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B-Cell Lymphoproliferation and Lymphomagenesis Are Associated with Clonotypic Intracellular Terminal Regions of the Epstein-Barr Virus

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We analyzed 17 B-cell lineages cloned from two patients with infectious mononucleosis and found that different B-cell lineages exhibited notable variation in the length of the fused Epstein-Barr virus (EBV) terminal region on intracellular EBV episomes. EBV termini in different B-cell clones from the same person differed by as many as 15 to 20 reiterations of the ca. 500-base-pair terminal repeat sequence. In contrast, analysis of seven B-cell lineages cloned from a patient with a fatal, oligoclonal lymphoma revealed that three of the cell clones had the same-sized EBV terminal region. These three clones had previously been shown, by immunoglobulin gene analysis, to be metastatic daughter cells descended from a common progenitor. Similarity of the EBV terminal regions in the three daughter clones suggested that EBV infected the progenitor cell before proliferation and metastasis. Individual, EBV-infected cells from a single individual showed sufficient heterogeneity in their EBV termini to allow use of terminal fragment size as a clonal marker in studies addressing the contribution of EBV to the clonal pathogenesis of tumors with which this virus has been associated.

The Epstein-Barr virus (EBV) causes infectious mononucleosis, a usually benign B-cell lymphoproliferative disorder, and is associated with several forms of human malignancy (for reviews, see references 15, 24, 30, 46). African Burkitt's lymphoma (BL) is an endemic, monoclonal B-cell lymphoma; in virtually all cases, the tumor cells harbor EBV and bear chromosomal translocations associated with transcriptional deregulation of the c-*myc* proto-oncogene (12, 13, 41). EBV is also found in the tumor cells of undifferentiated nasopharyngeal carcinoma (NPC), a tumor which lacks consistent cytogenetic abnormalities (46). The etiologic significance of EBV in BL and NPC has been difficult to ascertain. It has not been possible to determine the stage at which the virus enters the malignant cell lineages. Some have argued that EBV may be a passenger virus in BL and NPC, i.e., that EBV may enter the tumor cell lineage after malignant transformation and proliferation. A third clinical setting in which EBV is associated with human malignancy is in the development of fatal B-cell lymphoproliferative disorders and B-cell lymphomas in persons suffering from various forms of congenital or acquired immunodeficiency (1, 2, 10, 11, 19, 23, 32, 33, 37, 40).

In virus particles, the EBV genome is a linear, duplex strand of about 170 to 175 kilobase pairs (kb) of DNA. Upon entry into a susceptible cell, the viral genome circularizes via its terminal repeat (TR) sequences, is amplified in copy number, and resides in the cell nucleus mostly as a multicopy plasmid but perhaps with integrated copies as well (26, 28, 39). In EBV virion DNA, the terminal regions are heterogeneous owing to variation in the number of reiterations of the ca. 500-base-pair (bp) TR sequences at each end (20, 25).

We have analyzed the configuration of DNA restriction fragments corresponding to the EBV terminal region in intracellular DNAs extracted from multiple different, EBV-transformed B-cell lineages. The cell lineages analyzed here were previously cloned directly from two patients with infectious mononucleosis and one patient with a fatal, oligoclonal, EBV-related B-cell lymphoma. Our analyses revealed that distinct cell lineages from each patient exhibited heterogeneity with respect to EBV terminal region restriction fragments. Most of this heterogeneity was caused by variation in the sizes of the fused EBV termini present on circularized EBV genomes, associated with variation in the number of reiterations of the ca. 500 bp TR sequence. Analysis of EBV terminal regions in cell clones or in tumor DNAs provides a virus-specific, clonotypic marker for studies addressing the clonal pathogenesis of EBV-associated tumors.

MATERIALS AND METHODS

Cell clones and DNA preparations. Derivation of the cell clones has been described previously (9, 37). Multiple EBV-transformed cell clones were derived by direct soft-agar cloning of fresh, T-cell depleted lymphocytes from single blood samples of two patients with acute infectious mononucleosis; these are designated the IM-F set (10 clones studied) and the GG57 set (7 clones studied). Similarly, a set of EBV-transformed cell clones was previously derived at autopsy by soft-agar cloning of fresh mononuclear cells taken from the blood and spleen of a young girl who died from a fatal, oligoclonal B-cell lymphoma associated with primary EBV infection; this set of cell clones is designated the TS set (seven cell clones). None of the cell clones exhibited a rearranged c-*myc* oncogene in *Bam*HI digests (unpublished data). Each of the cell clones produces immunoglobulin composed of a single class of heavy chain and a single type of light chain (except TS-S1, which produces μ heavy chain and no light chain) (9, 37). Furthermore, each represents a genetically distinct B-cell lineage, evidenced by

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distinct and clonal patterns of immunoglobulin gene rearrangement (6, 7; unpublished data). The exceptions to the latter are the TS-L3, TS-S2, and TS-S4 cell clones which were derived from the spleen (TS-S2, TS-S4) and blood (TS-L3) of the patient with lymphoma. These three cell clones produce immunoglobulin M (μ and κ chains) and have the same pattern of immunoglobulin heavy- and light-chain gene rearrangement, indicating that they are daughter cells of a single, metastatic B-cell lineage present in the patient at the time of death (7).

Each cell clone was propagated in RPMI 1640 plus 10% fetal bovine serum and antibiotics (penicillin, 50 IU/ml; streptomycin, 50 μg/ml; amphotericin B, 1 μg/ml). Total cellular DNA was extracted from pellets of $\geq 10^8$ cells, essentially as described previously (27).

