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Epstein–Barr virus genomes in lymphoid cells: Activation in mitosis and chromosomal location

(*in situ* hybridization/cell cycle/integration/immunocytochemistry)

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ABSTRACT **Biotin-labeled Epstein–Barr virus (EBV)-specific DNA probes have been used to detect viral genomes by *in situ* hybridization. Immunocytochemically amplified signals produced by the hybridized probe allow visualization of viral DNA even in cells previously reported to contain only one or two EBV genomes. In EBV producer lymphoid cell lines, such as B95-8, P3HR-1, or Daudi, activation of latent EBV DNA could be observed in mitotic cells; in non-virus-producing cells of these same lines, EBV was found to be present in low copy numbers. Noninducible cell lines such as IB4, AW-Ramos, and Namalwa exhibited low but clearly positive hybridization. Unexpectedly, significant variations in the amounts of EBV DNA per cell were observed between individual cells of these lines. The EBV DNA in the cloned IB4 cell line was localized to chromosome 4 in metaphase cells, but in the noncloned converted line AW-Ramos, the location of integrated viral DNA was essentially random.**

The technique of *in situ* hybridization is potentially an invaluable method for the analysis of viral pathogenesis since it can, in a tissue sample, detect and quantitate viral genomes and transcripts in single cells that harbor the respective nucleic acid sequences. Most studies aimed at high sensitivity detection of virus genes *in situ* have utilized radioactively labeled DNA or RNA probes (1–5). Nonautoradiographic hybridization procedures, usually using biotinylated probes, have been applied in certain instances, although detection by these approaches has proved possible only in cells that have undergone lytic infection (6–8) or cells that otherwise contain viral genes at relatively high abundance (9–13). Similarly, only multicopy chromosomal genes have been identified by this technique. As part of a study to investigate the state of the Epstein–Barr virus (EBV) in viral producer and nonproducer cell lines, in an attempt to gain insight into latency (14), we have considerably increased the sensitivity of the *in situ* procedures using nonradioactive probes.

We report here that the genomic sequences of EBV present in single cells at low copy numbers can be detected *in situ* by using biotinylated double-stranded EBV-specific DNA probes. Several Burkitt lymphoma cell lines and EBV-transformed lymphoblastoid lines have been analyzed, ranging from those harboring replicative viruses to those found previously by solution hybridization to contain only a few (less than five) copies per cell of the viral genome. The resulting data substantiate the earlier findings of considerable variation of viral gene copy number among different cell lines, but further point to variation of EBV genome numbers within a particular cell line. Moreover, in producer lines, cells in mitosis appear to provide particularly rich sources of viral DNA. We also have used the modified biotinylated method to localize the EBV genome in the chromosomes of a cloned lymphoblastoid cell line in which the viral gene has been reported to be integrated into a specific chromosome site (2) and in a noncloned EBV-converted lymphoblastoid cell line not previously examined with regard to the site of viral integration.

MATERIALS AND METHODS

Cell Lines and Tumor Tissue. B95-8, P3HR-1, and Daudi are EBV producer lines in which a small number of cells spontaneously produce high copy numbers of viral DNA and undergo lysis (15). Raji and Namalwa, which are Burkitt lymphoma lines, contain 50–60 (16, 17) and 1 or 2 (18) EBV genome copies per cell, respectively. AW-Ramos, a converted line (19), and the cloned lymphoblastoid line IB4-D (20) have less than five copies of EBV DNA per cell. Ramos cells do not contain EBV DNA (21) and were used as negative controls.

Preparation of Cells and Chromosomes. The lymphoid cell lines were propagated as suspension cultures. During the exponential growth phase, they were washed in Dulbecco's phosphate-buffered saline (PBS, GIBCO), adjusted to a cell density of 10^6 cells per ml, and centrifuged onto precleaned but otherwise untreated microscope slides. The cells were then fixed in 4% (wt/vol) paraformaldehyde in PBS for 30 min, washed in PBS, dehydrated in an ethanol series, and stored at 4°C (6). Each slide always contained two identical sets of cells, one being used for hybridization with a recombinant DNA viral probe containing vector DNA and the other with vector only. Metaphase spreads of IB4-D, Namalwa, AW-Ramos, and Ramos cells were prepared as described by Lin *et al.* (22) with modifications. Cells were treated with 100–200 μg of 5-bromodeoxyuridine per ml (17 hr), washed twice with PBS, cultured in medium containing 250 μg of Colcemid per ml for 45 min, and fixed by the hypotonic method.

