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Epstein-Barr Virus Intrastrain Recombination in Oral Hairy Leukoplakia

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In laboratory lymphoblastoid cell lines and in natural human infections, Epstein-Barr virus (EBV) strains have been identified by DNA restriction fragment length polymorphisms of the *Bam*HI H fragment. Multiple, heterogeneous *Bam*HI H fragments have been detected in oral hairy leukoplakia (HLP), raising the question of EBV coinfection with multiple strains. To investigate whether the heterogeneous *Bam*HI H fragments represent different EBV strains or recombinant variants of the same strain, EBV DNA from HLP lesions was analyzed to characterize the viral strains and determine the source of possible recombinant variants. Clones of heterogeneous *Bam*HI H fragments from a single HLP lesion were determined to have strain identity on the basis of sequence identity of the EBNA-2 genes. Intrastrain homologous recombination within the IR2 internal repeat region and nonhomologous recombination of other sequences accounted for the heterogeneity of the *Bam*HI H fragments. PCR amplification from additional HLP specimens detected similar recombinant variants. A possible example of site-specific recombination joining the *Bam*HI Y portion of the EBNA-2 gene to sequences within the *Bam*HI S fragment was also detected in multiple HLP specimens. These data indicate that intrastrain recombination during productive replication confounds the use of restriction fragment length polymorphism analysis of the *Bam*HI Y and H fragments to identify EBV strains in HLP. In patients with permissive epithelial EBV infections, EBV strains could be more accurately distinguished by sequence identity or divergence within known regions of genetic strain variation.

Oral hairy leukoplakia (HLP), a lesion occurring on the lateral borders of the tongue in many patients infected with the human immunodeficiency virus, is caused by the productive replication of the Epstein-Barr virus (EBV) within mucosal epithelial cells (23). Some HLP lesions are coinfected with both EBV type 1 (EBV-1) and EBV-2, on the basis of typing of the Epstein-Barr nuclear antigen 2 (EBNA-2) gene, and some HLP lesions may be coinfected with multiple strains of the same EBV type (55). During productive replication, the EBV in HLP apparently recombines within the *Bam*HI WYH region, including the EBNA-2 gene, to generate viral variants that are capable of replicating efficiently and persisting within the lesion over time (55).

The EBV strains in lymphoblastoid cell lines (LCLs) can be differentiated by *Bam*HI H fragment size (6, 24), which differs among strains in the number of 125-bp repeat units within the IR2 internal repeat region. Based on these observations in LCLs, restriction fragment length polymorphism analysis of the *Bam*HI H fragment has been used as a marker of EBV strain identity and coinfection in studies of natural infections in patients. In HLP, multiple fragments hybridize to probes containing sequences from the *Bam*HI H portion of the EBNA-2 gene in the size range of 4.5 to 12 kb (55). These heterogeneous *Bam*HI H fragments could represent coinfection of HLP with different, genetically stable EBV strains, or they could represent recombinant viral variants that differ in the number of repeat units within IR2 (55). However, it was difficult to attribute the large size of some of the *Bam*HI H fragments in HLP (8 to 12 kb) only to IR2 variation, suggesting that additional rearrangements may have occurred elsewhere in the fragment.

The present study was designed to investigate the origin of the *Bam*HI H fragment heterogeneity in HLP by identifying and characterizing the EBV strains and recombinant variants present in clones from a single HLP specimen containing multiple *Bam*HI H fragments. Restriction mapping and sequence analysis of four clones demonstrated that multiple viral variants were generated through recombination within the *Bam*HI Y and H regions of a single EBV strain. Although an HLP lesion may be coinfected with multiple EBV types and strains, numerous variants of EBV arise through intrastrain recombination during productive viral replication, accounting for much of the restriction fragment heterogeneity of the *Bam*HI Y and H fragments.

MATERIALS AND METHODS

Cloning of HLP DNA. HLP biopsy specimens were obtained from human immunodeficiency virus-seropositive, volunteer patients, and the DNA was extracted as described elsewhere (18, 43). DNA to be cloned was digested with *Hin*dIII, ligated into cosmid vector Lorist B (34), and transfected into *Escherichia coli* NM554, which is a *recA* mutant and deficient for in vitro recombination (Stratagene Cloning Systems, La Jolla, Calif.). Subclones of the *Bam*HI H and *Bsa*MI fragments were ligated into plasmid vector pGem3Z (Promega, Madison, Wis.) and grown in *E. coli* DH5αF'IQ, which is also a *recA* mutant and deficient for in vitro recombination (Gibco BRL, Grand Island, N.Y.). The *Bam*HI H fragment from LCL B95-8 was cloned into plasmid vector pGem2 (Promega) and grown in *E. coli* DH5αF'IQ. Cosmid and plasmid DNA was extracted from bacterial cell cultures by alkaline lysis and was purified on Qiagen-tip columns (Qiagen Inc., Chatsworth, Calif.).

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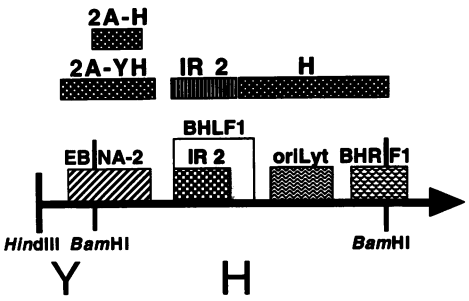

FIG. 1. Schematic diagram of the *Bam*HI YH region of the B95-8 EBV genome. The location of the EBNA-2 gene, the IR2 internal repeat sequences, the BHLF1 gene, oriLyt, and the BHRF1 gene are indicated. The hybridization domains of the probes used in the analysis of this region are shown above the diagram. All probes are RNA probes transcribed from viral DNA except IR2, which is a DNA oligonucleotide probe whose specific hybridization domain is shown in Fig. 3A. The 2A-H probe identifies the sequence from the *Bam*HI H region of the EBNA-2 gene, while the 2A-YH probe identifies both the *Bam*HI H and the *Bam*HI Y portions of EBNA-2. Probe H identifies sequences to the right of IR2, including oriLyt and the BHRF1 gene.

