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Epstein-Barr Virus DNA
XII. A Variable Region of the Epstein-Barr Virus Genome Is Included in the P3HR-1 Deletion

WALTER KING, TIMOTHY DAMBAUGH, MARK HELLER, JULIA DOWLING, AND ELLIOTT KIEFF*

Departments of Medicine, Microbiology, and Virology, The University of Chicago, Chicago, Illinois 60637

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The P3HR-1 subclone of Jijoye differs from Jijoye and from other Epstein-Barr virus (EBV)-infected cell lines in that the virus produced by P3HR-1 cultures lacks the ability to growth-transform normal B lymphocytes (Heston et al., Nature (London) **295**:160–163, 1982; Miller et al., J. Virol. **18**:1071–1080, 1976; Miller et al., Proc. Natl. Acad. Sci. U.S.A. **71**:4006–4010, 1974; Ragona et al., Virology **101**:553–557, 1980). The P3HR-1 virus was known to be deleted for a region which encodes RNA in latently infected, growth-transformed cells (Bornkamm et al., J. Virol. **35**:603–618, 1980; Heller et al., J. Virol. **38**:632–648, 1981; King et al., J. Virol. **36**:506–518, 1980; Raab-Traub et al., J. Virol. **27**:388–398, 1978; van Santen et al., Proc. Natl. Acad. Sci. U.S.A. **78**:1930–1934, 1980). This deletion is now more precisely defined. The P3HR-1 genome contains less than 170 base pairs (and possibly none) of the 3,300-base pair U2 region of EBV DNA and is also lacking IR2 (a 123-base pair repeat which is the right boundary of U2). A surprising finding is that EBV isolates vary in part of the U2 region. Two transforming EB viruses, AG876 and Jijoye, are deleted for part of the U2 region including most or all of a fragment, *Hin*fI-c, which encodes part of one of the three more abundant cytoplasmic polyadenylated RNAs of growth-transformed cells (King et al., J. Virol. **36**:506–518, 1980; King et al., J. Virol. **38**:649–660, 1981; van Santen et al., Proc. Natl. Acad. Sci. U.S.A. **78**:1930–1934).

The Epstein-Barr virus (EBV) genome is approximately 175,000 base pairs (bp) (for review, see references 5 and 6; E. Kieff, T. Dambaugh, M. Heller, W. King, A. Cheung, V. van Santen, M. Hummel, C. Beisel, S. Fennewald, K. Hennessy, and T. Heineman, J. Infect. Dis., in press). The linear double-stranded molecule has single-strand interruptions (13, 31). Direct tandem repeats of a 500-bp sequence, TR, are at both termini (4, 11, 13, 24). Tandem repeats of a 3,071-bp sequence, IR, divide the genome into a 15,000-bp unique region, US, and a 150,000-bp-long unique region, UL (4–6, 12; A. Cheung and E. Kieff, J. Virol., in press). Three other repeat sequences have recently been discovered in UL, a 123-bp tandem direct repeat (IR2), a simple direct repeat (IR3), and a 103-bp tandem direct repeat (IR4) (for review, see Kieff et al., in press). These repeats divide UL into unique domains U2, U3, U4, and U5 (Fig. 1; Kieff et al., in press).

The IR1-U2-IR2 region is one of three segments of EBV DNA which seem important in latent infection and growth transformation. One strain of EBV, P3HR-1, derived by cloning a culture of Jijoye Burkitt tumor cells (21), differs from Jijoye and all other isolates of EBV in that it lacks the ability to transform human B lymphocytes (27, 28, 34). The DNA of the P3HR-1 virus differs from other EBV DNAs in the deletion of part of U2 (2, 15, 22, 33). The P3HR-1 deletion includes the part of U2 from the *Hin*dIII site in *Bam*HI-X through *Hin*fI fragments c and d of *Bam*HI-H (Fig. 1; 15, 22). The size of the P3HR-1 *Bam*HI fragment formed by the joining of IR1 sequences presumably from the left part of *Bam*HI-X to the right portion of *Bam*HI-H is about 4,000 bp (15). The new P3HR-1 fragment is therefore about 4,000 bp smaller than the fragment(s) containing *Bam*HI-X and -H sequences in other EBV isolates (15). Other evidence that the deleted DNA is important in growth transformation comes from studies of EBV RNA in latently infected, growth-transformed, or Burkitt tumor cells (22, 23, 30, 42). cDNA to cytoplasmic or polyribosomal polyadenylated RNA hybridizes to IR1 and U2, including sequences to the right of the *Hin*dIII site in *Bam*HI-X and *Hin*fI fragments c and d of *Bam*HI-H (Fig. 1, 42). *Bam*HI-X and *Bam*HI-H

