The Only Domain Which Distinguishes Epstein-Barr Virus Latent Membrane Protein 2A (LMP2A) from LMP2B Is Dispensable for Lymphocyte Infection and Growth Transformation In Vitro; LMP2A Is Therefore Nonessential

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Received 30 June 1992/Accepted 6 August 1992

Using second-site homologous recombination, Epstein-Barr virus (EBV) recombinants were constructed which carry an LMP2A mutation terminating translation at codon 19. Despite the absence of LMP2A or LMP2A cross-reactive protein, the recombinants were able to initiate and maintain primary B-lymphocyte growth transformation in vitro. EBNA1, EBNA2, and LMP1 expression was unaffected by the LMP2A mutation. The LMP2A mutant recombinant EBV-infected lymphoblastoid cell lines (LCLs) were identical to wild-type recombinant EBV-infected control LCLs with respect to initial outgrowth, subsequent growth, sensitivity to limiting cell dilution, sensitivity to low serum, and growth in soft agarose. The permissivity of LCLs for lytic EBV infection and virus replication was also unaffected by the LMP2A mutation.

Epstein-Barr virus (EBV) causes infectious mononucleosis in normal adolescents and an infected B-lymphocyte proliferative disease in immunocompromised patients or in marmosets (23, 35). EBV is also etiologically associated with African Burkitt's lymphoma, nasopharyngeal carcinoma, some forms of Hodgkin's disease, and some unusual malignancies of epithelial or lymphatic lineages (2, 14, 16, 18, 22, 23, 35, 40, 48, 49, 60). In vitro, the EBV host range is restricted to B lymphocytes, which are largely nonpermissive for virus replication. Infected lymphocytes are driven to perpetually proliferate and are tumorigenic in SCID mice, marmosets, or immunocompromised humans (28, 44, 47, 54, 62). Infected lymphocytes express a restricted repertoire of EBV gene products, including two small RNAs, six nuclear proteins (EBV nuclear antigens [EBNAs]), and two integral membrane proteins (LMP1 and LMP2) (23). These viral gene products are presumed to mediate latent infection or the associated cell growth transformation.

The objective of these studies was to investigate the role of the amino-terminal cytoplasmic domain of LMP2 in EBV infection and lymphocyte growth transformation in vitro. The LMP2 gene is simultaneously transcribed under control of two spatially separated promoters (26, 46). The two transcripts are multiply spliced, and the two resultant mRNAs differ only in their first exons (26, 46). The LMP2A first exon contains an initiator of translation which results in an amino-terminal hydrophilic sequence. The LMP2B first exon lacks a methionine codon, and translation of LMP2B initiates at a methionine codon near the beginning of the second exon, which is common to both mRNAs. This latter methionine codon immediately precedes the first of the 12 transmembrane domains. LMP2A is expressed not only in latently infected B lymphocytes but also in nasopharyngeal carcinoma tumor cells (6, 9). LMP2A is implicated in transmembrane signal transduction because of its association

with LMP1 and with cellular tyrosine kinases in lymphocyte plasma membranes (7, 29). LMP1 induces the expression of lymphocyte activation-associated molecules (27, 56-58), whereas the *src* family tyrosine kinases with which LMP2A associates are implicated in immunoglobulin-mediated signal transduction (8, 61). The amino-terminal cytoplasmic domain of LMP2A is likely to be critical to tyrosine kinase interactions, since a mutant LMP2A consisting only of the amino terminus and the first two transmembrane domains still associates with B lymphocyte *src* family tyrosine kinases.

To investigate the role of the LMP2A unique amino-terminal cytoplasmic domain in lymphocyte infection, specifically mutated EBV recombinants were constructed with a stop codon inserted in the DNA encoding codon 19 of the LMP2A open reading frame. The mutant recombinant virus was then tested for the ability to infect and alter the growth of primary B lymphocytes and for the ability to replicate in the resultant transformed cells.

