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Non-Immortalizing P3J-HR-1 Epstein-Barr Virus: a Deletion Mutant of Its Transforming Parent, Jijoye

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The P3J-HR-1 strain of Epstein-Barr virus (EBV) fails to immortalize human lymphocytes. We wished to understand the nature of the genomic alterations which correlated with the loss of this ability. As a first step, the heterogeneity of DNA molecules in the P3J-HR-1 line was eliminated by cell cloning. Then a physical map was prepared of virion DNA from one cell clone, designated FF452-3. By comparison with the genomes of two EBVs, B95-8 and FF41, which are competent to immortalize lymphocytes, we identified a total of eight modifications of *Bam*HI and *Eco*RI restriction endonuclease fragments of EBV (FF452-3) DNA consisting of insertions, deletions, or loss of a restriction endonuclease recognition site. To determine which of these alterations might be responsible for the loss of transforming phenotype, we examined homologous DNA fragments of the Jijoye strain of EBV, the progenitor of the HR-1 strain which still retains the ability to immortalize lymphocytes. We also studied viral DNA in lymphocytes transformed in vitro by Jijoye virus. Six of the eight alterations were found both in Jijoye and in clonal HR-1 DNA and were presumably genomic traits characteristic of this lineage of EBV. A small deletion in the *Bam*HI-K fragment of HR-1 DNA was not found in Jijoye virion DNA, but this deletion was present in intracellular Jijoye DNA. Thus only one major genomic lesion in HR-1 DNA, a deletion of at least 2.4×10^6 molecular weight of DNA from a fused *Bam*HI-H-Y fragment, consistently distinguished Jijoye DNA from its non-immortalizing P3J-HR-1 derivative. This deletion is likely to affect EBV genes which are directly or indirectly involved in immortalizing lymphocytes.

P3J-HR-1 (HR-1) is an unusual laboratory strain of Epstein-Barr virus (EBV) which differs from all other strains examined thus far. HR-1 viruses fail to immortalize B lymphocytes in vitro and are unable to induce lymphomas in primates (9, 22, 25). HR-1 viruses are cytolytic to primary lymphocytes, and they interfere with immortalization (25, 35). In certain lymphoid cell lines HR-1 viruses induce synthesis of early antigens and, depending on the multiplicity, viral DNA and late products as well. Such behavior is not characteristic of transforming strains (17, 25, 32).

Stocks of HR-1 virus, unlike stocks of transforming virus, are heterogeneous in biological properties (12) and in genome structure. There seem to be several different types of HR-1 virion DNA, each with a characteristic partial denaturation pattern (7). Restriction enzyme digestion profiles of HR-1 DNA all contain a variety of submolar fragments, indicating a lack of clonality (2, 14, 16, 18, 21, 29, 36); the pattern of this heterogeneity differs in stocks prepared by different laboratories.

The heterogeneity of HR-1 DNA has been an obstacle to a molecular definition of the genomic alterations which are responsible for the unusual properties of this virus. Nonetheless considerable information has been obtained in efforts to delineate the biologically significant changes in HR-1 DNA. HR-1 DNA shows extensive homology (>90%) with DNA of immortalizing viruses by reassociation kinetics (36). Hybridization with restriction fragments of EBV DNA has also demonstrated considerable similarity in sequence organization (2, 16). However there seems to be a variety of structural features which characterize the HR-1 genome—these include a smaller *Eco*RI-A fragment (2, 16, 30), due to deletions in *Bam*HI fragments H and Y (16, 20); a novel *Eco*RI site in fragment G2 (2, 16); and fusions of *Bam*HI fragments B with G and W′ with I′ (16). Furthermore there are about 1,000 additional base pairs (bp) in the *Hin*dIII-E fragment of HR-1 DNA (2). Finally the HR-1 strain has a characteristic larger right-terminal form. The biological significance of these alterations is unknown.

We report here three types of experiments which were aimed at understanding how the unique biological properties of HR-1 virus correlated with changes in genome structure. First, clonal derivatives of the HR-1 line were isolated which release EBV which is homogeneous and lacks the heterogeneity of parental HR-1 DNA (18). Hence it was possible to construct an unambiguous map of the genome of a nontransforming EBV. Second, we compared the structure of HR-1 EBV DNA with that of its parent, Jijoye. Virus from the Jijoye line retains its ability to immortalize lymphocytes (27, 31). Therefore genomic lesions which distinguish HR-1 from Jijoye DNA are likely to be of significance in the process of lymphocyte immortalization. Finally, we have characterized intracellular viral DNA of lymphocytes immortalized in vitro by Jijoye virus. Any alterations which appear in the EBV genomes in such cells are not likely to be inimical to the transformation process.

MATERIALS AND METHODS

Cell lines. EBV virion DNA from several lymphoid lines was analyzed. Serving as sources of prototypes of lymphocyte immortalizing virus were lines B95-8 and FF41, cotton-top marmoset lymphocytes harboring EBV strains from patients with mononucleosis (8, 24). The Jijoye Burkitt lymphoma line was obtained from the American Type Culture Collection. Its clone, HR-1, originally isolated by Hinuma et al. (19), releases non-immortalizing EBV, as do two subclones of HR-1, FF452-3 and GG68-13, which were recently isolated in agarose (18). All cell lines except Jijoye were grown at 37°C; the latter was incubated at 33°C. Medium was RPMI 1640, 10% fetal bovine serum, and antibiotics.

Lymphocytes transformed in vitro. The EBV sequences in intracellular DNA of two lines of human neonatal lymphocytes immortalized in vitro were examined. One line (HH446-5) became established after exposure of fresh lymphocytes to a 1:10 dilution of filtered supernatant fluid from the Jijoye line which had been treated with 20 ng of 12-*O*-tetradecanoylphorbol-13-acetate per ml (40). Another line, FF467, was immortalized by cocultivation of neonatal human lymphocytes with human placental cell fibroblasts which had been transfected with 1 μg of EBV (FF41) DNA (23).

Virion DNA. EBV DNA was prepared from 20-liter batches of supernatant fluid of lines FF41, Jijoye, HR-1, FF452-3, and GG68-13, by procedures described previously (8). The yields of DNA were approximately 0.5 μg per liter of culture medium. DNA from virions of the B95-8 and HR-1 strains was provided by M. Nonoyama through the Office of Biologic Resources, National Cancer Institute.

Intracellular DNA. Total intracellular DNA was prepared from four lines, Jijoye, FF452-3, HH446-5, and FF467, by a modification of the procedure of Wahl et al. (38). Approximately 3×10^7 cells were centrifuged at 4°C for 10 min at 2,000 rpm. The pellet was suspended in phosphate-buffered saline (0.137 M NaCl, 0.0027 M KCl, 0.008 M Na_2HPO_4, 0.0015 M KH_2PO_4) and centrifuged at 2,000 rpm for 10 min. After three washes in phosphate-buffered saline the cell pellet was suspended in 5 ml of 0.1 M EDTA–0.2 M Tris (pH 8.5). The sample was made 1% in sodium dodecyl sulfate, and pronase was added to 1 mg/ml. The mixture was incubated for 2 h at 60°C and then chilled on ice. One-fifth volume of 5 M potassium acetate was added, and storage on ice was continued for 30 min. The lysate was centrifuged at 2°C for 15 min at 11,000 rpm, and the pellet was discarded. Two volumes of cold 95% ethanol was added to the supernatant, and the sample was immediately centrifuged at 10,000 rpm for 20 min at −20°C. The pellet, which contained both viral and cellular DNA, was suspended in 0.5 ml of 0.01 M Tris (pH 7.4)–0.001 M EDTA. About 5 μg of this material was used per lane in an agarose gel.

