An Epstein-Barr Virus Isolated from a Lymphoblastoid Cell Line Has a 16-Kilobase-Pair Deletion Which Includes gp350 and the Epstein-Barr Virus Nuclear Antigen 3A

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Epstein-Barr virus (EBV), a ubiquitous human gammaherpesvirus, has unique properties in activating resting B lymphocytes and transforming them into continuously proliferating lymphoblastoid cell lines (LCLs) (33, 35, 50). In these transformed cells, which resemble a phenotype of activated B cells by cognate antigen binding to the surface receptor (1, 34; A. B. Rickinson, Editorial, N. Engl. J. Med. 388:1461–1463, 1998), the episomes reside in the nuclei of cells as a latent episome and are actively engaged in continuous expression of a number of viral latent genes, thereby inducing and maintaining proliferation of transformed cells while presumably preventing them from terminal differentiation and apoptosis (4). These latent gene products include six nuclear antigens (EBNAs), three membrane antigens (LMPs), two small nonpolyadenylated RNAs (EBERs), and the BARF0 RNAs (9, 15, 20, 27, 28, 33, 40–42, 63–65). The continuous expression of these genes in LCLs suggests that many of them may play important roles not only in the initiation of the transforming process but also in the maintenance of the proliferative state of the transformed LCLs (33, 37, 51). In fact, biochemical and molecular genetic analyses involving recombinant EBV with mutations in the latent genes and further testing the ability of the resulting recombinant virus to transform primary B cells have demonstrated that EBNA1, EBNA2, EBNA3A, EBNA3C, EBNA-LP, and LMP1 are critical, whereas LMP2A and -2B, the EBERs, and EBNA3B are dispensable for EBV-induced B-cell transformation in vitro (9, 15, 19, 28, 33, 39, 41, 42, 63–65).

EBNA-1 may function in both the replication of viral episomes and the stable segregation and maintenance of viral episomes into daughter cells (68, 69). EBNA2 is a powerful transactivator of viral and cellular gene transcription (8, 67). This EBNA2-mediated transactivation drives the continuous expression of the EBV major latent genes and upregulation of cellular genes related to cell division and proliferation (5, 33, 35, 66, 67). LMP1 is essential for transformation of B lymphocytes and functions as an activated TNFR/CD40-like signaling receptor, constitutively transmitting activation signals in a ligand-independent manner (27, 44). In addition, blocking expression of LMP1 in LCLs by incubating with LMP1 antisense oligomers leads to apoptosis (4, 33). Moreover, cells transformed with a conditional EBV mutant for EBNA2 fused to an estrogen receptor were growth arrested in the absence of estrogen (31, 32). Of these essential genes, EBNA2 and LMP1 have been shown to be necessary for the initiation and maintenance of continuous LCL growth (9, 27). Further genetic analysis, which truncated EBNA3A and -3C proteins at amino acids 302 and 365, respectively, resulted in null recombinant EBV in terms of their ability to transform B lymphocytes (65).
Surprisingly, a separate report suggested that EBNA3A is not required for maintenance of the transformed phenotype (30). Mutants of EBV have been previously isolated containing deletions in essential genes required for virus infection, replication and transformation. Two of the strains, the P3HR-1 and Daudi strains, contain deletions in the critical transforming genes EBNA1, EBNA2 and EBNA2 (17, 36, 57, 60, 61, 71, 73). The B95-8 strain has a deletion of approximately 12 kb of viral DNA adjacent to the viral encoded DNA polymerase and gp100 open reading frames (ORFs) but is fully functional in terms of its ability to infect, replicate, and transform B lymphocytes (2, 13). The Raji strain has been characterized and has been shown to have two major deletions (3, 13, 26, 72): one in a region adjacent to the BARF1 oncogene and the BALF2 reading frame that encodes the single-stranded DNA-binding protein and another which lies within the EBNA3C gene critical for B-lymphocyte transformation (13). This strain is incompetent for transformation in addition to genome replication (12). However, it can be rescued for replication by providing the BALF2 cDNA in trans and also for transformation by rescuing the EBNA3C deletion by recombination, which incorporates the wild-type EBNA3C gene from an overlapping genomic DNA fragment (12, 54). This recombination event renders the resulting virus competent for B-lymphocyte transformation (54).

The two distinct types of EBV, I and II, share high sequence homology throughout their genomes, except for regions encoding EBNA2, -3A, -3B, and -3C, and also show small but type-related differences in EBER genes (2, 17, 59, 62, 70). Type-related genes easily characterized at the nucleotide or protein level have provided a means of type determination for EBV isolates. Here we report the identification of an EBV strain infecting a spontaneously derived LCL from a leukemia patient, with a deletion of a portion of its genome, which includes lytic and latent cycle-associated genes. Some of these genes, the gp350 glycoprotein, and the EBNA3A latent nuclear antigen are important for infection and transformation of human primary B lymphocytes. The identification of such an LCL, along with previous studies with recombinant EBV, suggests that EBNA3A may be critical but not essential in the early stages of transformation and may also be dispensable for maintenance of the transformation phenotype.

MATERIALS AND METHODS

Plasmids and cosmids. Plasmids pBS-EcoG2 and pBS-EcoF were constructed by cloning the B95-8 EcoRI G2 or F fragment derived from the EBV SalI C fragment into the EcoRI site of pBluescript SK(+) respectively. These constructs were used as a source of EcoRI G2 and F fragments as probes in Southern blot hybridization and analysis (SalI C cosmid was constructed by ligating the SalI C fragment of EBV into the SalI site of pDVCosA2 and packaging using the Stratagene Lambda gold packaging system. Cells and cell culture. B95-8 is a marmoset lymphoblastoid cell line immortalized by type I EBV (2). AG876 is a Burkitt's lymphoma (BL) cell line derived from an African BL and harbors the type 2 EBV (11, 17, 48, 49). BJAB is an EBV-negative BL cell line (49, 50, 52). SNU-265 and SNU-1103 LCLs are spontaneous EBV-transformed B-lymphocyte cell lines derived from non-EBV-related cancer patients and were described previously (10). All cell lines except SNU-265 were grown and maintained in RPMI 1640 medium (Gibco-BRL) with supplements of 2 mM L-glutamine, 5 μg of gentamicin (Gemini Bio-Products) per ml, and 10% heat-inactivated fetal bovine serum. SNU-265 was also grown in essentially the same medium described above with a 20% serum concentration.

