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Transformation by Epstein-Barr Virus Requires DNA Sequences in the Region of *Bam*HI Fragments Y and H

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Eight independent recombinant Epstein-Barr virus genomes, each of which was a transforming strain, were made by superinfecting cell lines containing Epstein-Barr virus DNA (Raji or B95-8 strain) with a nontransforming virus (P3HR1 strain). A knowledge of the constitution of each transforming recombinant allowed the localization of the defect in the genome of the nontransforming parent to a 12-megadalton sequence within the *Eco*RI A fragment. Within this region, the nontransforming virus has a deletion of the *Bam*HI Y fragment and about half of the sequences in the adjacent *Bam*HI H fragment. The present data suggest that this deletion is responsible for the nontransforming phenotype. Furthermore, mapping a deletion in one of the recombinant genomes allowed the conclusion that a sequence (comprising about 20% of the Epstein-Barr virus genome) from the center of *Bam*HI-D to *Bam*HI-I′ is not necessary for the maintenance of transformation by Epstein-Barr virus.

Epstein-Barr virus (EBV) is a herpesvirus which can cause infectious mononucleosis. For individuals with an impaired immune system, it may lead to polyclonal B-cell proliferation, e.g., fatal infectious mononucleosis or B-cell lymphoma. Burkitt's lymphoma and nasopharyngeal carcinoma are also associated with EBV. In addition, EBV causes fatal lymphoproliferation in some new world primates. In vitro, the oncogenic potential of EBV is demonstrated by its ability to transform lymphocytes into permanently growing cell lines. It transforms cells as part of its normal life cycle rather than by accident (14).

Study of the in vitro transformation of B lymphocytes by EBV is facilitated by the fortuitous event which produced the P3HR1 strain. P3HR1 is a spontaneous laboratory mutant (16) which acquired two biological properties which no other EBV isolate possesses. First, it is incapable of transforming B lymphocytes into permanent cell lines, even though it can both absorb to cells and direct synthesis of the viral nuclear antigens. Second, P3HR1 can cause an abortive infection when added to certain lymphoblastoid lines at a high multiplicity of infection. This property is best observed when the target cell is strain Raji, a cell line which has multiple episomal copies of EBV but which does not produce virus. Upon superinfection, the Raji cells produce large amounts of early antigens and viral DNA. Also, some late antigens are synthesized, and a few viral particles are secreted. It was discovered (8, 28) that some of these viruses had the ability to transform lymphocytes, but it was unclear whether these transforming progeny might contain DNA from the genome in Raji cells, from a transforming subset in the P3HR1 population, or from recombinants between the P3HR1 and the resident genomes.

A collection of several cell lines was made by using virus produced from such abortive infections. The target cells included strains Raji, NC37, and B95-8-BJAB (a result of the infection of the EBV-negative strain BJAB cell line with strain B95-8 of EBV). An examination of the *Hin*dIII fragments from the EBV DNA inside the in vitro transformed cell lines revealed that the progeny transforming virus was different from both parents and suggested that they were recombinant EBV (7, 9). We confirmed that the transforming progeny were recombinant EBV and report here the structure of a number of these recombinants. A knowledge of their composition suggests that one region of EBV DNA is essential for EBV to transform cells, whereas another region is not.

(This work was presented in part at the International Workshop on Herpesviruses, Bologna, Italy, 27 to 31 August 1981.)

MATERIALS AND METHODS

Transforming recombinant EBV was made by superinfection of either strain Raji (NC37) or B95-8-BJAB cells with strain P3HR1 of EBV, as previously described (7–9). Approximately 3 μg of DNA from each of eight independent virus-transformed cord blood cell lines made in such experiments was digested with a restriction endonuclease and electrophoresed in 0.5% agarose gels (thickness, 0.125 in. [0.318 cm]), except for *Msp*I digests for which 2% gels were used. The DNA was denatured in situ, transferred to nitrocellulose, and then hybridized with ^{32}P-labeled EBV DNA or cloned *Bam*HI fragments, as described by Skare and Strominger (25).

RESULTS

Structure of parental EBV genomes. To compare the recombinant EBV genomes with those of the input P3HR1 strain of EBV DNA and the resident viral genomes of the superinfected cells, it was necessary to have detailed maps of these EBV genomes to identify fragments which could

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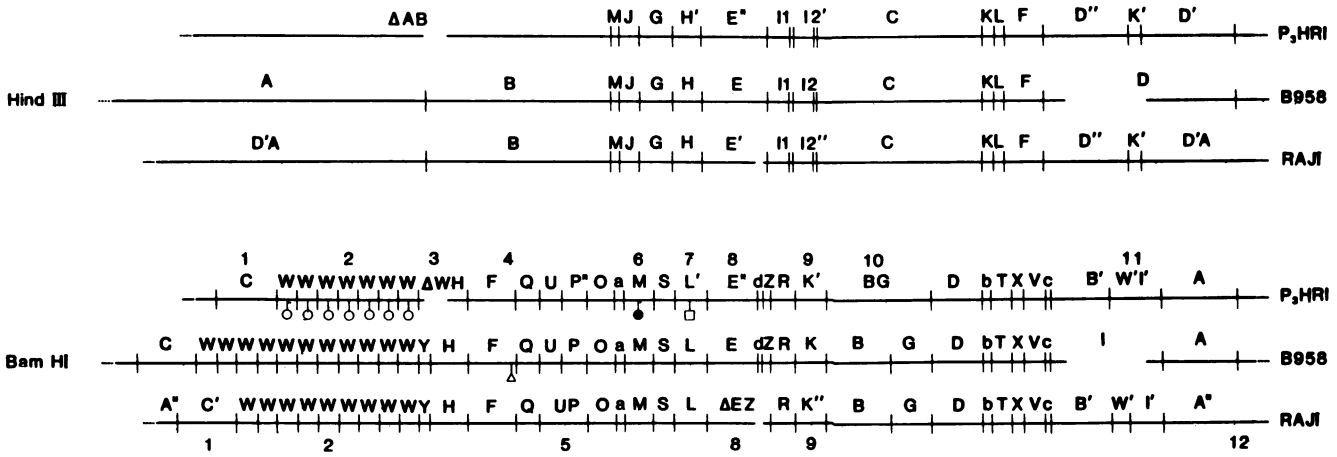


FIG. 1. Scale maps of the *Hin*dIII and *Bam*HI cleavage sites in the parental genomes. Symbols: ', the fragment is slightly smaller than that in the strain B95-8 prototype, except for fragments B', I', and W', which are not found in strain B95-8; *, the fragment is slightly larger than the corresponding fragment in strain B95-8. Numbers 1 through 12 refer to text paragraphs (i) to (xii), in which sequence differences are described. Distinguishing restriction enzyme cleavage sites are referred to as follows: ○, *Xho*I; ●, *Eco*RI; □, *Sal*I; △, *Sst*I.

distinguish among them. The EBV genomes in strain P3HR1 of EBV and the resident genomes in the target cells (strains NC37, Raji, and B95-8-BJAB) therefore were analyzed by the blot hybridization technique. The viral or cellular DNA was digested with restriction endonucleases, and the myriad fragments were electrophoresed on agarose gels. The fragments, separated on the basis of size, were denatured in the gel with alkali and then transferred to nitrocellulose filter sheets. The resulting blots were incubated with ^{32}P-labeled single-stranded EBV DNA under conditions which favored hybridization of the labeled probe to the homologous EBV fragments on the blot. Therefore, only the EBV fragments on the blot were visualized after autoradiography.

The recombinant genomes all have strain P3HR1 (the superinfecting virus) as one of their parents. The other parents are the EBV genomes inside the target cells, i.e., Raji, NC37, or B95-8-BJAB cells. The B95-8-BJAB cell line was produced by infecting the EBV-negative cell line BJAB with strain B95-8 of EBV (10). B95-8 EBV is the prototype virus whose *Bam*HI fragments have been cloned in *Escherichia coli* (1, 4, 5, 25). The EBV genomes in strains P3HR1, Raji, and NC37 needed to be characterized to the same degree of accuracy as that of strain B95-8 to determine the origin of the sequences in the recombinants. Genomes in NC37 and Raji cells appeared identical in analyses with seven different restriction endonucleases, viz., *Bam*HI, *Xho*I, *Hin*dIII, *Sal*I, *Kpn*I, *Xba*I, and *Eco*RI (data not shown). Data from another laboratory (23) also indicated that the NC37 and Raji cell lines are identical. The EBV genome in these cells will be referred to as Raji (NC37).

