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Episomal and Integrated Copies of Epstein-Barr Virus Coexist in Burkitt Lymphoma Cell Lines

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The Epstein-Barr virus genome is present in more than 95% of the African cases of Burkitt lymphoma. In this tumor, the viral genome is usually maintained in multiple episomal copies. Viral integration has been described only for Namalwa, a cell line lacking episomes. In this study, we have addressed the question of whether integrated and episomal copies can coexist in Burkitt lymphoma cells. Gel electrophoresis was used to demonstrate the presence of episomal as well as free linear DNA in three Burkitt lymphoma cell lines. The numbers of episomal copies per cell were estimated to be 5 to 10 in BL36 and BL137 cells and below 1 in BL60 cells, indicating that BL60 does not represent a homogeneous cell population. Fluorescence in situ hybridization was combined with chromosomal banding to study the association of the viral DNA with metaphase chromosomes. A symmetrical pattern of signals at both chromatids located at the same chromosomal sites in many if not all metaphases was taken as evidence for viral integration. In each of the three cell lines, one site of integration was identified: at chromosome 11p15 in BL36 cells, at chromosome 1p34 in BL137 cells, and at the site of a reciprocal t(11;19) translocation in BL60 cells. Integrated, episomal and linear copies of Epstein-Barr virus DNA thus coexist in Burkitt lymphoma cells. The biological significance of viral integration in Burkitt lymphoma cells remains to be elucidated.

Epstein-Barr virus (EBV), a ubiquitous human herpesvirus, is the causative agent of infectious mononucleosis and is associated with a number of human malignant diseases, including nasopharyngeal carcinoma, Burkitt lymphoma (BL), malignant lymphoproliferations arising in immunocompromised individuals, and some cases of Hodgkin's disease (9, 21). The contribution of the virus to the development of these diseases is still unclear. In vitro, EBV is a potent agent which infects resting human B cells latently (i.e., without producing virus particles) and drives the infected cells to unlimited proliferation (12). These EBV-immortalized cells harbor the viral genome in multiple episomal copies and express only a limited set of viral genes, including the genes coding for six nuclear antigens (EBNAs) and three membrane antigens (26).

BL is characterized by specific reciprocal chromosomal translocations involving the c-*myc* locus on the long arm of chromosome 8 and one of the immunoglobulin heavy- or light-chain loci on chromosome 14, 2, or 22 (3). Deregulated expression of the c-*myc* gene, brought about by juxtaposition of c-*myc* to the immunoglobulin loci, is known to be a necessary although not sufficient step in the development of the tumor (3). Other factors, such as mutations or deletions in tumor suppressor genes such as p53 appear to be required in addition (5, 6, 29). EBV is a candidate for contributing to the development of BL in a multistep process. The association of EBV with BL shows a geographic predisposition. EBV is found in almost all fresh BL tumors and established cell lines derived from BL in Africa and New Guinea but in only about 15 to 20% of cases in Caucasians (4).

The role of EBV in the development of BL is still not understood. In tumor cells, the viral DNA is maintained in multiple episomal copies as it is in EBV-immortalized cells established in vitro (15, 18). However, BL tumor cells and EBV-immortalized cells differ remarkably in the pattern of viral gene expression. In tumor biopsies, EBNA1 appears to be the only viral gene product which is present in detectable amounts (25). EBNA1 binds to the viral origin of episomal replication and is required for maintaining the viral genome as episomes (34). In contrast, the viral protein EBNA2, which is known to be absolutely required for immortalization of primary B cells (8), is not expressed at a detectable rate in tumor biopsies in vivo (25).

Several possibilities may account for these observations: (i) EBV might be a passenger maintained latently in a tumor cell which happens to proliferate for reasons not related to EBV, (ii) EBNA1 might be the only viral oncoprotein required for maintaining the transformed state of BL cells, (iii) the set of viral transforming proteins might be required in the initial stage of the disease but might be counterselected for by the immune system during tumor progression, and (iv) the viral DNA might integrate into the cellular genome in addition to being maintained episomally. Integration might lead to activation or inactivation of cellular genes involved in growth control or could disrupt expression of a viral gene.