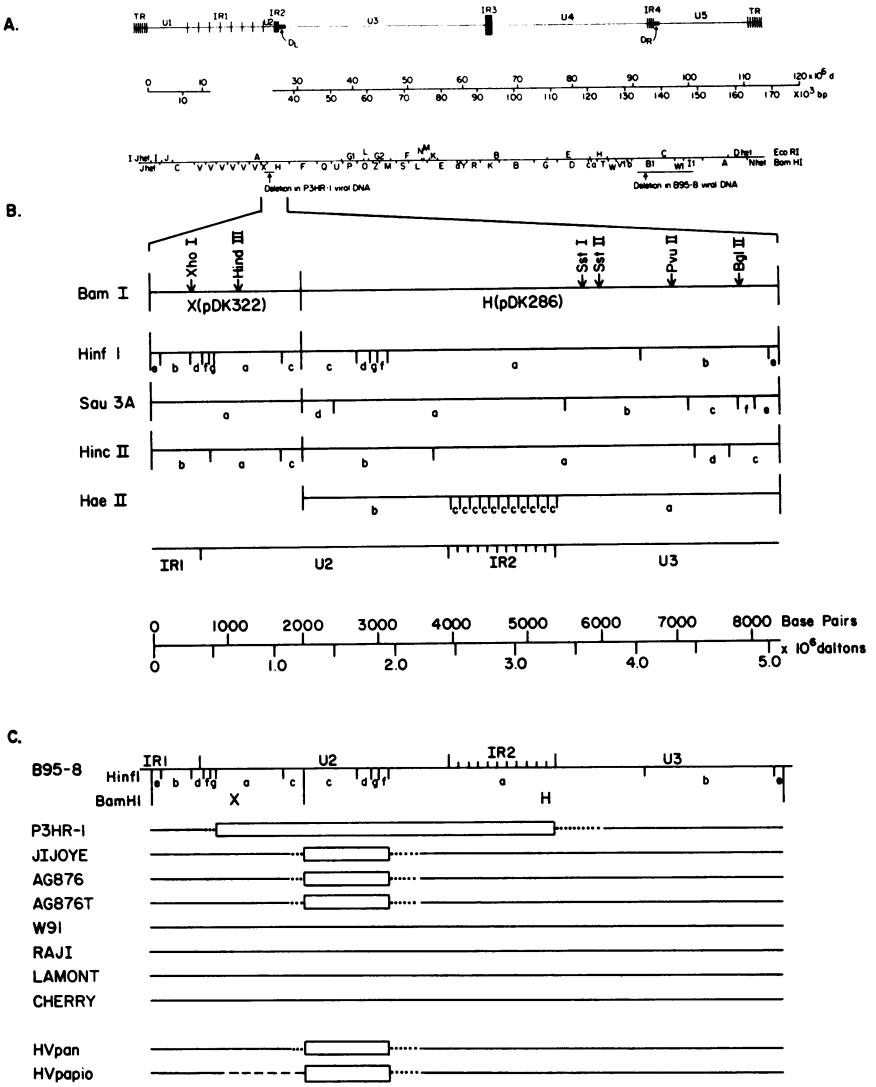

FIG. 1. EBV DNA maps and summary of results. (A) *Eco*RI and *Bam*HI maps (3, 33) and overall organization (Kieff et al., in press) of EBV DNA. TR, 500-bp tandem direct repeats at the termini. Regions of unique complexity, designated U1 through U5, are separated by tandem internal direct repeats IR1 (3,071 bp), IR2 (123 bp), IR3 (about 700 bp of a simple sequence array), and IR4 (103 bp). (B) *Hin*fI, *Sau*3A, and *Hin*cII maps of *Bam*HI fragments X and H from recombinant plasmids pDF322 and pDK286, respectively, and the *Hae*II restriction endonuclease map of *Bam*HI fragment H. *Xho*I, *Hin*dIII, *Sst*I, *Sst*II, *Pvu*II, and *Bgl*II sites are also indicated. *Hae*II cuts once in IR2, yielding *Hae*II fragment c. (C) Extent of conservation of *Bam*HI-X and -H among EBV, HV pan and HV papio DNAs. The open bar indicates no homology to prototype B95-8 sequences. The dotted lines indicate regions of uncertainty. The dashed line in HV papio DNA indicates an area of weak homology. In P3HR-1 DNA, the deletion results in a fragment (*Bam*HI-X-H) which contains the right end of IR1 and the left end of U3 and is 4,000 bp smaller than B95-8 *Bam*HI-H and -X. In AG876 and Jijoye DNA, the *Bam*HI site between *Bam*HI-X and -H is missing, resulting in fragment D1, which is equal in size to *Bam*HI-X and -H.

