Organization of the Epstein-Barr Virus DNA Molecule

II. Fine Mapping of the Boundaries of the Internal Repeat Cluster of B95-8 and Identification of Additional Small Tandem Repeats Adjacent to the HR-1 Deletion

S. DIANE HAYWARD,[1]* SONDRA G. LAZAROWITZ,[2] AND GARY S. HAYWARD[1]

Department of Pharmacology and Experimental Therapeutics[1] and Department of Molecular Biology and Genetics,[2] The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

We used cloned *Bam*HI fragments from Epstein-Barr virus strain B95-8 [EBV(B95-8)] DNA to obtain detailed restriction maps of the region of the genome adjacent to the large internal repeat cluster. These maps together with the results of hybridization experiments using a 3.1-kilobase repeat probe defined more precisely the location of the junctions between the internal repeat cluster and the flanking unique-sequence DNA. On one side (U_L), the repeat sequences extended 600 ± 80 base pairs (bp) into *Bam*HI-Y; on the other side (U_S), they extended $1,300 \pm 200$ bp into *Bam*HI-C. Therefore, EBV(B95-8) DNA contained a nonintegral number of 3.1-kilobase repeat units, namely, 12.6 copies. The mapping studies also revealed a second series of internal tandem repetitions in EBV(B95-8) DNA located within the *Bam*HI-H fragment. This cluster comprised 11 copies of a 135-bp repeat unit which contained a single site for the *Not*I restriction endonuclease. Hybridization to these cloned EBV(B95-8) fragments using total EBV(HR-1) DNA as probe indicated that the deletion in EBV(HR-1) removed all 3,000 bp of unique-sequence DNA which lay between the large 3.1-kilobase and the small 135-bp repeat clusters. Thus, the deletion which destroyed the transforming ability in the EBV(HR-1) virus was bounded on either side by tandem repetitions.

Information has been steadily accumulating on the structure of the Epstein-Barr virus (EBV) DNA molecule. The linear DNA isolated from virions varies in molecular weight from 110×10^6 to 120×10^6, depending on the isolate (2, 10, 12, 17). The DNA molecule contains a number of repetitive elements. The termini consist of a series of tandem repeats of a 500-base pair (bp) sequence (9, 16), and a cluster of tandem repetitions of a 3.1-kilobase (kb) sequence located between 0.710 and 0.915 map units divides the DNA molecule into large unique (U_L) and short unique (U_S) segments (4, 8, 10). Although all isolates so far studied adhere to this general structural pattern, sufficient variations occur within the genome to render each isolate individually distinguishable. Variations in size occur most frequently in restriction fragments encompassing the 3.1-kb repeat unit cluster which may comprise anywhere from 3 to 6 (HR-1) up to 12 (B95-8) copies of the repeats and in the region of the *Eco*RI-C fragment (0.065 to 0.166) which sustains its largest deletion (approximately 8×10^6 molecular weight) in B95-8 DNA (2, 5, 6, 10, 12, 17, 20, 21, 25). Smaller insertions and deletions have also been noted in other regions of the genome (2).

EBV transforms human and simian lymphocytes in vitro into continuously growing lymphoblastoid cell lines. The single exception is the nontransforming HR-1 isolate. A characteristic deletion in the genome of HR-1 appears to correlate with the inability of this isolate to transform lymphocytes. Superinfection of nonproducer EBV cell lines, such as Raji, with HR-1 virus can result in the regeneration of transforming virus. Analysis of the DNA of these new viruses indicates that in each case they represent recombinant molecules in which the sequences deleted in HR-1 DNA have been regained from the resident Raji genomes (7). The HR-1 deletion was initially described in general terms by Raab-Traub et al. (18) and defined in more detail by Bornkamm et al. (2, 5) and Heller et al. (12) and is located near coordinates 0.7 in the U_L region, adjacent to the 3.1-kb internal repeats.

In this paper, we describe the use of cloned fragments of B95-8 DNA to construct detailed restriction maps of the region surrounding the 3.1-kb repeat cluster. These mapping data were accumulated with the aim of pinpointing (i) the location of the junctions of the 3.1-kb repeat cluster within the U_L and U_S segments and (ii) the location of the deletion in HR-1 DNA. These

TABLE 1. Characteristics of the *Bam*HI
recombinant plasmids

Plasmid	Fragment	Orientation[a] and insert size (kb)	Map coordinates[a]
pSL3	*Bam*HI-Y	I, 1.85	0.686–0.696
pSL8	*Bam*HI-YW	I, 4.95	0.686–0.704
pSL9	*Bam*HI-W	P, 3.1	(0.696–0.913)/12
pSL76	*Bam*HI-WW	P, 6.2	(0.696–0.913)/6
pSL77	*Bam*HI-H	P, 5.8	0.652–0.686
pSL93	*Bam*HI-C	P, 8.9	0.913–0.967

[a] The prototype (P) and inverted (I) orientations and
fractional map units are both based on our standard
map of the EBV(B95–8) genome drawn with the S
segment on the right (10).

studies led to the discovery that B95-8 DNA
contains a nonintegral number of 3.1-kb repeat
units and to the identification of a new cluster of
internal tandem repeat sequences which map
within the *Bam*HI-H fragment.

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MATERIALS AND METHODS

Generation of plasmid clones of B95-8 DNA. Portions
of B95-8 DNA (1 μg) which had been extracted from
purified virions were cleaved with the *Bam*HI restriction
endonuclease and cloned into the *Bam*HI site of
the plasmid pBR322, using the shotgun approach.
Recombinant plasmids were transfected into *Escherichia
coli* HB101 by the calcium shock method. All
ampicillin-positive, tetracycline-negative colonies
were grown as minicultures (1 ml), the DNA was
extracted by the method of Birnboim and Doly (1), and
the size of the EBV DNA insert was estimated by
electrophoresis through 1% agarose slab gels, followed
by ethidium bromide staining. Recombinant plasmids
carrying the *Bam*HI-C, *Bam*HI-W, and *Bam*HI-Y inserts
were initially identified by their ability to hybridize
to an isolated *Bgl*II repeat fragment probe. Subsequently,
the identity of these plasmids and of the
*Bam*HI-H recombinant plasmid was confirmed by
hybridizing the in vitro-labeled plasmid DNAs to blots
of *Eco*RI- and *Hin*dIII-digested B95-8 viral DNA and
by comparison of the *Eco*RI, *Hin*dIII, and *Sal*I cleavage
patterns with those predicted from published
cleavage maps (4, 21). Table 1 gives the properties of
the plasmids used in the experiments described here,
and Fig. 1 shows ethidium bromide-stained profiles of
the cleaved plasmid DNAs.

Purification of viral and plasmid DNAs. B95-8 viral
DNA was extracted from virions purified by banding
in dextran T10 gradients as previously described (10).
The HR-1 DNA which was used to form in vitro-
labeled probes was extracted from crude virus pellets.
These pellets were obtained by centrifugation of clarified
HR-1 culture supernatant for 90 min at 9,000 rpm
in a Sorvall GS3 rotor. The DNA preparations obtained
in this way contained cellular DNA in addition
to EBV sequences.

