Epstein-Barr Virus DNA Recombination and Loss in Sporadic Burkitt's Lymphoma

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Epstein-Barr virus (EBV) antigens in tumor tissue define associations of virus with human malignancies and provide clues as to mechanisms of viral oncogenesis. In Burkitt's lymphoma, EBV markers are absent from 85% of sporadic cases and 4% of endemic (African) cases, raising questions as to the exact role of EBV in the disease. Standard screening criteria may be insufficient to determine the EBV status of all tumors. One of 9 tumors from American patients expressed EBV nuclear antigen 1 (EBNA1) and contained standard episomal EBV DNA, making this series consistent with the 15% EBV association traditionally ascribed to sporadic Burkitt's lymphoma. Surprisingly, 3 tumors without detectable EBNA1 contained partial EBV genomes. Identification of defective, integrated viral DNA in some tumors indicates greater involvement of virus in sporadic Burkitt's lymphoma than previously documented and suggests a process of viral DNA rearrangement and loss during malignant progression most consistent with an initiating role for EBV in tumorigenesis.

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus associated with Burkitt's lymphoma (BL), nasopharyngeal carcinoma, Hodgkin's disease, and tumors in immunodeficient patients [1]. Acceptance of EBV as oncogenic is confounded by the <100% association of the virus with tumors of similar histopathologic features, a discrepancy most apparent in lymphoid malignancies such as BL [2] and Hodgkin's disease [3]. Because of recent observations of loss of EBV DNA from cultured BL cell lines [4], we questioned whether a similar process may occur during in vivo lymphomagenesis. We sought to detect EBV DNA fragments in EBV nuclear antigen 1 (EBNA1)–negative sporadic BL tissue that lacks viral episomes; such findings could provide clinical evidence for a broader association of EBV with tumors that is reminiscent of a "hit-and-run" mechanism for viral oncogenesis [5].

Materials and Methods

Clinical samples. Frozen biopsy material from 9 American patients with BL seen between August 1981 and November 1992 was made available by the tumor bank at St. Jude Children's Research Hospital. Samples, obtained at diagnosis, consisted of 7 lymph node biopsies, 1 bone marrow biopsy, and cells derived from a malignant pleural effusion. Karyotypic analysis revealed the chromosomal translocation t(8;14)(q24;q32) in all 9 cases. Patients were human immunodeficiency virus–negative and included 6 boys and 3 girls with an age range of 5–18 years.

Control cells consisted of cultured BL cell lines Raji (containing 50–60 EBV episomes) [6], P3HR-1 clones 5 and 16 (EBV WZhet DNA–positive and –negative, respectively; provided by G. Miller, Yale University, New Haven, CT) [7], Akata [8], and EBV-infected lymphoblastoid cell lines B95-8 [9] and IB4 [10].

Immunofluorescence and immunoblotting for viral antigens. Frozen sections of tumors fixed in 1:1 methanol and acetone were examined by indirect immunofluorescence by standard procedures [11, 12] for the EBV latent protein EBNA1 and lytic protein BZLF1 (first leftward open-reading frame [ORF] of the *Bam*HI Z fragment of EBV DNA). EBNA1 expression was determined using pooled monoclonal antibodies 3E4 and 4D3 [13] and a well-characterized polyclonal human serum (JT) containing antibodies to latent EBV proteins [14]. The monoclonal antibody BZ.1 (gift of L. S. Young, University of Birmingham, UK) [12] was used to detect BZLF1.

For immunoblotting, cells were lysed in SDS-PAGE sample buffer containing 10% β-mercaptoethanol. Samples were sonicated, electrophoresed on SDS-polyacrylamide gels (8.5% for EBNA1; 10% for BZLF1), and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) using a semidry electroblotter (Bio-Rad, Richmond, CA). To detect EBNA1, blots blocked in 5% milk were incubated for 12 h with protein A–purified antibody from EBV-positive human serum and probed with 1 μCi of ^{125}I-labeled protein A (NEN, Boston) for 30 min. For BZLF1 detection, antibody BZ.1 was used at 1:1000 dilution, and binding was detected by chemiluminescence per manufacturer's recommendation (ECL kit; Amersham, Little Chalfont, UK).

