Mutants of Epstein-Barr Virus with a Selective Marker Disrupting the TP Gene Transform B Cells and Replicate Normally in Culture

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We have isolated mutants of Epstein-Barr virus (EBV) which carry a dominant selectable marker inserted into the third exon of the gene encoding two membrane proteins, TP1 and TP2 (or LMP2A and LMP2B), which are expressed in latently infected, growth-transformed B cells. One of the mutants also acquired a 260-bp deletion beginning in the first intron a few base pairs from the terminal repeats and removing most of the second TP exon, including the initial coding sequences of TP2. These EBV mutants transform human B cells in culture, and the transformed B-cell clones carrying them release EBV at approximately normal frequencies.

Epstein-Barr virus (EBV) is a human herpesvirus that has been implicated in several forms of cancer. In vitro, EBV efficiently infects human B cells latently and stimulates them to proliferate indefinitely in culture. Nine EBV-encoded proteins, six which localize to the nucleus and three which localize to the plasma membrane, are expressed in such transformed B-cell lines and are presumed to contribute to growth transformation or to latency (for reviews, see references 23 and 41).

Two related proteins expressed in latently infected B cells are of particular interest because of known biochemical properties or sequence features. These proteins, TP1 and TP2, the terminally encoded proteins (also called latent membrane proteins LMP2A and -2B), are expressed from mRNAs that are formed by transcription and splicing across the joined terminal repeats (TRs) of circularized viral genomes (26, 27, 48). As illustrated in Fig. 1, distinct promoters and initial exons for TP1 and TP2 on one side of the TRs are joined to eight common exons on the other side of the TRs. The common TP exons encode 378 amino acids that are mostly hydrophobic and could potentially form a tertiary structure which would span a lipid bilayer 12 times (27). Families of membrane transporter proteins appear to have 12 membrane-spanning domains but also have more prominent extracellular and intracellular extensions between the membrane-spanning segments than appear in the TP sequence (14, 44). While the first exon of TP2 is noncoding, the first exon of TP1 adds a hydrophilic cytoplasmic extension to this membrane protein.

TP1, through its amino-terminal cytoplasmic extension, has the ability to associate with tyrosine kinases of the *src* family and to serve as a substrate for these kinases in vivo and in vitro (6, 30). It was recently shown that the expression of TP1 in an EBV-negative B-lymphoma cell line could block calcium mobilization following cross-linking of cell surface molecules (40). TP-specific antisera, which primarily detected TP1 in cells, revealed punctate patching on the cytoplasmic membrane with the appearance of a polar localization on cells, a pattern that largely overlapped the localization of the other latently expressed EBV-encoded membrane protein, LMP (or LMP1). Another property common to TP1 and LMP is that each has a relatively short metabolic lifespan (1, 35, 47), suggesting that both proteins might be degraded through a similar pathway

related to the observed membrane patching. LMP can transform the growth and morphological properties of cells of fibroblast or epithelial lineages (2, 10, 12, 58, 61) and can induce activation-associated surface molecules when expressed in EBV-negative B-lymphoma cell lines, almost certainly implicating it in growth transformation of B cells by EBV (59). In contrast, there have been no reports of phenotypic changes related to cell growth having been caused by the introduction of TP genes into cells.

In recent years, methods have arisen to permit a genetic analysis of EBV's capacity to transform B cells (17, 28, 29, 36-38, 48, 54, 56, 60). To test whether TP1 and TP2 contribute to the phenotype of infection of B cells in culture, we constructed EBV mutants in which the TP gene was disrupted by the insertion of a 2.9-kb selective marker so that neither TP1 nor TP2 could be synthesized. These mutants exhibited normal phenotypes for transformation of B cells and replication in culture.