Fortunately, even though the genomes of strains Raji (NC37), P3HR1, and B95-8 are quite similar, there are sufficient minor differences to distinguish them at many locations. Several of these differences also have been noted by other investigators (2, 13, 22, 23). These differences will be described, starting from the left side in Fig. 1.

(i) *Bam*HI-C. A 0.2-megadalton deletion occurs in *Bam*HI-C of strain Raji (NC37) compared with *Bam*HI-C of strains B95-8 and P3HR1. The deletion lies within a 2.5-megadalton sequence near the center of *Bam*HI-C. Also, P3HR1 has a small insertion in *Bam*HI-C that can only be observed in an *Msp*I digestion; Raji and B95-8 lack this insertion. The insertion occurs in the *Msp*I B fragment derived from *Bam*HI-C. As a result, the derived *Msp*I A and B fragments comigrate in strain P3HR1.

(ii) *Bam*HI-W. To the right of *Bam*HI-C lie the *Bam*HI-W repeats. The number of tandem repeats differ in the parental genomes. It is difficult to precisely determine how many repeats there are, but we estimated the number based on apparent molar ratios of *Bam*HI-W (Fig. 1). This 2-megadalton repeat sequence contains one *Xho*I site in strains B95-8 and Raji but contains two such sites in strain P3HR1. In addition, *Bam*HI-W of B95-8 is distinguishable from those of both Raji and P3HR1 by one of its *Msp*I fragments. Strain B95-8 has a small deletion in its largest *Msp*I fragment compared with strains Raji and P3HR1.

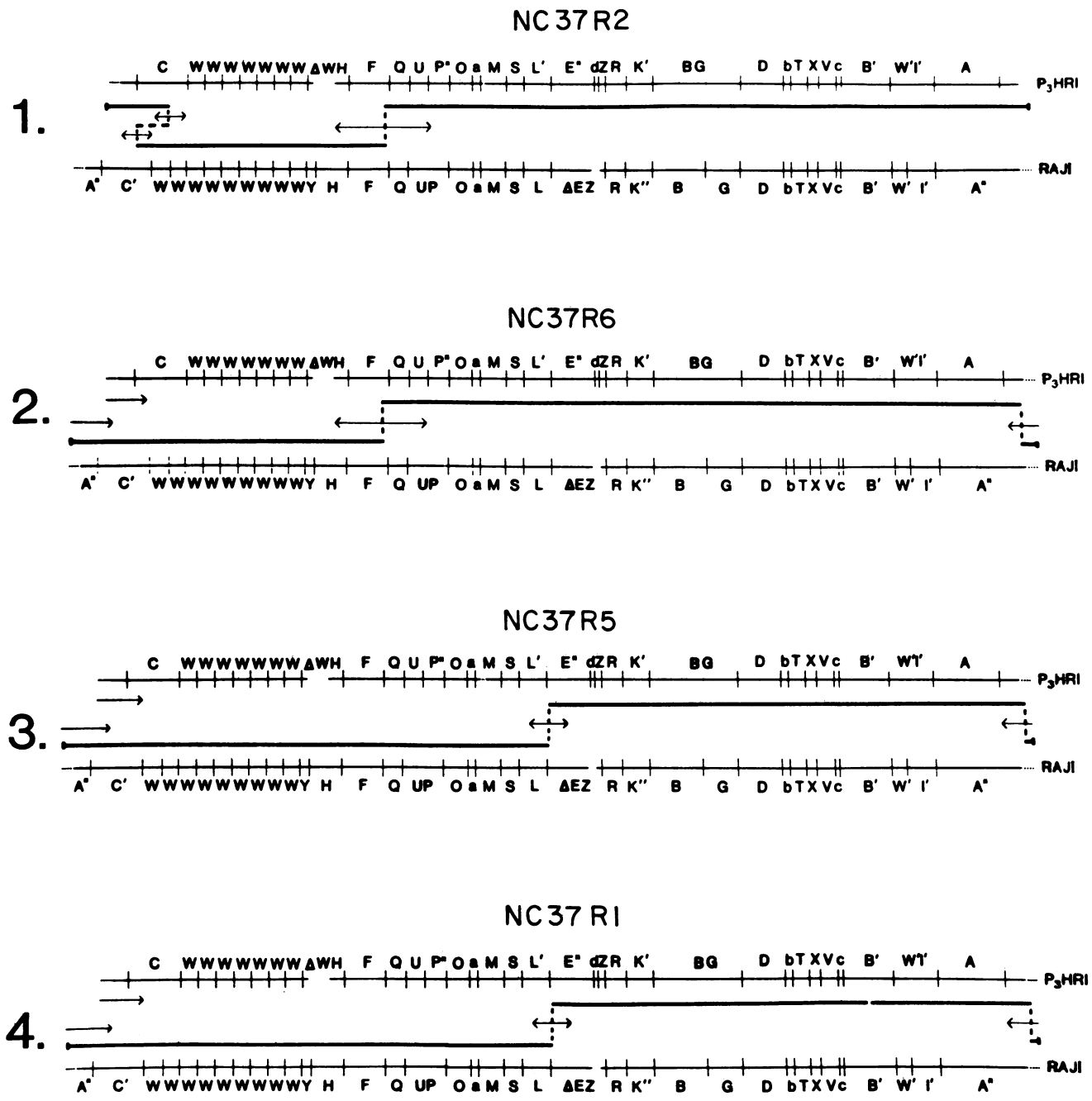
(iii) *Bam*HI-Y and -H. To the right of *Bam*HI-W, strain P3HR1 is unique in having lost about 4 megadaltons of DNA containing part of a *Bam*HI-W repeat, all of *Bam*HI-Y, and about half of *Bam*HI-H (3). This deletion, first mapped by electron microscopy, removes the *Hin*dIII site in *Bam*HI-Y from strain P3HR1 (2; also see ref. 21).

(iv) *Bam*HI-F. The *Bam*HI F fragment of strain P3HR1 also can be distinguished from that of strain Raji by digesting it with *Msp*I, in which case it appears to be missing one or two sites. The *Bam*HI F fragments from strains B95-8, P3HR1, and Raji differ in that B95-8 has an extra *Sst*I site 0.6 megadaltons from its right-hand end. As a result, a 1.6-megadalton *Sst*I fragment from strain P3HR1 begins in *Bam*HI-F and ends in *Bam*HI-Q, whereas strain B95-8 has a 0.3-megadalton *Sst*I fragment within *Bam*HI-F and a 1.3-megadalton fragment which begins in *Bam*HI-F and ends in *Bam*HI-Q.

(v) *Bam*HI-U and -P. The next difference resides in the region of *Bam*HI-U and -P. The Raji EBV genome lacks the *Bam*HI site that separates *Bam*HI-U and *Bam*HI-P. The result is a fragment that comigrates with *Bam*HI-F, which we designate UP. In addition, strain P3HR1 has a 0.1-megadalton insertion relative to strain B95-8 in *Bam*HI-P. We call the P3HR1 fragment *Bam*HI-P*. From *Sst*I mapping, it appears that *Bam*HI-P of strain B95-8 is also smaller than the corresponding sequence in strain Raji.

(vi) *Bam*HI-M. In the center of the *Bam*HI M fragment from strain P3HR1, there is an additional *Eco*RI site.

(vii) *Bam*HI-L. The *Bam*HI-L sequence of strain P3HR1 is different from that of strains B95-8 and Raji in two respects.



First, there is a 0.1-megadalton deletion, so we named the fragment from P3HR1 *Bam*HI-L'. (This deletion is also discernible in the size of the *Hin*dIII H fragment.) Second, a *Sal*I cleavage site is located 1.1 megadaltons from the left end of *Bam*HI-L'. As shown below, one of the recombinant genomes contains the *Sal*I site in *Bam*HI-L' but does not contain the deletion. Therefore, the deletion must lie in the left 1.1 megadaltons of *Bam*HI-L'.

(viii) *Bam*HI-E. Within the *Bam*HI E (or *Hin*dIII E) fragment, the *Eco*RI K fragment of strain P3HR1 has a 0.1-megadalton deletion not found in strain B95-8 or Raji. Despite this deletion, P3HR1 has a larger *Bam*HI E (or *Hin*dIII E) fragment, which we designate E*. The EBV genome in strain Raji is distinguished from those of strains B95-8 and P3HR1 in having a 0.9-megadalton deletion of part of the *Bam*HI E and Z fragments and all of the d fragment (Rymo et al., 1981). We called the fragment which results *Bam*HI-ΔEZ.

