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No evidence for differences in the Epstein–Barr virus genome carried in Burkitt lymphoma cells and nonmalignant lymphoblastoid cells from the same patients

(restriction enzyme length polymorphism/cloned Epstein–Barr virus DNA/chromosomal translocations/c-*myc* gene)

GEORG W. BORNKAMM*, MAGNUS V. KNEBEL-DOEBERITZ*, AND GILBERT M. LENOIR†

*Institut für Virologie, Zentrum für Hygiene der Universität, Hermann-Herderstrasse 11, 78 Freiburg, Federal Republic of Germany; and †International Agency for Research on Cancer, Lyon, France

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ABSTRACT **Epstein–Barr virus (EBV), although not an indispensable factor for the development of Burkitt lymphoma, is apparently associated with the 20-fold higher incidence of the disease in Equatorial Africa compared to the incidence in other parts of the world. To determine whether different EBV subtypes are associated with the appearance of the malignant phenotype, we have compared the EBV genomes carried in the Burkitt tumor cells with those carried in the nonmalignant lymphoblastoid cells from the same individuals. From three patients with EBV-associated Burkitt lymphoma, tumor cell lines as well as spontaneously established lymphoblastoid cell lines representing the nonmalignant counterparts were obtained. The viral DNA in these cell lines was analyzed by Southern blot hybridization, using a set of cloned EBV DNA fragments as probes that recognize polymorphic regions in the viral genome. Using a number of different polymorphic markers to distinguish one isolate from another, the virus genome found in the tumor cells could also be identified in the nonmalignant cells of the same patient. In one case, in which two independent lymphoblastoid cell lines were established, evidence was obtained that this patient was infected by at least two distinct EBV subtypes. These results strongly suggest that in Burkitt lymphoma, the risk associated with EBV is related to cofactors such as chronic malaria and the mode of infection rather than to peculiar viral subtypes. The situation seems to be totally different from papillomavirus-associated diseases, in which the risk of progression to malignancy appears to be associated with particular viral strains.**

Burkitt lymphoma is characterized by unique epidemiological features: it is one of the most frequently occurring malignant tumors in Equatorial Africa and New Guinea, but it occurs with much less frequency in other parts of the world (1, 2). In the so-called endemic areas, the disease coincides with holoendemic malaria, suggesting that a chronic malaria infection is a risk factor for the development of the tumor (3). In high incidence areas, most of the cases (96%) are associated with the Epstein–Barr virus (EBV), and malignant cells harbor multiple copies of its genome (4).

With an increasing number of Burkitt lymphoma cases reported from low incidence areas, however, it became apparent that EBV is not a "conditio sine qua non" for the development of Burkitt lymphoma. Only 15%–20% of all cases in Caucasians reported from the United States and Europe are associated with EBV (2, 5). The inconsistent association of EBV with Burkitt lymphoma has focused the main interest from the virus to the chromosomal translocations involving chromosome 8, which are invariably observed regardless of whether the disease is associated with EBV or not (6–8). The identification of the c-*myc* gene at the breakpoint of the chromosomal translocation has obviously opened an exciting era in cancer research (9–11).

Even though the activation of a cellular oncogene by the chromosomal translocation is probably the most critical step in the appearance of the malignant cell clone, the role of EBV cannot be disregarded. Epidemiological studies indicate that at least one important factor in the development of African Burkitt lymphoma is contributed by the virus (12). Furthermore, the virus is well known as having *in vitro* immortalizing properties and is capable of inducing lymphoma-like syndromes in immunosuppressed individuals (13).

The EBV genome found in virus particles is a linear double-stranded DNA molecule of ≈175 kilobase pairs (kbp). It consists of identical small terminal repeats and at least four clusters of tandem repeats of variable size. Two of the repeat clusters, ≈100 kbp apart, are closely related and are flanked by homologous sequences, which have the same orientation within the viral genome (for review see ref. 14). Comparison of the DNA of different viral strains revealed variabilities in the sizes of several restriction enzyme fragments (15). These fragments are the *Hin*dIII fragments A, D2, E, and I1, and the *Bam*HI fragment H. With the exception of *Hin*dIII E, the fragments are known to carry tandem repeats. The variability in size can be attributed to different numbers of tandem repeats in the respective fragments (15–18). Another polymorphic site is the region between the large internal 3.1-kbp repeats and the DS_L region. The transformation-defective EBV strain P3HR-1 was shown to have a deletion of 6.5 kbp, resulting in fusion of the 3.1-kbp repeats to the DS_L region (19). The analysis of recombinants between the nontransforming P3HR-1 virus with other EBV strains has clearly demonstrated the importance of this region for initiation of transformation (20).