hybridize to a 3-kilobase cytoplasmic polyadenylated RNA in latently infected, growth-transformed cells (42).

The initial objective of this work was to more precisely define the ends of the P3HR-1 deletion and to show that the parent virus, Jijoye, which is competent for growth transformation of lymphocytes, has the DNA for which P3HR-1 is deleted. Surprisingly, Jijoye was found to share part of the P3HR-1 deletion. Analysis of five other EBV isolates revealed that one isolate, AG876, is also deleted for the same B95-8 sequences as Jijoye, whereas four isolates are similar to B95-8 in this region. To evaluate the

biological significance of the AG876 deletion, cord blood lymphocytes were infected with AG876 virus. A growth-transformed cell line was established and found to contain DNA with the characteristic AG876-Jijoye deletion.

MATERIALS AND METHODS

Cell culture. The AG876 transformant cell line, AG876T, was established by infection of neonatal umbilical cord lymphocytes with supernatant medium from AG876 cells (22). The outgrowth of lymphoblastoid cells was evident after 6 weeks. All of the cells in the resultant AG876T line were positive for the EBV nuclear antigen (35). Cultures of EBV-, herpesvirus (HV) pan-, or HV papio-infected lymphoblastoid cell lines were maintained as previously described in complete medium consisting of RPMI 1640 medium (GIBCO Laboratories) with 10% fetal calf serum (K. C. Biologicals) (11). The AG876T cells were harvested for EBV DNA after 4 months of continuous culture.

Preparation of viral DNAs. DNAs from B95-8 (28), P3HR-1 (21), AG876 (29), or HV papio (16) cultures were extracted from supernatant virus (11). Viral DNAs from W91 (11, 27), Jijoye (34), and nonpermissive Raji, Lamont, Cherry (32), and HV pan (17) cultures and the AG876T-infected-cord blood line were enriched by three successive cycles of isopycnic centrifugation of cellular DNA and selection of the high-density fraction (15). The Cherry and Lamont lymphoblastoid cell lines are of American origin and are EBV nuclear antigen positive (15, 33; W. Henle and G. Henle, personal communication).

Recombinant plasmid DNAs pDF322 and pDK286, containing B95-8 *Bam*HI fragments X and H, respectively (4), or EBV DNAs were digested with restriction enzymes (Bethesda Research Laboratories and New England Biolabs) under the conditions suggested by the manufacturers and fractionated by agarose gel electrophoresis. For the isolation of EBV insert DNA, the appropriate fragment was electroeluted against a dialysis membrane. After removal of the running buffer, the DNA was washed from the membrane with a small volume of buffer (0.01 M Tris–0.001 M EDTA, pH 7.4) and precipitated with 2 volumes of ethanol after addition of NaCl to 0.1 M.

Mapping of restriction endonuclease sites in *Bam*HI-X (pDF322) and *Bam*HI-H (pDK286). The sizes of restriction endonuclease fragments were determined by electrophoresis in agarose gels relative to fragments of pBR322 (40), φX174 (37), and lambda (41) DNAs. Restriction endonuclease maps were derived by comparison of the sizes of fragments produced after digestion with one, two, or three restriction endonucleases. For sequential restriction endonuclease digestions, previous restriction enzymes were inactivated, and the buffer conditions were adjusted to those recommended by the manufacturer for the next enzyme. Restriction endonuclease sites were confirmed by analysis of partial restriction endonuclease digestion products (38). pDF322 DNA was 5′ end labeled at the *Xho*I site after removal of the terminal phosphate with bacterial alkaline phosphatase (26). pDK286 DNA was 3′ end labeled at the *Sst*I site, using terminal transferase and ^{32}P-labeled cordycepin (7, 26).