(ix) *Bam*HI-K. The *Bam*HI-K region is distinguishable for all three parental genomes. Both P3HR1 and Raji genomes have deletions with respect to the prototype B95-8. The deletion in strain P3HR1 is about 0.1 megadalton, and we call the fragment *Bam*HI-K'. In strain Raji, the deletion is 0.2 megadalton, and the fragment is called *Bam*HI-K''. These differences are also seen in the *Hin*dIII-I_2 fragment.

(x) *Bam*HI-B and -G. The *Bam*HI site between *Bam*HI-B

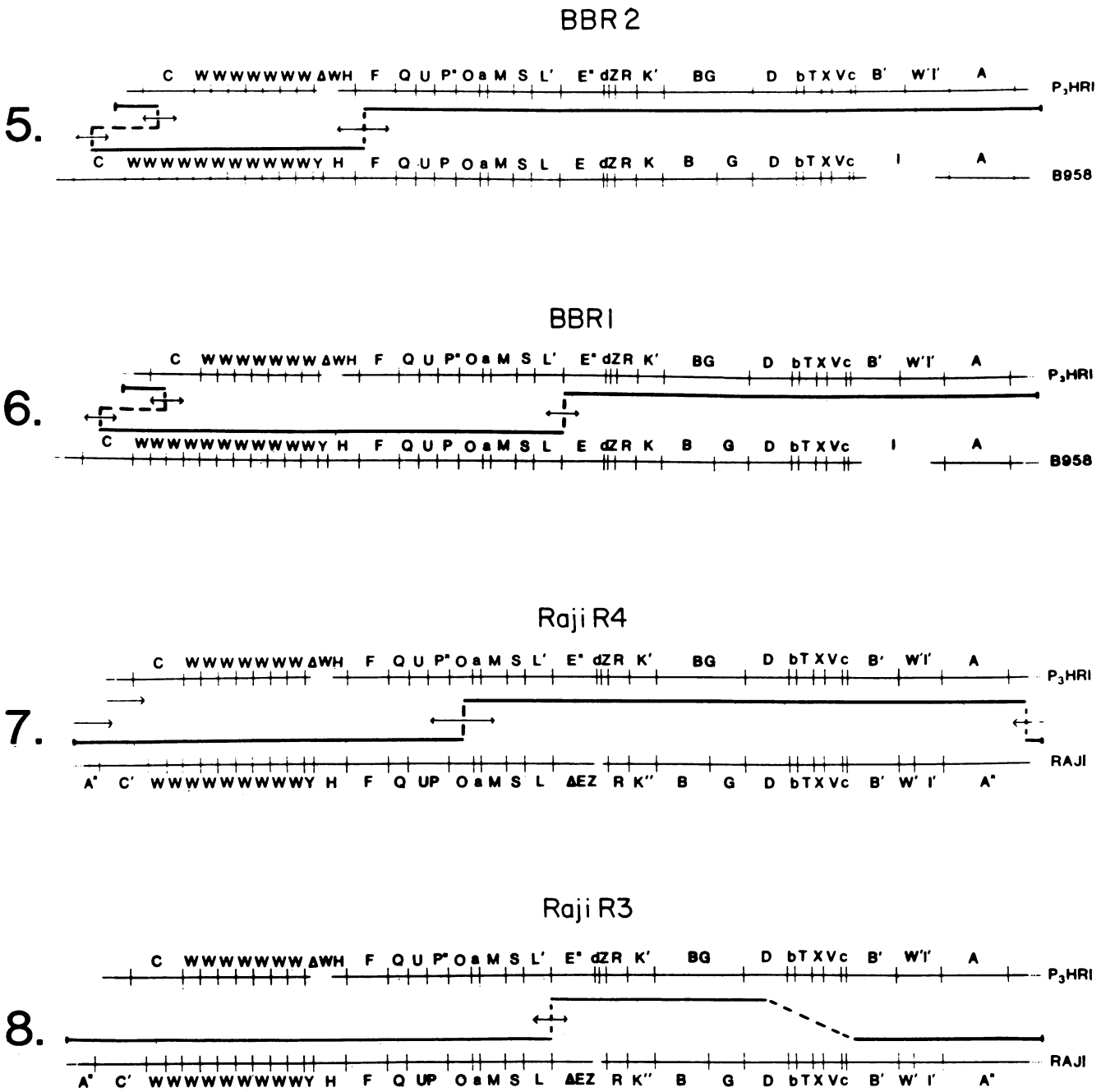


FIG. 2. Scale maps showing the composition of the recombinant EBV genomes. The length of the arrows indicate the uncertainty in the crossover points. 1, NC37R2; 2, NC37R6; 3, NC37R5; 4, NC37R1; 5, BBR2; 6, BBR1; 7, Raji R4; 8, Raji R3.

and -G is probably missing in strain P3HR1. In some digests the very large BG fusion product is less apparent than in others, and a *Bam*HI fragment that migrates at the position of *Bam*HI-G is sometimes seen, but we do not conclude from this that the *Bam*HI site between *Bam*HI-B and -G is intact in strain P3HR1.

(xi) *Bam*HI-W′ and -I′. The next observable difference in the parents is in the region deleted in strain B95-8. B95-8 acquired a 7.6-megadalton deletion (2, 20) soon after it originated (24). Within this region, a difference between strains P3HR1 and Raji exists. P3HR1 lacks the *Bam*HI site between *Bam*HI-W′ and -I′. Some digests of P3HR1 DNA have a fragment which migrates as if it were I′, but this must be a partial digestion product.

(xii) *Bam*HI-A. The right-hand end of the genome in strain Raji is easily distinguished from the ends of strains B95-8 and P3HR1. The rightmost *Bam*HI site is missing in Raji. Since the Raji genome is circular, *Bam*HI-A is fused to *Bam*HI Jhet, Nhet resulting in *Bam*HI-A*. Strains P3HR1 and B95-8 are also circular in nonproducing cells, and hence the ends are fused, resulting in NJhet. The *Hin*dIII site in the *Bam*HI A fragment of strain Raji is also lost. The *Hin*dIII D′

