JOURNAL OF VIROLOGY, July 1991, p. 3958–5963
0022-538X/91/073958-06$02.00/0
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Vol. 65, No. 7

The Prototypical Epstein-Barr Virus-Transformed Lymphoblastoid Cell Line IB4 Is an Unusual Variant Containing Integrated but No Episomal Viral DNA

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Received 10 December 1990/Accepted 2 April 1991

IB4 is a prototype, latently Epstein-Barr virus (EBV)-infected, lymphoblastoid cell line. We show here that IB4 contains only integrated EBV genomes. Episomal EBV DNA is not detected by Gardella gel analysis or in situ hybridization. Restriction enzyme mapping indicates that the EBV genomes first circularized and then integrated into and deleted part of the *Bam*HI C fragment. IB4 is therefore the only lymphoblastoid cell line described to date that lacks episomal EBV and has integrated EBV genomes with joined ends. Thus, the detection of joined EBV termini on Southern blots is not as reliable as the Gardella gel system for detecting episomal EBV DNA, and IB4 is not an ideal prototype cell line for the study of latent infection by EBV.

Epstein-Barr virus (EBV) is a human herpesvirus which usually infects small resting B cells (3, 10) and persists as multiple covalently closed, circular episomes (CCC) (19). CCC EBV DNA replicates autonomously through interaction of the origin of replication, Ori-p, with Epstein-Barr nuclear antigen 1 (25). Alternatively, when EBV infects EBV-negative BL cells, the virus most frequently persists as a unit copy molecule integrated into the host cell genome (9).

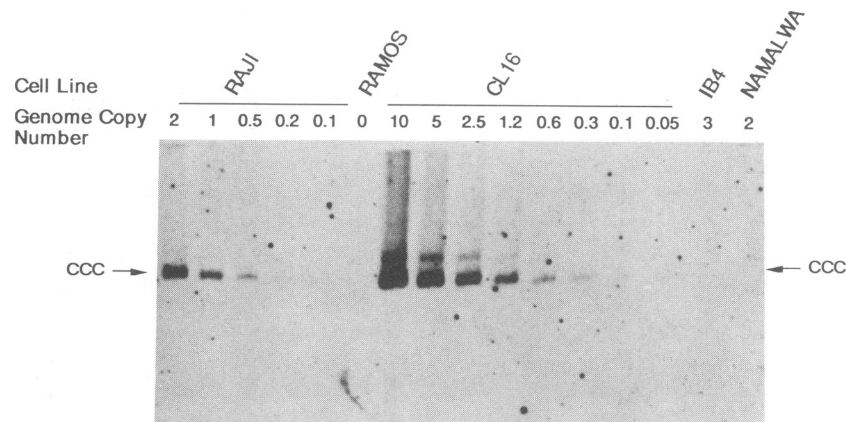

FIG. 1. Gardella gel analysis of IB4. The technique used was based on that originally described by Gardella et al. with modifications (7, 10). In this system, EBV-infected cells are lysed in the well of an agarose gel; cellular DNA and integrated viral DNA are too large to enter the gel and are excluded. Circular and linear EBV DNA will enter the gel, however, and can be distinguished on the basis of their differential mobilities. Raji cells (American Type Culture Collection) with 55 genome equivalents per cell (1) and CL16 (kind gift from George Miller) with 11 genome equivalents per cell (1) were serially diluted with EBV negative Ramos cells (American Type Culture Collection) and run as quantitation controls on all gels. The IB4 cell line (kind gift from Elliot Kieff) contains three to five genomes per cell, and Namalwa (American Type Culture Collection) contains two integrated genomes.

IB4 is a lymphoblastoid cell line derived from infection of umbilical cord blood B cells (11). It has been described as tightly latent and as such has served as a paradigm in studies of latent EBV infection. IB4 has been estimated to contain four to five EBV genomes (11). The termini of each of the viral genomes are joined, and no independently migrating EBV termini, indicative of integration of the linear viral genome, have been detected by restriction mapping and Southern blotting of IB4 DNA (11). However, EBV DNA has been detected on chromosome 4 by in situ hybridization (8), suggesting that at least one of the viral genomes in IB4 has integrated. It has been presumed that the other EBV genomes in IB4 are in the CCC form normally found in