Probes and Southern blots. RNA probes used for Southern blot hybridization were synthesized with [α-^{32}P]UTP (Amersham, Arlington Heights, Ill.) and either SP6, T3, or T7 RNA polymerase (Promega). Probes 2A-H and 2A-YH have been described previously (55). Probe H was derived from the subcloned *Bam*HI H fragment of B95-8 digested with *Not*I and synthesized from the right end of the fragment. The hybridization domains of these probes are illustrated in Fig. 1. Probes 2A-Y and S were derived from the insert of clone 4 (see Results), subcloned in opposite orientations, digested with *Sma*I to release either the *Bam*HI Y or the *Bam*HI S end of the fragment, and synthesized from the desired end of the remaining insert. Single-stranded DNA oligonucleotides were synthesized and subsequently labeled with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.) for use as the probes designated IR2, F, Q, U, P, and O. The sequences of these probes are given in Table 1. For hybridization to labeled probes, Southern blots of cloned DNA were performed by agarose gel electrophoresis (SeaKem agarose; FMC BioProducts, Rockland, Maine) and transfer of DNA to supported nitrocellulose membrane (Micron Separations Inc., Westboro, Mass.).

DNA sequencing. DNA sequences were obtained from cloned DNA by one of the following methods: (i) dideoxynucleotide chain termination using [α-^{35}S]ATP (Amersham) and Sequenase enzyme (United States Biochemical, Cleveland, Ohio) with denatured double-stranded DNA or with single-stranded DNA generated by asymmetric PCR or (ii) PCR dideoxynucleotide chain termination sequencing with *Taq* polymerase (Promega) and with direct incorporation of [α-^{35}S]ATP or with [γ-^{32}P]ATP (Amersham)-end-labeled oligonucleotide primers. Sequencing reaction products were run on 6 to 8% polyacrylamide gels (Long Ranger; AT Biochem Inc., Malvern, Pa.). SP6 and T7 RNA polymerase promoter primers (Promega) were used for sequencing reactions of subcloned DNA. Single-stranded DNA oligonucleotides synthesized for use as sequencing primers are listed in Table 1.

PCR amplification. PCR amplifications of HLP DNA were performed using 2 U of Vent DNA polymerase (New England Biolabs); a 1:10 dilution of 10× reaction buffer as supplied with the Vent polymerase; 200 μM (each) dATP, dGTP, dCTP, and dTTP; and a 0.5 pM concentration of each primer in 50- or 100-μl reaction volumes. In some cases, 7-deaza-dGTP (Boehringer Mannheim, Indianapolis, Ind.) was substituted for dGTP in a ratio of 3:1 to increase amplification efficiency. Amplification proceeded for a total of 40 cycles with denaturation at 98°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min. Single-stranded DNA oligonucleotides synthesized for use as PCR primers are listed in Table 1. PCR products were detected by hybridization with radiolabeled probes after Southern blotting as described above.

RESULTS

Identification of *Bam*HI-H heterogeneity. DNA from an HLP lesion containing multiple *Bam*HI H fragments of EBV-1 was digested with *Hin*dIII and cloned into the cosmid vector Lorist B. Hybridization with the 2A-YH probe (Fig. 1) identified four clones of *Hin*dIII-B, a fragment that includes part or all of the *Bam*HI Y, H, F, Q, U, P, and O fragments. Clones 1, 2, and 3 contained *Bam*HI H fragments of 6.4, 9.4, and 10.4 kb, respectively (Fig. 2). Clone 4 was only 2.2 kb in size and had

TABLE 1. Oligonucleotides used for PCR, sequencing, and probes

Oligonucleotide	Location	Sequence (5′–3′)	Coordinates
IR2 probe	IR2	CCGCCAGGTCCTGGGGCAGCCGGGGTTCCTGGCGCTCC	58578–50615
F probe	*Bam*HI-F	CTCAAGCTTGCCTAATATCCTACTGGCTCA	55031–55008
Q probe	*Bam*HI-Q	GTAATGTTGTCTGGTCGCTAGATGG	62359–62383
U probe	*Bam*HI-U	ATTTCCGGTAGGGCCATGAGCCGTT	66145–66169
P probe	*Bam*HI-P	GAACACTTCATCTTCGAACATGTCT	69434–69458
O probe	*Bam*HI-O	ACCGGCAGTATGCCTCGACGTCTGT	73502–73526
1	EBNA-2	CTCTCTAGACTATCTTGCGTTACATGGGGGACA	48515–48538
2	EBNA-2	TTGTGTCCAGGCATCCCTGCGCT	48827–48805
3	EBNA-2	AGGGATGCCTGGACACAA	48810–48827
4	EBNA-2	TGGGGTGCTTTGATG	49307–49293
5	EBNA-2	CATCAAAGCACCCCA	49293–49307
6	EBNA-2	AGGCATGCTAGGACTGGA	49652–49635
7	EBNA-2	TCCAGTCCTAGCATGCCT	49635–49652
8	EBNA-2	CTCCTGCAGCGAGGTCTTTTACTGGGTCC	49952–49932
9	U2	TGCATAGGAGTGCGAACATGG	50236–50216
10	U2	GTCTGAGCATTCCATGGGCAGCA	50541–50563
11	U3	CCTGGGGGAGGGAACCGGGT	52356–52337
12	U3	CTCTCTAGAGGTGCATCTGGAACGGCTTACCT	53870–53892
13	*Bam*HI-S	CTCCTGCAGGCGATACTCTCAGTTAACGAGCA	86036–86014