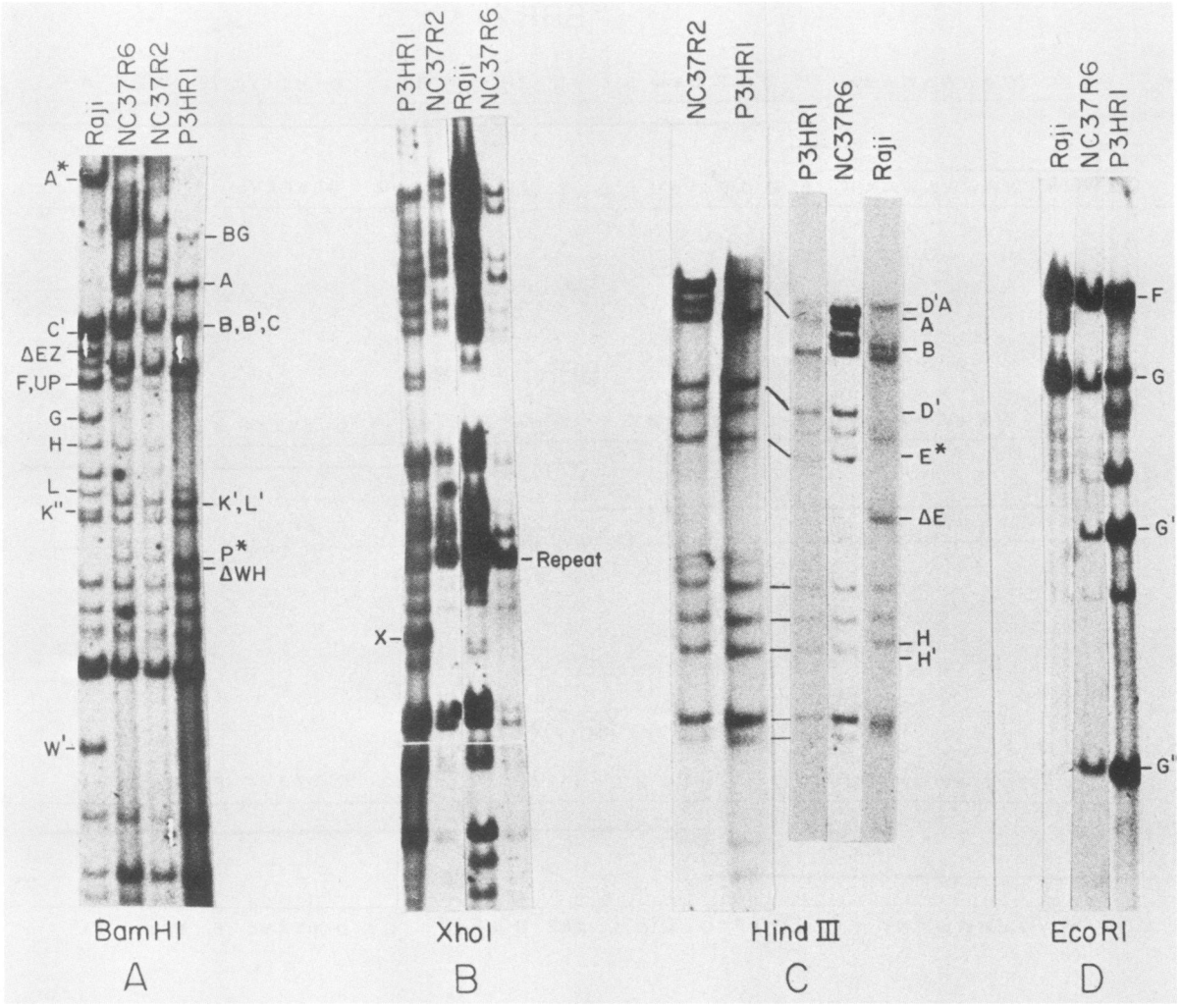


FIG. 3. Restriction endonuclease digests of strains NC37R2 and NC37R6. All digests in this and the following figures were probed with nick-translated strain P3HR1 viral DNA unless otherwise noted. The *Eco*RI digests (D) were probed with a mixture of nick-translated *Bam*HI-P, -O, -a, -M, -S, and -L.

fragment is therefore lost, and *Hin*dIII-A* is formed. The difference in the *Bam*HI-A region of Raji also can be seen in an *Xho*I digest.

Studies of recombinant EBV genomes. (i) NC37R2 (Fig. 2-1 and 3). In analyzing the genomes of the transforming recombinant strains, we focused on which fragments were derived from the NC37 endogenous genomes and which were derived from the superinfecting P3HR1 genome by using the differences described above. The cell line containing the recombinant NC37R2 EBV genome may contain the least amount of DNA from the Raji (NC37) parent and the most from P3HR1.

Starting from the left end of the physical map, strain NC37R2 has the *Bam*HI C fragment from strain P3HR1 rather than the *Bam*HI C′ fragment from strain Raji (Fig. 3A). The deletion in Raji *Bam*HI-C′ is to the right of the *Eco*RI J fragment so we know that at least the left 2 megadaltons of *Bam*HI-C in NC37R2 come from P3HR1.

The 2-megadalton tandem repeats (*Bam*HI-W) of strain NC37R2 are probably from strain Raji because they lack the additional *Xho*I site which earmarks strain P3HR1 (Fig. 1). Therefore, they contain a complete repeat fragment rather than the smaller *Xho*I fragments (Fig. 3B). It is remotely possible that NC37R2 has the *Xho*I site but cannot recognize it because it is completely methylated. However, the AW Ramos line, a long-established nonproducer cell line containing P3HR1 DNA, does have the cleavage site, and it is difficult to understand how the cellular methylase could completely methylate the site in NC37R2 but not methylate it in AW Ramos.

*Bam*HI-H (and also -Y which is difficult to see) of strain Raji (NC37) is present in strain NC37R2 (Fig. 3A), as well as in all the other recombinants (see Fig. 4 to 8). (It and all the other recombinants are also missing the *Bam*HI ΔWH fragment, characteristic of strain P3HR1.)

The next locus that distinguishes strain P3HR1 from strain Raji is the *Bam*HI site between fragments U and P (or P*). Strain Raji (NC37) lacks this site, but strain NC37R2 possesses it (Fig. 3A). The *Bam*HI site is present in NC37R2 because it contains fragment P* rather than UP. To the right of *Bam*HI-U, *Bam*HI-P*, -L′, and -K′ as well as *Hin*dIII-D′, -E*, -H′ (slightly smaller than fragment H), and other markers to the right of *Bam*HI-U (Fig. 3C) all typify strain P3HR1. Therefore, one crossover point lies near the center of *Bam*HI-C, and the other is somewhere within *Bam*HI-F, -Q, or -U (Fig. 2-1).

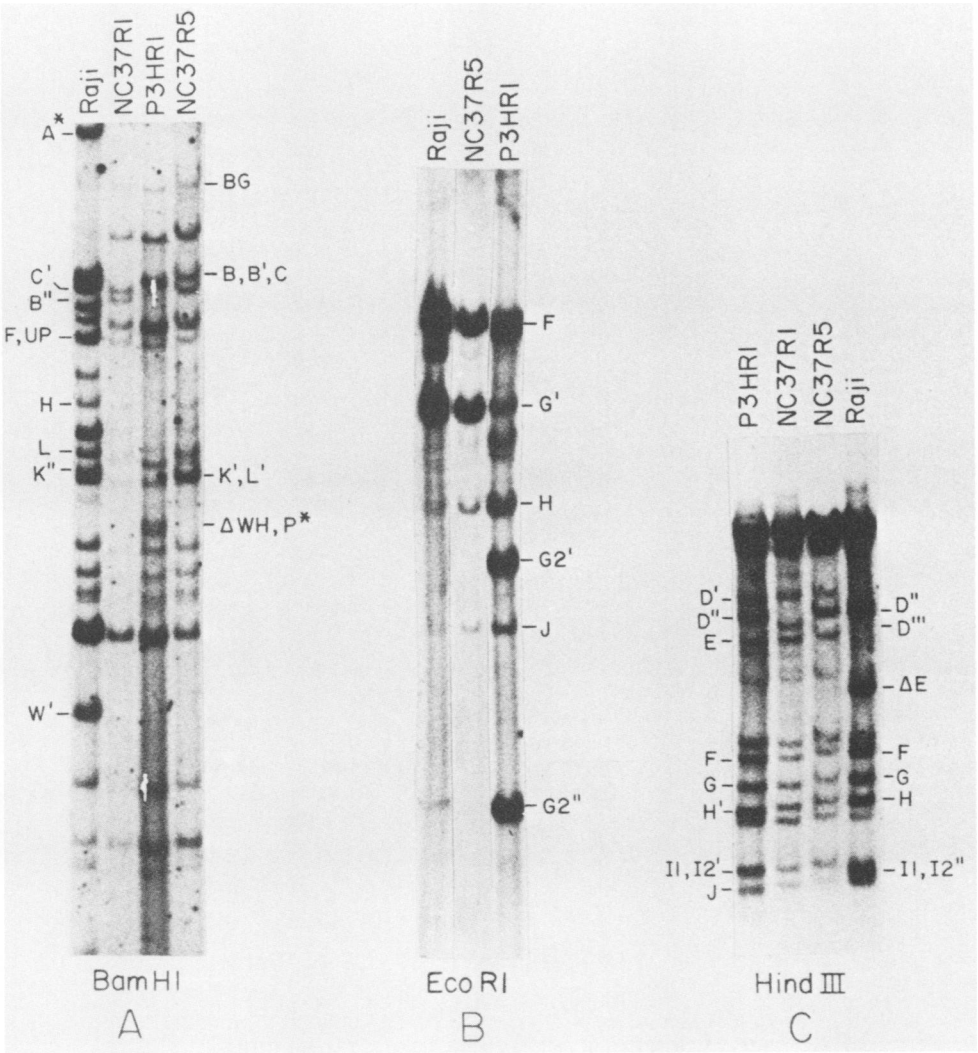


FIG. 4. Restriction endonuclease digests of strains NC37R5 and NC37R1. The *Eco*RI digests were probed with *Bam*HI-P, -O, -a, -M, -S, and -L.

