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Deletion of DNA Encoding the First Five Transmembrane Domains of Epstein-Barr Virus Latent Membrane Proteins 2A and 2B

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A recombinant Epstein-Barr virus (EBV) was constructed, with a positive-selection marker inserted at the site of a deletion of a DNA segment which encodes the first five transmembrane domains of LMP2A and LMP2B. Despite the mutation, the mutant recombinant EBV was able to initiate and maintain primary B-lymphocyte growth transformation in vitro. Cells transformed with the mutant recombinant were not different from wild-type virus transformants in initial or long-term outgrowth, sensitivity to limiting cell dilution, or serum requirement. Expression of EBNA1, EBNA2, EBNA3A, EBNA3C, and LMP1 and permissivity for lytic EBV infection were also unaffected by the LMP2 deletion mutation. These results complete the molecular genetic studies proving LMP2 is dispensable for primary B-lymphocyte growth transformation, latent infection, and lytic virus replication in vitro.

Of the nine Epstein-Barr virus (EBV) proteins expressed in latently infected growth-transformed lymphocytes, EBNA1, LMP1, and LMP2A or LMP2B are regularly expressed in Burkitt's lymphoma, nasopharyngeal carcinoma, or Hodgkin's disease tumor biopsies (2, 4, 8, 10, 20, 23–25, 31, 32, 38). EBNA1 is essential for EBV episome maintenance in latently infected cells (37). LMP1 is a key mediator of EBV effects on cell growth (1, 33–35). LMP2A associates with LMP1 and with *src* family tyrosine kinases (3, 13), is a tyrosine kinase substrate (13), and down modulates the effects of transmembrane signalling on calcium mobilization (19).

The LMP2 gene is simultaneously transcribed under the control of two promoters separated by 3 kb (Fig. 1, line 1) (11, 12, 29). The two LMP2 mRNAs have different 5′ exons followed by eight common exons (Fig. 1, line 2) (11, 12, 29). The LMP2A-predicted primary amino acid sequence includes 119 amino acids encoded by the LMP2A unique 5′ exon, 12 hydrophobic domains of at least 16 amino acids, each of which is likely to traverse a membrane, and a 27-amino-acid carboxyl-terminal domain. Both the amino and carboxyl termini are predicted to reside in the cytoplasm. The LMP2B first exon is not translated, and LMP2B initiates at a methionine which is 120 amino acids into LMP2A.

The purpose of the experiments reported here was to complete the formal EBV recombinant molecular genetic analysis of LMP2's role in lymphocyte infection in vitro. Deletion of the entire LMP2 gene from EBV recombinants has not been possible since the LMP2 gene is more than 12 kbp long and it spans the termini which are essential *cis*-acting elements for packaging of virions. Two specifically mutated LMP2 recombinants had been made to assess the importance of LMP2 in lymphocyte infection and subsequent growth transformation. One mutation inserted a nonsense codon after LMP2A codon 19 (15), thereby interrupting LMP2A expression. The second inserted a nonsense codon after the fifth transmembrane domain common to LMP2A and LMP2B (16), thereby truncating LMP2A and LMP2B after the fifth transmembrane domain. Neither mutation had a discernible effect on the ability of the mutant EBV recombinant to infect, transform, or replicate in primary B lymphocytes. In the experiments described here, a DNA segment which encodes the first five transmembrane domains was deleted, the only part of LMP2 not affected by the previous mutations. The effect of the mutation on the ability of EBV to infect, growth transform, and replicate in primary B lymphocytes was then evaluated.

Since the mutations affecting the rest of LMP2 had no effect on primary lymphocyte infection in vitro, deletion of DNA which encodes the first five transmembrane domains was likely to result in a virus which was still able to transform primary B lymphocytes. A marker selection experiment was therefore attempted with primary B-lymphocyte infection. A cloned EBV DNA fragment containing LMP2 genomic DNA was mutated by the deletion of a 643-bp *Eco*RI-to-*Sal*I fragment (Fig. 1, line 3). The deletion removes the second exon, the third exon, and a portion of the fourth exon common to both LMP2A and LMP2B (Fig. 1, lines 2 and 3). The deleted DNA encodes the first five transmembrane domains of LMP2A and LMP2B. The deleted DNA segment was replaced with the drug resistance gene, hygromycin phosphotransferase, under the control of the simian virus 40 (SV40) promoter. The cloned fragment was then transfected into B95-8 cells, a marmoset cell line latently infected with EBV (21, 22). The EBV Z immediate-early transactivator under the control of the SV40 promoter (pSVNaeZ [30]) was included in the transfection to induce lytic infection (5, 7, 27). Cell-free virus was prepared from the transfected cells and used to infect primary B lymphocytes (15, 16). The infected primary B lymphocytes were