EBV subgenomic probes and isotopic labeling. Schematic illustrations of the linear and circular forms of the EBV genome and of the probes used in this work are given (Fig. 1). The *Eco*RI-D_{het} probe was donated by John Arrand (Christie Hospital and Holt Radium Institute, Manchester, United Kingdom). For convenience, it will hereafter be called *Eco*RI-D. Briefly, it is a pHC79 cosmid clone carrying an inserted *Eco*RI fragment of about 13 kb which corresponds to sequences in the B95-8 genome from the rightmost *Eco*RI site through the TRs to the *Eco*RI-I junction on the left side of the genome (Fig. 1) (3). We created a probe for the latent membrane protein (LMP) gene from the *Eco*RI-D DNA by subcloning a 1.9-kb *Xho*I fragment from *Eco*RI-D into the *Sal*I site of plasmid vector pBR322, as described previously (17, 22, 27). We also created a probe spanning the DNA containing the LMP promoter region by gel purification of a ca. 1.1-kb *Bgl*II-*Sst*II fragment (positions 169,034 to 170,153 on the B95-8 DNA sequence [4]) from the DNA of the *Eco*RI-D cosmid. Our EBV *Eco*RI-I probe, containing sequences near the left end of the linear EBV genome, was donated by Elliott Kieff (University of Chicago, Chicago, Ill.). It represents 4.2 kb of EBV (B95-8) virion DNA cloned in plasmid vector pUC13 (14). E. Kieff also donated a probe for the EBV TR sequence; it is a ca. 530-bp *Sau*3A fragment subcloned in the pSP64 vector (29). George Miller (Yale University, New Haven, Conn.) donated a probe for the EBV *Eco*RI-B sequences; it was cloned from the FF41 EBV strain into plasmid vector pACYC184 (18). The relative positions of these cloned fragments of the EBV genome are illustrated (Fig. 1).

Plasmid and cosmid DNAs were prepared from the molecular clones by standard methods (27). Probe DNAs were radiolabeled with [α-^{32}P]dCTP to specific activities of $\geq 1 \times 10^8$ dpm/μg by nick translation or random priming (16, 36).

Preparation of DNA blots. Cell clone DNAs were digested with restriction endonucleases (5 U per μg of DNA) under the conditions recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). Spermidine (4 mM final concentration) was used in all reactions to promote complete digestion. Digested DNAs were loaded (10 μg per lane) into 0.6 to 1.8% agarose gels. Electrophoresis of the DNA was conducted at 1 to 1.5 V/cm for 20 to 42 h. The gels were then stained with ethidium bromide, photographed under UV light (254 nm), denatured in 0.4 M NaOH–0.8 M NaCl, neutralized in 0.5 M Tris–1.5 M NaCl (pH 7.5), and blotted to nylon membranes by the Southern method (27, 38).

Blot hybridization, washing, and autoradiography. Prehybridization, hybridization, and blot washing were conducted by the method of Wahl et al. (43). The hybridized blots were exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) by using Cronex Lightning-Plus intensifying screens (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) for 2 to 7 days.

RESULTS

Configuration and stability of EBV terminal region fragments in cell clones derived from patients with mononucleosis. Blots of *Bam*HI digests of DNAs from cell clones from the patients with mononucleosis were first hybridized with the *Eco*RI-D terminus probe (Fig. 2A). For both patients (IM-F and GG57), individual cell clones tended to exhibit a different pattern of hybridization when compared with other cell clones from the patient, i.e., there was clonal heterogeneity in the *Bam*HI restriction fragments detected with the *Eco*RI-D probe. Occasionally, there was some similarity, e.g., clones IM-F17 and IM-F18 appear to differ only by the fact that the topmost band (ca. 15 to 16 kb) seen with the *Eco*RI-D probe is clearly a doublet in IM-F17 and a singlet in IM-F18 (Fig. 2A).

The observed clonal heterogeneity in EBV termini was clearly not due to incomplete endonuclease digestion or

