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When Epstein-Barr Virus Persistently Infects B-Cell Lines, It Frequently Integrates

ELIZABETH A. HURLEY,[1] SUSAN AGGER,[1] JOHN A. McNEIL,[2] JEANNE B. LAWRENCE,[2] ALAIN CALENDAR,[3] GILBERT LENOIR,[3] AND DAVID A. THORLEY-LAWSON[1]*

Department of Pathology, Tufts University School of Medicine, 136 Harrison Ave., Boston, Massachusetts 02111[1]; Department of Cell Biology, University of Massachusetts Medical Center, Worcester, Massachusetts 01605[2]; and International Agency for Research on Cancer, 69372 Lyons Cedex 08, France[3]

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In this study we used Gardella gel analysis of intact DNA, Southern blotting of digested DNA, and fluorescence in situ hybridization to provide complementary and unequivocal information on the state of the Epstein-Barr virus (EBV) genome in persistently infected cells. The fluorescence in situ hybridization technique allowed us to directly visualize both integrated and episomal EBV DNA at the single-cell level. We show here that circularization of the EBV genome is rarely detected upon infecting activated normal B cells. The virus can persist upon infection of a different proliferating B-cell target, EBV-negative Burkitt's lymphoma tumor cell lines. Analysis of 16 such lines reveal again, that the virus infrequently persists as covalently closed episomes; rather, the virus preferentially persists by integrating into the host DNA (10 of 16 clones). The integrated virus is linear and usually intact, although 3 of 10 isolates have deletions from the left-hand end including the latent origin of replication. At the level of our analysis, no obvious relationship was seen between the integration sites. These studies provide, for the first time, a reproducible in vitro model system to study integration by EBV.

Epstein-Barr virus (EBV) preferentially infects resting B cells (2) in vitro, resulting in their immortalization and establishment as lymphoblastoid cell lines (18) that express several characteristic latency-associated proteins including six nuclear-associated proteins (EBNA-1 to EBNA-6) and three membrane-associated proteins (LMP, TP1, and TP2) (14). The infectious viral DNA is linear; however, upon infection, the resting B cell becomes activated (27), and as it initially traverses G_1, a single linear viral genome becomes joined at the ends to generate a covalently closed circle (CCC). This occurs even at high multiplicities of infection. Only cells (about 15 to 20% of whole peripheral blood B [PBL-B] cells) that have formed a CCC express all of the latency-associated proteins (5). Subsequently, these cells also express characteristic activation antigens such as CD 23 (12) and finally become immortalized, proliferating lymphoblasts. Once the cell has begun to proliferate, no further circularization events are detected. Rather, the single CCC replicates in unison with the cellular DNA for 1 to 2 weeks (12), indicating that amplification does not begin until long after the cells are morphologically transformed. Interestingly, this period of time coincides with the disappearance of linear input virus from the nucleus. Furthermore, it has been observed (29) that vectors based on origin of plasmid replication (ori-p) (30) and EBNA-1 alone also fail to amplify, suggesting a role for both viral and cellular factors in amplification. The CCC eventually begins to amplify so that, commonly, 10 to 500 copies per cell are detected after a few months in culture (25). The mechanism and function of amplification are unknown.

These observations have led us to suggest that the formation of CCCs may occur during a specific, early stage of activation and that CCCs are necessary to obtain the correct viral transcription required to push the cell on to become fully activated and proliferate. In support of this model is the observation that a substantial fraction (~40%) of PBL-B cells become infected; however, they fail to circularize a viral genome (12). Such cells express only a limited repertoire of latent genes, most notably lacking LMP, and fail to progress to become activated and proliferate (5, 12). Our hypothesis would predict that the formation of CCCs should not occur at a high frequency upon infection of cells that are already activated or proliferating. Such targets would include activated normal B cells and established EBV-negative B-cell lines. Recently, two of us (A.C. and G.L.) established a number of persistently infected cell lines through infection of EBV-negative Burkitt's lymphoma (EBV^- BL) B-cell lines (7). These EBV-converted subclones express latent viral genes and hybridize to virus-specific probes in whole-cell DNA dot blots (7, 19). We have used these lines to study the mechanism of persistence upon infection of a proliferating cell target and thereby test our hypothesis. We show that in the majority of these cell lines the EBV genome persists solely as a single integrated copy.

MATERIALS AND METHODS

Cells, cell lines, and viruses. The derivation of the EBV-converted BL cell lines used in this study has been described in detail elsewhere (7). Between 5 and 50% of the cells were EBNA positive by indirect immunofluorescence. This dropped to less than 1% at the time of cloning, 2 weeks postinfection. Of the clones obtained and of 200 screened, 20 to 25% were EBNA positive. The cells were maintained in RPMI 1640 plus 10% fetal calf serum and antibiotics. The notation used is as follows: for example, BL-41 B95-8 IA1 was derived by infecting the EBV^-BL line BL-41 with the B95-8 strain of EBV. After subcloning the infected cells, EBV-positive clones were derived, one of which was designated IA1. The viruses used were the transforming A types B95-8 and BL-72, B type AG-876, and the nontransforming B type P3HR-1. CL-16 is a nonproducer subclone of the P3HR-1 line and was a kind gift from George Miller.

* Corresponding author.

The EBV genome copy number was assessed for each line by using the quantitative dot blot method of Brandsma and Miller (6) as described previously (27) with RAJI and CL-16 cells as standards.

PBL-B cells were purified as described previously (12). Resting and activated populations of B cells were prepared by Percoll (Pharmacia) step gradient fractionation as described by the manufacturer. Cells that banded at 41% (fraction 1) are considered activated, whereas those at 62% are resting. The intermediate fractions, 48% (fraction 2), 54% (fraction 3), and 58% (fraction 4), yield mixtures of partially activated populations. Normal and EBV$^-$BL cells were infected with the B95-8 strain of EBV as described previously (12).

Gardella gels. The gel technique used was based on that originally described by Gardella et al. with modifications (8, 12). RAJI cells (55 genome equivalents per cell) (1) serially diluted with RAMOS cells (EBV negative) were routinely run as quantitation controls on all gels.