Recombinant DNA clones. Both the *Bam*HI and *Eco*RI restriction fragments of EBV (FF41) DNA have been cloned, the former into pBR322 (8) and the latter into pACYC184 (13). Detailed methods of ligation, transfection, and screening of recombinant plasmids have been described previously (8). Additional chimeric plasmids containing the *Eco*RI fragments of EBV (FF452-3) DNA were constructed by cloning into the unique *Eco*RI site of pACYC184 (4). Tentative identification of FF452-3 cloned fragments was based on comigration with plasmids containing cloned FF41 DNA fragments which had been digested with restriction enzymes. Southern blots containing analogous cloned FF41 and FF452-3 *Eco*RI DNA fragments, double digested with *Bam*HI and *Eco*RI, were probed with nick-translated FF41 *Eco*RI fragments for confirmation of fragment identity (see Fig. 1). Cloned regions of the FF452-3 DNA included *Eco*RI fragments B, E, F, G1, H, I, I2, J, and J2.

Restriction endonucleases and gel electrophoresis. The restriction enzymes *Bam*HI, *Eco*RI, and *Hin*dIII were purchased from New England Biolabs and Bethesda Research Laboratories. Digestions were carried out in the conditions specified by the suppliers. DNA fragments were separated by gel electrophoresis in 0.5% agarose (Sigma type II) containing 0.5 μg of ethidium bromide per ml. DNA fragments were transferred to nitrocellulose filters by the method of Southern (34).

Nick translation of DNA and hybridization to Southern blots. Recombinant plasmids carrying EBV (FF41) restriction fragments were labeled with ^{32}P by nick translation (28). The conditions used for hybridization have been described previously (8).

RESULTS

Structure of HR-1 virion DNA. The initial objective of these studies was to obtain a clear picture of the genome structure of a nontransforming EBV variant. We analyzed DNA of a virus released from a subclone of HR-1 cells, designated FF452-3. This was the first HR-1 cellular subclone from which we prepared viral DNA. Neither supernatant fluids nor lethally X-irradiated cells of the FF452-3 clone are able to immortalize human umbilical cord lymphocytes. In this way the clone resembles the parental HR-

1. However the virus from this clone, like nearly all clonal HR-1 variants we have studied, differs from parental HR-1 virus in its inability to induce early antigen in Raji cells (18). The yield of virion DNA from supernatant fluids of FF452-3 is increased about 12-fold after treatment with 12-*O*-tetradecanoyl-phorbol-13-acetate (from 0.1 to ~1.2 ng/ml), so this clone falls into the "moderately inducible," but not "superinducible," category.

Preliminary experiments indicated that the DNA of FF452-3 was homogeneous; the pattern of DNA fragments obtained after digestion with *Eco*RI and *Bam*HI did not contain submolar bands of the unique sequences. (These heterogeneous fragments are usually referred to as "het" bands.) Several probes which were homologous to multiple fragments in HR-1 parental DNA identified only single fragments in FF452-3 DNA (18). However the termini of the cloned HR-1 DNA are heterogeneous due to variable numbers of the terminal reiterated sequences.

We employed two general methods to analyze the genome structure of virus from the HR-1 cell clone. The first consisted of digesting DNA from virions with a restriction endonuclease and probing Southern blots of this virion DNA with a cloned *Bam*HI or *Eco*RI fragment of EBV (FF41) DNA. For reference we analyzed simultaneously virion DNA from two strains (B95-8 and FF41) whose physical map is known in some detail (6, 8, 33). We also studied parental (nonclonal) HR-1 DNA at the same time. The second, confirmatory, method was to analyze *Eco*RI fragments of FF452-3 DNA which had been inserted into the pACYC184 plasmid. Subdigestions of cloned *Eco*RI fragments of EBV (FF41) and EBV (FF452-3) DNA were compared. *Bam*HI fragments which comigrated, were homologous by nucleic acid hybridization, and were contained within the same *Eco*RI fragment were assumed to occupy similar positions on the EBV physical map (Fig. 1).

We identified a total of 31 *Bam*HI fragments of EBV (FF452-3) DNA, whose combined molecular weight (MW) was about 104×10^6, assuming that there are six copies of the internal reiteration (*Bam*HI-W) (Table 1). Of these 31 fragments, 22 were homologous to a corresponding fragment of the same size in DNA from the transforming strain FF41. Nine were homologous to fragments of different size in B95 and FF41.

In the course of these initial experiments we were interested to learn which *Bam*HI fragments identified heterogeneity in the parental HR-1 DNA. Such fragments are homologous to fragments of their own molecular weight in *Bam*HI digests of the clonal HR-1 DNA and in parental DNA; however they are homologous to additional (het) fragments which are present only in parental HR-1 DNA. At least eight *Bam*HI fragments of clonal HR-1 DNA were represented in the heterogeneous bands of parental HR-1 DNA; these fragments were scattered throughout the physical map of EBV DNA. Thirteen *Bam*HI fragments did not seem to be contained within heterogeneous *Bam*HI fragments; our information is incomplete about 10 fragments.

On the basis of the blot hybridization experiments and by comparison with DNA of B95-8 and FF41 a physical map of *Bam*HI and *Eco*RI fragments of the HR-1 clone was constructed (Fig. 2). There was general similarity of genome organization of the transforming and nontransforming viral DNAs.

Differences between HR-1 and the B95-8 and FF41 genomes. During the course of this analysis we identified a total of eight genomic locations at which the HR-1 clone and the HR-1 parent differed from the lymphocyte immortalizing viruses. These alterations, deletions, insertions, and mutated restriction enzyme cleavage sites are enumerated in Table 2 and illustrated in Fig. 3 and 4.

The largest alteration was a deletion of about 3,800 bp from the *Eco*RI-A fragment in *Bam*HI fragments H and Y. Using the *Bam*HI-Y fragment as a probe we detected a $\sim 2.7 \times 10^6$-MW fragment [*Bam*HI-YH(h)] in HR-1 and clonal HR-1 virus and a single *Bam*HI-Y fragment (1.17×10^6 MW) in the transforming strains (Fig. 3). *Bam*HI-Y detects the internal repeat in all strains since the repeat ends in *Bam*HI-Y (5, 15). The same probe hybridized to two submolar het fragments (6.2×10^6 and 1.3×10^6 MW) in parental HR-1 DNA. Similar results were obtained when *Bam*HI-H was the probe. A full-sized H fragment was seen in the transforming DNA, and a deleted YH (h) was seen in HR-1 and its clone. Since *Bam*HI-H had partial homology with *Bam*HI-B', this fragment was identified with the *Bam*HI-H probe in all strains except B95-8, in which B' was deleted. The *Bam*HI-H probe detects heterogeneity in the parental HR-1 DNA; at least five additional fragments were identified. Four of these were about the size of *Bam*HI-B', and there was an additional submolar band of 4.7×10^6 MW.

