JOURNAL OF VIROLOGY, May 1992, p. 2893–2903
0022-538X/92/052893-11$02.00/0

Vol. 66, No. 5

Use of Second-Site Homologous Recombination To Demonstrate That Epstein-Barr Virus Nuclear Protein 3B Is Not Important for Lymphocyte Infection or Growth Transformation In Vitro

BLAKE TOMKINSON* AND ELLIOTT KIEFF

Department of Microbiology and Molecular Genetics and Medicine, Harvard University, 75 Francis Street, Boston, Massachusetts 02115

Received 28 October 1991/Accepted 3 February 1992

Recombinant Epstein-Barr viruses with a stop codon inserted into the nuclear protein 3B (EBNA 3B) open reading frame were generated by second-site homologous recombination. These mutant viruses infected and growth transformed primary B lymphocytes, resulting in the establishment of lymphoblastoid cell lines (LCLs). Polymerase chain reaction analysis and Southern hybridizations with infected cell DNA demonstrated the presence of the mutant EBNA 3B and the absence of wild-type EBNA 3B. Immunoblot analysis of the LCLs with affinity-purified EBNA 3B antibodies confirmed the absence of EBNA 3B cross-reactive protein. Virus was reactivated from two of these infected LCLs and serially passaged through primary B lymphocytes. The newly infected cells contained only the mutant recombinant virus. No difference was noted between mutant and wild-type recombinants, derived in parallel, in latent (other than EBNA 3B) or lytic cycle-infected cell virus protein expression or in the growth of the latently infected transformed cell lines. These data indicate that the EBNA 3B protein is not critical for primary B-lymphocyte infection, growth transformation, or lytic virus infection in vitro.

Epstein-Barr Virus (EBV) infection of B lymphocytes is primarily latent, causing cell activation and growth transformation (17, 45). The resulting transformed lymphoblastoid cell lines (LCLs) are similar to antigen-activated primary B lymphocytes except for their continued proliferation (3, 14, 34, 57, 60). Although EBV has 80 to 100 genes, only 10 genes are expressed in latent infection (reviewed in references 24 and 25). These are likely to be involved in the initiation or maintenance of latent virus infection or the concomitant B-lymphocyte growth transformation. Six virus genes expressed in latent infection encode nuclear proteins (EBNA) EBNA 1, EBNA 2, EBNA 3A, EBNA 3B, EBNA 3C, and EBNA LP. Two genes encode integral membrane proteins LMP 1 and LMP 2, and two genes encode EBV-encoded nonpolyadenylated small RNAs (EBERS).

Recent advances in EBV recombinant molecular genetics have made possible the assessment of the importance of specific EBV genes in virus infection and cell growth transformation (7, 8, 15, 32, 33, 56). One approach is to transfect an EBV-infected cell line with an EBV DNA fragment and to induce virus replication so that the replicating endogenous genome can undergo homologous recombination with the transfected DNA (7, 8, 15, 32, 33, 56). When P3HR-1 cells, whose resident EBV genome contains a single deletion involving EBNA LP and EBNA 2 (4, 11, 16, 18, 21, 26, 46, 47) rendering it unable to transform B lymphocytes (35, 38), were transfected with cloned EBV DNA which spanned the deletion, recombinants arose which were restored for the deleted DNA and for the ability to transform primary B lymphocytes (7, 8, 15). Since mutations within the EBNA 2 or EBNA LP open reading frames altered the ability of the transfected DNA to restore transformation, the critical role of EBNA LP and the essential role of EBNA 2 in transformation could be established (7, 8, 15, 32). This strategy is centered around the P3HR-1 deletion and was therefore initially limited in application to the construction of EBNA LB or EBNA 2 mutants. Subsequent experiments demonstrated that this strategy could be extended to genes which map near the P3HR-1 deletion and which could therefore be included in a large cosmid DNA fragment which spanned the deletion (33, 56). Since there was strong positive selection for the P3HR-1-deleted DNA, linked mutations in adjacent genes which did not adversely affect transformation could be recovered in the resultant LCLs. Using this latter strategy, recombinant mutant EBVs were constructed which were deleted for the EBERs (56). Despite the 40-kb distance between the EBERs and the P3HR-1 deletion, 15% of all recombinants which had acquired the EBNA LP and EBNA 2 exons from the transfected DNA and were therefore transformation competent had also acquired the linked EBER mutation (56). Surprisingly, these EBER-deleted recombinant viruses did not differ from wild-type control recombinant viruses in their ability to infect, growth transform, or replicate in B lymphocytes (56). On the basis of these experiments, the number of EBV genes potentially necessary for lymphocyte growth transformation was reduced from 10 to 8.

The high frequency (15%) of coincorporation of the EBER mutation and the EBNA LP- and EBNA 2-encoding DNAs, which are necessary to restore P3HR-1's deletion and a transforming phenotype, was likely to be due to the physical linkage in the transfected cosmid between the EBER and the EBNA LP- and EBNA 2-encoding DNAs. However, the EBERs are separated from the deleted EBNA LP- and EBNA 2-encoding DNA by multiple copies of a 3-kb DNA element which was likely to be highly recombinogenic. We therefore began to consider the possibility that the incorporation of the EBER mutation into P3HR-1 genomes which were engaged in recombination with the EBNA LP and EBNA 2 genes might not be dependent on the physical linkage between the EBERs and EBNA LP- and EBNA

* Corresponding author.

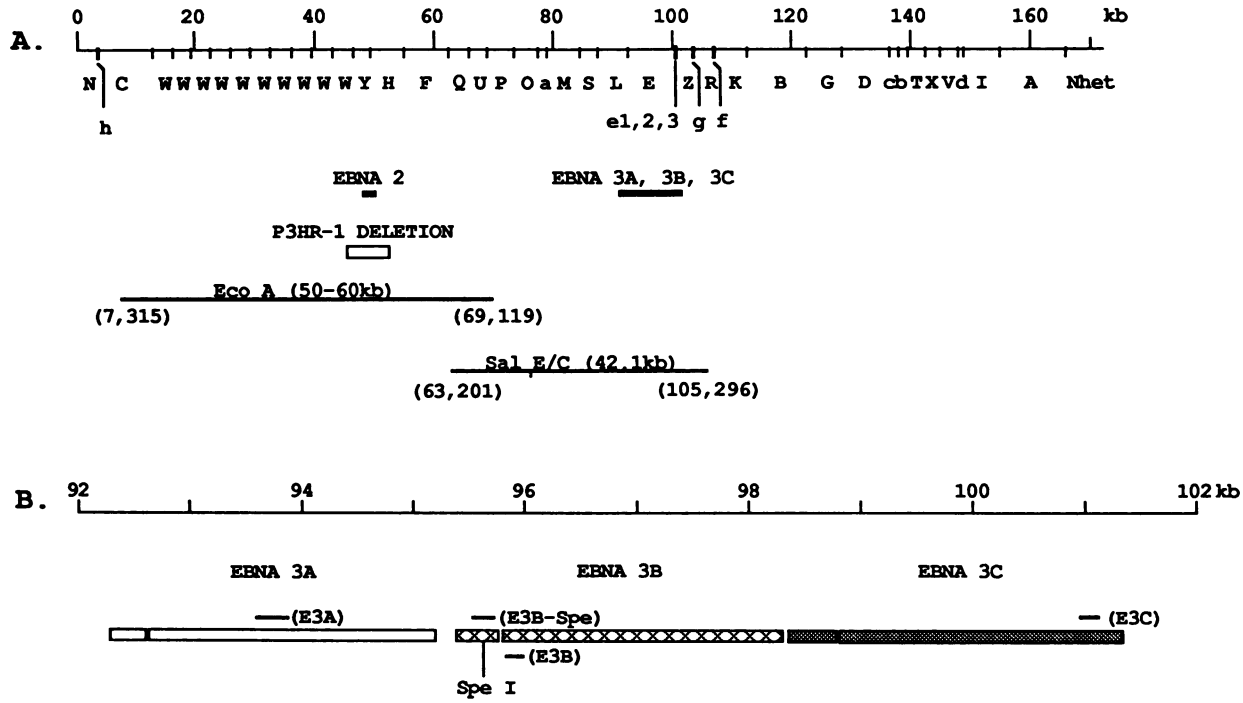

FIG. 1. (A) Schematic representation of the EBV genome indicating the location of the EBNA 2 and EBNA 3 genes and the P3HR-1 deletion. Also shown are the EBV DNA fragments in the pDVEcoRI A and pDVSECWT cosmids. (B) Schematic representation of the *Bam*HI-E region indicating the locations of the EBNA 3A, 3B, and 3C genes and the fragments amplified by PCR and the EBNA 3B *Spe*I site used to terminate translation of the EBNA 3B open reading frame.