Radioactive labeling of DNA and hybridization to nitrocellulose filters. Approximately 5 nmol of the 123-bp *Hae*II fragment c was radiolabeled with [^{32}P]dCTP (Amersham Corp.) and 50 U of terminal deoxynucleotidyl transferase from calf intestine (P-L Biochemicals, Inc.) (7). All other DNAs were radiolabeled by nick translation with DNA polymerase I from *Escherichia coli* (Boehringer Mannheim Corp.) (36). All radiolabeled DNAs were hybridized to nitrocellulose blots as previously described (15, 39).

RESULTS

Maps of fragments of B95-8 *Bam*HI-X and -H. Restriction endonuclease maps (Fig. 1B) were derived for B95-8 fragments of *Bam*HI-X and -H as described in Materials and Methods. The presence of tandem repeats of a 123-bp sequence in *Bam*HI-H, designated IR2, is indicated by the following: (i) the size of the *Bam*HI-H insert in pDK286 varies between 3×10^6 and 4×10^6 d in a step ladder of fragments differing by 123-bp increments, (ii) a partial *Hae*II digest of end-labeled pDK286 yields a step ladder of fragments differing by 123 bp, (iii) a complete *Hae*II digest of pDK286 yields a single supramolar 123-bp fragment, and (iv) the end-labeled, 123-bp *Hae*II fragment is a unique nucleotide sequence (T. Dambaugh and E. Kieff, manuscript in preparation).

Hybridization of EBV DNAs to fragments of B95-8 *Bam*HI-X and -H. Previous analyses of the *Bam*HI fragments of EBV DNAs indicate that W91, Raji, Cherry, and Lamont have *Bam*HI fragments similar in size and homology to B95-8 *Bam*HI-X and -H (15), that AG876 has a single fragment (designated *Bam*HI-D1) homologous to both *Bam*HI-H and -X which is equal in size to the combined fragments (15), and that P3HR-1 has a single fragment consisting of part of IR1 (presumably from the left part of *Bam*HI-X) joined to the right part of *Bam*HI-H and designated *Bam*HI-X-H (15). P3HR-1 *Bam*HI-X-H is 4,000 bp smaller than the size of B95-8 *Bam*HI fragments X and H combined.

To map the boundaries of the P3HR-1 deletion and to show that other EBV DNAs, including Jijoye, have these sequences, labeled EBV DNAs were hybridized to Southern blots of *Hin*fI fragments of B95-8 *Bam*HI-X and -H (Fig. 2 and 3, respectively). Jijoye, W91, Raji, Cherry, Lamont, and AG876 DNAs hybridized to all of the *Hin*fI fragments of *Bam*HI-X (Fig. 2). P3HR-1 DNA hybridized to *Hin*fI fragments b and e of *Bam*HI-X, relatively less to fragment d, and not at all to fragment a or c (Fig. 2). *Hin*fI fragment d is 150 bp and consists of approximately 140 bp of IR1 and 10 bp of U2 (3). The lack of homology of P3HR-1 DNA to *Hin*fI-a indicates that the P3HR-1 deletion begins to the left of *Hin*fI fragment a and that P3HR-1 contains less than 170 bp (size of *Hin*fI-f and -g