Induction and PCR analysis of virus progeny. To determine whether SNU-265 is competent for virus replication and can produce progeny, we collected 5 million exponentially growing cells, which were centrifuged and resuspended in 5 ml of fresh medium containing phorbol ester at a concentration of 20 ng/ml of medium. Cells were further incubated for 4 days at 37°C with 5% CO2. The supernatant was then collected, and viral particles were spun down at 15,000 rpm for 30 min. The pellet was resuspended in 25 μl of 0.2 M phosphate-buffered saline (PBS), heated to 95°C for 15 min, and then switched to 56°C for 1 h with protease K treatment (10 mg/ml). The enzyme was then killed by treatment at 95°C for 30 min. A 5-μl portion of virus lysate was used for PCR amplification of the EBNA3C, region for 40 cycles, and standard protocols were followed using primers that can detect the type 1 or type 2 differences within the EBNA3C gene.

Southern blot analysis. DNA was prepared from 10 million cells using a modified Hirt fractionation method (8, 58). Briefly, cells were washed in PBS and lysed in 2 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA: 0.65% sodium dodecyl sulfate [SDS]). NaCl was added to a final concentration of 1.0 M, and the cell lysate was incubated overnight at 4°C to selectively precipitate high-molecular-weight, bulky nuclear DNA, which was then removed by a high-speed centrifugation (12,000 rpm) for 20 min at 4°C. DNA in the resulting Hirt supernatant was then extracted with phenol-chloroform (1:1) and chloroform-isoamyl alcohol (24:1), precipitated by adding 2 volumes of absolute ethanol, dried, and resuspended in 100 μl of Tris-EDTA (pH 7.5). For Southern blotting, 10 μg of each DNA sample was digested to completion with EcoRI or BamHI and then 5-μg aliquots were fractionated by electrophoresis through two 0.7% ME-agarose gels (53). DNA in each gel was blotted onto a nylon membrane (GeneScreen Plus; NEN-Dupont), which was hybridized with 32P-labeled probes prepared from either the HindIII E or the EcoRI G2 plus F DNA fragments of B95-8 EBV type I DNA. DNA labeling was done using [α-32P]dCTP (ICN) and the Prime-It II random labeling kit (Stratagene) according to the protocol provided by the manufacturer. The nylon membranes were washed using stringent conditions and exposed to X-ray film. For repropbing, the membranes were stripped as described previously and further hybridized with either the 4.0-kbp KpnI-KpnI or the 1.6-kbp KpnI-EcoRI fragment derived from EcoR1 G2 (2).

PCR and sequencing analyses. For amplification of sequences encompassing the SNU-265 deletion-specific junction, PCR was carried out using a pair of primers, 265DF1 (5′-79453 to 79472)-3′ and 265DR1 (5′-79475 to 79457)-3′, designed to target sequences flanking the junction. The reaction mixtures of a final volume of 50 μl were set up in 0.25-ml microtubes and contained 0.1 to 0.25 μl of Hirt-fractionated cell DNA, a 0.2 mM concentration of each dNTP, 100 pmol of each primer, 20 μM KCI, 1.5 mM MgCl2 and 0.5 μl of recombinant Taq polymerase. The tubes were subjected to heating at 95°C for 5 min and subsequently to 40 cycles, each composed of 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min, followed by further incubation at 72°C for 10 min, in a PTC-100 programmable thermal cycler (MJ Research, Inc.). Next, 5-μl aliquots of PCR products were assayed by electrophoresis on a 2.0% ME-agarose gel containing ethidium bromide (56). The PCR product was expected to have a BamHI site 85 bp away from its 5′ end and an EcoRI site at the very 3′ end that were introduced at the end of the EBV-specific primer sequences in the 265DR1 primer. For sequencing analysis, amplified DNA was digested with EcoRI and BamHI and ligated to the corresponding sites of pBluescript SK(+) to yield pBS-265D. Sequencing was carried out using T7 primer and the Thermo Sequenase radiolabeled (35S) terminator cycle sequencing kit (Amersham Life Sciences). Labeled products were then resolved on a 6% sequencing gel. The gel was dried on a sheet of Whatman 3 MM filter paper and subjected to autoradiography at 80°C. Based on the sequencing data, an EcoRI site was present in the insert, which was confirmed to be unique in the plasmid pBS-265D. To get deletion-junction sequences from the T7 primer, a deletion derivative, p265D-nNcoI/EcoRI, was made from pBS-265D by deleting its sequences between EcoRI and NcoI, blunting ends, and self-ligation. The nucleotide sequences obtained were analyzed by the Editseq program in DNASTAR and searched against the entire EBV type 1 sequence.

Western blot analysis. Cells (2 x 106) were harvested, washed with Dulbecco PBS (Gibco-BRL), and lysed in 400 μl of SDS loading buffer (114 mM Tris-HCl, pH 6.8, 4% SDS; 18.2% glycerol, 0.02% thiazolblu (pore-size) nitrocellulose membrane. The membrane was probed with a human polyclonal serum capable of detecting the repertoire of EBNA proteins expressed by EBV during latent infection, the S12 monoclonal antibody for the gp350-EBNA3A deletion (6, 43). The membrane was stripped as described previously and washed extensively in PBS with Tween 20 before being probed with the subsequent antibodies. Signals were detected by chemiluminescence using standard procedures (6).
Transformation of primary B lymphocytes by EBV. Virus supernatants from B95-8 derived LCL and SNU-265 were filtered through a 0.45-μm-pore-size sterile filter. Primary B lymphocytes were harvested by rosetting from peripheral blood mononuclear cells using a Lymphoprep gradient. The enriched B-lymphocyte population was washed and infected with virus supernatant at 5 million B lymphocytes per infection and plated at 50,000 cells in 150 μl of complete medium per well in a 96-well tissue culture plate. Phorbol ester-induced viral supernatants from B95-8 derived LCL and SNU-265 were used for the infections, and supernatant from BJAB-treated cells was used as a negative control. All 96-well plates were incubated at 36°C with 5% CO₂ for 4 to 8 weeks. The plates were treated once after 8 days of incubation with another 100 μl of complete medium.