Southern analysis of EBV DNA. Total cellular DNA from tumors and control BL cell lines was digested with the *Bam*HI restriction endonuclease, loaded at 10 μg of DNA per well, and separated by electrophoresis in 0.8% agarose gels, then transferred by the Southern method to nylon membranes for hybridization

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Informed consent was obtained from all patients or their parents, and the study was done in accordance with guidelines of the St. Jude Institutional Review Board.

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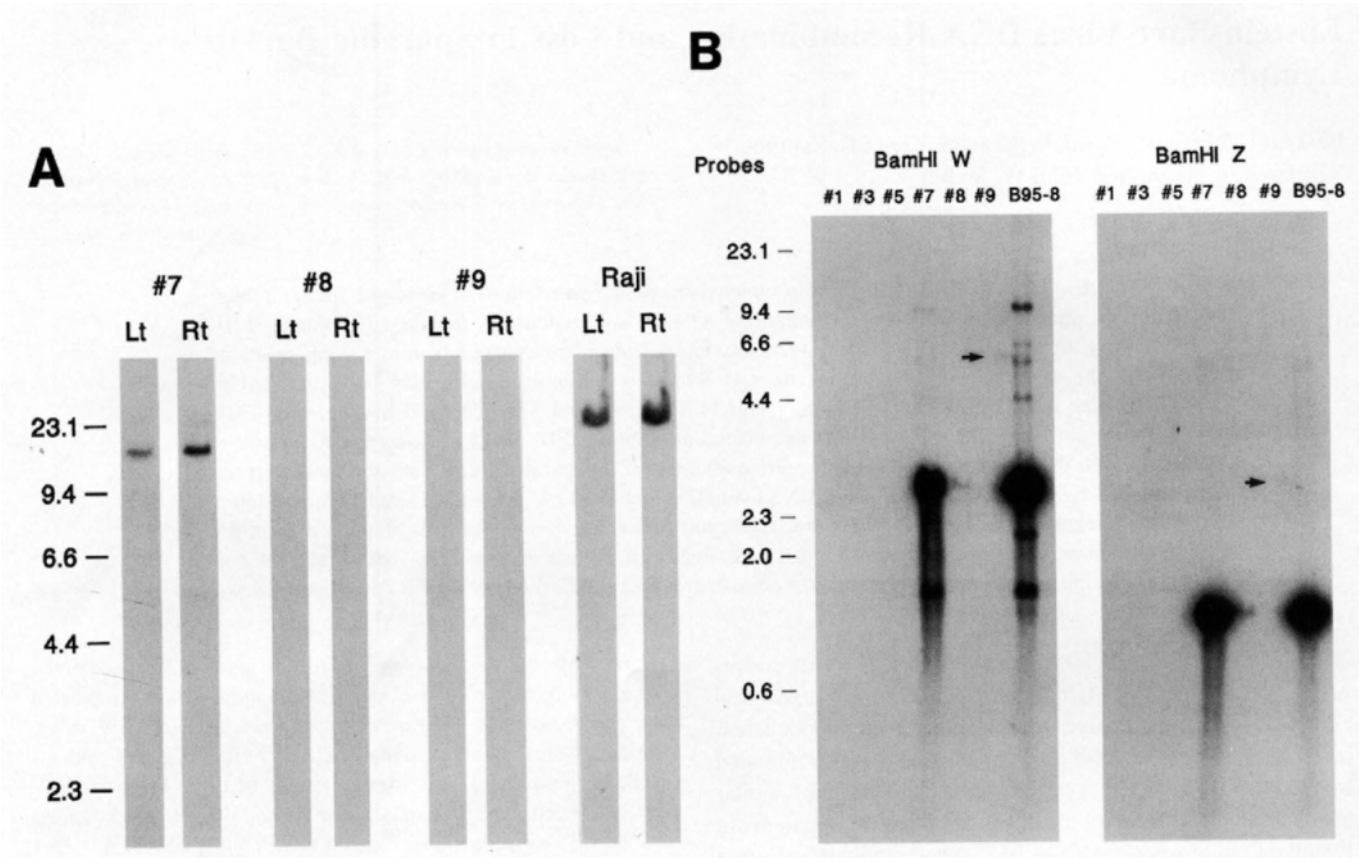


