JOURNAL OF VIROLOGY, Oct. 1985, p. 328–332
0022-538X/85/100328-05$02.00/0

Vol. 56, No. 1

Random Association of Epstein-Barr Virus Genomes with Host Cell Metaphase Chromosomes in Burkitt's Lymphoma-Derived Cell Lines

ANN HARRIS,*† BRYAN D. YOUNG, AND BEVERLY E. GRIFFIN‡
Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, England

Received 20 December1984/Accepted 24 April 1985

The random association of Epstein-Barr virus DNA with host cell metaphase chromosomes of all sizes in Burkitt's lymphoma-derived cell lines was demonstrated by two substantially different techniques, namely fluorescence-activated chromosome sorting and in situ hybridization. The nature and potential importance of this association are discussed.

It is still not clear whether integration of Epstein-Barr virus (EBV) into the host cell genome is an important primary event during the genesis of Burkitt's lymphoma (BL). Each individual BL-derived cell line carries a characteristic number of copies of the EBV genome that remains constant from one cell generation to the next but may vary widely among different cell lines; the majority, if not all, of these copies are episomal in nature (2). Attempts to study integration are hindered by the fact that when chromosomal DNA is isolated from BL-derived cell lines by a variety of methods it is contaminated with EBV DNA; this has generally been assumed to be due to the fact that most methods of isolating cellular DNA yield shear-produced fragments that are about the same size as linearized EBV DNA (172 kilobase pairs; reference 7) and may copurify with the latter. However, the possibility that one or a few copies of the genome may be integrated cannot be ruled out.

Indirect evidence in favor of EBV integration exists (3, 12). Further, direct evidence has been obtained, at least in the case of Namalwa (16, 19) and Raji (4, 25) isolates; these data, however, suffer from the disadvantage that both cell lines show gross chromosomal rearrangements (16; A. Harris, data not shown). The latter is a common feature of most of the long-established BL-derived cell lines, some of which have been in culture for up to 20 years (17, 29; A. Harris et al., unpublished observations). In the presence of such chromosomal instability it is therefore possible that any detected site of EBV integration might not be of primary importance in the genesis of the tumor.

The purpose of the present study was to determine whether any EBV DNA is genuinely covalently linked to (that is, integrated into) the cellular DNA in newly established BL lines. We analyzed three recently isolated and characterized BL-derived cell lines (8, 9, 14) that show few, if any, chromosomal abnormalities other than the t2;8, t8;14, and t8;22 reciprocal translocations that are characteristically associated with Burkitt's lymphoma. The major problem in such an analysis is the presence of many episomal copies of the virus in these, as in most, BL lines. Two methods, aimed at separating extrachromosomal EBV and chromosomal DNAs, are described here, namely, fluorescence-activated chromosome sorting and in situ hybridization under a variety of denaturing conditions. The basis of the former method is that after staining with ethidium bromide the relative fluorescence value of each chromosome correlates quite closely with its DNA contents. Hence, a flow karyotype can be constructed in which discrete peaks of fluorescence intensity correspond to individual or groups of chromosomes with similar DNA contents. These can then be sorted separately and purified (11, 20, 21, 28). Since the amount of DNA in episomal forms of EBV is so much smaller than in the smallest human chromosome, we anticipated that this technique might be capable of removing episomal EBV from host cell chromosomal DNA.

Fluorescence-activated chromosome sorting was carried out broadly as described by Young et al. (28). Cells were blocked in metaphase by exposure to colchicine (Colcemid; GIBCO Laboratories) at 0.05 mg/ml of culture medium for 16 h and then harvested by centrifugation at 900 × *g* for 15 min. The cell pellet was washed once in phosphate-buffered saline, suspended after centrifugation in 0.075 M KCl, then spun at 600 × *g* for 8 min, and suspended in polyamine buffer (15 mM Tris hydrochloride, 0.2 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 0.5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 80 mM KCl, 20 mM NaCl, 14 mM β-mercaptoethanol [pH 7.2]). The suspension was spun at 900 × *g* for 12 min and then suspended in 0.1% digitonin in polyamine buffer. Cells were vortexed for 10 s to release metaphase chromosomes, which were stained with ethidium bromide at 0.5 mg/ml. Chromosomes were analyzed on a Becton Dickinson Fluorescence Activated Cell Sorter II. A Spectra Physics 164-05 laser producing 1.5 W of power output at 514.5 nm was used to excite chromosomal fluorescence, and a Schott OG550 emission filter blocked scattered laser light. Dual parameter analysis of scatter and fluorescence signals allowed discrimination of chromosomes from certain background signals.