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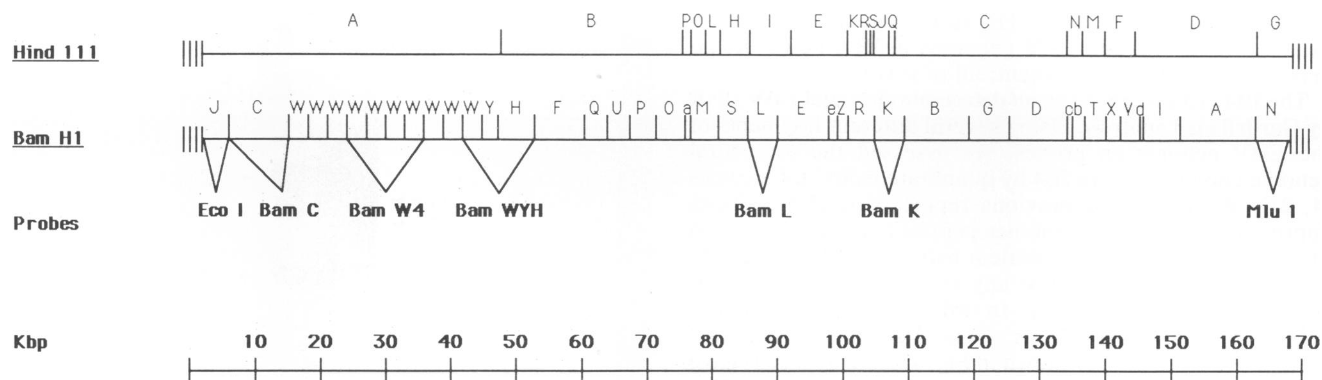
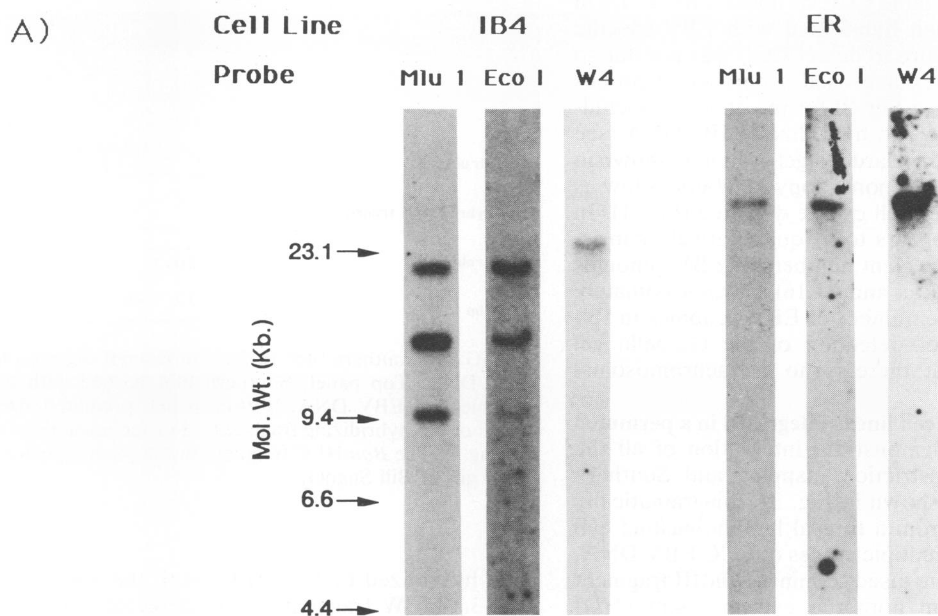


FIG. 2. *Hin*dIII and *Bam*HI restriction maps of the EBV genome (5) and probes used in this study.