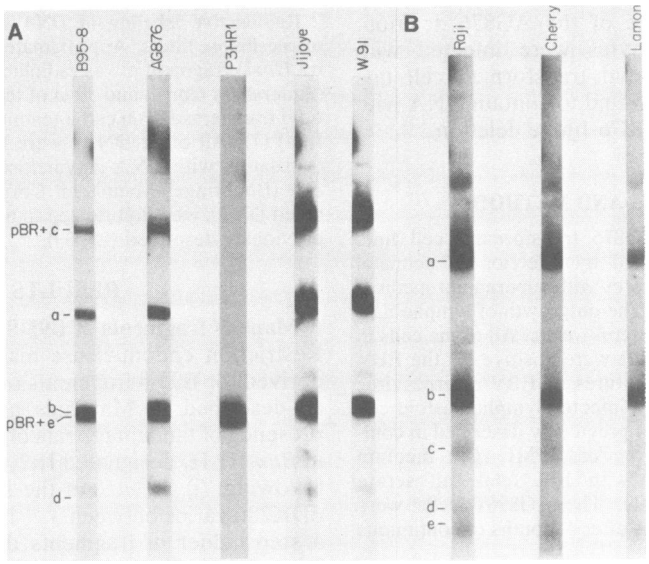

FIG. 2. B95-8 *Bam*HI-X sequences in EBV isolates. Southern blots of *Hin*fI fragments of pDF322 (*Bam*HI-X) were hybridized to ^{32}P-labeled B95-8, AG876, or P3HR-1 viral DNA or Jijoye or W91 density-selected cellular DNAs (A). *Bam*HI *Hin*fI fragments of pDF322 were hybridized to ^{32}P-labeled Raji, Cherry, or Lamont density-selected cellular DNAs (B). Longer exposure did not reveal *Hin*fI-a and -c sequences in P3HR-1 DNA.

combined; Fig. 1B) of the U2 part of *Bam*HI-X.

B95-8 *Bam*HI fragment H consists of 11 tandem direct repeats of a 123-bp sequence, IR2, and regions of apparently unique sequences (U2 and U3; Fig. 1). All labeled EBV DNAs hybridized to *Hin*fI fragments a, b, and e of *Bam*HI-H, which map in the right part of *Bam*HI-H (Fig. 3). B95-8, W91, Raji, Cherry, and Lamont DNAs also hybridized to *Hin*fI fragments c and d. Labeled AG876, Jijoye, and P3HR-1 DNAs did not hybridize to *Hin*fI fragment c or d.

The EBV AG876 and Jijoye isolates have the

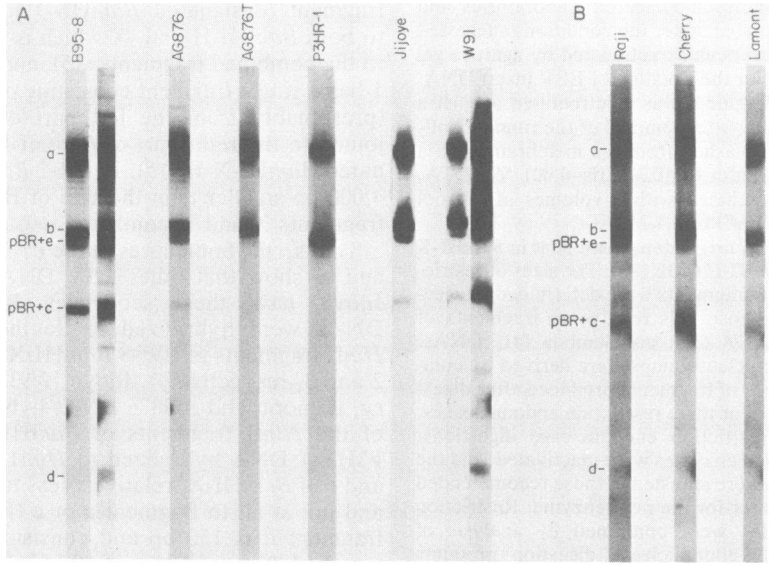

FIG. 3. B95-8 *Bam*HI-H sequences in EBV isolates. Southern blots containing *Hin*fI fragments of pDK286 (*Bam*HI-H) were hybridized to ^{32}P-labeled B95-8, AG876, or P3HR-1 viral DNAs or ^{32}P-labeled AG876T, Jijoye, or W91 density-selected intracellular DNA (A) or to ^{32}P-labeled, density-selected DNA from Raji, Cherry, or Lamont cells (B). Radiofluorographic exposure was normalized to achieve comparable levels of signal.

ability to growth-transform human B lymphocytes (29, 34). The data therefore suggest that *Hin*fI fragments c and d of B95-8 *Bam*HI-H are not necessary for growth transformation. To exclude the possibility that the transforming activity is associated with a subpopulation of DNA molecules which contain *Hin*fI-c and -d, neonatal cord blood lymphocytes were growth-transformed by AG876 infection. The intracellular EBV DNA was extracted and labeled in vitro. The labeled intracellular AG876 DNA hybridized to all of the *Hin*fI fragments of *Bam*HI-X (data not shown) and to *Hin*fI fragments a, b, and e of *Bam*HI-H but not to *Hin*fI fragment c or d of *Bam*HI-H (Fig. 3).