Southern blots. Genomic DNA was prepared by standard techniques (16). DNA (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 specifications of the manufacturer. The digested DNA was fractionated on 0.3 or 0.6% agarose gels as appropriate, transferred to Zeta probe, and hybridized by standard methods as described previously (16).

Probes. For the localization of the probes, see Fig. 5A. The *Mlu*I (right-hand) probe in the vector pHSI-LM (28) and the *Eco*RI fragment 1 (left-hand) probe in the SP65 vector were kind gifts from Elliott Kieff. The probes, *Bam*HI fragments C and W4 and the *Hin*dIII fragments B, C, D, and E, all in the pkan-2 vector (26), were kind gifts from Bill Sugden. 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 specifications of the manufacturer (Boehringer-Mannheim).

Fluorescence in situ hybridization. A detailed account of the fluorescent in situ hybridization methodology was described previously (15).

RESULTS

Infection of activated B cells. PBL-B cells were fractionated into resting and activated populations on Percoll gradients. The fractions were infected with transforming (B95-8) EBV and analyzed by the Gardella gel technique for the presence of CCCs at 36 h postinfection (Fig. 1). CCCs were detected in the high-density population; none were detected in the low-density population, although approximately half of the cells were successfully infected as judged by the expression of EBNA detected by indirect immunofluorescence (5, 26) (data not shown). This is consistent with previous reports that activated cells internalize virus as efficiently as resting cells (3). Similarly, a higher level of linear genomes, as detected by Gardella gels, were associated with the high-density cells at 36 h postinfection. This occurred despite the fact that similar levels of virus were bound to the surface of both cell populations at time zero (data not shown). This suggests that the viral DNA is less stable in the activated population. These results indicate that the population previously identified as EBNA positive but lacking episomes (5, 12) is derived from the infected low-density B cells and that failure to form a CCC may, in part, explain the failure of these cells to immortalize.

One set of proliferating B cells which are known to be

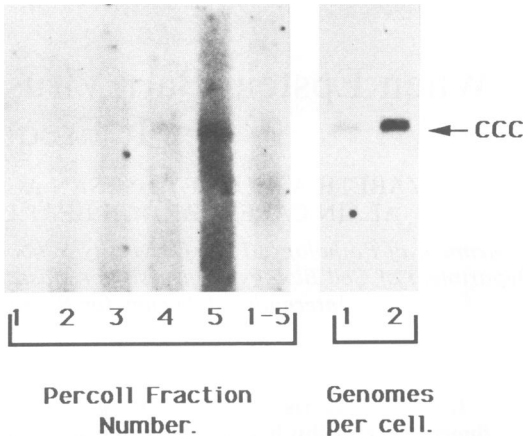


FIG. 1. Analysis of EBV DNA on day 2 postinfection in cells separated by buoyant density prior to infection. (Left) Percoll-purified fractions of PBL-B cells were infected with EBV and analyzed on day 2 postinfection by Gardella gels. Lane 1 corresponds to the most activated fraction, and lane 5 corresponds to the most resting fraction. Lane labeled 1–5 corresponds to pooled fractions 1 to 5. (Right) Quantitation of the Gardella gel. RAJI cells were serially diluted with EBV-negative cells (RAMOS) to yield mixtures corresponding to one-fourth to eight circular EBV genomes average per cell. The blots for one and two genomes only are shown.

infectable by EBV are established EBV$^-$BL cell lines. Figure 2 shows a time course of the appearance of CCC upon infection of such a cell line, BL-2, compared with normal resting B cells. As we have described previously, CCCs were detected in the resting population by 24 h postinfection and the signal increased in intensity thereafter. In comparison, no CCCs were detected at any time in the BL-2 cells although this technique can routinely detect as little as 0.1 genome on average per cell. The sensitivity of the Gardella gel technique is shown in Fig. 3 for two cell lines of known genome copy number (RAJI and CL-16).

We have attempted to infect several EBV$^-$BL lines with

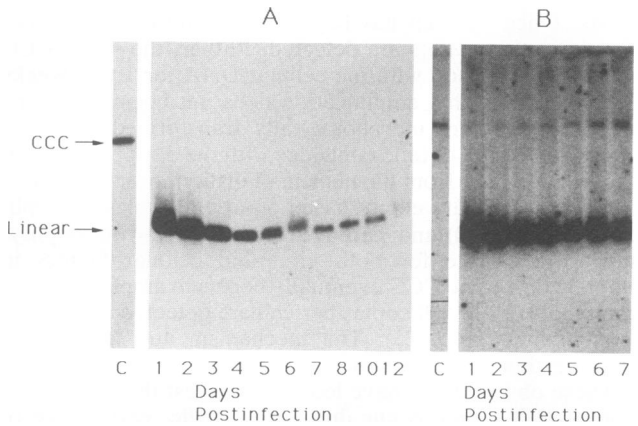


FIG. 2. Analysis of EBV$^-$BL BL-2 (A) and the normal B cells (B) for the presence of CCC at various times postinfection. Cells were infected with the B95-8 strain of EBV, harvested at various times postinfection, and analyzed by the Gardella gel technique. The two analyses were performed at different times. Representative lanes equivalent to one genome per cell from the RAJI standards are shown for each experiment. C, Control.

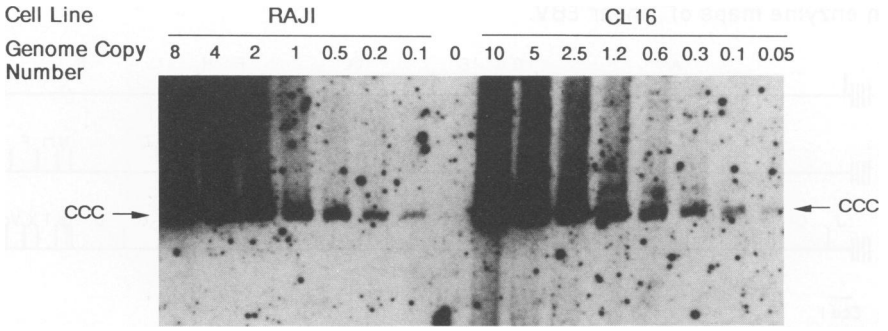


FIG. 3. Demonstration of the sensitivity of the Gardella gel technique. Cells from the RAJI (55 genome equivalents per cell) and CL-16 (11 genome equivalents per cell) lines (2) were serially diluted with RAMOS (EBV-negative) cells to give the average EBV genome equivalents shown. RAMOS cells were used as a negative control (genome copy number 0). The film was overexposed to demonstrate the maximum sensitivity. Note that as few as 0.1 genome per cell can be detected on the basis of either standard cell line.

the same results, i.e., no detectable CCCs even though, at early time points, 10 to 50% of the cells were EBNA positive and, therefore, contained and expressed EBV DNA.