RESULTS

PCR analyses suggest that the EBV genome maintained in the SNU-265 LCL contains a deletion in the region of EBNA3A and its adjacent ORFs encoding latent and lytic genes. Two distinct types of EBV strains maintain characteristic sequence differences in four EBV nuclear antigen genes (EBNA2, -3A, -3B, and -3C) (33, 59). The specific types of EBV isolated from patients can be determined by exploiting such type-specific differences in these EBNA genes either at the nucleotide level or at the amino acid level. During PCR analyses of a panel of EBV isolates from spontaneous LCLs derived from cancer patients to determine their genotypes, we found an EBV isolate, SNU-265, which was consistently negative for amplification of the EBNA3A gene despite positive amplifications for the EBNA2, -3B, and -3C genes (see Fig. 1 for the positions of the EBNA genes on the EBV genome). A representative of such PCR-mediated genotyping analyses using primers specific for each of type-distinct EBNA genes is shown in Fig. 2. All of the typing primers employed—E2-1 and E2-2 for the EBNA2 gene, A1 and A2 for the EBNA3A gene, B1 and B2 for the EBNA3B gene, and C1 and C2 for the EBNA3C gene (Table 1)—were derived from sequences which flank type-distinct re-

FIG. 1. Schematic illustration showing a linear EBV genome with the relative positions of the EBNA2, -3A, -3B, and -3C and gp350 genes. These genes were analyzed by PCR to determine the genotypes and deletion ends of the SNU-265 genome. The respective gene-specific primers employed are indicated over each ORF (denoted by open bars) by a combination of names and arrowheads. Vertical lines and filled bars indicate the BamHI recognition sites and the terminal repeats, respectively. Also shown are the replication origin of viral episome, OriP, and the position of the EBNA1 and LMP1 genes (2, 13, 14).

FIG. 2. PCR analyses of the EBNA2, -3A, -3B, and -3C genes in SNU-265 to determine the allelic types of each of these EBNA genes. Aliquots of cell DNA samples obtained from SNU-265, as well as control DNAs from B95–8, AG986, SNU-1103, and BJAB, were subjected to 40 cycles of PCR amplification, using primers that were specific for EBNA2 (E2-1–E2-2) (A), EBNA3B (B1-B2) (B), EBNA3A (A1-A2) (C), and EBNA3C (C1-C2) (D). For the primer sequences, see Table 1. Aliquots of PCR products were resolved on a 2.5% ME-agarose gel and visualized by ethidium bromide staining. Note that the SNU-265 genome is type 1 for EBNA2, -3B, and -3C and has no signal for the EBNA3A gene compared to the prototypic B95–8 type 1 genome and the AG876 prototypic type 2 genome. BJAB was used as an EBV-negative control in this assay.
regions in each EBNA gene and are shown to be conserved in both type 1 and type 2 viruses. As expected, B95-8, the prototypic type 1 strain, and SNU-1103, a type 1 strain isolated from a leukemia patient, yielded type 1-specific bands, respectively. AG876, the prototypic type 2 strain, yielded the type 2 allelic bands for all four EBNA genes analyzed. However, SNU-265 failed to give any PCR product (using the specific primer sets A1 and A2 for the EBNA3A gene), although it gave type 1-allelic bands for other EBNA genes (Fig. 2, compare panels A and D). The A1 and A2 specific amplification is shown in Fig. 2C.

One possibility for such a failure is that there could be sequence variations in the EBNA3A primer binding sites in SNU-265, thereby preventing the primers from engaging in efficient priming. To address this possibility, we prepared another set of EBNA3A-specific primers (A3 and A5, Table 1; see also Fig. 1), which flank the same EBNA3A type-specific region, as do the EBNA3A typing primers, but would target different type-conserved sequences outside those by the latter primer set. Amplifications with the new EBNA3A primers resulted in expected 378-bp type 1 and 350-bp type 2 bands from B95-8 and AG876, respectively, but once again failed to amplify SNU-265 (Fig. 3A and Table 1). Different combinations of these primers, such as A1-A5 and A3-A2, did give expected PCR products from B95-8 and AG876 but resulted in no bands with SNU-265 (data not shown), providing further support for the view that the failure in the EBNA3A amplification may be due to the deletion of the gene rather than to sequence alterations in SNU-265.

IB4 cells have four integrated EBV genomes per cell and have been used as a copy control for the EBV genome. To estimate the sensitivity of our PCR analysis, we then made serial 10-fold dilutions of 10⁴ IB4 cells with the same number of BJAB cells, and DNA preparations made from the undiluted or diluted IB4 cells were used as templates in the EBNA3A PCR using A3 and A5 primers. A faint signal where a single IB4 cell out of the background of 10⁴ BJAB cells was detected under our experimental conditions, which represents four copies of EBV out of 10⁴ cells (Fig. 3A showing ethidium bromide staining). The signal for 10 IB4 cells, which represents 40 copies of the genomes in DNA lysate from 10⁴ cells, however, is clearly seen. The blot was then transferred to GeneScreen nylon membrane and probed for the EcoRI K fragment of EBV, which includes EBNA3A. As expected, the specific band was detected representing the EBNA3A signal (Fig. 3B).

Thus, these results suggest that there is a possible deletion of the EBNA3A gene itself rather than inefficiency of the EBNA3A PCR amplification.