Recombinant plasmid DNAs were prepared as follows.
Bacterial cultures (1 liter) were grown at 37°C in
TY broth (1% tryptone, 0.5% yeast extract, 0.5%
NaCl, and 0.1% glucose, pH 7.2) to an optical density
at 600 nm of 0.6, at which time chloramphenicol was
added at 50 μg/ml to obtain plasmid amplification, and
the incubation was continued overnight. Extraction of
the plasmid DNA followed the methods of Clewell and
Helinski (3), and the DNA was purified by banding to
equilibrium in cesium chloride-ethidium bromide density
gradients.

End labeling of DNA. The cloned *Bam*HI-H fragment
was cleaved at the *Bgl*II site, treated with
bacterial alkaline phosphatase (Bethesda Research
Laboratories), and end labeled as described by Smith
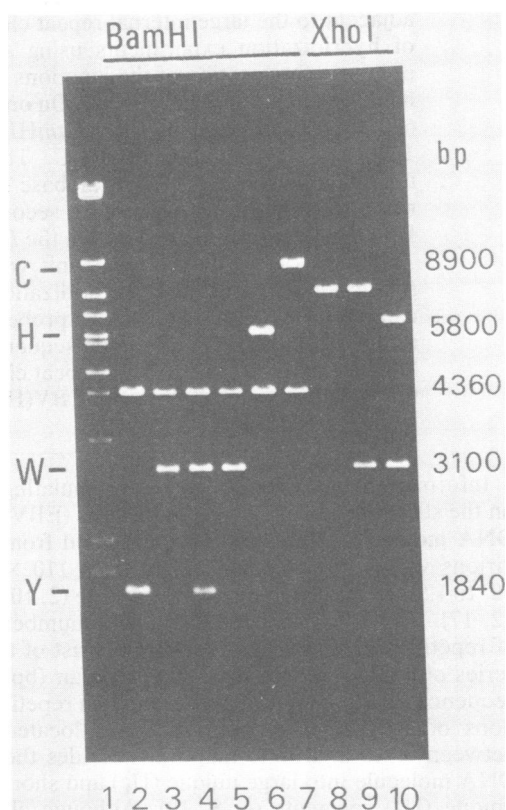and Birnstiel (23), using polynucleotide kinase (Be-

FIG. 1. Characterization of the molecularly cloned
*Bam*HI fragments from EBV (B95-8) DNA used in this
study. Plasmid DNAs were cleaved with restriction
enzymes, electrophoresed through a 1% agarose gel
for 750 V × h, and stained with ethidium bromide. The
size markers (slot 1) consist of a mixture of the *Eco*RI
and *Hin*dIII cleavage products from phage lambda
DNA. The samples in slots 2 through 7 were each
cleaved with *Bam*HI. Slot 2, pSL3 (*Bam*HI-Y); slot 3,
pSL9 (*Bam*HI-W); slot 4, pSL8 (*Bam*HI-YW); slot 5,
pSL76 (*Bam*HI-WW); slot 6, pSL77 (*Bam*HI-H); slot
7, pSL93 (*Bam*HI-C). Samples in slots 8 through 10
were each cleaved with *Xho*I. Slot 8, pSL9 (*Bam*HI-
W); slot 9, pSL76 (*Bam*HI-WW); slot 10, pSL8
(*Bam*HI-YW).

thesda Research Laboratories) and [γ-³²P]ATP (New England Nuclear Corp.).

Restriction enzyme digestion and gel electrophoresis. Restriction enzymes were purchased from either Bethesda Research Laboratories or New England Biolabs. Cleaved plasmid DNAs (100 to 500 ng) were analyzed by electrophoresis in Tris-phosphate buffer through agarose gels (11) in the form of either vertical slabs or horizontal submarine gels. The DNA was visualized by staining with ethidium bromide (1 μg/ml). Partial digests of BamHI-H DNA were obtained as follows. The reaction mix (50 μl) contained 5 μg of sonicated calf thymus DNA, 1 μg of ³²P-labeled BamHI-H DNA, and 0.5 U of the selected restriction enzyme. After 5, 10, 15, 30, and 90 min of incubation at 37°C, 10-μl portions of reaction mix were removed into stop buffer (10 mM Tris, pH 7.8, 20 mM EDTA, 10% glycerol, and 0.01% bromophenol blue). Half of each sample was analyzed by electrophoresis through a 1% agarose gel, and those samples giving an appropriate range of partial digestion products were subsequently pooled and analyzed on a master gel. The 3.1-kb BamHI repeat fragment was isolated from a BamHI digest of pSL9 (10 μg) which had been electrophoresed through a 20-cm cylindrical gel of 0.8% agarose. The gel was stained briefly with ethidium bromide, and the DNA was visualized under long-wave UV illumination. The appropriate band was excised, extracted in 5 M sodium perchlorate at 60°C, and absorbed onto hydroxylapatite (13). The DNA was eluted with 0.4 M potassium phosphate buffer and concentrated by ethanol precipitation.

In vitro labeling and hybridization. The probes used consisted of portions (0.5 to 1.0 μg) of HR-1 or B95-8 viral DNA, recombinant plasmid DNA, or isolated EBV DNA fragments which were labeled in vitro with [³²P]TTP and [³²P]dCTP (New England Nuclear) by nick translation (19).

Electrophoretically separated DNA fragments were transferred from the agarose gels to nitrocellulose filters as described by Southern (24). The filters were sealed in plastic bags and preincubated at 63°C in hybridization buffer (0.8 M NaCl, 0.05 M Tris, pH 7.4, 0.001 M EDTA, 0.2% sodium dodecyl sulfate, 20% formamide, and 1× Denhardt solution [0.02% Ficoll, 0.02% polyvinyl pyrrolidone, and 0.02% bovine serum albumin]) plus 50 μg of sheared, denatured calf thymus DNA per ml. Hybridization was carried out at 63°C for 24 to 48 h with 1 × 10⁶ to 5 × 10⁶ cpm of denatured probe in 8 ml of hybridization buffer. After annealing, filters were washed successively with 2× SSC (SSC = 0.15 M NaCl plus 0.015 sodium citrate), fresh hybridization buffer at 63°C, 2× SSC, and finally 0.01× SSC. Autoradiography was carried out for 1 to 14 days at −70°C with enhancing screens.

RESULTS

Relative organization of cloned BamHi-H, -Y -W, and -C fragments in the viral genome. In our previous studies on the internal repeat cluster and its flanking sequences in EBV(B95-8) DNA using cleaved viral DNA and isolated fragment probes, we suggested that the outside physical termini of the repeat units lay closer to the BglII site than to the BamHI site on the Uₛ side of the

cluster and closer to the BamHI site than the BglII site on the Uₗ side (10). This interpretation was based on the assumption that the cluster contained an exactly integral number of copies of the tandemly duplicated 3.1-kb sequence. However, subsequent studies indicated that the structure was more complicated. In particular, double cleavages with PvuII plus BamHI or BglII revealed that BamHI mapped closer to the termini than did BglII at both ends of the cluster and that BamHI released an extra complete copy of the repeat unit (i.e., 13 × 3.1 kb) relative to BglII (12 × 3.1 kb). To examine this phenomenon further, we constructed pBR322 plasmids containing the cloned 3.1-kb repeats and flanking sequences from EBV(B95-8) virion DNA. The plasmids selected for use in the mapping work described here contained either single BamHI fragment inserts or combinations of adjacent BamHI fragments from a partial digest (Fig. 1). Table 1 summarizes their characteristic sizes, orientation, and map coordinates.