Hybridization Probes. EBV-specific probes were derived from cloned *Bam*HI-digested fragments of the B95-8 strain of EBV DNA (23). The *Bam*HI W fragment, 3.1 kilobases (kb) in length, was used for cytohybridization studies. In addition to fragment W, *Bam*HI fragments A, B, C, and E (11.9, 9.7, 9.2, 7.9 kb, respectively) were used for chromosomal localization. Probes were biotinylated by nick-translation with biotin-11-dUTP (Bethesda Research Laboratories) according to the manufacturer's instructions; separated from low molecular weight material by using a spin column filled with Sephadex G-50 in 50 mM Tris·HCl/1 mM EDTA/0.1% $NaDodSO_4$, pH 7.5; concentrated to 20 μg/ml; and stored at −20°C until use.

Cytological Hybridization. Treatment of cells prior to hybridization was based on the method of Brigati *et al.* (6): samples were treated with 0.02 M HCl for 10 min, washed

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Abbreviation: EBV, Epstein–Barr virus.

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twice in PBS (5 min each) permeabilized with 0.01% Triton X-100 in PBS (1.5 min), washed, digested with Pronase at 0.25 mg/ml in 0.05 M Tris·HCl, pH 7.6/5 mM EDTA (4–8 min), and then washed twice in PBS containing 2 mg of glycine per ml (5 min each). The samples were then treated with RNase A (100 μg/ml) for 1 hr at 37°C, washed twice in PBS, post fixed in 4% paraformaldehyde in PBS (5 min), washed twice in PBS containing 2 mg of glycine per ml, dehydrated through an ethanol series, and air-dried.

The hybridization cocktail consisted of 50% formamide, 10% (wt/vol) dextran sulfate, 2× SSC, (1× SSC = 0.15 M NaCl/15 mM sodium citrate), 25 mM sodium phosphate (pH 6.5), 2× Denhardt's solution (1× = 0.02% polyvinylpyrrolidone/0.02%Ficoll/0.02% bovine serum albumin), 250 μg of sonicated herring sperm DNA per ml, and 0.2 μg of probe DNA per ml. Ten microliters of the mixture was applied to each sample under a glass coverslip and the edges were sealed with rubber solution.

Target and probe DNA were denatured simultaneously by heating the slides in a convection oven for 10 min at 100°C and cooling rapidly. Hybridization was carried out at 37°C for 16–20 hr. Slides were sequentially washed in 2× SSC (three times for 10 min each at room temperature), 2× SSC (once for 30 min at 60°C), 0.2× SSC (three times for 5 min each at room temperature), and 0.2× SSC (once for 30 min at 42°C) and immersed in PBS.

Slides containing metaphase spreads and interphase cells were incubated with RNase A in 2× SSC (100 μg/ml) for 1 hr at 37°C, dehydrated in an ethanol series, denatured in 70% (vol/vol) formamide/2× SSC for 2 min at 70°C, and air-dried as described (24). The hybridization mix, of the same composition as that used for tissue hybridization, was boiled for 5 min, plunged in ice, and applied to the slides. Hybridization was also carried out at 37°C for 16–20 hr. Thereafter, slides were washed in 50% formamide/2× SSC (once for 10 min at 45°C) and then in 2× SSC (5 times for 10 min each) and were immersed in PBS.

Immunocytochemical Detection. Slides removed from PBS were washed for 15 min with buffer A (PBS containing 5% bovine serum albumin and 0.1% Triton X-100) and were incubated sequentially at 37°C in goat anti-biotin antiserum (Sigma) (diluted 1:10,000) for 60 min, in biotinylated anti-goat IgG (Sera-lab, Crawley Down, England) (diluted 1:200) for 30 min, and in avidin DH–biotinylated horseradish peroxidase H complex (Vector Laboratories, Burlingame, CA) (prepared according to the manufacturer's instructions) for 60 min. Intervening washes were made in buffer A for 15 min at room temperature. After the third incubation step, the slides were washed at room temperature in PBS containing 0.1% Tween 20 (30 min) followed by PBS (5 min), treated with PBS containing 0.1% 3,3′-diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide (5 min), and washed twice in PBS. Gold–silver amplification of the 3,3′-diaminobenzidine signal was made by the method of Burns *et al.* (25). Briefly, with intervening rinsing in deionized water, slides were sequentially incubated at room temperature for 5 min each in 2.5 mM sodium gold chloride, 0.1 M sodium sulfide (pH 7.5), and a light-insensitive physical developer consisting of 0.24 M sodium carbonate, 0.013 M ammonium nitrate, 6.0 mM silver nitrate, 1.5 mM dodecatungstosilicic acid, and 0.025%