Form of the genome in EBV-converted BL-41 cell lines analyzed by Gardella gels. Although it was not possible to detect CCCs in the newly infected EBV$^-$BL cells, EBV DNA must be retained at some frequency since stably converted sublines have been derived from such infected cultures (7). We have analyzed the status of the viral genome in a large panel of such converted cell lines, including a series derived from the BL-41 cell line. In the first experiment, the sublines were analyzed by the Gardella gel technique. Figure 4 shows the results for the BL-41 set. CCCs could only be detected in one of the sublines, namely, BL-41 72, which contains approximately 10 genome equivalents per cell. The remaining six independently derived lines all contained no detectable CCC. Essentially 100% of the cells in these six lines are EBNA positive and contain, on average, approximately 1 genome per cell as assessed by quantitative dot blots (7) (data not shown). Thus, they all contain viral genetic information but not in the form of intact episomes. This suggests the possibility that the virus is integrated.

Form of the genome in EBV-converted BL-41 cell lines analyzed by Southern blot. An alternative approach which can be used to determine the status of the genome in an infected cell is to perform Southern blot analysis with probes derived from unique sequences at the ends of the linear viral genome (17, 21). Figure 5A shows the restriction enzyme digestion map of EBV for the three enzymes we used in our studies. Figure 5B shows the expected outcome for such an analysis performed on whole-cell DNA containing either CCCs or intact linearly integrated genomes. Irrespective of the restriction enzyme used, digestion of a single CCC will generate a single fused terminal fragment that will hybridize to probes from either end of the linear genome. In comparison, digestion of integrated EBV will generate distinct fragments which hybridize to probes from either the left- or right-hand ends, and the size of the hybridizing fragments will depend on the amount of linked cellular flanking sequences and the enzyme used.

DNAs were prepared from the panel of converted BL-41 cell lines and digested with *Bam*HI. The resulting gel was transferred in both directions to generate a pair of identical blots. These were then probed with either a left (*Eco*RI fragment 1) or a right (*Mlu*I) end probe (Fig. 5). The results are shown in Fig. 6. For ease of comparison, the blots from both hybridizations were cut into strips and the strips corresponding to each cell line are shown side by side. Each cell line was tested a minimum of two times by this technique to confirm the conclusions. A summary of the fragment sizes is presented in Table 1. As expected for a cell line that contains CCCs, BL-41 72 revealed the same-sized fragment when hybridized with either left or right end probes. Of the six that had no detectable CCC, four showed clear evidence of integration, with the two probes failing to hybridize to the same-sized fragments. Interestingly, three of these had deleted the left end of their genomes (see below). Two of the lines (BL-41 P3HR-1 IA1 and BL-41 AG876) that lacked detectable CCC, however, showed similar-sized end fragments. The presence of integrated EBV in these two lines was, therefore, confirmed by repeating the analysis after

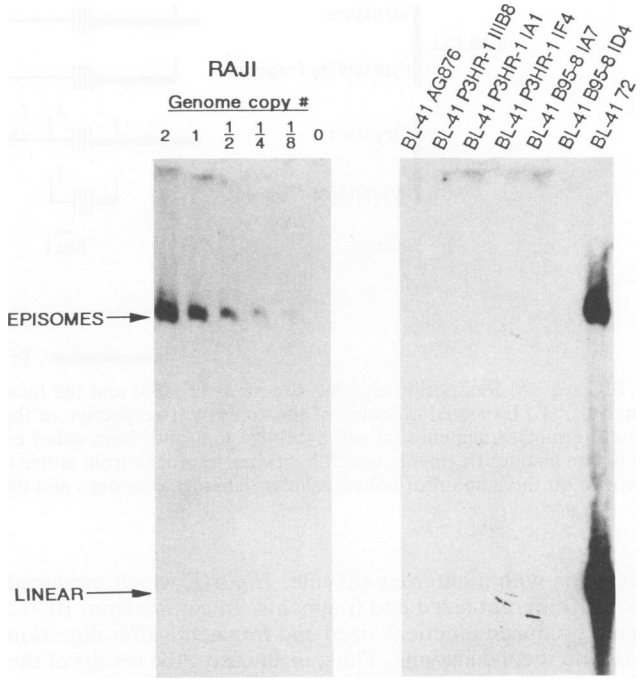
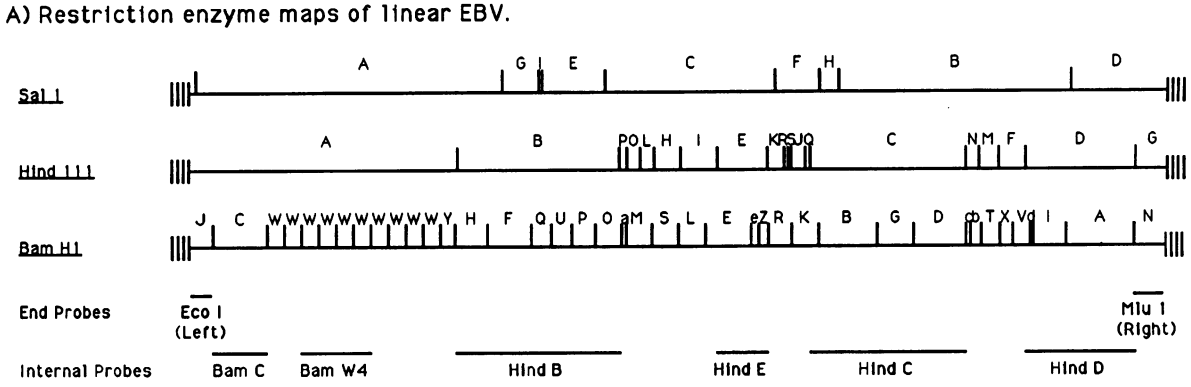


FIG. 4. Analysis of EBV-converted sublines of BL-41 for the presence of CCC. The cell lines were analyzed by the Gardella gel technique. For an explanation of the cell line nomenclature, see the legend to Fig. 6 and Materials and Methods. On the left is the RAJI cell standard.