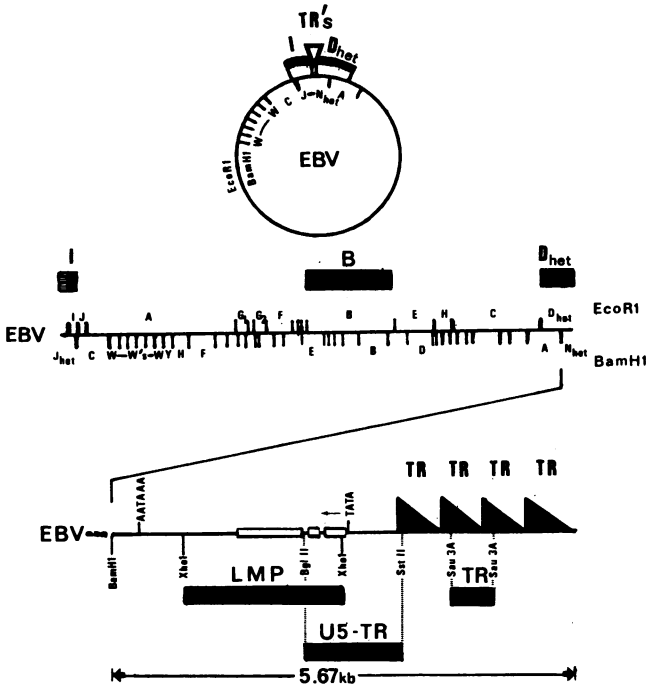
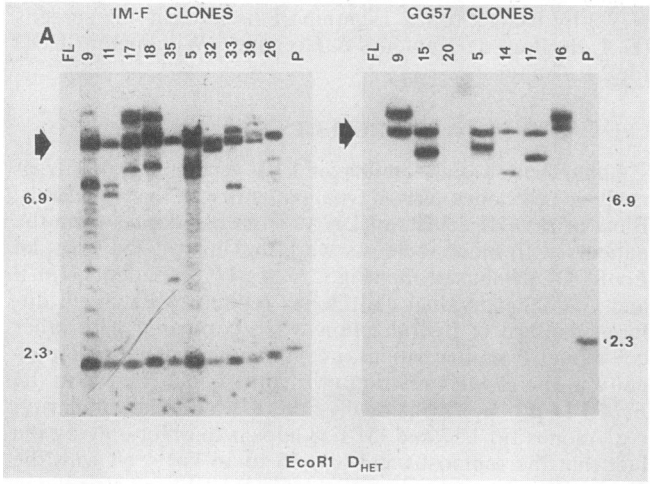
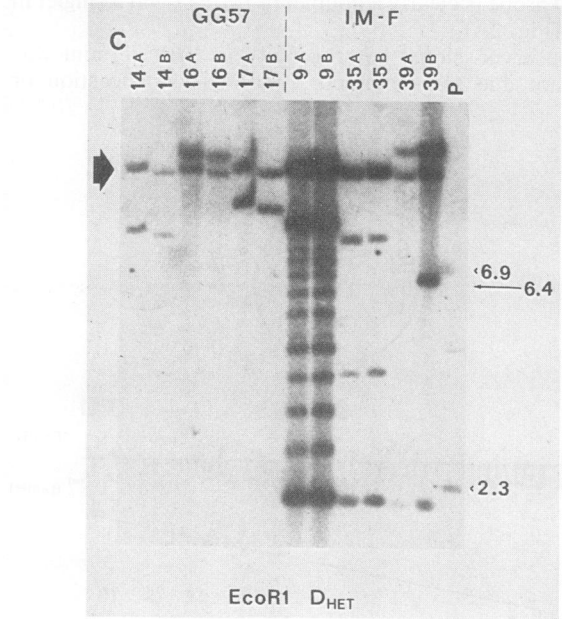


FIG. 1. Linear and circular forms of the EBV genome and the probes used in this work. (Middle) The linear (virion) form of the EBV genome, with *Eco*RI sites along the top and *Bam*HI sites along the bottom. Some of the larger restriction fragments are labeled. The *Eco*RI I (I) and D_{het} fragments used as probes are depicted above the left and right ends of the linear genome. Hatched area on the left of *Eco*RI-I indicates a region that is included on our *Eco*RI-D probe which was derived from circularized (plasmid) EBV DNA (3). Position of the internal *Eco*RI-B probe (B) is also indicated. (Top) Plasmid EBV genome circularized via its TRs. (Bottom) Schematic expansion of some of the features of the rightward end of the EBV (B95-8) genome, including the LMP gene, the LMP promoter region (contained on U5-TR), and the TRs. The LMP gene is illustrated as three open boxes between its 5′ TATA promoter motif and its 3′ polyadenylation signal (AATAAA) (4, 17, 22). Fragments used in this work were a 1.9-kb *Xho*I fragment (LMP probe), a 1.1-kb *Bgl*II-*Sst*II fragment (U5-TR probe), and a 0.5-kb *Sau*3A fragment (TR probe).