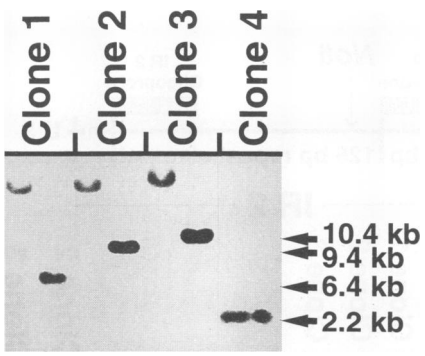

FIG. 2. Identification of the *Bam*HI H fragment in four HLP *Hin*dIII-B clones. Each clone was digested with *Hin*dIII or *Hin*dIII plus *Bam*HI in paired lanes. Hybridization with the 2A-YH probe demonstrated a large (approximately 30-kb) *Hin*dIII B fragment which contained a distinct *Bam*HI H fragment in three of the clones: clone 1, 6.4 kb; clone 2, 9.4 kb; and clone 3, 10.4 kb. Clone 4 contained only a 2.2-kb *Hin*dIII B fragment that lacked a *Bam*HI restriction enzyme site.

lost the *Bam*HI restriction site between *Bam*HI-Y and -H (Fig. 2). Clones 1 to 3 were further analyzed by *Bam*HI digestion and hybridization with oligonucleotide probes to individually identify the *Bam*HI F, Q, U, P, and O fragments to the right of the *Bam*HI H fragment. These fragments were identical in size for each clone, matching the predicted size based on the B95-8 genome (5) (data not shown). Thus, in these three clones, the *Bam*HI H fragment was the only variable fragment within the approximately 30-kb *Hin*dIII B region.

EBV strain identification. To identify the EBV strains represented, the sequences of the EBNA-2 genes were determined for clones 1 to 3. All three clones contained identical EBNA-2 sequences, indicating that a single EBV strain was the source of the variants; thus, the *Bam*HI H fragment heterogeneity in this HLP developed through intrastrain recombination. The HLP EBNA-2 sequence was similar to that of W91 and distinct from that of B95-8, the two prototype EBV-1 LCL strains (Table 2) (11, 13). The HLP EBNA-2 sequence contained a 24-bp deletion of sequence from the polyproline region, a 3-bp insertion, and a 3-bp deletion of sequence, each preserving the open reading frame of the gene. Single-nucleotide substitutions usually resulted in conservative or similar amino acid changes in the predicted protein sequence (Table 2). The sequence changes in the HLP EBNA-2 gene would not disrupt the known EBNA-2 functional domains involved in transcriptional transactivation and B-lymphocyte growth transformation (11).

Recombination within the IR2 repetitive sequences. After the *Bam*HI H fragments of clones 1 to 3 were subcloned into plasmid vectors, the IR2 regions of the three clones were analyzed to determine the number of repeat units present in each. The restriction enzyme *Not*I cuts only once within each 125-bp unit of IR2 and rarely within the rest of the EBV genome. Partial digestion with *Not*I and hybridization with the IR2 oligonucleotide probe generated a ladder array of bands differing in size by 125 bp for each clone such that the total number of repeat units in IR2 equaled one plus the number of bands present in the ladder (Fig. 3). B95-8 demonstrated 10 bands, consistent with the presence of 11 repeat units (6, 14,

TABLE 2. HLP EBNA-2A coding sequence compared with those in B95-8 and W91 strains

B95-8 coordinate(s)	EBNA-2 codon	Nucleotide change	Amino acid change	Conserved or similar amino acid	EBNA-2 functional domain	HLP strain	W91 strain
48687–48689	62	CTC → —[a]	Leu → —	−	Polyproline	+	Similar
48690–48692	63	CCA → —	Pro → —	−	Polyproline	+	Similar
48693–48695	64	CCA → —	Pro → —	−	Polyproline	+	Similar
48696–48698	65	CCC → —	Pro → —	−	Polyproline	+	Similar
48699–48701	66	CCC → —	Pro → —	−	Polyproline	+	Similar
49702–49704	67	CCA → —	Pro → —	−	Polyproline	+	Similar
49705–49707	68	CCA → —	Pro → —	−	Polyproline	+	Similar
49708–49710	69	CCA → —	Pro → —	−	Polyproline	+	Similar
49722	73	C → T	Pro → Pro	+	Polyproline	−	+
49731	76	C → T	Pro → Pro	+	Polyproline	+	+
49740	79	C → T	Pro → Pro	+	Polyproline	+	+
49749	82	C → T	Pro → Pro	+	Polyproline	+	+
49759	86	C → T	Pro → Ser	+	Polyproline	+	+
48990	163	A → G	Arg → Glu	−	Divergent	+	+
48991	163	G → T	Arg → Val	−	Divergent	+	+
48998	165	C → A	Val → Val	+	Divergent	+	+
49057	185	A → G	Gln → Arg	−	Divergent	+	+
49091	196	G → T	Met → Ile	+	Divergent	+	+
49113	204	A → T	Thr → Ser	+	Divergent	+	+
49136/49137	211/212	— → CTC	— → Leu	−	Divergent	+	+
49170	223	C → T	Leu → Leu	+	Divergent	+	+
49214	237	A → C	Glu → Asp	+	Divergent	+	−
49449	316	C → A	His → Asn	−	Protein binding	+	+
49603	367	C → T	Pro → Leu	−	Nonessential	+	−
49613	370	T → A	Pro → Pro	+	Nonessential	+	+
49754	417	G → A	Thr → Thr	+	Nonessential	+	+
49913	470	A → T	Ser → Ser	+	Acidic	+	+
49917–49919	472	GAA → —	Glu → —	−	Acidic	+	−
49923	474	T → C	Tyr → Asn	−	Acidic	−	+
49930	476	A → G	Glu → Gly	−	Nuclear localization	+	+
49956	485	T → C	Ser → Pro	+	Nuclear localization	+	+

[a] —, deleted.

24, 25), and clone 1 demonstrated 15 bands, indicating the presence of 16 repeat units in IR2.