Generation of TP mutants. We tested whether the intact TP gene was important for transformation of B cells by EBV by attempting to isolate B-cell transformants infected by EBV mutants carrying a selective marker inserted into the TP gene. This was done by first constructing a plasmid carrying the TP gene with a G418 resistance marker inserted into its third exon and then using the plasmid to transfer the mutation to the EBV genome by homologous recombination in virus-producing B95-8 cells, by methods established previously (28). In p575, shown in Fig. 1, the third exon of the TP gene has the CMVIE-neo selective marker inserted at a former *PvuII* site; flanking EBV sequences extend past the fused TRs on one side and just past oriP on the other side. The plasmid was constructed to carry the EBV lytic replication origin because this was found to increase the frequency of homologous recombination between introduced plasmids and EBV (28).

B95-8 cells were electroporated with a mixture of p575 and pCMV-BZLF1 (16), the latter serving to induce lytic replication in successfully electroporated cells. Culture medium was harvested from the cells 4 days later, passed through a 0.2- μ m-pore-size filter, and used to infect T-cell-depleted leukocytes obtained from adult peripheral blood. Leukocytes were cultured in 24-well plates over gamma-irradiated human fibroblasts at 7×10^4 leukocytes per well and 0.1 ml of supernatant from electroporated B95-8 cells per well. After 2 to 3 weeks, when the growth of transformed B cells had acidified the

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FIG. 1. Structures of the EBV genome in the vicinity of the TP gene and of the plasmid, p575, which was used to generate TP-disrupted mutants. (A) The ends of the EBV genome are shown joined with four TRs. Exons for the TP and LMP genes are indicated by black rectangles if they code for the protein product and by open rectangles if they are noncoding and are joined together by lines to indicate the patterns of transcription and splicing. Bent arrows pointing left or right indicate the positions and orientations of latent promoters. OriP and part of the first IR1 repeat are also indicated. Below, the positions of cleavage sites for *EcoRI* (E), *BamHI* (B), and *HindIII* (H) are indicated. DNA fragments used as hybridization probes, probe 1 (*StuI-SalI*) and probe 2 (*SalI-BamHI*), are shown. (B) p575 is shown linearized at an *EcoRI* site (restriction sites are indicated as in panel A). It includes EBV sequences spanning the fused TRs, from 169,397 (*StuI*) to 9,134 (*HpaI*). The CMVIE-neo selective marker (28) was inserted at a *PvuII* site (nucleotide 434) in the third TP exon, generating *BamHI* sites at both ends of the inserted DNA. Bacterial vector sequences are indicated by closer parallel lines with vertical bars. To construct p575, cloned EBV DNA segments were assembled from plasmid clones (53) and from a bacteriophage lambda clone, EB0-8 (5), and placed on the cosmid vector p553, which carries *oriLyt* of EBV (28).

medium, some of the medium in each well was replaced with medium containing G418 to achieve a final concentration of 750 μ g (activity) per ml. Eight G418-resistant B-cell clones appeared from a total of 600 wells tested. Five clones (A through E) were analyzed; the corresponding EBV isolates carrying the CMVIE-neo marker were called B95-575A through B95-575E.

Structures of TP-disrupted EBV genomes. To investigate the structures of the viral genomes present in the selected B-cell clones, DNA from the expanded clones was cut with *EcoRI* and subjected to Southern analysis. As shown in Fig. 2A, DNA from B-cell clone A lacked the normal 4.2-kb *EcoRI* I fragment that spans most of the TP gene; in its place was observed the altered *EcoRI* fragment carrying the CMVIE-neo insertion in exon 3 (I-neo fragment), present at the level of several copies per cell. DNA from clone D contained the I-neo fragment at multiple copies per cell, but the normal *EcoRI* I fragment was also weakly visible, indicating that this cell line carried parental B95-8 genomes at a fraction of a copy per cell in addition to carrying multiple TP-disrupted genomes per cell (Fig. 2B).