In this study, we have readdressed the question of whether EBV DNA is integrated in BL cells. In 1976, when the episomal state of EBV DNA was discovered, Lindahl and collaborators provided circumstantial evidence suggesting that a small number of integrated copies of the viral genome might be present in BL and nasopharyngeal carcinoma cells in addition to episomal viral DNA (15, 18). The high number of viral genome copies in most EBV-positive BL cell lines has, for technical reasons, prevented a systematic search for viral integration in BL cells up to now. Therefore, for most BL cell lines, direct evidence for or against viral integration is still missing. We have now used

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fluorescence in situ hybridization of metaphase chromosomes combined with chromosomal banding to detect integrated viral copies apart from the episomal and linear viral DNA in BL cells.

MATERIALS AND METHODS

Cell lines. The status of EBV was studied in the three North African Burkitt cell lines BL36, BL60, and BL137 (kindly provided by G. Lenoir, Lyon, France). Controls included the EBV-negative cell line DG 75 (2), BL41/P3HR1 cells containing an integrated copy of EBV (13), the BL cell line Raji, which contains episomal but no free linear viral DNA (23, 36), and the P3HR1 subclone HH514, induced to virus production by (20 ng/ml) tetradecanoyl phorbol acetate and containing episomal as well as linear viral DNA (35). All cells were grown in RPMI 1640–10% fetal calf serum.

Gardella gels. Episomal and linear form of EBV DNA were distinguished by electrophoresis according to Gardella et al. (7). The gel was prepared essentially as described previously (7) except that proteinase K was used instead of pronase in the lysis gel. Each well was loaded with 10^6 cells in an equal volume of phosphate-buffered saline–Ficoll 15%. Electrophoresis was carried out at 30 V for 3 h and then at 130 V for 14 h at 4°C. The separated DNA was blotted onto a nylon membrane (Hybond N+) and then hybridized and washed according to standard protocols (19), using the EBV cosmid cM-SalI-A as a probe (24).

Preparation of the probe. Three cosmids spanning the majority of the EBV genome (cM-SalI-A, cM-SalI-C, and cM302-21) (24) and plasmid pM-B2-B were nick translated in the presence of biotin-11-dUTP.

Chromosome preparation. In vitro-cultured BL cell lines were synchronized by incubation in a excess of thymidine (0.5 mg/ml) for 14 h (27). After two washings in normal medium, 5-bromodeoxyuridine (10 μg/ml) was added for 8 h. Colcemid (0.5 μg/ml) was added 1 h before harvesting. For swelling, the cells were treated with one of two hypotonic solutions, using either 0.075 M KCl or fetal calf serum (15% [vol/vol] in water) for 15 min. The cells were then fixed in ethanol-acetic acid (3:1). After spreading, slides were kept at −20°C until hybridization.

Banding of the chromosomes. R banding of the chromosomes was obtained by incubating the slides with a mounting medium containing *p*-phenylenediamine (1 mg/ml) and propidium iodide (0.5 μg/ml) at pH 11 (17). R banding was observed with a Zeiss Axiophot microscope equipped for epifluorescence and the following set of filters: Zeiss LP510 plus KP560, FT580, and LP590. Microphotographs were taken with Ilford PanF 50 ASA black-and-white film. When chromosomes were difficult to identify, G banding (GTG) was done prior to in situ hybridization.

In situ hybridization. After four washes for 10 min each in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% Tween 20, slides were treated with RNase A (100 μg/ml in 2× SSC) at 37°C for 1 h and dehydrated in ethanol of increasing concentration (50, 75, and 100%). Chromosomes were denatured by incubation in 2× SSC–70% formamide (pH 7.0) for 2 min at 70°C. After being rinsed for 2 min in 2× SSC, slides were dehydrated as described above. Probes diluted in hybridization buffer (2× SSC, 50% formamide, 10% dextran sulfate) were then denatured at 75°C for 5 min, chilled on ice, placed on the slide, covered by a coverslip, and sealed with rubber cement. For hybridization, slides were placed at 37°C overnight in a moist chamber.

Probe detection and observation of the signals. Slides were washed four times for 5 min in 2× SSC–50% formamide at 42°C and three times for 10 min in 1× SSC. To obtain a better signal-to-background ratio, the last washes were performed at either 60 or 42°, depending on whether the cells had been pretreated with KCl or fetal calf serum, respectively.

After preincubation in 4× SSC–3% bovine serum albumin (BSA) at 37°C for 20 min, hybridization was revealed by incubation with avidin-conjugated fluorescein isothiocyanate (5 μg/ml; Vector Laboratories) in 4× SSC–3% BSA–0.1% Tween 20 for 30 min at 37°C. Slides were washed three times for 5 min in 4× SSC–0.1% Tween 20 at 42°C. Signals were observed with the filters (BP450-490, FT510, and LP520). Microphotographs were taken with Kodak Ektachrome 400 ASA color film.