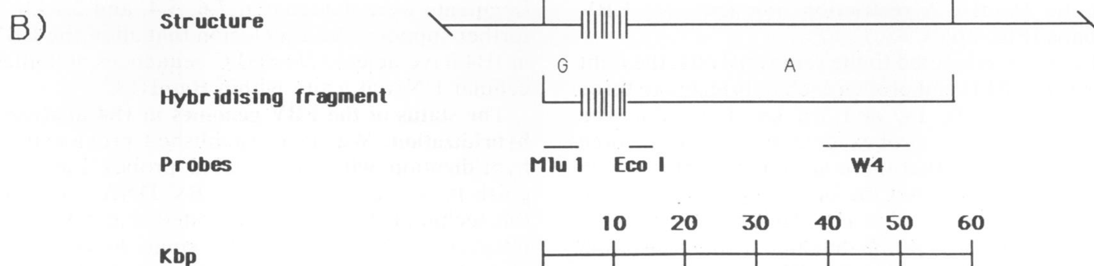


FIG. 3. Southern blot analysis of *Hin*dIII-digested IB4 and ER DNA. (A) Southern blots. The first two strips for each cell line were prepared by two-way blotting (9, 15) and therefore are exact duplicates. The third strip was from a separate blot that was lined up on the basis of the migration of the size markers. Size markers are shown (in kilobases) at the left. (B) Predicted size and structure of the hybridizing fragments. Genomic DNA was prepared by standard techniques (15), and 0.25 to 10 μg (depending on the EBV genome copy number of the cell line) was digested with the appropriate restriction enzymes according to the manufacturer's specifications. The digested DNA was fractionated on 0.3 or 0.6% agarose gels as appropriate, transferred to Zeta probe (Bio-Rad), and hybridized by standard methods as described previously (15). ER is a normal lymphoblastoid line and was derived in this laboratory as described previously (21). For the probes used and their localization in the EBV genomes, see Fig. 2. The *Mlu*I (right-end) probe in the vector pHSI-LM (22), the *Eco*RI I (left-end) probe in the SP65 vector, and the *Bam*HI K and L fragments were kind gifts from Elliott Kieff. The W4 fragment in the pkan-2 vector (20) was the kind gift of Bill Sugden. The Ori-p probe was derived by digestions of the *Bam*HI C fragment. Probes were removed from the vectors by digestion with the appropriate restriction enzymes, gel purified, and labeled by the random priming method according to the manufacturer's specification (Boehringer-Mannheim).

lymphoblastoid cell lines (6, 11). In this article, we confirm that there are multiple EBV genomes in IB4; however, no episomal EBV DNA is present: all of it is integrated.

The IB4 cell line contains no detectable episomal EBV DNA by Gardella gel analysis. Using several different fragments of the EBV genome as probes, we assessed the total viral genome copy number in IB4 by quantitative dot blot analysis (4, 21). We confirmed previous reports that IB4 contains approximately 4.0 viral genomes per cell (standard deviation of 1.4 based on four independent estimates). This suggests that the cell line has not lost any viral genomes because of long-term passage in culture. In order to detect and quantitate unintegrated EBV DNA within IB4, the Gardella gel system was used (7). As shown in Fig. 1, Namalwa cells gave no signal corresponding to CCC or linear EBV DNA. This finding was expected since all EBV DNA in Namalwa cells is integrated into cellular DNA (8, 13, 16). Surprisingly, IB4 cells also showed no detectable CCC or linear EBV DNA in Gardella gels (Fig. 1) when hybridized with EBV-specific probes. To ensure that failure to detect CCC was not due to deletions in IB4 DNA, we have used a number of probes across the viral genome (see Fig. 2) for the analysis, including probes which are known to hybridize to IB4 DNA (see Fig. 4). The sensitivity of the Gardella gel system is shown in parallel to demonstrate that genome copy numbers as low as 0.1 CCC EBV genomes per cell can be detected (Fig. 1). In addition, the technique appears to be quantitatively consistent, as the signals for equivalent numbers of EBV genomes in both control cell lines (Raji and CL16) are approximately equal. Therefore, since the number of EBV genomes in IB4 is well above the limit of detection of the Gardella gel system, we conclude that there is no extrachromosomal EBV DNA in IB4.

All EBV DNA in the IB4 cell line is integrated in a permuted form. We attempted to demonstrate integration of all the EBV DNA in IB4 by restriction mapping and Southern blotting of IB4 DNA. As shown in Fig. 3A (diagramatically in Fig. 3B), when DNA from a typical lymphoblastoid cell line, ER, which contains multiple copies of CCC EBV DNA, is digested with *Hin*dIII, the fused terminal *Hin*dIII fragment hybridizes to probes from both the extreme right (*Mlu*I hybridizing to *Hin*dIII G) and left (*Eco*RI I hybridizing to the left end of *Hin*dIII A) ends of the linear EBV genome (see Fig. 2). In addition, the 60-kb *Hin*dIII fused terminus is detected by a probe for *Bam*HI W (W4), which lies at the right end of the *Hin*dIII A restriction fragment (see EBV restriction maps [Fig. 2]).