DNA from clone B contained the normal *EcoRI* fragment, indicating the presence of a normal B95-8 genome as well as an altered *EcoRI* I-homologous fragment close to 20 kb in length (Fig. 2A and B). To separate the mutant EBV from the parent virus carried in clone B, secondary B-cell transformants were

obtained by cocultivating lethally gamma-irradiated cells of clone B with peripheral blood B cells. DNA from four G418-resistant secondary transformants was analyzed and found in each case to lack the normal *EcoRI* I fragment but to contain the large I-homologous fragment in sizes that varied by as much as 2 kb (Fig. 2B). Reprobing the blot of Fig. 2B sequentially with labeled CMVIE-neo DNA and TR DNA identified the same large, variably sized band as containing these sequences (data not shown), suggesting that the *EcoRI* site at the beginning of the B95-8 sequence was missing in this EBV mutant, fusing the I-neo fragment to the terminal joint fragment, D-het, in the circularized viral genome. When these same DNAs were cut with *BamHI* and probed with the TRs, the terminal joint fragments were observed in the expected variable sizes (Fig. 2C). The *BamHI* joint fragments were made smaller by the CMVIE-neo insertion because *BamHI* sites flank the inserted DNA (Fig. 1). Careful comparison of the size of these joint fragments with the ladder of B95-8 terminal fragments indicated that a deletion in B95-575B between the TRs and the inserted marker, presumably including the *EcoRI* site in this region, was close to half the length of one TR (or 1.5 or 2.5, etc., times the length).

With primers specific to TR sequences and sequences at the site of the inserted marker, this region of DNA was amplified by the polymerase chain reaction (PCR) from one of the secondary transformants of B95-575B and sequenced. The

FIG. 2. Southern analysis of EBV genomes carried in four primary (1°) B-cell clones obtained with B95-575 mutant EBV (clones A, B, C, and D) and four secondary (2°) clones obtained with B95-575B. Total DNAs from the cell clones were digested with restriction enzymes, separated on 0.7% agarose gels, transferred to nylon membranes, and probed with randomly prime-labeled probe 1 or 2, shown in Fig. 1. Included for comparison are DNA from DF2, a B-cell clone immortalized with B95-8 virus; DNA from B95-8 cells (42) and from Akata cells (55); and p575 DNA loaded in amounts corresponding to 1, 3, and 10 molecules per cell-equivalent of DNA (p575 Stds). (A and B) A total of 6 μ g of each DNA (or 0.6 μ g with DF2) was digested with *EcoRI* and detected with probe 2, a *Sall*-to-*BamHI* fragment contained within the *EcoRI* I fragment (Fig. 1). The 4.2-kb *EcoRI* I fragment, the 7.0-kb I-neo fragment, and the ~20-kb D-het-I-neo fusion fragment are indicated. (C) A total of 3 μ g of each DNA (0.3 μ g with DF2) was digested with *BamHI* and detected with probe 1, which spans the TRs (Fig. 1). *HindIII* was also added to the digestions to reduce the amount of human DNA fragments of large molecular size that lead to distortions in migration. The positions of the *BamHI* fragments containing the fused termini are indicated at the right for B95-8 genomes and for the mutant B95-575 genomes. The 3.5-kb *BamHI* fragment to the right of the CVMIE-neo insert is also detected because it overlaps probe 1 by about 200 bp. *HindIII* was omitted for the two secondary clones of B95-575B shown on the left. The minor hybridizing fragment seen near the position of 18 kb in these two lanes is believed to be due to partial digestion at the *BamHI* site separating the 12-kb *BamHI* A fragment at the right end of the genome from the terminal *BamHI* fragment. The positions of size markers, *HindIII* fragments of bacteriophage lambda DNA, are shown at the left.

sequence revealed a 260-bp deletion beginning only eight nucleotides from the TRs and removing most of the second TP exon (Fig. 3A). The deletion joined nucleotide 172,237 of the B95-8 sequence to nucleotide 217. The absence of a functional TP exon 2 in mutant B95-575B prevents the expression of possible truncated forms of TP1 and TP2 that might be