MATERIALS AND METHODS

Cells. LCL1 and LCL2 are EBV-transformed fetal (LCL1) and adult (LCL2) lymphoblastoid cell lines (LCL) which are not permissive for viral replication. BJAB is an EBV-negative B-lymphoma cell line (34). The HH514-16 subclone of the original EBV-infected P3HR1 (P3JHR1) Burkitt's lymphoma cell line (a gift from G. Miller) has only EBV genomes with a single type of DNA which is deleted for a segment which encodes EBNA2 and the last two exons of EBNA1P (20). B95-8 is an EBV-infected marmoset cell line that is partially permissive for viral replication (36, 38). Primary human mononuclear cells were obtained from blood of healthy donors by centrifugation over a cushion of Ficoll-Paque (Pharmacia). For EBV-positive donors, the mononuclear cells were depleted by rosetting mononuclear cell preparations with sheep erythrocytes treated with 2-aminoethyl isothiuronium bromide (Sigma) as previously de-

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FIG. 1. Schematic representation of the locations of the EBV LMP2A and LMP2B genes in the EBV genome. In line 1, the LMP2 genes begin in unique region U5 at the right end of the linear virion DNA and are transcribed across the terminal repeats (TR) into unique region U1. Line 2 depicts the splicing which results in the LMP2A and LMP2B mRNAs. Open exons represent noncoding segments of the mRNA; solid exons represent coding regions. Line 3 indicates the locations of primers used in PCR analysis. Lines 4 and 5 depict the EBV DNA in plasmid clones used for construction of the LMP2A mutants. Relevant restriction sites are as shown. The nonsense linker which contains an *Xba*I site was inserted into the unique *Bam*HI site of pRL49 by blunt-end ligation, resulting in destruction of the *Bam*HI site. In line 6, the *Bgl*III fragment used as a probe in Fig. 3 is indicated.

scribed (32). Mononuclear cells were used without further purification. All LCLs created in this study were maintained in RPMI 1640 medium supplemented with 15% inactivated fetal bovine serum and gentamicin (2 μ g/ml). All other cell lines were maintained in RPMI 1640 medium supplemented with 10% inactivated fetal bovine serum and gentamicin (2 μ g/ml).

Construction of plasmids and cosmids. pSVNacZ, used to induce lytic EBV replication, and *Eco*RI-A, used to rescue the EBNA2 and EBNA1 mutations contained in the P3HR1 cell line, were described previously (32, 51). pRL49 contains a *Sa*I (B95-8 bp 155773)-to-*Eco*RI (B95-8 bp 1) fragment from B95-8 EBV DNA and has a small deletion from bp 168399 to 168480. This spontaneous 81-bp deletion removes 27 codons from the large DNA repeat which encodes a repeat domain near the carboxy terminus of LMP1. A stop codon was introduced in codon 19 of LMP2A (pLMP2A19S) by cloning an *Xba*I linker (CTAGTCTAGAC TAG) containing stop codons in all three frames into the unique *Bam*HI site of pRL49. The *Bam*HI ends were first filled in with the Klenow fragment of DNA polymerase I.

Transfections. All DNA used for transfections was banded twice on CsCl equilibrium density gradients. For generating recombinant genomes in P3HR1 cells, 2×10^7 cells were resuspended at room temperature in 0.4 ml of complete culture medium containing 25 μ g of supercoiled pSVNacZ BZLF1 expression plasmid, 5 μ g of the *Eco*RI-A cosmid, and 25 μ g of pLMP2A19S and transferred to an electroporation cuvette having a 0.4-cm electrode gap (Bio-Rad). The cells were then pulsed once with a Gene Pulser (Bio-Rad) set at 210 V and 960- μ F capacitance. The cells were immediately diluted in 10 ml of complete RPMI medium. EBV replication was induced in LCLs with pSVNacZ in a similar fashion but with a 240-V pulse. The LCLs were then diluted in 12-O-tetradecanoylphorbol-13-acetate (TPA) per ml.