Flow karyotypes of the non-BL cell line Ball-1 and the BL cell lines LY47, LY91, and BL8 are shown in Fig. 1; novel peaks due to the t8;22, t2;8, or t8;14 translocations in the BL cell lines are indicated. Ball-1 has a normal diploid male human karyotype, and the BL cell lines, except for the translocations, show similar patterns. Sorting experiments were only carried out when flow karyotypes were as clear as those shown in Fig. 1, that is, with very low background fluorescence levels and discrete peaks contributed by spe-

* Corresponding author.
† Present address: Paediatric Research Unit, Guy's Hospital Medical School, Guy's Tower, London Bridge, London, SE1 9RT, England.
‡ Present address: Department of Virology, Royal Postgraduate Medical School, London W12, England.

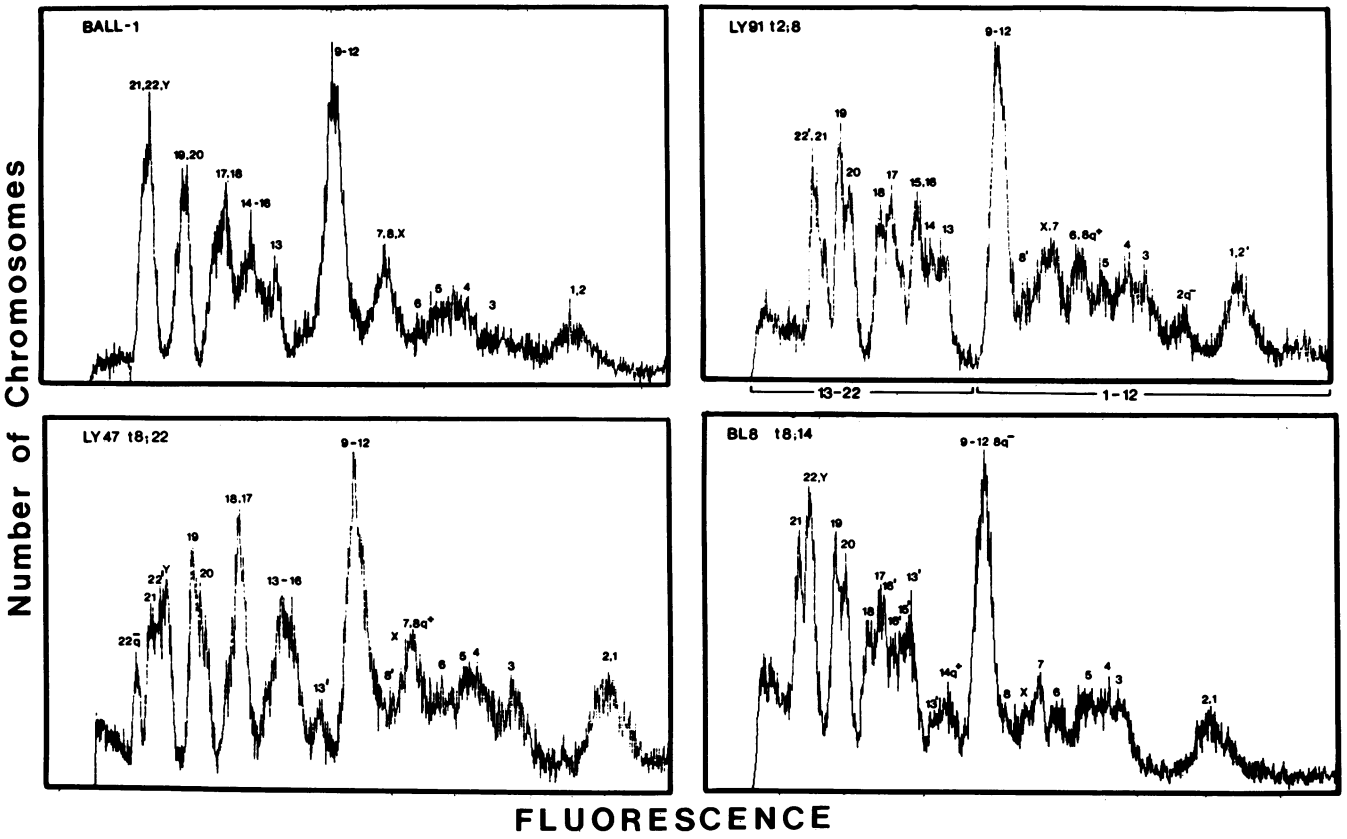

FIG. 1. Flow karyotypes of BL cell lines LY47, LY91, BL8, and Ball-1, an EBV-negative, B cell lymphoma-derived cell line (16a). LY47 carries the t8;22 chromosome translocation, LY91 carries the t2;8 translocation, and BL8 carries the t8:14 translocation characteristic of BL-derived cell lines. LY47 and BL8 are derived from males while LY91 is derived from a female. The chromosome contribution of each peak is marked. Ball-1 has a normal male human karyotype. Relative to this, the $22q^-$, $2q^-$, and $14q^+$ translocated chromosomes in LY47, LY91, and BL8, respectively, were observed to produce novel peaks. The reciprocals of these translocations altered the area under other peaks contributed by normal chromosomes. Size sorts of chromosomes 1 to 12 and 13 to 22 are shown in the LY91 panel.

cific chromosomes. Under conditions in which a cell line has inconsistent chromosomal changes or there are many broken chromosomes, such discrete flow karyotypes are not obtained and sorting would not be carried out. Two sets of experiments were carried out on sorted material. In the first, total sorts were made; that is, pure chromosomal material was isolated from the full human karyotype, collecting all chromosomes from 1 to 22 into a single sample. In the second set, material from LY91 was sorted into two samples, containing