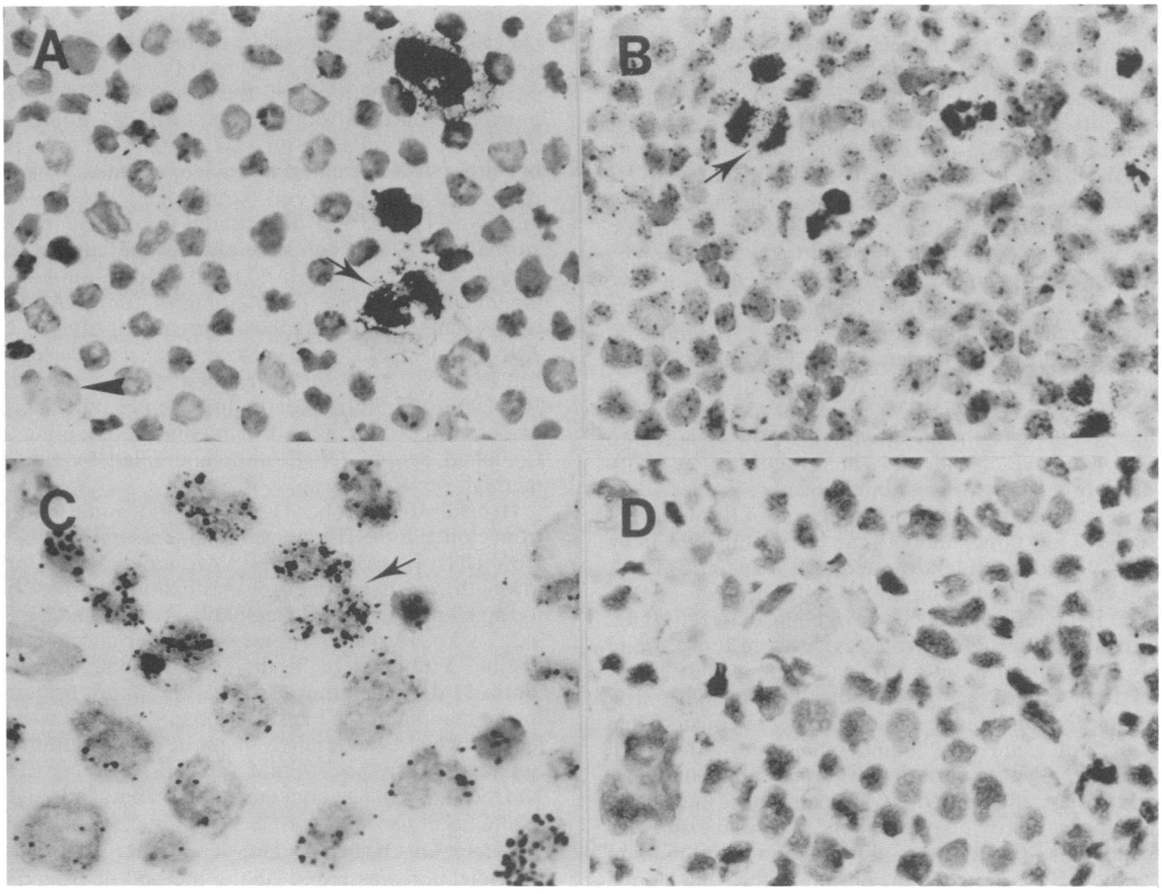

FIG. 1. *In situ* hybridization of the EBV *Bam*HI W probe to B95-8, Raji, and Ramos cells. (*A* and *B*) Detection of EBV probe DNA in nuclei of B95-8 cells with an anti-biotin antibody diluted 1:10,000 (*A*) or 1:1000 (*B*). (×550.) (*C*) Detection of EBV DNA in nuclei of Raji cells. (×1350.) (*D*) Ramos cells were probed in the same manner. (×550.) Arrows (*A*, *B*, and *C*) point to labeled cells at mitosis. The arrowhead in *A* (bottom left) points to a weakly labeled cell at mitosis.