A

B

C

FIG. 3. Structure and expression of disrupted TP genes. (A) Structures of the wild-type and mutant genomes extending from the TRs through TP exon 4 for the wild type and into the inserted CMVIE-neo DNA, which is inserted into TP exon 3, for the mutants. Splicing of RNA transcripts is indicated by lines connecting the exons from above. Mutants A and B refer to viral mutants B95-575A and B95-575B. (B) The sequence of the altered exon 3 found in cDNAs from mutants A and B. Splicing occurs after 44 nucleotides of the linker DNA (in box), located at the beginning of the 2.9-kb insert within exon 3, to join this altered third exon to TP exon 4, creating a shift in the translational reading frame. For mutant B, the splice from exon 1 to exon 3 is out of frame. (C) Truncated TP proteins that could be expressed by the mutants. Polypeptide chains are depicted with the 12 putative membrane-spanning regions shown as six loops and the amino-terminal cytoplasmic domain for TP1 shown at the lower left. The thickened extensions for proteins of mutant A represent the out-of-frame amino acids at the carboxy termini.

expressed by the simple insertion mutants, as is discussed below.

DNA from clone C contained both the normal *EcoRI* I and the mutant I-neo fragments, apparently at an equimolar ratio

(Fig. 2B). Other blots, not shown, revealed that clone C carried bacterial vector sequences of p575 (while clones A, D, and B did not) and indicated that the entire plasmid p575 had been inserted into the left end of the EBV genome and on the 3' side of the position of disruption within the TP gene, with the result that the entire TP transcription unit remained intact. A single reciprocal crossover between a circular plasmid and the second DNA molecule results in the fusion of the two DNAs, and plasmids as large as p575 can be inserted into the B95-8 genome in this way without exceeding the packaging limit of EBV (28). The fifth clone (clone E, not shown) carried an EBV genome in which the wild-type TP gene had been replaced by the disrupted TP gene, but the mutant EBV genome also carried sequences of the bacterial plasmid vector in an undetermined arrangement; clone E was not investigated further.

Expression of the disrupted TP genes. While intact TP proteins could not be expressed by the 575A or 575B mutants, we examined B-cell clones transformed by the mutants for aberrant TP gene transcripts. In an initial test with a reverse transcription-PCR assay (reverse transcription of extracted RNA, followed by amplification by PCR [references 22 and 45]), spliced transcripts containing TP exons 7 and 8, which are located on the 3' side of the insertion, were detected in poly(A)-containing RNA from B cells immortalized by either mutant virus (data not shown). This suggested that processed transcripts of the TP gene were made from the mutant genomes despite the 2.9-kb insertion into exon 3 and that most of the inserted sequences were probably being spliced out since internal exons in vertebrate mRNA are very seldom more than 300 nucleotides long (18). To test whether processed transcripts crossed the inserted marker, primers specific to either first exon (of TP1 or TP2) were used with primers from exon 4 or exon 5 for reverse transcription-PCR. For each pair of primers, the major TP-specific amplified cDNA that was observed with mutant 575A was about 20 bp longer than the corresponding wild-type cDNA (data not shown). The amplified cDNA from mutant 575A was sequenced and was found to contain an altered exon 3 that includes 44 nucleotides of the linker sequence that joins the CMVIE-neo marker to the *PvuII* site in exon 3. As illustrated in Fig. 3, splicing from the linker sequences to exon 4 gives an altered exon 3 for mutant 575A that is 20 nucleotides longer than normal TP exon 3. The linker sequence present upstream from the cytomegalovirus enhancer was derived from pLink322 (49), and the nucleotide sequence at the splice junction is CTGgtaggt (34), which conforms well with a splice donor consensus, (C/A)AGgt(a/g)agt (43), where intron nucleotides are shown in lowercase and the nearly invariant GT at the beginning of the intron is underlined.