chromosomes 1 to 12 and 13 to 22, respectively, as illustrated (Fig. 1). The number of chromosomes sorted in each experiment and the amount of DNA isolated are summarized in Table 1. In all cases, DNA was extracted from sorted chromosomes cleaved with *Bam*HI restriction endonuclease, and digested material was loaded onto 0.66% horizontal agarose gels. Fragments were separated by electrophoresis in buffer containing 50 mM sodium acetate (pH 7), 1 mM EDTA, and 0.01% ethidium bromide. The DNA was transferred to nitrocellulose (27), baked, and probed with ^{32}P-labeled (26) EBV DNA fragments. Blots were washed in 2 liters of 0.3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) in 0.1% sodium dodecyl sulfate and then in 2 liters of 0.1× SSC in 0.1% sodium dodecyl sulfate.

TABLE 1. Summary of chromosome sorting experiments

Cell line	No. of chromosomes sorted (10^6)	Estimate of amt of DNA (ng)[a]
BL8	2.7	500
LY91 (all chromosomes)	2.6	490
LY47	2.4	450
LY91 (chromosomes 1-12)	2.4	600
LY91 (chromosomes 13-22)	1.8	200

[a] As estimated by Davies et al. (11) and Mendelsohn et al. (22), 2×10^6 copies of the X chromosome are approximately equal to 400 to 500 ng of DNA. Mean DNA content of a human chromosome was estimated from the average of all chromosomes. EBV copy numbers: LY47, 35; LY91, 120; BL8, 220 (14).

When sorted material (Fig. 2a) was cleaved with *Bam*HI and probed with the large repetitive sequence encompassed by B95-8 (23) *Bam*HI fragment W (Fig. 2d), the pattern of restriction fragments hybridizing to the probe in each case was qualitatively identical to that seen in *Bam*HI-cleaved total cellular DNA from the same cell line (Fig. 2b). Further, allowing for differences in specific activity of probes used in Fig. 2a and b, there was no substantial reduction in the amount of EBV associated with 500 ng of sorted chromosomes, compared with that found in an equivalent amount of total cellular DNA from the same cell line. It is clear from these results that, contrary to expectation, the technique of fluorescence-activated cell sorting did not separate episomal EBV from human chromosomes; there appeared to be a