2-encoding DNA. To investigate the possibility that genomes which participate in one recombination event, thereby picking up EBNA LP- and EBNA 2-encoding DNA, are more likely than other genomes to recombine with another DNA fragment, thereby picking up EBER-encoding DNA, we investigated the frequency of coincorporation of a nonlinked marker (58). P3HR-1 cells, which are deleted for EBV DNA extending from 30 to 45 kb, were transfected with a cosmid extending from 7 to 69 kb and with a second cosmid extending from 75 to 105 kb relative to the prototype EBV genome (2, 10). The frequency of the first recombination as assayed by the number of resultant transformation-competent virus recombinants was approximately 200. Of these, about 10% had incorporated DNA from the second cosmid (58). Both DNA segments were incorporated into the correct locations in the EBV genome, presumably by homologous recombination. The second cosmid included the EBNA 3A, 3B, and 3C genes. Since the EBNA 3A, 3B, and 3C genes differ between type 1 EBV isolates and type 2 isolates such as P3HR-1 (50, 52), the strategy was used to create recombinant EBVs which differed in having the P3HR-1 type 2 EBNA 3 genes or type 1 EBNA 3 genes from the transfected cosmid DNA (58). Although lymphocytes infected with type 1 EBV strains differ from those infected with type 2 strains in their growth (48), primary B lymphocytes infected with recombinant EBVs carrying type 1 or type 2 EBNA 3 genes grew similarly. These data indicated that second-site homologous recombination occurred with a sufficient frequency that recombinant EBVs could be made with mutations in the EBNA 3 genes and that type 1 and type 2 EBNA 3s are equivalent in their effects on in vitro cell growth or virus infection (58).

The experiments reported here used this latter EBV recombinant molecular genetic strategy to assess the role of EBNA 3B in B-lymphocyte infection, growth transformation, and virus replication. EBNA 3A, 3B, and 3C are distantly homologous and are tandemly placed in the EBV genome (19, 22, 23, 41, 43, 49, 54). Each consists of a short and a long exon. The proteins are remarkably hydrophilic overall, contain unusual clusters of charged amino acids, and have similarly positioned clusters of negatively charged amino acids (19, 23, 41, 43). The type 1 EBNA 3A, 3B, and 3C genes encode 145-, 165-, and 155-kDa proteins, respectively (19, 23, 41, 43, 49). In all latently infected growth-transformed B lymphocytes, these proteins localize to clumps within the nucleus but spare the nucleolus (19, 22, 41–43). Although nothing is known about the role of EBNA 3B in B-lymphocyte infection, single-gene transfer experiments with EBNA 3C in non-EBV-infected B-lymphoma cells revealed that cells expressing EBNA 3C also expressed higher levels of CD21, suggesting that EBNA 3C and the other EBNA 3s proteins are transactivators of B-lymphocyte gene expression (61). Thus, EBNA 3B mutants might be expected to differ from wild-type virus in their effects on B-lymphocyte growth or gene expression.

MATERIALS AND METHODS

Plasmid and cosmid construction. Plasmid pSVNaeI Z (32, 33, 56) was used to induce lytic EBV replication (9). Cosmid *Eco*RI-A (33) was used to obtain recombinants restored for the last two EBNA LP exons and the EBNA 2 exon. Cosmid *Sal*I-E/C (pDVSECWT) (58) was used to obtain recombinants at the EBNA 3B allele (Fig. 1). A stop codon was introduced after codon 109 of EBNA 3B in pDVSECWT by partial digestion with *Spe*I. The overhanging *Spe*I ends were

filled in with T4 DNA polymerase. A 14-bp *Xba*I linker, CTAGTCTAGACTAG, with termination codons in all three reading frames was blunt-end ligated into the *Spe*I site and packaged into bacteriophage lambda by using an in vitro packaging extract (Stratagene). Cosmid clones obtained following infection of *Escherichia coli* with packaged lambda were screened with *Xba*I restriction enzyme digestion to identify clones with linker inserted into the *Spe*I site in EBNA 3B. Analysis with additional restriction enzymes demonstrated no other changes in the DNA.

Cells and cell culture. The HH514-16 subclone of P3HR-1 (c16) (a gift from George Miller, Yale University) contains a type 2 EBV genome deleted for the EBNA 2 gene and part of the EBNA LP gene, rendering it nontransforming (20). B95-8 is a marmoset B-cell line immortalized with type 1 EBV (37, 39). BJA-B is an EBV-negative B-lymphoma cell line (36). Loukes is an EBV-negative Burkitt's lymphoma cell line (59). Human peripheral blood B lymphocytes were obtained from adult seronegative or seropositive donors. T cells were depleted with *S*-(2-aminoethyl)isothiuronium bromide (Sigma)-treated sheep erythrocytes (33). Cell lines were maintained in complete medium which consisted of RPMI 1640 medium supplemented with 10% inactivated fetal bovine serum, glutamine, and 10 μg of gentamicin per ml.

Cosmid transfection. P3HR-1 c16 cells were transfected with cloned type 1 EBV *Eco*RI A and wild-type or mutant *Sal*I C DNA fragments after release from cosmid vector DNA. Ten micrograms of cosmid *Eco*RI-A DNA and 50 μg of wild-type or mutant cosmid *Sal*I-E/C DNA were mixed with 25 μg of pSVNaeI Z and used to electroporate 10^7 P3HR-1 c16 cells. For electroporation, the cells were harvested during log-phase growth, washed once with complete medium, and resuspended in 400 μl of complete medium with DNA in a cuvette (0.4-cm gap; Bio-Rad). Following a 10-min incubation at 25°C, the culture was pulsed with 200 V at 960 μF. Cells were immediately diluted into 20 ml of complete medium. Lytic EBV infection was activated in LCLs by transfection with 25 μg of pSVNaeI Z under similar conditions.

Primary B-lymphocyte infections. Primary human B lymphocytes were infected with filtered (0.45-μm pore size) culture supernatant obtained from P3HR-1 c16 cells 3 days after transfection. Intracellular virus was first released into the medium by three cycles of freezing and thawing. The virus preparation was used directly or concentrated by centrifugation at 8,800 × *g* for 2 h. Virus was incubated with 10^7 primary B-lymphocyte-enriched human peripheral blood mononuclear cells for 2 h at 37°C. Infected cells were resuspended in complete medium at a concentration of 3.3×10^5 cells per ml, and 150 μl (5×10^4 cells) was distributed into each well of a 96-microwell plate. The cultures were fed at 14 days after plating with 100 μl of complete medium. LCLs were macroscopically visible 3 to 5 weeks after plating. Recombinant virus from LCLs was passaged by cocultivation of lethally irradiated EBV-infected B lymphocytes with human T-cell-depleted mononuclear cells or by infection of T-cell-depleted mononuclear cells with filtered (0.45-μm pore size) culture supernatant from EBV-infected cells. For cocultivation, 3 days after pSVNaeI Z transfection, 5×10^4 irradiated (8,800 rads) LCLs were mixed with 5×10^4 T-cell-depleted mononuclear cells in a 96-microwell plate. The cultures were fed at 14 days after plating with 100 μl of complete medium. LCL outgrowth was visible 3 to 5 weeks after plating. Irradiated LCLs (5×10^4) were also plated separately to serve as irradiation controls.

PCR and DNA sequence analyses. (i) Primers. Oligonucleotide primers for amplification of distinctive fragments from type 1 versus type 2 EBNA 3A, 3B, or 3C (52) or from mutant versus wild-type EBNA 3B were synthesized on an Applied Biosystem model 391 oligonucleotide synthesizer. The EBNA 3B *Spe*I-specific primers GAGCACAATGGTG GTGACGA and TCAAGGAATAAACTGCCTAG correspond to bases 95590 to 95609 and 95825 to 95806 of the prototype EBV DNA sequence, respectively (2). These primers amplify a 236-bp fragment from type 1 EBV DNA and a 254-bp fragment from mutant type 1 EBV DNA containing the *Xba*I linker. The type 2 EBNA 3C-specific primers GTAGGTGACTATGGTTTTGT and AAATCTA GAGCAGCCGGAAG correspond to bases in a 69-bp insertion (inserted following base 100759 in the B95-8 sequence) and a 27-bp insertion (inserted following base 100775 in the B95-8 sequence), respectively, in type 2 EBNA 3C (52). These primers amplify a 110-bp fragment from type 2 EBV.

DNA amplification and electrophoresis. DNA was prepared from 0.5×10^5 to 1×10^5 cells harvested from microwell cultures. Cells were resuspended in 0.2× phosphate-buffered saline, boiled for 10 min, and digested with 0.1 volume of 10-mg/ml proteinase K (Sigma) for 30 min at 55°C. Proteinase K was inactivated by incubation at 95°C for 20 min. DNA was amplified in a Perkin-Elmer thermal cycler machine with 10 to 20 μl of DNA in a 50-μl reaction mixture. Polymerase chain reaction (PCR)-amplified DNA was analyzed by electrophoresis with 1% SeaKem ME–2% NuSieve agarose (FMC Corp.) gels and visualized by staining with ethidium bromide.

(ii) DNA sequencing. PCR products were sequenced by the dideoxy method (53).

Immunoblot and Southern blot analyses. Expression of latent or lytic-cycle EBV proteins was evaluated by electrophoresis of denatured infected cell proteins in 7% denaturing polyacrylamide gels and immunoblotting with human EBV immune sera or mouse monoclonal antibodies. EBV latent proteins were detected by incubation (2 h at room temperature) of the blots with a 1/10 dilution of a human serum specific for type 1 proteins. EBNA 3B and 3C proteins were specifically detected by incubation of blots with a 1/10 dilution of EBNA 3B- and EBNA 3C-specific antibodies, respectively, affinity purified from the type 1-specific serum (41, 43). LMP 1 protein was detected by using the monoclonal antibody S12 (31). For Southern blots, 15 μg of cell DNA was digested with *Xba*I and *Bcl*I restriction enzymes in 200 μl. DNA was then precipitated, dried briefly in a vacuum dryer, and resuspended in 40 μl of loading buffer. The DNA fragments were size fractionated by electrophoresis through a 0.8% agarose gel. DNA was then transferred to an activated nylon membrane (GeneScreen Plus; New England Nuclear). The filter was hybridized with a ^{32}P-labeled EBNA 3B cDNA probe (30). For detection of linear and episomal EBV DNA in cells, in situ lysing gel analysis was performed by loading 2×10^6 cells into a gel well with 1% sodium dodecyl sulfate and proteinase K (12). The gel was run at 0.5 V/cm for 4 h to achieve lysis, and episomal and linear EBV DNA was fractionated by increasing the voltage to 3.3 V/cm for 18 h (12). The DNA was transferred to an activated nylon membrane and probed with a ^{32}P-labeled EBV *Sal*I C DNA fragment.