Figure 2 is a composite diagram illustrating the arrangement of the appropriate BamHI fragments relative to the internal repeat cluster and its flanking regions on both the Uₗ and Uₛ sides in the parent viral DNA molecule. The observation that the pSL8 plasmid, which contains a single copy each of both the BamHI-Y and BamHI-W fragments, released a complete 3.1-kb "repeat unit" after cleavage with XhoI (Fig. 1, slot 10) confirmed our earlier evidence that these two fragments map adjacent to one another at the Uₗ/repeat junction in the parent viral genome (4, 8, 10). In addition, this "partial" BamHI-YW clone provided key information for establishing the correct orientations of both the BamHI-Y and BamHI-W fragments relative to the left (Uₗ) and right (Uₛ) ends of the intact EBV DNA molecule. We previously identified the flanking fragment on the Uₗ side as BamHI-C (10), and the orientation of this fragment was deduced from the positions of the internal EcoRI sites (8). Skare and Strominger (22) and Dambaugh et al. (4) reported that BamHI-H is the next fragment adjacent to BamHI-Y on the Uₛ side of the repeats. The orientation of BamHI-H shown in Fig. 2 is the only arrangement which can accommodate the 10.3-kb BglII-C species known to be the Uₛ flanking fragment in a BglII digest (10). These deductions indicate that the orientation originally assigned to the BglII-R-repeat unit in Fig. 11d of Hayward et al. (10) needs to be reversed.

Location of the Uₗ and Uₛ repeat junctions by restriction site mapping. Figure 3 summarizes the physical maps of plasmids containing BamHI-H, -Y, -W, -YW, and -C fragments of EBV(B95-8) DNA. These were constructed by performing single, double, and triple restriction
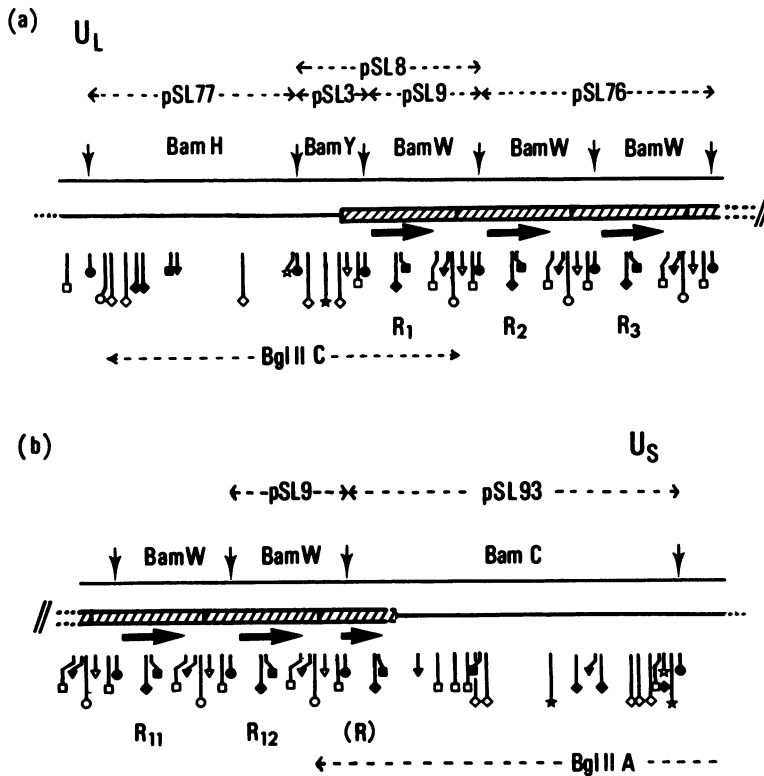
FIG. 2. Arrangement and orientation of *Bam*HI-H, -Y, -W (repeat), and -C in B95-8 virion DNA and localization of the junctions between the unique sequences and 3.1-kb internal repeat cluster by restriction site analysis. (a) U_L side between map coordinates 0.650 to 0.750; (b) U_S side between coordinates 0.870 to 0.970. Hatched bars and horizontal arrows indicate the positions of individual 3.1-kb repeats at the edges of the internal repeat cluster. Symbols are used to illustrate the pattern of mapped restriction sites within the cloned fragments. Only those cleavages which are most relevant for determining the repeat/unique junctions are included. (●) *Bam*HI; (□) *Pst*I; (△) *Xho*I; (○) *Bgl*II; (▲) *Sac*I; (■) *Sac*II; (◆) *Pvu*II; (◇) *Hinc*II; (☆) *Bst*EII; (★) *Hind*III in (a) and *Eco*RI in (b).

enzyme digests and measuring the size of the DNA fragments generated by using known molecular weight markers. To ensure as great a degree of accuracy as possible, many different enzyme combinations were used to determine the location of each restriction site. The double-cut mapping technique provided adequate information for the subsequent hybridization studies. However, because it is difficult to map regions in which the sites for a given enzyme occur less than 200 to 300 bp apart by this procedure, the map locations of the smallest fragments, particularly those in *Bam*HI-C, should be considered tentative. In the case of *Bam*HI-H, some cleavage sites were also mapped by using the technique of partial digestion of end-labeled DNA described by Smith and Birnsteil (23). We had previously described the relative position of 10 cleavage sites within the 3.1-kb *Bam*HI repeat (10), and these data were confirmed by using a cloned monomer *Bam*HI-W-repeat unit and a cloned dimer of the repeat.

Inspection of the cleavage sites within *Bam*HI-Y (Fig. 2a) revealed that the first three sites (*Bam*HI, *Pst*I, and *Xho*I) of the eight-site repeat pattern were present, but the fourth site, *Bgl*II, was missing. This suggested that the repeat sequences extended into *Bam*HI-Y to a position no further than 200 bp beyond the *Xho*I site. A similar examination of the U_S/repeat junction at the opposite end of the cluster (Fig. 2b) showed that, of the eight cleavage sites present in the repeat unit, only two (*Pvu*II and *Sac*II) were present in *Bam*HI-C; the third would be the *Pst*I site. Therefore, the repeat sequences within the flanking *Bam*HI-C fragment extended no further than 1,000 bp beyond the *Sac*II site.