RESULTS

Linear and episomal copies of EBV DNA are present in BL36, BL60, and BL137 cells. To examine whether BL36, BL60, and BL137 cells contain linear as well as episomal DNA, cells were lysed in the slots of the gel and then electrophoresed as described by Gardella et al. (7). Under these conditions, linear DNA migrates more rapidly than episomal DNA. The viral DNA of Raji cells served as a control for EBV episomal DNA, and DNA of tetradecanyol phorbol acetate-treated P3HR1 cells (subclone HH514) served as a control for episomal as well as linear viral DNA. Lysis of the cells in the gel liberates chromosomal DNA, most of which persists as high-molecular-weight DNA and does not enter the gel. A small amount is, however, sheared and produces a faint smear which migrates just below the linear forms of EBV. Integrated copies of EBV DNA remain associated with the chromosomal DNA and give rise to a signal in the wells after hybridization with an EBV-specific probe. Cellular lysis and deproteinization obtained by this technique are probably incomplete such that part of the linear and episomal copies stay noncovalently associated with the chromosomal DNA and cellular debris present in the wells. Moreover, the high DNA concentration in the wells after lysis leads to nonspecific hybridization. Loading of 10^6 cells of the negative control BL41 thus gave rise to a signal comparable to that obtained with BL41/P3HR1 cells, which contain one integrated EBV DNA copy.

As shown in Fig. 1, cell lines BL36, BL60, and BL137 contained variable amounts of free linear viral DNA. All three cell lines also contained episomes. The number of circular molecules was much higher in BL36 and BL137 cells than in BL60 cells. In BL60 cells, only a faint signal was obtained after loading of 5×10^6 cells and 2 days of exposure. In BL36 and BL137 cells, episomal DNA was readily detected after loading of 10^6 cells and 6 h of exposure. By comparing the signal intensity of episomal DNA in BL60, BL36, and BL137 cells with that of Raji cells, the numbers of episomal copies per cell were estimated to be in the range of about 5 to 10 in BL36 and BL137 cells and well below 1 in BL60 cells. The linear viral DNA should be derived from a minority of virus-producing cells, which presumably also carry viral episomes. Since only a minority of cells within the BL60 cell population harbor episomal viral DNA, the majority of BL60 cells should carry only integrated, if any, EBV DNA. The low to moderate number of viral genome copies in BL36, BL60, and BL137 cells greatly facilitated detection of integrated viral copies, as will be described below.

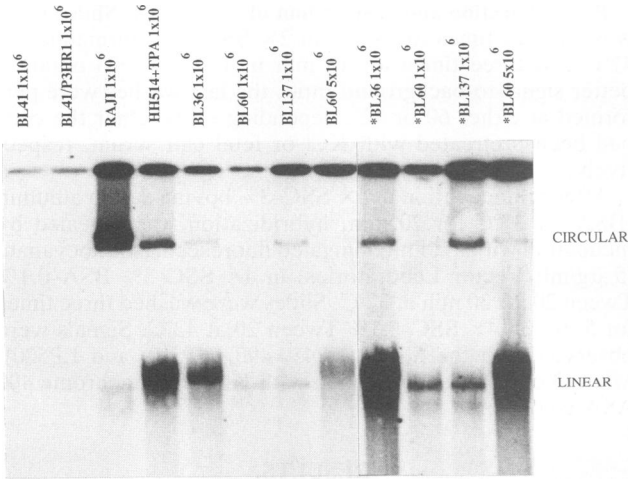

FIG. 1. Evaluation of the status of EBV-free DNA in BL36, BL60, and BL137 cells by Gardella gel analysis, using cosmid cM-SalI-A as a probe. For each cell line tested as well as for the controls, 10^6 cells per slot were loaded. For BL60 cells, 5×10^6 cells were also analyzed. Controls included BL41 as an EBV-negative BL lymphoma cell line, BL41-P3HR1 (a BL cell line containing one integrated copy of EBV DNA), Raji (a BL cell line which contains a large amount of episomes), and HH514 (a subclone of the virus producer cell line P3HR1 after tetradecanoyl phorbol acetate treatment containing linear as well as episomal copies of the viral genome). The asterisks denote a longer exposure of the same autoradiogram.