(ii) NC37R6 (Fig. 2-2 and 3). Since strain NC37R6 has the *Bam*HI-C′ and H fragments of strain Raji but the A fragment of strain P3HR1 (Fig. 3A), the left-hand crossover point of NC37R6 must lie to the right of the A fragment of P3HR1 and to the left of the deletion in *Bam*HI-C′ of Raji. It is clearly different from that of NC37R2. NC37R6 is like NC37R2 in that it has a crossover site between the middle of *Bam*HI-H and the right end of *Bam*HI-U. Moreover, an *Eco*RI site in *Bam*HI-M which characterizes strain P3HR1, producing the G′ and G″ fragments, is present in strain NC37R6. All the markers to the right of *Bam*HI-M are like P3HR1. Note that the *Hin*dIII A fragment from NC37R6 is larger than that of NC37R2 (and also most other recombinants), reflecting variability in the number of the *Bam*HI-W repeats (Fig. 3C).

(iii) NC37R5 (Fig. 2-3 and 4). The NC37R5 and NC37R1 (below) recombinants differ from all other siblings in that the cells they infected produce measureable amounts of virus. The NC37R5 recombinant genome has a larger contribution from the NC37 (Raji) parent than do the siblings described above. About half of its sequences originate from strain Raji. Strain NC37R5 has *Bam*HI-C′ from Raji (Fig. 4A). The *Xho*I site in *Bam*HI-W, characteristic of P3HR1, is not found in NC37R5. *Bam*HI-P* of P3HR1 is not found, and therefore, it has *Bam*HI-UP (obscured by *Bam*HI-F). The additional *Eco*RI site in *Bam*HI-M of strain P3HR1 is not found in strain NC37R5, and thus, the *Eco*RI G2′ and G2″ fragments are not formed (Fig. 4B). Also, the deletion in *Bam*HI-L′ of P3HR1 is not present in NC37R5 which has the L fragment. (This also manifests itself in the *Hin*dIII H fragment [Fig. 4C].) However, the additional *Sal*I site in *Bam*HI-L′ of strain P3HR1 is found in strain NC37R5. This means that the junction between Raji DNA and P3HR1 DNA is in the left 1.1 megadaltons of *Bam*HI-L. All of the sequences to the right of *Bam*HI-L (starting with the *Eco*RI K′ fragment within *Bam*HI-E) appear to be derived from P3HR1. Like NC37R6 and Raji R4 (below), the second crossover site is somewhere near the termini of the molecule (between *Bam*HI-A of P3HR1 and *Bam*HI-C′ of Raji).

(iv) NC37R1 (Fig. 2-4 and 4). Physically, strain NC37R1 has been distinguished from strain NC37R5 only by the absence of about 0.4 megadalton of DNA in its *Bam*HI B′ fragment, called B″ (Fig. 4A). This deletion gives rise to a shortened *Hin*dIII D″ fragment (7), called *Hin*dIII-D‴ (Fig. 4C).

(v) BBR2 (Fig. 2-5 and 5). The BBR2 recombinant was made in B95-8-BJAB cells superinfected with strain P3HR1.

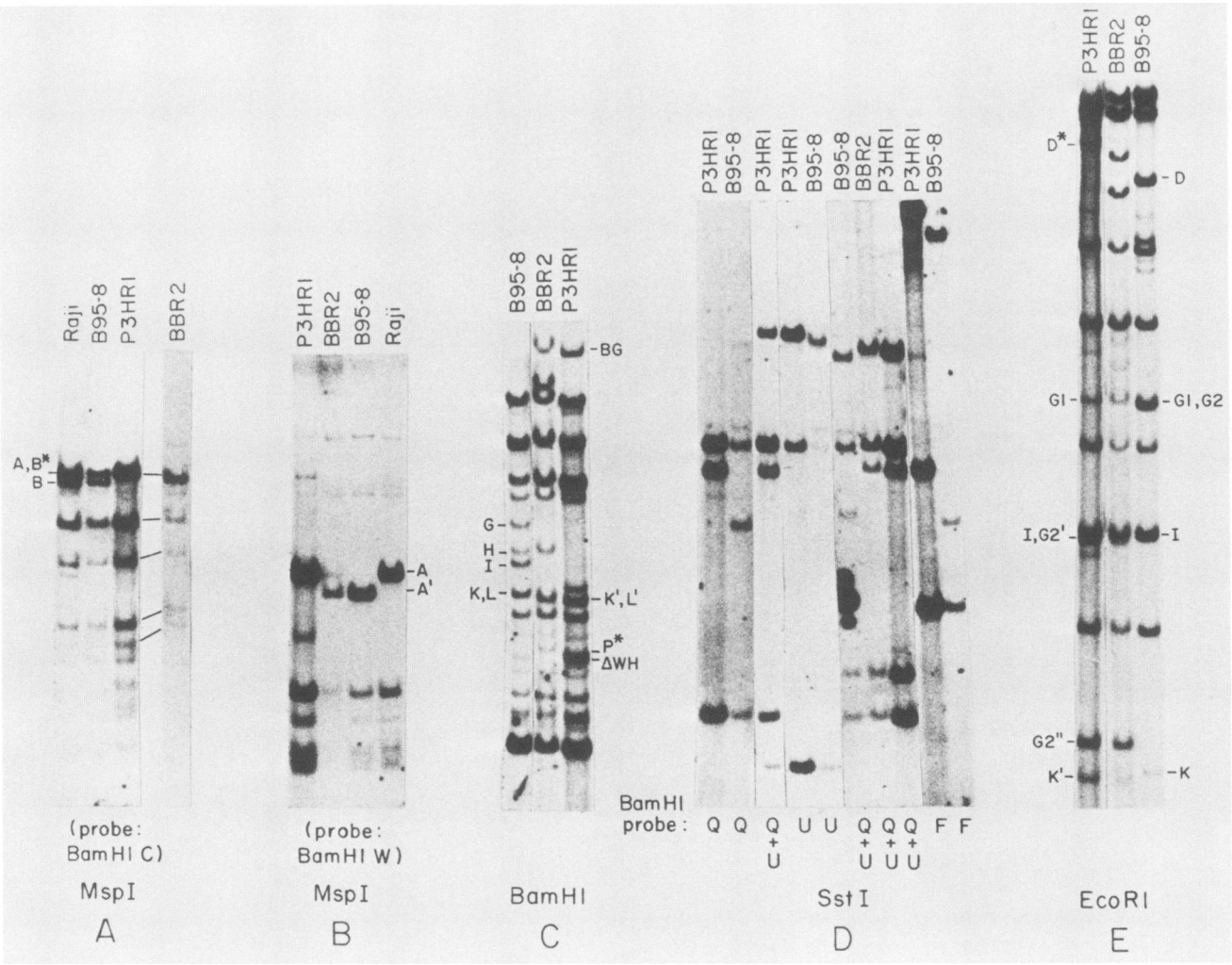


FIG. 5. Restriction endonuclease digests of strain BBR2.

Thus, this and the following recombinant BBR1 differ from all of the others in that the parental genomes are B95-8 EBV DNA (in the target cell rather than Raji EBV DNA) and P3HR1 EBV DNA. The target cell was derived from an EBV-negative B-lymphoma cell line which was subsequently infected with the B95-8 strain of EBV. Like that of Raji, the B95-8 genome is distinguished from the P3HR1 genome by a number of fragments (Fig. 1). Starting from the left end of the genome, BBR2 has the small insertion in *Bam*HI-C that can be seen in the *Msp*I digest of P3HR1 yielding the B* fragment, rather than the B fragment of B95-8 (Fig. 5A) (i.e., BBR2 and P3HR1 have two large *Msp*I A and B* comigrating fragments, whereas in B95-8, fragments A and B are resolved). Therefore, *Bam*HI-C of strain BBR2 is like that of strain P3HR1. However, *Bam*HI-W is derived from strain B95-8, based on *Msp*I digestion (Fig. 5B, A and A'). Therefore, one crossover occurred within *Bam*HI-C. BBR2 also has the *Bam*HI Y and H fragments from B95-8 rather than ΔWH of P3HR-1 (Fig. 5C).