Location of U_L and U_S repeat junctions by blot hybridization. Proof that these matching cleavage patterns do represent homologous sequences came from Southern blot hybridization experiments in which a repeat DNA probe was hybridized to a variety of restriction enzyme
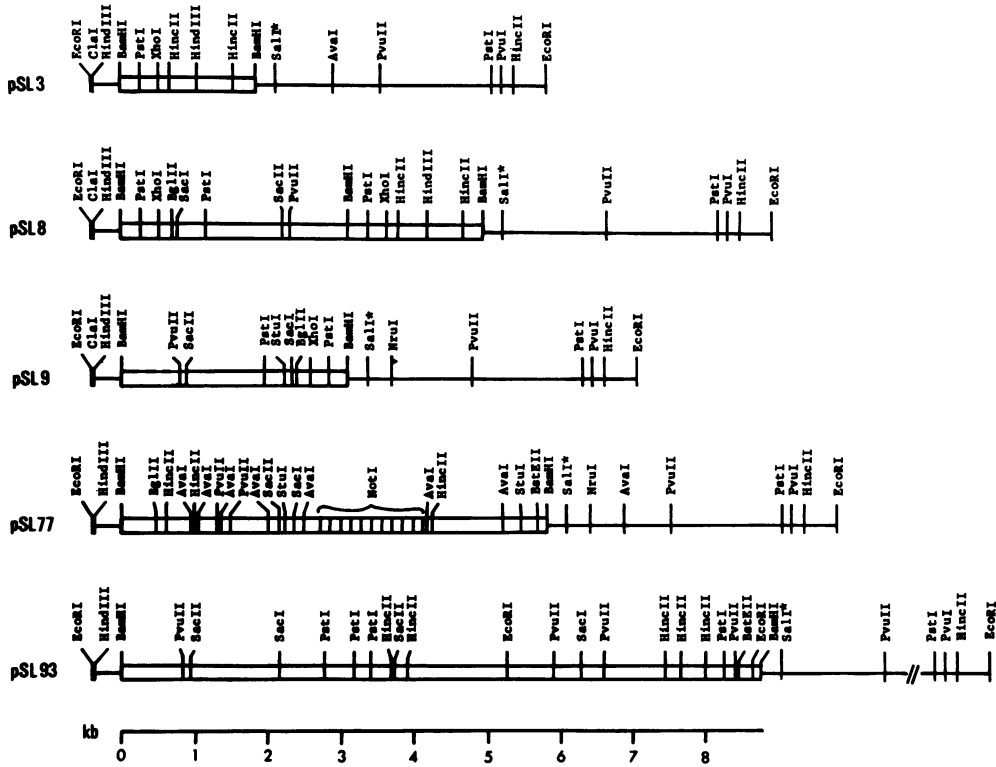
FIG. 3. Restriction site maps for pSL3 (*Bam*HI-Y), pSL8 (*Bam*HI-YW), pSL9 (*Bam*HI-W), pSL77 (*Bam*HI-H), and pSL93 (*Bam*HI-C). The regions drawn as double lines represent EBV DNA sequences; the plasmid pBR322 sequences are represented by a single line. The asterisk indicates that the *Sal*I site is also a *Hin*cII site. The size scale for insert DNA is given in kilobases. The following restriction enzymes do not cleave these fragments. *Bam*HI-Y, no sites for *Ava*I, *Bgl*II, *Bst*EII, *Eco*RI, *Hpa*I, *Kpn*I, *Pvu*I, *Pvu*II, *Sac*I, *Sac*II, *Sal*I, and *Xba*I; *Bam*HI-repeat, no sites for *Bst*EII, *Cla*I, *Eco*RI, *Hin*cII, *Hin*dIII, *Hpa*I, *Kpn*I, *Not*I, *Nru*I, *Pvu*I, *Sal*I, and *Xba*I; *Bam*HI-H, no sites for *Cla*I, *Hin*dIII, *Kpn*I, *Nru*I, *Pst*I, *Pvu*I, *Sal*I, *Xba*I, and *Xho*I; *Bam*HI-C, no sites for *Bgl*II, *Hin*dIII, *Hpa*I, *Kpn*I, *Pvu*I, *Sal*I, *Xba*I, and *Xho*I.

digests of the *Bam*HI-Y and *Bam*HI-C fragments. This approach also addressed the possibility that homologous but diverged sequences, lacking the characteristic repeat-unit cleavage sites, may have extended beyond the boundaries suggested above. An example of the results obtained by hybridizing restriction digests of the *Bam*HI-Y fragment with a *Bam*HI-repeat probe is shown in the upper panel of Fig. 4. Our interpretations of the results of this and similar blot hybridization experiments (not shown) are summarized in tabular form in the lower panel of Fig. 4. The use of a large set of different restriction fragments has the advantage of minimizing potential misinterpretations which could arise from occasional hybridization artifacts in individual tracks or from aberrant hybridization properties associated with localized unusual DNA structure (see later). Relevant observations include the following: (i) the cloned *Bam*HI-Y fragment hybridized with the *Bam*HI-W-repeat probe as expected; (ii) the hybridiza-

tion within *Bam*HI-Y occurred within sequences which map directly adjacent to *Bam*HI-W in virion DNA; (iii) the ratio of hybridization to *Bam*HI-Y relative to *Bam*HI-W in the pSL8 plasmid was consistent with flanking sequence homology totaling in the order of 400 to 1,000 bp; (iv) the region giving hybridization appeared to be confined to a contiguous stretch of sequences containing sites for *Bam*HI, *Hin*fI, *Pst*I, and *Xho*I; and (v) a boundary between homologous and nonhomologous sequences could be detected between the *Xho*I and *Hin*cII sites located 520 and 680 bp from the *Bam*HI site. We conclude that a contiguous 600-bp segment of the flanking *Bam*HI-Y fragment (U_L side) consists of repeat-unit sequences and that no additional diverged "repeat-like" sequences exist beyond the characteristic cleavage sites identified in the previous section.

Figure 5 presents similar analyses with restriction digests of the *Bam*HI-C fragment to examine the extent of homology between the *Bam*HI-
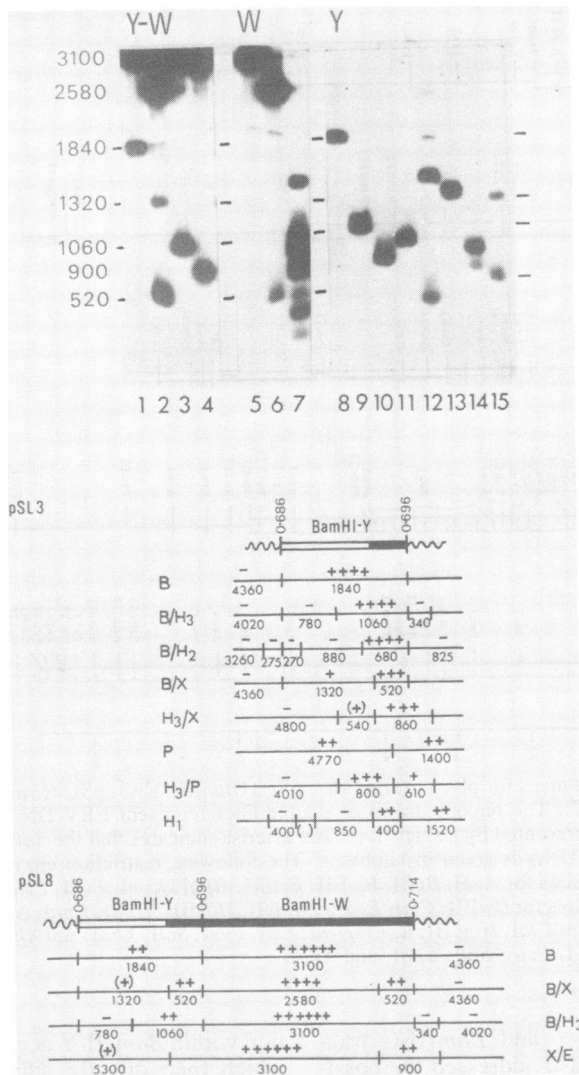
FIG. 4. Localization of the U_L/3.1-kb repeat junction by hybridization analysis. The autoradiograph shows the hybridization of an in vitro-labeled BamHI-repeat fragment probe to restriction enzyme digests of pSL8 (BamHI-YW), pSL9 (BamHI-W), and pSL3 (BamHI-Y) DNAs which had been electrophoresed through a 1.4% agarose gel for 800 V × h. Only that region of the gel containing fragments of less than 3,100 bp is presented. The numbers indicate fragment sizes in base pairs. Individual tracks contain: pSL8 DNA digested with (1) BamHI, (2) BamHI/XhoI, (3) BamHI/HindIII, and (4) EcoRI/XhoI; or pSL9 DNA digested with (5) BamHI, (6) BamHI/XhoI, and (7) HinfI; or pSL3 DNA digested with (8) BamHI, (9) BamHI/HindIII, (10) BamHI/HincII, (11) HindIII/ XhoI, (12) HinfI, (13) PstI, (14) PstI/HindIII, and (15) BamHI/XhoI. A summary and interpretation of the results from this and other similar hybridization experiments are also presented. Solid bars depict those portions of the cloned inserts that contain sequences homologous to the repeat unit probe, and open bars represent regions lacking repeat sequence homology. Wavy lines denote pBR322 sequences. Individual fragments are identified by their measured sizes in base pairs, and those which hybridize to the BamHI-W-repeat probe are assigned (+), (++), or (+++), etc., notations, depending on hybridization intensity. Lack of hybridization is indicated by (−). The abbreviations for the restriction enzymes used to cleave the DNA are as follows: B, BamHI; E, EcoRI; H1, HinfI; H2, HincII; H3, HindIII; P, PstI; X, XhoI.