Figure 1. EBV DNA configuration in sporadic Burkitt's lymphoma (BL) tissue. **A**, Autoradiograph from DNA blot of *Bam*HI-digested tumor DNA successively probed for unique DNA sequences located at right (Rt) or left (Lt) end of EBV genome. Single high-molecular-weight bands of equal size (tumor 7 and BL-derived Raji cell control) indicate joined ends in their episomal configuration characteristic of EBV DNA in monoclonal tumors [17]. Hybridization signals were not detected from remaining 8 tumors (2 are shown here), even on gross overexposure of autoradiographs (not shown). **B**, Southern blots of tumor DNA hybridized to probes specific for sequences internal to EBV termini. *Bam*HI W and Z probes detected standard 3.0- and 1.8-kb fragments, respectively, in tumor 8, tumor 7, and control B95-8 virus. Tumor 9 contained abnormal-sized fragments (arrows) consistent with recombination. DNA from tumors 1, 3, and 5 is representative of 6 tumors that did not hybridize to any probe. Molecular weight markers (kb) are at left.

[15]. ^{32}P-labeled probes were derived from cloned EBV fragments spanning representative segments throughout the length of the viral genome (*Bam*HI C, W, Y, H, M, S, Z, R, K; *Eco*RI C, Dhet) (random primed DNA labeling kit; Boehringer Mannheim, Indianapolis) [16]. The molecular configuration of EBV termini was determined by the method of Raab-Traub and Flynn [17], using riboprobes to regions of unique DNA (*Xho*Ia and the *Bam*HI J portion of *Eco*RI I) flanking EBV's terminal repeats. T7 RNA polymerase (Promega, Madison, WI) was used to incorporate ^{32}P into RNA templates of EBV DNA restriction fragments cloned into pGEM2 vector [18]. The integrated optical density of bands was quantitated with an image analysis system (Visage 110; Bio-Image Products, Ann Arbor, MI).

Detection of EBV DNA rearrangements by polymerase chain reaction (PCR) analysis. PCR amplification was done on 10, 100, and 800 ng of total cellular DNA with Taq polymerase for 25–35 cycles on a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) as described [19]. Primers were selected that framed the junction of rearranged DNA in WZhet EBV (5′-GCACATTAG-CAATGCCTGTG-3′ and 5′-GTCCAGCGCGTTTACGTAAG-3′; base coordinates 1381 and 1649, respectively) [20] or were specific to regions within the BZLF1 ORF (5′-AGTGGTTTCCTTG-TACGTCG-3′ and 5′-TCCCAGTCTCAGACATAACC-3′; base coordinates 750 and 954, respectively) [20]. PCR products electrophoresed in a 3% NuSieve/1% Seakem agarose gel (FMC Bioproducts, Rockland, ME) were hybridized after Southern transfer to ^{32}P-labeled probes specific for sequences internal to the primers.

Results

Molecular configuration of EBV DNA in sporadic BL tumor tissue. The structure of EBV termini has been used to provide evidence for clonality of EBV neoplasms, to indicate viral integration, and to suggest the state of viral activation in cells: replicating (linear configuration) versus latent (circular forms) [17]. Restriction endonuclease digestion of EBV DNA with *Bam*HI preserves the terminal repeat sequences, which vary in number with each virus isolate, but cleaves flanking unique DNA. In EBV's episomal configuration, fused terminal sequences provide a band of higher molecular weight than single unjoined ends of the linear EBV genome. Clonal expansion of one cell carrying a single virus can be distinguished from an

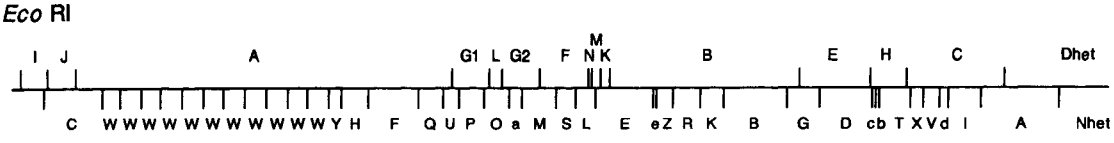
oligoclonal or polyclonal lymphoproliferative process involving mixed populations of EBV genomes, each with distinct numbers of terminal repeats, by the detection of single versus multiple high-molecular-weight episomal bands.