However, both clone 2 and clone 3 demonstrated two distinct ladders each, with 9 and 15 bands and 12 and 23 bands, respectively. The double ladder sets in the *Bam*HI H fragments of these two clones indicated the presence in each clone of two separate IR2 regions, separated by a unique sequence. In this partial-digestion analysis, the lower ladder represented the IR2 region with the most repeats, whereas the upper ladder represented the total number of 125-bp repeat units present within both IR2 regions. Thus, clone 2 contained IR2 regions of 10 and 6 repeat units, and clone 3 contained IR2 regions of 13 and 11 repeat units. These data indicated that some of the *Bam*HI H fragment size variation in HLP was due to homologous recombination in the IR2 repeat regions. Duplication of the IR2 regions in clones 2 and 3 indicated that additional rearrangements had occurred in the unique sequences of the *Bam*HI H fragment.

Recombination and rearrangement of the *Bam*HI H fragment. To characterize the rearrangements in the unique sequences, the *Bam*HI H fragments from clones 1 to 3 were mapped with three restriction enzymes that conveniently cut at sequences between EBNA-2 and IR2 and within sequences of the BHLF1 gene and the lytic origin of viral replication (oriLyt) (Fig. 4). Replicates of each digest were hybridized to probes 2A-H, IR2, and H (Fig. 1). Each of the three enzyme digestions generated subfragments of the three clones that were compared with those of the B95-8 *Bam*HI H fragment (Fig. 4), thereby allowing derivation of the organizational structure of the three HLP *Bam*HI H fragments. Clone 1 (Fig. 5) demonstrated the typical *Bam*HI H fragment structure, differing from B95-8 in the total number of 125-bp repeat elements in IR2 and in the absence of the *Bsa*MI site in the U3 region to the right of IR2. Clones 2 and 3 departed markedly from the typical *Bam*HI H fragment structure but differed from each other only in the sizes of their IR2 regions (Fig. 6). As predicted by the *Not*I digestions, they each contained duplicate IR2 regions, separated by 3 kb of unique sequence derived from the *Bam*HI H fragment.

To further define the structure of clones 2 and 3, the two large *Bsa*MI fragments containing the duplicated sequences were subcloned. Repeated *Not*I partial-digestion analysis of the two *Bsa*MI subclones of clone 2 confirmed the presence of 10 and 6 repeat units in the IR2 regions of clone 2 (data not shown). Sequence analysis confirmed the structural organization of clones 2 and 3, including the order of the IR2 regions and the recombination site between the U3 sequence to the right of oriLyt and the EBNA-2 sequence (Fig. 6). In both clones, nonhomologous recombination occurred, linking bp 54208 in U3 to bp 49802 in the carboxy-terminal end of EBNA-2. This event resulted in the complete duplication of the BHLF1 gene and the sequences of oriLyt.

The *Bam*HI H fragments of clones 1 to 3 each lost the *Bsa*MI site in BHLF1 to the right of IR2 but differed in the number of repeat units within IR2 (Fig. 5 and 6). Therefore, the unique DNA sequences from U2 and U3 immediately surrounding IR2 were examined in the three clones. All three clones contained identical sequences around the U2-IR2 junction, matching B95-8 and including the first 38-bp incomplete repeat unit of IR2. Examination of the sequence around the IR2-U3 junction in clone 1 demonstrated a 225-bp in-frame deletion linking the 25th base pair of the last repeat of IR2 with bp 52240 of U3. This recombination took place at an 8-bp sequence of homology between the two sites: CCGCCGGT. Similarly, at all four IR2-U3 junctions within clones 2 and 3, there was a 117-bp in-frame deletion linking the 105th base pair of the last repeat of IR2 with bp 52212 of U3. The

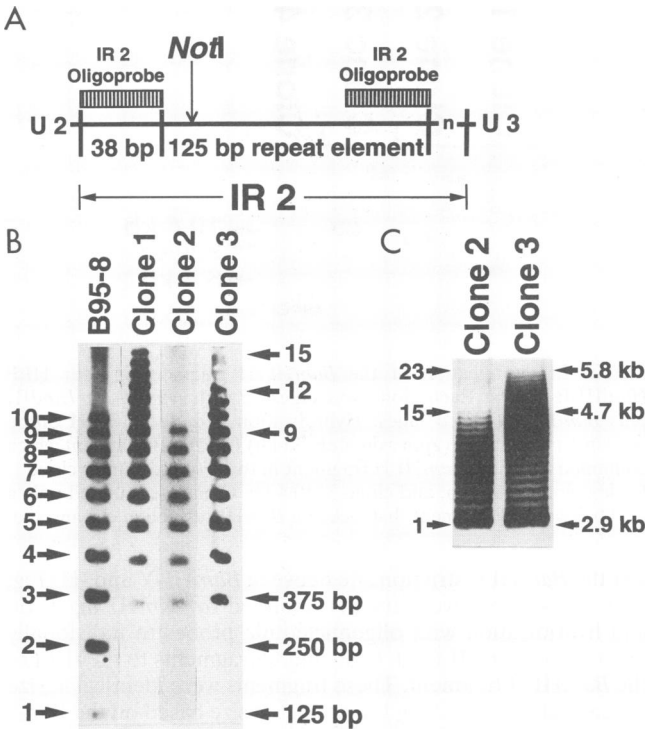

FIG. 3. *Not*I partial digestion analysis of the *Bam*HI H fragment subclones. (A) Partial digestion with *Not*I produced ladders of bands differing in size by multiples of 125 bp. Flanked by unique sequences U2 and U3, IR2 in the B95-8 strain contains a 38-bp incomplete copy of the repeat unit before the series of complete units. Given the location of the *Not*I restriction site and the hybridization domain of the oligoprobe, the number of copies of the repeat unit is one more than the maximum number of bands in the ladder. (B) B95-8, with 11 copies of the repeat unit, demonstrated 10 bands in this assay. The ladder in clone 1 contained 15 bands, indicating 16 copies. Clones 2 and 3 demonstrated ladders of 9 and 12 bands, respectively. (C) Clones 2 and 3 each contained a second ladder of bands, indicating two separate copies of the IR2 repeat region. In clone 2, the second ladder of 2.9- to 4.7-kb fragments contained 15 bands, indicating a total of 16 copies in both IR2 regions in this clone. In clone 3, the second ladder of 2.9- to 5.8-kb fragments contained 23 bands, indicating a total of 24 copies in both IR2 regions in this clone.

recombinations in clones 2 and 3 took place at a 7-bp sequence of homology between the two sites: CTGGGGC. These data indicated that homologous recombination of the IR2 sequences may also include nearby unique sequences and suggested that some degree of site specificity is involved.