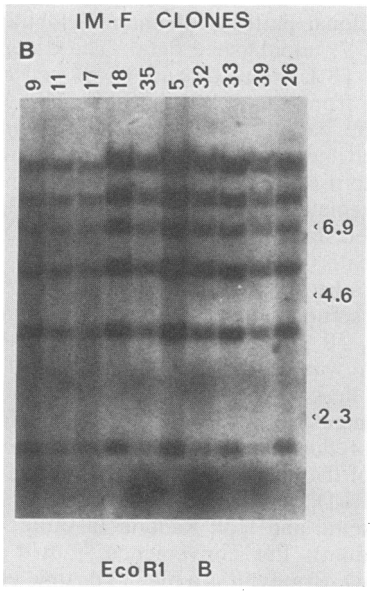


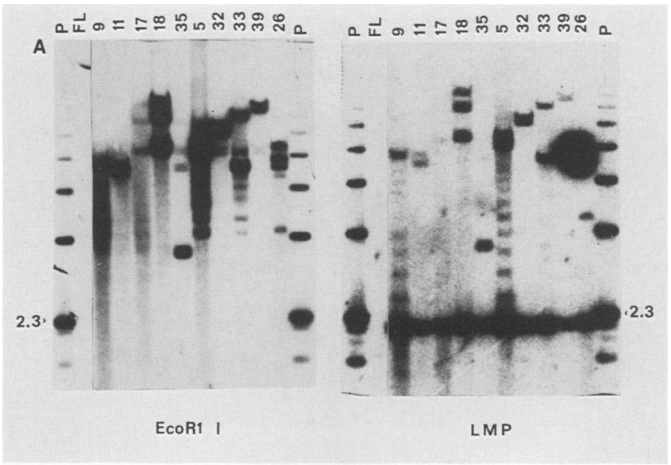
FIG. 2. (A) Clonal variation in *Eco*RI-D-hybridizing fragments, in *Bam*HI digests of the cell clone DNAs. FL in human fetal lung DNA (EBV negative) and P is a plasmid marker lane, consisting of oligomers of a plasmid of monomeric length, equal to 2.3 kb (6). Two reference points (6.9 and 2.3 kb) are indicated for the plasmid lane. Arrows, Position of *Bam*HI-A-like fragments for the cell clones (see Fig. 1). A 2-kb fragment is present in all the IM-F cell clones DNAs but not in the GG57 cell clone DNAs, indicating a strain-related polymorphism in the EBV genomes infecting the two patients. (B) Hybridization of the same blot used in panel A for the IM-F cell clones with the EBV *Eco*RI-B probe. (C) Stability of the clonally heterogeneous *Eco*RI-D fragments in six propagated cell clones. The two DNA preparations for each cell clone (A and B) were temporally separated by 4 to 12 months of continuous culture (about 15 to 50 cell generations). The 6.9- and 2.3-kb positions are from the plasmid marker lane (P). Arrow, Position of the invariant *Bam*HI A fragment.

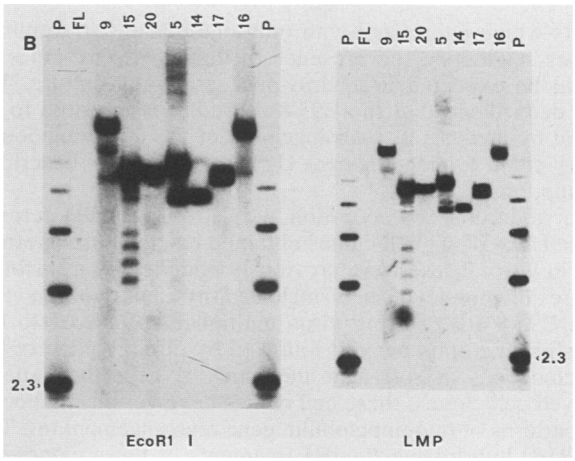
artifacts of electrophoresis. The patterns of hybridization were reproducible, and similar variations were not seen on the same or other blots with probes for internal regions of the EBV genome nor with probes for nonrearranged cellular genes. For example, the *Eco*RI-D probe was washed from the blot used to produce the data for the IM-F clones in Fig. 2A, and the same blot was then hybridized with a cloned probe for a large internal region of the EBV genome (the *Eco*RI-B probe) (Fig. 2B). The *Bam*HI fragments comprising the *Eco*RI-B-like sequences in the intracellular EBV genomes within the IM-F cell clones were clearly well digested and migrated approximately equally from lane to lane. Similar results were achieved for the blot of the GG57 cell clone DNAs shown in Fig. 2A (radiogram not shown).

The stability of in vitro maintenance of the clonotypic arrays of terminal region fragments was investigated by analysis of serial DNA preparations from six of the mononucleosis cell clones, three from each patient. For each of these six cell clones, two different DNA preparations were made which were temporally separated by 15 to 50 cell generations (4 to 12 months in culture). *Bam*HI digests of these serial DNA preparations were then hybridized with the *Eco*RI-D probe. Five of the six cell clones maintained their clonotypic array of terminal region fragments with complete accuracy (Fig. 2C). Cell clone IM-F39 exhibited faithful maintenance of three previously observed fragments, with evolution of a different-sized fourth fragment (a 6.4-kb band) in the second DNA sample (FIg. 2C).

Nature of clonal heterogeneity in EBV terminal region fragments. The clonal heterogeneity in *Bam*HI fragments containing EBV terminal region DNA sequences was investigated further.

First, we used probes for the opposite ends of the linear EBV genome, e.g., the *Eco*RI-I probe derived from the left end and the LMP probe derived from the right end of the linear EBV (B95-8) genome. We reasoned that if the clonally variable EBV *Bam*HI fragments identified with the *Eco*RI-D probe were present on circularized EBV genomes, the *Eco*RI-I and LMP probes would identify the same *Bam*HI fragments which corresponded to fused EBV terminal regions. As illustrated (Fig. 3A and B), this proved to be the case. The EBV *Eco*RI-I and LMP probes cohybridized to many of the same positions on the same Southern blots of *Bam*HI digests of the cell clone DNAs. As expected, these blot positions corresponded to positions which hybridized with the *Eco*RI-D probe, and they showed marked clonal variation. These results indicated that the clonal heterogeneity observed first with the *Eco*RI-D probe was due to the presence in the different B-cell clones derived from each