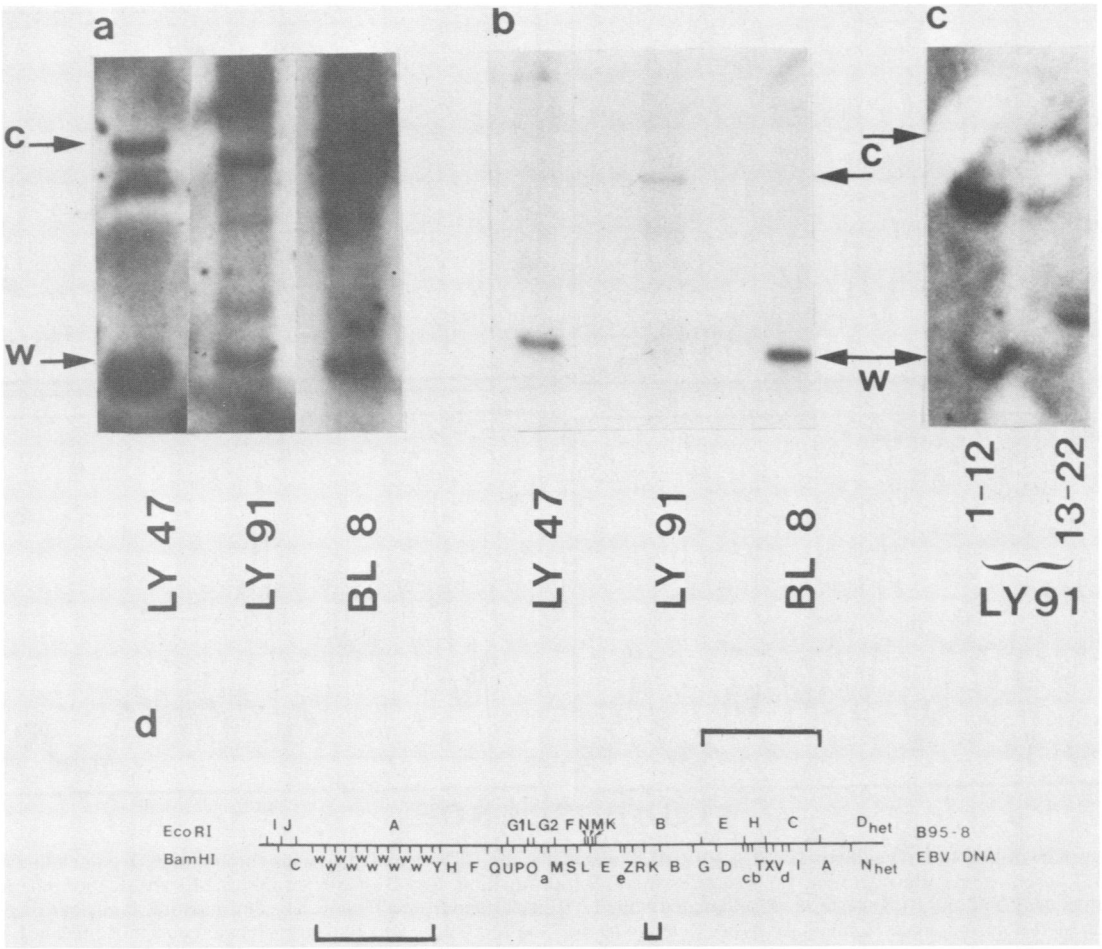

FIG. 2. Hybridizations of sorted chromosomal material (a and c) from cell lines LY47, LY91, and BL8 as marked (amounts of DNA shown in Table 1), and 500 ng of total cellular DNA (b) from the same cell lines. Data obtained by hybridization with the cloned *Bam*HI W restriction fragment of B95-8 EBV DNA are shown. This probe also hybridizes to the *Bam*HI C and Y restriction fragments of EBV DNA (18). Novel restriction fragments hybridizing to *Bam*HI-W in the LY91 cell line (migrating between *Bam*HI fragments C and W) may be accounted for by a major defective species within this cell line (14). High background hybridization levels are due to the long exposure times required to detect specific hybridization signals in small amounts of sorted material. Weak hybridization at the position of *Bam*HI fragment C in lane marked 1-12 shown in panel c is due to a technical problem and does not reflect a genuine difference between the two lanes. Panel d shows *Eco*RI and *Bam*HI restriction maps of B95-8 EBV DNA (5). Probes used in Southern hybridizations and in situ hybridization (see the text) are marked.