With this approach, tumor DNA was examined for EBV episomes characteristic of virus-associated lymphomas. When Southern blots of *Bam*HI-digested cellular DNA were hybridized to riboprobes specific to the left and right termini of EBV, only one (no. 7) of 9 tumor DNAs contained EBV (figure 1A). As with the Raji cell control [17], single and intense high-molecular-weight bands of equal size indicated tumor clonality and presence of multiple copies of a uniform EBV episome. In contrast to this result, preliminary PCR screening for sequences specific to the *Bam*HI Z fragment of EBV DNA showed virus in 4 of 9 tumor DNAs: 3 lymph node biopsies (nos. 7–9) and 1 pleural effusion (no. 1) (data not shown).

Because integrated fragments of viral DNA have been detected in the absence of the complete viral genome after in vitro B cell infection [21], we probed blots for EBV sequences internal to viral termini. With probes specific for EBV DNA fragments *Bam*HI W and Z (figures 1B and 2), intense hybridization signals were obtained with DNA from tumor 7, consistent with its multiple episomal copy number. However, in 2 additional tumors (nos. 8 and 9), low-intensity bands (<1 genome equivalent/cell) were detected: one had appropriately sized restriction fragments, while the second revealed bands of abnormal size (arrows, figure 1B), suggesting intragenomic rearrangements or recombination of W and Z fragments with cellular DNA.

Hybridizations with probes spanning much of the EBV genome are summarized in figure 2. The tumor with episomal EBV DNA (no. 7) contained normal-sized restriction fragments, consistent with the presence of the standard EBV genome. Analysis of tumor 8 also indicated presence of the standard viral genome except for DNA deletions at each terminus. Absence of the *Bam*HI C fragment at the left end and evidence for an abnormally sized 10-kb *Eco*RI C fragment on the right end are consistent with integration of EBV into cellular DNA at those sites (figure 2). A third tumor (no. 9) contained not only the abnormal *Bam*HI W and Z fragments (figure 1B) but also a single 7.5-kb band when hybridized to the *Eco*RI C probe, which normally spans 5 *Bam*HI fragments in the B95-8 prototype virus (see restriction map, figure 2). Last, the tumor DNA derived from pleural effusion (no. 1), identified as EBV-positive by PCR screening, did not hybridize in Southern blot analyses.

Defective EBV genome that disrupts latency. Rearrangements of EBV DNA, called heterogeneous (het) DNA [23, 24], are found in a defective viral genome known to activate the EBV lytic replication cycle [25–27]. The intragenomic rearrangement results in the constitutive expression of the BZLF1 protein [28], a transcriptional transactivator [29, 30] that has also been shown to bind the cellular tumor suppressor protein, p53 [31]. To determine if abnormally sized W and Z EBV DNA fragments shown here were juxtaposed with inversion of the BZLF1 ORF typical of WZhet [20, 24], we used PCR primers to flank the aberrant junction of the two DNA fragments, separated by 55 kb in standard EBV. Of the 9 tumors, 2 (nos. 1 and 9) contained rearranged WZhet DNA as evidenced by PCR products that hybridized to both Z- and W-specific oligonucleotide probes (figure 3). WZhet DNA sequences were not detected in the remaining 7 patient samples



	Eco RI I	Bam HI C	Bam HI W	Bam HI Y	Bam HI H	Bam HI M	Bam HI S	Bam HI Z	Bam HI R	Bam HI K	Eco RI C	Eco RI Dhet
Tumor #7	+	+	+	+	+	+	+	+	+	+	+	+
Tumor #8	-	-	+	+	+	+	+	+	+	+	+*	-
Tumor #9	-	-	+*	-	-	-	-	+*	-	-	+*	-

Figure 2. Integrity of EBV genome in 3 sporadic Burkitt's lymphomas: hybridization patterns of tumor DNA (below) in relation to restriction map of standard EBV genome [22] (above). Tumor 7 contained standard EBV episomes; tumor 8 carried EBV DNA deleted for end sequences that is consistent with integration; tumor 9 had rearranged, deleted viral DNA. +, present; −, not detected; * abnormal-molecular-weight band compared to standard EBV restriction fragment.