Hybridization of fragments of B95-8 *Bam*HI-H to Southern blots of EBV DNAs. To further evaluate the extent of similarity of the left and central regions of B95-8 *Bam*HI-H to AG876 and Jijoye *Bam*HI-D1 and to P3HR-1 *Bam*HI-X-H, labeled *Hin*fI-c, *Hin*fI-d, *Hin*cII-b, *Hae*II-c (IR2), or *Sau*3A-b (see Fig. 1B for map locations) was hybridized to blots of *Bam*HI digests of AG876, Jijoye, and P3HR-1 DNAs (Fig. 4A through E, respectively). Blots of a *Bam*HI digest of pDK286 containing an equivalent amount of *Bam*HI-H DNA or of B95-8 EBV DNA were included as controls. The *Hin*fI-c and -d probes identified *Bam*HI-H in B95-8 DNA but did not hybridize to AG876, Jijoye, or P3HR-1 DNA (Fig. 4A and B). The *Hin*cII-b probe hybridized less to *Bam*HI-D1 of AG876 and Jijoye DNA than to B95-8 *Bam*HI-H and not at all to P3HR-1 DNA (Fig. 4C). In contrast, the IR2 (*Hae*II-c) and *Sau*3A-b fragment probes hybridized similarly to AG876 and Jijoye *Bam*HI-D1 and to B95-8 *Bam*HI-H (Fig. 4D and E), indicating that IR2 and *Sau*3A-b are highly conserved among AG876, Jijoye, and B95-8 DNAs, whereas the *Hin*cII-b region (especially the *Hin*fI-c and -d part) is poorly conserved. IR2 probe did not hybridize to P3HR-1 *Bam*HI-X-H at a level of a single copy of the IR2 repeat (Fig. 4D). Only the *Sau*3A-b probe identified homologous sequences in P3HR-1 *Bam*HI-X-H (Fig. 4E). Taken together, these results indicate that P3HR-1 is deleted for all of U2 in *Bam*HI-H and all of IR2.

These experiments also extend previous knowledge of the duplication of *Bam*HI-H sequences in *Bam*HI-B1 of AG876 and Jijoye (15, 32). *Bam*HI-B1 maps in the right portion of UL (15, 32). IR2 and the unique sequences to the right of IR2, in *Sau*3A fragment b, hybridize extensively to AG876, Jijoye, and P3HR-1 *Bam*HI-B1 DNA (Fig. 4). This is the first description of Jijoye *Bam*HI fragments B1 and D1 and of their similarity in size and sequence content to AG876 *Bam*HI fragments B1 and D1 (15, 32).

Conservation of B95-8 *Bam*HI-H and -X sequences in DNAs of HV pan and HV papio. Analysis of sequence homology among related viruses can indicate evolutionary origin and suggest function. HV papio and HV pan DNAs have 40% homology to EBV DNA (8, 10).