To examine the scope of the possible internal deletion in SNU-265, we expanded our PCR analyses to the regions immediately upstream or downstream of the EBNA3A gene, such as those encoding gp350 and the amino terminus of EBNA3B, respectively. Amplifications for the gp350 gene with two sets of gp350-specific primers (G2-G5 and G3-G4) resulted in the expected 1.4-kb PCR products from B95-8 but no PCR bands from SNU-265 (Fig. 1 and Table 1). Similar amplifications for the EBNA3B gene using the typing reverse primer (B2) and a forward primer targeting 944 bp (B5) or 514 bp (B7) upstream of the B1 binding site gave rise to PCR bands of expected sizes from B95-8 but no signal from SNU-265 (Fig. 1 and Table 1). Taken together, the PCR data were most consistent with the possibility that SNU-265 might have a substantial deletion of its genome, including not only the EBNA3A gene but also its neighboring genes such as gp350 and the amino-terminal region of the EBNA3B gene, with respect to the B95-8 prototypic type 1 virus.

Southern blot analyses confirmed a large deletion in SNU-265 localized between BamHI M and E regions relative to the

### Table 1. Primers used for analysis of the genomic region deleted in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Coordinates</th>
<th>Size (bp) of PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBNA2</td>
<td>E2-1</td>
<td>AGGCTGCCCCCACCTGTAGGAT</td>
<td>48170–48189</td>
<td>170 186 170</td>
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<tr>
<td></td>
<td>E2-2</td>
<td>GCCACCTGGCGACCTCTAAAG</td>
<td>48339–48320</td>
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<tr>
<td>EBNA3A</td>
<td>A1</td>
<td>GAAACCAAGACCAGGTCC</td>
<td>93596–93615</td>
<td>273 237 ND</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>TCCCAAGGCCCCAGAAATAGG</td>
<td>93871–93857</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td>CACCTTATGGCAACACCTAG</td>
<td>93566–93583</td>
<td>378 339 ND</td>
</tr>
<tr>
<td></td>
<td>A5</td>
<td>ACCTGTTCTACTGCGATG</td>
<td>93943–93926</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A6</td>
<td>GACAGACCTACAGGCAAGG</td>
<td>93627–93646</td>
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<tr>
<td>EBNA3B</td>
<td>B1</td>
<td>CCGTTGCGGATGGCAAGG</td>
<td>97333–97352</td>
<td>125 149 125</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>GCGCTGATATGGAATGTGCCC</td>
<td>97457–97438</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B5</td>
<td>TGACGACGAAAGTGGAGGC</td>
<td>96504–96523</td>
<td>944 NA ND</td>
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<tr>
<td></td>
<td>B7</td>
<td>GACGATGAAGCTAGGAGGCA</td>
<td>96964–96983</td>
<td>514 NA NA</td>
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<tr>
<td></td>
<td>B2</td>
<td>GGCTGATATGGAATGTGCCC</td>
<td>97457–97438</td>
<td></td>
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<tr>
<td>EBNA3C</td>
<td>C1</td>
<td>AGAAGGGAGCGCTGTTG</td>
<td>99939–99958</td>
<td>153 246 153</td>
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<tr>
<td></td>
<td>C2</td>
<td>GGCTGCTTGGTGTGAGTCGCGC</td>
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<td>gp350</td>
<td>G2</td>
<td>AAGGTACCGGTGCGAGCAATGAGGACAG</td>
<td>92164–92144</td>
<td>1,433 ND ND</td>
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<tr>
<td></td>
<td>G5</td>
<td>GGTTGACATCGCGCTGGGATAC</td>
<td>90732–90752</td>
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<tr>
<td></td>
<td>G3</td>
<td>TTTCTAGACTTTATACATAGGTGTCGTGCTACTGATA</td>
<td>89426–89456</td>
<td>1,327 NT ND</td>
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<td>G4</td>
<td>GTATCCACGCCGGTGGATGTCACC</td>
<td>90752–90732</td>
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* ND, not detected; NA, not applicable; NT, not tested.
To explore and confirm possible deletions of the EBNA3A gene and other adjacent genes in SNU-265, we carried out Southern blot analyses, using two nonoverlapping probes which were derived from the B95-8 EBV SalI C fragment (Fig. 4A). The two probes, designated EcoRI G2+F and HindIII E, consisting of either an equimolar mixture of the EcoRI G2 and F fragments or the HindIII E fragment, respectively, allowed us to screen a 26.3-kb contiguous stretch (B95-8 positions 76596 to 102891) of the EBV genome (2) except for a small gap between the two probes representing the first 400 bp of EcoRI N. The regions covered by the probes thus contain not only the EBNA3 family of genes but also a number of ORFs involved in the lytic replication cycle, such as BLLF1a and -1b (gp350/220), BMRF1, and BSLF1. Cell DNA preparations enriched for EBV episomes were made from SNU-265, SNU-1103, B95-8, and BJAB cells by Hirt fractionation and were used to prepare two duplicate GeneScreen nylon membranes (NEN-Dupont). Each filter contained pairs of 5 μg of each cell DNA preparation per lane, which had been digested with EcoRI or BamHI, respectively, and fractionated through a 0.7% agarose gel. The filters were then hybridized with the 32P-labeled EcoRI G2+F or HindIII E probe and were processed for autoradiography (Fig. 4). Results obtained with the HindE probe detected all expected bands for EcoRI fragments N, M, K, and B and also those for BamHI fragments L, E, e1, e2, e3, and Z from B95-8, as expected (Fig. 4C). The bands for EcoRI N and BamHI e1 fragments became more prominent after a longer period of exposure (data not shown). A faint 9.0-kb EcoRI band was also detected at a lower intensity, suggesting partial digestion of the EBV genomic DNA.

Similar EcoRI and BamHI restriction patterns were also obtained with SNU-1103, a spontaneous LCL carrying a type 1 EBV isolate, except for the BamHI Z band, which ran as 2.1 kb, unlike the B95-8 counterpart of 1.7 kb. Band intensities of SNU-1103 and SNU-265 were much weaker than those of B95-8 counterparts, presumably reflecting lower copy numbers of their episomal genomes loaded on the membrane compared to those of B95-8. Densitometric analyses indicated that at least 10-fold more B95-8 compared to SNU-265 or SNU-1103 copy numbers of viral episomes were represented on the nylon membrane. In addition, the expected bands were detected from SNU-1103, demonstrating the integrity of the employed probes and procedures and of the DNA isolation procedures used in the Southern analysis.