A smaller deletion in parental HR-1 DNA occurred at the junction of *Bam*HI-I' and *Bam*HI-W', resulting in a *Bam*HI-W'I' fragment of 4.89×10^6 MW. The fused *Bam*HI-W'I' was approximately 370 bp smaller than the sum of the individual fragments. FF41 DNA possesses these fragments unfused, whereas B95-8, due to a deletion of 8×10^6 MW in this region, has a smaller *Bam*HI-I fragment with partial homology to *Bam*HI-I' and lacks *Bam*HI-W' entirely (8, 26). A similar fused *Bam*HI-W'I' fragment in

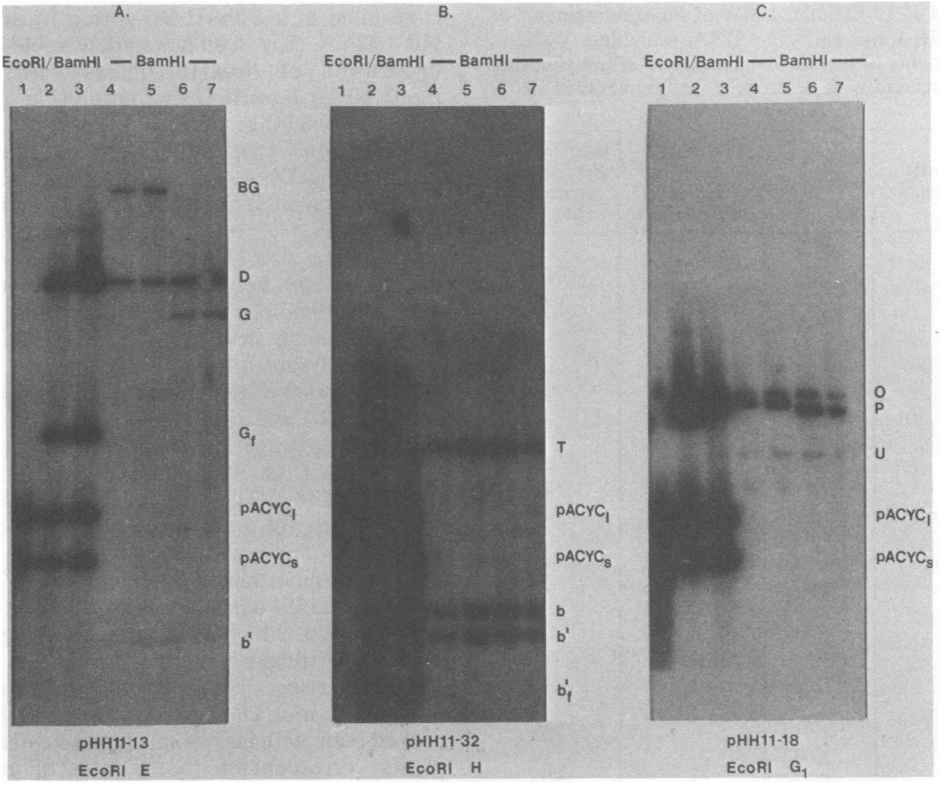

FIG. 1. Method of analysis of genome organization of the nontransforming HR-1 virus, FF452-3, with Southern blots containing *Eco*RI and *Bam*HI digests of cloned *Eco*RI fragments (lanes 2 and 3) or *Bam*HI digests of virion DNA (lanes 4 through 7). Blots were probed with the indicated cloned *Eco*RI fragments of FF41 DNA. Lanes: 1, pACYC184 digested with *Bam*HI and *Eco*RI; 2, pACYC184 containing a cloned *Eco*RI fragment of FF41 DNA; 3, pACYC184 containing a cloned *Eco*RI fragment of FF452-3 DNA; 4, FF452-3 virion DNA; 5, parental HR-1 virion DNA; 6, B95-8 virion DNA; 7, FF41 virion DNA. In (A), (B), and (C), the cloned fragments represent *Eco*RI-E, *Eco*RI-H, and *Eco*RI-G1, respectively. Note in (A) that *Bam*HI-D fragments are homologous and of the same size in all viruses; the same is true of *Bam*HI-T, *Bam*HI-b, and *Bam*HI-b' in (B) and *Bam*HI-O and *Bam*HI-U in (C). Note also that the method identifies differences among the viruses which are described in the legends to subsequent figures. Clone designations are given at the bottom of each gel.

HR-1 DNA was observed by Heller et al., although they reported no deletion (16).

Two additional small deletions were found in HR-1 DNA (Fig. 4). A loss of approximately 80 bp was detected in *Bam*HI-L; the HR-1 *Bam*HI-K fragment was approximately 150 bp smaller than the corresponding fragment in B95-8 or FF41 virion DNA. The *Hin*dIII-I1 fragment of HR-1 DNA which was partially colinear with *Bam*HI-K showed a corresponding decrease in size; therefore the deletion mapped to sequences at the left side of the *Bam*HI-K fragment.

Insertions of DNA occurred at two sites in the HR-1 genome by comparison with B95-8 and FF41. The major insertion of about 1,700 bp was located in the *Hin*dIII-E fragment. This insertion contained two additional *Bam*HI sites and was responsible for two small *Bam*HI fragments (c' and d') which were seen in the DNA of HR-1, but not FF41, virus. However these fragments were revealed by probing with a cloned *Eco*RI-B fragment of FF41 DNA; therefore the new fragments contained some sequences which were already represented in the *Eco*RI-B fragment of FF41. A smaller insertion of about 100 bp was present in the *Bam*HI-P DNA fragment of HR-1 and its clone, relative to the FF41 and B95 strain (Fig. 4). The origin of these sequences is not known.

A third type of genome alteration involved the acquisition or loss of a restriction enzyme site. Since these alterations occur with no apparent change in MW they may involve point mutations or insertions or deletions too small to detect by agarose gel electrophoresis. Loss of the *Bam*HI restriction site separating *Bam*HI-B and *Bam*HI-

TABLE 1. Estimated MW of *Bam*HI fragments of HR-1 clone FF452-3 DNA—homology with fragments of identical size in DNA of transforming strains and with heterogeneous fragments in HR-1 parental DNA

*Bam*HI fragment	MW ($\times 10^6$)	Present as homologous fragment in B95 FF41 DNA	Homology with het fragments in parental HR-1 DNA
BG	10.68	−	−
A	6.34	+	ND[a]
B′	6.34	+[b]	+
C	6.19	+	+
D	5.38	+	−
E	5.25	+	−
F	4.94	+	−
W′I′	4.89	−	−
K	3.29	−	−
L	3.29	−	−
M	3.11	+[c]	+
N (terminal)	3.02	−[d]	+[e]
O	2.88	+	−
YH(h)	2.73	−	+
P	2.73	−	−
Q	2.54	+	ND
R	2.36	+	+
S	2.25	+	+
T	2.22	+	−
U	2.16	+	−
V	2.07	+	ND
W	2.00	+	+
X	1.33	+	ND
Z	1.12	+	ND
a	1.06	+	ND
b	0.82	+	−
b′	0.72	+	−
c	0.70	+	ND
c′	0.66	−	ND
d′	0.46	−	ND
d	0.45	+	ND

[a] ND, Not determined.
[b] B′ is not present in B95-8.
[c] Contains a new *Eco*RI site.
[d] Detected with *Bam*HI-A.
[e] Detected with *Eco*RI "terminal" probe provided by J. Arrand (1).