To further evaluate variation among clones 1 to 3, the sequences of the partially duplicated EBNA-2 genes from clones 2 and 3 were examined. Codon 472 (GAA) that had been deleted from the complete EBNA-2 sequences of clones 1 to 3 (Table 2) was present in the partially duplicated EBNA-2 sequences of clones 2 and 3. Thus, the deletion of codon 472 represented another intrastrain recombination event that occurred independently of the U3–EBNA-2 recombination and the deletions at the IR2-U3 junction. These structural and sequence variations among clones 1 to 3 (Fig. 7) indicated that a complex series of recombination events gave rise to several different intrastrain viral variants, accounting for much of the *Bam*HI H-fragment heterogeneity in this HLP.

***Bam*HI H-fragment recombination in permissive infection in vivo.** To determine if *Bam*HI-H duplications occurred in

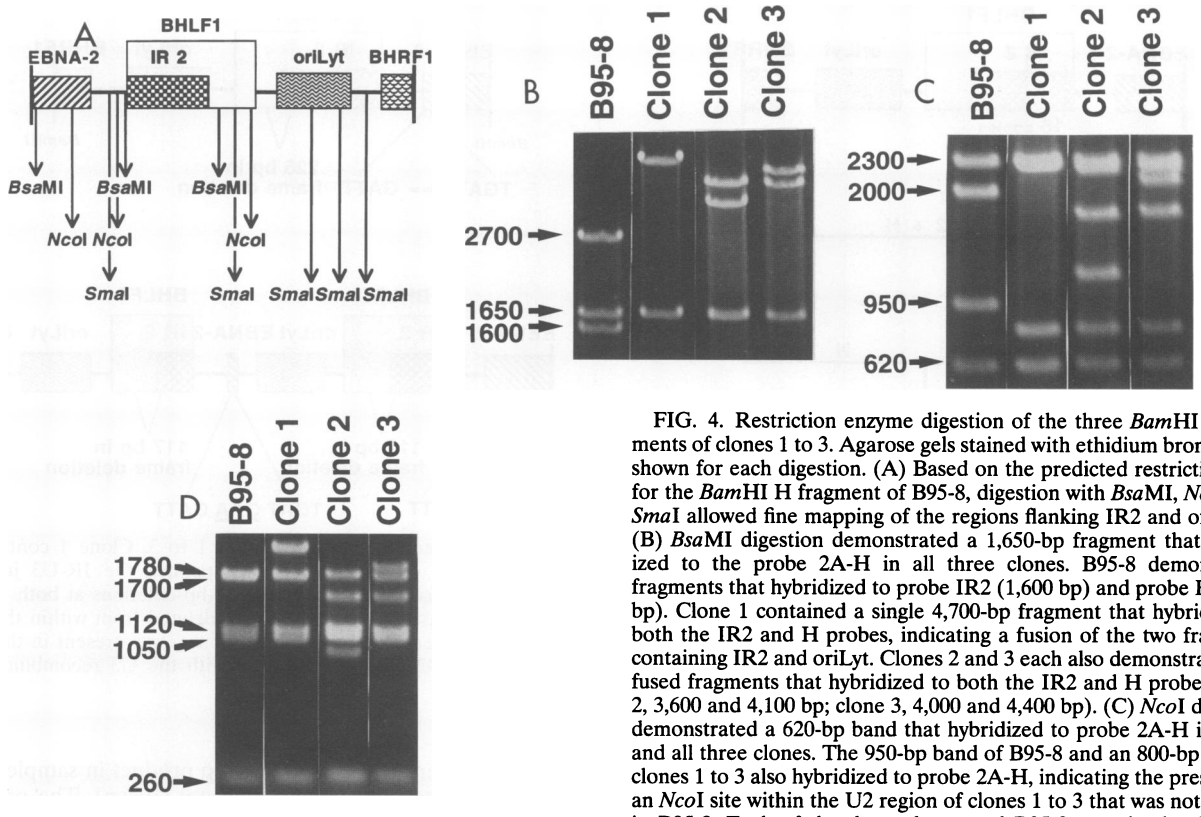

FIG. 4. Restriction enzyme digestion of the three *Bam*HI H fragments of clones 1 to 3. Agarose gels stained with ethidium bromide are shown for each digestion. (A) Based on the predicted restriction sites for the *Bam*HI H fragment of B95-8, digestion with *Bsa*MI, *Nco*I, and *Sma*I allowed fine mapping of the regions flanking IR2 and of oriLyt. (B) *Bsa*MI digestion demonstrated a 1,650-bp fragment that hybridized to the probe 2A-H in all three clones. B95-8 demonstrated fragments that hybridized to probe IR2 (1,600 bp) and probe H (2,700 bp). Clone 1 contained a single 4,700-bp fragment that hybridized to both the IR2 and H probes, indicating a fusion of the two fragments containing IR2 and oriLyt. Clones 2 and 3 each also demonstrated two fused fragments that hybridized to both the IR2 and H probes (clone 2, 3,600 and 4,100 bp; clone 3, 4,000 and 4,400 bp). (C) *Nco*I digestion demonstrated a 620-bp band that hybridized to probe 2A-H in B95-8 and all three clones. The 950-bp band of B95-8 and an 800-bp band in clones 1 to 3 also hybridized to probe 2A-H, indicating the presence of an *Nco*I site within the U2 region of clones 1 to 3 that was not present in B95-8. Each of the three clones and B95-8 contained a 2,300-bp fragment that hybridized to probe H. Clones 2 and 3 each contained an additional 2,200-bp fragment that also hybridized to probe H. Fragments that hybridized to probe IR2 were detected in B95-8 (2,000 bp), clone 1 (2,250 bp), clone 2 (1,150 and 1,650 bp), and clone 3 (1,650 and 2,050 bp). (D) *Sma*I digestion demonstrated fragments in B95-8 and clone 1 of 260, 1,050, and 1,120 bp that hybridized with probe H. These same three fragments were detected in clones 2 and 3 at double the intensity of fluorescence, indicating duplication of these fragments in these two clones. B95-8 and all three clones also contained a 1,700-bp fragment that hybridized to probe 2A-H. Fragments that hybridized to probe IR2 were detected in B95-8 (1,780 bp), clone 1 (2,150 bp), clone 2 (940 and 1,500 bp), and clone 3 (1,525 and 1,850 bp).