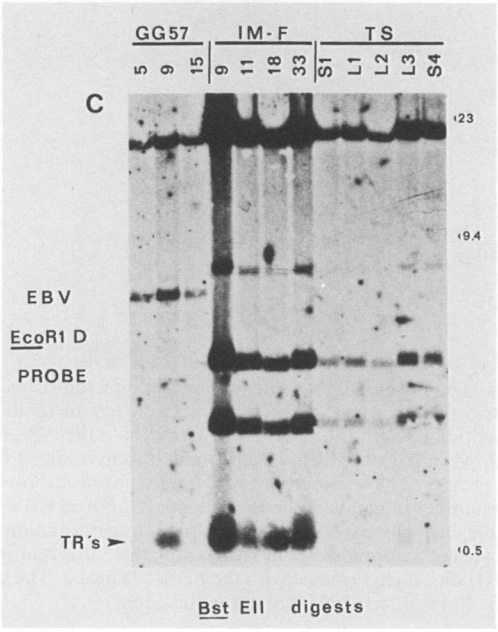


FIG. 3. Hybridizations of *Bam*HI-digested cell clone DNAs with the EBV *Eco*RI-I and LMP probes. (A) Analysis of the IM-F clones with the two probes. Radiograms are displayed adjacently to illustrate that the two probes identify many of the same fragments in each cell clone. (B) Similar analyses of the GG57 cell clone DNAs, again indicated similarity of the *Eco*RI I and LMP fragments for each of the cell clones. For both panels A and B, lane FL contains *Bam*HI-digested human fetal lung DNA (EBV negative); P, plasmid marker lane. The position of the 2.3-kb plasmid monomer is indicated. (C) Reduction of clonal heterogeneity to variation in intensity of the TR bands when cell clone DNAs are digested with endonucleases which cleave within the TR sequences. Illustrated are *Bst*EII digests hybridized with the *Eco*RI-D probe. Some strain-associated *Bst*EII site polymorphisms are apparent among the three sets of cell clones. Within a given set, clonal heterogeneity is evident only as variation in the intensity of the ca. 0.5-kb TR band. Fragment sizes at the right (in kilobase pairs) are from a λ bacteriophage marker lane.

patient of circularized EBV plasmids having variable-sized *Bam*HI fragments corresponding to the fused EBV terminal regions.

Next, we asked whether the variation among restriction fragments containing the EBV terminus was primarily due to variation in the number of reiterations of the TR sequence. We digested the cell clone DNAs with two groups of restriction enzymes: those which are known to cut within the TR sequence and those which do not, based upon published nucleotide sequence data (4). Enzymes of the first type included *Sma*I, *Sst*II, and *Bst*EII. Enzymes of the latter type included *Eco*RI, *Xho*I, and *Sst*I. A typical result is shown (Fig. 3C). This figure illustrates the hybridization analysis of *Bst*EII digests of 12 of the cell clone DNAs with the *Eco*RI-D probe. *Bst*EII cuts within the TR sequence (4). For a given patient, the only clonal variation evident in these digests was the intensity of hybridization at the ca. 0.5-kb blot position, a position which cohybridizes with the purified TR probe (radiograms not shown). Similar results were achieved with *Sma*I and *Sst*II digests (radiograms not shown). Other analyses revealed that the cell clones which showed multiple fused EBV termini in *Bam*HI digests also showed multiple, different-sized EBV termini after digestion with other restriction endonucleases which do not cut within the TR sequence (*Eco*RI, *Xho*I, and *Sst*I). The latter results confirmed that these cell clones contained subsets of viral episomes having different-length terminal regions rather than *Bam*HI site polymorphisms in the viral terminal regions (radiograms not shown).

Taken together, the above results indicated that most of the heterogeneity observed in the EBV termini in our cell clones was due to the presence of circularized viral genomes containing variable numbers of TR reiterations. It was also interesting to note that some of the cell clones contained multiple, different-sized EBV termini. This result suggested the presence of subsets of plasmid EBV genomes having different numbers of TR reiterations in the DNA of some of the propagated cell clones. To help interpret this observation, we performed EBV terminal fragment analyses of three prototype EBV-containing cell lines, Daudi, Raji, and B95-8. The first two are BL lines thought to be latently infected with EBV (24, 30). B95-8 is the prototype virus-producer cell line cloned from an EBV-transformed marmoset lymphoid culture (31). EBV terminal fragment analyses of the DNAs of these three cell clones are shown (Fig. 4A). Daudi and Raji contained single *Bam*HI fragments hybridizing with terminal region probes. B95-8 contained multiple (at least six) *Bam*HI fragments which cohybridized with probes for the left and right ends of the linear EBV genome (*Eco*RI-I and LMP probes, respectively). Thus, our B95-8 cell culture contained at least six subsets of circularized EBV genomes having different-sized terminal regions. As indicated (Fig. 4A), the B95-8 cellular DNA also contained several *Bam*HI frag-

ments which hybridized with only one of the two terminus probes, indicating the presence of linear viral genomes as would be expected in a virus-productive cell culture. We also determined that the B95-8 cell culture appeared to be clonal by analysis of rearrangement of the immunoglobulin heavy-chain joining segment (J_H), as previously described (6; unpublished data).

For additional corroboration, we also studied EBV termini in the DNA of six cell clones obtained by clonal transformation in vitro. These were previously obtained by transforming fresh lymphocytes of a single healthy adult donor in vitro with B95-8 EBV, using virus multiplicities of 0.01 to 0.1 transforming units per cell followed by direct plating of the infected cells in soft agar medium (8). Like the patient-derived cell clones, these cell clones have various monoclonal patterns of immunoglobulin gene rearrangement (6). The *Eco*RI-D-hybridizing *Bam*HI fragments of these clones are shown (Fig. 4B). Although these cell lines have maintained their clonal states of immunoglobulin gene rearrangement and were transformed by low multiplicities of EBV, two (GG29-10 and GG73-5) have developed evidence of multiple, different-sized EBV termini (Fig. 4B).