Assignment of EBV DNA hybridization signals to metaphase chromosomes in BL36, BL60, and BL137 cells. Fluorescence in situ hybridization was used to determine the status of EBV DNA in BL cell lines at the single-cell level. We assume that episomes should be associated with chromosomes randomly, if at all, whereas integrated copies should give rise to symmetrical hybridization signals at distinct chromosomal sites in the majority of the metaphases.

Hybridization of labeled viral DNA to Raji, Jijoye, and P3HR1 cells revealed the expected high number of viral DNA copies (data not shown). Raji cells contained on average between 50 and 100 copies, with considerable variation of the copy number per cell, as reported by Yamamoto et al. in 1979 (33). Jijoye and P3HR1 cells showed similarly high copy numbers in the majority of the cells and a particular accumulation of label in a minority of virus-producing cells. Because of the high degree of chromosome aneuploidy and the high load of viral DNA copies, we did not make any further attempt to discriminate between integrated and nonintegrated copies of the viral DNA in Raji, Jijoye, and P3HR1 cells. We therefore focused our attention on BL36, BL60, and BL137 cells carrying low to moderate numbers of episomal viral DNA.

About 40 mitoses of each cell line were analyzed. In BL36 and BL137 cells, most signals on metaphase chromosomes appeared to be unique, nonsymmetrical signals. In situ hybridization directly confirmed the conclusion drawn from the Gardella gel analysis and demonstrated that BL36 and BL137 cells contain about 5 to 10 viral DNA copies per cell. About 5% of BL36 cells and less than 1% of BL137 and BL60 cells underwent a productive cycle. In situ hybridization also confirmed the finding that the majority of BL60 cells lack episomal copies.

In addition to unique, nonsymmetrical hybridization signals, all three cell lines exhibited doublet signals present on both chromatids of a given chromosome. Only signals which were truly symmetrically oriented at both chromatids were regarded as doublet signals. Signals located asymmetrically to each other were taken as two individual signals which happened to colocalize. To determine whether the association of doublet signals with metaphase chromosomes was random or recurrent in the three cell lines, in situ hybridization was combined with chromosomal R banding so that in each individual mitosis, every doublet signal could be assigned to a specific chromosomal site (Fig. 2 and 3). In more than 95% of all mitoses, doublet signals were observed at chromosomal sites specific for each cell line (Fig. 4). Recurrent signals were observed on the short arm of chromosome 11p15 in BL36 cells (Fig. 2), on chromosome 1p34 in BL137 cells (Fig. 3 top), and at the breakpoint of a reciprocal t(11;19) translocation in BL60 cells, resulting in a der (19) t(11;19) (q14;q13.3 or q13.4). In each of the three cell lines, tetraploid mitoses were observed at a variable extent. On two of the four homologous chromosomes, tetraploid mitoses displayed the same symmetrical doublet signals observed on diploid mitoses, indicating that viral integration preceded the duplication of the cellular genome (Fig. 3, bottom). Besides the consistent sites to which the viral DNA hybridized in the three cell lines, a remarkable portion of signals appeared as symmetrical doublet signals without mapping to a specific chromosomal site. This finding suggests that also episomal viral DNA may be associated with chromosomes and may segregate to daughter cells during mitosis in a highly ordered fashion. The intensity of randomly distributed doublet signals and of unique signals was variable and generally much weaker than the intensity of the signal at sites of viral integration. This finding is consistent with the assumption that closed circular DNA will reanneal immediately after denaturation, thus precluding efficient hybridization to the exogenously added hybridization probe.

Comparison of early- and late-passage BL60 cells. Studying the tumorigenicity of hybrids between the BL cell line BL60 and an EBV-immortalized lymphoblastoid cell line obtained from the same patient, Wolf and coworkers (30) have recently analyzed the viral genome maintained in hypoxanthine-guanosine phosphoribosyltransferase-negative subclones of BL60 cells into which a dominant selectable marker had additionally been introduced. They found the viral genome to be partially deleted and showed that this deleted copy of the viral genome is integrated into the cellular genome, thus precluding the onset of the lytic cycle of the virus in these cells (32). An extension of the study to the BL60 cell stock from which the hypoxanthine-guanosine phosphoribosyltransferase-negative subclones had been selected demonstrated that EBV DNA was integrated in these cells as well. Integration was assigned to the same chromosomal locus as reported here (31). The analysis of BL60 cells by Gardella gel electrophoresis provided evidence for heterogeneity within our BL60 cell population; only a small fraction of cells apparently contained episomal viral DNA, and this small fraction presumably gave rise to viral replication. Given the heterogeneity of the BL60 cell population, the question arose as to whether integration of viral sequences had occurred during prolonged cultivation in vitro. To answer this question, early-passage BL60 cells were studied and the patterns of integration were compared in cells of early versus late passage. Cells frozen in the third passage were made available to us by G. Lenoir. Metaphase chromosomes were prepared at passage 6 and hybridized to labeled EBV DNA. Early- and late-passage BL60 cells