close association of EBV episomes with host cell metaphase chromosomes. Moreover, a restriction enzyme analysis of viral DNA associated with host chromosomes of different sizes (Fig. 2c), that is, chromosomes 1 to 12 or 13 to 22, suggested that this was not confined to a limited number of sites on specific chromosomes. Rather, episomal EBV appeared to be anchored to host cell metaphase chromosomes of all sizes, equally distributed between large (1 to 12) and small (13 to 22) chromosomes, as is shown in Fig. 2c, bearing in mind the amount of sorted chromosomal DNA in each lane (Table 1).

This result was confirmed by direct analysis of the localization of EBV in one of the BL cell lines, BL8, by in situ hybridization. In all in situ hybridization experiments, a probe for human satellite III DNA, located in the centromeric region of chromosome 1, was used as a positive control (10). In situ hybridization was carried out as follows. Metaphase chromosomes were prepared by standard procedures and spread on glass slides. They were then dehydrated through an ethanol series, twice in 70 and once in 90% ethanol, air dried, and RNase treated for 1 h at 20°C (RNase type IIIA at 100 μ/ml; Sigma Chemical Co.) in 2× SSC, pH 7. After being washed twice in 2× SSC, slides were acetylated by addition of acetic anhydride (5 ml/liter in 0.1 M triethanolamine) and, after 5 to 10 min, again dehydrated. Chromosomes were denatured by immersion in 60% formamide–0.2 M EDTA at 50 or 55°C for 4 min, and denaturation was quenched by immersion in 70% ethanol, followed by dehydration through the ethanol series as before. Hybridization probes were labled with ^{125}I (26) to specific activities of 5×10^7 to 1×10^8 cpm/μg of DNA.

Denatured, radioactively labeled probe solution (40 μl) at 100 ng/ml (in 50% formamide, 2× SSC [pH 7 to 7.5], 5 mM NaH_2PO_4, 1× Denhardt solution, 50 μg of denatured salmon sperm DNA per ml, 0.1 mM KI, and 10% dextran sulfate) was placed on each slide, and the solution was overlaid with a cover slip. The slides were placed in an environment saturated with hybridization buffer at 42°C for 15 h, then submerged in 2× SSC to remove the cover slips, and washed four times in 2× SSC at 25°C, twice in 2× SSC at 65 to 68°C