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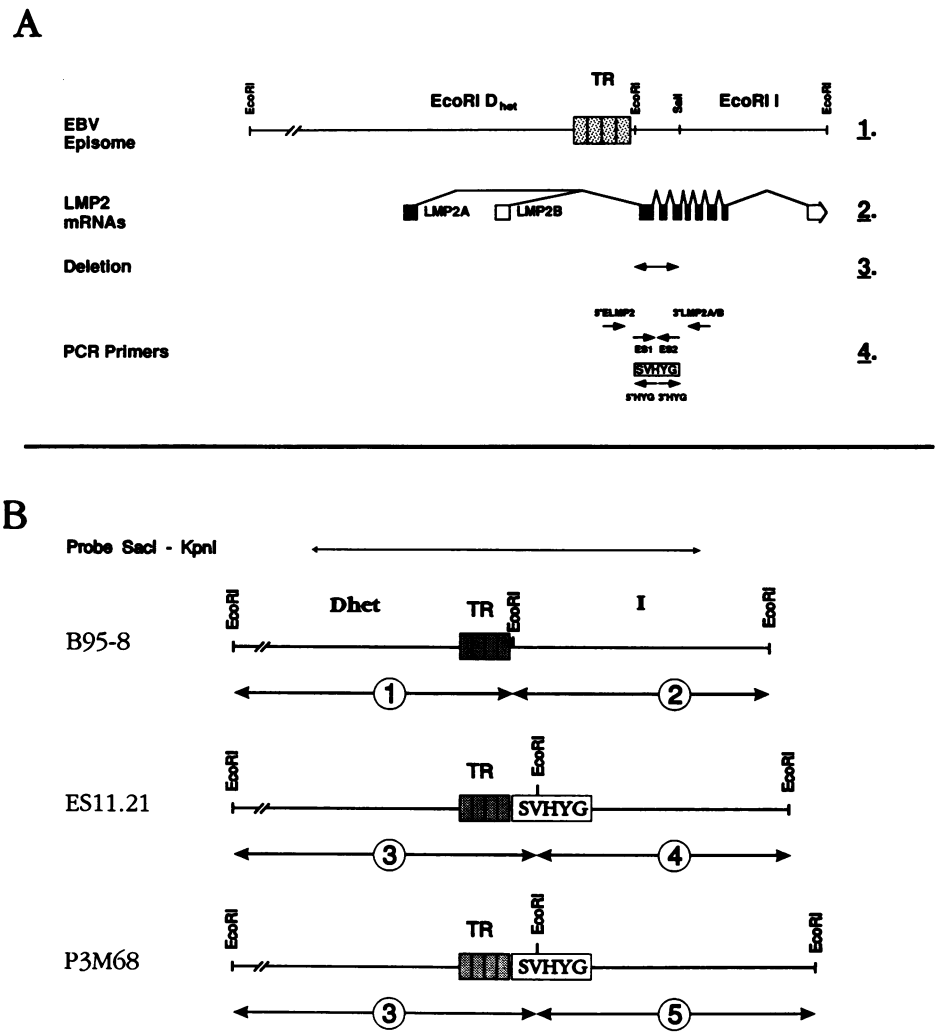

FIG. 1. Schematic representations of the EBV LMP2A and LMP2B genes. (A) (Line 1) The EBV episome circularized through joining of the terminal repeats (TR). The rightmost *Eco*RI fragment, Dhet, and the leftmost terminal fragment, I, are indicated. (Line 2) The spliced LMP2A and LMP2B mRNAs are shown. Open exons do not encode protein. (Line 3) The LMP2 deletion in plasmid pLMP2ES is depicted. (Line 4) The primers used in PCR analysis are indicated. (B) Arrangement of the terminal repeat region in B95-8, ES11.21-, or P3M68-infected cells, indicating the sites for the insertion of the hygromycin (HYG) gene under the control of the SV40 promoter. Insertion of the SV40 HYG cassette results in destruction of both the *Eco*RI and *Sal*I sites in the ES11.21 construct and the *Eco*RI site in the P3M68 construct. The *Sac*I-to-*Kpn*I fragment used as a probe in the experiment shown in Fig. 3 is above. The expected *Eco*RI restriction fragments for each virus are numbered and correspond to those shown the Southern blot in Fig. 3.