LCL growth. Cell growth was determined in quadruplicate by seeding cells at serial twofold dilutions from 2×10^5 cells per ml (4×10^4 cells per well) to 3×10^3 cells per ml (6×10^2 cells per well) in microwells and measuring thymidine incorporation 3 days later (48). Cells were pulsed with 0.625 μCi of [^{3}H]thymidine (5 Ci/mM) for 6 h and harvested, and the

TABLE 1. EBNA 3B recombination frequency

Expt	No. of type 1 EBNA 3B recombinants (no. of P3HR-1 coinfected)/ total no. of LCLs analyzed	
	Mutant type 1 EBNA 3B	Type 1 EBNA 3B[a]
1	5 (3)/65	NT[b]
2	4 (1)/19	NT
3	9 (4)/87	9 (6)/68
4	9 (7)/89	11 (10)/91
Total	27 (15)/260	20 (16)/159
Total % E3B recombinants	10	12
Total % P3HR-1 coinfected	56	80

[a] Results are taken from a previous study (58) which was performed in parallel with this study and served as a baseline control for EBNA 3B mutant analysis.

[b] NT, not tested.

incorporation of [^{3}H]thymidine was quantitated. LCL outgrowth from conditions of low-inoculum cell density was determined in quadruplicate by serially diluting to 6×10^2 cells per well and maintaining the cultures in growth medium until macroscopic growth was visible or until the cells in the well had died. Saturation density was determined by plating cells at 10^5/ml in 400 μl in triplicate microwells. At 3- to 4-day intervals, 20 μl was removed and the cell concentration was determined.

RESULTS

EBV recombinants with an EBNA 3B stop codon mutation. Otherwise isogenic EBV recombinants with wild-type or mutant EBV type 1 EBNA 3B were created by homologous recombination with the EBV genome in P3HR-1 cells. P3HR-1 cells were transfected with *Eco*RI-A, which spans the P3HR-1 deletion and restores the deleted EBNA LP and EBNA 2 genes, with wild-type or mutant type 1 *Sal*I-C, which contains the exons for EBNA 3B, and with pSVNaeI Z to induce virus replication. The EBNA 3B mutation was made by inserting a 14-bp oligonucleotide into the *Spe*I site located after EBNA 3B codon 109 in a cloned type 1 *Sal*I-E/C cosmid (Fig. 1) (58). The 14-bp oligonucleotide has translational termination codons in all three reading frames and a new *Xba*I cleavage site. Virus from filtered culture supernatant obtained from transfected P3HR-1 cells was used to infect human primary B lymphocytes. Infected cells were plated out in microwells to establish growth-transformed lymphoblastoid cell lines (LCLs). The resultant LCLs were screened by PCR analysis, using primers which distinguish between type 1 and type 2 EBV to identify those LCLs which were infected with recombinant virus in which the type 2 EBNA 3B gene of the P3HR-1 parent had been replaced by the EBNA 3B gene of the transfected mutant or wild-type *Sal*I-E/C cosmid.

Approximately 12% of the transformation-competent recombinants resulting from cotransfections with the wild type 1 *Sal*I-C were recombinant for the type 1 EBNA 3B gene, while approximately 88% had the type 2 EBNA 3B gene of the P3HR-1 parent (Table 1). These results have been reported as part of a previous study characterizing type 1 EBNA 3 recombinants and the high frequency of second-site homologous recombination (58). The results are partially restated here because the EBNA 3B mutant transfections were done in parallel with these other studies so that the frequency of recombination of wild-type EBNA 3B could serve as a control for the expected frequency of derivation of mutant EBNA 3B recombinants if EBNA 3B were to be unimportant in B-lymphocyte infection or growth transformation. Of the LCLs infected with virus from transfections with *Sal*I-E/C DNA carrying the EBNA 3B stop codon mutation, 12% were recombinant for the type 1 EBNA 3B stop codon mutation (Table 1). Some of these were coinfected with parental nonrecombinant P3HR-1 virus, and some were singly infected with virus containing the type 1 EBNA 3B stop codon mutation (Table 1 and Fig. 2). The similar frequency of derivation of mutant and wild-type EBNA 3B recombinants is consistent with the hypothesis that EBNA 3B is nonessential to B-lymphocyte growth transformation in vitro.

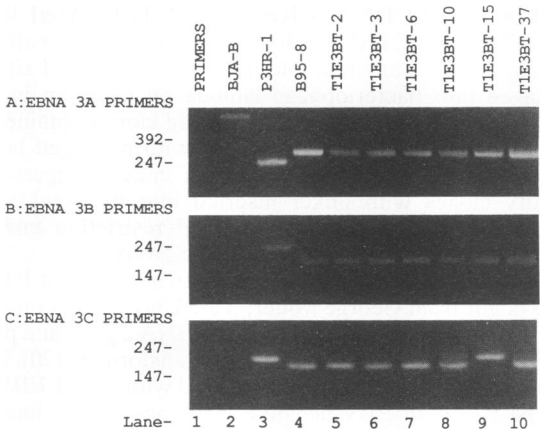

FIG. 2. PCR analysis of the EBNA 3 genes in LCLs immortalized by mutant recombinant EBVs with a termination codon in EBNA 3B. (A, B, and C) Ethidium bromide-stained PCR-amplified fragments, shown by using primers which distinguish between type 1 and 2 EBNA 3A, 3B, and 3C genes, respectively (52). The EBNA 3A primers amplify a 237- or 276-bp fragment from type 2 or type 1 DNA, respectively. The EBNA 3B primers amplify a 218- or 183-bp fragment from type 2 or type 1 DNA, respectively. The EBNA 3C primers amplify a 179- or 158-bp fragment from type 2 or type 1 DNA, respectively. The location of these DNA fragments is shown in Fig. 1. Lanes 1 and 2 are negative control amplifications with primers only and EBV-negative BJA-B genomic DNA. Lanes 3 and 4 are positive control amplifications of P3HR-1 (type 2) and B95-8 (type 1) genomic DNA. Lanes 5 to 10 are amplifications of mutant EBNA 3B (T1E3BT) LCL genomic DNA transformed by viruses taken from P3HR-1 cells transfected with pSVNaeI Z plasmid, *Eco*RI-A, and mutant EBNA 3B *Sal*I-C DNAs. Size markers (base pairs) of *Rsa*I fragments of φX174 DNA are shown on the left.

PCR was used to initially characterize the EBV genomes in LCLs infected with EBNA 3B mutant recombinant virus (Fig. 2 and 3). PCR with the type-specific EBNA 3B primers resulted in amplification of a 218-bp fragment from type 2 P3HR-1 EBV DNA (Fig. 2B, lane 3) and a 183-bp fragment from type 1 B95-8 EBV DNA (Fig. 2B, lane 4). For each of the five recombinant EBNA 3B mutant-infected LCLs, only a fragment the size of the type 1 EBNA 3B was amplified. No larger type 2 EBNA 3B-sized fragment was amplified (Fig. 2), indicating the absence of coinfecting P3HR-1 virus. Although not apparent from these analyses, initial analysis of early passages of the T1E3BT-10 and -37 LCLs demonstrated amplification of type 2 DNA, indicating that these LCLs were initially coinfected with wild-type P3HR-1 virus (see below). The T1E3BT-10 LCL later had little or no type

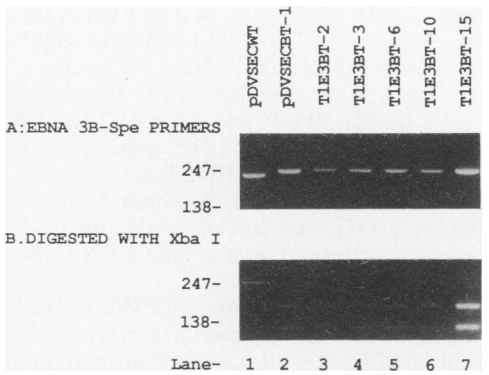

FIG. 3. (A) PCR analysis of the mutant EBNA 3B-infected LCLs with primers which amplify a 254-bp fragment from DNA with the mutated EBNA 3B *Spe*I site or a 236-bp fragment from DNA with the wild-type *Spe*I site. Amplifications of the wild-type pDVSECWT (lane 1) or the EBNA 3B mutant pDVSECBT (lane 2) cosmid are shown as controls. Amplifications from five T1E3BT LCLs are shown in lanes 3 to 7. In all cases, the size of the amplified product is the same as the pDVSECBT mutant cosmid PCR fragment, demonstrating the insertion the *Xba*I-termination codon linker into the EBV genome. (B) The PCR fragments shown in panel A were digested with *Xba*I to confirm the presence of the linker. Digestion of the 254-bp PCR fragments amplified from the pDVSECBT cosmid DNA or five T1E3BT LCL DNAs resulted in two smaller fragments (lanes 2 to 7), while the 236-bp pDVSECWT PCR fragment was not digested with *Xba*I (lane 1).