*Bam*HI-Q hybridizes to *Sst*I fragments of 1.8, 1.6, and 0.65 megadaltons in strain P3HR1 (Fig. 5D). For strain B95-8, the sizes are 1.8, 1.3, and 0.65 megadaltons. The 1.8-megadalton fragment is homologous to *Bam*HI-U. Both the 1.6-megadalton fragment from P3HR1 and the 1.3-megadalton fragment from B95-8 are homologous to *Bam*HI-F, whereas the 0.65-megadalton fragment is entirely within *Bam*HI-Q. Digestion of the B95-8 *Bam*HI-F clone reveals that an *Sst*I site lies 0.6 megadalton from one end and that a 0.3-megadalton fragment could map adjacent to the site. *Bam*HI-Q has a site 0.7 megadalton from *Bam*HI-F. Therefore, the *Sst*I site 0.6 megadalton from the right-hand end of *Bam*HI-F is absent in strain P3HR1, thus giving rise to a 1.6-megadalton *Sst*I fragment instead of a 1.3-megadalton fragment. BBR2 DNA probed with *Bam*HI-Q and -U shows that it lacks the *Sst*I site that is 0.6 megadalton within *Bam*HI-F. This means that at least the right-hand 0.6 megadalton of *Bam*HI-F originated from the P3HR1 genome. Therefore, a crossover event occurred in the 6 megadaltons between the deletion in *Bam*HI-H and the *Sst*I site in *Bam*HI-F (Fig. 2 to 5). The entire right side of strain BBR2 is from strain P3HR1, i.e., it has (i) *Bam*HI-P* of strain P3HR1, (ii) the additional *Eco*RI site of P3HR1 that is within *Bam*HI-M (Fig. 5E), (iii) *Bam*HI-L' of P3HR1, (iv) *Eco*RI-K' within *Bam*HI-E* (Fig. 5E), (v) *Bam*HI-K' from P3HR1, (vi) *Bam*HI-BG of P3HR1, and (vii) *Bam*HI-B' and -W'I' from P3HR1 (the region in which the 7.6-megadalton deletion of strain B95-8 lies, resulting in the I fragment). This recombinant is the most informative of the genomes in that it

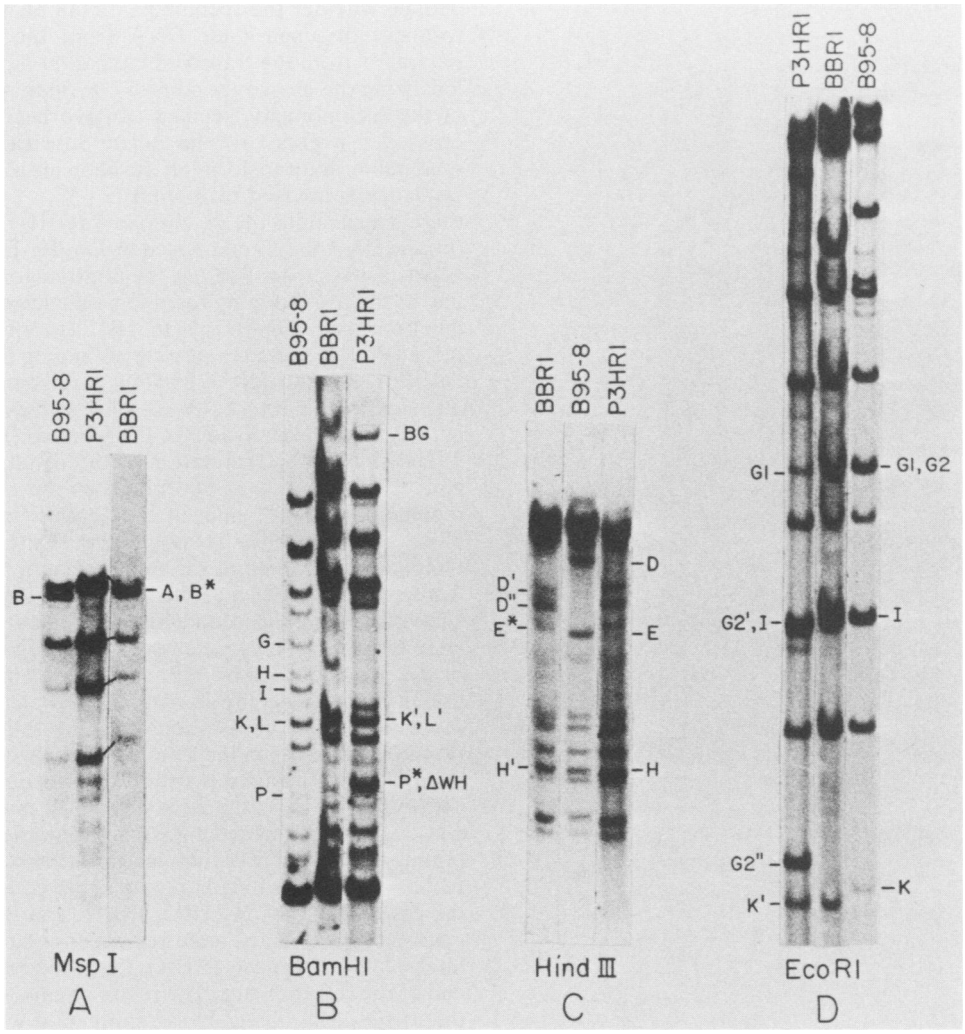


FIG. 6. Restriction endonuclease digests of strain BBR1.

has the largest residual DNA from the transformation-defective P3HR1 and the smallest contribution from a transforming parent B95-8.

(vi) BBR1 (Fig. 2-6 and 6). The BBR1 recombinant was made in the same way as BBR2, but its composition is different. Strain BBR1 has the very small insertion in *Bam*HI-C that is characteristic of strain P3HR1 and is seen in an *Msp*I digest (Fig. 6A, B*). It has B95-8 DNA from *Bam*HI-C to *Bam*HI-E, i.e., it has the *Bam*HI H, P, M, and L (*Hin*dIII H) fragments of strain B95-8 and is missing the ΔWH fragment (Fig. 6B and C). It does not have the *Eco*RI site in *Bam*HI-M that characterizes strain P3HR1 and gives rise to the *Eco*RI G2′ and G2′′ fragments (Fig. 6D), i.e., strain BBR1 has a normal G2 fragment. However, it does have the small deletion that creates *Eco*RI-K′ of P3HR1 (within *Bam*HI-E) (Fig. 6D). The deletion in *Bam*HI-L′ of P3HR1 lies to the left of the *Sal*I site (not found in BBR1) which is 1 megadalton from the left end of *Bam*HI-L′. This then localizes the crossover to a region containing 2 megadaltons of *Bam*HI-L and 1 megadalton of *Bam*HI-E. Strain BBR1 does not contain the deletion found in strain B95-8 (as indicated by the presence of the *Hin*dIII D′ and D′′ fragments characteristic of strain P3HR1, rather than the D fragment from B95-8 [Fig. 6C]). The left-hand crossover event lies to the right of the small insertion that strain P3HR1 has in *Bam*HI-C as in the case of strain BBR2.

(vii) Raji R4 (Fig. 2-7 and 7). The *Bam*HI digest of strain Raji R4 reveals that it has the *Bam*HI C′ fragment of strain Raji (Fig. 7A). Raji R4 seems to lack *Bam*HI-P* from strain P3HR1, but it inherited the *Eco*RI site in *Bam*HI-M from P3HR1 (Fig. 7B), so there is a crossover event between *Bam*HI-U and the center of *Bam*HI-M. P3HR1 fragments constitute the right-hand portion of Raji R4 (i.e., *Bam*HI-L′, -E*, -K′, -BG, -W′I′, and -A) so the other crossover must have occurred between *Bam*HI-A of P3HR1 and the small deletion in *Bam*HI-C′ of Raji.