Infection of primary cells. Cell-free supernatant virus was

prepared from transfected P3HR1 cells or LCLs by one freeze-thaw cycle, sonication, centrifugation of cell debris, and filtration through a 0.45- μ m-pore-size filter. Virus in the filtered supernatant was used to infect purified mononuclear cells at 37°C for 1 to 2 h. Cells were infected with 2 ml of virus, virus diluted 1:10, or virus diluted 1:100. Infected cells were pelleted and suspended in 15 ml of RPMI 1640 medium with 15% serum, and 0.15-ml aliquots were distributed per well into one flat-bottom 96-well culture plate (5×10^4 to 10×10^4 cells per well). Cultures were fed with 0.1 ml of fresh medium 10 to 14 days after initial plating. LCLs emerged at between 3 and 5 weeks after plating.

PCR analysis and sequencing of PCR-amplified DNA. Three LMP2A oligonucleotides (see Fig. 1, line 3, for location and orientation) were synthesized on an Applied Biosystems synthesizer and purified. Oligonucleotide 5'LMP2A (CTGC TGCAGCTATGGGGTCC) corresponds to bases 166550 to 166569 of the B95-8 sequence. Oligonucleotides 3'LMP2A (ATTAGATTCACGTTCTCAT) and WTLMP2A (CGT ACCCATCCGGATCCCCG) are complementary to bases 166700 to 166719 and bases 166611 to 166630, respectively, of the B95-8 sequence. Genomic DNA for polymerase chain reaction (PCR) analysis was prepared as described previously (51). Twenty-five-microliter PCR reactions were amplified for 35 or 40 cycles with annealing at 55°C for the primer pair 5'LMP2A-3'LMP2A and annealing at 58°C for the primer pair 5'LMP2A-WTLMP2A. PCR reaction products were electrophoresed in a mixture of 1% Seakem ME and 3% Nu-Sieve agarose (FMC Corp.) in 1 $\times$ Tris acetate buffer.

The integrity of the linker stop codons in recombinant virus-transformed LCLs was verified by sequencing gel-purified PCR-amplified products with a kit purchased from Pharmacia.

Assays of growth of EBV-infected cells. EBV-infected LCLs at early passage were counted and resuspended at various concentrations in fresh complete medium or in

medium supplemented with lower concentrations of fetal calf serum; 0.1-ml aliquots of each dilution were plated in a 96-well tissue culture plate. Growth was scored 10 days after plating by noting acidification, cell number, and cell clumping. Growth in soft agar was assayed in 12-well tissue culture dishes as previously described (50). Macroscopic growth was scored at 4 weeks.

Additional techniques and reagents. Positive-pressure Southern transfer (Stratagene) onto GeneScreen Plus (DuPont), hybridization to cloned ^{32}P -labeled EBV DNA, autoradiography, sodium dodecyl sulfate-polyacrylamide gel analysis, and immunoblotting were done as previously described (1, 30, 59). Affinity-purified polyclonal antibodies against LMP2A were as described previously (30). S12 is a monoclonal antibody directed against LMP1 (31).

RESULTS

Construction of LMP2A mutant EBV recombinants. The strategy for deriving mutant LMP2A recombinant EBV utilizes P3HR1, a cell line infected with a transformation-incompetent, replication-competent EBV deletion mutant (5, 19, 21, 24, 33, 37, 43). P3HR1 is deleted for a DNA fragment encoding the last two EBNA2 exons and the EBNA2 exon. Transfection of cells infected with P3HR1 EBV with a cloned wild-type EBV DNA fragment spanning the deletion, such as *EcoRI*-A used here, and induction of lytic replication result in a low but reproducible frequency of homologously recombinant progeny virus that is restored for the previously deleted DNA and is now able to transform primary B lymphocytes (12, 17). A distal cloned EBV DNA fragment transfected with *EcoRI*-A into such cells recombines with the recombinant genomes with a relatively high frequency. As many as 12% of the recombinants are double recombinants (52, 53). The EBV DNA fragment carrying the LMP2A mutation was therefore cotransfected with *EcoRI*-A into P3HR1 cells with the expectation that recombined P3HR1 genomes restored for EBNA2 might also incorporate the LMP2A mutation. If the LMP2A mutation does not adversely affect the ability of the recombinant virus to transform primary B lymphocytes, transformed LCLs infected with the doubly recombinant virus should result. Since a vast excess of nonrecombinant P3HR1 is also produced in such an experiment, the recombinant virus-infected primary B lymphocytes are often initially coinfecting with nonrecombinant P3HR1 virus (52, 53). The nonrecombinant P3HR1 virus could functionally transcomplement an EBNA2(WT) [wild type]-EBNA2(WT)-LMP2A stop codon mutant recombinant virus if LMP2A is essential to growth transformation.