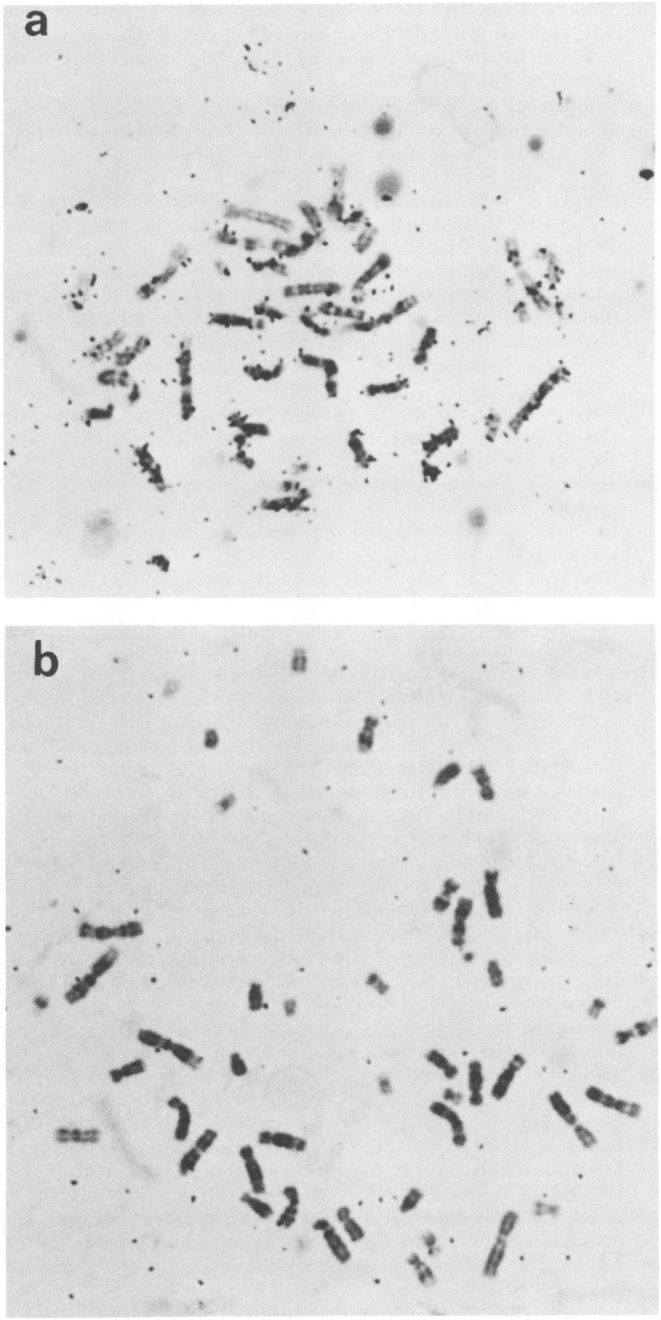

FIG. 3. In situ hybridization on metaphase chromosomes from the BL8 cell line, with ^{125}I-labeled B95-8 *Eco*RI combined fragments C, E, and H as a probe. Chromosomes were banded with Wright stain. (a) Chromosome denaturation was carried out at 50°C, and strong hybridization signals were seen on nearly all metaphase chromosomes, while the background levels of nonspecific hybridization were low. (b) Chromosome denaturation was carried out at about 55°C, and only a few hybridization signals were seen over metaphase chromosomes, while background levels of nonspecific hybridization were similar to those in panel a.

for 15 min each, once in 0.1× SSC at 50°C for 15 min, and then five times in 0.1× SSC at 25°C. Finally, the slides were dehydrated through an ethanol series (as above), air dried, and dipped in a 1:1 (wt/vol) dilution of Kodak NTB-2 autoradiographic emulsion in water. After 3 days to 1 month, slides were developed in Kodak D19 developer and Kodafix.

Under the conditions of low background labeling (Fig. 3a), strong hybridization signals were seen along most metaphase chromosomes when they were probed with a mixture of ^{125}I-labeled B95-8 *Eco*RI fragments C, E, and H (Fig. 2d). Although in the particular metaphase spread shown in Fig. 3a not all chromosomes have autoradiographic grains, analysis of many metaphase spreads showed on the average equal levels of hybridization on all chromosomes. Since *Eco*RI fragments C, E, and H have not been observed to hybridize nonspecifically to host chromosomal DNA (6, 24), the data (Fig. 3a) reveal the presence of episomal EBV attached to metaphase chromosomes. In similar studies, the *Bam*HI K restriction fragment of EBV DNA has been shown to hybridize to at least one region of every human chromosome except the Y chromosome (15); however, *Bam*HI-K has been shown to have substantial homology to cellular DNA (6, 15).

To search for specificity, the in situ hybridization protocol used in Fig. 3a was modified such that the temperature of chromosome denaturation was increased by about 5°C. Under these conditions, with the same mixed *Eco*RI fragments C, E, and H probe, the vast majority of episomal EBV molecules attached to metaphase chromosomes were found to have been removed (Fig. 3b). No effect on the overall chromosome architecture, as observed by Wright's banding, was evident. This result suggests that, although most episomal molecules of EBV become anchored to host cell chromosomes at metaphase, this interaction does not involve covalent linkage since it may be disrupted under appropriate experimental conditions during in situ hybridization.

We have thus demonstrated close association of episomal EBV DNA with metaphase chromosomes of BL-derived cell lines by two entirely different techniques. Moreover, there was no obvious selectivity in this association; there may be specific chromosomal sites for EBV DNA anchorage, but the in situ hybridization data suggest that these occur at random throughout the genome.