plated in microwells (15, 16) and incubated with a toxic level of hygromycin B (50 μg/ml). Eleven clones emerged, and the presence of the hygromycin gene was confirmed by Southern blot and polymerase chain reaction (PCR) analysis (data not shown). One clone, ES11 was readily reactivated to permissivity for lytic EBV infection by pSVNaeZ transfection and treatment with tetradecanoyl phorbol acetate (15, 16). Cell-free virus from ES11 was used to infect primary B lymphocytes. Clones were again selected and expanded as described above. One clone, ES11.21, readily grew in hygromycin and was easily activated into lytic virus infection and was therefore further analyzed.

The primary clone ES11.21 and passages from this clone were subjected to PCR analysis as shown in Fig. 2. Passages are indicated by a period. To demonstrate that the SV40 hygromycin gene had been inserted into the appropriate site in the EBV genome, two primers specific for the hygromycin cassette were synthesized. Both primer 5'HYG, TGC AGCGCCACTCAA, and primer 3'HYG, TCAGCCAG CAACTCG, point outwardly from the hygromycin gene (Fig. 1, line 4) and should amplify fragments only when paired with primers containing the adjacent EBV DNA. In amplifications with the primers 5'HYG and 5'ELMP2, GGTCGGATTTCGCCCTTATT, a 656-bp product was detected only in lymphoblastoid cell lines (LCLs) infected with the LMP2ES deletion recombinant and not in wild-type B95-8-infected LCLs (LCL1), P3HR1-16 infected cells, BJAB cells, or reactions containing only primers and no cell DNA (Fig. 2A). Similarly, using the primers 3'HYG and 3'LMP2A/B (16), a 304-bp product was detected only in LCLs infected with the LMP2ES deletion recombinants (Fig. 2B). As expected, this product was not apparent in control amplifications with wild-type EBV DNA (Fig. 2B). To verify that LMP2ES mutant-infected LCLs were not

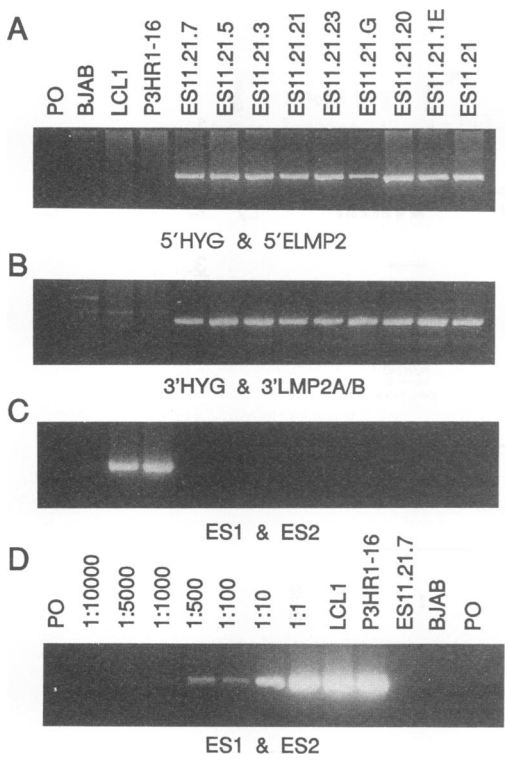

FIG. 2. Ethidium bromide-stained PCR products from wild-type- and LMP2 mutant recombinant-infected LCLs (15). (A) PCR products using a primer at the 5′ side of the SV40 hygromycin (5′ HYG) selectable marker and an EBV-specific primer 5′ of the *Eco*RI site at bp 1 of the B95-8 sequence. BJAB, LCL1, and P3HR1-16 lack SV40 HYG DNA. The LMP2ES mutant recombinant-infected cells contain the expected amplified product of 656 bp. (B) PCR products using a primer at the 3′ side of the SV40 HYG selectable marker and an EBV-specific primer 3′ of the *Sal*I site at bp 643 of the B95-8 sequence. BJAB, LCL1, and P3HR1-16 lack the SV40 HYG selectable marker. Cell lines infected with the ES11.21 mutant all contain the expected amplified 304-bp product. (C) PCR products using primers ES1 and ES2 within the *Eco*RI-to-*Sal*I deletion. The wild-type 240-bp product is observed in LCL1 or P3HR1-16 DNA and not from ES11.21-infected cell lines. (D) To assess the sensitivity of detection of wild-type DNA using the ES1 and ES2 primers, dilutions of 10^5 wild-type-infected LCL DNA with EBV-negative BJAB cells were added to 10^5 LMP2ES-infected LCLs. PO indicates control with primers only and no cell DNA.