TABLE 2. Genotype and immunophenotype of EBNA 3B recombinants

Clone	Genotype[a]	Immunophenotype[b]	Coinfection
Mutant EBNA 3B recombinant LCLs			
T1E3BT-2	T1/T1/T1	T1/T1/T1	No
T1E3BT-3	T1/T1/T1	T1/T1/T1	No
T1E3BT-6	T1/T1/T1	T1/T1/T1	No
T1E3BT-10	T1-T2/T1-T2/T1-T2[c]	T1/T1/T1	Yes
T1E3BT-15	T1/T1/T2	T1/T1/T2	No
Type 1 EBNA 3 recombinant LCLs			
T1E3-26	T1/T1/T1-T2	T1/T1/T1	No
T1E3-28	T1/T1/T2	T1/T1/T2	No
T1E3-35	T1/T1/T1	T1/T1/T1	No
T1E3-100G	T1/T1/T2	T1/T1/T2	No
Type 2 EBNA 3 LCLs[d]			
T2E3-3	T2/T2/T2	T2/T2/T2	NT[e]
T2E3-9	T2/T2/T2	T2/T2/T2	NT
T2E3-14	T2/T2/T2	T2/T2/T2	NT
T2E3-6H	T2/T2/T2	T2/T2/T2	NT

[a] Type 1 EBNA 3A/type 1 EBNA 3B/type 1 EBNA 3C.
[b] Did not test for type 2 EBNA 3 protein expression.
[c] T1-T2 indicates the presence of type 1 and type 2 EBNA 3 genes.
[d] LCLs taken from a previous study (58) which was performed in parallel with this study and served as a baseline for EBNA 3B mutant analysis.
[e] NT, not tested.

2 EBV DNA (Fig. 2). The loss of coinfecting P3HR-1 virus over time has been observed in previous studies (7, 32, 33, 56, 58) and is consistent with the fact that the wild-type P3HR-1 virus is nontransforming and therefore dispensable for LCL growth (35, 38). The recombinant EBNA 3B-infected LCLs were also analyzed with primers for the type 1 or 2 EBNA 3A and 3C genes (Fig. 2A and C). Four type 1 EBNA 3B mutant recombinant-infected LCLs had type 1 EBNA 3A and 3C amplification products only (Fig. 2A and C, lanes 5 to 9), indicating that the recombination in these LCLs occurred 5′ to EBNA 3A and 3′ to EBNA 3C. Amplification of DNA from LCL T1E3BT-15 resulted in a type 1 EBNA 3A fragment (Fig. 2A, lane 10) and a type 2 EBNA 3C fragment (Fig. 2C, lane 10), indicating that the recombination occurred 5′ to EBNA 3A and between EBNA 3B and EBNA 3C. The EBNA 3 genotypes of these recombinants and the control LCLs are summarized in Table 2.

PCR amplification with oligonucleotide primers flanking the stop codon linker insertion site revealed the expected size fragments from wild type- and mutant-infected LCLs (Fig. 3). Type 1 EBNA 3B DNA resulted in the amplification of a 236-bp fragment (Fig. 3, lane 1), while *Xba*I linker mutant EBNA 3B resulted in the amplification of a 254-bp fragment due to an increase of 18 bp from the blunt-end ligation of the 14-bp *Xba*I linker into the EBNA 3B site. The larger size of the mutated fragment is reflected by its slower migration (Fig. 3A, lane 2). PCR amplification of DNA from the five LCLs with type 1 EBNA 3B resulted in the amplification of the larger 254-bp DNA fragment (Fig. 3A, lanes 3 to 7), consistent with the insertion of the *Xba*I linker in all five recombinant EBNA 3B LCLs. This was confirmed by digestion of the PCR-amplified fragments with *Xba*I, which resulted in two smaller fragments (Fig. 3B, lanes 2 to 7). The wild-type PCR-amplified fragment was not digested by *Xba*I (Fig. 3B, lane 1).

The amplified linker insertion site DNAs from five recombinant mutant EBNA 3B virus-infected LCLs were sequenced to further confirm the persistence of the mutation in the infected LCLs. The sequence of all five DNAs was exactly that expected from the previously determined EBV DNA sequence (2) and the methods used to construct the mutation (data not shown).

Southern hybridization with an EBNA 3B-specific probe confirmed that the mutant EBNA 3B was in the expected context in the EBV genome, consistent with the expected product of doubly reciprocal homologous recombination between the transfected mutant *Sal*I-C DNA and the P3HR-1 EBV genome. DNAs from P3HR-1, B95-8, five mutant EBNA 3B (T1E3BT) recombinant virus-infected LCLs, one type 2 EBNA 3 recombinant virus-infected LCL (T2E3-3), and one type 1 EBNA 3 recombinant virus-infected LCL (T1E3-35) were digested with *Bgl*I and *Xba*I and probed with ^{32}P-labeled type 1 EBNA 3B cDNA. Figure 4A shows a schematic representation of the type 1 and 2 EBV genome around EBNA 3B, the EBNA 3B linker insertion (*Spe*I) site, and the relevant *Bgl*I and *Xba*I sites in wild-type and mutant EBV DNA. Type 1 EBV has a 1.6-kb fragment and a 1.4-kb fragment when digested with *Bgl*I and *Xba*I and hybridized with an EBNA 3B cDNA probe (Fig. 4B, lanes 3 and 10). Type 2 EBV (P3HR-1) lacks a *Bgl*I site at 96827 within the EBNA 3B open reading frame and therefore generates a 3.0-kb fragment when digested with *Bgl*I and *Xba*I and hybridized with an EBNA 3B cDNA probe (Fig. 4B, lanes 2 and 4). The faint 4.3-kb fragment seen in the P3HR-1 DNA is due to weak hybridization of the probe to the 4.3-kb *Bgl*I fragment to the right of the 3.0-kb *Bgl*I-*Xba*I fragment (Fig. 4A). In mutant EBNA 3B DNA, the 1.4-kb fragment includes the *Xba*I linker at the *Spe*I site so that the 1.4-kb fragment should be cleaved into a 1.1- and a 0.3-kb fragment. Genomic DNA from all five mutant

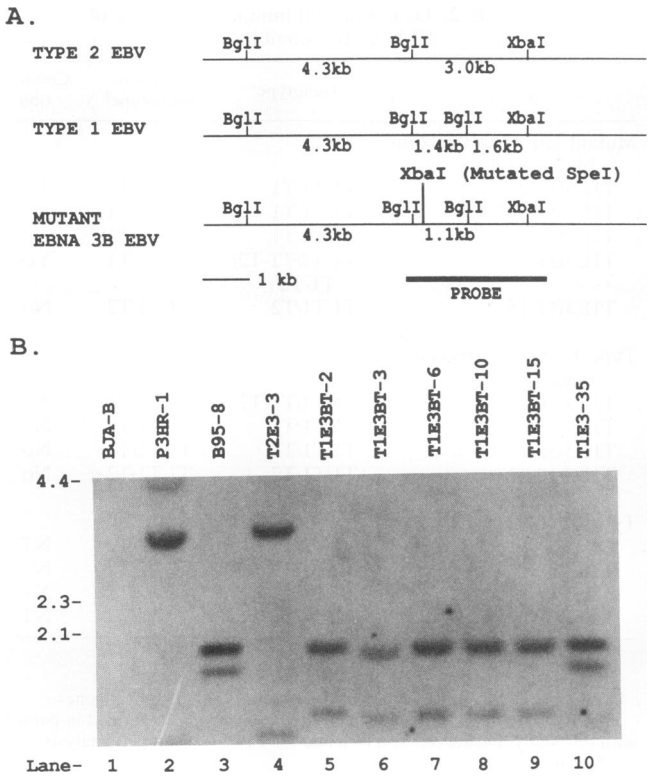

FIG. 4. Southern hybridization of genomic DNA from mutant EBNA 3B LCLs to demonstrate homologous recombination. Genomic DNA from representative LCLs was digested with *BglI* and *XbaI* and probed with ^{32}P-radiolabeled B95-8 EBNA 3B open reading frame cDNA. (A) Schematic representation of the type 1 and type 2 EBNA 3B region, indicating the EBNA 3B *SpeI* site and the expected *BglI-XbaI* fragments from type 2 P3HR-1 DNA, type 1 B95-8 DNA, and mutant type 1 EBNA 3B (T1E3BT) DNA. Type 2 DNA lacks a *BglI* site within the EBNA 3B open reading frame (96827) and has a different *BglI* fragment pattern compared with type 1 DNA when probed with an EBNA 3B cDNA probe. The type 1 1.4-kb fragment contains the EBNA 3B *SpeI* site. Mutant EBNA 3B DNA is digested by *XbaI* to generate 1.1- and 0.3-kb fragments. (B) Southern blot hybridizations of representative LCLs. A negative control digest of EBV-negative BJA-B genomic DNA is shown (lane 1). Digests of genomic DNA from P3HR-1 and B95-8 (lanes 2 and 3), one wild-type type 2 EBNA 3 (T2E3-3) LCL (lane 4), one wild-type type 1 recombinant (T1E3-35) LCL (lane 10), and five T1E3BT LCLs (lanes 5 to 9) are shown. Size markers (kilobases) of *Hin*dIII fragments of lambda DNA are shown on the left.