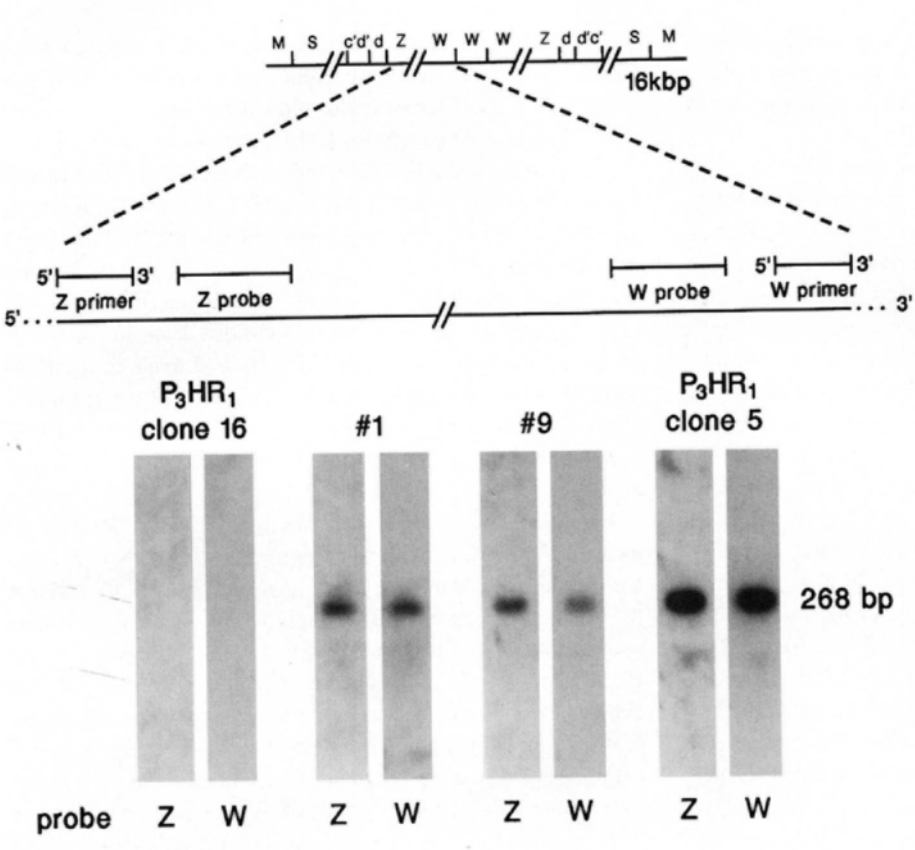


Figure 3. Defective (WZhet) EBV DNA in sporadic Burkitt's lymphomas. Polymerase chain reaction amplification strategy based on organization of 16-kb central palindrome of WZhet EBV DNA (top) [32] yielded 268-bp products from tumors 9 and 1. On Southern blot analysis (bottom), each contained abnormally juxtaposed W and Z sequences (see restriction map [figure 2] for organization of prototype genome) when hybridized to ^{32}P-labeled W- and Z-specific oligonucleotides. P3HR-1 cellular clone 5 contains WZhet DNA plus nontransforming EBV strain; P3HR-1 cellular clone 16 contains only nontransforming EBV without WZhet DNA.

(not shown) or in the negative control, P3HR-1 cellular clone 16 (figure 3).

Expression of viral proteins. Of 9 tumors, only the specimen that contained EBV episomes expressed EBNA1 when examined by immunofluorescent staining and immunoblot techniques (figure 4A, B). Notably, tumor 8, which retained the EBNA1-encoding *Bam*HI K DNA fragment but lacked terminal sequences that regulate latent gene expression [33] (figures 1 and 2), did not express EBNA1. The lytic protein BZLF1 was not detected in any of the 4 tumors with EBV DNA, including those containing WZhet DNA (figure 4C).