G resulted in a *Bam*HI-BG fusion fragment in HR-1 DNA. This fusion was detectable by hybridization of *Bam*HI digests with either *Bam*HI-B or *Bam*HI-G as a probe (Fig. 3).

From *Eco*RI digestions we observed that HR-1 lacked one fragment (*Eco*RI-G2) found in transforming DNA, but instead possessed two smaller fragments, *Eco*RI-I_2 (2.8×10^6 MW) and *Eco*RI J2 (1.5×10^6 MW), the sum of which equalled the size of *Eco*RI-G2. *Bam*HI-M hybridized to both *Eco*RI-I2 and *Eco*RI-J2, indicating it has homology with both fragments; therefore there is a new *Eco*RI site in the HR-1 *Bam*HI-M fragment (2).

Identification of genomic alterations which distinguish HR-1 and Jijoye DNA. The eight modifications of genome structure which distinguish HR-1 DNA from B95-8 and FF41 are summarized in Table 2. The question at this point was which, if any, of these changes were shared by Jijoye virus and by viral DNA within cells which had been immortalized by Jijoye virus. Therefore we focused our analysis on the eight genomic regions at which the nontransforming DNA could be distinguished from that of the transforming variants. We prepared Southern blots containing virion DNA or intracellular DNA and probed them with individual cloned *Bam*HI fragments corresponding to the sites of genome alteration (Fig. 5, 6, and 7).

Six of the eight modifications in HR-1 virus DNA, namely, deletion and fusion of *Bam*HI-W′ and *Bam*HI-I′, deletions in *Bam*HI-L and *Bam*HI-P, insertions in *Hin*dIII-E and *Bam*HI-P, loss of the *Bam*HI site between *Bam*HI-B and *Bam*HI-G and the additional *Eco*RI site in *Bam* HI-M, were all present in Jijoye virion DNA, in intracellular DNA from the Jijoye cell line, and in DNA from a line of umbilical cord lymphocytes transformed in vitro by Jijoye virus (Fig. 5). The modifications were all seen in intracellular DNA prepared from FF452-3 and in virion DNA prepared from another cell clone of

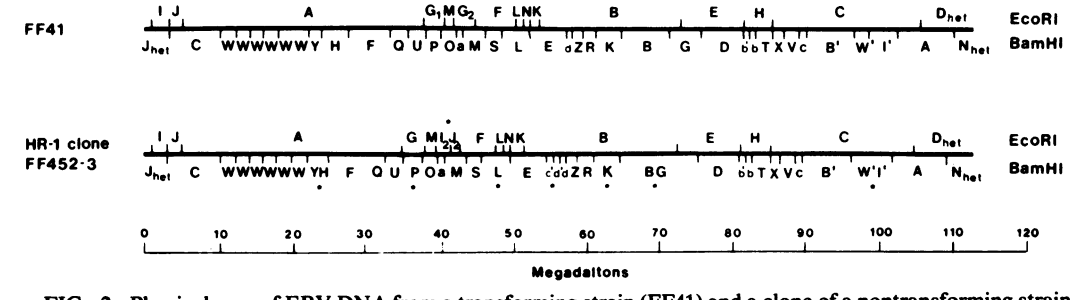

FIG. 2. Physical map of EBV DNA from a transforming strain (FF41) and a clone of a nontransforming strain (FF452-3 clone of HR-1). The number of *Bam*HI-W reiterations in the HR-1 clone has not been measured directly. The location of *Bam*HI fragments c′ and d′ within the *Eco*RI-B fragment of HR-1 has not been determined. The sites of differences in genome structure between the two viruses are indicated with asterisks.

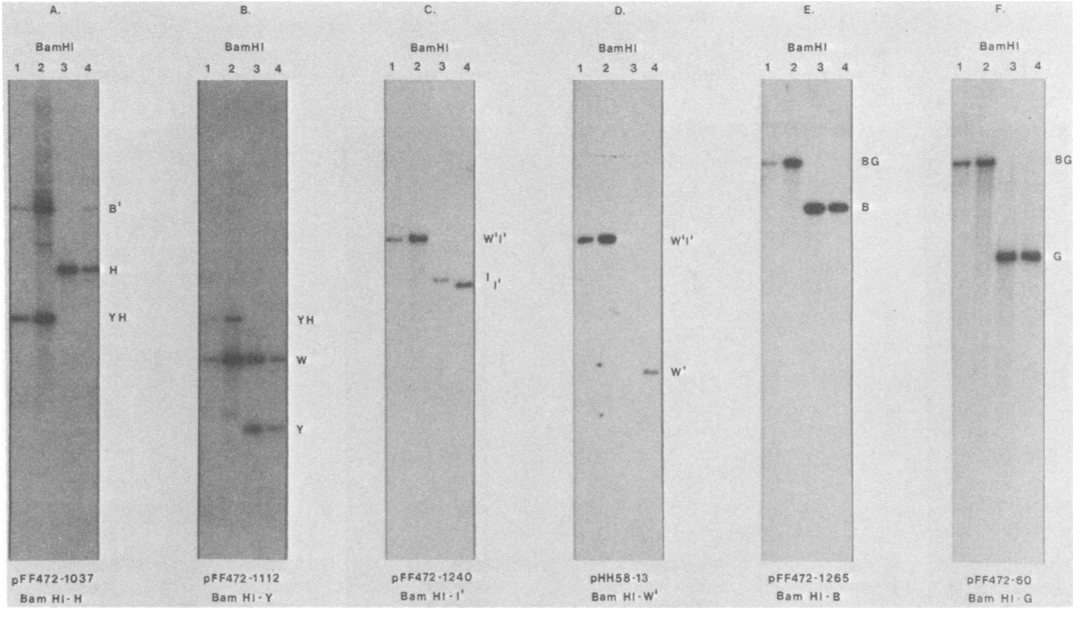

FIG. 3. Southern blot hybridizations of DNA of transforming and nontransforming EBVs with cloned EBV (FF41) DNA *Bam*HI fragments. The lanes contained virion DNA from the following: 1, HR-1 clone FF452-3; 2, HR-1 parent virus; 3, B95-8 (transforming); 4, FF41 (transforming). Each panel illustrates a site of sequence variability between the nontransforming viruses (lanes 1 and 2) and the transforming viruses (lanes 3 and 4). Heterogeneity in parental HR-1 DNA was demonstrated with probes of *Bam*HI-H and *Bam*HI-Y, but not with the other fragments. B95-8 DNA lacks fragments B', W', and I' (8, 26). Clone and EBV probe designations are given at the bottoms of the gels in this and all subsequent figures.

HR-1 (GG68-13). None of the modifications was identified in DNA from FF41 virions or in intracellular DNA from umbilical cord lymphocytes immortalized by FF41 DNA.