EBNA 3B LCLs (Fig. 4B, lanes 5 to 9) yielded fragments of 1.6 and 1.1 kb when digested with *BglI* and *XbaI*, demonstrating the presence of the *XbaI* linker. The 3.0-kb fragment characteristic of the type 2 parent DNA was absent from all mutant EBNA 3B LCLs, consistent with replacement of the P3HR-1 parent type 2 EBNA 3B by reciprocal homologous recombination. The absence of the 3-kb fragment also confirms the PCR, which did not detect coinfecting parental nontransforming P3HR-1 virus.

To detect more sensitively possible coinfecting P3HR-1 virus, we synthesized primers specific for type 2 EBNA 3C and used them to detect type 2 EBNA 3C in the mutant EBNA 3B LCLs. To assess the sensitivity of detection of type 2 virus by these primers, we mixed a constant amount of type 1 EBV DNA with fivefold dilutions of type 2 EBV. Type 2 EBNA 3C sequences were detected to a sensitivity of 1 part of type 2 EBV in 625 parts of type 1 EBV. Under these conditions, no type 2 EBNA 3C DNA was amplified from mutant EBNA 3B LCLs T1E3BT-2, -3, and -6. Type 2 EBNA 3C DNA was amplified in the coinfected mutant EBNA 3B LCLs T1E3BT-10 and -37 and T1E3BT-15, which has type 2 EBNA 3C (Fig. 2). The absence of the type 2 EBNA 3C PCR product in T1E3BT-2, -3, and -6, further confirms the above PCR and Southern hybridization results—that these LCLs are free of coinfecting P3HR-1 virus and are infected with only pure mutant EBNA 3B recombinant virus.

Immunoblot analysis of the mutant EBNA 3B LCLs with human EBV-immune serum specific for type 1 latent proteins and purified EBNA 3B-specific human antibodies confirmed the absence of EBNA 3B and did not detect any antigenically related cross-reactive proteins. Protein lysates from representative LCLs were separated on 7% polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with a human immune serum and antibodies affinity purified from the serum which specifically react with type I EBV latent proteins (1, 41, 43, 58). Figure 5 shows immunoblots of protein lysates from representative LCLs reacted with the whole human type 1-specific immune serum (Fig. 5A), affinity-purified EBNA 3B-specific antibodies (Fig. 5B), and affinity-purified EBNA 3C-specific antibodies (Fig. 5C). The type 1 specificity of the serum is shown by its strong reactivity with the type 1 EBNA 3A, 3B, and 3C of B95-8 (Fig. 5A, lane 3) and its absence of reactivity with the type 2 EBNA 3A, 3B, and 3C of P3HR-1 (Fig. 5A, lane 2) and T2E3-3 (Fig. 5A, lane 4). The specificity of the E3B-specific antibodies is shown by their strong reactivity with EBNA 3B expressed from the BJpZE3B-2 expression vector (Fig. 5B, lane 12) and their nonreactivity with vector-expressed EBNA 3A (Fig. 5B, lane 11) and EBNA 3C (Fig. 5B, lane 13). Similarly, the specificity of the EBNA 3C-specific antibodies is shown by their strong reactivity with the vector-expressed EBNA 3C (Fig. 5C, lane 13). No proteins of any size were detected by the EBNA 3B-specific antibodies in the protein lysates of the five mutant EBNA 3B LCLs (Fig. 5B, lanes 5 to 9). EBNA 3B protein expression was detected in B95-8 and T1E3-35 (Fig. 5B, lanes 3 and 10) but not in P3HR-1 or T2E3-3 (Fig. 5B, lanes 2 and 4). As expected, EBNA 3C could be detected in the mutant recombinant virus-infected LCLs with affinity-purified type 1 EBNA 3C-specific antibodies (Fig. 5C) (43). The type 1 EBNA 3C-specific antibodies detected type 1 EBNA 3C protein in each of the mutant EBNA 3B LCLs except T1E3BT-15, which by PCR contains type 2 EBNA 3C DNA. These data demonstrate the absence of full-length or truncated EBNA 3B in the mutant recombinant virus-infected LCLs and the effectiveness of the linker insertion in terminating EBNA 3B translation. These data indicate that EBNA 3B is not required by EBV for maintenance of B-lymphocyte latent infection or growth transformation.

EBV gene expression, episome maintenance, and growth of LCLs infected with EBNA 3B mutant recombinant virus. The absence of EBNA 3B protein expression in the mutant recombinant virus-infected cell lines was not associated with abnormal expression of other latent or lytic infection-associated viral proteins. The mutant recombinant EBNA 3B-infected LCLs expressed latent proteins EBNA 1, 2, LP, 3A, and 3C (Fig. 5A, lanes 5 to 9) at levels which were indistinguishable from those of type 1 B95-8 or T1E3-35 recombinant virus-infected LCLs (Fig. 5A, lanes 3 and 10). Similarly, no difference was seen in LMP 1 (Fig. 6A) or lytic virus infection-associated protein (Fig. 6B) expression be-

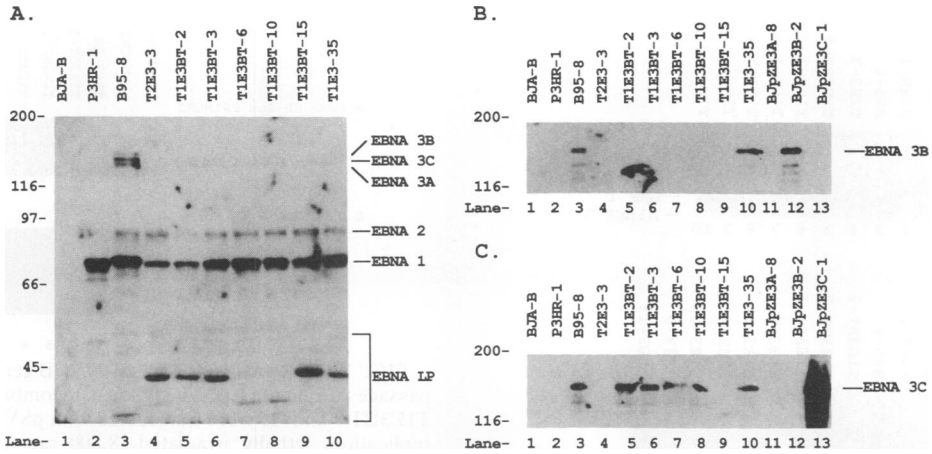

FIG. 5. EBNA protein expression in mutant EBNA 3B LCLs. Triplicate immunoblots were incubated with a 1/10 dilution of a human EBV immune serum (1, 41, 43, 58) specific for type 1 latent proteins (A), a 1/10 dilution of EBNA 3B-specific antibodies (41) purified from the immune serum (B), or a 1/10 dilution of EBNA 3C-specific antibodies (43) purified from the immune serum (C). Human immunoglobulin was detected with radiolabeled ^{125}I-protein A. A negative control from the EBV-negative BJA-B is shown in lane 1. Protein lysates from P3HR-1 and B95-8 cells are shown in lanes 2 and 3. The antigen specificity of the purified EBNA 3B-specific antibody is shown by its strong reactivity with EBNA 3B expressed from the BJpZE3B-2 expression vector and lack of reactivity with EBNA 3A (panel B, lane 11) or EBNA 3C (panel B, lane 13). Similarly, the specificity of the EBNA 3C-specific antibody is shown by its specific reactivity to the vector-expressed EBNA 3C (panel C, lane 13). Protein lysates from one T2E3 LCL (T2E3-3, lanes 4), one T1E3 LCL (T1E3-35, lanes 10), and five T1E3BT LCLs (lanes 5 to 9) are shown. Size markers (kilodaltons) of protein molecular mass standards are shown on the left of each gel.

tween the mutant EBNA 3B recombinant virus-infected LCLs and control virus-infected LCLs.

The EBNA 3B mutation was not associated with a difference in EBV episome copy number in infected LCLs. EBNA 3B mutant and T1E3 and T2E3 wild-type control recombinant virus-infected LCLs were analyzed by Southern blot analysis of in situ cell-lysing gels (12) with a ^{32}P-labeled EBV *Sal*I-C probe to characterize the EBV genome in these LCLs. DNAs from two mutant EBNA 3B-, two T1E3-, and two T2E3-infected LCLs were analyzed. The EBV DNA in all six LCLs was primarily episomal, with various amounts of linear EBV DNA for each LCL. No difference was observed between the mutant EBNA 3B LCLs and the control LCLs in the EBV episome number (data not shown).

Five EBV type 1 EBNA 3B mutant recombinant virus-infected LCLs did not differ from six type 1 EBNA 3B or from six type 2 wild-type control recombinant virus-infected LCLs derived in parallel in the time to initial outgrowth, growth rate, saturation density, or outgrowth from cultures seeded at 600 cells per well.