When IB4 DNA is subjected to the same analysis, the right (*Mlu*I) and left (*Eco*RI I) end probes each hybridized to three distinct fragments of 20, 14, and 9.6 kb (Fig. 3A). The findings that each of the probes detects the same three restriction fragments and that there are no fragments which hybridize to one probe but not the other indicate that all of the viral DNA has circularized in IB4. However, hybridization to the *Bam*HI W (W4) probe detected a size restriction fragment different from that of the *Eco*RI I probe, indicating that the right and left ends of the *Hin*dIII A fragments are not joined to each other and therefore have integrated somewhere in *Hin*dIII A.

DNA is deleted from the *Bam*C fragment. Digestion of IB4 DNA with *Bam*HI and hybridization to the *Mlu*I and *Eco*RI I probes yield fused terminal *Bam*HI fragments which are within the expected size range (data not shown). Therefore, the genomes must have circularized and broken apart at sites in either the *Bam*HI C or W fragment (see Fig. 2). As shown in Fig. 4, when Raji and ER DNAs are digested with *Bam*HI and hybridized to *Bam*HI C, both the 10-kb *Bam*HI C and the 3.1-kb W fragments were detected, as expected, since the repeat sequence containing the *Bam*HI site in the W repeats begins in *Bam*HI C. In IB4 DNA, however, while the 3.1-kb *Bam*HI W fragment was detected, no 10-kb C fragment was present. Instead, three anomalously migrating fragments were detected at 7.6, 5.4, and 2.1 kb. This result further supports the conclusion that all of the EBV genomes in IB4 have deleted *Bam*HI C sequences and integrated into cellular DNA at a site within *Bam*HI C.

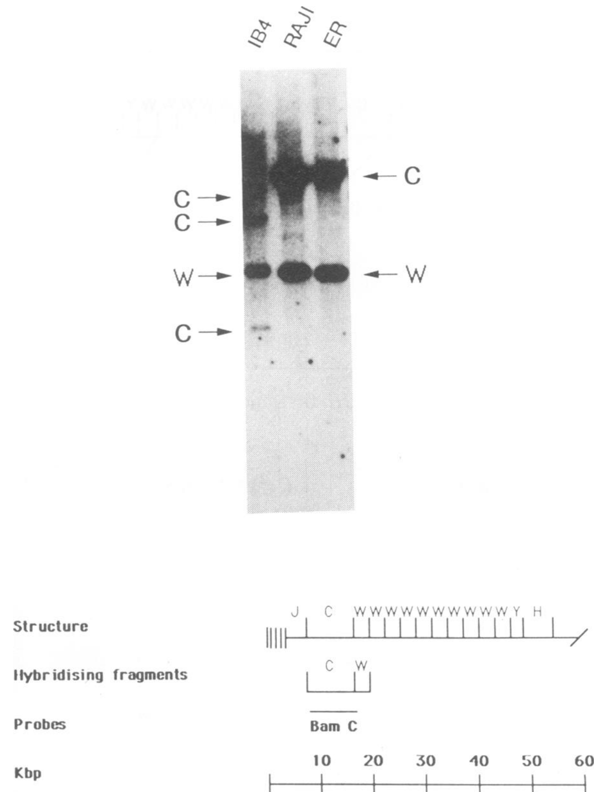


FIG. 4. Southern blot analysis of *Bam*HI-digested IB4, ER, and Raji DNA. Top panel, Southern blot probed with the *Bam*HI C fragment of EBV DNA. Bottom panel, predicted sizes and structures of the hybridizing fragments. For technical details, see legend to Fig. 3. The *Bam*HI C fragment in the pkan-2 vector (20) was the kind gift of Bill Sugden.

The status of the EBV genomes in IB4 analyzed by in situ hybridization. We have established previously that in situ hybridization with biotinylated probes can readily distinguish integrated from CCC EBV DNA (9). Application of this technique to IB4 demonstrated (Fig. 5A) the presence of integrated EBV genomes at a single location on a B-group chromosome, confirming previous reports that integration has occurred on chromosome 4 (8). This site was labeled in over 90% of the nuclei and no other chromosome showed sister chromatid labeling, indicating that all of the integrated genomes were present at this location. Analysis of a large number of cells failed to demonstrate any signal that would be expected because of the several CCC previously reported to be present in IB4 (6, 11). We have also shown previously that cells with multiple episomes show multiple tracks of viral RNA synthesis, whereas a cell line that has only integrated viral DNA (Namalwa) shows only one or occa-