Configuration and stability of EBV terminal region fragments in cell clones derived from the B-cell lymphoma patient. *Bam*HI digests of the seven TS cell clones were hybridized with the *Eco*RI-D, *Eco*RI-I, and LMP probes (Fig. 5A and B). Three cell clones had the same configuration of EBV terminal region fragments. These three cell clones have been previously shown by analysis of immunoglobulin gene rearrangements to be clonal siblings (TS-L3, TS-S2, and TS-S4) (7, 37). Another cell clone, TS-S3, exhibited a configuration of terminal fragments similar to those observed in TS-L3, TS-S2, and TS-S4, but the size of the lower band seen in TS-S3 DNA with the *Eco*RI-D probe appears to be consistently slightly less than that of the lower band present in the latter cell clones. The impression that TS-S3 has a shorter EBV terminal region was confirmed by the observation that the *Eco*RI-D-hybridizing fragment in *Eco*RI digests of the DNA of TS-S3 is about 0.5 kb shorter than that in the TS-L3-S2-S4 lineage, as shown on the right side of Fig. 5A. A similar observation was made in *Sst*I digests (not shown). In Fig. 5A, we tested two preparations of TS-L1 DNA because the two top bands seen in TS-L1 DNA with the *Eco*RI-D probe are close in size to the two bands seen in the DNAs of TS-L3, TS-S2, and TS-S4. The third (lower) band seen in TS-L1 DNA is not present in the latter but is consistently present in both TS-L1 DNA preparations. Further analyses revealed that the ca. 10.2- and 6.2-kb *Bam*HI fragments in TS-L1 hybridized to the LMP and TR probes. Also, the ca. 10.2-kb fragment, but not the 6.2-kb fragment, hybridized with the EBV *Eco*RI-I probe. Furthermore, we could not detect two such related fragments in other digests of TS-L1 DNA with other enzymes which do not cut in the TR sequence (e.g., *Eco*RI, *Xho*I, *Sst*I). Thus, the most likely interpretation of the two EBV terminal region bands seen in *Bam*HI digests of TS-L1 DNA was that this cultured cell clone contains two subpopulations of EBV genomes, with a *Bam*HI site polymorphism in the terminal region of one subpopulation. Since both terminal region fragments in TS-L1 hybridized with the *Eco*RI-D and LMP probes, but only one (the ca. 10.2-kb band) cohybridized with the *Eco*RI-I probe, the polymorphism appears to be in the right end of a viral subpopulation (i.e., in the U5 region). This restriction site polymorphism may have arisen during proliferation of the TS-L1 clone. Finally, it was of interest that none of the individual DNAs of the cell clones of the patient

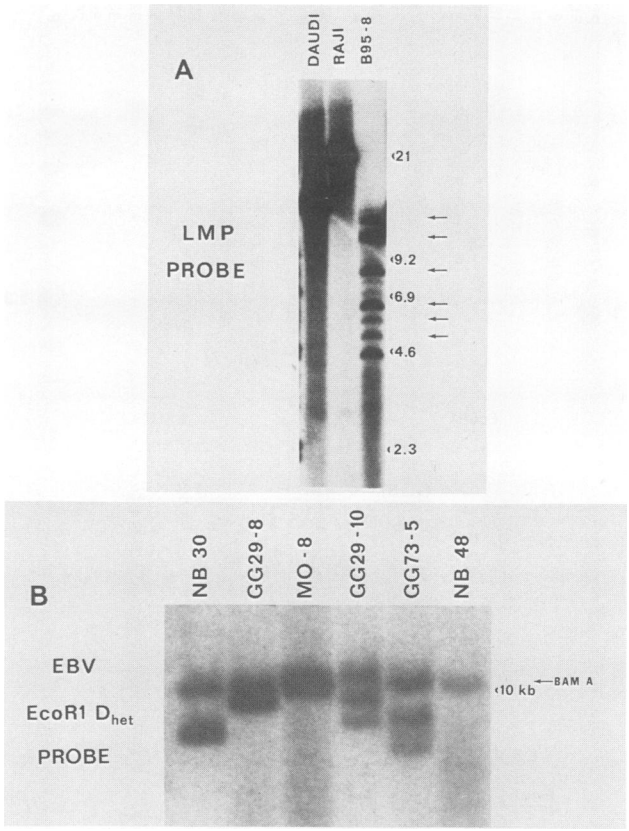


FIG. 4. (A) Fused EBV termini in latently infected (Daudi, Raji) and virus-productive (B95-8) cells. *Bam*HI digests of total cellular DNAs hybridized with the LMP probe. Arrows to the right indicate the LMP-hybridizing bands in B95-8 which also hybridized with the *Eco*RI-I probe. The radiogram was overexposed to reveal the maximal number of bands in the B95-8 lane. (B) Fused EBV termini in isogenic cell clones obtained by clonal transformation of the lymphocytes of a normal adult in vitro, using low virus multiplicities (8). *Bam*HI digests hybridized with the *Eco*RI-D probe. The position of the ca. 10-kb *Bam*HI A fragment is indicated.

with lymphoma contained evidence of multiple, different-length EBV termini, as had been seen in some of the cell clones of the patients with mononucleosis.

The identity of the EBV terminal regions in the three metastatic TS clones (i.e., TS-L3, TS-S2, and TS-S4) was further established by *Eco*RI-D hybridizations of other digests of these cell clone DNAs. In repeated *Bam*HI, *Hin*dIII, and *Eco*RI digests and in several DNA preparations from the three cell clones, the sizes of the EBV terminal fragments were the same (Fig. 5B).