Replication and infection of primary B lymphocytes with mutant EBNA 3B recombinant virus. Since the EBNA 3B recombinant virus-infected LCLs had been infected with virus from transfected P3HR-1 cells, they could have been initially coinfected with P3HR-1 virus which could have initially supplied wild-type EBNA 3B in *trans*. Thus, although the absence of P3HR-1 from the recombinant virus-infected LCLs indicates that EBNA 3B is not essential for the maintenance of growth transformation, EBNA 3B could be essential for the initiation of growth transformation. To exclude this unlikely possibility, lytic infection was activated in LCLs which contained only type 1 mutant or wild-type recombinant EBV, and the LCLs were cocultured with primary human B lymphocytes. The resultant mutant or wild-type recombinant virus-infected progeny LCLs grew out equally rapidly. The mutant EBNA 3B viruses did not differ from type 1 or type 2 wild-type EBNA 3 recombinant viruses passaged in parallel in their ability to infect and growth transform primary B lymphocytes. Three mutant EBNA 3B virus-infected LCLs derived from two different parental mutant EBNA 3B virus isolates as well as control recombinant virus-infected LCLs were expanded. PCR amplification of EBNA 3B DNA with the E3B-Spe primers flanking the codon linker insertion site resulted in the expected 254-bp DNA fragment from the mutant virus-infected LCLs (Fig. 7A, lanes 5 to 7 and 9 to 11). Digestion of the 254-bp DNA fragment with *Xba*I resulted resulted in two smaller fragments, indicating the persistence of the *Xba*I linker at this site in the mutant EBNA 3B virus genome in the infected LCLs (Fig. 7B, lanes 5 to 7 and 9 to 11). Amplification of DNA from control wild-type recombinant virus-infected LCLs resulted in a smaller fragment (Fig. 7A, lanes 3 and 12) which was not digested by *Xba*I (Fig. 7B, lanes 3 and 12). PCR analysis of the progeny virus-infected LCLs with the type-specific EBNA 3A, 3B, or 3C primers demonstrated that the progeny LCLs were infected with the same virus as their parent LCL (Fig. 8C to E). In addition, progeny clones T1E3BT-3F, T1E3BT-3C, T1E3BT-15A, and T1E3BT-15C were reactivated by transfection with pSV-NaeI Z, and filtered (0.45-μm pore size) culture supernatants were used to establish tertiary clones under conditions of limiting dilution. Progeny mutant EBNA 3B LCLs did not differ from progeny control recombinant LCLs in time to outgrowth. PCR analysis of the clones from tertiary passage of pure mutant EBNA 3B recombinant virus again demonstrated the persistence of the *Xba*I linker in the *Spe*I site in the EBNA 3B gene (data not shown). Tertiary passage of the mutant EBNA 3B virus further excludes the possibility of coinfection by wild-type P3HR-1 and ensures passage of pure mutant EBNA 3B virus. The ability of pure EBNA 3B mutant recombinant virus to infect and growth transform primary B lymphocytes confirms that EBNA 3B is nonessential for initiation or maintenance of transformation.

Immunoblot analysis of the progeny mutant EBNA 3B-infected LCLs demonstrated the absence of EBNA 3B and

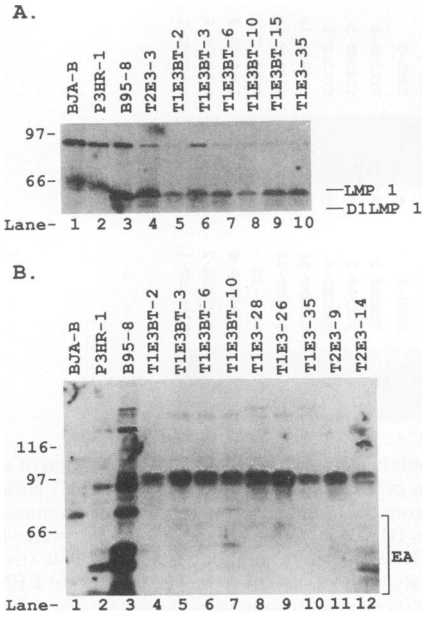

FIG. 6. LMP 1 and lytic protein expression in mutant EBNA 3B LCLs. (A) LMP 1 expression was examined by incubation with S12 anti-LMP 1 monoclonal antibody (31), a rabbit anti-mouse secondary antibody, and ^{125}I-protein A. LMP 1 and the lytic cycle product of the LMP 1 gene, D1LMP 1, are indicated. Both are expressed in B95-8 cells. EBV infection in recombinant LCLs, unlike B95-8, is primarily latent, and D1LMP 1 is not detected. Protein lysates from the EBV-negative BJA-B (lane 1), P3HR-1 (lane 2), B95-8 (lane 3), one T2E3 (lane 4), and one T1E3 (lane 10) control LCLs and six T1E3BT LCLs are shown. (B) Lytic antigen expression was examined by incubation with a 1/100 dilution of serum from a patient with chronic mononucleosis. Human immunoglobulin was detected with radiolabeled ^{125}I-protein A. The location of the major 40- to 60-kDa early antigen (EA) group is shown on the right. A protein lysate from EBV-negative BJA-B cells is shown in lane 1. Protein lysates from P3HR-1 (lane 2), B95-8 (lane 3), two T2E3 (lanes 11 and 12), three T1E3 (lanes 8 to 10), and four T1E3BT (lanes 4 to 7) LCLs are shown. Size markers (kilodaltons) are indicated on the left.

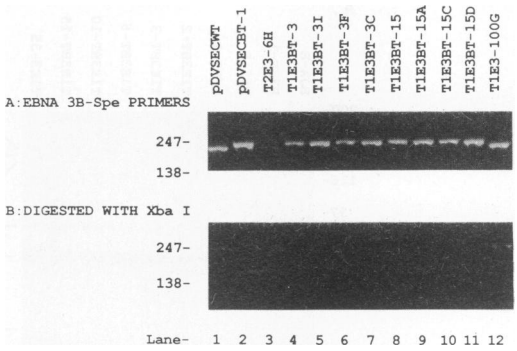

FIG. 7. PCR analysis of the EBNA 3B genes in LCLs derived by passage of mutant EBNA 3B EBV recombinants. Parental clones T1E3BT-3 and -15 were transfected with pSVNaeI Z to induce virus replication, lethally irradiated (8,800 rads), and cocultured with human B cells. Transformed lymphocytes were visible 2 to 4 weeks after plating, similar to the outgrowth of T2E3 and T1E3 progeny clones. (A) The PCR-amplified *Spe*I-site DNAs from three progeny mutant virus-infected LCLs (lanes 5 to 7) derived from the T1E3BT-3 parent LCL (lane 4), three progeny mutant virus-infected LCLs (lanes 9 to 11) derived from the T1E3BT-15 parent LCL (lane 8), one T1E3 progeny virus-infected LCL (T1E3-100G, lane 12), and one T2E3 progeny virus-infected LCL (T2E3-6H lane 3) amplified with the EBNA 3B-Spe primers are shown. (B) These PCR-amplified DNAs were digested with *Xba*I. Size markers (base pairs) of *Rsa*I fragments of ϕX174 DNA are shown on the left.

of EBNA 3B cross-reactive protein. Protein lysates from the T1E3BT-3 and T1E3BT-15 progeny LCLs were analyzed with the EBV type 1-specific immune human serum, and no EBNA 3B protein was detected in the mutant virus-infected LCLs (data not shown). The expression of other EBNA proteins, lytic proteins, and LMP 1 protein in these LCLs was indistinguishable from expression in the type 1 and 2 EBNA 3B control recombinant virus-infected LCLs (data not shown).

DISCUSSION

These studies yield the surprising result that EBNA 3B is not critical for EBV latent infection, cell growth transformation, or lytic virus replication in B lymphocytes in vitro. The conclusion is based on a stop codon mutation after codon 109 of EBNA 3B. The stop codon is in the appropriate context in the recombinant EBV genomes. The infected cells do not contain any wild-type EBNA 3B genes. The mutant recombinant EBVs could be freely passaged without any possible contamination with virus carrying a wild-type EBNA 3B gene. The frequency of isolation of the EBNA 3B stop codon recombinant genomes was as high as would be expected for an indifferent mutation excluding the possibility of second-site reversion (58). Investigations of the possible effects of the mutation on latent and lytic EBV gene expression, on EBV episome copy number, on initiation or maintenance of cell growth transformation, on the sensitivity of transformed LCLs to growth at limiting dilution, and on LCL growth rate were all negative. Three issues then require consideration: Is the stop codon mutation effective in interrupting EBNA 3B expression? What then is the likely function of EBNA 3B? What are the implications for the role of EBNA 3A and 3C?

The stop codon mutation used in these experiments interrupts EBNA 3B expression but would permit expression of

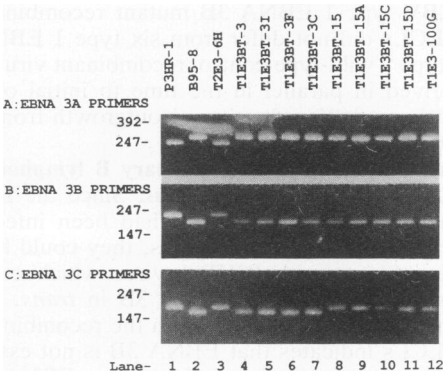

FIG. 8. Type-specific PCR analysis of EBNA 3A (A), EBNA 3B (B), or EBNA 3C (C) genes in progeny LCLs infected with the mutant EBNA 3B, T1E3, or T2E3 recombinant EBVs. The PCR analysis of three progeny mutant virus-infected LCLs (lanes 5 to 7) derived from the T1E3BT-3 parent LCL (lane 4) and of three progeny mutant virus-infected LCLs (lanes 9 to 11) derived from the T1E3BT-15 parent LCL (lane 8) is shown. Also shown is the PCR analysis of a T1E3 progeny virus-infected LCL (T1E3-100G, lane 12) and a T2E3 progeny virus-infected LCL (T2E3-6H, lane 3).