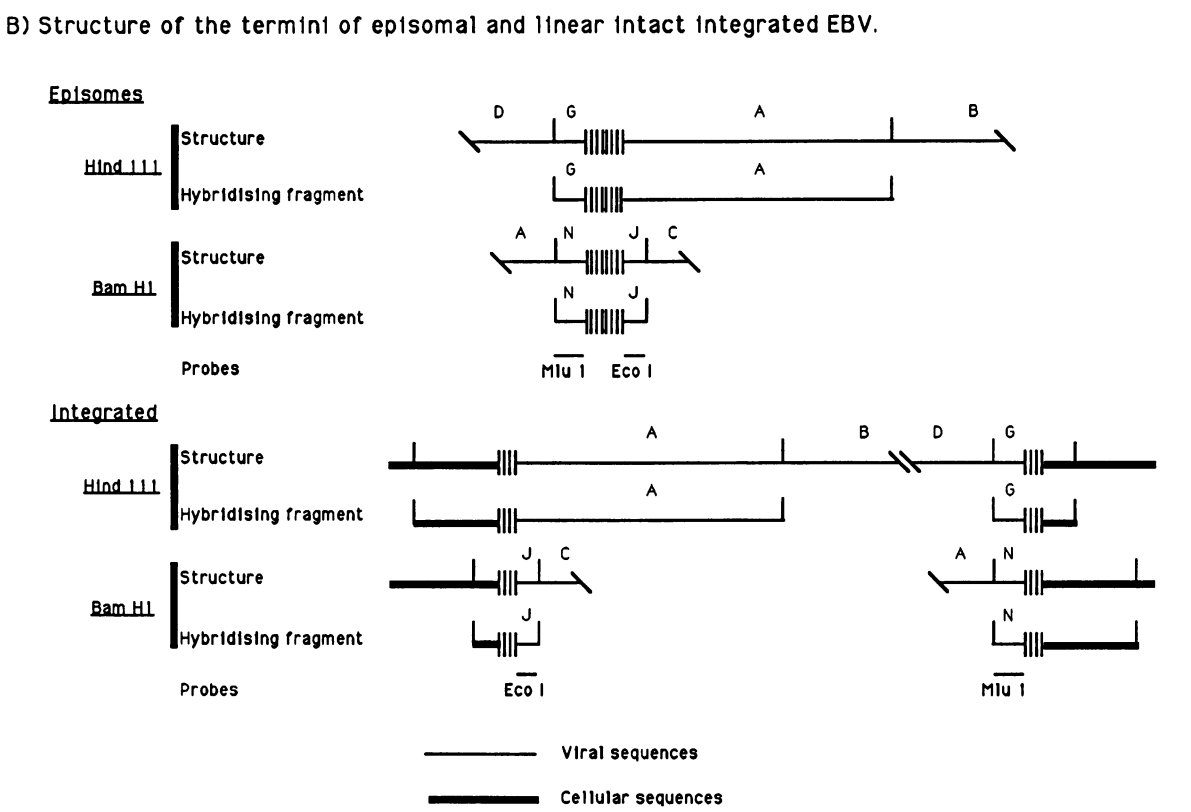


FIG. 5. (A) Restriction enzyme site maps of EBV and the location and terminology used to describe the probes for the Southern blot analysis. (B) Expected outcome of the analysis. Irrespective of the restriction enzyme used, digestion of CCC DNA will generate a single fused terminal fragment that will hybridize to probes from either end of the linear genome. In comparison, digestion of integrated EBV will generate distinct fragments which hybridize to probes from either the left- or right-hand ends, and the size of the hybridizing fragments will depend on the amount of linked cellular flanking sequences and the enzyme used.

digestion with a different enzyme, *Hin*dIII, which produced clearly different-sized end fragments. In comparison, BL-72 again produced identical-sized end fragments after digestion with the second enzyme. Thus, in all cases, the results of the Gardella gel were confirmed by the Southern blot analysis.

Form of the genome in other panels of EBV-converted BL cell lines. The only cell line for which a large number of converted lines was available was BL-41. However, limited panels were obtained from several cell lines by using a variety of viral isolates. We have analyzed a number of these, and the results of the Gardella gels and the Southern blots are summarized in Table 1 and Fig. 7. In summary, the results were not significantly different from those obtained with BL-41, although the frequency of lines with integrated genomes was somewhat lower (4 of 9 compared with 6 of 7). Taken together with the results from the BL-41 panel, it was clear that persistence as a CCC or as an integrated copy was not dependent on the virus strain used. However, there may