(vol/vol) formaldehyde. The slides were then washed for 15 min in 0.17 M acetic acid followed by distilled water. Cells were counterstained with hematoxylin, washed in tap water, dehydrated, air-dried, and mounted in DePex. Chromosomes were stained with Hoechst 33258 at 1 μg/ml (15 min), immersed in 2× SSC, exposed under a Philips 40-W cool white lamp at a distance of 5 cm for 16 hr, rinsed in water, and further stained in a 7% Giemsa solution (in Sorensen's buffer at pH 6.8) for 15 min.

RESULTS

Producer Lymphoid Cell Lines. The appearance of cells of the EB virus producer lines after the hybridization-amplification detection process described above does not qualitatively differ from that produced from previous studies using less-sensitive methods (10, 15). That is, a minority of the cell population label strongly compared to the majority of cells. Hence, in our experiments, 5–10% of B95-8, 2–5% of P3HR-1, and about 1% of Daudi cells exhibited intense labeling. Fig. 1*A* shows the typical appearance of a field of B95-8 cells; similar data (not shown) were obtained with P3HR-1 and Daudi cells. Interestingly, the nuclei of strongly signaling cells of these lines frequently included cells in mitosis. In the case of B95-8, 20–40% of the strongly signaling cells were found to be in the anaphase or telophase stages of the mitotic cycle (Fig. 1 *A* and *B*); whether the remainder of the strongly signaling cells were diploid or were at the earlier stages of mitosis could not be determined. Not all cells at anaphase or telophase were labeled strongly, however, as exemplified in Fig. 1*A*.

The majority of cells of producer lines did not label intensely. On the contrary, nuclei of these cells exhibited weak positive signals or no apparent signals (Fig. 1*A*). When the primary antibody was applied at higher concentrations, positive discrete signals could be seen in most nuclei with some increase in background (Fig. 1*B*). The EBV genome numbers in nonproducer cells in these populations did not appear to differ significantly from those seen in cells of lymphoblastoid lines carrying low numbers of EBV genomes, such as IB4 (see Fig. 2*C*).

Latently Infected Cell Lines. Cells from the Raji line (containing about 50 EBV genomes per cell) displayed multiple discrete signals that filled the nuclei, regardless of the dilution of anti-biotin antibody used; the labeling intensity among nuclei was distinctly heterogeneous (Fig. 1*C*). Nuclei of the EBV-negative Ramos lines showed no significant labeling (Fig. 1*D*).

In the B-cell lines IB4, AW Ramos, and Namalwa (which carry low copy numbers of EBV genomes), positive hybridization could be seen over the nuclei of most cells after incubation with the recombinant EBV DNA-vector probe (Fig. 2 *A*, *C*, and *D*). These signals were distinctively more abundant and different from the occasional ones seen over nuclei of cells hybridized with vector sequences only, as shown in Fig. 2*B*. To determine the average number of discrete signals per nucleus, five 1000-cell counts from different preparations of each cell line were made. After