In addition, alterations in many individual restriction enzyme sites, presumably caused by single base-pair changes, are found in many EBV strains and can be used for the characterization of the viral DNA. The viral genome and the sites of variability are shown in Figs. 1 and 2. These different types of polymorphic markers show that each individual virus isolate has its own characteristic pattern.

Comparison of EBV isolates from different origins has so far not allowed us to assign EBV isolates to a given disease such as Burkitt lymphoma, nasopharyngeal carcinoma, or infectious mononucleosis (15).

The possibility of characterizing each viral isolate by a number of different polymorphic DNA markers, as well as the possibility of comparing in a given individual the EBV genome carried in malignant and nonmalignant cells, led to an in-depth evaluation of the possibility that viral substrains

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Abbreviations: EBV, Epstein–Barr virus; kbp, kilobase pair(s); LCL, lymphoblastoid cell line(s).

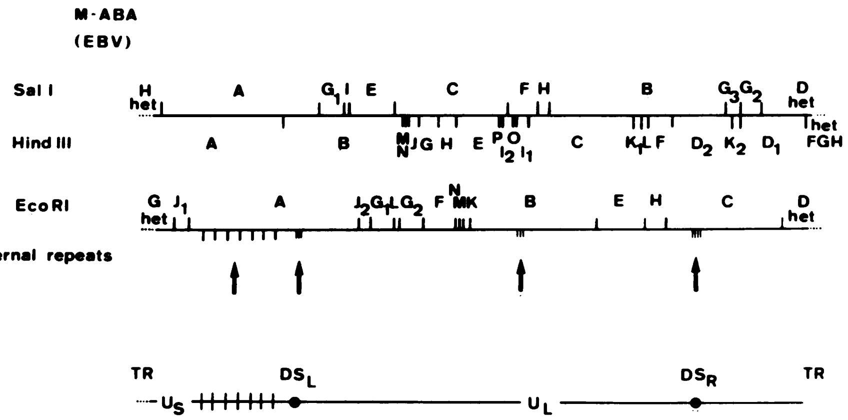

FIG. 1. Schematic representation of the EBV genome with the restriction sites for *Eco*RI, *Hin*dIII, and *Sal* I. The linear viral genome is ≈175 kbp. The genome carries multiple arrays of tandem repeats with variable numbers of the repeat unit. The four repeat clusters giving rise to restriction enzyme-length polymorphism are marked by arrows. TR, terminal repeats; U$_S$ and U$_L$, short and long unique region; DS$_L$ and DS$_R$ designate the region duplicated in the wild-type genome.

could be associated with the appearance of the malignant phenotype. This approach has been stimulated by the finding that within 24 human papilloma virus types only 7 are associated with the progression from benign lesions to malignancy (21, 22).

We have, therefore, studied the EBV genome carried in Burkitt lymphoma cell lines and compared its structural organization with the genome of the virus carried persistently in the normal peripheral lymphocytes of the same patients. The latter virus became accessible to molecular analysis after establishing spontaneously outgrowing lymphoblastoid cell lines (LCL) from patients with Burkitt lymphoma. These lymphoblastoid cells do not show any chromosomal aberrations at the onset of the culture period and represent a normal B-cell counterpart of Burkitt lymphoma cells.

MATERIALS AND METHODS

Establishment of Cell Lines and Analysis of the Karyotype. The cell lines were established at the International Agency for Research on Cancer (IARC) from samples (biopsy or blood or bone marrow) received from three patients with EBV-associated Burkitt lymphoma (Table 1). Patients 1 and 2 were French boys, 3 and 9 yr old, respectively, with abdominal tumors. Patient 3 was a 4-yr-old Algerian girl with a maxillary tumor. Two tumors showed a t(8;22) translocation (IARC BL 37 and BL 60) and the other showed a t(8;14) (BL 54 and BL 59). The lymphoblastoid cell lines were established spontaneously from the peripheral blood or bone marrow and had a normal karyotype.

All cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum/penicillin (100 units/ml)/streptomycin (100 μg/ml).

Gel Electrophoresis and Blot Hybridization. DNA was extracted from frozen cell pellets of 10^8 cells. After thawing, cells were resuspended in isotonic buffer containing 2 mM EDTA and then lysed and extracted as described (19). DNA (5 μg) was digested by *Bam*HI or *Hin*dIII (Boehringer Mannheim and New England Biolabs) in a total vol of 20 μl in the buffers indicated by the manufacturers, and the fragments were separated on 0.5% vertical agarose gel. Fragments were transferred to nitrocellulose as described by Southern (23). DNA probes for hybridization were labeled with [^{32}P]TTP (400 Ci/mmol; 1 Ci = 37 GBq; Amersham) by nick-translation (24). The cloning of the EBV DNA fragments used as probes and the conditions of hybridization and washing of the filters have been described (19, 25).

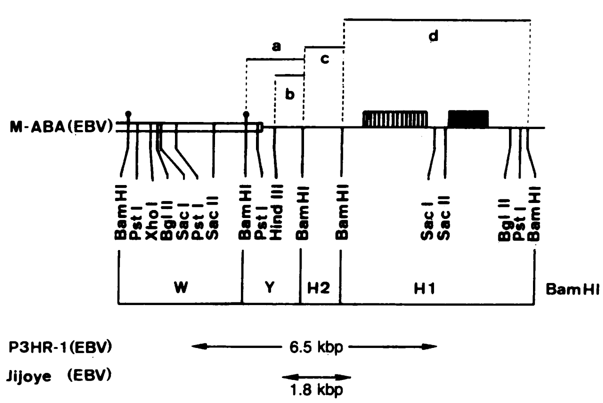

FIG. 2. Boundary of the large internal repeat to the long unique region in M-ABA virus DNA. The labeled probes used for hybridization in Fig. 4 are designated here as *a* and *b*, those used in Fig. 5 are *c* and *d*. The clone containing the insert b is designated pM 765-2. The large arrow describes the deletion of P3HR-1 virus, the small arrow shows the region replaced in Jijoye virus as well as in some other EBV strains.

RESULTS

Polymorphism Detected by *Hin*dIII I1. The *Hin*dIII I1 fragment carries a cluster of repeats with homology to cellular DNA (17) and is part of the *Bam*HI K fragment that codes for EBV nuclear antigen (26). Transfer of *Hin*dIII I1 into recipient cells also leads to expression of EBV nuclear antigen (unpublished observation). The size of *Hin*dIII I1 varies in different EBV strains between 3.1 and 4.5 kbp (15), but it was always found to be constant within a given strain.

To compare the *Hin*dIII I1 fragments in the paired Burkitt lymphoma/LCL samples from the same patients, the DNA of the various cell lines was digested with *Hin*dIII, and the fragments were separated on 0.5% agarose gels. After transfer of the fragments to nitrocellulose filters the *Hin*dIII I1 fragments were visualized by hybridization with ^{32}P-labeled cloned M-ABA (EBV) *Hin*dIII I1. As shown in Fig. 3, the *Hin*dIII I1 fragments of EBV carried in BL 37 and IARC 176 B cells (patient 1) are both 3.8 kbp (lanes 1 and 2). In the three cell lines from patient 2 (BL 54, BL 59, and IARC 247), the *Hin*dIII I1 fragments are larger (4.0 kbp) and, again, are identical in size among each other (lanes 3–5). The pattern is more complicated in the cell lines from patient 3. Here, two independent LCLs have been established (IARC 261 and IARC 277) in addition to the Burkitt lymphoma line (BL 60). In one of the LCL lines (IARC 261), two *Hin*dIII I1 fragments of different size (3.7 and 4.2 kbp) were detected by the labeled probe (lane 7), the larger fragment being the same size as the fragment of the IARC 277 cell line (lane 8), and the smaller one being the same size as the corresponding fragment of the tumor cell line BL 60 (lane 6).