In contrast, however, with the SNU-265 EcoRI digest, the Hind E probe detected only a single EcoRI band (marked by a dot), which was slightly larger than the 30-kb EcoRI B of B95-8 and SNU-1103 (Fig. 4C). No other bands for EcoRI N, M, and K fragments were detected even upon a longer period of exposure in the corresponding cell line, indicating that these three consecutive EcoRI fragments were deleted in SNU-265. The absence of these EcoRI fragments was thus consistent with our failure in the amplification of the EBNA3A and the amino-terminal sequence of gp350 from SNU-265 and was further confirmed by the lack of both BamHI L and E bands from its BamHI digest (Fig. 4C), which overlap with these EcoRI fragments. The same probe, however, could detect a 1.7-kb BamHI Z band in B95-8 and a novel 4.4-kb BamHI band (marked by an asterisk) in SNU-265 (Fig. 4C). Upon a longer exposure, the BamHI e1 to e3 bands could also be detected in SNU-265, as well as in SNU-1103 (data not shown). Given that SNU-265 yielded type 1-allele-specific EBNA3B and -3C bands in geno-
typing analyses (Fig. 2), these Southern blot data clearly indicated that SNU-265 lacks EcoRI N, M, and K fragments and carries partially deleted EcoRI B and BamHI E fragments. Thus, both the larger >30-kb EcoRI and 4.4-kb BamHI bands detected in SNU-265 represent novel bands that may be specific for the deletion. With the EcoRI G2+F probe, we could readily detect not only bands for EcoRI fragments F and G2 but also bands for BamHI fragments O, a, M, S, and L from B95-8 and also from SNU-1103 (Fig. 4B). The intensities of the bands for SNU-1103 and SNU-265 were again much weaker than those for B95-8, presumably due to the relatively lower copy numbers of viral genomes loaded. However, the corresponding signals from B95-8 are all seen in SNU-1103 when the membrane was exposed for a longer period to X-ray film.

**FIG. 4.** Southern blotting analysis of the SNU-265 genome using the HindIII E or EcoRI G2+F fragments as probes to determine the position of the SNU-265 deletion. (A) Map of the genomic region analyzed, indicating the fragments generated by EcoRI digestion (top) and those generated by BamHI digestion (bottom) from a region of the EBV genome cloned as a SalI C fragment (75,601- to 105,296-bp EBV coordinates based on the B95-8 genome). The solid lines above show the EcoRI G2+F (76,596- to 91,421-bp) and HindE (91,821- to 102,891-bp) fragments used as 32P-labeled probes in panels B and C. (B) Results of Southern blot analysis of the SNU-265 genome digested with EcoRI (R) and BamHI (B) probed with 32P-labeled EcoRI G2+F fragments. The fragments shown on the left of the panel are the BamHI fragments detected and those on the right of the bands on the panel are the EcoRI fragments. In panel B, the leftmost panel is a shorter exposure of the blot to clearly show the B95–8 bands used as controls in this analysis. The right panel is a longer exposure to detect the EBV genomes in the SNU-265 and SNU-1103 with a smaller number of EBV genome copies. The closed circle in the EcoRI digest of SNU-265 and the asterisk in the BamHI digest of SNU-265 indicate the new fusion bands created by the deletion, respectively. Note that the EcoRI G2+F fragments and the BamHI L, O, and S fragments are missing in SNU-265. The BamHI a fragment is detected. Analysis using the Hind E probe also shows the fusion fragments on EcoRI and BamHI digestion, suggesting fusion of the EcoRI B fragment and the BamHI E fragment, respectively, with the left end in the BamHI a region (53).
Interestingly, probe EcoRI G2+F detected the same larger 30-kb EcoRI band and the 4.4-kb BamHI bands from SNU-265, as did the Hind E probe (Fig. 4, compare panels B and C). Hence, the detection of both bands by these two nonoverlapping probes provides strong evidence that these two bands were indeed novel ones, presumably derived from a region specific for the SNU-265 deletion, and thus may contain the deletion-specific junction containing EBV DNA from the EcoRI G2 region and the BamHI E region. In addition to these bands, the probe could detect a 1.7-kb BamHI a band but no bands for BamHI M, S, and L fragments, indicating that the novel larger 30-kb EcoRI and 4.4-kb BamHI bands were detected by the EcoRI G2 component of the probe. The absence of the BamHI L band was consistent with our inability to PCR amplify the gp350 gene. Taken together, the Southern blot data indicate that SNU-265 lacks BamHI S and L (or EcoRI F) fragments and has the BamHI M (or EcoRI G2), as well as the BamHI E (or EcoRI B), fragment partially deleted. Therefore, the 5′ end of the SNU-265 deletion should lie within EcoRI G2 or, more precisely, within the region overlapping BamHI M and to the right within the EcoRI B, more specifically, the BamHI E, fragment (Fig. 4A).

To demonstrate that these novel larger 30-kb EcoRI and 4.4-kb BamHI bands are the deletion junction-containing fragments carrying a region of BamHI M as the 5′ component of the junction and also to more precisely map the 5′ endpoint of the deletion, we stripped off the bound probes on both membranes and reprobed them with either of the two leftmost KpnI subfragments (Fig. 5A) of EcoRI G2 (the 4.0-kb KpnI/KpnI and 1.6-kb KpnI/EcoRI fragments). The 4.0-kb probe hybridized to both novel bands (indicated on the gel by the dot and the asterisk), as well as to the 1.7-kb BamHI a band in SNU-265 (Fig. 5B), whereas the 1.6-kb probe did not give any signal with SNU-265 though it could detect the EcoRI G2 and BamHI M bands of B95-8 and SNU-1103 (Fig. 5C). These results therefore unambiguously proved that the two novel bands are most likely derived from the deletion junction and indicate that the 5′ end of the SNU-265 deletion should lie within the first 1.8 kb of BamHI M.