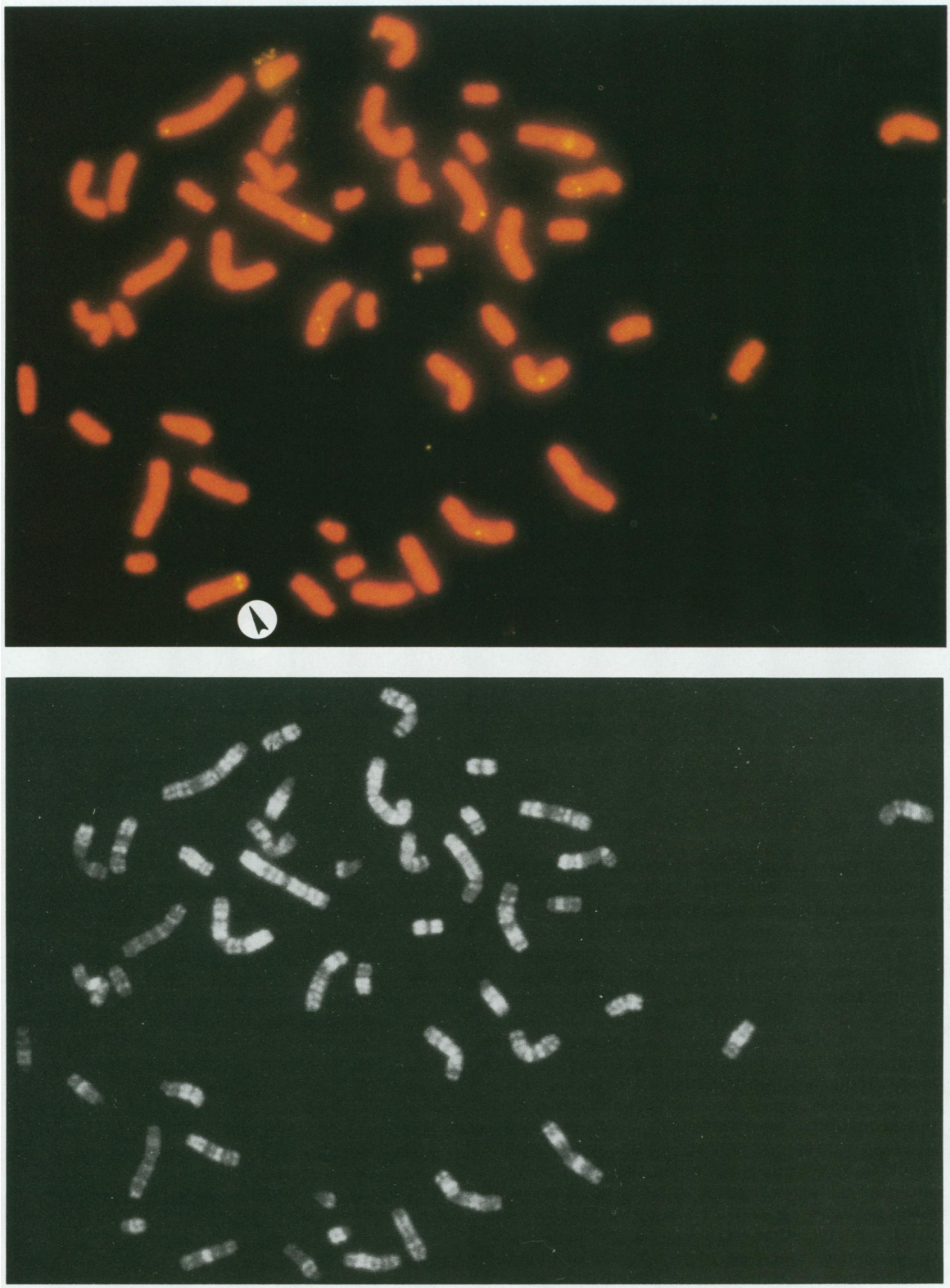

FIG. 2. (Top) In situ hybridization on BL36 chromosomes. Fourteen signals are present along the chromosomes, mostly as single spots and sometimes as doublets. Only the doublet situated at the bottom left of the picture is symmetrically present at homologous sites of both chromatids (arrowhead). Note the higher intensity of this doublet signal. (Bottom) R banding of the same mitosis allows assignment of the hybridization signal to the short arm of chromosome 11.