Polymorphism at the Boundary Between the Large Internal Repeat and the Long Unique Region. At the boundary between the large internal 3.1-kbp repeat and the long unique region, a number of differences can be observed in different virus strains. First, the number of 3.1-kbp repeats may vary significantly in different isolates. Second, the boundary it-

Table 1. Cell lines

Patient	Geographic origin, sex, and age	Burkitt lymphoma lines	Chromosomal translocation	Cell line established from	Spontaneously established lymphoblastoid line	Chromosomal aberration	Cell line established from
1	French, male, 3 yr	BL37	**(8;22)**	Bone marrow	IARC 176 B	—	Blood
2	French, male, 9 yr	BL54	**(8;14)**	Ascite	IARC 247	—	Bone marrow
		BL59	**(8;14)**	Biopsy			
3	North African, female, 4 yr	BL60	**(8;22)**	Maxillary biopsy	IARC 261	—	Bone marrow
					IARC 277	—	Blood

self between the large internal repeat and the long unique region is different in M-ABA virus giving rise to a *Bam*HI Y fragment, which is about 300 base pairs smaller than that in B95-8 virus (19). Third, the *Hin*dIII site between *Hin*dIII A and B is absent in at least two more strains (Daudi and QIMR-GOR; unpublished observation) in addition to the transformation-defective P3HR-1 virus. Some viral strains harbor apparently unrelated or distantly related sequences of 1–2 kbp between the large internal 3.1-kbp repeat and the *Not* I repeats (19, 27). This is remarkable, because this region is required for initiation of transformation (20) and codes for the 3′ part of a messenger RNA transcribed in EBV-transformed cells (28). These variabilities in the organization of the viral genome can be visualized by using a *Bam*HI Y fragment, or part of it, as a probe for hybridization to blots containing separated *Hin*dIII or *Bam*HI fragments.

Fig. 4*a* shows the hybridization of cloned labeled *Bam*HI Y to separated *Hin*dIII fragments. *Bam*HI Y contains sequences of the large internal repeat and spans over the *Hin*dIII site between *Hin*dIII fragments A and B. In all cell lines, the *Hin*dIII fragments A and B were separately visible, indicating that in none of the lines is this *Hin*dIII site deleted. Because of the large size of these fragments, the resolution, however, is limited and does not show small differences. No differences in the size of *Hin*dIII A and B fragments were observed by comparing the tumor lines with the corresponding lymphoblastoid cell lines, with the exception of the lines from patient 3. In IARC 277 (lane 8), the *Hin*dIII A fragment is significantly smaller than in the Burkitt lymphoma line BL 60 (lane 6) from the same patient. In the second LCL line from this patient, IARC 261 (lane 7), the pattern seems to be heterogeneous, suggesting that both types of *Hin*dIII A fragments are represented in this cell line. *Bam*HI Y also hybridized to a number of other bands because of homology with sequences in the cellular DNA, as described by Peden *et al.* (29).

A similar pattern was also observed after hybridization of labeled pM 765-2 DNA to a blot containing *Bam*HI fragments (Fig. 4*b*). The probe used here contains the right part of *Bam*HI Y and is devoid of sequences from the large internal 3.1-kbp repeat. In addition to the hybridization to cellular sequences (29), this clone visualized *Bam*HI Y of identical size (1.8 kbp) in all cell lines examined. This indicates that in all these cell lines, the boundary between the large internal 3.1-kbp repeat and the long unique region is organized in the same fashion as in B95-8 virus.

Polymorphism Detected by *Bam*HI H1 and H2. Two types of polymorphism are related to the *Bam*HI H2 fragment. The first is concerned with the presence or absence of the respective sequences in a viral strain. As described above, in some EBV isolates the sequences of *Bam*HI H2 are substituted by distinct but related sequences (19, 27). The second polymorphism is characterized by the presence or absence of the *Bam*HI site between *Bam*HI H1 and H2, giving rise either to two fragments of 1.05 and ≈4.8 kbp or to a single *Bam*HI H fragment of ≈5.9 kb. The latter fragments are polymorphic in size, because they contain the DS_L region with the cluster of variable numbers of *Not* I repeats (18, 30). Finally, because *Bam*HI H1 (or *Bam*HI H) carries the DS_L region with homology to DS_R, the labeled fragment also visualizes the fragment containing the DS_R region. Due to a variable number of DS_R repeats, this region is also polymorphic in size (16).