Restriction site configuration of the LMP gene and LMP promoter region in the cell clones. As a possible functional correlate of terminal region variability, we wished to determine whether individual cell clones might exhibit restriction-site variation in the region of the LMP gene or its promoter (44, 45). Hybridization analyses of numerous digests of the cell clone DNAs with the LMP and the U5-TR probes failed to reveal any evidence of clonal variation in restriction sites in these regions, although strain-associated site polymorphisms were observed for each of the viruses from the three patients. The restriction endonucleases used included *Msp*I, *Bst*EII, *Sst*II, *Sma*I, and *Xho*I (radiograms not shown).

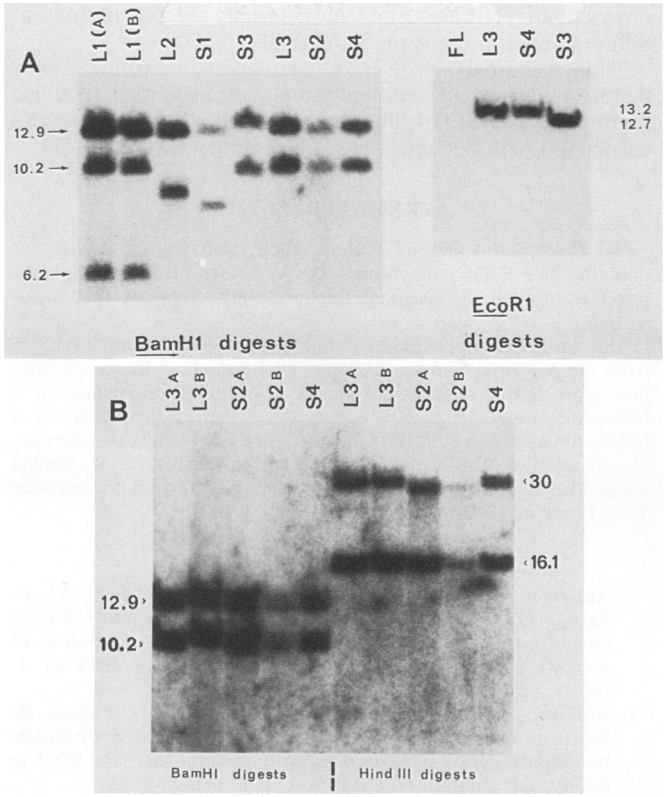


FIG. 5. (A) Left side indicates hybridization with the *Eco*RI-D probe of *Bam*HI digests of the seven cell clones from the patient with lymphoma (patient TS). The invariant ca. 12.9-kb band hybridized with a probe for the *Bam*HI-A region. The TS-L1 cell clone has two terminal region bands at ca. 10.2 and 6.2 kb (see text). Both fragments are present in two DNA extractions from TS-L1, separated by a year of propagation. TS-L3, TS-S2, and TS-S4 have the same-sized EBV terminal regions and were previously shown to be sibling clones of a metastatic cell lineage by immunoglobulin gene analysis (7). TS-S3 has a slightly shorter EBV terminus. In *Bam*HI digests, the result was equivocal, but hybridization of *Eco*RI digests indicated that the EBV terminal region in TS-S3 was ca. 0.5 kb shorter than that in the TS-L3-S2-S4 lineage. (B) Identity of the EBV termini in the metastatic cell lineage represented by the TS-L3, TS-S2, and TS-S4 cell lineages. *Bam*HI and *Hin*dIII digests were hybridized with the *Eco*RI-D probe. Serial DNA preparations were made for the TS-L3 and TS-S2 cell clones.

DISCUSSION

By analysis of multiple, isogenic cell lines cloned directly from EBV-infected patients, we found that the EBV terminal regions among individual B-cell clones isolated from a single patient were present as clonotypic arrays of restriction fragments when the cellular DNAs were cut with restriction enzymes that did not cut within the TR sequences. In our studies, *Bam*HI proved to be the most useful restriction endonuclease for demonstrating this phenomenon. We also found that virtually all of the observed variation in length of the EBV terminal region was due to variation in the number of reiterations of the ca. 500-bp TR sequence among the circularized viral episomes present within the various cell clones, as was also suggested by data from previous reports (34, 39).

The extent of the length heterogeneity in the termini of intracellular EBV genomes among different cell clones from a given patient can be rather large. For example, cell clones IM-F39 and IM-F35 exhibited long (14.7 kb) and short (4.2 kb) EBV termini, respectively. The net difference of ca. 10.5 kb indicates that fused EBV termini among different cell clones from patient IM-F differed by as many as ca. 21 reiterations of the TR sequence. For patient GG57 from whom only seven cell clones were analyzed, the maximal observed difference among EBV termini corresponded to a difference of ca. 15 reiterations of the TR sequence (e.g., cell clones GG57-16 and GG57-14). It seems likely that the clonal heterogeneity in fused, intracellular EBV terminal regions is related to the reiterative variation of the TR sequence on the ends of the infecting virion genomes, which could result in variable-length fused terminal regions; however, this idea requires further experimental analysis.

We also observed that separate B-cell lineages exhibiting different clonal states of immunoglobulin gene rearrangement and expression can sometimes exhibit similar- or identical-length EBV termini. This was exemplified by the apparent difference of one TR exhibited in the EBV termini of the TS-S3 lineage compared with that of the metastatic TS-L3-S2-S4 lineage and the possible identity of the terminal region length of the EBV episomes in TS-L1 compared with that of the TS-L3-S2-S4 lineage. Also, inspection of the results from cell clones of the patients with mononucleosis (Fig. 2A and 3A and B) reveals occasional similarities. Thus, analysis of B-cell neoplasms potentially related to EBV should always include both immunoglobulin gene and EBV terminal-fragment analyses to allow surer inferences regarding clonal pathogenesis.