(viii) Raji R3 (Fig. 2-8 and 8). The Raji R3 recombinant is the most unusual in the entire series. All of the sequences from *Bam*HI-C′ through *Bam*HI-L (*Hin*dIII-H), including *Bam*HI-H, -UP (P* absent), and -M, are characteristic of strain Raji (Fig. 8A and B). It is clear that strain Raji R3 did not inherit the deletion in *Bam*HI-E that is characteristic of strain Raji. This can be seen from the mobilities of *Hin*dIII-E and *Bam*HI-E of Raji R3. However, the *Eco*RI K fragment of Raji R3 is derived from Raji (Fig. 8C). Therefore, the crossover event took place between *Eco*RI-K (within *Bam*HI-E) and the deletion in *Bam*HI-E. *Bam*HI-K′ and *Bam*HI-G are from strain P3HR1. The peculiar structure of Raji R3 is

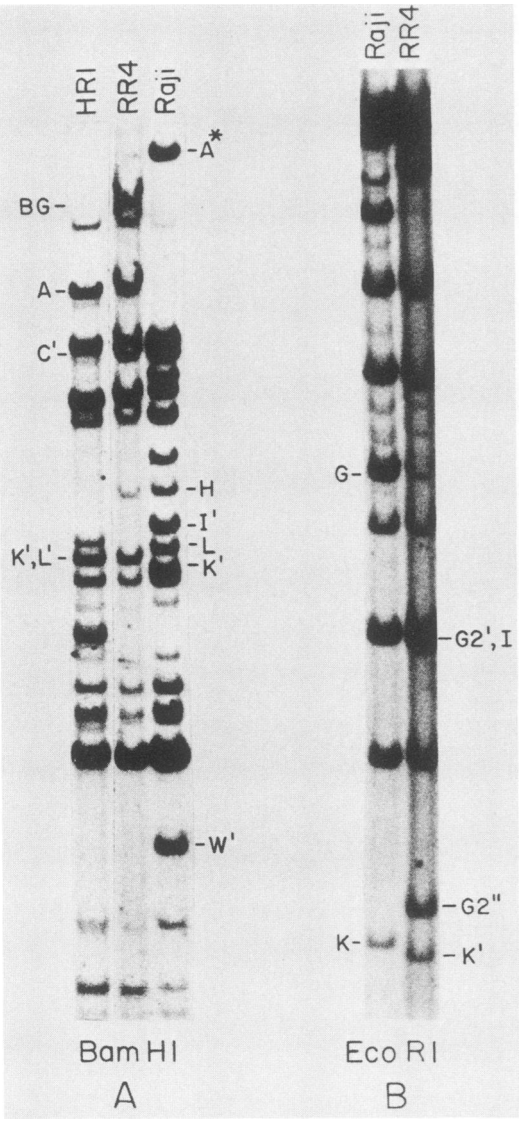


FIG. 7. Restriction endonuclease digests of strain Raji R4.

demonstrated by its absence of *Hin*dIII-F, -D', and -D'' (Fig. 8B). In *Eco*RI digests, *Eco*RI-E and -H are absent. Also, *Bam*HI-X is missing, and *Bam*HI-ΔDB' of 6.8 megadaltons is formed. There appears to be a deletion in Raji R3 from within *Bam*HI-D to within *Bam*HI-B', and the resultant fragment is *Bam*HI-ΔDB' (Fig. 8A). Cleavage of Raji R3 DNA with *Sst*I and probing with *Bam*HI-D or -B' reveals that almost half of *Bam*HI-D is missing and part of *Bam*HI-B' is gone (Fig. 8D). To the right of the deletion are the Raji *Bam*HI W', I', and A* fragments (Fig. 8A). These data indicate that Raji R3 has P3HR1 DNA to the left of the deletion and Raji DNA to the right.

DISCUSSION

The genomes of a number of recombinants between a nontransforming mutant (P3HR1) of EBV and two other strains (Raji and B95-8) have been characterized. Restriction endonuclease analysis revealed several small insertions and deletions and the loss of some restriction endonuclease cleavage sites which serve to distinguish one parent of the recombinants from the other. Therefore, it was possible to deduce whether the recombinants (all of which were transforming) obtained their DNA from the nontransforming mutant or from the transforming parent at several loci, thus localizing the crossover points. The findings were that (i) all of the recombinants seemed to have been created by two crossover events; (ii) the defect in strain P3HR1 which renders it nontransforming resides in 12 megadaltons of sequence composed of *Bam*HI-G, -W, -Y, -H, and -F (and therefore includes the deletion in *Bam*HI-Y and -H) (Fig. 9); (iii) the DNA that is composed of *Bam*HI-E through *Bam*HI-D was always from P3HR1, the nontransforming parent; (iv) one of the transforming recombinants acquired a substantial deletion from *Bam*HI-D to -B'; (v) the number of 2-megadalton tandem repeats varied among the recombinants; and (vi) the Raji (NC37) parent has some substituted sequences in the *Bam*HI-A region. (These findings are discussed below in the same order as above.)

The simplest interpretation of the results is that homologous recombination at two points in the genome generated recombinant EBV, and then infection of umbilical cord blood selected for the subpopulation that could transform B lymphocytes. (Some of the recombinants had lost either an *Xho*I site in *Bam*HI-S or the *Sal*I site in *Bam*HI-L [data not shown].) From these data one could conclude that there were several closely spaced crossover points near the center of the genome in some of the recombinants. However, we prefer to attribute the loss of *Sal*I or *Xho*I sites in some recombinants to methylation of the cytosine at the recognition site. *Sal*I sites in the genome of nonproducing cells have been noted to become resistant to digestion with time (19). The observation that there is never just one crossover point was expected, since the resident genome is a covalently closed circle. Two recombination events are required to reconstruct a unit-length genome that can be packaged. In the cases of strains NC37R2, BBR1, and BBR2, the P3HR1 parental genome must have been in circular form (replicative intermediate) because P3HR1 sequences are found at each end of the recombinant. The recombinants would seem to be the result of homologous recombination with the exception of Raji R3, which may have been formed by illegitimate recombination between sequences in *Bam*HI-D of P3HR1 and *Bam*HI-B of Raji, resulting in a recombinant that lacked sequences from *Bam*HI-D to *Bam*HI-B'.

The data of strains NC37R2 and BBR2 show that a recombinant which had Raji or B95-8 DNA from somewhere near the center of *Bam*HI-C to a point 4 megadaltons to the right of *Bam*HI-H but had the remainder of its genome from strain P3HR1 could transform B lymphocytes. Since the right half of *Bam*HI-C is largely composed of *Bam*HI-W sequences, we consider it most likely that all the sequences to the left of the *Bam*HI-W repeat in these recombinants are derived from strain P3HR1. Therefore, the defect in P3HR1 must lie within that 11-megadalton sequence. Within this sequence, P3HR1 has lost *Bam*HI-Y and half of *Bam*HI-H, including the 123-base-pair tandem repeats (3, 12). Moreover, Jijoye, the parental cell line of P3HR1, does not have this deletion (22). These data, therefore, lend strong support to the hypothesis of Fresen et al. (9) that the deletion is the defect which results in loss of transforming ability in strain P3HR1. Marker rescue with the ligated *Bam*HI YH fragment would provide unambiguous proof of this hypothesis. Of course, additional loci outside the 11-megadalton sequence may also be necessary for transformation.

Some gene(s) in the *Bam*HI W, Y, H, or F fragment may be required for either the initiation or the maintenance of transformation. One might argue that this gene provides