109 of the 119 codons of the first EBNA 3B exon. The resultant 109-residue polypeptide would contain 24 acidic residues and lack a nuclear localization signal. Thus, it would not be expected to fulfill putative nuclear functions of EBNA 3B. Since we have no antiserum to this part of EBNA 3B, we cannot determine whether it accumulates in the mutant recombinant virus-infected cells. The next downstream potential strong translational reinitiation site in EBNA 3B which has the requisite A or G at the −3 position (27, 29) is 313 bases into exon 2 and out of frame. Five other out-of-frame potential strong translational initiation sites occur before the first in-frame potential initiation site with an A or G at −3. This later site is 666 bases into exon 2 and would result in the translation of the carboxyl-terminal 573 amino acids of EBNA 3B. Reinitiation at this site would be after translation of a 109-amino-acid protein fragment, after reading through several weak initiation sites (no A or C at the −3 position), after initiation at any of several strong out-of-frame sites, and after termination before the putative strong in-frame initiation site. Reinitiation of translation at this site is likely to be inefficient, in part because of the translation of the 109-codon open reading frame and in part because of the distance between the 109-codon open reading frame and the next strong initiation site (13, 28, 29, 62). Moreover, 487 of the putative 593 residues of the anticipated open reading frame downstream of the site at 666 in exon 2 would be part of the fusion protein used to purify EBNA 3B-reactive antibody from human sera (41), and no cross-reactive protein was detected in mutant recombinant virus-infected cells. EBV-infected cells contain few copies of the EBNA 3 RNAs (51), and alternative splice donor or acceptor sites are not evident in nuclease protection experiments (23). Thus, the stop codon mutation is highly likely to be a null mutation for EBNA 3B expression.

The implications of this study for the role of EBNA 3B, 3A, or 3C in EBV infection are more difficult to assess. The EBNA 3s are likely to be important in EBV infection in vivo. These genes encode more than half of the translated open reading frames in latently infected, growth-transformed lymphocytes (for reviews, see references 24 and 25). EBNA 3A, 3B, and 3C are likely to be expressed in vivo since they are recognized by EBV-immune cytotoxic T lymphocytes (5, 6, 39a, 40, 40a). The persistence of these genes in the EBV genome in the face of the negative selective pressure of cytotoxic T lymphocytes also indicates that they play an important role in EBV infection. Further, in a previous study of recombinants between the Raji EBV genome and the P3HR-1 EBV genome, the transforming recombinants had the *Bam*HI E fragment from P3HR-1 (55). Since the Raji EBV genome has an unusual deletion in *Bam*HI-E which includes the EBNA 3C-coding region (44), these data suggest that EBNA 3C is critical for latent growth-transforming infection in vitro. Moreover, expression of EBNA 3C by single-gene transfer was associated with increased CD21 expression in the BJA-B B-lymphoma cell line, suggesting that EBNA 3C transinduces cell gene expression (61). Thus, the EBNA 3s are likely to be transactivators of B-lymphocyte genes and important for infected cell survival or proliferation.

Despite this evidence that the EBNA 3s are important in EBV infection, EBNA 3B may only be critical for latent growth-transforming infection in vivo, while EBNA 3A or 3C may be critical for latent, growth-transforming infection in vivo and in vitro. EBNA 3B was not expressed in one EBV-transformed LCL which has been extensively passaged in vitro, while EBNA 3A and 3C are uniformly expressed in such cells (19, 41, 43). Despite the similarities noted above, there is little precise amino acid sequence conservation among the EBNA 3 genes. Ongoing analyses of the results of transfections of P3HR-1 cells with cosmid constructs carrying stop codon mutations in the EBNA 3A or 3C genes are consistent with these genes being essential for latent growth-transforming B-lymphocyte infection in vitro.

ACKNOWLEDGMENTS

Wonkeun Lee and Jennifer Lee contributed valuable assistance, and George Miller generously provided the HH514-16 clone of P3HR-1.

This research was supported by Public Health Service grant CA00449 from the National Cancer Institute. B.T. was supported by postdoctoral research awards from the American Cancer Society (grant PF-3561) and the Baxter Foundation.

REFERENCES

1. **Alfieri, C., M. Birkenbach, and E. Kieff.** 1991. Early events in Epstein-Barr virus infection of human B lymphocytes. Virology **181:**595–608.
2. **Baer, R., A. Bankier, M. Biggin, P. Deininger, P. Farrell, T. Gibson, G. Hatfull, G. Hudson, S. Satchwell, C. Sequin, P. Tuggnell, and B. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr genome. Nature (London) **310:**207–211.
3. **Bird, A. G., S. Britton, J. Ernberg, and K. Nilsson.** 1981. Characteristics of Epstein-Barr virus activation of human B lymphocytes. J. Exp. Med. **154:**832–839.
4. **Bornkamm, G. W., J. Hudewentz, U. K. Freese, and U. Zimber.** 1982. Deletion of the nontransforming Epstein-Barr virus strain P3HR-1 causes fusion of the large internal repeat to the DSL region. J. Virol. **43:**952–968.
5. **Burrows, S., I. Misko, T. B. Sculley, C. Schmidt, and D. J. Moss.** 1990. An Epstein-Barr virus-specific cytotoxic T-cell epitope present on A- and B-type transformants. J. Virol. **64:**3974–3976.
6. **Burrows, S., T. Sculley, S. Misko, C. Schmidt, and D. J. Moss.** 1990. An Epstein-Barr virus-specific cytotoxic T cell epitope in EBV nuclear antigen 3 (EBNA 3). J. Exp. Med. **171:**345–349.
7. **Cohen, J. I., F. Wang, and E. Kieff.** 1991. Epstein-Barr virus nuclear protein 2 mutations define essential domains for transformation and transactivation. J. Virol. **65:**2545–2554.
8. **Cohen, J. I., F. Wang, J. Mannick, and E. Kieff.** 1989. Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. Proc. Natl. Acad. Sci. USA **86:**9558–9562.
9. **Countryman, J., H. Jenson, R. Seibl, H. Wolf, and G. Miller.** 1987. Polymorphic proteins encoded within BZLF1 of defective and standard Epstein-Barr viruses disrupt latency. J. Virol. **61:**3672–3679.
10. **Dambaugh, T., C. Beisel, M. Hummel, W. King, S. Fennewald, A. Cheung, M. Heller, N. Raab-Traub, and E. Kieff.** 1980. EBV DNA. VII. Molecular cloning and detailed mapping of EBV (B95-8) DNA. Proc. Natl. Acad. Sci. USA **77:**2999–3003.
11. **Dambaugh, T., K. Hennessy, L. Chamnaukit, and E. Kieff.** 1984. U2 region of Epstein-Barr virus may encode Epstein-Barr nuclear antigen 2. Proc. Natl. Acad. Sci. USA **81:**7632–7636.
12. **Gardella, T., P. Medveczky, T. Sairenji, and C. Mulder.** 1984. Detection of circular and linear herpesvirus DNA molecules in mammalian cells by gel electrophoresis. J. Virol. **50:**248–254.
13. **Geballe, A., and E. Mocarski.** 1988. Translational control of cytomegalovirus gene expression is mediated by upstream AUG codons. J. Virol. **62:**3334–3340.
14. **Gordon, J., L. Walker, G. Guy, G. Brown, M. Rowe, and A. Rickinson.** 1986. Control of human B-lymphocyte replication. II. Transforming Epstein-Barr virus exploits three distinct viral signals to undermine three separate control points in B cell growth. Immunology **58:**591–595.
15. **Hammerschmidt, W., and B. Sugden.** 1989. Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. Nature (London) **340:**393–397.