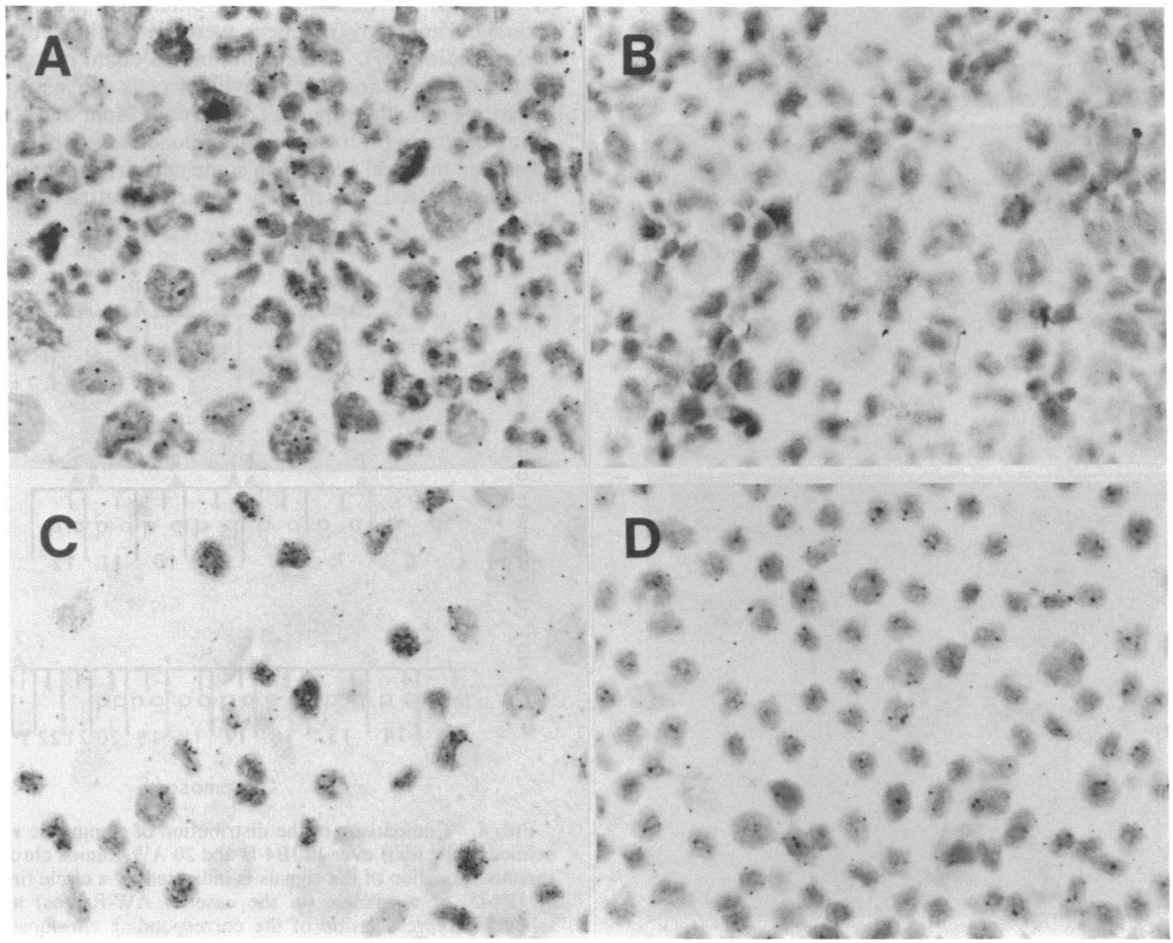

FIG. 2. Detection of low copies of the EBV genome in B-lymphocyte cell lines. Experimental conditions were as in Fig. 1*A*. (*A*, *C*, and *D*) Nuclei of Namalwa, IB4, and AW-Ramos cells, respectively, after hybridization with a recombinant EBV DNA probe that also contained vector sequences. (×550.) Note that IB4 nuclei (*C*) exhibit marked heterogeneity in size and staining intensity and that the density of hybridized signals was higher in the smaller, more darkly-staining nuclei. (*B*) Nuclei of Namalwa cells after hybridization with vector only (×550.)