coinfected with wild-type B95-8 genomes, ES11.21-infected LCL DNA was amplified with primers specific for the DNA deleted from ES11.21 (Fig. 1) (ES1, CATTTACAATGCAT GATGTT; ES2, TAAAGTTGACGTCATGCCAA). As expected, a 240-bp fragment was detected in B95-8-infected LCLs (LCL1) or in P3HR1-16-infected cells but not in LCLs infected with the LMP2ES deletion recombinants (Fig. 2C). PCR with these primers is sufficiently sensitive to detect 1 in 500 wild-type LMP2 DNAs (Fig. 2D).

The LMP2ES deletion and hygromycin marker insertion were further confirmed by Southern blot hybridization with DNA from cells infected with ES11.21-derived virus. Duplicate DNA samples of DNA from representative mutant- or wild-type-infected LCLs were cut with *Eco*RI, electrophoresed in agarose gels, transferred, and hybridized with probes from the *Sac*I-to-*Kpn*I fragment of EBV DNA (Fig. 1B) or from the hygromycin resistance gene (36). The resulting autoradiograms are shown in Fig. 3. The expected *Eco*RI fragments are numbered in Fig. 1B, and the fragments are numerically designated in Fig. 3. The wild-type 12.9-kbp *Eco*RI D_{het} (Fig. 1B and 3A, band 1) and 4.2-kbp *Eco*RI I (Fig. 1B and 3A, band 2) DNA fragments were detected with the *Sac*I-to-*Kpn*I probe in wild-type EBV-infected LCL (LCL1) or P3HR1-16 cells. *Eco*RI D_{het} can vary in size because of various numbers of the terminal repeat sequences found at the right end of this fragment (Fig. 1, line 1). As expected, the hygromycin probe did not hybridize to DNA from these two cell lines (Fig. 3B). In the LMP2ES mutant-infected LCLs, the *Eco*RI I fragment was 430 bp larger because of the deletion of 643 bp between the *Eco*RI and *Sal*I sites and the addition of 1,073 bp from hygromycin DNA (Fig. 1B and 3A, band 4). As expected, the hygromycin and the EBV *Sac*I-to-*Kpn*I probes both hybridized to this fragment (Fig. 1B and 3A and B, band 4). No fragment of wild-type size was visible even upon longer exposure of the blot (data not shown). Because of the large size of the *Eco*RI D_{het} fragment, the addition of 840 bp from the SV40 promoter and the 5′ part of the hygromycin DNA is not noticeable (Fig. 1B and 3A and B, band 3). As a control, a previously described EBV recombinant, P3M68.2 (17), containing an insertion of the SV40 promoter and hygromycin gene into the *Eco*RI site in the same orientation as the LMP2ES mutation, was digested and probed in parallel. As expected, the DNA fragment corresponding to *Eco*RI I from this strain was 643 bp larger than the *Eco*RI I fragment from the LMP2ES mutants and 1,716 bp larger than wild-type *Eco*RI I since the *Eco*RI-to-*Sal*I fragment of *Eco*RI I was not deleted (Fig. 1B and 3A and B, band 5). Also as expected, the *Sac*I-to-*Kpn*I fragment and the hygromycin probe hybridized to this band (Fig. 3A and B, band 5). The probes did not hybridize to fragments in the EBV-negative B-lymphoma cell line, BJAB (Fig. 3).

The absence of LMP2A in the LMP2ES mutant-infected LCLs was confirmed in immunoblots with crude membrane preparations (19), using a polyclonal affinity-purified rabbit serum reactive with the amino-terminal cytoplasmic domain of LMP2A (Fig. 4). The LMP2ES mutants lacked detectable LMP2A or LMP2A cross-reactive proteins (CRP), whereas LMP2A was readily detected in wild-type control-infected LCLs (Fig. 4, WT.111.5) or B95-8 cells (Fig. 4, lane B95-8). As expected, the affinity-purified antibody did not react with the EBV-negative BJAB cell line. The expression of LMP2B CRP in these cells could not be monitored, since the rabbit sera is only reactive with the amino-terminal domain unique to LMP2A.