The present Southern blot data obtained by the use of the two long nonoverlapping probes clearly indicated that four EcoRI fragments, F, N, M, and K, have been completely deleted and that EcoRI G2 and B have been partially deleted in SNU-265, presumably leading to the generation of the large 30-kb EcoRI fusion band. Alternatively, BamHI S and L have been completely deleted and BamHI M and E have been partially deleted in SNU-265, thereby giving rise to the 4.4-kb BamHI fusion fragment.

Analysis of the fusion junction revealed that a number of critical EBV genes, including EBNA3A and gp350, have been deleted in SNU-265. To amplify the SNU-265 deletion-specific junction, we decided to PCR amplify, clone, and sequence the junction to precisely determine the deletion and map the endpoints which created the fusion. In designing SNU-265 deletion-specific primers, we took advantage of the fact that this virus carried an intact BamHI a fragment and that a portion of its EBNA3B sequence could be amplified. A forward primer, 265DF1, was designed to target a sequence within BamHI a, which was 85 bp away from the 3′ BamHI site of BamHI a, and the EBNA3B typing reverse primer was chosen as a reverse primer, 265RF1, with an EcoRI recognition site at its 5′ end (Fig. 6A). Thus, while targets of these two primers would be separated by 18 kb from each other in wild-type viruses such as B95-8, they are separated by only 1.3 kb in an internal-deletion-containing virus such as SNU-265 (Fig. 6B). Indeed, amplifications with these primers resulted in the approximately 1.3-kb PCR band from SNU-265 but not from B95-8 and BJAB, an EBV-negative cell line (Fig. 6B).

For sequencing analysis, the 1.3-kb PCR product was then digested with BamHI and EcoRI, and the resulting 1.2-kb DNA was cloned in the corresponding sites of pBluescript SK(+) to obtain pBS-265D. Restriction digestion analyses showed several enzyme recognition sites, which appeared to be unique in the insert and the plasmid. The sites included a PstI site and an NcoI site, presumably derived from the BMRF1 and the EBNA3B genes, respectively (Fig. 7B). Sequencing analysis confirmed the presence of the EBNA3B NcoI site, which was about 240 bp away from its EcoRI end. In order to target the deletion junction, a construct with a deletion at the Ncol site, p265D-dNco/EcoRI, was made from pBS-265D by removing sequences between NcoI and EcoRI and religating the blunted ends. Subsequent sequencing of p265D-dNco/EcoRI (Fig. 7A) and analysis of determined nucleotide sequences (Fig. 7B) revealed that the SNU-265 deletion lies between position 80299 in the BMRF1 gene and position 96998 in the EBNA3B gene with respect to the EBV coordinates derived from the B95-8 sequence. This deletion eliminates a 16.7-kbp portion of EBV DNA. This deletion created an out-of-frame fusion between the BMRF1 codon 134 and the EBNA3B codon 523, which would produce a predicted polypeptide of 155 amino acids with the BMRF1 amino-terminal 134 amino acids and a novel 21-amino-acid carboxy-terminal tail from the EBNA3B protein sequence. These results thus confirmed that SNU-265 lacks all genes or ORFs located between the BMRF1 gene and EBNA3B and includes the EBNA3A and gp350/220 genes (Table 2).

Western blot analysis indicates that the major essential latent antigens, including the EBNA3C protein but not EBNA3A and -3B, are expressed in SNU-265. The Southern blot analyses described above clearly demonstrated that SNU-265 contains a large deletion of its genome, including the regions coding for EBNA3A and the amino-terminal ~300-amino-acid portion of EBNA3B. To determine which of the EBNA3 molecules is expressed in SNU-265, we performed Western blots for detection of all the type 1 EBNA3 molecules. Lysates from SNU-265, as well as control cell lines expressing the EBNA3s and an EBV-negative B cell line, BJAB, were fractionated and transferred to nitrocellulose membranes. The results clearly demonstrate that a single band which migrated in the region similar to that of the EBNA3 proteins was detected by a human polyclonal serum with reactivity to the three EBNA3 proteins (Fig. 8A). The polypeptide migrated slightly above that of the B95-8 EBNA3C band, indicating a variation in the EBNA3 proteins in this type 1 EBV strain. The EBNA3 family members are known for their highly repetitive nature, and it is possible that the slower mobility is due to the increased size of the repetitive region of the EBNA3C polypeptide. Since it was determined that EBNA3A and EBNA3B were affected by the deletion, the detected band was most likely due to EBNA3C expression. To confirm that this band is
in fact EBNA3C, the membrane was stripped and reprobed with a monoclonal antibody to specifically detect EBNA3C (43). Again, the results indicate that this larger migrating band was EBNA3C (Fig. 8B). Other experiments using antibody against EBNA3A did not reveal any signal in SNU-265 (data not shown). Therefore, these data strongly support the previous Southern blot analyses, which demonstrated that the EBNA3A and EBNA3B polypeptides were affected by the deletion.

Since it was expected that EBNA3s regulate the expression of other latent genes from the major latent promoters, we wanted to determine if LMP1 expression was altered. As described above, equivalent amounts of lysates were fractionated and transferred to membrane and further blotted for LMP1 signal using a monoclonal antibody that specifically recognizes LMP1. LMP1 was clearly expressed in all EBV-positive cell lines. However, we did observe a consistently increased amount of LMP1 in SNU-265. In our Southern blot analysis we estimated, based on densitometric readings, that there were approximately 10-fold more copies of EBV genomes in B95-8

FIG. 5. Southern bloting assay of SNU-265 using KpnI subfragments of EcoRI G2 as probes to roughly map the 5′ endpoint of the deletion. (A) The nylon membrane used in Fig. 4 was stripped and reprobed with subfragments in the EcoRI G2 region. (B) Southern blot with the 4-kb KpnI fragment showing the BamHI O and a fragments but not BamHI M, which migrates above BamHI O, indicated by the asterisk. The solid circle in the EcoRI-digested lane suggests a fusion of the EcoRI G2 and B fragments creating a larger migrating band. (C) Southern blot using a probe of 1.6 kb from the BamHI M fragment. As shown, no signal was seen in the SNU-265 EcoRI- and BamHI-digested lanes. However, the EcoRI G2 and BamHI M fragments are clearly seen in the B95-8 and SNU-1103 control lanes. These results indicate that the region on BamHI M is deleted in the SNU-265 genome.
compared to SNU-265. However, by Western blot with equivalent amounts of lysate loaded, approximately twofold more LMP1 signal was consistently seen in SNU-265. This suggests a potential dysregulation of LMP1 expression, which may in part be due to the absence of two major latent proteins EBNA3A and -3B, which were deleted from the SNU-265 genome.

