (Fig. 1; Table 1), with the exception of *Hin*dIII fragment F, which was measured from virion DNA. Since no fragment larger than 11.8 kb (*Eco*RI-E) was used to generate the map, all measured fragments migrated within the linear portion of the gel, and their measured lengths were as accurate as those of the standards used. *Sal*I-B (43 kb), for example, was measured by summing its double-digestion prod-

ucts from four overlapping phage inserts. Assuming that there were 10 copies of the internal repeat, 1 terminal repeat at the left terminus, and 4 at the right terminus, the B95-8 strain of EBV DNA was 175 kb (117 megadaltons) in length.

When pBR1 was hybridized to strip blots, not only were the fragments containing the internal repetition detected, but *Sal*I-fragment F and *Hin*dIII fragment J were also detected (Fig. 1C). This site of homology mapped to *Eco*RI-fragment B, which was not resolved from *Eco*RI-fragment A to which pBR1 hybridized strongly. *Bam*HI digested within this second site of homology to yield two fragments detected on longer exposures. Although the pBR1 plasmid was homologous to two distant sites, it appeared to be pure and to contain a single *Bam*HI fragment of virion DNA. Digestion of the plasmid DNA with several endonucleases indicated that a single fragment was inserted into the pBR322 vector (data not shown). In addition, when the purified insert isolated from pBR1 was labeled in vitro and hybridized to strip blots containing virion DNA, the same virion fragments were detected as when the entire plasmid was labeled and used as a probe (Fig. 1C).

Four independent phages which contain either a portion or all of *Hin*dIII fragment J were isolated from our bank, and each had homology to pBR1. Phage EB59-68 hybridized to the 3.2-kb *Bam*HI fragment and to the *Eco*RI, *Hin*dIII, and *Sal*I A fragments, which contained the internal repetition (Fig. 1F). Phages EB53-61 and EB61-72 contained portions of *Hin*dIII fragment J of virion DNA, and each shared some homology with pBR1 (Fig. 3). Phage EB59-68 contained all of *Hin*dIII fragment J (Fig. 1 and 2) and suffered deletions within that region which occurred during propagation (data not shown). Similarly, phage EB61-72 suffered deletions within the portion of the *Hin*dIII J fragment it contained, and these smaller fragments still had some homology to pBR1 (Fig. 3). A fourth phage, EB57-65, had an apparently stable deletion of 500 within *Hin*dIII fragment J and retained some homology to pBR1 (data not shown). We cloned the *Sal*I fragment F of EBV DNA into pBR322. This fragment encompassed all of *Hin*dIII fragment J (Fig. 2) and contained homology to pBR1 (Fig. 3).

Eleven recombinant phages plus one recombinant plasmid appeared to contain all of the sequences found in the virion DNA of EBV (Fig. 2, Table 1). Within the limits of our analysis, these recombinant DNAs were equivalent to their virion counterparts. However, in the procaryotic hosts and vectors used, no Charon phage contained a complete copy of one unit of the large internal repetition, and all phages which contained 80% or more of *Hin*dIII fragment J suffered deletions in that region upon

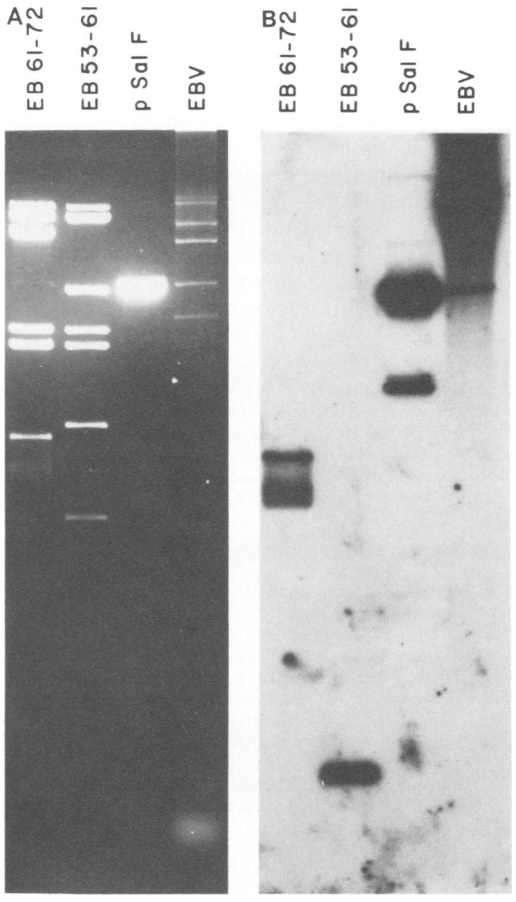

FIG. 3. *Identification of a site of homology to the large, internal repetition and a region of instability in the recombinant DNA. Phages EB53-61 and EB61-72 were digested with EcoRI and HindIII. The insert in the plasmid carrying SalI fragment F was isolated after digestion with SalI. Virion DNA was digested with SalI. All DNAs were electrophoresed in a 0.7% agarose gel, visualized by staining with ethidium bromide (A), and transferred to nitrocellulose. Sites of homology to the large, internal repetition were detected by hybridizing labeled pBR1 DNA (2×10^7 cpm) to the transfer and visualized by autoradiography (B). The right terminal fragment (700 bp) of EB53-61 ending within the HindIII fragment is detected in (B), as is the left terminal fragment (3,200 bp) of EB61-72 which ends in HindIII fragment J and has suffered multiple deletions. SalI fragment F from the recombinant plasmid and the virion DNA is detected, as is the SalI fragment A of virion DNA. The fast-moving fragment in the plasmid SalI fragment F lane is a small amount of pBR322 DNA which contaminates the isolated insert (it is not visible in [A]).*

propagation (Fig. 3). In addition, *Hin*dIII fragment J in virion DNA and in recombinant DNAs contained homology to the large internal repetition (Table 1; Fig. 1 and 3). This site of homology to the large internal repetition mapped near or overlapped a site in the viral DNA which was unstable when it was propagated as recombinant phage DNA in a procaryotic host. This homology differs from that reported in other strains of EBV in which a region adjacent to the right end of the large internal repetition shares sequences with a region absent from the DNA of the B95-8 strain of EBV (5, 11).

We thank Robert T. Schimke, in whose laboratory the initial phage construction was done.

Our work was supported by Public Health Service grants CA-09135, CA-22443, and CA-97175 from the National Cancer Institute. B.S. was supported by American Cancer Society Faculty Research Award FRA-203.

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