With mutant 575B, amplified cDNAs that spanned from either first exon to exon 4 or 5 were each about 160 bp shorter than the corresponding cDNAs from 575A, consistent with the absence of the exon 2 sequences due to the deletion in this mutant removing most of exon 2 and its splice acceptor sequence. The DNA sequence of an amplified cDNA from mutant 575B confirmed splicing from exon 1 to exon 3 and showed it to have the identical splice from the linker sequence within exon 3 to exon 4 that was found for mutant 575A (Fig. 3).

The structures of truncated TP proteins that could be expressed from mutants 575A and 575B are shown in Fig. 3C. The first three putative transmembrane segments would be expressed by mutant 575A, joined to 17 mostly hydrophobic amino acids encoded by the juxtaposed linker DNA and a few nucleotides at the beginning of exon 4 from an incorrect translational frame. A recently reported EBV mutant in which

a nonsense codon was inserted into exon 4 of the TP gene after sequences encoding the fifth putative membrane-spanning segment was shown to express a truncated TP1 protein (32). The amino-terminal cytoplasmic domain of TP1 linked only to the first two membrane-spanning regions was shown to be as effective as full-length TP1 in associating with tyrosine kinases and serving as a substrate (6), and so the truncated form of TP1 that could be expressed from mutant 575A might be able to perform some of the normal functions of TP1.

The deletion in mutant 575B removes the initial coding sequences of TP2 in exon 2 (26, 48) and also has the effect that transcripts that initiate from the TP1 promoter are spliced from exon 1 to exon 3, shifting the translational reading frame. For TP1, translation would terminate after a few out-of-frame codons in exon 3 so that the cytoplasmic domain would not be attached to a membrane anchor. For TP2, which normally initiates translation at the beginning of exon 2, a methionine codon in a context favorable for translational initiation (25) occurs near the beginning of exon 3 but in an incorrect reading frame which terminates after a few codons. Mutant 575B is therefore not expected to allow significant levels of expression of the transmembrane portion of either TP1 or TP2. Unfortunately, an antiserum suitable for detection of TP2 is not available to test this prediction. Recently, Longnecker et al. reported an EBV mutant similar to mutant 575B in that a selective marker was inserted in place of the exons encoding the first five putative transmembrane regions of TP proteins (33). With this mutant, the amino-terminal cytoplasmic domain of TP1 could not be detected with a TP1-specific antiserum, and so either the cytoplasmic domain of TP1 might be unstable without a membrane anchor or a stable mRNA might not have been expressed from the disrupted gene.

Propagation of TP mutants by repeated transformation of B cells. The absence of detectable wild-type TP DNA in the primary B-cell transformant of 575A and in several secondary transformants of 575B indicated that these EBV mutants could sustain the transformed state of the infected B cells. However, because parental B95-8 virus was present when these B-cell clones were being established, it was necessary to show that the mutant viruses could establish immortalized B-cell lines in the absence of parental B95-8 virus. This was shown with a cocultivation assay (29) with three different B-cell clones carrying TP-disrupted EBV genomes and lacking detectable B95-8 DNA: the primary transformant of B95-575A, a secondary transformant of B95-575B, and a secondary transformant of B95-575D (which has a simple insertion mutation like B95-575A). Two of these clones released transforming EBV at a frequency just below 10^{-5} (one virus-releasing cell per 10^5 cells), and one clone released transforming virus at a frequency just over 10^{-5} . One secondary transformant of B95-575B did not give rise to transformants in the assay (no virus detected from 2×10^6 cells). For comparison, four B-cell clones immortalized by a derivative of B95-8 having a selective marker inserted on the 3' side of the EBNA1 gene in a manner that avoided disrupting any viral genes (29a) were tested for the release of virus by the same assay and with similar results: one failed to release virus and three released virus at frequencies between 10^{-4} and 10^{-5} . These rates of spontaneous replication and release of EBV, with variation from clone to clone, are rather typical of B-cell clones transformed by B95-8 (29, 51). However, a larger number of clones carrying mutant and wild-type EBV would have to be tested quantitatively to rule out minor effects of the TP mutations on rates of virus replication and release.