repeat probe and the flanking DNA on the U_S side of the 3.1-kb cluster. The results of these experiments provided evidence that: (i) again, all of the hybridization was restricted to the region directly adjacent to the repeat cluster; (ii) in this case, the homology ended in a poorly defined segment between the SacII site 915 bp beyond BamHI and the SacI site 2,100 bp away
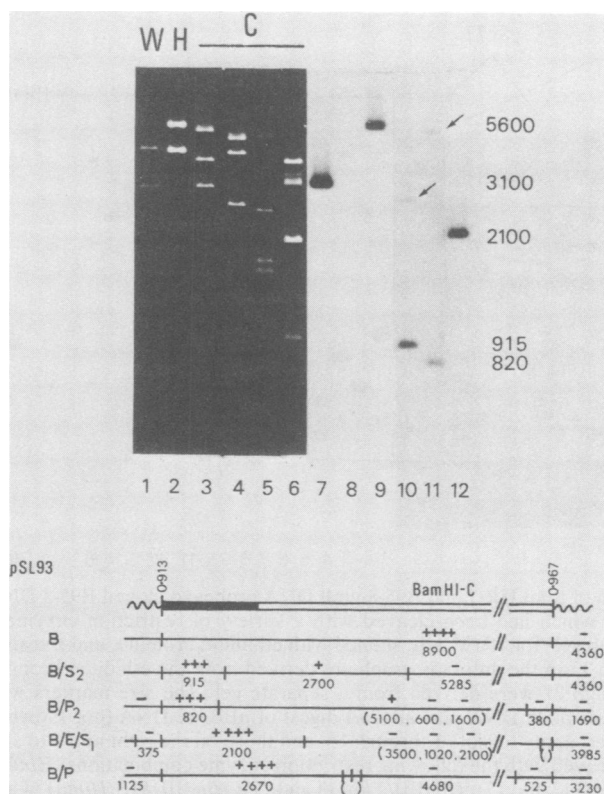
FIG. 5. Localization of the U_S/3.1-kb repeat junction by hybridization analysis. The autoradiograph illustrates hybridization of an in vitro-labeled, isolated BamHI-repeat fragment probe to a blot of the cleaved pSL9 (BamHI-W), pSL77 (BamHI-H), and pSL93 (BamHI-C) DNAs shown in the adjacent ethidium bromide stain. The DNA samples were electrophoresed through a 1.4% agarose gel for 700 V × h. Strongly positive fragments are identified by their sizes in base pairs, and weakly hybridizing fragments are indicated by arrows. Individual tracks contained the following digests: (1 and 7) pSL9, BamHI; (2 and 8) pSL77, BamHI; (3 and 9) pSL93, BamHI/EcoRI; (4 and 10) pSL93 BamHI/SacII; (5 and 11) pSL93, BamHI/PvuII; (6 and 12) pSL93 BamHI/EcoRI/SacI. The figure includes a diagrammatic summary of the results from this and other additional hybridization experiments. The format and symbols used are the same as in Fig. 4. Restriction enzyme sites are abbreviated as follows: B, BamHI; E, EcoRI; P, PstI; P2, PvuII; S1, SacI; S2, SacII.

from BamHI; and (iii) based on the relative hybridization intensity to nearby fragments, no more than 250 to 500 bp in the DNA beyond the SacII site formed hybrids. Therefore, a presumably contiguous segment of approximately 1,300 bp of the flanking fragment BamHI-C (U_S side) has homology with the repeat unit probe.

In conclusion, the results of the detailed cleavage site mapping together with the hybridization experiments clearly confirm that the sequences forming the physical termini of the repeat cluster on the U_L side come from a different position within the 3.1-kb repeat unit than do those on the U_S side; i.e., there is a nonintegral number of copies of the repeat unit in the cluster.

Localization of the HR-1 deletion. Previous work has indicated that the deletion in HR-1 encompasses the HindIII site between HindIII-A and -B on the U_L side of the repeat cluster (2, 11, 17), i.e., presumably the single HindIII site within BamHI-Y. To further define the limits of the deletion within the U_L sequences, Southern transfers containing restriction enzyme digests of the cloned BamHI-Y and BamHI-H fragments from EBV(B95-8) were hybridized with in vitro-labeled probes of total B95-8 and HR-1 DNAs (Fig. 6). The location of the deletion in HR-1 was defined by those restriction fragments which hybridized with a B95-8 probe but not with an HR-1 probe.