Fig. 5*a* shows the hybridization of labeled *Bam*HI H2 to a blot containing separated *Bam*HI fragments. In both cell lines from patient 1, the labeled probe detected fragments of 1.05 kbp (*Bam*HI H2) (lanes 1 and 2). In the three lines from patient 2, the fragments visualized by the *Bam*HI H2 probe had the same size of 6.0 kbp (*Bam*HI H) (lanes 3–5). In the three cell lines from patient 3, the pattern was heterogeneous. In the Burkitt lymphoma line BL 60, a fragment of 1.05 kbp was observed (lane 6); in the LCL IARC 277, a fragment of 13.1 kbp was observed (lane 8). The second LCL IARC 261 contained both fragments observed in BL 60 and IARC 277 (lane 7). A principally identical pattern was observed when *Bam*HI H1 was used as a probe (Fig. 5*b*). In both lines from patient 1, it detected fragments of 4.9 kbp (*Bam*HI H1) and 9.4 kbp (*Bam*HI B1) (lanes 1 and 2), in the three lines from patient 2 fragments of 6.0 kbp (*Bam*HI H) and 10.7 kbp (*Bam*HI B1) were observed (lanes 3–5). In BL 60 and IARC 277, two fragments of 4.9 and 10.5 kbp, and of 10.5 and 13.1 kbp, respectively, were observed (lanes 6 and 8). IARC 261 contained all fragments found in BL 60 and IARC 277 (lane 7). Since the 13.1-kbp fragment was also visualized by the *Bam*HI H2 probe, we assumed that this fragment is a fusion fragment of *Bam*HI H and the neighboring fragment *Bam*HI F. By using the labeled *Bgl* II X fragment as a probe, which is included in *Bam*HI F, this could indeed be demonstrated. As shown in Fig. 6, this probe hybridized to *Bam*HI fragments of 13.1 kbp in IARC 261 and IARC 277 (lanes 7 and 8) and to fragments of 7.2 kbp (*Bam*HI F) in all other cell lines examined.

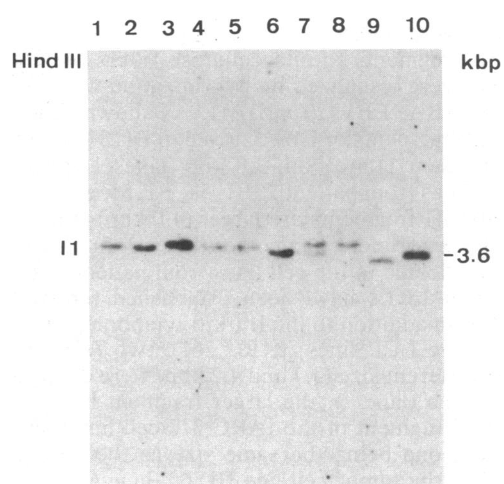

FIG. 3. Hybridization of ^{32}P-labeled cloned *Hin*dIII I1 to a filter containing separated *Hin*dIII fragments of BL 37 (lane 1), IARC 176B (lane 2), BL 54 (lane 3), BL 59 (lane 4), IARC 247 (lane 5), BL 60 (lane 6), IARC 261 (lane 7), and IARC 277 (lane 8). B95-8 virus DNA (lane 9) and M-ABA virus DNA (lane 10) served as controls.