B-cell clones immortalized with TP mutants exhibit normal growth characteristics. Growth properties of several B-cell

TABLE 1. Growth properties of B-cell clones immortalized by TP mutants of EBV

Clone	Mutant type	Doubling time (days)	Cloning efficiency (%)
JK	wt ^a		1.3
P2B2	wt		1.3
ML1	wt	1.9	
PB1	wt	1.8	
PBA2°1	A	2.0	3.5
PBA2°2	A	2.2	2.6
XZD3°1	A	1.7	
XZD3°2	A	1.6	
JYB2°1	B	1.6	2.8
JYB2°2	B	1.5	3.5
JYB2°3	B		1.3
JYB2°4	B		0.6
PBB3°14	B	2.0	
PBB3°15	B	2.0	

^a wt, wild type.

clones transformed by TP mutants were assessed by measuring the doubling times of cell populations growing in liquid medium and the efficiencies with which the different clones could form colonies in 0.35% agarose over a feeder layer of lethally irradiated fibroblasts (50). The measurements were made for each cell line after 2.5 to 3 months in culture following initial infection. At this stage, under the conditions used in this laboratory, EBV-immortalized B-cell clones have population doubling times ranging from 1.5 to 3 days with close to 2 days being most typical. As shown in Table 1, the B-cell clones established with TP mutants grew at typical rates. The efficiencies with which several B-cell clones immortalized with the TP mutants formed colonies in agarose ranged from 0.6 to 3.5%. These values, as well as the values obtained with two B-cell clones immortalized with B95-8 virus, were within the normal range for the assay (Table 1), which is typically 0.5 to 5% (50).

Possible significance of TP proteins. The results of the studies indicate that intact TP proteins are not required for EBV either to transform B cells in culture or to spontaneously execute the lytic phase of infection in those cells. The numerical data further suggest that TP proteins do not contribute greatly to the efficiency of either event or to the growth rates of the transformed cells.

Longnecker et al. reported the isolation of EBV mutants deficient in expression of TP1 because of placement of a nonsense codon after the nineteenth codon of exon 1 (31). The availability of a TP1-specific antiserum for this study allowed Longnecker et al. to show that a linker-insertion mutant failed to express the protein and to rule out any obvious contribution of TP1 to EBV's phenotype of infecting B cells in vitro (31). Expression of TP2 in cells infected by these mutants should have been normal. After the submission of the manuscript, additional studies describing a mutant with a nonsense codon placed in TP exon 4 (32) and another mutant with a selective marker substituted for exons 2 and 3 and part of exon 4 (33) were reported. These latter two mutants are likely to be similar to our 575A and 575B insertion mutants, respectively, in the kinds of truncated TP proteins that could be expressed. All of these mutants with disruptions in the TP gene appeared to transform B cells normally and to replicate in transformed B cells normally, as was found for the mutants of the present study. Without specific antibodies capable of recognizing TP2, it is not possible with any of these mutants to rule out the

expression of carboxy-terminal fragments of TP2 due to reinitiation of protein synthesis at internal methionine codons located downstream of the disruption. The fact that four different ways of disrupting the exons encoding the membrane-spanning segments of TP all gave similar phenotypes strengthens the conclusion that TP2 is also dispensable for virus replication and for immortalization of B cells.

Consistent with a lack of involvement of TP proteins in transforming B cells, no phenotypic change compatible with such a function has been reported to result from expression of TP proteins in EBV-negative cell lines. While it is clear that TP1 associates with specific tyrosine kinases and is a substrate for them in transfected cells (6, 30), no evidence has been presented to indicate that the association of TP1 with these kinases increases enzymatic activity in any way. Middle T antigen of polyomavirus contributes to oncogenic transformation of cells by binding to the tyrosine kinase, pp60^{src}, an association which activates the kinase, greatly increasing its maximum velocity (8). Conceivably, the role of TP proteins might be to limit signal transduction or to alter it in some way for a purpose either of preserving or of changing the state of latency. The recent report that expression of TP1 in BJAB cells blocks the changes in intracellular free calcium that would otherwise result from cross-linking of receptors on the cell surface supports is consistent with such a role (40). It is unclear how TP2, or the membrane portion of TP1, which resembles transporter proteins in possible tertiary structure (14, 27, 44), might contribute to this function.