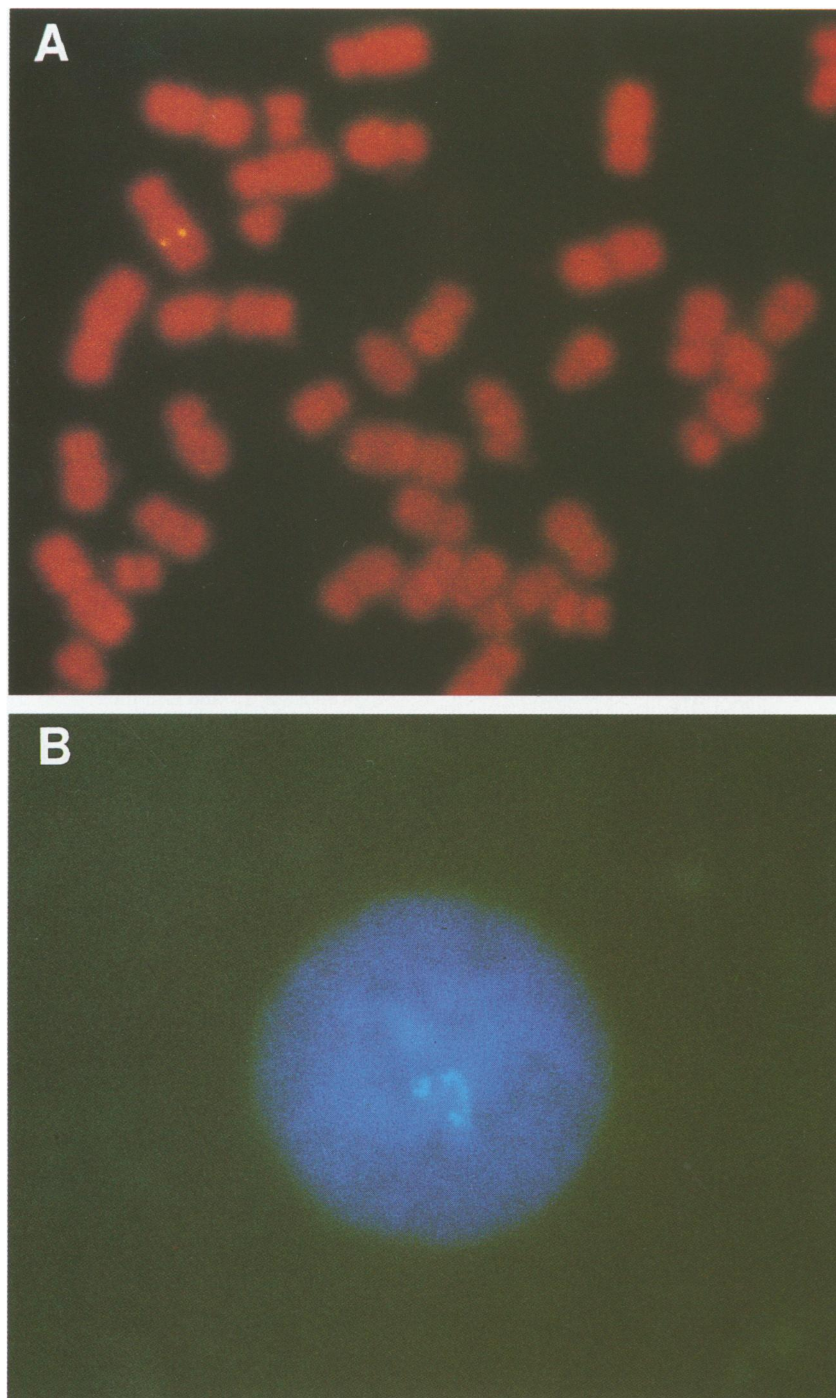


FIG. 5. Analysis of IB4 by fluorescence in situ hybridization. (A) Chromosomal hybridization to the *Bam*HI W fragment of EBV DNA. A single site of hybridization at the same site on each sister chromatid of the short arm of this B-group chromosome (chromosome 4 or 5) was seen in essentially all cells. Note that in a previous study, we have shown that the presence of sister chromatid labeling is characteristic of integrated viral DNA (13). By comparison, CCC DNA, although associated with the chromosomal DNA, demonstrates variation in the sites of hybridization from cell to cell, and sister chromatid staining is not generally seen (9). Hybridization signals characteristic of CCC were not detected in the IB4 cells analyzed. A detailed account of the fluorescent in situ hybridization methodology has been described previously (9, 13). (B) Detection of EBV RNA within interphase nuclei. The IB4 cells consistently showed one or occasionally two tracks of viral nuclear DNA when the *Bam*HI W fragments were used as a probe. In this regard, they appeared essentially identical to Namalwa cells, which do not carry episomal DNA (14). In contrast, cells containing episomes, such as B9-58, have been previously shown to have many foci or tracks of RNA in the nucleus (24). These results support the conclusion that the only viral DNA present is integrated at a single site.