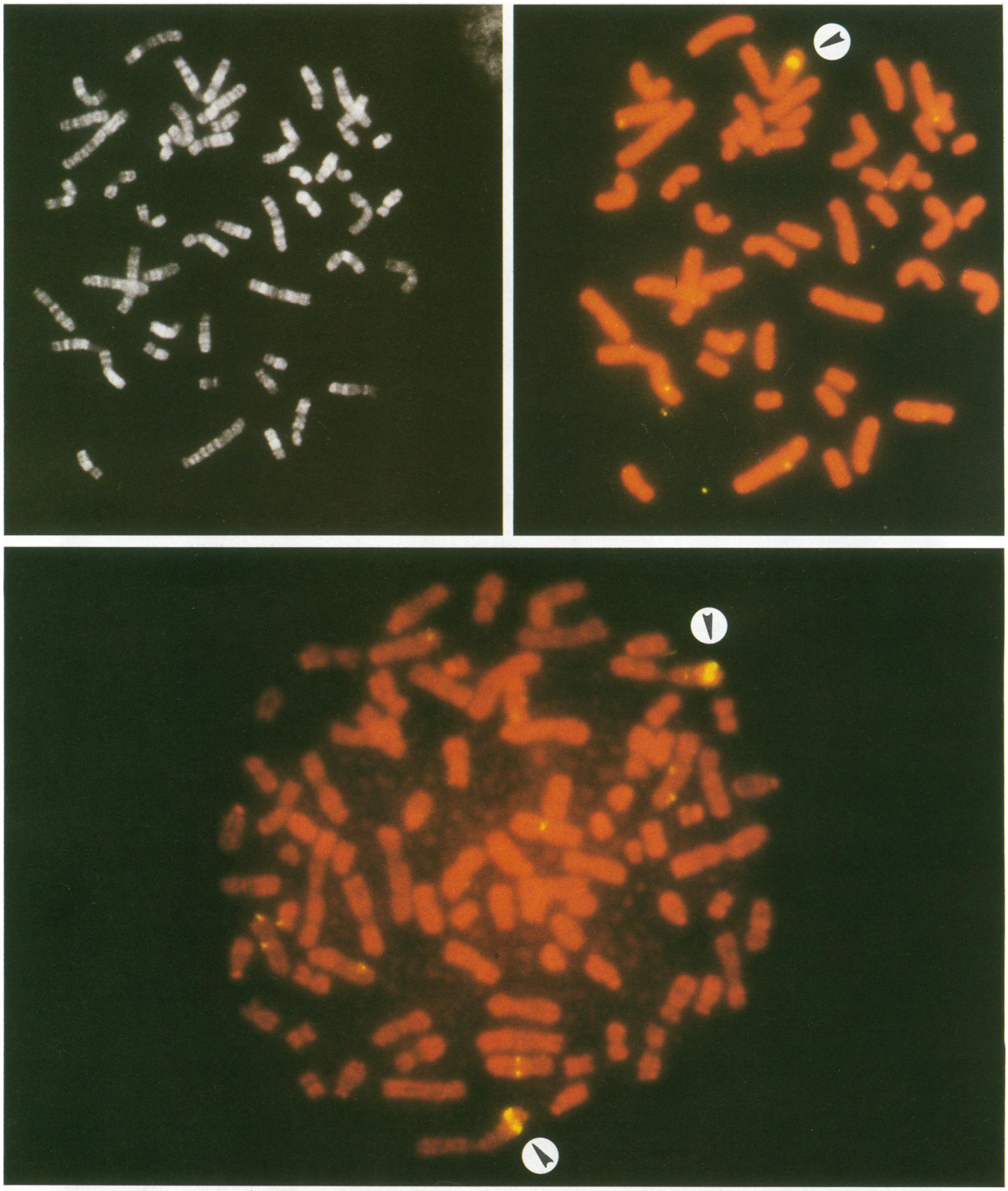

FIG. 3. (Top) In situ hybridization on BL137 chromosomes, using EBV DNA as a probe and R banding of the same mitosis. Episomes appear as single or doublet spots along the chromosomes. A much more intense doublet signal is found on the short arm of chromosome 1 (arrowhead). The intensity of this doublet signal is so high that it is not possible to clearly distinguish the two signals on both chromatids. (Bottom) In situ hybridization on a tetraploid mitosis from BL137 cells. Two strong doublet signals are present on two copies of chromosome 1 (arrowheads), apart from fainter unique or doublet signals which are indicative of episomes. Note the symmetrical doublet signal just above the integrated copy of EBV on chromosome 1 at the bottom. The chromosomal localization of this doublet has not been found to be recurrent on several analyzed mitoses, excluding integration and suggesting the presence of two episomes opposite each other on both chromatids.