other HLP samples, PCR primers that flanked the U3–EBNA-2 junction site were chosen. Amplification of DNA from clone 2, and from the original HLP specimen from which clone 2 was obtained, generated the predicted product size of 442 bp (Fig. 8). HLP specimens from several additional patients were examined by PCR amplification with these primers. Notably, the PCR products detected in these HLP specimens ranged in size from 442 to 1,600 bp (Fig. 8). The 442-bp PCR product did not hybridize to the probe 2A-H because of the small amount of EBNA-2 sequence between the PCR primer and the recombination junction with the U3 sequence. Many but not all PCR products larger than 442 bp did hybridize to probe 2A-H, indicating a greater portion of EBNA-2 sequence in these products. Similarly, many but not all PCR products hybridized with probe H. These data suggested variation among and within HLP specimens for the site of recombination in both the EBNA-2 and U3 sequences. Thus, the EBNA-2–U3 recombinations in HLP were quite variable and both nonhomologous and non-site specific. In three additional patients, some of the PCR product bands were detected in sequential biopsy samples from the same patient (data not shown), suggesting that some of the viral variants replicated and persisted. These data are consistent with Southern blot hybridizations demonstrating large *Bam*HI H fragments (8 to 12 kb) persisting within serial HLP biopsy samples from the same lesion (55).

Defective EBV and detection in permissive infection in vivo. Clone 4 contained a 2.2-kb insert with the EBNA-2 sequence that lacked the expected *Bam*HI site between the *Bam*HI Y and H fragments (Fig. 2). Subcloning and sequencing clone 4 demonstrated recombination between the *Bam*HI Y portion of EBNA-2 and distant EBV sequences from the *Bam*HI S region. The EBNA-2 sequence in clone 4 matched identically with that in clones 1 to 3. Thus, clone 4 represented another variant of the same original strain, arising through recombination within the HLP lesion. The recombination took place within the polyproline region of the EBNA-2 gene, with bp 48737 of EBNA-2 linked to bp 85777 of *Bam*HI-S (Fig. 9). Similar to the IR2-U3 recombinations, this recombination site also shared an 8-bp sequence homology between *Bam*HI-Y and BamHI-S: CTCCACCA. As a result of this recombination, the EBNA-2 open reading frame was truncated by a stop codon 24 bp beyond the recombination site. The BSLF1 gene was also truncated by a stop codon in *Bam*HI-Y, preserving less than half of its potential amino acid sequence (Fig. 9).

An RNA probe synthesized from the entire insert of clone 4 was hybridized by Southern blot back to the HLP DNA from which it was cloned. Both the standard *Bam*HI Y and *Bam*HI S fragments were easily detected by this probe, but a 2.2-kb fragment was not detected (data not shown), indicating a low copy number of the EBNA-2–*Bam*HI-S recombinant molecule within the HLP lesion.

To determine if this EBNA-2–*Bam*HI-S recombination had occurred in other HLP lesions, PCR primers that flanked the

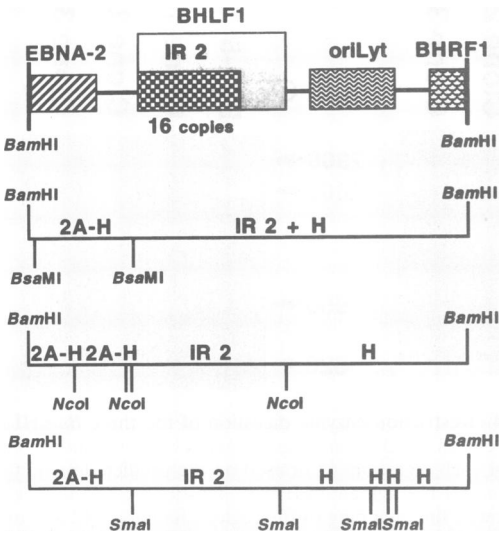

FIG. 5. Organizational structure of the *Bam*HI H fragment of clone 1. For each restriction enzyme digestion (*Bsa*MI, *Nco*I, and *Sma*I), hybridization with probes 2A-H, IR2, and H identified the fragments as shown. Clone 1 demonstrated the prototype *Bam*HI-H structure, differing from B95-8 only in the number of repeat elements present in IR2 and the loss of the *Bsa*MI site in BHLF1.

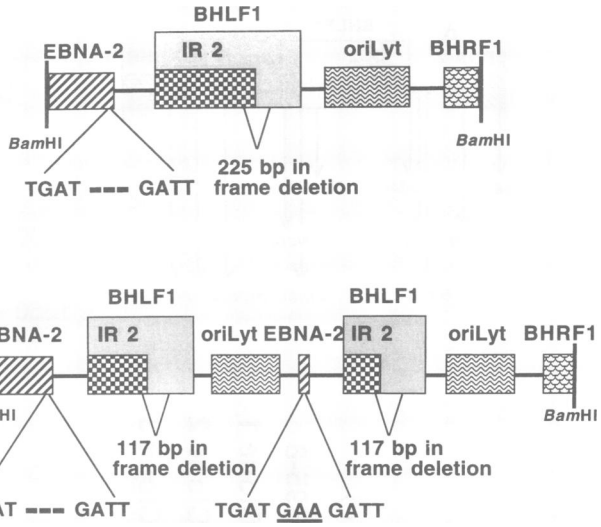

FIG. 7. Sequence analysis of clones 1 to 3. Clone 1 contained a 225-bp deletion within BHLF1 that spanned the IR-U3 junction. Clones 2 and 3 contained identical 117-bp deletions at both of their IR2-U3 junctions. Codon 472 (GAA), deleted from within the complete EBNA-2 sequence of all three clones, was present in the small piece of EBNA-2 sequence involved with the U3 recombination of clones 2 and 3.