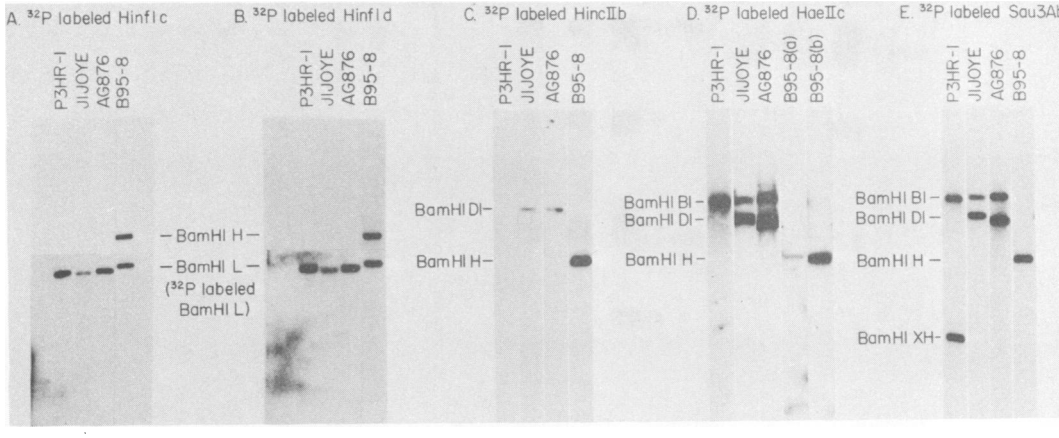

FIG. 4. Conservation of *Bam*HI-H sequences in EBV DNAs. Blots containing approximately 200 ng of *Bam*HI digests of P3HR-1, Jijoye, AG876, or B95-8 EBV DNA were hybridized to the following ^{32}P-labeled fragments of *Bam*HI-H: *Hin*fI-c (A), *Hin*fI-d (B), *Hin*cII-b (C), *Hae*II-c (D) or *Sau*3A-b (E) DNAs. The relative amount of EBV DNAs in the set of blots hybridized to *Hin*fI-c or -d was revealed rehybridizing the blots to ^{32}P-labeled *Bam*HI-L (A and B). *Bam*HI-B1, which maps near the right end of EBV DNA, contains the duplication, DR, of part of *Bam*HI-H (Fig. 1) and is an internal control for the relative amount of EBV DNAs in the second set of blots (C, D, and E). One tenth the amount of *Bam*HI-H DNA was included (D, lane A) to indicate the level of hybridization expected for one copy of the IR2 repeat. ^{32}P-labeled *Sau*3A-b hybridized to the same fragments as *Hae*II fragment c but also hybridized to the P3HR-1 *Bam*HI-X-H fusion fragment.

Almost every *Bam*HI fragment of EBV DNA, including *Bam*HI-X and -H, hybridizes to the fragment of HV pan or HV papio DNA which is colinear with EBV in their respective genomes (16–18, 25). To evaluate the extent of conservation of homology within *Bam*HI fragments H and X, labeled HV pan or HV papio DNAs were hybridized to Southern blots of *Hin*fI fragments of B95-8 *Bam*HI-X or -H. The labeled HV pan or HV papio DNAs hybridized to all of the *Hin*fI fragments of B95-8 *Bam*HI-X and to *Hin*fI fragments a, b, and e of *Bam*HI-H (Fig. 5). The labeled DNAs did not hybridize to *Hin*fI fragment d of *Bam*HI-H and only weakly to *Hin*fI fragment c (Fig. 5). These data indicate that the left end of B95-8 *Bam*HI-H is not conserved in HV pan and HV papio DNAs.

Labeled fragments of EBV *Bam*HI-H were also hybridized to Southern blots of fragments of HV pan and HV papio DNAs. The labeled *Hin*fI fragments c and d did not hybridize to HV pan or HV papio DNA (data not shown), whereas labeled IR2 hybridized to the HV papio and HV pan DNA fragments which map in the left end of UL in *Xba*I-I and *Bam*HI-A, respectively, and to homologous *Xba*I-A and *Bam*HI-KI, respectively, which map near the right end of UL (Fig. 5C; 16–18, 25).

DISCUSSION

These experiments were undertaken to further evaluate the hypothesis that the left portion of UL, now designated U2 (Fig. 1), is an essential region for EBV-induced cellular growth transformation. The hypothesis is supported by correlative data that U2 is part of one of the three regions of the EBV genome which function in latent transforming infection (22, 23, 30, 42) and that the P3HR-1 deletion includes all or almost all of U2 and all of IR2 (Fig. 1C; 15, 33; Kieff et al., in press). Part of U2 deleted from P3HR-1, the *Hin*fI fragments c and d of *Bam*HI-H, is also deleted from the AG876 and Jijoye DNAs which are capable of initiating growth transformation