Treatment of SNU-265 with phorbol esters results in no observable induction of progeny virus. To determine if SNU-265 with an approximately 16-kbp deletion was capable of efficiently producing viral progeny and transforming primary B lymphocytes, we treated SNU-265 cells with phorbol esters, which induce EBV replication. Supernatants from the induced cells were filtered through a 0.45-μm (pore size) filter and used for infection of fresh human primary B lymphocytes. PCR analysis of the supernatant did not show any detectable signal for EBV when amplified at the EBNA3C loci (Fig. 9B). Uninduced cells were also analyzed to show that we can in fact amplify the DNA. Figure 9A shows that EBNA3C was detected in both SNU-265 and the B95-8-derived LCL, with both analyses done in duplicate. However, compared with the LCL, SNU-265 had no signal of virus production after treatment with phorbol ester after 40 cycles (Fig. 9B), suggesting that the SNU-265 did not efficiently produce any detectable virus progeny by our PCR assay.

To further test whether or not any virus progeny was produced from SNU-265 and were able to infect primary B lymphocytes, we used the remaining supernatant to infect fresh primary B lymphocytes. As expected, no transformed LCLs were seen after 8 weeks in culture in three separate experiments (Table 3) compared to the results from the infection with the positive control, wherein LCLs were observed as soon as 4 weeks in culture. These results suggest that SNU-265 is not capable of efficiently producing infectious competent virus progeny.

**DISCUSSION**

A number of naturally occurring mutant EBV genomes with deletions in a number of documented regions have been previously isolated from EBV-infected cell lines (3, 17, 26, 57, 62). Some of these deletions occur in regions encoding genes critical for the replication, infection, and transformation of primary B lymphocytes (7, 12, 16, 22–25, 47, 55). In this study we have identified an EBV genome infecting an LCL isolated...
from a leukemia patient (38) that has a deletion in a major region of the genome encoding the gp350 gene product, the EBV glycoprotein important for targeting the CD21 cellular receptor (45). Another major latent gene previously known to be critical for EBV-mediated B-lymphocyte transformation, EBNA3A, was also deleted in this particular strain of EBV (30).

One commonly known EBV deletion mutant, lacking a 12-kb region (genomic position 152012 EBV coordinates), is fully competent for replication and transformation (2, 47). This B95-8 genome has been fully sequenced, and the junction of the deletion has been identified (47). The reading frames encoded within this deletion may be involved in the regulation of lytic reactivation of EBV by antisense transcription, which may hinder transcription of the essential lytic genes, including BALF2, BALF4, and BALF5. Two other mutants, Daudi and P3HR-1, lack a major transforming region of the genome, which encodes the EBNALP and EBNA2 (13, 14). The dele-

<table>
<thead>
<tr>
<th>ORF</th>
<th>Coordinates</th>
<th>Protein and/or potential function(s)*</th>
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</tr>
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<tbody>
<tr>
<td>BMRF1</td>
<td>79899–81113</td>
<td>Diffuse early antigen, BALF5 DNA polymerase accessory protein, and a transactivator for the early BHLF1 promoter</td>
<td>CT truncated at codon 134</td>
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<tr>
<td>BMRF2</td>
<td>81118–82191</td>
<td>Early reading frame</td>
<td>Del</td>
</tr>
<tr>
<td>BMLF1</td>
<td>82743–84059</td>
<td>Diffuse early antigen; homologous to RF4 VZV and IE63 HSV</td>
<td>Del</td>
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<tr>
<td>BSLF2</td>
<td>84229–84288</td>
<td>Early reading frame; BSLF2+BMLF1 is also called EB2</td>
<td>Del</td>
</tr>
<tr>
<td>BSLF1</td>
<td>84257–86881</td>
<td>Primase; with BBLF4 and BBLF2/3 forms a helicase-primase complex</td>
<td>Del</td>
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<tr>
<td>BSRF1</td>
<td>86924–87580</td>
<td>Late reading frame</td>
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</tr>
<tr>
<td>BLLF3</td>
<td>87638–88474</td>
<td>Early reading frame; homologous to RF8 (VZV) and dUTPase (HSV)</td>
<td>Del</td>
</tr>
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<td>BLRF1</td>
<td>88547–88855</td>
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<td>Del</td>
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<td>88925–89413</td>
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</tr>
<tr>
<td>BLLF2a and BLLF1b</td>
<td>89430–92153</td>
<td>gp350/220 major envelope glycoprotein, serving as a viral ligand for the cell receptor CD21</td>
<td>Del</td>
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<tr>
<td>BLLF2</td>
<td>89567–90013</td>
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<td>Del</td>
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<td>BLRF2 and BERF1</td>
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<td>EBNA3A; essential for the initiation of B-cell transformation</td>
<td>Del</td>
</tr>
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<td>BERF2a and BERF2b</td>
<td>95353–95709, 95788–98247</td>
<td>EBNA3B; dispensable for B-cell transformation</td>
<td>NT truncated at codon 523</td>
</tr>
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* VZV, varicella-zoster virus; HSV, herpes simplex virus.