Figure 7 presents a summary and interpretation of all of the results of hybridization experiments to define the HR-1 deletion. Our analysis for BamHI-Y indicated the following: (i) HR-1 lacked sequences located within the BamHI-Y fragment of B95-8; (ii) the deletion included an apparently contiguous region of 1,300 bp which
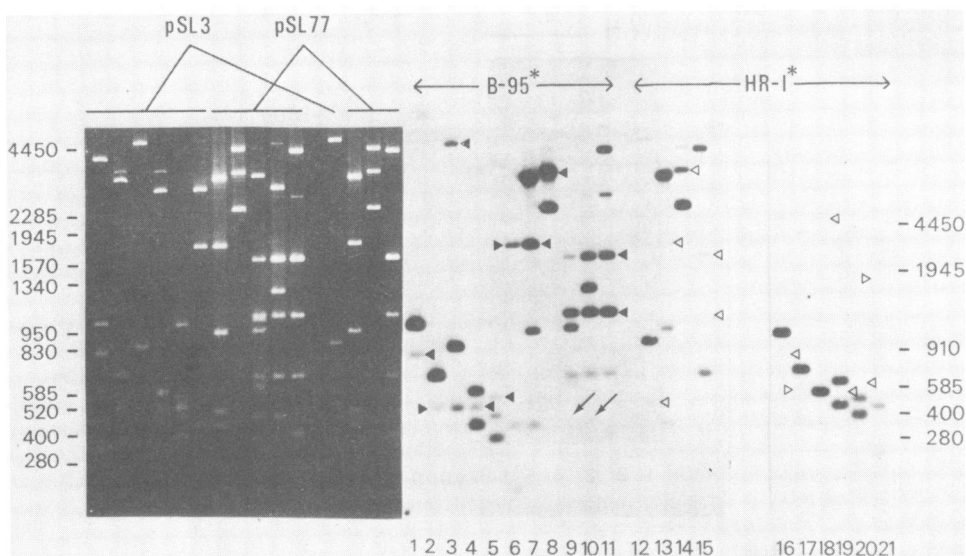
FIG. 6. Hybridization of total HR-1 and B95-8 viral DNA probes to cloned B95-8 DNA fragments. BamHI-Y and BamHI-H plasmids which had been cleaved with a variety of restriction enzymes were electrophoresed through 1.5% agarose slab gels for 750 V × h, stained with ethidium bromide, and transferred onto nitrocellulose sheets. Tracks 1 through 15 in the autoradiograph are derived from the ethidium bromide-stained gel shown to the left. Tracks 16 through 21 were derived from a separate gel. The size markers were HindIII and EcoRI/HindIII digests of phage lambda DNA plus an AluI digest of pBR322 DNA (not shown). Note that the sizes in base pairs given on the left apply to slots 1 through 15, and those on the right apply to slots 16 through 21. pSL3 (BamHI-Y) DNA was cleaved with the following restriction enzyme combinations: HindIII/BamHI (tracks 1 and 16); HindIII/BamHI/HincII (2 and 17); HindIII/XhoI (3 and 12); HindIII/PstI/HincII (4 and 19); HindIII/HinfI (5 and 20); HindIII/AvaI (6 and 21); and HindIII/BamHI/XhoI (18). pSL77 (BamHI-H) was digested with: HindIII/HincII (slots 7 and 13); BamHI/SacI (8 and 14); BamHI/AvaI (9); HindIII/AvaI (10); and SacI/AvaI (11 and 15). Tracks 1 through 11 were hybridized with in vitro-labeled B95-8 viral DNA, and tracks 12 through 21 were hybridized with an in vitro-labeled HR-1 viral DNA probe. The triangles indicate bands which form hybrids with the B95-8 probe (solid) but are either negative or hybridize with reduced intensity when the probe is HR-1 DNA (open). The arrows indicate the position of a 480-bp AvaI viral DNA fragment which does not hybridize to the B95-8 probe (see text). Note that with B95-8 DNA probes, those portions of BamHI-Y that contain sequences from the 3.1-kb repeat unit hybridize more strongly than do the unique-sequence portions because each B95-8 DNA molecule contains 12 copies of the 3.1-kb repeat. This phenomenon is less obvious with the HR-1 DNA probe because of the lower copy number for the repeats in HR-1.

in B95-8 contained sites for the BamHI, HindIII, HincII, and HinfI enzymes; and (iii) those sequences within BamHI-Y which did form hybrids with the HR-1 probe began between the XhoI and HincII sites at the repeat/unique junction identified in the previous section. Therefore, the HR-1 deletion removed all unique-sequence DNA from the BamHI-Y fragment. Note that because HR-1 is known to retain several copies of the 3.1-kb BamHI repeats, these data do not necessarily define the actual boundary of the deletion.

A similar analysis was performed on the adjacent BamHI-H fragment. Hybridization with an in vitro-labeled HR-1 DNA probe indicated that the homology extended beyond the SacI site (Fig. 6, slots 8 and 14) because fragments mapping immediately to the right of the SacI site were positive, although hybridizing with reduced intensity. Use of the HincII enzyme (Fig.

6, slots 7 and 13) localized the deletion boundary to within the region between the SacI site 4,300 bp from the BamHI-H to -Y junction and the HincII site 2,450 bp away from it. In the hope of mapping the U_L side of the deletion more precisely, we searched for enzymes which cleaved within this large undefined segment of BamHI-H. Although numerous enzymes were screened (including those with 4- and 5-bp recognition sites such as AluI, HinfI, MboII, and TaqI), only two, AvaI and NotI, cleaved within the 1,830-bp SacI/HincII fragment. AvaI removed approximately 200 bp, but digestion with NotI revealed that the sequence complexity within this region was much less than anticipated (see below). This accounted for the paucity of restriction enzyme cleavage sites.

Detection of new small tandem repeats in BamHI-H. Cleavage of the pSL77 plasmid containing BamHI-H with NotI yielded two bands,
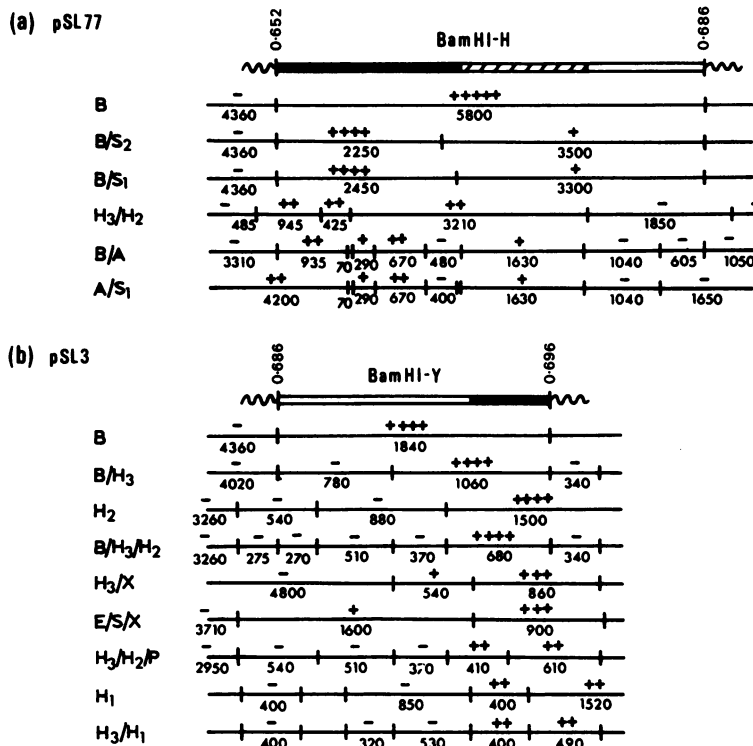
FIG. 7. Summary and interpretation of the hybridization results which define the extent of the HR-1 deletion. Some of the data originate from the experiments shown in Fig. 6. The solid bars represent DNA sequences within the cloned B95-8 *Bam*HI-H and -Y fragments that are retained in the HR-1 genome. Open bars indicate deleted sequences, and the hatched bar denotes the region of uncertainty in *Bam*HI-H. Wavy lines indicate pBR322 sequences. Fragment sizes are given in base pairs, and hybridization (or lack of hybridization) with an HR-1 probe is indicated by (+) and (−), respectively. The aberrant hybridization of the 480-bp *Ava*I fragment from pSL77 is discussed in the text. The restriction enzymes used to cleave the DNA are given at the left of the diagram: A (*Ava*I); B (*Bam*HI); E (*Eco*RI); H1 (*Hin*fI); H2 (*Hin*cII); H3 (*Hin*dIII); P (*Pst*I); S (*Sal*I); S1 (*Sac*I); S2 (*Sac*II); and X (*Xho*I).