sionally two tracks (14, 24). When IB4 was examined by this technique, it demonstrated the same pattern as Namalwa, namely the consistent presence of one or occasionally two tracks in each nucleus. Thus, the results from both of these techniques are most consistent with IB4 containing only integrated EBV and with all the genomes having integrated in close proximity. Therefore, we conclude that the IB4 cell line, which has been used extensively as a model to study latent EBV transcription, is in fact atypical of EBV-immortalized cell lines. It is the only EBV-positive, immortalized primary B-cell line studied to date in which extrachromosomal (CCC) EBV DNA cannot be detected.

We have recently described 10 EBV integration events in EBV-converted BL lines (9). In every case, the genomes had, as in Namalwa, integrated as linear molecules. Thus, again IB4 is unique in having integrated genomes with joined ends.

It could be argued that at the time of its derivation, IB4 had episomes which were subsequently lost; however, this is probably not correct since the conclusions drawn in this paper were based on studies with two IB4 cultures that had been grown independently in different laboratories for several years. Furthermore, the original line was described as tightly latent and uninducible, like the Namalwa line (11). This suggests that the form of the persistent EBV DNA was established soon after the cell line was derived, that it is stable, and that integrated EBV DNA cannot be reactivated.

The original description of IB4 concluded the presence of multiple CCC on the basis of the detection of fused terminal joint fragments (11). However, our observations indicate that this form of analysis, which has become prevalent recently, is only consistent with, not proof of, the presence of CCC. The technique of Gardella, on the other hand, is reliable and sensitive for the detection of CCC EBV DNA.

Deletion in the *Bam*HI C fragment of IB4 has been described previously (23). In that study, it was shown that the entire Cp promoter was absent. We have probed blots of *Bam*HI-digested IB4 with an Ori-p probe, since Ori-p also resides in *Bam*HI C (25), and have found that all three *Bam*HI C fragments hybridize (data not shown). It is not clear, however, whether these origins of replication are functional.

Since the *Bam*HI C promoter is deleted in IB4, the *Bam*HI W promoter is utilized to produce the EBNA gene products (23), with the exception of EBNA 4, which is absent from IB4 (2). Therefore, the primary EB nuclear antigen transcript, spanning approximately 70 kb of EBV DNA (18), is largely intact. Integration at the viral termini would preclude expression of the latent membrane proteins TP-1 and TP-2, whose transcription proceeds across the episomal joint (12) and may also interfere with latent membrane protein (LMP) expression. The viral genomes in IB4 have circumvented this problem by circularizing prior to integration; thus, TP-1, TP-2, and LMP are expressed in IB4 (17). Therefore, while IB4 contains no CCC EBV DNA, it has integrated in such a way as to allow the expression of the latent viral genes essential for the maintenance of the immortalized state. Presumably, it would be a rare event for integration not to inactivate these genes since they span such a large area of the EBV genome. This may be one reason why IB4 is the only EBV-immortalized primary B-cell line thus far described which contains only integrated EBV DNA.

Our analysis does not give a clear picture of how the integration events occurred in IB4. It is particularly intriguing that, just as with Namalwa, there are multiple integration events very close together. Since only metaphase spreads were analyzed in detail by in situ hybridization, it was not possible to resolve these sites as independent signals. However, preliminary analysis of interphase spreads, which did not rigorously exclude the presence of G2 cells, did indeed reveal multiple sites of integration. It is highly unlikely that this happened by chance. The simplest model would be that a single genome integrated and underwent brief amplification, replicating from the viral origin. This is consistent with the known ability of EBV episomes to amplify within infected cells.

ACKNOWLEDGMENTS

We thank Cindy Welch for typing the manuscript and Gerry Parker for photographic work.

This work was supported by Public Health Service Grants AI-15310 and CA-28737.

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