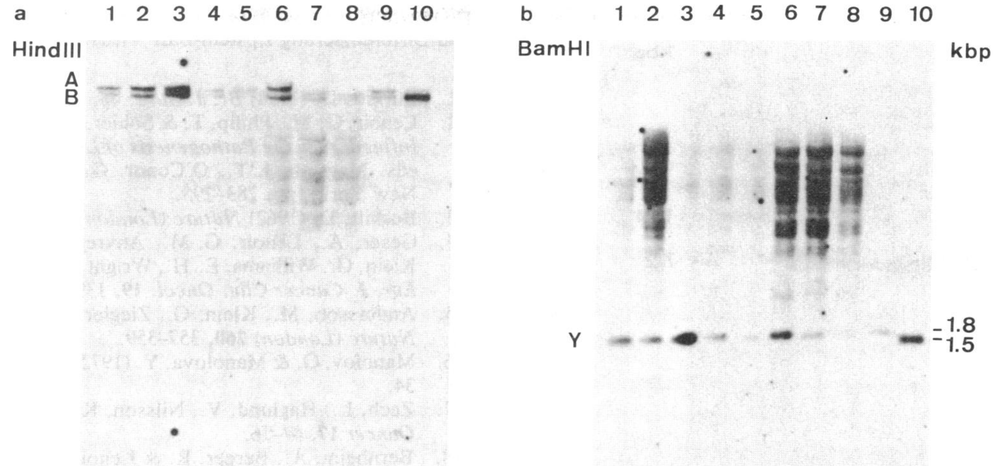

FIG. 4. Hybridization of ^{32}P-labeled cloned *Bam*HI Y to a filter containing separated *Hin*dIII fragments (*a*) and of ^{32}P-labeled pM 765-2 DNA (see Fig. 2) to a filter with separated *Bam*HI fragments (*b*) of different cell lines. The lane numbers represent the same cell lines as in Fig. 3.

DISCUSSION

EBV, although not a mandatory factor in the development of Burkitt lymphoma, is implicated in the development of tumors in the high incidence areas. In contrast, the chromosomal translocations are invariably observed, regardless of whether the tumor developed in or outside the endemic area and regardless of its association with EBV. Therefore, it seems likely that the activation or deregulation of the c-*myc* gene by the chromosomal translocation is the key event leading to the malignant phenotype. The role of EBV is more difficult to consider. Since the virus is a potent lymphoproliferation-inducing agent, it seems likely that viral functions involved in growth stimulation may also contribute to the malignant proliferation of the cell. This function, however, can be replaced by a rare unknown agent or process. Taking into account the specific epidemiological features of African EBV-associated Burkitt lymphoma, it is conceivable that infection with EBV early in life and concomitantly with chronic malaria increases the risk for development of the disease.

To test whether differences can be observed between the virus in malignant versus nonmalignant cells, we have compared, in three cases, the virus carried by the Burkitt lymphoma cells and by normal cells from the same individual. By using a variety of different polymorphic markers to distinguish practically any EBV isolate from another, it was impossible to find differences in the viral genomes carried in the Burkitt versus lymphoblastoid cell lines from two different patients. Also, in the cell lines from the third patient, the virus carried in the tumor line was detected in one of both LCL. In this case, however, evidence could be presented that the patient was infected by at least two different viruses, of which one was found in BL 60 and IARC 277, and both were found in the lymphoblastoid cell line IARC 261. We cannot differentiate whether both viruses are carried in each cell or in two different subpopulations of the cell line. In view of the fact that the spontaneous outgrowth is a polyclonal event induced by EBV released *in vitro* (31), the second possibility appears more likely. The fact that one individual can be infected by two different virus isolates is an interesting observation regarding the mechanism of natural infec-