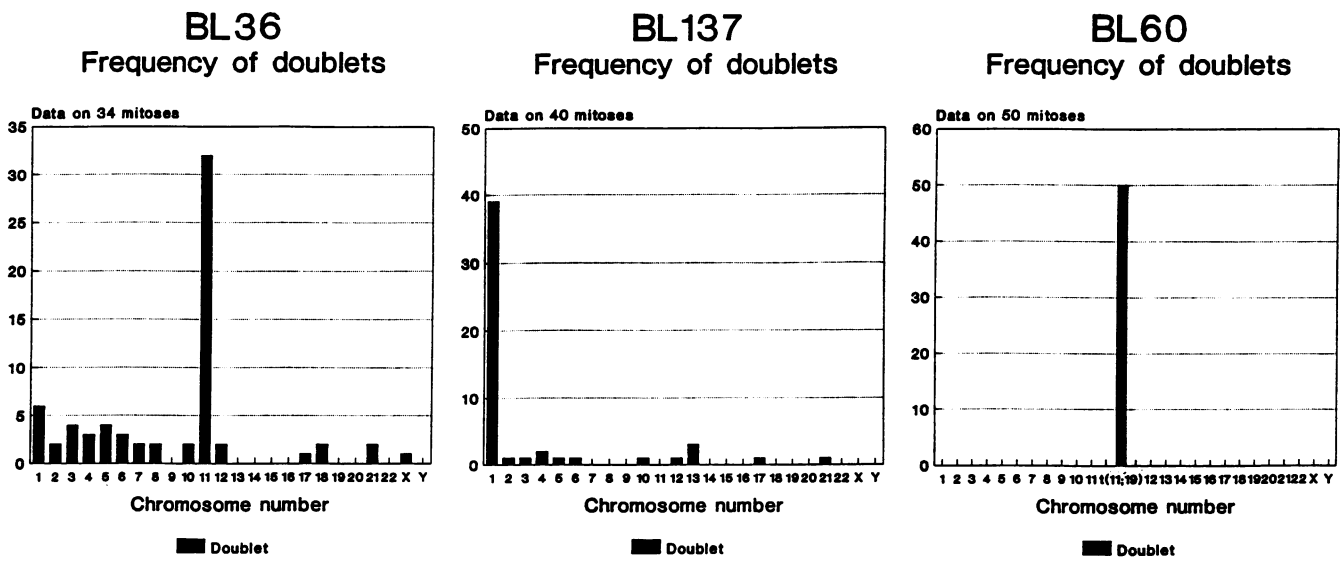

FIG. 4. Distribution of doublet signals on chromosomes from BL36, BL60, and BL137 cells after fluorescence in situ hybridization using EBV DNA probes.

showed the same pattern of integration of EBV on the rearranged chromosome 11.

EBV is not integrated in two cord blood lymphoblastoid cell lines. To determine whether viral integration is also a common feature of EBV immortalization, two human cord blood B-lymphocyte lines were studied for viral integration. The cell lines had been established in our laboratory by infection of cord blood mononuclear cells with the B95-8 and M-ABA strains of EBV, respectively. After in situ hybridization of EBV DNA to metaphase chromosomes, no consistent hybridization pattern emerged, fulfilling the criterion of integration in both cell lines. This finding indicates that integration is not required for immortalization, even though integration may be observed in EBV-immortalized cells (11, 14).