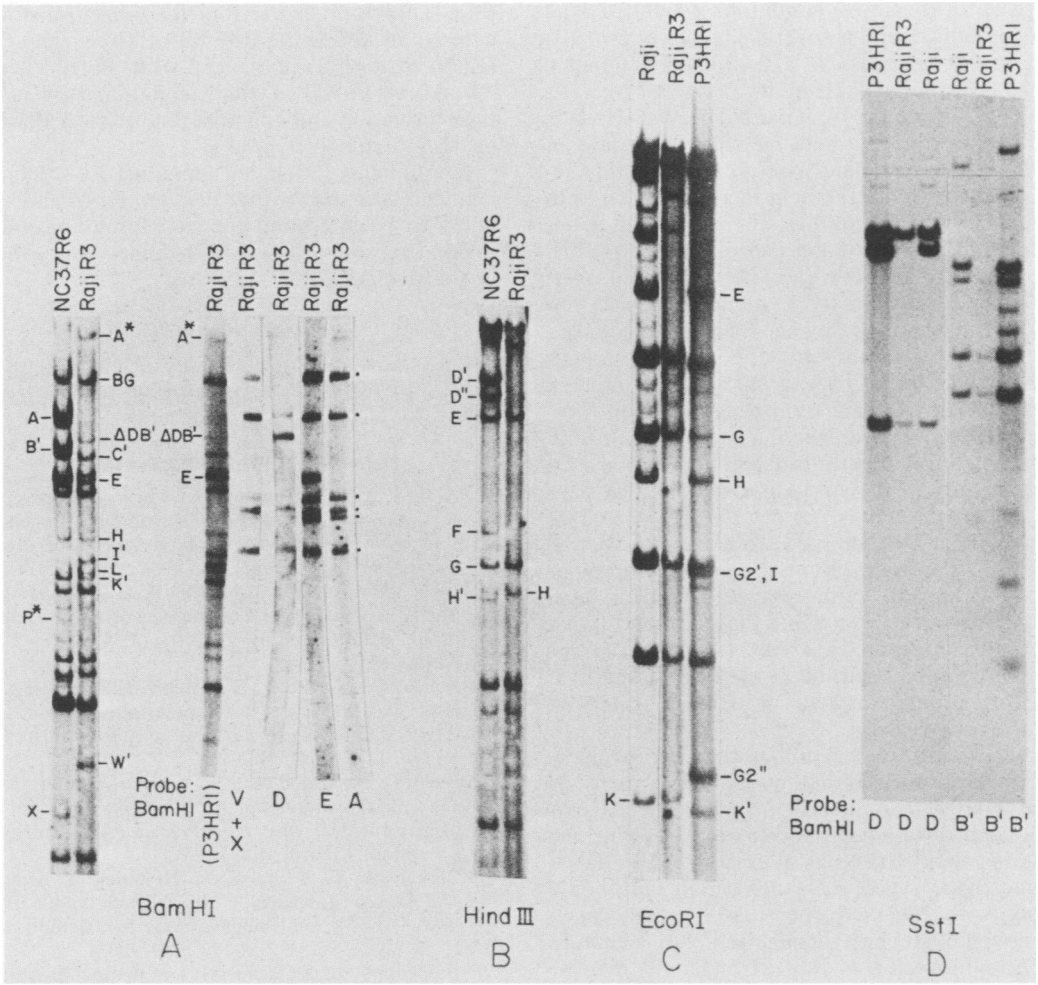


FIG. 8. Restriction endonuclease digests of strain Raji R3. The fragments indicated with dots in panel A, lane a, are probably mitochondrial DNA that is partly homologous to *E. coli* DNA in our probes.

some function required for the initiation of transformation because the cell line carrying the P3HR1 genome is transformed even though P3HR1 virus can no longer transform. However, it is possible that, in addition to the defective episomal P3HR1 genomes, this line may also carry a nondefective integrated genome responsible for transformation or, more likely, that some mutation(s) may have occurred in the cellular DNA of this cell line, eliminating the need for the EBV transforming gene(s) (as presumably occurs on BJAB or RAMOS cells, both derived from EBV-negative Burkitt's lymphoma). Latently infected (i.e., transformed) lymphocytes produce a small amount of a 3-kilobase (kb) mRNA that seems to result from splicing a primary transcript with a 5′ end about 1.8 kb into the *Bam*HI W fragment and a 3′ end that is about 1.3 kb into the *Bam*HI H fragment (27). This transcript clearly will be absent in P3HR1-infected cells and would be an excellent candidate for a function needed in the maintenance of transformation,

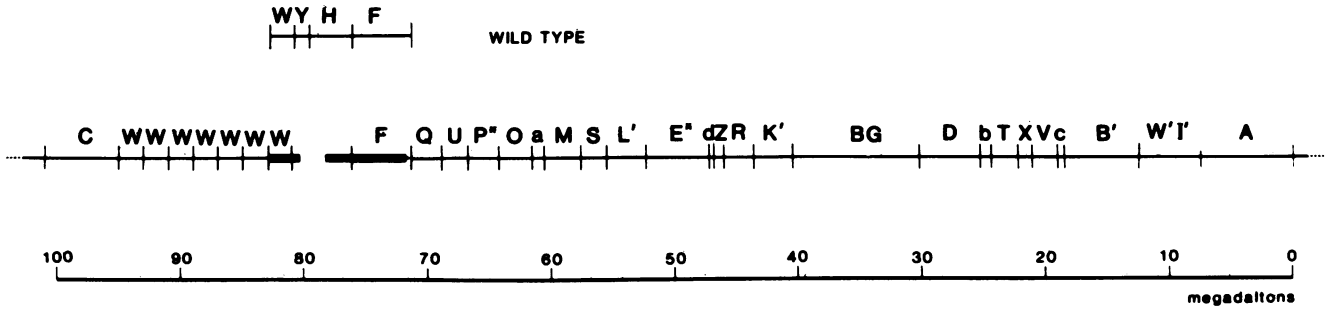


FIG. 9. Localization of the defect which renders strain P3HR1 nontransforming.

provided that either of the above conditions is fulfilled. The mature mRNA probably contains several portions of *Bam*HI-W totalling about 0.9 kb and also a contiguous sequence corresponding to 0.6 kb of *Bam*HI-Y and 1.3 kb of *Bam*HI-H. The portion of the 3-kb mRNA derived from *Bam*HI-H is not likely to code for a protein because different strains of transforming EBV have completely different nucleotide sequences from a point in *Bam*HI-Y near its right-hand end to a point more than 1.2 kb into *Bam*HI-H (3, 18). (mRNA is also transcribed from the right-hand end of *Bam*HI-H [17], but it seems to be a lytic function rather than a latent one.) The first EBV-induced protein to be found in latently infected cells was the nuclear antigen EBNA (also called EBNA 1). There are two reasons to suppose that the 3-kb mRNA does not code for EBNA. First, P3HR1-infected cells have EBNA, as judged by anticomplement immunofluorescence. Second, transfection of cells with both the *Bam*HI K and M fragments results in expression of a nuclear antigen detectable by anti-EBNA-containing sera (11, 26), and strain P3HR1 has normal *Bam*HI K and M fragments. The 3-kb mRNA derived from *Bam*HI-W, -Y, and -H could specify a protein with poor immunogenicity, or this mRNA could act in transformation without specifying a protein. However, recent data indicate that a second transformation-specific protein called EBNA 2 is encoded in this region (6, 15). This protein is absent in the P3HR1 cell line. Since only occasional antisera have anti-EBNA 2 titers, it may be of low immunogenicity.

An unexpected finding is that all of the recombinants (except Raji R3, as discussed below) obtained their sequences from *Bam*HI-E to *Bam*HI-A from the P3HR1 parent. Strain Raji itself does not produce virions (despite the fact that it contains about 60 copies of episomal EBV DNA per cell) and may have a defect in this region that would prevent progeny with Raji sequences from transforming lymphocytes or would prevent packaging of Raji genomes into virus; the small deletion in Raji ΔE2 (Fig. 1) may be significant in this connection. Strain Raji is a nonproducer cell line, even on stimulation with phorbol esters. However, the two recombinants between strains B95-8 and P3HR1 have a similar construction to the Raji-P3HR1 recombinants, despite the fact that the genome in B95-8-BJAB should have no defects. It would seem odd that none of the viruses which transformed the umbilical cord lymphocytes were derived entirely from the resident (presumably transforming) genomes since they should have been encapsidated as well as the recombinant genomes. The region from *Bam*HI-E to *Bam*HI-A may be repressed in the resident genome of strain Raji (as a result of methylation or nucleosome structure for example), and the recombinant may survive only if it has derepressed DNA in this region. Some of the recombinants inherited both their *Bam*HI M and K fragments from strain P3HR1, which suggests that P3HR1 has functional EBNAs.

The deletion in Raji R3 runs from *Bam*HI-D to -B′ and thus is the largest deletion yet described in EBV. This deletion may have occurred either before or after the recombinant viruses transformed its host cell. If it occurred before transformation, then the region from *Bam*HI-D to *Bam*HI-B′ is not necessary for transformation. If it occurred after the cell was transformed, then at least these sequences cannot be involved in maintenance of the transformed state. In addition, the deletion in B95-8 from *Bam*HI-B′ to *Bam*HI-I′ (Fig. 1) has no effect on transformation. Therefore, a continuous sequence of more than 16 megadaltons from *Bam*HI-D to -I′ is not necessary for transformation. The Raji R3 genome gets its *Bam*HI W′, I′, and A* fragments from Raji. Therefore, if the Raji R3 virus transformed lymphocytes to establish the Raji R3 cell line, then the *Bam*HI-A* region of Raji (as compared with the A fragment in strain P3HR1 or B95-8) is either functional or unnecessary for transformation and amplification of the EBV genome to the usual 5 to 20 copies per cell.

In summary, detailed mapping of recombinant EBV genomes has shown that the *Bam*HI-Y and -H regions are likely to be important for transformation and that a large region from *Bam*HI-D to -I′ is unnecessary for maintenance of transformation.

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