The LMP2ES deletion is predicted to result in splicing of the LMP2 first exon to exon four and fusion of the amino-terminal 119-amino-acid polypeptide to 13 amino acids encoded by the fourth exon which is out of frame with LMP2A. To further explore the possibility that the 132-amino-acid polypeptide might be expressed as a CRP in LMP2ES-infected cells, whole cell lysates were solubilized in urea sample buffer (19), resolved in polyacrylamide gels, and reacted in Western immunoblots with LMP2A antibodies. Although LMP2A was detected in wild-type LCLs, no CRP of the predicted or aberrant size was detected in the LMP2ES mutant-infected cells (data not shown). The amino-terminal 119 amino acids of LMP2A are therefore probably rapidly degraded in the LMP2ES mutant-infected LCLs.

Aside from the difference in LMP2 expression in the LMP2ES mutant recombinant-infected LCLs, expression of the other EBV latent genes was identical to that of wild-type controls. A human serum sample reactive with the EBNAs

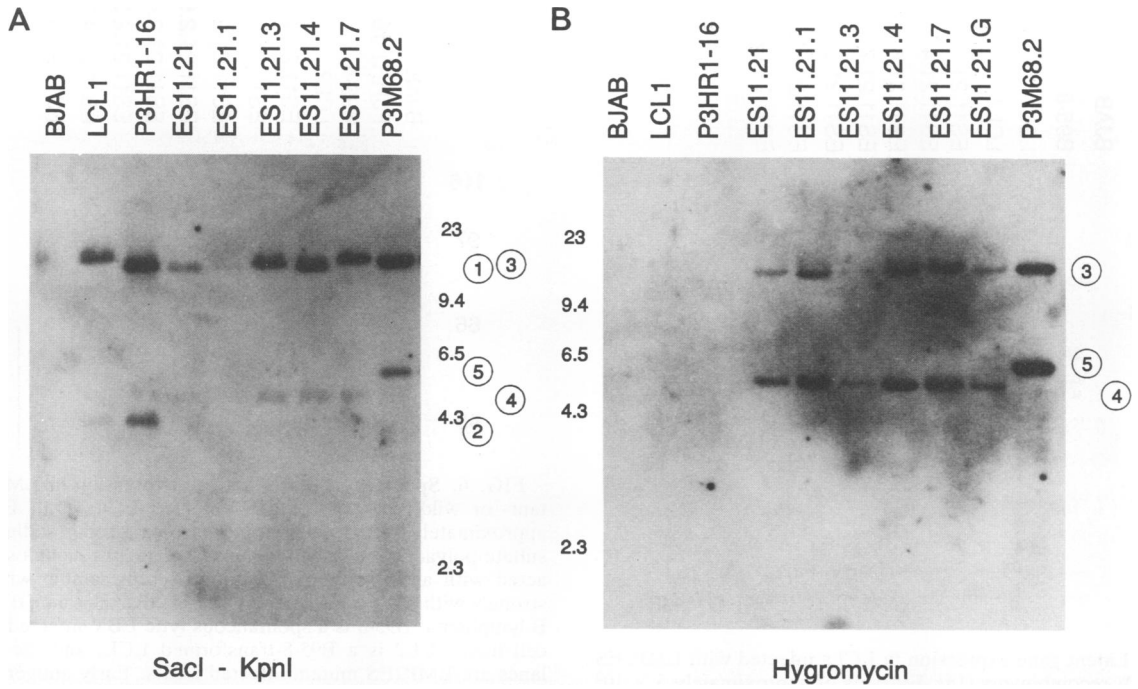

FIG. 3. Southern blot analysis of wild-type and mutant LMP2 DNA from infected LCLs, demonstrating homologous insertion of the SV40 hygromycin selectable marker into the EBV genome. DNA from representative LCLs was digested with *Eco*RI and probed with the *Sac*I-to-*Kpn*I fragment indicated in Fig. 1B (A) or with the SV40 hygromycin selectable marker (B). Circled numbers refer to predicted fragments shown in Fig. 1B. Sizes of DNA molecular size markers are indicated in kilobase pairs.

demonstrated similar EBNA1, EBNA2, EBNA3A, and EBNA3C expression in wild-type- and LMP2ES mutant-infected LCLs (Fig. 5A). EBNA3B was not detected in the wild-type control LCLs or in any of the LMP2ES mutant-infected LCLs (Fig. 5A) but was detected in the B95-8-transformed marmoset cell line (Fig. 5A). Similar results with this human serum have been observed with some other B95-8-transformed LCLs.