Discussion

Primary B lymphocytes immortalized by EBV harbor multiple episomal copies of the EBV genome and express six nuclear antigens and three membrane proteins, most of which have been shown to be critical for growth transformation [33]. By contrast, only EBNA1 (which appears to have no intrinsic growth-enhancing potential but is required for episome maintenance) [34, 35] is expressed in virus-associated BL tumors [36]. Thus, viral genes critical to growth transformation and proliferation of primary B cells (and perhaps to initial steps of the oncogenic process) appear nonessential for maintenance of the malignant phenotype.

Our findings now suggest that the viral genome itself may be dispensable at some stage of tumor development. We detected EBV DNA evidence in 3 of 8 sporadic BL tumors that would otherwise be classified as virus-negative by standard criteria. Presence of EBV DNA in clinical samples without detectable viral antigen expression (to include EBNA1) has not been previously reported [37]. That viral sequences we identified might represent infiltration of the tumor mass with EBV-infected lymphocytes or denote secondary infection of a subset of EBV-negative tumor cells seems unlikely. Circulating B cells carry prototype EBV genomes, heterogeneous primarily with respect to number of terminal repeats. Detection solely of integrated or rearranged WZhet viral sequences is inconsistent with either polyclonal lymphoid infiltration or de novo infection of the tumor mass with a mixed virion population and may reflect susceptibility of EBV DNA to recombinogenic processes underlying chromosomal translocations and cellular genomic instability.

Although observed in vitro [21, 38–41], integration of EBV into cellular DNA has been assumed to occur rarely in natural disease states [42, 43]. Our examination of sporadic BL lacking EBV episomes removed the technical difficulty of detecting a single integrated sequence against the background of a high episomal copy number and suggests an approach to a systematic search for EBV integration. Indeed, selection of cell lines specifically for low episomal copy number facilitated the demonstration of coexistent integrated EBV [44]. With increasing evidence to suggest that cellular recombination machinery is usurped for essential needs of the virus [45, 46], EBV integra-