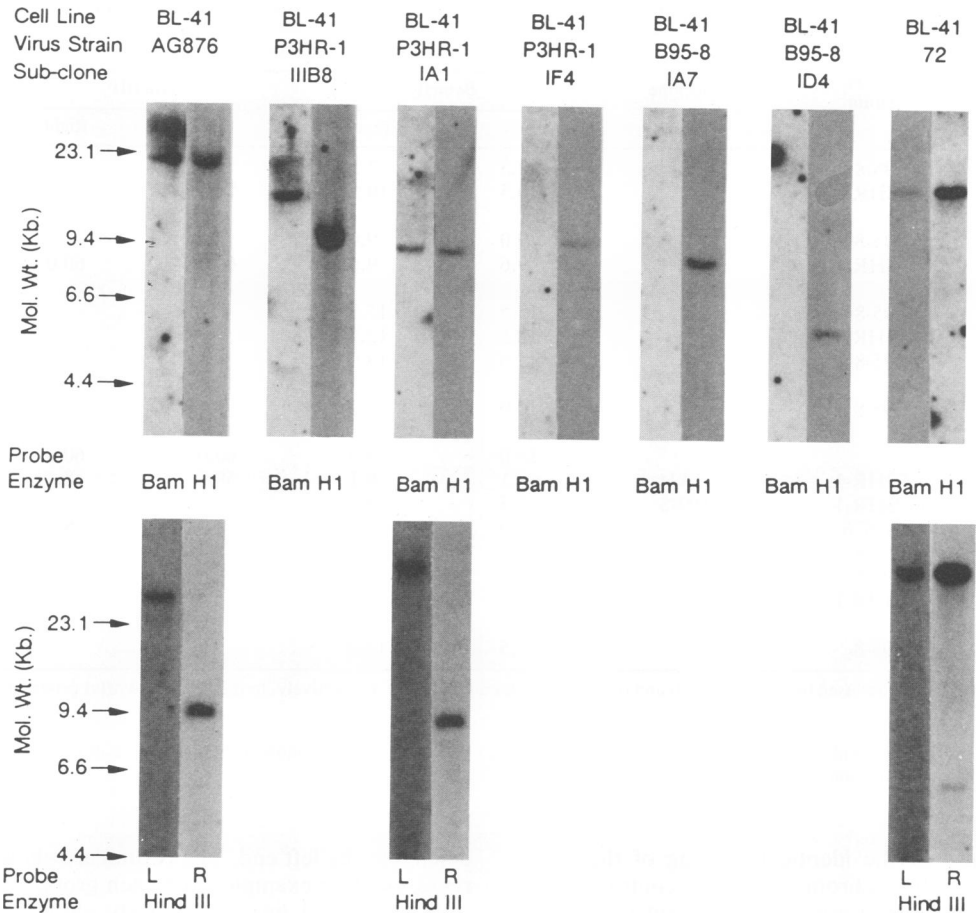


FIG. 6. Southern blot analysis of the converted sublines of BL-41. For the probes and strategy, see the legend to Fig. 5. Whole genomic DNA from each of the cell lines was digested and fractionated on agarose gels. The digested DNA was then transferred in two directions to generate duplicate blots. The blots were hybridized with either the *Mlu*I right (R) or *Eco*RI fragment 1 left (L) end probes. The resulting photographs were sliced and remounted so that right and left probes could be readily compared for each cell line. DNAs that gave similar-sized right and left end fragments after digestion with *Bam*HI were reanalyzed after digestion with *Hin*dIII.

be some predisposition in certain cell lines; for example, BL-30 and BL-31 gave a preponderance of lines with CCCs, whereas BL-2 and BL-41 gave mostly lines with integrated genomes.

The Southern blot analysis revealed that, in every case, integration appeared to involve a single linear molecule, since only one fragment hybridized to each end probe, and that each integration site was unique since the size of the flanking fragments was different in each case.

Lastly, comparison of the viral genome copy number with the form of the persistent genome revealed that in every case where the copy number was low (0.5 to 2), CCCs were absent and the virus had integrated. Conversely, when the copy number was high (10 to 40), CCCs were always present. Whether or not integrated genomes were also present in these cells is unclear.

Deletions in the integrated genomes. As mentioned above, three of the cell lines studied did not show hybridization to the left end probe, suggesting that part of their genome had been deleted. Since the ori-p lies near the left end of the genome (28), it was of interest to analyze the extent of the deletions. DNA from these three lines and two others with apparently intact integrated EBV DNA (BL-2 P3HR-1 and BL-41 P3HR-1 IA1) were digested with *Sal*I and probed with a series of internal probes that allowed the entire genome to be assessed. Figure 5A shows the *Sal*I restriction map and the internal probes used. This analysis revealed no gross deletions (data not shown). To more precisely map the left end deletions, *Bam*HI digests were analyzed with the probes *Bam*HI fragments C and W4 (Fig. 5A). This analysis revealed (Fig. 8) that all three lines had deleted all of the *Bam*HI fragment C (detectable down to about 150 bp; data not shown) but had retained *Bam*HI fragment W repeats. Thus, in all three lines, the ori-P, which lies in *Bam*HI fragment C, has been deleted. Curiously, we were unable to detect aberrant-sized fragments that should be generated by the joining of *Bam*HI fragment W or C sequences to cellular flanking sequences. The most likely explanation may be that in all three cases, integration occurred close to the *Bam*HI site in the fragment W repeats.

Structure of the viral genome by in situ hybridization. The molecular analysis described above suggests that, in the majority of in vitro converted BL lines that we have analyzed, EBV persists as a single integrated linear copy. To provide an independent verification of this observation, six of the cell lines were analyzed by fluorescence in situ hybridization with biotinated probes (15). This technique can readily demonstrate single-copy integrated genomes in meta-

TABLE 1. Analysis of the joint fragment sizes for a collection of EBV-converted BL cell lines

Cell line infected	Virus strain	Subclone	Fragment size (kbp)[a]				Form of the genome[b]
			*Bam*HI[c]		*Hin*dIII[c]		
			Left	Right	Left	Right	
BL-2	B95-8		12.5	7.5			I
	P3HR-1		10.5	10.5	58.0	7.3	I
BL-30	B95-8		9.0	9.0			E
	P3HR-1		9.6	9.6	60.0	60.0	E
BL-31	B95-8	B	15.5	15.5			E
	P3HR-1		12.5	12.5			E
	B95-8	A	5.0	19.0			I
BL-40	B95-8		10.0	10.0			E
BL-41	72		13.0	13.0	60.0	60.0	E
	P3HR-1	IA1	8.1	8.1	58.0	8.1	I
	P3HR-1	IIIB8	11.5	9.4			I
	AG876		17.0	16.5	42.0	8.3	I
	B95-8	ID4		5.5			I
	B95-8	IA7		7.7			I
	P3HR-1	IF4		8.5			I
BL-70	B95-8		7.5	17.0			I

[a] Left and right refer to the probes used to analyze the left and right joint fragments and refer, respectively, to the *Eco*RI and *Mlu*I probes described in the legend to Fig. 6.