Raab-Traub and Flynn (35) recently reported different work providing some similar implications while the present work was being completed (5; N. A. Brown, C. Liu, Y.-F. Wang, and C. R. Garcia, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 319, 1986). They demonstrated that DNA from several tumor biopsies, including specimens of NPC and BL, contained single, fused EBV termini, implying a clonal origin for these tumor samples. These authors, however, did not assess the variability of EBV terminal-fragment length among different cell lineages from the same patient, and they did not analyze different daughter clones of a metastatic cell lineage from an individual patient, as we have done here. Furthermore, some of our results provide different implications, as discussed below.

Serial analyses of immunoglobulin gene markers indicated that our cell clones maintained the same pattern of immunoglobulin gene rearrangement during in vitro propagation, with no evidence of emergence of other cellular subpopulations by immunoglobulin gene analysis (6, 7; unpublished data). Nevertheless, some (but not all) of the cell clones exhibited multiple, different-sized EBV termini, as noted above (Fig. 2A and 3A and B). Also, we found that among several different cell clones created from a healthy adult donor by clonal transformation in vitro, using low viral multiplicities, at least two clones exhibited multiple fused EBV termini (Fig. 4B). This result differs from related results described by Raab-Traub and Flynn and by previous workers, which largely involved analyses of BLs or neonatal cord lymphocytes transformed with low viral multiplicities (25, 35). Except for the TS clones, our analyses involved lymphoid clones from adolescents and adults. The patient with lymphoma was 4 years old, and it was interesting that her clones contained single-length EBV termini, similar to the results obtained with Daudi and Raji cells here and with those obtained by others for transformed neonatal cord lymphocytes (25, 35). These results may provide further evidence for ontogenetic variation between neonates and

adults in the ability to maintain EBV genomes in a nonproductive, tightly latent state (30). Our interpretation of the results with those of our clonally derived, cell lines from patients with mononucleosis which showed multiple fused EBV termini is that such cell clones have probably undergone occasional episodes of virus production and reinfection of cellular subpopulations during propagation. Such a mechanism could also explain the multiple fused termini seen in the DNA of the producer cell line (B95-8) compared with the single fused terminus seen in the nonproducer cell lines (Daudi and Raji) (Fig. 4A). Two other mechanisms could also account for the observation of multiple fused viral termini in a cell clone. (i) Some cells may have been infected by more than one virion, resulting in circularized intracellular EBV genomes with different-length termini, or (ii) the viral terminal regions within a single cell might have occasionally undergone DNA-DNA recombination events, resulting in EBV plasmid subpopulations with different-length, fused termini. The latter two notions are not favored by the results in Fig. 4A and B, however. Two of six cell clones created from healthy adult lymphocytes by in vitro transformation with low viral multiplicities developed evidence of multiple fused EBV termini (Fig. 4B). And the fused EBV termini in the nonproducer cell lines (Daudi and Raji) did not exhibit heterogeneity despite years of in vitro propagation (Fig. 4A). Thus, we favor the idea that multiple termini can develop in a cell clone through occasional episodes of virus production and reinfection. Consistent with this idea is the fact that the IM-F9 cell clone, which showed the most extensive ladder of fused EBV termini (Fig. 2A and C and 3A), was also the only cell clone which was found to have an appreciable fraction (ca. 5%) of cells staining positive for the EBV viral capsid antigen (21). It was also interesting that different B-cell lineages propagated from the same mononucleosis patient showed variation in the propensity to exhibit multiple fused EBV termini, implying that there may be clonal heterogeneity in the ability of individual B cells from the same person to maintain a latent state of the EBV genome.

Our results support the idea that if a clonal pattern of immunoglobulin gene rearrangement and a single-sized EBV terminus are observed in a B-cell lymphoid tumor lineage, the likely implication is that the tumor population evolved from a single progenitor B cell which was infected with EBV before it started to proliferate. The rationale for this inference is similar to that presented by Raab-Traub and Flynn (35). Although there are no independent clonal markers for epithelial cell carcinomas associated with EBV (e.g., NPC), the observation of a single EBV terminus in such tumor masses may also indicate evolution of the carcinoma from a single EBV-infected cell (35).

Terminal-fragment analysis does not implicate EBV as the sole agent responsible for the malignant behavior of a tumor lineage. But such analyses can be used to address the passenger virus notion of EBV in oncogenesis, which postulates that EBV enters malignant cell lineages after they have begun to proliferate and metastasize. If multiple different tumor sites from an afflicted individual consistently show the same-sized EBV terminal region, then it is likely that EBV entered the tumor lineage before its proliferation and metastasis. In our study, we found that the metastatic lineage represented by the TS-L3, TS-S2, and TS-S4 cell clones maintained a single-sized EBV terminus. The importance of analyzing metastatic tumor samples from the same individual is illustrated by the work of Cleary et al. who showed that individual tumor foci can be oligoclonal or monoclonal yet can represent different cell populations at different sites in the same individual (10, 11).

Thus, the analysis of EBV termini in metastatic tumor deposits can provide etiologic implications similar to those for the clonotypically integrated genomes found in tumors associated with hepadnaviruses and retroviruses (42, 47).

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