The 150-bp deletion in the *Bam*HI-K fragment of HR-1 virus DNA was not seen in DNA prepared from Jijoye virions. However this deletion was present in intracellular DNA from Jijoye cells and from cells transformed by Jijoye virus (Fig. 6). Similarly the *Hin*dIII-I1 fragment, which is colinear with the *Bam*HI-K fragment, was smaller in HR-1, in intracellular Jijoye DNA, and in DNA from cells transformed by Jijoye virus than in Jijoye virion DNA (Fig. 6).

The only deletion which consistently distinguished DNA from the nontransforming HR-1 variants (parent and clones) from DNA of transforming virus was the 3,800-bp deletion from *Bam*HI fragments Y and H. In HR-1 and its subclones the deleted *Bam*HI-YH(h) fragment had a MW of 2.73×10^6. In B95-8 and FF41 *Bam*HI-Y and *Bam*HI-H were present as individual fragments. In Jijoye virion and intracellular DNA there was a loss of the *Bam*HI site, giving rise to a single fragment which we designated *Bam*HI-YH(j) (Fig. 7). An identical sized fragment was found in neonatal cells transformed by Jijoye virus. The MW of this fragment, 5.12×10^6, was equal to the sum of the molecular weights of *Bam*HI-Y and *Bam*HI-H. Therefore, the absence of the *Bam*HI site between *Bam*HI-Y and *Bam*HI-H in Jijoye DNA reflects a minor change in DNA structure.

DISCUSSION

Although several alterations in genome structure distinguish the nontransforming HR-1 EB virus from immortalizing strains such as FF41 and B95-8, only one of these modifications differentiated HR-1 from its transforming parent, Jijoye. This was a deletion of about 2.4×10^6 MW from sequences mapping to the *Bam*HI-Y and *Bam*HI-H fragments. A full-sized *Bam*HI-YH(j) fragment (5.1×10^6 MW) was found in Jijoye virion DNA and in intracellular viral DNA of the Jijoye line and of neonatal lymphocytes immortalized by Jijoye virus. The deleted fragment *Bam*HI-YH(h) was seen in the HR-1 line and in two of its subclones which lack the capacity to transform lymphocytes.

The exact limits of the deletion which distinguishes HR-1 from Jijoye have not been defined in this study. This will require further fine mapping and sequencing in this region. Because of the homology between *Bam*HI-W and *Bam*HI-Y, our data do not reveal whether the deletion

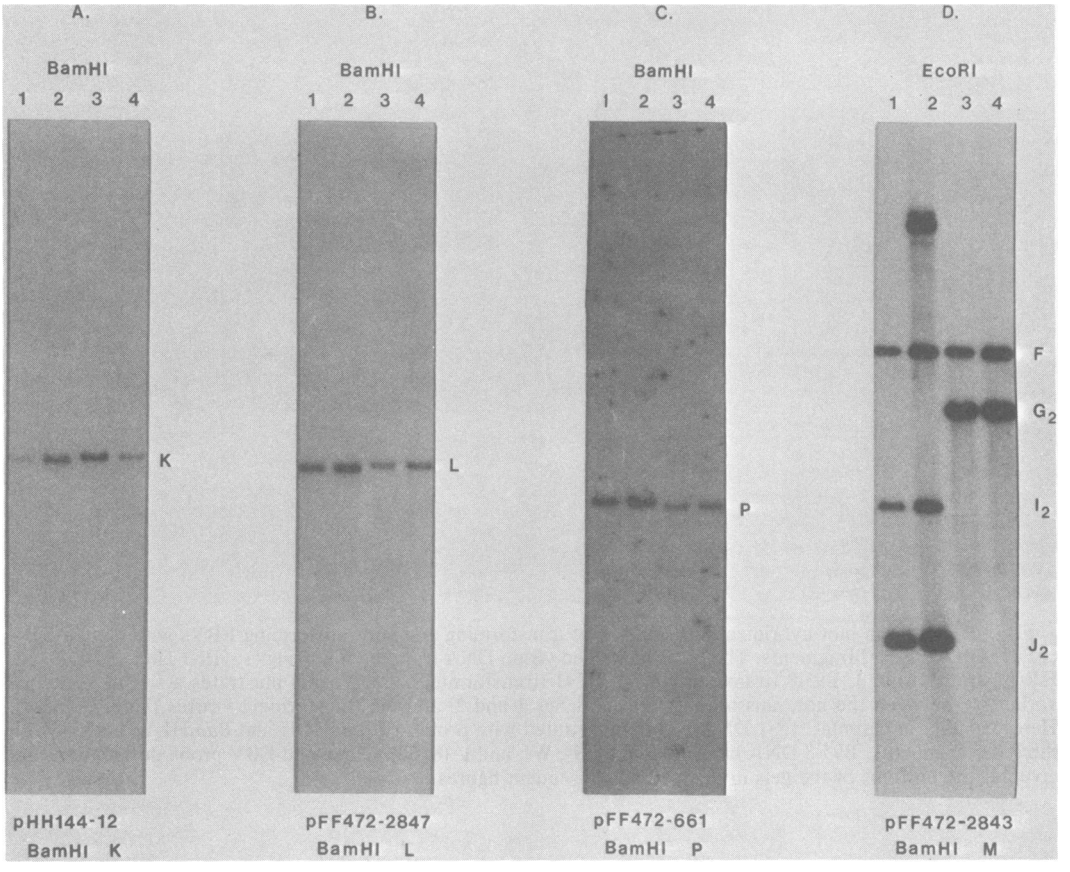

FIG. 4. Small deletions, insertions, and additional restriction endonuclease recognition sites which distinguish HR-1 and its clone from the two transforming viruses. Lanes: 1, FF452-3; 2, HR-1; 3, B95-8; 4, FF41. *Bam*HI-M reveals heterogeneity in parental HR-1 DNA.

begins within the internal repeat (*Bam*HI-W) or within the homologous sequences present at the left end of *Bam*HI-Y.

The biological significance of this deletion is supported by two other types of experimental evidence. First, the *Bam*HI-Y and *Bam*HI-H region codes for a 3.1-kilobase mRNA in transformed cells which are not permissive for virion synthesis (20, 37). Second, a correlation between deletion of the *Bam*HI-Y and *Bam*HI-H fragments and loss of the transforming phenotype has been suggested by analysis of transformation-competent viruses formed after recombination between HR-1 DNA and the endogenous EBV DNA in Raji cells (10, 11, 39). Those recombinants which retain lymphocyte immortalizing ability are found to contain genetic sequences characteristic of Raji viral DNA including *Bam*HI fragments W, Y, H, and F (J. Skare and J. Strominger, personal communication). Thus this region of EBV DNA is likely to encode one of the several genome functions which appear to be necessary to initiate and maintain permanent lymphoid cell growth. Nothing is yet known of the nature of the product(s) of this region.

Jijoye DNA contains a "fused" *Bam*HI-YH fragment, and B95-8 and FF41 each contain individual *Bam*HI-Y and *Bam*HI-H fragments. The genome of another transforming virus of Burkitt lymphoma origin, AG876, also contains a fused *Bam*HI-YH fragment (16, 26). Thus alterations of this *Bam*HI site do not appear to impair the transformation function.