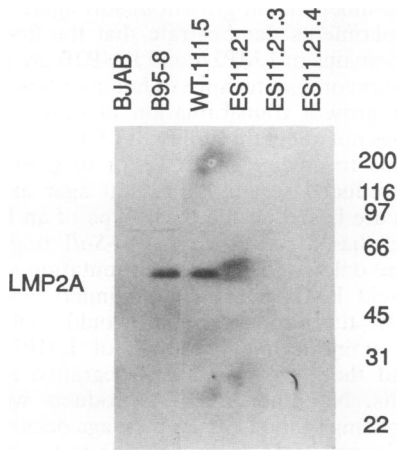

FIG. 4. LMP2A in wild-type- but not LMP2ES mutant recombinant-infected LCLs. Crude membrane preparations were prepared, solubilized, electrophoresed, transferred to nitrocellulose, and reacted to purified LMP2A antibodies as previously described (19). BJAB is an EBV-negative B-lymphoma cell line (18); B95-8 is an EBV-infected marmoset cell line (21, 22) that is partially permissive for viral replication; WT.111.5 is a wild-type-transformed LCL; and ES11.21, ES11.21.3, and ES11.21.4 are LMP2ES mutant-infected LCLs. Migration of the LMP2A protein is indicated. Sizes of protein standards in kilodaltons are indicated on the right.

LMP1 expression was similar in both wild-type control and LMP2ES mutant-infected LCLs (Fig. 5B). However D1LMP1 expression was increased in the LMP2ES mutant-infected LCLs despite their largely latent infection state. Increased D1LMP1 expression has been previously observed with EBV recombinants containing the SV40 hygromycin expression cassette in the *Eco*RI site (17) and is likely due to the effect of the SV40 enhancer sequences on the D1LMP1 promoter, which is otherwise active only in lytic infection. Anomalous D1LMP1 expression is not likely to be an effect of the LMP2 deletion since previously described LMP2 mutants which lacked the SV40 hygromycin marker were not associated with increased D1LMP1 expression (15, 16).

Spontaneous lytic gene expression in the LMP2ES mutant-infected cells was examined in Western immunoblots, using a human serum sample reactive with early lytic cycle proteins. A representative blot is shown in Fig. 6. Of 26 LMP2ES mutant-infected LCLs, 9 spontaneously expressed some early lytic antigen. This level of permissitivity is within the range observed for wild-type control-infected LCLs (data not shown).

LMP2ES mutant EBV recombinants were similar to wild-type EBV in initial transformation efficiency of primary B lymphocytes and in the growth characteristics of the transformed B lymphocytes. EBV replication was induced in the LMP2ES mutant or in wild-type control-infected LCLs. Virus was studied from LCLs which had similar levels of permissivity as assayed by EBV early antigen expression on immunoblot. Dilutions of the resultant virus were filtered

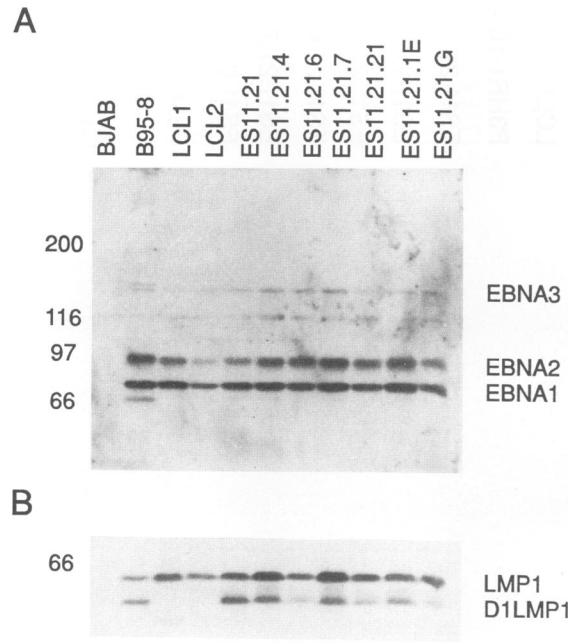

FIG. 5. Latent gene expression in LCLs infected with LMP2ES mutant EBV recombinants (15). Extracts of approximately 5×10^5 cells were separated in sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose, and blotted with either an EBV immune human serum sample (A) or the S12 monoclonal antibody directed against LMP1 (B). BJAB is an EBV-negative B-lymphoma cell line, B95-8 is an EBV-negative marmoset cell line that is partially permissive for viral replication, LCL1 and LCL2 are B95-8 EBV-transformed fetal (LCL1) and adult (LCL2) LCLs, and ES11.21s are LMP2 mutant EBV recombinants. Migration of EBNA3, EBNA2, EBNA1, LMP1 and DLMP1 and size markers in kilodaltons are indicated.