In a recent study by Qu and Rowe employing PCR to detect spliced RNA transcripts of EBV genes in B cells from peripheral blood, spliced TP1 transcripts were detected readily while spliced transcripts could not be detected for LMP, TP2, or any of the EBNA's (45). This finding would seem to imply a role for TP1 during a restricted form of latent infection that exists in humans. TP transcripts have also been detected in nasopharyngeal carcinomas (4, 7). Antibodies against TP1 have been detected in nearly 40% of sera from patients with nasopharyngeal carcinoma but were never found in sera of EBV-infected individuals who had infectious mononucleosis or Burkitt's lymphoma or were asymptomatic (13). These findings suggest a possible role for TP proteins in the infection of epithelial cells.

A deletion adjacent to the TRs. The deletion that arose during the isolation of B95-575B might be of interest because the left boundary of the deletion is only seven nucleotides from the terminal repeats (TRs), suggesting the possibility that the deletion arose during circularization of the viral genome after infection. For this to have happened, the right terminus of the genome that participated in forming the deletion would have needed to extend at least seven nucleotides into unique sequences at the right end of the genome. It is not known precisely where cleavage of tandem EBV genomes occurs within the TR prior to packaging, but the mechanisms involved are believed to be similar for all herpesviruses (11, 15). For herpesviruses with TRs, conserved sequences, pac1 (near one end of the TR) and pac2 (near the other end), are each presumed to direct cleavage at a distance at either end of the TR (11, 57). A cleavage a short distance from the end of a TR that would place unique sequences at the terminus, perhaps an aberrant event, could then lead to these added sequences becoming embedded between adjacent TRs by successive cycles of cleavage and rejoining of termini. Interestingly, the first 23 bp of unique sequence at the left end of the B95-8 genome (172,231 to 172,253) are present, except for two nucleotide changes, at the end of each TR of the integrated EBV genome carried by the Namalwa Burkitt's lymphoma cell

line, with the distance between the *pac1* and *pac2* homologous sequences of adjacent TRs being 18 bp longer than it is for most adjacent TRs of the B95-8 sequence (3, 39). One TR of the B95-8 sequence is 15 bp shorter than the others at one end, but the deletion did not extend into the nearby *pac1* homolog. These observations suggest that sequence changes at the boundaries of the EBV TR may occur rather frequently through errors in cleavage and/or rejoining.

Must EBV have joined termini to transform B cells? In vitro infection by EBV of B-cell lines derived from B-cell tumors often results in integration of the EBV genome into a human chromosome involving the unjoined viral termini (19). In contrast, circularized EBV genomes with fused termini are almost always carried in multiple copies in EBV-immortalized B-cell lines (21, 24). The only known exception is the cell line IB4, which only carries integrated viral genomes (20), and, curiously, the EBV genomes present in IB4 have joined termini and apparently became integrated after ends were joined (20). These observations led to a suspicion, now ruled out, that a functional TP gene, which spans the TRs, might be required for efficient immortalization of B cells. It is possible that the probability of the EBV termini becoming joined relative to the probability of them recombining with a cell chromosome is higher for infection of normal B cells than it is for infection of the B-lymphoma cell lines in which frequent integration has been observed. It is also conceivable that circularization might be important for optimal expression of the LMP gene by bringing it within a functional range of the enhancer of *oriP* (46, 52). If so, joining of the termini would present a selective disadvantage for newly infected B-lymphoma cell lines for which LMP gene expression can retard growth (9).

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