Our experiments suggest that this deletion has occurred while the Jijoye virus was being propagated in the laboratory. Jijoye DNA contains all of the variations seen in HR-1 DNA, except the Y-H deletion. This, in fact, is verification of the true lineage of the Jijoye strain which was used for comparison. Since Jijoye DNA does not seem to be heterogeneous, it is unlikely that a

TABLE 2. Summary of genomic alterations in HR-1 (clone FF452-3) EBV DNA by comparison with three transforming viruses, FF41, B95-8, and Jijoye[a]

Alteration no.	DNA fragment, affected	Designation of altered HR-1 fragment	Description of the alteration	Alteration confirmed in a recombinant FF452-3 *Eco*RI fragment	Alteration present in the following:			
					B95-8 virion	FF41 virion	Jijoye virion	Jijoye cellular
1	*Bam*HI-H, -Y	*Bam*HI-YH(h)	Deletion of ~3,800 bp of sequences from *Bam*HI-Y and -H	+	−	−	−	−
2	*Bam*HI-K	*Bam*HI-K	Deletion of ~150 bp from left side of this fragment	+	−	−	−	+
3	*Bam*HI-W', -I'	*Bam*HI-W'I'	Deletion of ~370 bp including the *Bam*HI site between *Bam*HI-W' and -I'	+	−	−	+	+
4	*Bam*HI-L	*Bam*HI-L	Deletion of ~80 bp	ND[b]	−	−	+	+
5	*Hin*dIII-E	*Hin*dIII-E, *Bam*HI-c',-d'	Insertion of ~1,700 bp of sequences of viral origin, includes two new *Bam*HI sites	+	−	−	+	+
6	*Bam*HI-P	*Bam*HI-P	Insertion of ~100 bp of sequences of unknown origin	+	−	−	+	+
7	*Bam*HI-B, -G	*Bam*HI-BG	Loss of *Bam*HI site	+	−	−	+	+
8	*Bam*HI-M	*Eco*RI-I2, -J2	Acquisition of a new *Eco*RI site	+	−	−	+	+

[a] The nomenclature of fragments in B95-8 (6, 33) and FF41 (8) DNA was as described previously.
[b] ND, Not determined.

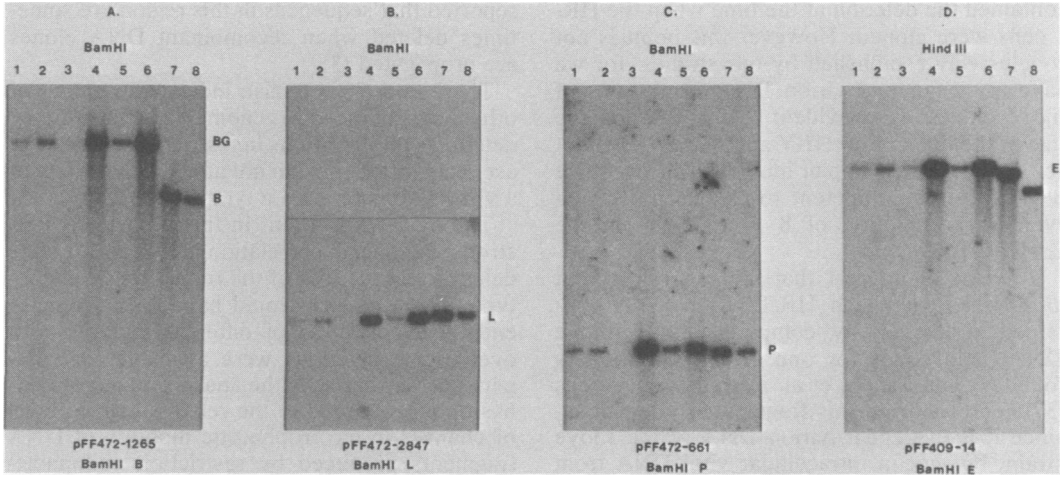

FIG. 5. Genomic alterations which are present in HR-1 DNA as well as in Jijoye DNA. Lanes: 1, Jijoye virion DNA; 2, Jijoye intracellular DNA; 3, intracellular DNA from lymphocytes transformed in vitro by Jijoye virus; 4, parental HR-1 virion DNA; 5, intracellular DNA from HR-1 clone FF452-3; 6, virion DNA from HR-1 clone GG68-13; 7, virion DNA from FF41; 8, intracellular DNA from lymphocytes transformed by FF41 DNA. Jijoye DNA contains the fusion of *Bam*HI-B and *Bam*HI-G (panel A), the deletion in *Bam*HI-L (panel B), the insertion in *Bam*HI-P (panel C), and the larger *Hin*dIII-E fragment (panel D) which are characteristic of HR-1 DNA. The *Hin*dIII-E fragment appears deleted in lymphocytes transformed by FF41 DNA.

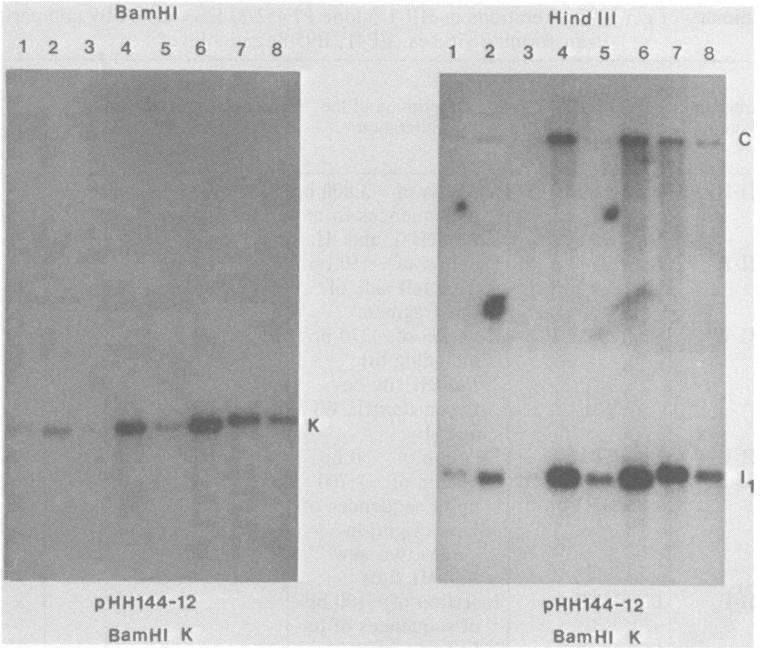

FIG. 6. Variation in size among *Bam*HI-K and *Hin*dIII-I1 fragments in Jijoye, HR-1, and FF41 DNAs. DNAs were digested with restriction endonucleases as indicated. *Bam*HI-K was the probe. Lanes: 1, Jijoye virion DNA; 2, Jijoye intracellular DNA; 3, DNA from lymphocytes transformed by Jijoye virus; 4, parental HR-1 virion DNA; 5, intracellular FF452-3 DNA; 6, HR-1 clone GG68-13 virion DNA; 7, FF41 virion DNA; 8, intracellular DNA from lymphocytes transformed by FF41 DNA. Note that the deletion in the *Bam*HI-K and *Hin*dIII-I1 fragments is present in HR-1 and its subclones, in intracellular Jijoye DNA, and in intracellular DNA from lymphocytes transformed by Jijoye virus, but not in virion DNA from Jijoye.

subpopulation of Jijoye DNA molecules already contained the deletion at the time when the HR-1 cells were cloned. However this point is not conclusively established by our studies, for we have not examined virion DNA in a series of Jijoye clones. It is evident that a deletion the size of the one in *Bam*HI-Y and *Bam*HI-H is not per se sufficient to impair immortalization, since B95-8, a virus competent to transform, has an even larger deletion of 8×10^6 MW in the *Eco*RI-C fragment.