one very large and the other containing DNA measuring only 135 bp in size (Fig. 8, slot 2). Additional cleavage with *Sac*I and *Sac*II showed that a *Not*I site was located 385 and 580 bp from the *Sac*I and *Sac*II sites, respectively (Fig. 8, slots 3 through 6). However, in these digests the intense staining of the 135-bp band relative to the two flanking fragments indicated that it must contain multiple copies of the 135-bp fragment. To determine the exact number of copies of this fragment and to further define the locations of the *Not*I sites, pSL77 DNA which had been end labeled with [^{32}P]phosphate at the *Bgl*II site was first cleaved to completion with *Hin*dIII and then subjected to partial digestion with *Not*I, *Sac*I, or *Hin*cII. As shown in Fig. 8 (slots 12 and 16), the *Not*I partial digest resulted in a ladder of 11 bands with sizes increasing in increments of 135 bp. Thus, the HR-1 deletion terminated within an 1,830-bp *Sac*I/*Hin*cII fragment which, in B95-8, contained 11 adjacent copies of a 135-bp repetition. (The presence of multiple copies

of the 135-bp *Not*I fragment within *Bam*HI-H is unlikely to have arisen as an artifact of cloning, since the *Bam*HI-H fragment derived from pSL77 was identical in size to that isolated from B95-8 virion DNA.)

In an attempt to determine whether the HR-1 deletion includes part or all of the small repeat cluster, HR-1 and B95-8 DNA probes were hybridized to blots of *Not*I-cleaved *Bam*HI-H DNA. Unfortunately, both the HR-1 and B95-8 DNA probes failed to anneal to the 135-bp *Not*I fragment (Fig. 8, slot 7). However, the HR-1 probe did hybridize to both the 580-bp *Sac*II/*Not*I fragment and the 385-bp *Sac*I/*Not*I fragment (not shown), indicating that sequences within these fragments were apparently retained in HR-1. The possible low-efficiency transfer to the nitrocellulose of such small-sized fragments could not alone account for the lack of hybridization to the 135-bp *Not*I repeats because in parallel experiments, similarly sized pBR322 fragments gave positive results with a pBR322
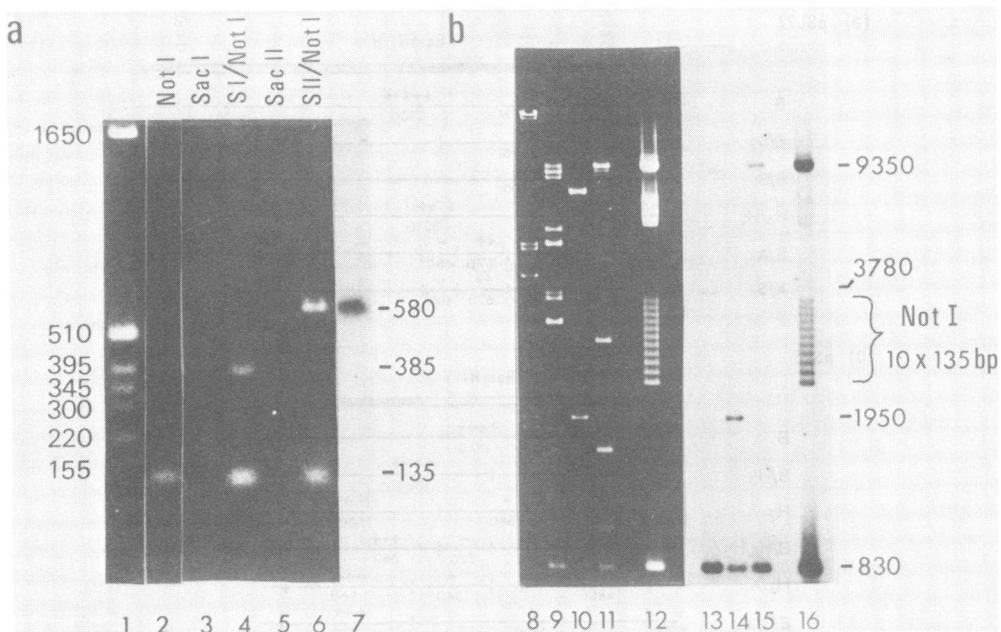
FIG. 8. Cleavage of the BamHI-H fragment using the NotI restriction enzyme and demonstration of the NotI repeat "ladder." (a) Ethidium bromide stain of pSL77 (BamHI-H) DNA cleaved with NotI (slot 2), SacI (slot 3), SacI/NotI (slot 4), SacII (slot 5), and SacII/NotI (slot 6). The autoradiograph (slot 7) shows hybridization of a total B95-8 DNA probe to a SacII/NotI digest of pSL77. The size references (slot 1) are HinfI fragments of pBR322 DNA. The DNA samples were electrophoresed through a 1.6% agarose gel for 600 V × h. Only the lower-molecular-weight region of the gel is shown. (b) Ethidium bromide stain and autoradiograph of pSL77 DNA which had been end labeled with ^{32}P at the BglII site, cleaved with HindIII, and subjected to partial digestion with HincII (slots 9 and 13), SacI (slots 10 and 14), AvaI (slots 11 and 15), or NotI (slots 12 and 16). The DNA samples were electrophoresed through a 1% agarose gel for 1,000 V × h. The constant band at 830 bp represents the HindIII/BglII fragment. Size markers were provided by an EcoRI/HindIII double digest of phage lambda DNA (slot 8).

DNA probe. Apparently, the 135-bp fragment itself has some unusual structure or base composition which interferes with either the blotting or the hybridization reaction. A further example of anomolous hybridization behavior could be seen in AvaI digests of BamHI-H (Fig. 7, slots 9 and 10). Unexpectedly, the 480-bp AvaI fragment located 3,280 to 3,760 bp from the BamHI-H to -Y junction did not self-hybridize with a B95-8 probe even though fragments which mapped on either side of it were positive (Fig. 6). This lack of hybridization was also observed with an HR-1 probe (not shown). Presumably, the 480-bp AvaI fragment also contains unusual structural features which inhibit either blotting or hybridization with the probe DNAs.

Since the HR-1 sequences extend up to the SacI site on one side of the repeat cluster and are absent 145 bp beyond the cluster on the other side (i.e., after the HincII site), it seems probable that the deletion terminates somewhere within the small repeat units themselves. Thus, the HR-1 deletion removes single-copy DNA which is bounded on either side by tandemly reiterated sequences: on the one side by

the previously described 3.1-kb BamHI repeat cluster and on the other by a newly identified set of 135-bp NotI repeats. Figure 9 presents our current understanding of the location of the HR-1 deletion and the structure of the flanking DNA. Further definition of the physical boundaries of the deletion with each set of tandem repeats will require direct analysis of cloned DNA fragments from HR-1 virus.