The nature of the linkage between EBV episomes and metaphase chromosomes is unknown. However, since the nonspecific RNase digestion step of the in situ hybridization procedure did not destroy it, RNA probably does not form an important part of the linkage. Therefore, we suggest that some thermolabile DNA-protein or DNA-DNA interaction may be involved. In this context, it is interesting to note that the EBV nuclear antigen I encoded within the *Bam*HI K restriction fragment of the virus has been shown to be invariably present on the chromosomes in metaphase preparations of mouse LTK cells and Raji cells (13). The precise function(s) of EBV nuclear antigen I is still unclear, although it may have some role in EBV replication (27a; J. Yates, personal communication). It is possible that EBV nuclear antigen I or other viral or cellular proteins are important in this episome-to-chromosome interaction.

Finally, the possible role of anchorage of EBV episomes on host cell chromosomes in the viral life cycle seems worthy of comment. It is known that although the EBV DNA copy number may vary widely from one BL-derived cell line to another, within any specific line that number will remain approximately constant from one cell generation to the next (1). It is not clear how this numerical constancy is achieved, but it seems a reasonable hypothesis that through anchorage to host cell metaphase chromosomes, the virus could ensure that approximately equal numbers of episomes

become segregated on each side of the metaphase plate and hence in each daughter cell.

We thank G. Lenoir, International Agency for Cancer Research, Lyon, France, for BL cell lines LY47, LY91, and BL8; Pete Harris for help with chromosome sorting and analysis; and Georgina Briody and Audrey Symons for help with preparation of the manuscript.

The chromosome sorting experiments were supported by grants from the Cancer Research Campaign.

LITERATURE CITED

1. **Adams, A.** 1979. The state of the virus genome in transformed cells and its relationship to host cell DNA, p. 158–159. *In* M. A. Epstein and B. G. Achong (ed.), The Epstein-Barr virus. Springer-Verlag, Berlin.
2. **Andersson, M., and T. Lindahl.** 1976. Epstein-Barr virus DNA in human lymphoid cell lines: *in vitro* conversion. Virology **73**:96–105.
3. **Andersson-Anvret, M., and T. Lindahl.** 1978. Integrated viral DNA sequences in Epstein-Barr virus-converted human lymphoma lines. J. Virol. **25**:710–718.
4. **Anvret, M., A. Karlsson, and G. Bjursell.** 1984. Evidence for integrated EBV genomes in Raji cellular DNA. Nucleic Acids Res. **12**:1149–1161.
5. **Arrand, J. R., L. Rymo, J. E. Walsh, E. Bjork, T. Lindahl, and B. E. Griffin.** 1981. Molecular cloning of the complete Epstein-Barr virus genome as a set of overlapping restriction endonuclease fragments. Nucleic Acids Res. **9**:2999–3014.
6. **Arrand, J. R., J. E. Walsh-Arrand, and L. Rymo.** 1983. Cytoplasmic RNA from normal and malignant human cells shows homology to the DNAs of Epstein-Barr virus and human adenoviruses. EMBO J. **2**:1673–1683.
7. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310**:207–211.
8. **Bernheim, A., R. Berger, and G. Lenoir.** 1981. Cytogenetic studies of African Burkitt's lymphoma cell lines: t(8;14), t(2;8) and t(8;22) translocations. Cancer Genet. Cytogenet. **3**:307–315.
9. **Bernheim, A., R. Berger, and G. Lenoir.** 1983. Cytogenetic studies on Burkitt's lymphoma lines. Cancer Genet. Cytogenet. **8**:223–229.
10. **Cooke, H. J., and J. Hindley.** 1979. Cloning of human satellite III DNA: different components are on different chromosomes. Nucleic Acids Res. **6**:3176–3197.
11. **Davies, K. E., B. D. Young, R. G. Elles, M. E. Hill, and R. Williamson.** 1981. Cloning of a representative genomic library of the human X chromosome after sorting by flow cytometry. Nature (London) **293**:374–376.
12. **Griffin, B. E., E. Bjorck, G. Bjursell, and T. Lindahl.** 1981. Sequence complexity of circular Epstein-Barr virus DNA in transformed cells. J. Virol. **50**:11–19.
13. **Grogan, E. A., W. P. Summers, S. Dowling, D. Shedd, L. Gradoville, and G. Miller.** 1983. Two Epstein-Barr virus nuclear neo-antigens distinguished by gene transfer, serology and chromosome binding. Proc. Natl. Acad. Sci. USA **80**:7650–7653.
14. **Harris, A., J. E. Walsh-Arrand, G. M. Lenoir, and B. E. Griffin.** 1984. Molecular characterisation of the Epstein-Barr virus DNA in three new Burkitt's lymphoma-derived lines. Mol. Biol. Med. **2**:135–150.
15. **Heller, M., A. Henderson, and E. Kieff.** 1982. Repeat array in Epstein-Barr virus DNA is related to cell DNA sequences interspersed on human chromosomes. Proc. Natl. Acad. Sci. USA **79**:5916–5920.
16. **Henderson, A., S. Ripley, M. Heller, and E. Kieff.** 1983. Chromosome site for Epstein-Barr virus DNA in a Burkitt tumor cell line and in lymphocytes growth-transformed *in vitro*. Proc. Natl. Acad. Sci. USA **80**:1987–1991.