EBNA-2–*Bam*HI-S junction site were chosen. Amplification of DNA from clone 4, and from the original HLP lesion from which clone 4 was obtained, generated the predicted product size of 477 bp (Fig. 10). PCR amplification of additional HLP biopsy specimens produced a 477-bp product in samples from 7 of 15 patients (Fig. 10 and data not shown). The products hybridized with both probes 2A-Y and S. In three cases, the EBNA-2–*Bam*HI-S variants were detected in sequential HLP biopsy specimens from the same patient (Fig. 10 and data not shown). Unlike the U3–EBNA-2 recombinations, the EBNA-2–*Bam*HI-S recombination demonstrated no heterogeneity in product size, suggesting that CTCCACCA may constitute a site-specific sequence for EBV recombination generating defective EBV in permissive infection.

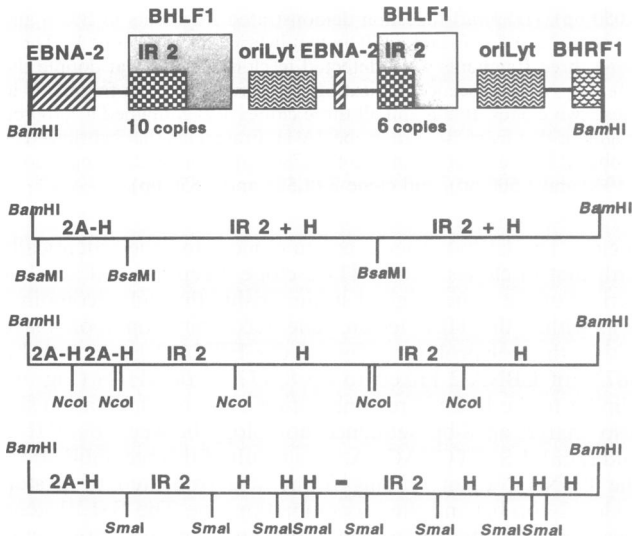

FIG. 6. Organizational structure of the *Bam*HI H fragments of clones 2 and 3. For each restriction enzyme digestion (*Bsa*MI, *Nco*I, and *Sma*I), hybridization with probes 2A-H, IR2, and H identified the fragments as shown. As predicted by the *Not*I digestion, clones 2 and 3 contained a duplication of the IR2 region arising from a recombination between U3 sequence to the right of oriLyt and EBNA-2 sequence. This recombination also resulted in a complete duplication of oriLyt and BHLF1. The *Bsa*MI site was absent from both copies of BHLF1. Clones 2 and 3 differed only in the number of repeat units present in each copy of IR2. Clone 2 contained 10 and 6 copies, while clone 3 contained 13 and 11 copies.

DISCUSSION

Accurate identification of EBV types, strains, and recombinant variants is required for the study of the epidemiology and natural history of EBV infection. EBV-1 and -2 are distinguished by regions of sequence divergence within several EBNA genes that differentially hybridize to type-specific probes (1, 13, 30, 46, 49, 50, 55). In LCLs, EBV strains have been identified by restriction fragment length polymorphism analysis of EBV fragments containing internal repetitive sequences. Each strain is characterized by unique and stable differences in the number of repeat units present, even after induction of lytic replication (6, 12, 14, 24, 25). Similarly, many of the EBNA proteins expressed in LCLs vary in size among strains because of variable repeat regions within their coding sequences, resulting in a unique EBNotype signature by Western blot (immunoblot) for each strain (17). Based on these observations in LCLs, epidemiological studies of EBV isolates from natural infections have relied on restriction fragment length polymorphism and EBNotype analysis to determine multiplicity of infection and to monitor strains through time and through sequential infections in both healthy patients (19, 28, 35–37, 49, 56) and immunocompromised patients (7, 20–22, 27, 28, 49, 55).

However, homologous recombination within repetitive sequences frequently generates variation (3, 38), and homolo-

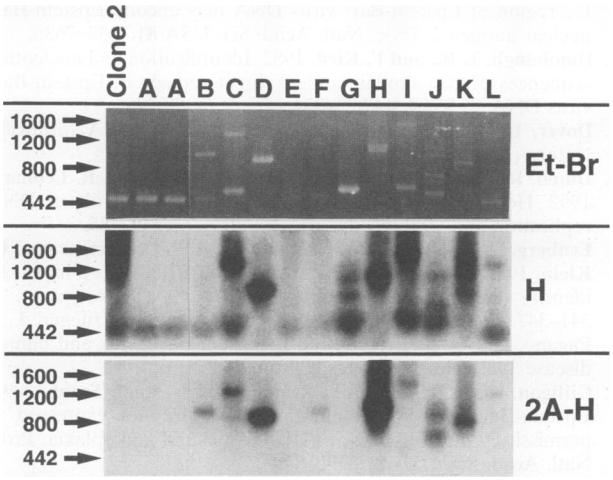

FIG. 8. PCR amplification of the U3–EBNA-2 recombination junction from the HLP samples of 12 patients. The PCR products were amplified with oligonucleotide primers 8 and 12 and are shown stained with ethidium bromide (Et-Br) and after hybridization with probes H and 2A-H. Clone 2 was a positive control and demonstrated the predicted 442-bp product. Patient A, from whom clone 2 was obtained, also demonstrated the 442-bp product from biopsy samples taken at two different time points. Patients B to L demonstrated a heterogeneous variety of PCR product sizes.