b Del, deleted; CT, carboxy terminus; NT, amino terminus.
tion in Daudi and P3HR-1 renders these viruses incapable of transforming human B lymphocytes (22, 25). However, they can efficiently infect B lymphocytes and be stably maintained as episomal DNA in the infected B-cell lines (34). Another strain, Raji, contains two major deletions in its genome which render the virus incompetent for transformation, as well as for replication (16). Ooka et al. have demonstrated that this genome can be rescued for replication through the transient or stable expression of the single-stranded DNA-binding protein encoded by the EBV ORF BALF2 (12, 46). Raji virus progeny was produced and shown to infect B lymphocytes in vitro (12). Additionally, further experiments which utilized BALF2 stably transformed Raji cells were able to rescue the EBNA3C deletion (54). This approach took advantage of a cosmid clone, which overlaps the region deleted in EBNA3C recombining with the parental Raji genome to create a recombinant Raji virus with wild-type EBNA3C. This indicated that competent virus could be produced for the transformation of B lymphocytes, leading to continually proliferating LCLs. However, this process was less efficient than the well-utilized P3HR-1 system, wherein the EBNALP and EBNA2 deletions are rescued by recombinant events using the EcoRI A cosmid overlapping this region of the genome (54).

Recent developments with bacterial artificial chromosomes in an F plasmid-based system now introduce another technology for mutagenesis of the EBV genome in Escherichia coli, which can then be tested in B-lymphocyte transformation assays (29, 30). One such minichromosome created picked up a mutation in the EBNA3A gene indicating that the loss of EBNA3A expression did not affect the maintenance of the LCLs generated in long-term growth of the culture (30). How-

![FIG. 8. Western blot analysis to determine the major latent gene products expressed by the SNU-265 LCL. (A) Western blot of cell lysates from SNU-265, two EBV-positive cell lines (B95-8 and LCL1), and an EBV-negative control cell line using a human polyclonal serum which recognizes all of the major EBNA proteins for the type 1 EBV strains. The lines on the right indicates the positions of the EBNA3 proteins migrating approximately 160 kDa and the EBNA2 and EBNA1 signals, which are approximately 80 to 90 kDa. (B) Same blot as in panel A but stripped and reprobed with EBNA3C-specific monoclonal antibody A10. This blot indicates that the signal expressed in the SNU-265 cell line is an EBNA3C-specific signal. (C) Further stripping and reprobing of the blot with another monoclonal antibody specific for LMP1, S12, shows the expression of LMP1 in SNU-265.]

![FIG. 9. PCR analysis of phorbol ester-treated SNU-265 shows no observable induction in virus production. (A) PCR analysis from uninduced DNA lysates prepared from SNU-265, an LCL as a positive control, and BJAB as a negative control prepared from 50,000 cells. (B) PCR analysis of the virus progeny produced from the same cells as in panel A but induced with phorbol ester for 4 days. No signal was seen with SNU-265, whereas a signal was seen from both LCL lanes with the parental Raji genome.]

<table>
<thead>
<tr>
<th>Expt</th>
<th>Transformation of B lymphocytes (no. of colonies)</th>
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<tr>
<td></td>
<td>BJAB</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
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* Result from in vitro transformation experiments as indicated after plating of 50,000 primary B lymphocytes infected with a B95-8-derived LCL and incubation for 30 days in culture at 37°C with 5% CO₂.

* Result from in vitro transformation experiments as indicated after plating of 50,000 primary B lymphocytes infected with supernatant from tetradecanoyl phorbol acetate-induced SNU-265 and incubated for 60 days in culture at 37°C with 5% CO₂.
ever, other studies by Tomkinson et al. have shown that insertion of a stop codon at position 302 in the EBNA3A reading frame rendered the recombinant virus incompetent for the transformation of human B lymphocytes (65). Moreover, the LCLs generated as coinfected cell lines did not survive long term in culture, suggesting a potential dominant-negative effect (65). This indicates that EBNA3A is important for EBV-mediated transformation of primary B lymphocytes. These experiments were clearly not conclusive and further experiments will be needed to determine the importance of EBNA3A in the transformation event. Other developments have indicated that gp350 required for binding to the cellular receptor CD21 can be deleted and that the resulting recombining virus infects and transforms primary B lymphocytes in vitro, albeit at a lower transforming frequency compared to a prototypic type 1 EBV (21).

Our finding, which maps a deletion in an EBV genome infecting an LCL derived from a leukemia patient (38), is a rare example of an EBV genome lacking major latent (as well as a lytic) EBV genes. Other genomes identified with deletions in essential lytic and latent genes have been identified from BLs but not from spontaneous LCLs derived from patients. Although it is expected that spontaneous deletions may occur in the EBV genome, it is unlikely that such deletions would occur prior to injection of the B cell. In Raji cells, for example, the virus is null for replication competency, which can be rescued by introduction of the essential gene product, BALF2, in trans (12, 16). Therefore, this virus would not be capable of propagating itself through replication in the host without complementation of the deleted essential lytic gene. Hence, the deletion probably occurred postinfection, and the resulting deleted episome was stably maintained in the infected cell.

In the case of the SNU-265 deletion, the genes are critical for replication and transformation. However, some of these genes may not be essential, and the functions may be complemented in trans through the addition of growth factors or by plating the infected cells on feeder cultures, which would provide essential factors in culture. The deletion of the gp350 gene and the subsequent infection and transformation of primary B cells in vitro suggests that this is possible, although the efficiency is expected to be extremely low. Further experiments are needed to determine if the infection and transformation properties of this SNU-265 virus would provide some clues as to the essential nature of these deleted genes. We are currently investigating this line of experimentation to evaluate this possibility and its potential for the development of another cell line for recombination assays within the EBNA3 family of genes.

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REFERENCES

palindrome of EBV (P3HR-1) heterogeneous DNA. J. Virol. 61:1495–506. (Erratum, 61:2950.)


42. Jones, M. D., L. Foster, T. Shedy, and B. E. Griffin. 1984. The EB virus genome in Daudi Burkitt’s lymphoma cells has a deletion similar to that observed in a non-transforming strain (P3HR-1) of the virus. EMBO J. 3:1513–1521.