16a. **Hiraki, S., I. M. Yoshi, H. Masuji, I. Kubonishi, Y. Matsuda, T. Nakayama, H. Kishimoto, P. Chen, and I. Kimura.** 1977. Establishment of an Epstein-Barr virus nuclear antigen-negative human B-cell line from an acute lymphoblastic lymphoma. J. Natl. Cancer Inst. **59**:93–94.

17. **Jarvis, J. E., G. Ball, A. Rickinson, and M. A. Epstein.** 1974. Cytogenetic studies on human lymphoblastoid cell lines from Burkitt's lymphomas and other sources. Int. J. Cancer **14**:716–721.
18. **Jones, M. D., and B. E. Griffin.** 1983. Clustered repeat sequences in the genome of Epstein Barr virus. Nucleic Acids Res. **11**:3919–3937.
19. **Klein, G., and L. Dombos.** 1973. Relationship between the sensitivity of EBV-carrying lymphoblastoid lines to superinfection and the inducibility of the resident viral genome. Int. J. Cancer **1**:327–337.
20. **Krumlauf, R., M. Jean-Pierre, and B. D. Young.** 1982. Construction and characterization of genomic libraries from specific human chromosomes. Proc. Natl. Acad. Sci. USA **79**:2971–2975.
21. **Lebo, R. V., A. V. Carrano, K. Burkhart-Schultz, A. M. Dozy, L. C. Yu, and Y. W. Kan.** 1979. Assignment of human β-, γ- and δ-globin genes to the short arm of chromosome 11 by chromosome sorting and DNA restriction enzyme analysis. Proc. Natl. Acad. Sci. USA **76**:5804–5808.
22. **Mendelsohn, M. L., B. H. Mayall, E. Bogart, D. H. Moore, and B. H. Perry.** 1973. DNA content and DNA-based centromeric index of the 24 human chromosomes. Science **179**:1126–1129.
23. **Miller, G., and M. Lipman.** 1973. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. Proc. Natl. Acad. Sci. USA **70**:190–194.
24. **Peden, K., P. Mounts, and G. S. Hayward.** 1982. Homology between mammalian cell DNA sequences and human herpes virus genomes detected by a hybridization procedure with high complexity probe. Cell **31**:71–80.
25. **Pulvertaft, R. J. V.** 1965. A study of malignant tumours in Nigeria by short term tissue culture. J. Clin. Pathol. **18**:261–273.
26. **Rigby, P., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling DNA to a high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–252.
27. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.

27a. **Yates, J., N. Warren, D. Reisman, and B. Sugden.** 1984. A *cis* acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids. Proc. Natl. Acad. Sci. USA **81**:3806–3810.

28. **Young, B. D., M. A. Ferguson-Smith, R. Sillar, and E. Boyd.** 1981. High-resolution analysis of human peripheral lymphocyte chromosomes by flow cytometry. Proc. Natl. Acad. Sci. USA **78**:7727–7731.
29. **Zech, L., U. Haglund, K. Nilsson, and G. Klein.** 1976. Characteristic chromosomal abnormalities in biopsies and lymphoid-cell lines from patients with Burkitt's and non-Burkitt lymphomas. Int. J. Cancer **17**:47–56.