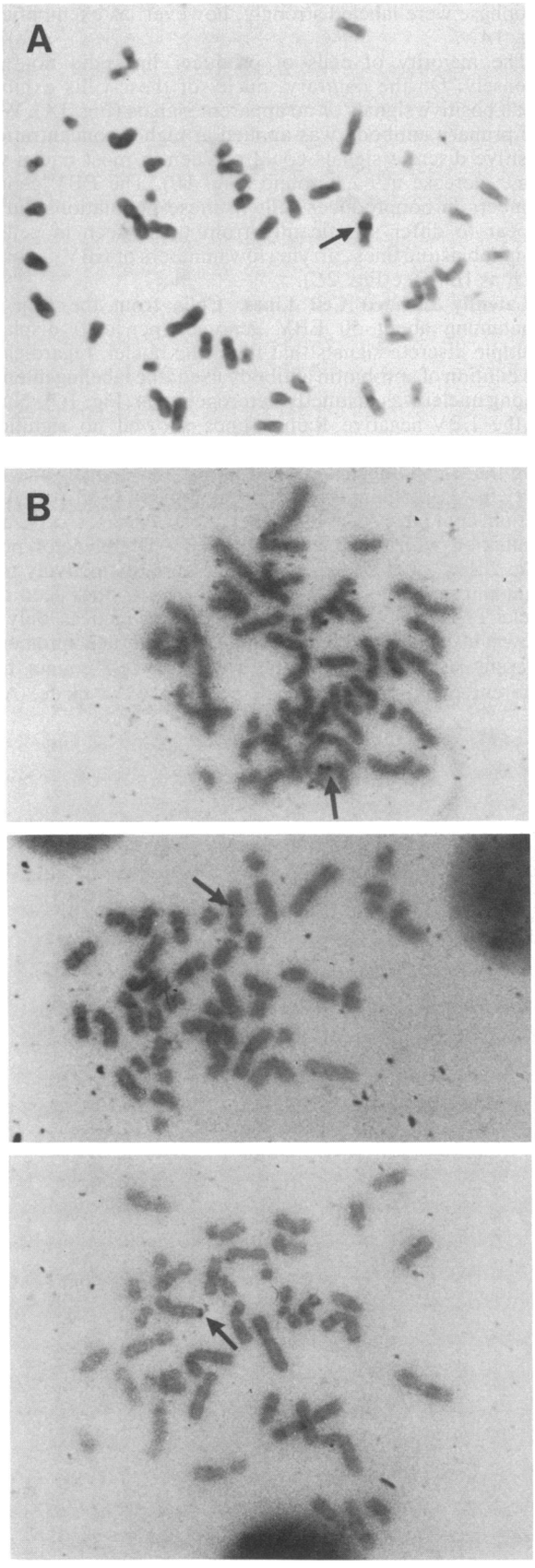

FIG. 3. (*A*) Chromosomal spread of IB4-D after hybridization with EBV *Bam*HI A, B, C, E, and W fragment probes, showing symmetric labeling at 4q2 (indicated by an arrow). (*B*) Spreads of AW-Ramos after hybridization under the same conditions as *A*, illustrating different sites of labeling as indicated by arrows.

correcting for counts obtained from corresponding numbers of cells of the same line hybridized with vector DNA only, the average number of signals per nucleus of IB4, AW-Ramos, and Namalwa were, respectively, 2, 1, and 2 (to the nearest unit).

Chromosomal Localization. Fifty metaphase spreads from the cloned IB4-D cell line were examined after hybridization with the EBV DNA probes. Of these, 19 metaphase spreads were symmetrically labeled at both chromatids on q2 of one or both chromosomes 4 (Fig. 3*A*). Such symmetric signals were not seen over other chromosome regions nor over 4q2 of Ramos metaphase cells used as a control. When Ramos metaphase cells were hybridized with biotinylated DNA probes for glucose-6-phosphate dehydrogenase (G6PD), the *G6PD* gene could be localized to the telomeric region of the long arm of chromosome X, as expected (ref. 26; data not shown). Similar studies on Namalwa cells when hybridized with EBV DNA probes were inconclusive because of the large number of aberrant chromosomes in this line (14). Examination of metaphase spreads from the noncloned converted line AW-Ramos (Fig. 3*B*) gave results indicative of random integration. That is, of 87 spreads examined, 20 exhibited symmetric labeling over at least one chromosome homolog per spread. In contrast to IB4-D, labeling sites were randomly located (Fig. 3*B* and 4).

DISCUSSION

The state of the EBV genome in a variety of lymphoid and lymphoblastoid lines has been examined previously, and average viral copy numbers were determined by solution hybridization (27). These experiments could not specify the status of the genome in individual cells nor ask questions regarding growth properties of the cell. Moar and Klein (15), using *in situ* hybridization and radioactively labeled probes, observed the viral genome in some cells from producer lines, such as P3HR-1, which they tentatively attributed to repli-

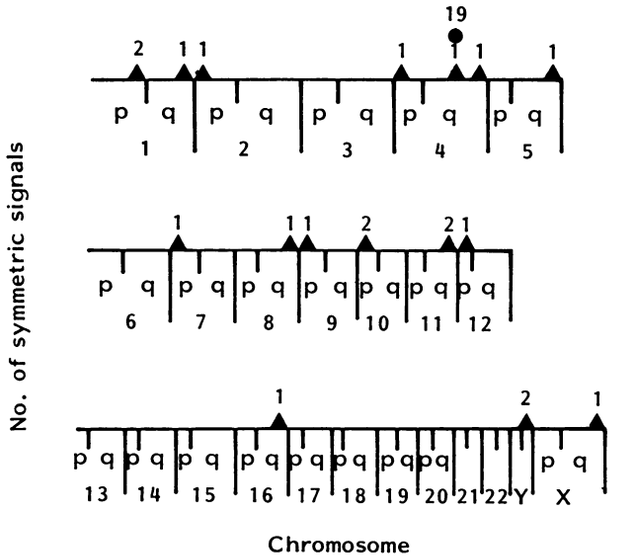

FIG. 4. Comparison of the distribution of symmetric signals (as defined in the text) over 19 IB4-D and 20 AW-Ramos chromosomal spreads. Location of the signals is indicated by a circle (in the case of IB4-D) or a triangle (in the case of AW-Ramos) above the schematic representation of the corresponding chromosome. The number above each circle or triangle denotes the frequency of labeling at the corresponding site. Aberrant chromosomes of AW-Ramos—that is, those with t(8;14), t(7;16), and t(6;17)—are not represented here. No symmetric labeling was found on these chromosomes.