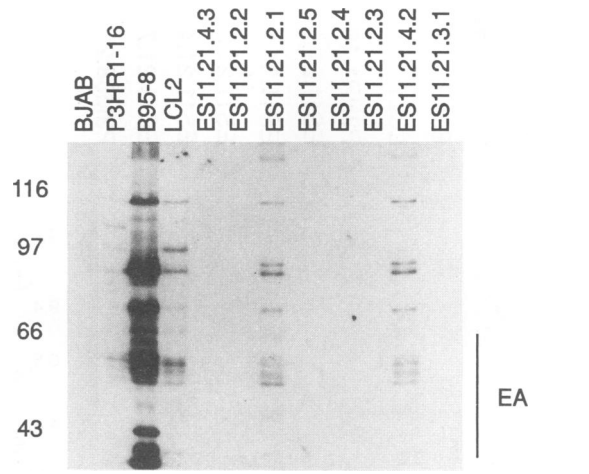

FIG. 6. Spontaneous early antigen expression in LMP2ES mutant- or wild-type control EBV-infected LCLs (15). Extracts of approximately 5×10^5 cells were electrophoresed in sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose, and reacted with an EBV human immune serum sample which reacts strongly with EBV early antigens (15, 16). BJAB is an EBV-negative B lymphoma, B95-8 is a spontaneous lytic EBV-infected marmoset cell line, LCL2 is a B95-8-transformed LCL, and the remaining lanes are LMP2ES mutant-infected LCLs. Early antigen (EA) and sizes in kilodaltons of marker proteins are indicated.

and used to infect primary B lymphocytes. The infected cells were plated into microwells to assay transforming activity. LMP2ES mutant recombinant and wild-type virus were similar in the numbers of wells positive for LCL outgrowth, in the time to first macroscopic LCL outgrowth, and in the growth characteristics of the LCLs as they were expanded into larger cultures. Three mutant-infected LCLs released 1.8×10^2, 3.0×10^2, and 4.6×10^2 virus-transforming units per percent gp350 positive cells, whereas two wild-type control LCLs released 3.8×10^2 and 5.0×10^2 virus transforming units per percent gp350 positive cells. PCR analysis of the resultant LCLs confirmed the presence of the LMP2ES deletion and insertion of the hygromycin selectable marker.

To further investigate the effect of the LMP2ES mutation on the growth of transformed LCLs, newly transformed wild-type or LMP2ES mutant recombinant virus-infected LCLs were plated at various cell concentrations and in medium supplemented with 10%, 1%, 0.1%, or 0% fetal bovine serum (Table 1). Growth was then assayed 10 days later by microscopic examination. At a seeding density of 5×10^3 cells per well, both the LMP2ES mutant and wild-type LCLs were unable to grow in medium containing less than 10% serum. At a seeding density of 2.5×10^4 cells per well, the LMP2ES mutant-infected LCLs grew slightly better in 1% and 0.1% serum than did the wild-type control-infected LCLs. At 10^5 cells per well, some of the LMP2ES mutant- and wild-type control-infected LCLs were able to grow in wells with only 0.1% serum. Additionally, anchorage-independent growth of six wild-type- and six LMP2ES-infected LCLs was assayed by plating them in soft agar over fibroblast feeder layers. Wild-type- and LMP2ES-infected LCLs had similar cloning efficiencies, as determined by the number of macroscopic colonies after 4 weeks of culture. Overall, mutant recombinant-infected LCLs were similar to wild-type LCLs in their resistance to plating at low cell density or in medium supplemented with low serum concentrations or in anchorage-independent growth in soft agar.