[b] I, Integrated; E, episomal.

[c] Whole-cell DNA was digested with *Bam*HI, and the terminal fragment sizes were analyzed by Southern blot. If the results were contradictory to those obtained by Gardella gels, then the analysis was repeated after digestion with *Hin*dIII.

phase cells because of the identical labeling of the sister chromatids in the same chromosome. In contrast, cells containing episomes, which are always present in multiple copies, should demonstrate multiple single hybridization signals, randomly associated with the chromosomes (10, 20). The six lines analyzed included two with episomes, three with intact integrated DNA, and one with integrated DNA deleted at the left end. The results are shown in Fig. 9 for a representative example from each group.

The four cell lines, previously shown by Gardella and Southern analysis to contain an integrated genome, demonstrated a single site of hybridization visible as an intense signal at the same position on each of the sister chromatids. Over 90% of metaphase spreads showed label, and in any

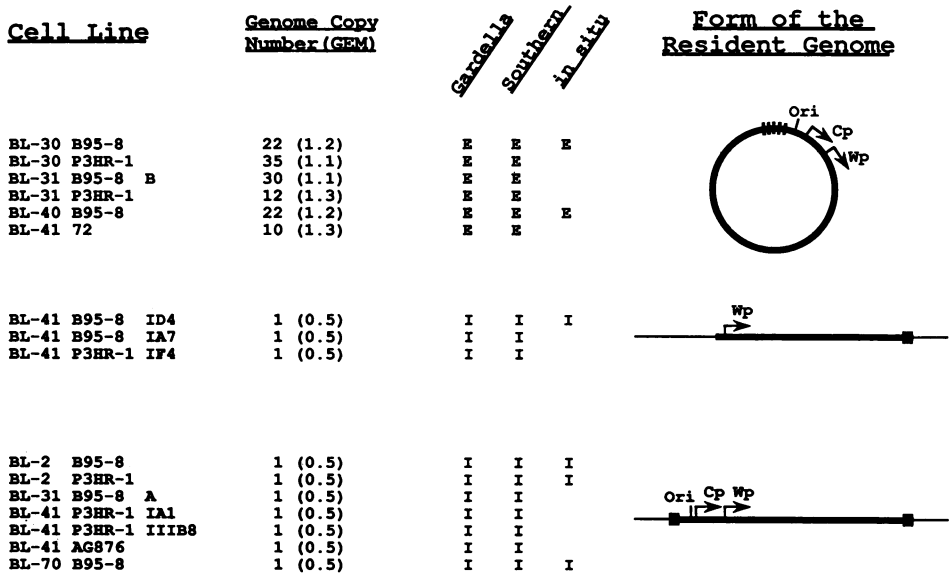


FIG. 7. Summary of the cell lines used and the conclusions drawn about the form of the persistent genome. The EBV genome copy number was assessed by quantitative dot blot analysis (6, 26). Geometric error of the mean (GEM) was estimated from standard curves. Abbreviations and symbols: I, integrated; E, episomal; Ori, origin of replication; Cp, C promoter; Wp, W promoter; ——, cellular DNA; ▬, viral DNA.

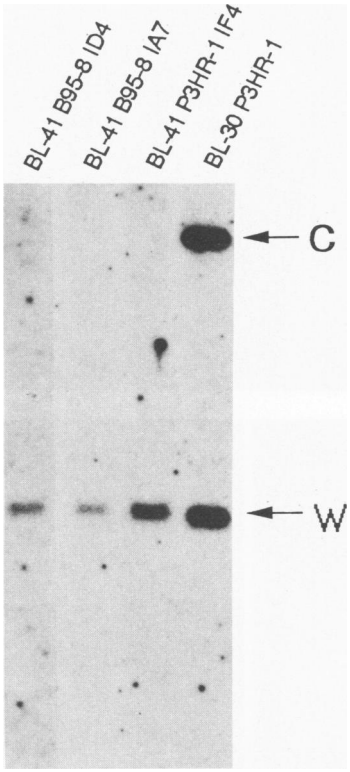


FIG. 8. Analysis of the partially deleted, integrated genomes for the presence of the *Bam*HI fragments C and W. Whole genomic DNA was digested with *Bam*HI and fractionated on an agarose gel. The four cell lines analyzed were the three BL-41 converted lines that lacked a left-hand end (Fig. 6) and BL-30 P3HR-1, which has multiple CCCs, as a positive control. The blots were probed with both the *Bam*HI fragments C and W4 (Fig. 5). The position of migration of the *Bam*HI fragments C and W are marked with arrows.

given cell line the same chromosomal site was consistently labeled. The hybridization efficiency and signal-to-noise ratio of this approach is extremely high, such that greater than 95% of fluorescence signals are associated with a single specific chromosomal location. Results from BL-70 B95-8 and BL-2 P3HR-1 are illustrated in Fig. 9A and B. Figure 9C illustrates, for the BL-2 B95-8 line, that the morphology of the chromosome and the position of the label are always the same from cell to cell for a given clonal line. In contrast, different cell lines showed labeling of a different chromosomal site, indicating that integration is not occurring at a preferential site.

Two cell lines, independently shown to carry CCCs by Gardella gel and Southern analysis, gave a very different pattern of hybridization. They were hybridized in the same experiment and under identical conditions by using the same probe preparation as for the lines described above. The BL-30 B95-8 and BL-40 B95-8 cell lines showed numerous single hybridization signals consistent with the 20 to 30 episomal copies that we have independently estimated to be present in these cells. Up to 50 fluorescent signals were observed in individual metaphase spreads (which have a G_2 DNA content) (Fig. 9F to I). Both the chromosomal location as well as the number of signals varied among different metaphase spreads. As expected, the episomal signals were associated with the chromosomes, particularly along the chromosome periphery. The episomes sometimes appeared slightly clustered or paired; however, it was clear that the vast majority were not present in the same position on both sister chromatids but were aligned along the chromosome length. Occasionally, a pair of signals were present in identical positions on each sister chromatid, consistent with the presence of integrated DNA; however, further analysis would be required to unequivocally determine if this in fact represents the coexistence of integrated and episomal DNA.