16. **Heller, M., T. Dambaugh, and E. Kieff.** 1981. Epstein-Barr virus DNA. IX. Variation among viral DNAs from producer and nonproducer infected cells. J. Virol. **38:**632–648.
17. **Henle, W., V. Diehl, G. Kohn, H. zur Hausen, and G. Henle.** 1967. Herpes type-virus and chromosome marker in normal leukocytes after growth with irradiated Burkitt cells. Science **157:**1064–1065.
18. **Hennessy, K., and E. Kieff.** 1985. A second nuclear protein is encoded by EBV in latent infection. Science **227:**1238–1240.
19. **Hennessy, K., F. Wang, E. Woodland-Bushman, and E. Kieff.** 1986. Definitive identification of a member of the Epstein-Barr virus nuclear protein 3 family. Proc. Natl. Acad. Sci. USA **79:**5693–5697.
20. **Heston, L., M. Rabson, N. Brown, and G. Miller.** 1982. New Epstein-Barr virus variants from cellular subclones of P3J-HR-1 Burkitt lymphoma. Nature (London) **295:**160–163.
21. **Jeang, K.-T., and S. D. Hayward.** 1983. Organization of the Epstein-Barr virus DNA molecule. III. Location of the P3HR-1 deletion junction and characterization of the *Not*I repeat units that form part of the template for an abundant 12-*O*-tetradecanoylphorbol-13-acetate-induced mRNA transcript. J. Virol. **48:**135–148.
22. **Joab, I., D. Rowe, M. Bodescot, J. Niccolas, P. Farrell, and M. Perricaudet.** 1987. Mapping the gene coding for Epstein-Barr virus-determined nuclear antigen EBNA 3 and its transient overexpression in a human cell line by using an adenovirus expression vector. J. Virol. **61:**3340–3344.
23. **Kerdiles, B., D. Walls, H. Triki, M. Perricaudet, and I. Joab.** 1990. cDNA cloning and transient expression of the Epstein-Barr virus-determined nuclear antigen EBNA3B in human cells and identification of novel transcripts from its coding region. J. Virol. **64:**1812–1816.
24. **Kieff, E., and D. Liebowitz.** 1990. Epstein-Barr virus and its replication, p. 1889–1920. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsh, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), Virology, 2nd ed. Raven Press, New York.
25. **Kieff, E., F. Wang, M. Birkenbach, J. Cohen, J. Sample, B. Tomkinson, S. Swaminathan, R. Longnecker, A. Marchini, J. Mannick, S. Tsang, C. Sample, and M. Kurilla.** 1991. Molecular biology of lymphocyte transformation by Epstein-Barr virus, p. 563–576. *In* J. Brugge, T. Curran, E. Harlow, and F. McCormack (ed.), Origins of human cancer: a comprehensive review. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **King, W., T. Dambaugh, M. Heller, J. Dowling, and E. Kieff.** 1982. Epstein-Barr virus DNA. XII. A variable region of the EBV genome is included in the P3HR-1 deletion. J. Virol. **43:**979–986.
27. **Kozak, M.** 1981. Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acids Res. **9:**5233–5252.
28. **Kozak, M.** 1987. Effects of intercistronic length on the efficiency of reinitiation by eukaryotic ribosomes. Mol. Cell. Biol. **7:**3438–3445.
29. **Kozak, M.** 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. **266:**19867–19870.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Mann, K. P., D. Staunton, and D. A. Thorley-Lawson.** 1985. Epstein-Barr virus-encoded protein found in plasma membranes of transformed cells. J. Virol. **55:**710–720.
32. **Mannick, J. B., J. I. Cohen, M. Birkenbach, A. Marchini, and E. Kieff.** 1991. The Epstein-Barr virus nuclear protein encoded by the leader of the EBNA RNAs is important in B-lymphocyte transformation. J. Virol. **65:**6826–6837.
33. **Marchini, A., B. Tomkinson, J. I. Cohen, and E. Kieff.** 1991. BHRF1, the Epstein-Barr virus gene with homology to Bcl2, is dispensable for B-lymphocyte transformation and virus replication. J. Virol. **65:**5991–6000.
34. **Masucci, M. G., R. Szigeti, I. Ernberg, C.-P. Hu, S. Torsteinsdottir, R. Frade, and E. Klein.** 1987. Activation of B lymphocytes by Epstein-Barr virus/CR2 receptor interaction. Eur. J. Immunol. **17:**815–820.
35. **Menezes, J., W. Leibold, and G. Klein.** 1975. Biological differences between Epstein-Barr virus (EBV) strains with regard to lymphocyte transforming ability, superinfection and antigen induction. Exp. Cell Res. **92:**478–484.
36. **Menezes, J., W. Leibold, G. Klein, and G. Clements.** 1975. Establishment and characterization of an Epstein-Barr virus (EBV)-negative lymphoblastoid B cell line (BJA-B) from an exceptional, EBV-genome-negative African Burkitt's lymphoma. Biomedicine **22:**276–284.
37. **Miller, G., and N. Lipman.** 1973. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. Proc. Natl. Acad. Sci. USA **70:**190–194.
38. **Miller, G., J. Robinson, L. Heston, and M. Lipman.** 1974. Differences between laboratory strains of Epstein-Barr virus based on immortalization, abortive infection, and interference. Proc. Natl. Acad. Sci. USA **71:**4006–4010.
39. **Miller, G., T. Shope, H. Lisco, D. Stitt, and M. Lipman.** 1972. Epstein-Barr virus: transformation, cytopathic changes, and viral antigens in squirrel monkey and marmoset leukocytes. Proc. Natl. Acad. Sci. USA **69:**383–387.
39a. **Moss, D., et al.** Unpublished data.
40. **Murray, R., M. Kurilla, H. M. Griffin, J. M. Brooks, M. Mackett, J. A. Arrand, S. R. Burrows, D. J. Moss, E. Kieff, and A. B. Rickinson.** 1990. Human cytotoxic T-cell responses against Epstein-Barr virus nuclear antigens demonstrated by using recombinant vaccinia virus. Proc. Natl. Acad. Sci. USA **87:**2906–2910.
40a. **Murray, R., et al.** Unpublished data.
41. **Petti, L., and E. Kieff.** 1988. A sixth Epstein-Barr virus nuclear protein (EBNA3B) is expressed in latently infected growth-transformed lymphocytes. J. Virol. **62:**2173–2178.
42. **Petti, L., C. Sample, and E. Kieff.** 1990. Subnuclear localization and phosphorylation of Epstein-Barr virus latent infection nuclear proteins. Virology **176:**563–574.
43. **Petti, L., J. Sample, F. Wang, and E. Kieff.** 1988. A fifth Epstein-Barr virus nuclear protein is expressed in latently infected growth-transformed lymphocytes. J. Virol. **62:**1330–1338.
44. **Polack, A., H. Delius, U. Zimber, and G. Bornkamm.** 1984. Two deletions in the Epstein-Barr virus genome of the Burkitt lymphoma nonproducer line, Raji. Virology **133:**146–157.
45. **Pope, J. H., B. Achong, and M. Epstein.** 1968. Cultivation and pure structure of virus bearing lymphoblasts from 2nd, N. G. Burkitt lymphoma. Int. J. Cancer **3:**171–182.
46. **Raab-Traub, N., R. Pritchett, and E. Kieff.** 1978. DNA of Epstein-Barr virus. III. Identification of restriction enzyme fragments which contain DNA sequences which differ among strains of EBV. J. Virol. **27:**388–398.
47. **Rabson, M., L. Gradoville, L. Heston, and G. Miller.** 1982. Nonimmortalizing P3J-HR-1 Epstein-Barr virus: a deletion mutant of its transforming parent, Jijoye. J. Virol. **44:**834–844.
48. **Rickinson, A., L. Young, and M. Rowe.** 1987. Influence of the Epstein-Barr virus nuclear antigen EBNA 2 on the growth phenotype of virus-transformed B cells. J. Virol. **61:**1310–1317.
49. **Ricksten, A., B. Kallin, H. Alexander, J. Dillner, R. Fahraeus, G. Klein, R. Lerner, and L. Rymo.** 1988. BamH1 E region of the Epstein-Barr Virus genome encodes three transformation associated nuclear proteins. Proc. Natl. Acad. Sci. USA **85:**995–999.
50. **Rowe, M., L. Young, K. Cadwallader, L. Petti, E. Kieff, and A. Rickinson.** 1989. Distinction between Epstein-Barr virus type-A (EBNA 2A) and type-B (EBNA-2B) isolates extends to the EBNA 3 family of nuclear proteins. J. Virol. **63:**1031–1039.
51. **Sample, J., and E. Kieff.** 1990. Transcription of the Epstein-Barr virus genome during latency in growth-transformed lymphocytes. J. Virol. **64:**1667–1674.
52. **Sample, J., L. Young, B. Martin, T. Chatman, E. Kieff, A. Rickinson, and E. Kieff.** 1990. Epstein-Barr virus types 1 and 2 differ in their EBNA-3A, EBNA-3B, and EBNA-3C genes. J. Virol. **64:**4084–4092.
53. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequenc-

ing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

54. **Shimizu, N., M. Yamaki, S. Sakuma, Y. Ono, and K. Takada.** 1988. Three Epstein-Barr virus (EBV)-determined nuclear antigens induced by the BamHI E region of EBV DNA. Int. J. Cancer **41:**744–751.
55. **Skare, J., J. Farley, J. L. Strominger, K. O. Fresen, M. S. Cho, and H. zur Hausen.** 1985. Transformation by Epstein-Barr virus requires sequences in the region of *Bam*HI fragments Y and H. J. Virol. **55:**286–297.
56. **Swaminathan, S., B. Tomkinson, and E. Kieff.** 1991. Recombinant Epstein-Barr virus with small RNA (EBER) genes deleted transforms lymphocytes and replicates *in vitro*. Proc. Natl. Acad. Sci. USA **88:**1546–1550.
57. **Thorley-Lawson, D. A., and K. P. Mann.** 1985. Early events in Epstein-Barr virus infection provide a model for B cell activation. J. Exp. Med. **162:**45–49.
58. **Tomkinson, B., and E. Kieff.** 1992. Second-site homologous recombination in Epstein-Barr virus: insertion of type 1 EBNA 3 genes in place of type 2 has no effect on in vitro infection. J. Virol. **66:**780–789.
59. **van Santen, V., A. Cheung, and E. Kieff.** 1981. Epstein-Barr virus RNA: size and direction of transcription of virus specific cytoplasmic RNAs in a transformed cell line. Proc. Natl. Acad. Sci. USA **78:**1930–1934.
60. **Walker, L., G. Guy, G. Brown, A. Milner, and J. Gordon.** 1986. Control of human B lymphocyte replication. I. Characterization of novel activation stages which proceed the entry of B cells into cycle. Immunology **58:**583–589.
61. **Wang, F., C. Gregory, C. Sample, M. Rowe, D. Liebowitz, R. Murray, A. Rickinson, and E. Kieff.** 1990. Epstein-Barr virus latent membrane protein (LMP1) and nuclear protein 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. J. Virol. **64:**2309–2318.
62. **Wang, F., L. Petti, D. Braun, S. Seung, and E. Kieff.** 1987. A bicistronic Epstein-Barr virus mRNA encodes two nuclear proteins in latently infected, growth-transformed lymphocytes. J. Virol. **61:**945–954.