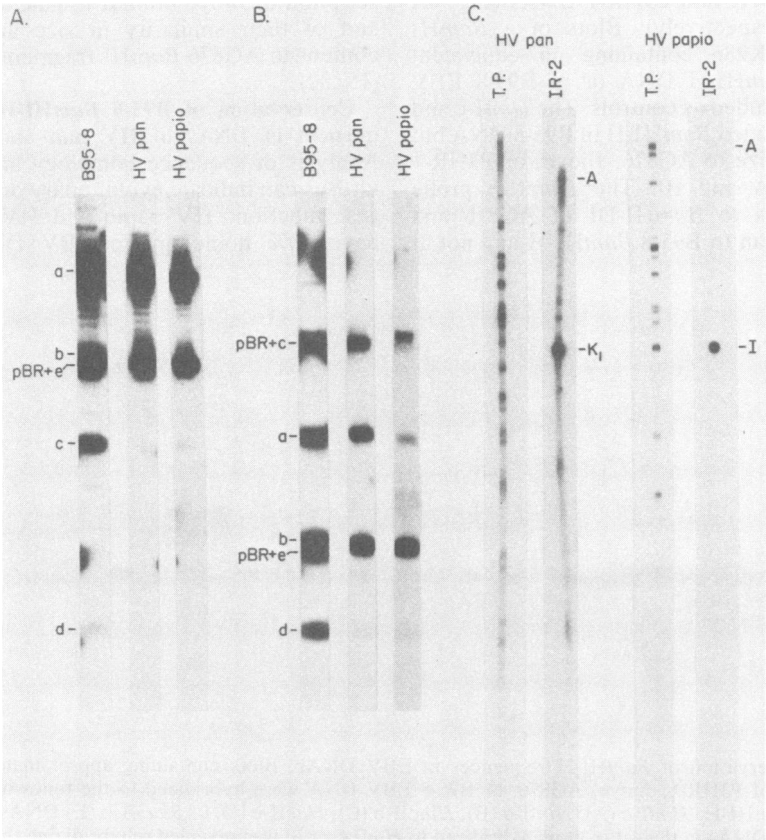

FIG. 5. Conservation of B95-8 *Bam*HI-H and -X sequences in primate EBV DNAs. Southern blots of *Hin*fI digests of B95-8 *Bam*HI-H of pDK286 (A) or B95-8 *Bam*HI-X of pDF322 (B) were hybridized to ^{32}P-labeled B95-8, HV pan, or HV papio DNA. To delineate the extent of conservation of IR2 in HV pan or HV papio DNA (C), Southern blots of a *Bam*HI digest of HV pan or *Xba*I digest of HV papio DNA were hybridized to ^{32}P-labeled IR2 (*Hae*II-c) DNA or ^{32}P-labeled HV pan or HV papio DNA (T.P.).

(Fig. 1C). *Hin*fI-c and -d DNA is also divergent or missing from HV pan and HV papio (Fig. 1C). In AG876, the B95-8 *Hin*fI-c and -d sequences are replaced with other DNA unrelated to *Hin*fI-c and -d (Dambaugh and Kieff, manuscript in preparation). Since *Hin*fI-c encodes part of the 3-kb cytoplasmic RNA transcribed from IR1 and U2 (42), *Hin*fI-c would not be expected to vary among transformation-competent viruses unless the *Hin*fI-c region encodes a nonessential part of the 3-kb RNA, there are alternative transforming products encoded by the IR1-U2 region such as those which occur when retroviruses incorporate different cellular DNA competent for growth transformation (1, 14), or the U2 DNA and the 3-kb RNA are not necessary for growth transformation.

With regard to the first possibility, *Hin*fI-c is near the 3′ end of the 3-kb RNA encoded by IR1 and U2. The more extensive P3HR-1 deletion includes at least one additional U2 exon (V. van Santen and E. Kieff, manuscript in preparation) which could be an essential part of the 3-kb RNA. Evaluation of the second possibility will rest in part on demonstrating homology between cell DNA and B95-8 *Hin*fI fragment c of *Bam*HI or the equivalent region of Jijoye or AG876 *Bam*HI-D1. With regard to the third possibility, P3HR-1 DNA could also differ in another region of the EBV genome which is necessary for the establishment of latent growth-transforming infection. The significance of the P3HR-1 U2-IR2 deletion as one important region in growth transformation can be established if P3HR-1 transformation competence can be restored through the addition of the deleted DNA (9). A correlate of the inability of P3HR-1 to transform is its ability to induce early antigen and to interfere with transformation (19, 20, 27, 28). Virus produced by clones of P3HR-1 cells retains these properties (20). Thus, the inability of P3HR-1 to transform could result from inappropriate turn-on or inadequate shut-off of viral replicative functions rather than a deletion of a direct transforming function.

The difference in U2 between the B95-8, Raji, Lamont, or Cherry DNAs and AG876 or Jijoye DNAs (Fig. 1C) is an example of significant unique sequence variation among EBV DNAs. Since Raji, AG876, and Jijoye are African Burkitt tumor isolates, whereas B95-8, Cherry, and Lamont are isolates from seropositive Americans, the difference does not correlate with disease or geographic origin. Further, the P3HR-1 strain is also deleted for IR2. Since P3HR-1 can replicate, IR2 is not essential for EBV DNA replication.

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