DISCUSSION

It is well established that EBV DNA is maintained in multiple episomal copies in BL and nasopharyngeal carcinoma cells. However, it is still unclear whether integration of EBV DNA is a common feature of EBV-associated tumors and whether integrated and episomal copies may coexist within the same tumor cell, as suggested by Lindahl and coworkers 16 years ago (18). Since then, these questions have not been properly addressed, mainly for technical reasons. Searching for an integrated copy on the background of a large number of episomal and linear viral DNA by cloning, for example, seemed to be a futile task that researchers were not prepared to undertake as long as integration of EBV remained uncertain. Integration has therefore been studied in only a few cell lines with an exceptionally low number of viral copies. The best-studied example is the African BL line Namalwa, which carries only two integrated and no episomal copies of the viral genome (11, 16, 20) and thus not representative of EBV-positive BL cells. In addition, integration has been observed in one EBV-immortalized cell line (14), which has been in culture for a long period of time, and in EBV-negative BL cell lines which had been infected by EBV in vitro and converted to EBV positivity (13). The only BL line which was reported to maintain episomal as well as integrated copies of the viral genome is the African BL line Raji (1). This report, however, dates back to 1984 and has not resulted in molecular characterization of the presumably integrated copy of EBV DNA.

Making use of the fluorescence in situ hybridization technique combined with chromosomal banding, we have provided evidence for the coexistence of integrated as well as episomal and linear EBV DNA in three BL cell lines. In each of the three cell lines studied, we found one chromosomal site which hybridized consistently with the viral probe. The symmetrical orientation of the doublet signals on both chromatids and the consistent assignment of the signals to specific chromosomal sites were taken as evidence that the viral genome or pieces thereof are integrated. An additional argument in favor of integration came from the fact that metaphases of tetraploid cells revealed identical pairs of signals on both derivative chromosomes. In the three cell lines analyzed, integration was found at three different chromosomal sites: on chromosome 11p15 in BL36 cells, at the breakpoint of a t(11;19) translocation in BL60 cells, and on chromosome 1p34 in BL137 cells.

An approach similar to that reported here was used by Harris et al. a few years ago and did not provided evidence for viral integration in BL cells (10). There are a number of reasons to explain the discrepancy between our results and those of Harris et al. First, we used different cell lines, and it is apparently not possible to generalize positive or negative findings. Second, Harris et al. used conventional radioactive in situ hybridization, which has an intrinsic high background and makes assignment of hybridization signals to specific chromosomal sites difficult. We have also found that it is extremely difficult to study integration in cells with a high number of viral genome copies (e.g., Raji cells), in cell lines with a high load of lytic virus production and eventually reinfection of the cells as described for P3HR1 cells (22), or in cells exhibiting a high degree of aneuploidy. Coincidence of several of these parameters may render the detection of integrated copies virtually impossible.

Two urgent questions now arise. First, is viral integration a phenomenon which is clonal and has occurred in vivo, or

did the virus integrate in vitro when the tumor cells had been established as cell lines in culture? Infection of EBV-negative BL cell lines with EBV in vitro may give rise either to integration or to episomal maintenance of the viral DNA (13, 28). Remarkably, upon in vitro infection of BL cell lines, integration and episomal maintenance appeared to be mutually exclusive (13, 28). Provided that this finding could be generalized and applied to the in vivo situation, coexistence of integrated and episomal copies would argue for EBV infection being an early event in Burkitt lymphomagenesis. The clonal pattern of EBV integration and the presence in early-passage BL60 cells additionally argue for integration having taken place in vivo rather than in vitro. The definitive proof will, however, await the analysis of metaphase spreads from fresh tumor biopsies. In this context, it is remarkable that Marke's disease virus, an oncogenic herpesvirus of chickens, is invariably found integrated in the primary lymphomas that it causes (unpublished data) as well as in cell lines established from these tumors (3a).

The second important question is concerned with the specificity of the cellular or viral site of integration. Is integration random, or does it occur at specific chromosomal loci? The finding that EBV integrated at three different sites in three cell lines might argue for random integration, but we consider it too early to speculate on the cellular sites of integration as long as so little information is available. It is our second aim to collect data on a much larger series of BL cell lines once we have definite proof that EBV integration reflects the situation in the primary tumor. The next step then will be to investigate viral-cellular junctions representing the integration site and to determine whether integration has affected cellular or viral genes involved in control of cell proliferation.

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