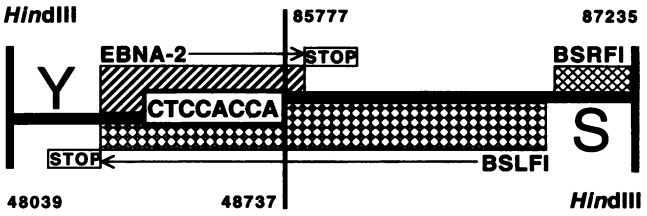

FIG. 9. Sequence analysis of the 2.2-kb *Hin*dIII insert of clone 4. Recombination between the *Bam*HI Y portion of EBNA-2 and sequences of the *Bam*HI S region linked bp 48737 to bp 85777 at an 8-bp sequence homology between *Bam*HI-Y and *Bam*HI-S: CTCCACCA. The EBNA-2 open reading frame was truncated by a stop codon 24 bp beyond the recombination site, and the BSLF1 open reading frame was truncated by a stop codon in *Bam*HI-Y.

gous recombination in EBV is closely linked with productive replication (54). In the present study of HLP, homologous recombination within the IR2 sequences contributed to the size variation of the *Bam*HI H fragment, indicating that *Bam*HI-H variation does not actually distinguish strains. Similarly, repetitive sequences within the latent membrane protein 1 gene recombine during replication in HLP to generate intrastrain variation (41). By analogy, other repetitive sequences within the *Bam*HI K and B1 fragments and the EBNA genes may also experience intrastrain homologous recombination (55). Thus, in HLP, intrastrain recombination would obscure the identification of EBV strains based on variation in these fragments or gene products. Even without HLP, immunocompromised patients demonstrate continuous, high-level EBV replication and shedding of virions in the oropharynx (2, 8, 52). Given this degree of viral replication, homologous recombination could produce EBV intrastrain variation in these patients as well. Thus, in permissive epithelial infections of immunocompromised patients, EBV strains could be more accurately distinguished by sequence identity or divergence within known regions of genetic strain variation, such as the EBNA-2 or latent membrane protein 1 gene (11, 41).

In the present study, variation of the 125-bp repeat units in IR2 resulted from unequal pairing of homologous DNA strands with subsequent crossover recombination. However, the recombination events at the IR2-U3 junction involved short (7- to 8-bp) sequence homologies, suggesting some degree of site specificity for the recombination event. In vitro recombination studies have shown for mammalian cells that greater than 200 bp of homology are required for efficient homologous recombination but that 14 to 25 bp are adequate, if very inefficient (4, 33, 45). In herpes simplex virus type 1, random double-stranded DNA breaks in the semirepetitive *a* sequences and homologous recombination are responsible for genome isomerization during viral replication and packaging (16, 48). Consequently, several "illegitimate" recombination events generating defective viral variants involving the *a* sequences with other regions of the herpes simplex type genome having short sequence homologies of 10 to 12 bp have been previously described (29, 42). A similar illegitimate mechanism may account for the two different IR2-U3 deletion patterns demonstrated for clone 1 (225 bp) and clones 2 and 3 (117 bp).

The EBNA-2–*Bam*HI-S recombination also involved an 8-bp sequence homology at the recombination site. However, the independent occurrence of this specific recombination in multiple HLP specimens suggests a site-specifically mediated recombination mechanism. The CTCCACCA recombination site shares features with many of the published recombination recognition sequences of prokaryotic and eukaryotic systems, including an 8-bp length and a high G+C content (15).

The P3HR-1 cell line, originally selected for high virus production, is an example in which nonhomologous recombination between normally distant regions of the EBV genome generated defective molecules in lymphocytes (9, 39). These molecules, which include juxtapositions of *Bam*HI-W with *Bam*HI-Z (WZhet), have been shown to be responsible for the unusual biologic properties of the P3HR-1 strain (40). The exact sequences involved at most of the P3HR-1 recombination sites have not been determined. However, the data presented here indicate that nonhomologous, non-site-specific recombination frequently occurs between nonrepetitive DNA sequences during EBV replication in vivo. Similar to the U3–EBNA-2 recombinations, WZhet DNA and deletions of the EBNA-2 gene detected in HLP specimens also demonstrate variability in their sites of nonhomologous recombination (26, 43, 51).

In addition to the presence of defective genomes, the P3HR-1 strain is also deleted for the EBNA-2 gene, and viral

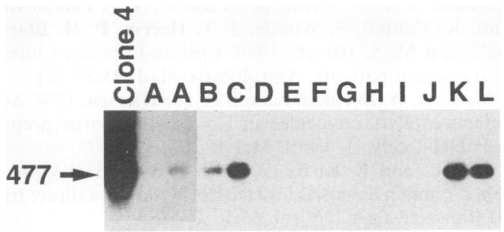

FIG. 10. PCR amplification of the EBNA-2–*Bam*HI-S recombination junction from the HLP samples of 12 patients. The PCR products were amplified with oligonucleotide primers 1 and 13 and are shown after hybridization with probe 2A-Y. Clone 4 was a positive control and demonstrated the predicted 477-bp product. The 477-bp product was detected at two different time points in patient A, from whom clone 4 was obtained, and in patients B, C, K, and L.

variants defective for EBNA-2 are commonly detected in HLP (51, 55). EBNA-2 is an important regulatory gene early in B-lymphocyte infection and is essential for B-lymphocyte transformation (11, 44). Thus, the viral variants with the EBNA-2–*Bam*HI-S recombination would be incapable of establishing latency and immortalizing B lymphocytes (10, 11, 31, 32). The role of EBNA-2 in epithelial infection is less clear. Latently infected nasopharyngeal carcinoma cells do not express EBNA-2 (57), and EBNA-2 is not required for productive replication in the oropharynx (51). It is unclear whether the EBNA-2 gene product is expressed in HLP (47, 53). However, the prevalence and persistence of the EBNA-2–*Bam*HI-S recombinant and other EBNA-2-defective variants in HLP (51, 55) suggest a selective advantage for these viral variants in a permissive environment. Thus, the role of the defective variants in the pathogenesis of HLP merits further investigation.

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