It seems of interest that the other fragment (*Bam*HI-K), in which HR-1 virion DNA contained a deletion by comparison with Jijoye DNA, also codes for one of the six mRNAs found by van Santen et al. in transformed cells (37). The lesion in this fragment is anomalous, since it is evident in virion DNA of the Jijoye strain, but not in intracellular viral DNA from Jijoye cells or from cells transformed by Jijoye virus. A valid hypothesis for this finding eludes us at present, but it appears that this region is highly variable. Heller et al. found a truncated *Bam*HI-K fragment in Raji cells (16), and Bornkamm et al. reported three size classes of *Hin*dIII-I1 fragments in an examination of six different EBV strains (2). Further, Buell et al. reported that sequences in this region are sometimes deleted when recombinant DNA clones are propagated (3).

These experiments also indicate a number of other areas of the viral genome where insertions, deletions, or alterations in restriction endonuclease recognition sites do not affect the capacity of a virus to immortalize a lymphocyte.

The evidence present in this report provides strong biological correlation between a single deletion and the loss of the transforming phenotype. The evidence would have been strengthened if a collection of different mutants with overlapping deletions were available for comparison. Furthermore the analysis in our studies has only proceeded to the relatively gross level of changes in electrophoretic mobility of DNA fragments produced by restriction endonucleases. It is obvious that additional biologically important lesions may exist in HR-1 viral DNA which will be demonstrable only after detailed nucleotide sequence analysis.

If the hypothesis is correct that the deletion of *Bam*HI-H and *Bam*HI-Y in HR-1 is the only important modification, then it should be possi-

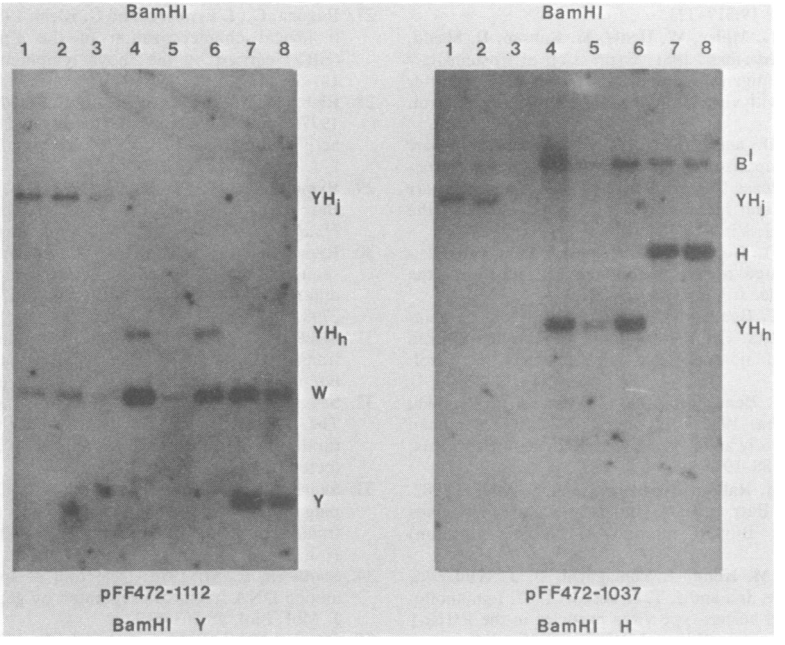

FIG. 7. The major genomic deletion which distinguishes HR-1 DNA from Jijoye DNA. Shown are Southern blots probed with *Bam*HI-Y (left panel) and *Bam*HI-H (right panel) fragments of EBV (FF41) DNA. Lanes: 1, Jijoye virion DNA; 2, Jijoye intracellular DNA; 3, intracellular DNA from lymphocytes transformed in vitro by Jijoye virus; 4, parental HR-1 virion DNA; 5, intracellular DNA from FF452-3; 6, virion DNA from HR-1 clone GG68-13; 7, virion DNA from FF41; 8, intracellular DNA from lymphocytes transformed by FF41 DNA. Intracellular DNA from Jijoye cells, from lymphocytes transformed by Jijoye virus, and from Jijoye virions all contained a large *Bam*HI-YH(j) fragment (5.2×10^6 MW); whereas HR-1 and its clones have a deleted *Bam*HI-YH(h) fragment $\sim 2.7 \times 10^6$ MW. In FF41 *Bam*HI-Y and *Bam*HI-H are present as separate fragments. *Bam*HI-H has homology to *Bam*HI-B' in all samples (right panel).

ble to provide a biological proof of this idea. This would consist of repairing the lesion in HR-1 virus by transfer of the missing DNA sequences from the parental Jijoye DNA.

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LITERATURE CITED

1. **Arrand, J., L. Rymo, J. E. Walsh, E. Bjorck, T. Lindahl, and B. E. Griffin.** 1981. Molecular cloning of the complete Epstein-Barr virus genome as a set of overlapping endonuclease fragments. Nucleic Acids Res. **9**:2999–3014.
2. **Bornkamm, G. W., H. Delius, U. Zimber, J. Hudewentz, and M. A. Epstein.** 1980. Comparison of Epstein-Barr virus strains of different origin by analysis of the viral DNAs. J. Virol. **35**:603–618.
3. **Buell, G. N., D. Reisman, C. Kintner, G. Crouse, and B. Sugden.** 1981. Cloning overlapping DNA fragments from the B95-8 strain of Epstein-Barr virus reveals a site of homology to the internal repetition. J. Virol. **40**:977–982.
4. **Chang, A. C., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
5. **Cheung, A., and E. Kieff.** 1981. Epstein-Barr virus DNA. X. Direct repeat within the internal direct repeat of Epstein-Barr virus DNA. J. Virol. **40**:501–507.
6. **Dambaugh, T., C. Beisel, M. Hummel, W. King, S. Fennewald, A. Cheung, M. Heller, N. Raab-Traub, and E. Kieff.** 1980. Epstein-Barr virus DNA. VII. Molecular cloning and detailed mapping of EBV (B95-8) DNA. Proc. Natl. Acad. Sci. U.S.A. **77**:305–309.
7. **Delius, H., and G. W. Bornkamm.** 1978. Heterogeneity of Epstein-Barr virus III. Comparison of a transforming and a nontransforming virus by partial denaturation mapping of their DNAs. J. Virol. **27**:81–89.
8. **Fischer, D. K., G. Miller, L. Gradoville, L. Heston, M. W. Weststrate, W. Maris, J. Wright, J. Brandsma, and W. C. Summers.** 1981. Genome of a mononucleosis Epstein-Barr virus contains DNA fragments previously regarded to be unique to Burkitt's lymphoma isolates. Cell **24**:543–553.
9. **Frank, A., W. Andiman, and G. Miller.** 1976. Epstein-Barr virus and non-human primates: natural and experimental infection. Adv. Cancer Res. **23**:171–210.
10. **Fresen, K. O., M.-S. Cho, L. Gissmann, and H. zur Hausen.** 1979. NC37-R1 EB virus: a possible recombinant between intracellular NC37 viral DNA and superinfecting P3HR-1 EBV. Intervirology **12**:303–310.
11. **Fresen, K. O., M.-S. Cho, and H. zur Hausen.** 1978. Recovery of transforming EBV from non-producer cells after superinfection with non-transforming P3HR-1 EBV. Int. J. Cancer **22**:378–383.
12. **Fresen, K. O., B. Merkt, G. W. Bornkamm, and H. zur Hausen.** 1977. Heterogeneity of Epstein-Barr virus originating from P3HR-1 cells. I. Studies on EBNA induction.