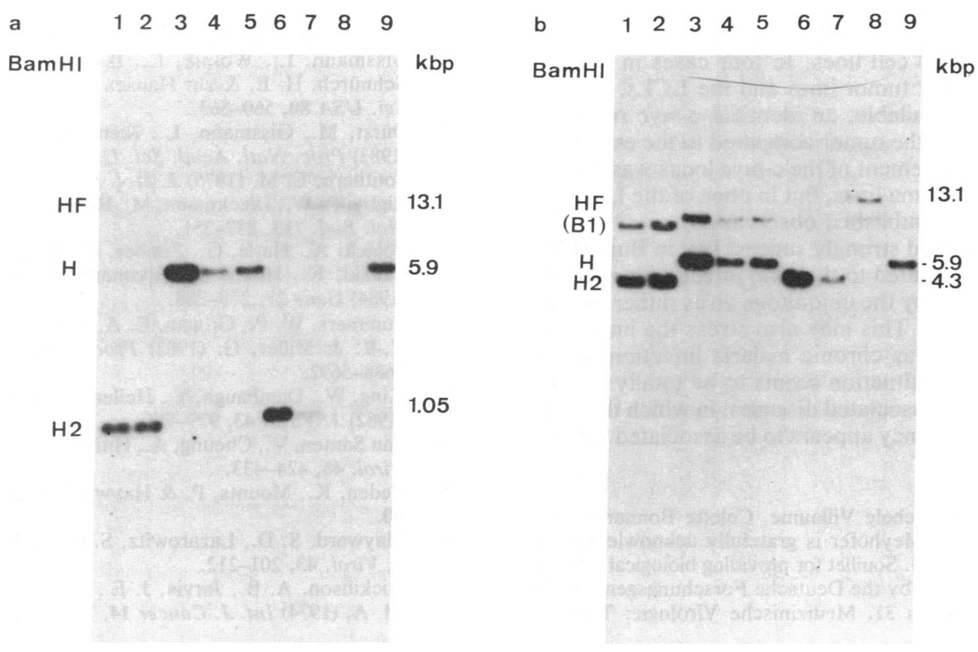

FIG. 5. Hybridization of ^{32}P-labeled cloned *Bam*HI H2 (*a*) and ^{32}P-labeled cloned *Bam*HI H1 (*b*) to filters containing separated *Bam*HI fragments of different cell lines. The lane numbers represent the same cell lines as shown in Fig. 3.

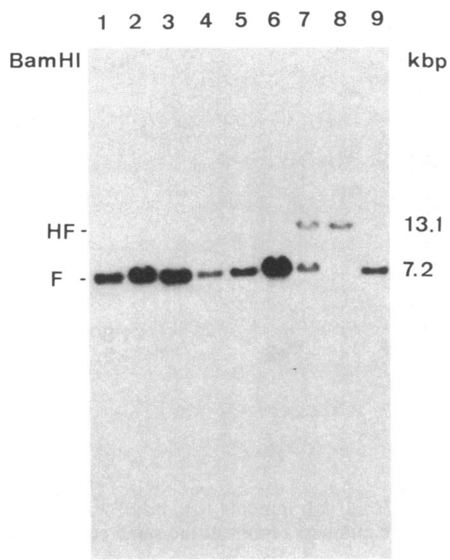

FIG. 6. Hybridization of ^{32}P-labeled cloned *Bgl* II X to a filter containing separated *Bam*HI fragments of different cell lines. The lane numbers represent the same cell lines as shown in Fig. 3.

tion. It is compatible with the clinical history of the patient that the infection with the second EBV type was the consequence of blood transfusion.

Since the virus carried in the tumor cells is indistinguishable from the virus in the nonmalignant cells, it is very unlikely that, in analogy to human papillomaviruses, specific types of EBV are associated with the malignant disease and others with the nonmalignant conditions induced by the virus. However, since we have only used polymorphic markers to characterize and compare the viral genomes, minor changes (e.g., point mutations) cannot be excluded, which may have modified the biological properties of the virus. Since the viral genome is present in the tumor cells in many copies, even cloning and sequencing the whole genome would principally not rule out point mutations in one or a few copies of the viral DNA.

In contrast to EBV, the analysis of the c-*myc* locus has revealed obvious differences between Burkitt lymphoma versus lymphoblastoid cell lines. In four cases in which in addition to the Burkitt tumor lines and the LCLs, fresh tumor biopsies were available, an identical c-*myc* rearrangement was observed in the tumor compared to the established tumor line. A rearrangement of the c-*myc* locus was found in 7 of 11 Burkitt lymphoma lines, but in none of the LCL from the same patients (unpublished observation).

The results presented strongly suggest that in Burkitt lymphoma, the risk associated to the viral infection is related to the mode of infection by the ubiquitous virus rather than to a peculiar viral subtype. This may also stress the importance of the cofactors such as chronic malaria infection in high-incidence areas. This situation seems to be totally different from papillomavirus-associated diseases, in which the risk of progression to malignancy appears to be associated with particular virus strains.

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