Thus, the in situ analysis provides direct and complementary evidence to support our conclusion that EBV is integrating in these cell lines. Furthermore, to our knowledge this is the first direct visualization of episomal EBV on a single-cell basis and provides a direct demonstration of the power of the fluorescence in situ technique to reveal the relationship of persistent EBV DNA with the cellular genome.

DISCUSSION

In this paper we show that EBV can, under certain conditions, reproducibly integrate after infection. This depends upon the nature of the target cell.

It has been shown previously that EBV infects and transforms resting B cells, persisting as multiple, covalently closed episomes (i.e., CCCs) (3, 22). In our previous studies we demonstrated that circularization of a single linear genome occurs sometime in early G_1 (12). Cells that contain a CCC transcribe all of the latent EBV proteins (5), express an activated B cell phenotype, and proceed to proliferate (12). No further circularization events are detected despite the persistence of multiple linear genomes for several days postinfection. We suggested that EBV preferentially transforms resting cells because passage through cellular activation (G_1) was required for the genome to attain the form in which it persists, i.e., the CCC. The current studies lend support to this conclusion, namely, that when EBV infects PBL-B cells that are already activated, it does not form detectable CCCs. Similarly, if EBV stably infects already established EBV^-BL lines, it does not usually persist as a CCC; rather, it has to integrate in order to persist. This could reflect an unfavorable environment either for CCC formation or stable retention in an activated cell. For example, proteases and nucleases could be more active in these cells, resulting in degradation of the incoming capsid and its DNA. This would also account for the more rapid loss of linear viral DNA from infected, activated cells. Furthermore, integration may be more likely in an activated cell since the DNA may be more open or available for integration than in a resting cell.

EBV infects at least 5% of the cells in an EBV^-BL line, as judged by EBNA expression, but stably converts them with a low efficiency, approximately 1 in 10^3 to 10^4 (15a). The failure of these lines to form stable CCCs has allowed us to detect stable integration of EBV into cellular DNA. The low frequency of stable conversion and the observation that only one integrated genome is detected in each cell would suggest that integration is a relatively rare event in this system. This would be difficult to detect if it occurred in normal B cells infected with EBV, where greater than 50% of the resting cells can circularize and amplify a genome (12). The low frequency of integration would also rule out a role for integration in primary transformation.

The resulting form of the persisting genome in the converted BL cell lines may, therefore, reflect heterogeneity in the target cell population. BL cells in vivo are thought to be related to the germinal center centroblast (9), an immature