These experiments demonstrate that the first five transmembrane domains of LMP2A and LMP2B are not essential for the initiation or maintenance of latent or lytic lymphocyte infection or growth transformation in vitro. Further, the mutation does not affect the ability of LCLs infected with the mutated virus to emerge as LCLs or to grow at low cell density, in reduced serum, or in soft agar assays. These conclusions are based on the phenotype of an EBV recombinant which has a 643-bp *Eco*RI-to-*Sal*I fragment of the EBV genome deleted. This deletion mutation truncates the 497-amino-acid LMP2A protein at amino acid 119. This LMP2A truncation product, which would contain only the hydrophilic amino-terminal domain of LMP2A, was not detected and therefore was likely degraded in the EBV-infected cells. No other LMP2A products would be expected. If splicing of the LMP2A message occurred from the first splice donor site 5′ of the deletion to the first 3′ splice acceptor site after the deletion, the remaining LMP2A protein would not be in frame and would result in only 13 additional amino acids. Furthermore, the deletion mutation includes the first LMP2B coding exon and deletes the initiation methionine for LMP2B translation. If splicing of the LMP2B message occurred around the SV40 hygromycin insert, the first potential initiation site would be at LMP2B amino acid 205. Since antibody to the carboxyl-terminal part of LMP2 is not available, expression of this possible amino-

TABLE 1. Serum- and cell-dependent growth of LMP2A and LMP2B mutants

Cell line	Growth at following cell number and serum concentration (%)[a]											
	10^5				2.5×10^4				5×10^3			
	10	1	0.1	0	10	1	0.1	0	10	1	0.1	0
ES11.21.1	+++	++	+	−	+++	+	−	−	+++	−	−	−
ES11.21.2	+++	+++	++	+	+++	++	−	−	+++	−	−	−
ES11.21.3	+++	++	+	+	+++	+	−	−	+++	−	−	−
ES11.21.5	+++	++	+	−	+++	+	+	−	++	−	−	−
ES11.21.7	+++	+	+	+	+++	+	+	−	++	−	−	−
111.WT.5	+++	++	+	+	+++	−	−	−	−	−	−	−
111.WT.2	+++	+++	+	−	+++	−	−	−	+++	−	−	−
111.WT.3	+++	+++	++	+	+++	−	−	−	+	−	−	−
111.WT.1	++	+	−	−	+	−	−	−	−	−	−	−
111.WT.4	+++	+	−	−	+	−	−	−	+	−	−	−

[a] +++ large clumps, acidified medium, clear growth; ++ medium clumps, possible acidification of medium, probable growth; + small clumps, neutral or basic medium, little growth; − no clumps, neutral or basic medium, no growth.

terminally truncated LMP2B CRP could not be directly evaluated.

Three different mutations have been constructed in LMP2 and recombined into the viral genome. The LMP2A unique amino-terminal cytoplasmic domain (15), the last seven transmembrane domains common to both LMP2A and LMP2B (16), and the first five transmembrane domains common to LMP2A and LMP2B (this study) are each nonessential for initiation or maintenance of latent or lytic lymphocyte infection or growth transformation in vitro.

The apparent lack of in vitro phenotype of LMP2 mutant virus or LCLs infected with LMP2 mutant virus is surprising since LMP2 is consistently expressed in latent EBV infection in vitro (14). LMP2 is expressed in infected B lymphocytes in vivo (26) and in tumor tissue from patients with nasopharyngeal carcinoma, Hodgkin's disease, and EBV-related lymphoproliferative disease (2, 4, 8, 10, 20, 23–25, 31, 32, 38). Patients with nasopharyngeal carcinoma have antibodies to LMP2 (6). Cytotoxic T lymphocytes from some humans recognize LMP2 epitopes (23). These cytotoxic T lymphocytes should select against EBV genomes that express LMP2 in humans if LMP2 is not important for EBV in vivo infection. In addition, LMP2A interacts with *src* family tyrosine kinases (3), a 70-kDa protein that may be the B-cell specific *syk* kinase (3, 13), and LMP1 (13). LMP1 has been demonstrated to be essential for EBV in vitro transformation (9). Finally, LMP2A interrupts cell surface signalling through cell surface receptors in EBV-negative B cells expressing LMP2A, as tested by calcium mobilization (19). LMP2's in vivo role is likely to be related to these known biochemical properties. LMP2 may be important in modulating the effects of LMP1 or transmembrane signalling in EBV-infected B lymphocytes or epithelial cells. The LMP2-induced block in receptor-mediated calcium mobilization could provide a mechanism by which reactivation of EBV is down regulated in certain tissues in vivo. Although LCLs infected with LMP2 mutant virus reactivated similarly to wild-type controls (15, 16, and this study) in response to the Z transactivator and treatment with tetradecanoyl phorbol acetate, these in vitro stimuli to reactivation likely bypass normal stimuli which might be present in vivo. Such hypotheses will require subsequent testing in experimental models.

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