A cloned EBV DNA fragment extending from the *SalI* site at bp 155773 of the B95-8 sequence across the terminal repeat of the viral genome to the *EcoRI* site at bp 1 of the B95-8 sequence (Fig. 1, line 4, pRL49) was used to construct a mutant LMP2A with a nonsense linker cloned into a unique *BamHI* site (Fig. 1, line 5, pLMP2A19S). The truncated LMP2A protein encoded by this mutant would terminate at an amber nonsense codon after 24 amino acids, 5 of which are encoded by the linker. The next potential translation reinitiation site is the initiation site for LMP2B at codon 120. Thus, virus carrying this mutation would express LMP2B but not LMP2A. (Reinitiation of translation of some LMP2A mRNAs at codon 120 might result in a small increase in LMP2B). The starting EBV DNA *SalI*-*EcoRI* fragment used for constructing the LMP2A mutant has an in-frame 81-bp deletion which removes 2.5 of the 5.5 DNA repeats which

encode a repeating domain within the carboxy terminus of the LMP1 protein. Naturally occurring EBV isolates vary in the extent of this repeat so that the deletion was not likely to affect function. Since this deletion results in an LMP1 protein smaller than that encoded by P3HR1 EBV, LMP1 size could be used to screen the virus-infected LCLs for second-site recombinants that had incorporated the mutant LMP1 fragment. These would likely have incorporated the LMP2A mutation which is 1,784 bp away.

Recombinant viruses were generated by homologous recombination following transfection of P3HR1 cells with *EcoRI*-A, pLMP2A19S, and the Z gene under control of a heterologous promoter to induce lytic replication (pSVNaeZ). Culture supernatants were harvested 6 days posttransfection, filtered, and used to infect primary B lymphocytes at a multiplicity of one to five nonrecombinant virus per cell. The infected cells were plated at various dilutions likely to yield less than one LCL per microwell.

The resulting LCLs were screened by PCR analysis, using primers flanking the site of insertion of the nonsense codon (Fig. 1, line 3, 5'LMP2A and 3'LMP2A). Approximately 6% (5 of 80) of the LCLs were infected with virus which had the nonsense linker at the *BamHI* site, as evidenced by the amplification of a 192-bp DNA fragment, using primers specific for DNA on either side of the stop codon insertion site (Fig. 2A, lanes 19S74 and 19S79). All six also had the 170-bp PCR product expected from wild-type LMP2A (Fig. 2A, lanes 19S74 and 19S79). The third DNA noted in the stained gel of the mixedly infected clones is a heteroduplex of LMP2A mutant and wild-type DNA, formed as a consequence of the denaturation and reannealing steps of the PCR protocol. The same three bands are evident in an amplification of an artificial mixture of wild-type (pRL49) and mutant (pLMP2A19S) (Fig. 2A, lane mixed). Under denaturing conditions, only wild-type- or mutant-size DNA is detected (data not shown). These data were consistent with the working hypothesis that the cells were coinfecting with an EBNA2(WT)-EBNA2(WT)-LMP2A19S double recombinant and with a parental P3HR1 EBV. The uniform finding of P3HR1 coinfection could simply be the consequence of the excess of nonrecombinant P3HR1 virus over the number of primary B lymphocytes infected in these experiments. Alternatively, only primary B lymphocytes coinfecting with P3HR1 may have grown into LCLs if the LMP2A mutation inactivated an essential transforming gene and nonrecombinant P3HR1 was required to provide LMP2A *in trans*.