DISCUSSION

We have constructed relatively detailed restriction enzyme maps for the region of the EBV genome adjacent to the 3.1-kb repeat unit cluster. The BamHI-Y and -C fragments which flank the 3.1-kb internal repeats were shown to contain approximately 600 and 1,300 bp, respectively, of repeat-sequence DNA. The two flanking BamHI fragments thus contain only 60% of a complete repeat sequence. When EBV(B95-8) DNA is subjected to cleavage with the BglII restriction endonuclease, the two flanking fragments are BglII-C and BglII-A (10), and these two fragments together contain 5,050 bp of re-
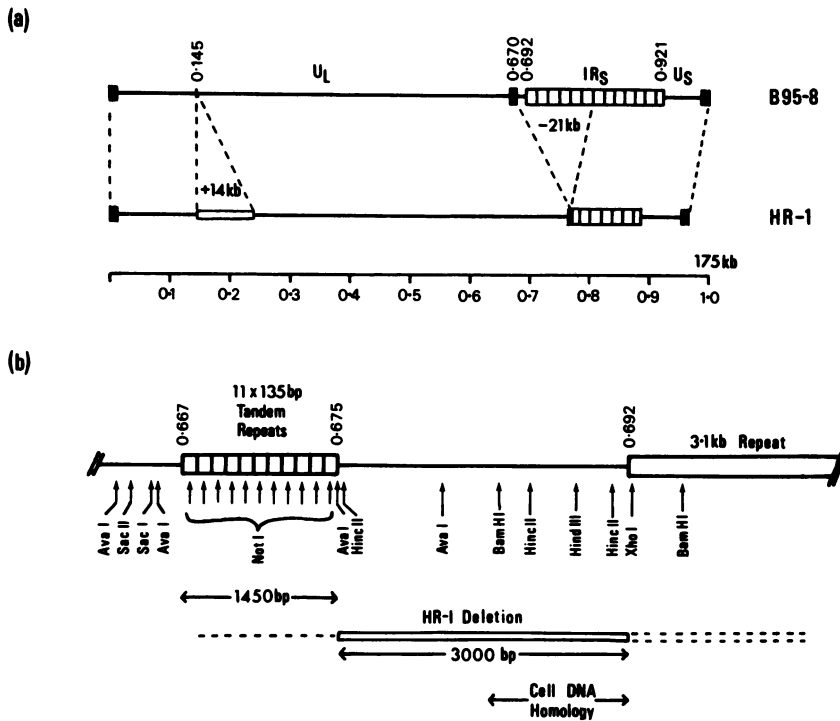
FIG. 9. Map location of the 135-bp *Not*I repeats in B95-8 DNA relative to the HR-1 deletion and 3.1-kb large internal repeats. (a) Comparison of the total size and structure of EBV(B95-8) and EBV(HR-1) genomes. The exact number of 3.1-kb repeat units missing in the HR-1 DNA is not known. In the electron microscope measurements on partially denatured molecules by Bornkamm et al. (2), the HR-1 DNA molecules can be interpreted as having four fewer repeats than B95-8. On the other hand, Rymo and Forsblum (20) suggest a difference of six copies, with HR-1 retaining only three or four. We have arbitrarily drawn six repeats in HR-1 DNA. (b) Expanded view of the region of the U$_L$ side of the 3.1-kb repeats in EBV(B95-8) that is implicated in transformation of B-lymphocytes (7). Only the position of unique-sequence DNA deleted in the HR-1 nontransforming isolate is depicted. The boundaries of the deletion within the *Not*I repeats and the 3.1-kb internal repeat sequence cluster cannot be determined by the procedures used in this work.

peat-sequence DNA or 1.6 repeat units. Thus, the 3.1-kb repeat unit cluster in B95-8 contains 12.6 tandemly duplicated copies of the repeat unit. Although the total number of 3.1-kb repeats varies from isolate to isolate, both of the isolates that we have studied in detail contain a nonintegral repeat unit number (12.6 in the case of B95-8 and 7.6 in the case of EBV [MCUV]; 10), with similar locations for the repeat-unique sequence boundaries. Whether the presence of this extra 60% of a repeat unit is important for proper gene expression from (or maintenance of) the repeat DNA cluster is unclear, but the fact that this feature is retained even when the total repeat unit number varies is suggestive of a functional significance.

EBV(HR-1) virus lacks the ability to transform lymphocytes, and this loss of "immortalizing" function appears to correlate with a characteristic deletion in the U$_L$ segment of the HR-1 genome. This HR-1 deletion terminates on one side within the 3.1-kb large internal repeat sequences, reducing the number of repeat unit copies from 12.6 in B95-8 to between 3 and 6 in HR-1 and possibly also altering the overall structure of the *Bam*HI repeat complex. In addition, the deletion removes all unique-sequence DNA from *Bam*HI-Y plus a large portion of the adjacent *Bam*HI-H fragment. We have determined that HR-1 has lost a total of 3,000 bp of single-copy DNA from U$_L$ plus an unknown amount of repeat-sequence DNA and that the deletion is bounded on either side by tandemly repeated sequences. Thus, the deletion eliminates all sequences lying between the 3.1-kb internal repeat cluster and a newly identified but apparently unrelated set of 11 135-bp repeats. We wish to emphasize that our experiments at present provide no information about whether any or all of the *Not*I repeats remain in HR-1. Since the *Bam*HI-W, -Y, and -H fragments are known to be major templates for transcription in EBV-transformed lymphocytes (13, 14), the deletion of coding sequences from within this region may

be sufficient to account for the loss of the transforming function in HR-1 virus.

The viral DNA in and around the HR-1 deletion has a number of unusual features which indicate a complex structural organization. In addition to the 3.1-kb repeats and the newly discovered *Not*I repeats, there are segments which exhibit homology to either mammalian cell DNA or other portions of the viral genome. The cloned EBV *Bam*HI-Y fragment contains sequences which hybridize with middle repetitive cellular DNA from both human and mouse genomes, and these map within the single-copy viral sequences that are lost in the HR-1 deletion (K. Peden, P. Mounts, and G. Hayward, submitted for publication). Our preliminary mapping data show that the region of *Bam*HI-H which has homology with the "additional" viral DNA at coordinate 0.14 in the W91, AG876, and FF41 isolates (6, 12, 17) lies adjacent to the *Not*I repeats but on the opposite side from the deletion. Finally, since both the B95-8 *Not*I repeats and a nearby region of *Bam*HI-H defined by a 480-bp *Ava*I fragment fail to form hybrids on filters with homologous B95-8 viral DNA probes, further unusual structural arrangements can be expected.

ADDENDUM IN PROOF

Nucleotide sequence data indicate that the *Not*I repeat unit consists of 126 bp and has a base composition of 83% guanine plus cytosine. Direct mapping of cloned EBV(HR-1) DNA fragments places the boundary of the deleted region between the *Sac*II and *Sac*I sites of EBV(B95-8) *Bam*HI-H (J. Hudewentz, U. K. Freese, U. Zimber, and G. W. Bornkamm, J. Virol., in press; D. Hayward, unpublished data). We thank Georg Bornkamm for exchanging preprints of unpublished work.

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