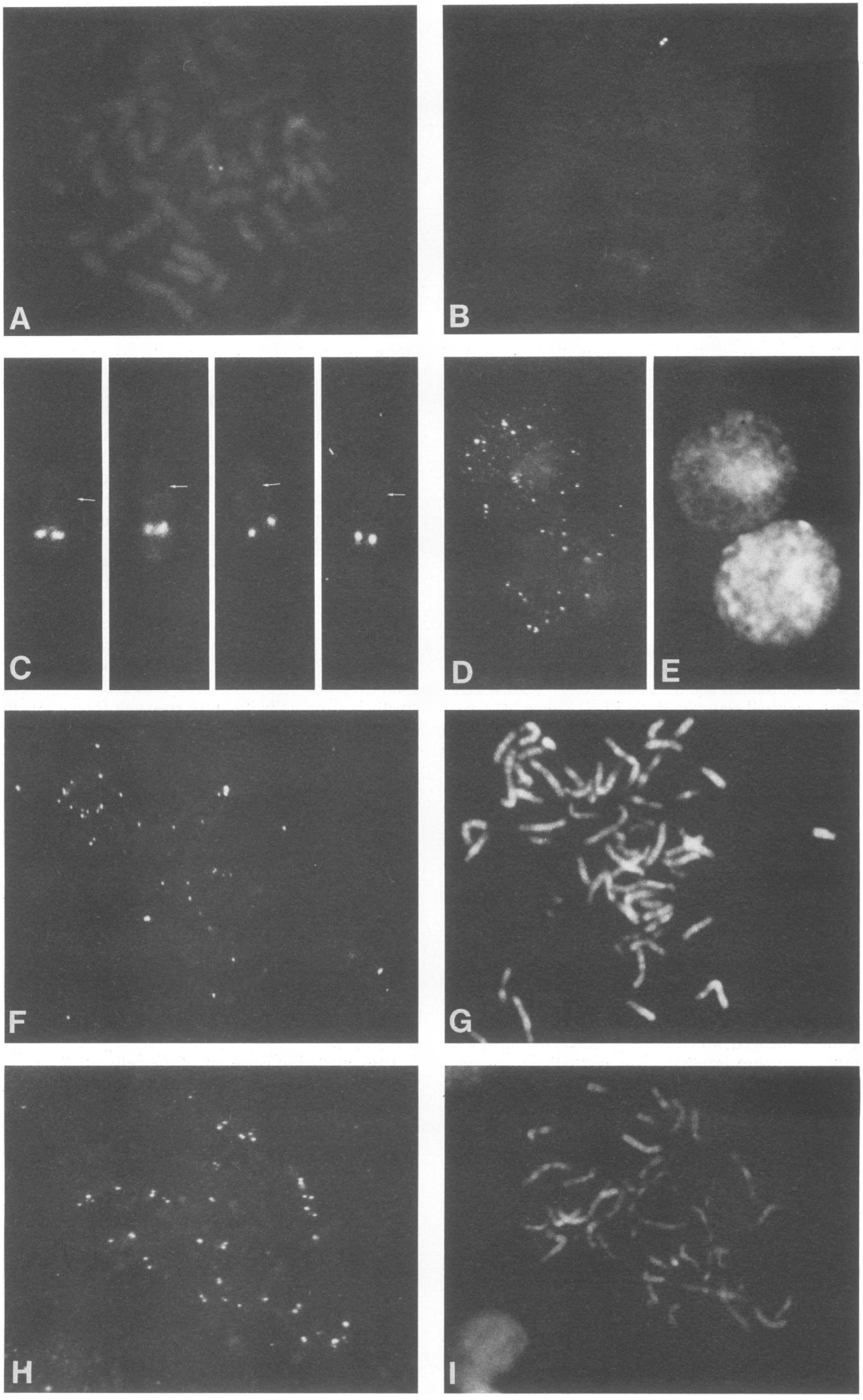

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FIG. 9. Visualization of the EBV genome by high-resolution in situ hybridization. (A) Hybridization of EBV *Bam*HI fragment W to the BL-70 B95-8 cell line shows a single site of EBV integration with paired signals on sister chromatids. This metaphase spread is counterstained with propidium iodide to outline the chromosome morphology. The signal is located just below the centromere. (B) BL-2 P3HR-1 cells show hybridization consistently to both sister chromatids of a single chromosomal site distinct from that in BL-70 B95-8. (C) Details of chromosomes from four different BL-2 B95-8 cells. The integration is consistently at the same site on the same chromosome distinct from that seen in panels A and B. Arrow indicates the centromere as a morphological landmark. (D) Interphase nucleus of a BL-40 B95-8 cell shows hybridization to numerous episomal sites. Some variability in the number of sites is observed in both interphase and metaphase. This is in contrast to integrated genomes in the absence of episomes where one or two signals are normally observed, depending on the total DNA content of the cell (15). (E) DAPI staining of total DNA shows the morphology of the nuclei in panel D. (F) Hybridization to a BL-40 B95-8 metaphase spread shows numerous episomal sites associated with the chromosomes. Comparison with the DAPI-stained image reveals that signals which are close together or which appear to be paired are generally positioned along the length of the chromatid and are not paired in identical positions across the width of the chromosome, as is seen with integrated DNA. (G) DAPI staining shows the morphology of the chromosome in the metaphase spread from panel F. (H) BL-30 B95-8 gives a pattern of hybridization similar to that of BL-40 B95-8 consistent with episomes. This film was overexposed to make the chromosomes more readily apparent. (I) DAPI staining of the nucleus from panel H.

proliferating B cell that phenotypically resembles a resting rather than an activated B cell. However, the phenotype of BL cells is known to drift towards an activated cell phenotype in culture (23). Thus, cultured EBV$^-$BL cells could be heterogeneous. On the basis of our hypothesis, EBV infection of the cells with a more resting phenotype may favor formation of CCCs compared with cells of a more activated phenotype, where integration would be favored. It should be possible to test this idea by selecting target cells on the basis of their phenotype, prior to infection.

In every example of integration that we have studied, the DNA appears to have integrated as a linear genome. Southern blot analysis revealed no gross rearrangements or deletions, with the exception of three lines that have all deleted a portion of the left end of the genome sufficient to remove the origin of replication. It is apparent that integration of deleted genomes may be occurring at a significant frequency, 30% of the total cases we have studied and 50% of the BL-41 cases, raising the possibility that it could be extremely high in certain cell types. Furthermore, the deletions we have seen have removed one of the major latent promoters (the C promoter). Further leftward deletion would remove the other (the W promoter) and could result in failure to express EBNA. Since all of the converted lines we have used were selected by staining for EBNA expression, such integration events would have been missed in our analysis. It is conceivable, therefore, that integration is occurring at a higher frequency and may involve more extensively deleted genomes. Cells carrying such integrated genomes would have only one partial copy of the virus and could be expressing EBNA aberrantly or not at all.

Nevertheless, it is surprising that all of the deletions we analyzed had lost a similar amount of DNA from the same end and that they were all BL-41 converts. This raises the possibility that, in some cases, the presence of ori-p could be detrimental such that integrated EBV could only persist if the ori-p were deleted. Since, as discussed above, BL cell lines are heterogeneous, it is possible that in some cells the ori-p may be functional and interfere with cellular replication; thus, only integrated DNA lacking ori-p could persist. On the other hand, intact integrated DNA could persist in cells where ori-p was nonfunctional. This could arise if certain cellular factors were absent that are required for ori-p function, perhaps through interaction with EBNA-1. Indeed, it would be interesting to know how the ori-p behaves in the cells with intact genomes and how overreplication, which characterizes the amplification of the CCC form, is suppressed.

The application of the fluorescence in situ hybridization technique not only allows the direct confirmation of integration events, but also allows the direct visualization of the CCC on a single-cell basis (24; this paper). This is possible since the technique, unlike traditional isotopic methods, does not rely on statistical analysis to locate the hybridizing signal. Furthermore, the method appears to be representative, since the number of hybridizing signals seen corresponded remarkably well with our estimates of genome copy number, based on traditional blotting techniques, implying no significant loss of CCC during the procedure. By careful scanning of metaphase spreads from a number of cells of the same cell line, it should now be possible to determine if there are integrated genomes in cells that carry multiple CCCs. This is possible since only an integrated copy would be present on both sister chromatids at the same location in every cell.

Since it was apparent that the integration events we have analyzed by in situ hybridization were at various locations on morphologically distinct chromosomes, we did not attempt to map them more accurately. Both the in situ and the Southern blot analyses revealed nothing obvious in common between the integration sites. It will, therefore, be important to study the sites of integration at the DNA sequence level to gain some insights into the possible mechanism of integration.

Earlier studies suggested that integrated EBV DNA may be present in a variety of EBV-infected cell types (4, 13); however, the only integration event that has been studied in detail, at the molecular level, is the one in the Namalwa cell line (11, 15, 17). The recognition that Namalwa is an old established cell line with many chromosomal abnormalities, combined with the failure to demonstrate integration, reproducibly, in any in vitro or in vivo system, casts doubt upon the significance of these findings. Our results provide the first reproducible system to study EBV integration and again raise the possibility that integrated viral genomes could be present in tumor cells both in the presence and absence of CCCs. Fragmented integrated genomes especially could give rise to aberrant expression of viral, cellular, or fusion proteins, thus providing potential oncogenic activities. This suggests that a reassessment of the presence of integrated intact and fragmented genomes in traditional EBV-associated and other neoplasms is now warranted.

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