cating cells, but they did not detect EBV DNA in nonproducer lines, such as Raji. Sixbey *et al.* (28) used cytohybridization with biotinylated probes and identified EBV DNA in Raji cells. We show here that the sensitivity of this approach can be increased to such a degree that the state of the virus in cells containing single copies of EBV genomes can be detected; further, the method has allowed the nature of the "virus factories" in some lytically infected lines to be analyzed.

The results obtained when the average number of discrete signals over nuclei were determined on low-copy-number lymphoid cell lines (IB4, AW-Ramos, and Namalwa) by this technique were consistent with copy numbers determined previously by filter hybridization or reassociation kinetics (18–20). Such congruence suggests that each discrete signal (Fig. 2) corresponds to a single copy of target DNA sequence. (The target sequence when the EBV *Bam*HI W repetitive fragment is used as probe is about 10–40 kb in length, depending upon the exact number of reiterations of this major internal repeat within the viral genome; refs. 29 and 30.) The novel finding of heterogeneity in the hybridization signals of nuclei in the latent lymphoid lines examined (Raji, IB4, AW-Ramos, and Namalwa) refutes the generally accepted notion that every cell of the same line contains the same number of EBV genome copies. On the contrary, our data show that as much as 10-fold variation can be observed among cells within a single line, a finding that could not be attributed to variation in copy numbers of the large internal repetitive sequence. The heterogeneity observed for Raji cells in particular could not be inferred from previous data (15, 28) and agrees with a report showing that single-cell clones from the Raji line contain different copy numbers of the EBV genome (31).

An unexpected and interesting finding came from the study of the viral producer lines. That is, a substantial proportion of the small number of cells that scored as strongly EBV positive were found to be in mitosis (Fig. 1 *A*, *B*, and *C*). This suggests that the activation of the EBV genome may be intimately linked with the growth cycle, at least in the case of some of the cells in the population. It should be noted, however, that not all cells in mitosis scored as "virus factories" (Fig. 1*A*), a point relevant to the maintenance of the culture and indicative of a mixed population of cells. What distinguishes between these classes of cells is unclear, but by using this sensitive hybridization technique, it should be possible to investigate this aspect further. In the bulk of the cells from producer lines (as exemplified by B95-8), low-copy-number cells, reminiscent of those in nonproducer lines (Fig. 2), were found that showed heterogeneity both in terms of the intensity and number of grains exhibited by the labeling procedure.

We extended this technique to include an examination of metaphase chromosomes from low-copy-number cells to investigate the somewhat controversial question of viral DNA integration. Our data on the cloned IB4 line (Fig. 3*A*) were consistent with the studies of Henderson *et al.* (2). These authors showed a chromosomal localization of EBV DNA at 4q2 in IB4 using radiolabeled probes. (They also observed specific integration at chromosome 1 in Namalwa cells, a finding we were unable to confirm). An independent study (32) localized a site of viral DNA integration at 4q22–25, along with five other sites in EBV-transformed lymphoblastoid B-cell lines from patients with Bloom syndrome. These data are consistent with the notion that the EBV genome may integrate at preferred sites in human chromosomes. In an attempt to confirm this notion, we have investigated the state of the EBV genome in the converted line AW-Ramos because this is an uncloned line containing an average of one genome copy per cell (Fig. 2), the viral DNA has been shown to be integrated (33), restriction enzyme analyses indicate the genome to be complete (C. Carlsson and T. Lindahl, personal communication), and no extrachromosomal DNA could be detected (34). The data from these studies (Figs. 3*B* and 4) suggest that EBV can integrate in a variety of sites in addition to those previously identified (2, 32) and, at least in converted lines, that integration is probably a largely random event. It will be of considerable interest to use this technique to investigate newly established Burkitt lymphoma cells, should a line with low copy numbers of the viral genome become available.

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