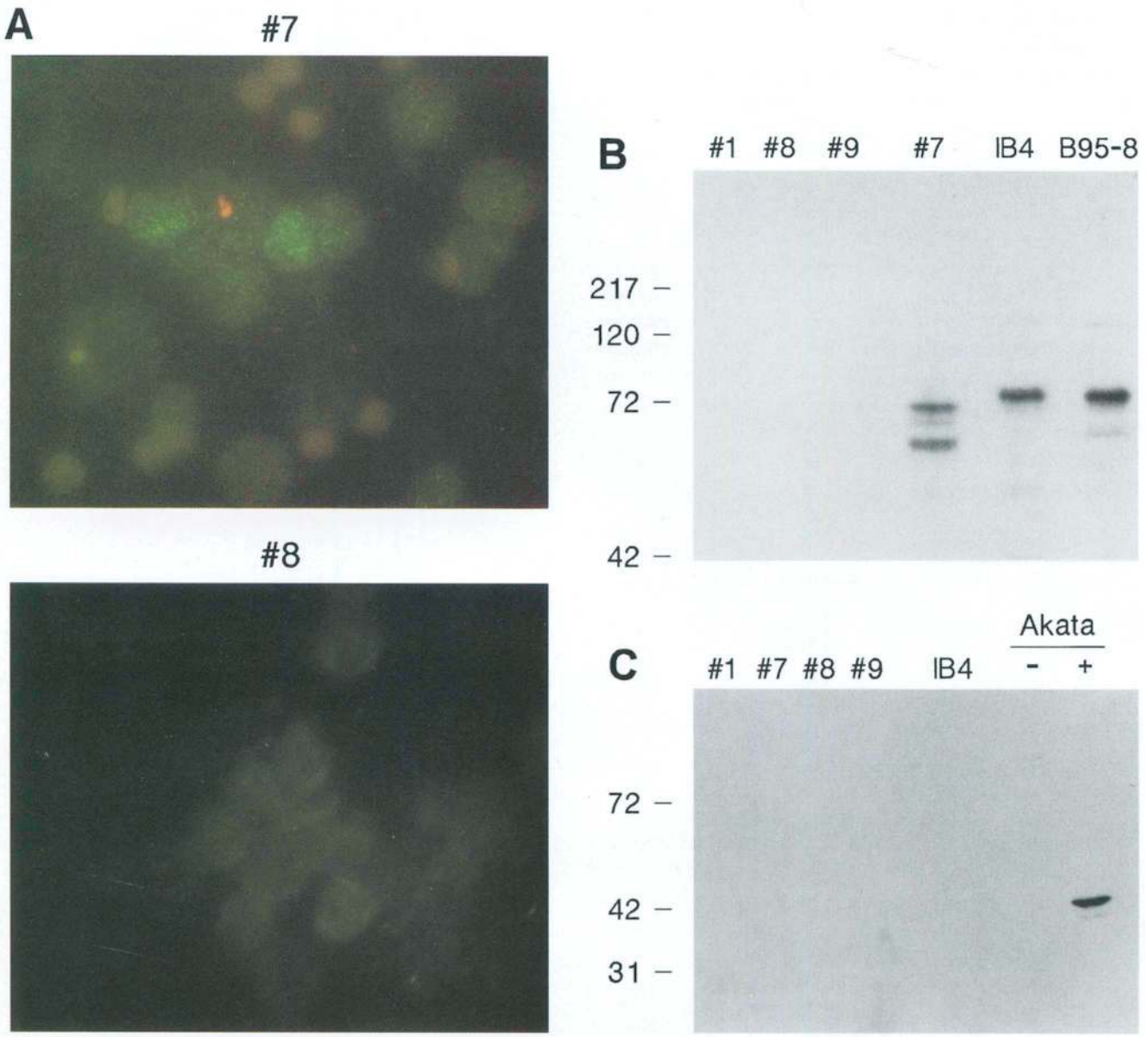


Figure 4. Lack of viral antigen expression in tumors with integrated, rearranged EBV DNA. Granular pattern of immunofluorescent staining characteristic of EBV nuclear antigen 1 (EBNA1) was detected with monoclonal antibody in tumor containing EBV episomes (**A**, tumor 7), not in tissue that retained the EBNA1-encoding *Bam*HI fragment as integrated DNA (tumor 8). Similarly, immunoblots probed with antibodies to EBNA1 (**B**) or BZLF1 (**C**) did not show proteins in tumors bearing incomplete EBV genomes (1, 8, 9). Double EBNA1 band (**B**, tumor 7) may represent partially degraded protein. IB4 and B95-8 are EBV-infected cell controls. Akata (−) is latently infected Burkitt's lymphoma cell line induced into lytic cycle (+) by treatment with anti-human immunoglobulin as described [8].

tion and intragenomic rearrangement [43, 47], as found in these tumors, may be patterned mechanistically after comparable cellular processes [48, 49].

The presence of WZhet EBV DNA without the prototype genome has not been previously observed in vitro [24, 25] or in vivo [19, 50, 51]. Components of WZhet DNA, visible on Southern blot, were confirmed by separate PCR techniques that amplified sequences in the BZLF1 ORF as well as across abnormally juxtaposed W and Z fragments. Presumably dependent on standard EBV for replication [52], the defective genome would not be maintained in cells lacking helper virus unless integrated, which the abnormally sized DNA bands (tumor 9, figure 1B) imply has occurred. Detection of the defective genome unaccompanied by standard EBV implies it is both infectious [52] and intrinsically pathogenic. Alternatively, intracellular generation of WZhet may have induced the loss of the parental virus, an interpretation consistent with the experimental reduction of EBV episomal copy number by transfection of the BZLF1 gene into latently infected cells [53]. Although we could not demon-

strate BZLF1 protein in the tumors examined, expression may have preceded integration.

The less-than-complete association of EBV with BL has made the virus's contribution to this disease unclear. Either factors other than EBV are responsible for tumorigenesis or, as our findings now suggest, EBV DNA may in some instances be lost after tumor initiation. Unlike other human DNA viruses, for which support for "hit-and-run" mechanisms of viral oncogenesis rests largely on negative evidence [5], EBV has both well-known oncogenic potential and an irregular association with tumors of common histologic type. Viral DNA footprints in a subset of BL tumors expands the scope of EBV participation in this disease and implies that EBV DNA recombination and loss, previously observed in cultured lymphocytes, may also operate in human disease processes.

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