Int. J. Cancer 19:317–323.
13. **Grogan, E., G. Miller, W. Henle, M. Rabson, D. Shedd, and J. C. Niederman.** 1981. Expression of Epstein-Barr viral early antigen in monolayer tissue cultures after transfection with viral DNA and DNA fragments. J. Virol. 40:861–869.
14. **Hayward, S. D., and E. Kieff.** 1977. 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. J. Virol. 23:421–429.
15. **Hayward, S., L. Nogee, and G. Hayward.** 1980. Organization of repeated regions within the Epstein-Barr virus DNA molecule. J. Virol. 33:507–521.
16. **Heller, M., T. Dambaugh, and E. Kieff.** 1981. Epstein-Barr virus DNA. IX. Variation among viral DNAs from producer and non-producer infected cells. J. Virol. 38:632–648.
17. **Henle, G., W. Henle, B. Zajac, G. Pearson, R. Waubke, and M. Scriba.** 1970. Differential reactivity of human serums with early antigens induced by Epstein-Barr virus. Science 169:188–190.
18. **Heston, L., M. Rabson, N. Brown, and G. Miller.** 1982. New Epstein-Barr virus variants from cellular subclones of P3J-HR-1 Burkitt lymphoma. Nature (London) 295:160–163.
19. **Hinuma, Y., M. Kohn, J. Yamaguchi, D. J. Wudarski, J. R. Blakeslee, Jr., and J. T. Grace, Jr.** 1967. Immunofluorescence and herpes-type virus particles in the P3HR-1 Burkitt lymphoma cell line. J. Virol. 1:1045–1051.
20. **King, W., A. L. Thomas-Powell, N. Raab-Traub, M. Hawke, and E. Kieff.** 1980. Epstein-Barr virus RNA. V. Viral RNA in a restringently infected, growth-transformed cell line. J. Virol. 36:506–518.
21. **Lee, Y. S., Y. Yajima, and M. Nonoyama.** 1977. Mechanism of infection by Epstein-Barr virus. II. Comparison of viral DNA from HR-1 and superinfected Raji cells by restriction enzymes. Virology 81:17–24.
22. **Menezes, J., W. Leibold, and G. Klein.** 1975. Biological differences between Epstein-Barr virus strains with regard to lymphocyte transforming ability, superinfection and antigen induction. Exp. Cell Res. 92:478–484.
23. **Miller, G., E. Grogan, L. Heston, J. Robinson, and D. Smith.** 1981. Epstein-Barr viral DNA: infectivity for human placental cells. Science 212:452–455.
24. **Miller, G., and M. Lipman.** 1973. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. Proc. Natl. Acad. Sci. U.S.A. 70:190–194.
25. **Miller, G., J. Robinson, L. Heston, and M. Lipman.** 1974. Differences between laboratory strains of Epstein-Barr virus based on immortalization, abortive infection and interference. Proc. Natl. Acad. Sci. U.S.A. 71:4006–4010.
26. **Raab-Traub, N., T. Dambaugh, and E. Kieff.** 1980. DNA of Epstein-Barr virus. VIII. B95-8, the previous prototype, is an unusual deletion derivative. Cell 22:257–267.
27. **Ragona, G., I. Ernberg, and G. Klein.** 1980. Induction and biological characterization of the Epstein-Barr virus (EBV) carried by the Jijoye lymphoma line. Virology 101:553–557.
28. **Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. 113:237–251.
29. **Rymo, L., and S. Forsblum.** 1978. Cleavage of Epstein-Barr virus DNA by restriction endonucleases *Eco*RI, *Hind*III, and *Bam*HI. Nucleic Acids Res. 5:1387–1402.
30. **Rymo, L., T. Lindahl, and A. Adams.** 1979. Sites of sequence variability in Epstein-Barr virus DNA from different sources. Proc. Natl. Acad. Sci. U.S.A. 76:2794–2798.
31. **Sairenji, T., and Y. Hinuma.** 1980. Re-evaluation of a transforming strain of Epstein-Barr virus from the Burkitt lymphoma cell line, Jijoye. Int. J. Cancer 26:337–342.
32. **Seignevrin, J. M., M. Vuillaume, G. Lenoir, and G. de-Thé.** 1977. Replication of Epstein-Barr virus: ultrastructural and immunofluorescent studies of P3HR-1 superinfected Raji cells. J. Virol. 24:836–845.
33. **Skare, J., and J. L. Strominger.** 1980. Cloning and mapping of *Bam* HI endonuclease fragments of DNA from the transforming B95-8 strain of Epstein-Barr virus. Proc. Natl. Acad. Sci. U.S.A. 77:3860–3864.
34. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.
35. **Steinitz, M., T. Bakacs, and G. Klein.** 1978. Interaction of the B95-8 and P3HR-1 substrains of Epstein-Barr virus with peripheral human lymphocytes. Int. J. Cancer 22:251–257.
36. **Sugden, B., W. C. Summers, and G. Klein.** 1976. Nucleic acid renaturation and restriction endonuclease cleavage analyses show that the DNAs of a transforming and a non-transforming strain of Epstein-Barr virus share approximately 90% of their nucleotide sequences. J. Virol. 18:765–775.
37. **van Santen, V., A. Cheung, and E. Kieff.** 1981. Epstein-Barr virus RNA VII. Size and direction of transcription of virus specified cytoplasmic RNAs in a transformed cell line. Proc. Natl. Acad. Sci. U.S.A. 78:1930–1934.
38. **Wahl, G. M., R. A. Padgett, and G. R. Stark.** 1979. Gene amplification causes overproduction of the first three enzymes of UMP synthesis in N-(phosphonacetyl)-L-aspartate-resistant hamster cells. J. Biol. Chem. 254:8679–8689.
39. **Yajima, Y., B. Marcynska, and M. Nonoyama.** 1978. Transforming activity of Epstein-Barr virus obtained by superinfection of Raji cells. Proc. Natl. Acad. Sci. U.S.A. 75:2008–2010.
40. **zur Hausen, H., F. J. O'Neill, U. K. Freese, and E. Hecher.** 1978. Persisting oncogenic herpesvirus induced by tumor promoter TPA. Nature (London) 272:373–375.