Segregation of LMP2A mutant EBV recombinants. To further investigate the effect of the LMP2A mutation on EBV activity in *in vitro* infection or cell growth transformation, attempts were made to segregate the viral genomes by inducing lytic virus replication in the LCLs and infecting primary B lymphocytes with the resultant virus. Two of the six coinfecting LCLs responded positively to transactivation of lytic virus infection. LCLs arose following infection with cell-free filtered virus supernatants from both cell lines. The resulting LCLs were subjected to PCR analysis, using the primers specific for a small DNA fragment inclusive of the stop codon insertion site. LCLs infected with three types of viral genomes were identified. Some LCLs contained only mutant or wild-type LMP2A DNA, whereas others contained both DNAs. LCLs infected only with viral genomes having the LMP2A19S mutation were of particular interest, since such a finding would indicate that LMP2A is nonessential for maintenance of cell growth transformation *in vitro* and probably nonessential for initiation of cell growth transformation *in vitro*. The PCR products of LCLs singly in-

FIG. 2. PCR analysis of wild-type and mutant LMP2A in EBV recombinant-infected LCLs. (A) Ethidium bromide-stained PCR products from LCL DNA, using primers which flank the *Bam*HI site at which the stop codon was inserted into LMP2A. Lanes P3HR1-16, LCL1, and LCL2 contain the expected wild-type product of 170 bp. Lanes 19S74 and 19S79 are the results from two initially derived LCLs which are coinfecting with an EBNA1P-EBNA2-LMP2A19S double recombinant and parental P3HR1 which is wild type for LMP2A. The three PCR products from these coinfecting LCLs are wild-type DNA amplified from the P3HR1 genome, LMP2A mutant DNA amplified from the double-recombinant genome, and a heteroduplex between the mutant and wild-type amplified products formed during denaturation and reannealing steps. Lanes 19S74.B7, 19S74.B7.1.34, 19S74.A.31, 19S74.3.35, 19S79.62, 19S79.62.7, and 19S79.0 have only the amplified products of 192 bp, as expected from LCLs singly infected with EBV carrying only LMP2A19S DNA. Lanes pLMP2A19S and pRL49 are the results of control amplifications of the wild-type (pRL49) and mutant (pLMP2A19S) plasmids. Lane Mixed contains the result of amplification with an artificial mixture of pLMP2A19S and pRL49. Sizes (in base pairs) of the relevant *Hae*III restriction fragments of ϕ X174 are indicated at the right. (B) To assess the sensitivity of detection of LMP2A wild-type alleles in a background of LMP2A19S mutants, dilutions from 10^5 wild-type-infected LCL (LCL1) with EBV-negative BJAB cells were added to 10^5 LMP2A19S mutant-infected LCLs and amplified for 40 cycles, using a wild-type LMP2A insertion site-specific primer pair which has a two-nucleotide mismatch with the LMP2A19S DNA at its 3' end. (C) Amplifications of LCL DNA, using the LMP2A wild-type-specific primer.

infected with LMP2A19S virus from the 19S74 or 19S79 cell line are shown in Fig. 2A, lanes 19S74.B7, 19S79.62, and 19S79.0. As shown in Fig. 2C, no wild-type LMP2A DNA could be detected in these cell lines, using primers which are specific for the wild-type DNA. PCR with these primers is sufficiently sensitive to detect 1 in 1,000 wild-type LMP2A DNAs (Fig. 2B).

LMP2A19S mutant EBV recombinants can transform primary B lymphocytes. To exclude the possibility that these

singly progeny LMP2A19S mutant virus-infected LCLs might have been initially coinfecting with P3HR1 virus, which would have provided wild-type LMP2A in *trans*, singly LMP2A19S-infected cells from the 19S74 or 19S79 lineage were induced to lytic virus infection, and the resultant virus was used to infect primary B lymphocytes under conditions in which there was less than one transforming virus per five microwells. LMP2A wild-type recombinant virus was used to infect primary B lymphocytes in parallel. LMP2A19S-infected LCLs grew out as rapidly as did wild-type-infected cells and were indistinguishable in the growth from wild-type recombinant virus-infected LCLs. As expected, LCLs arising following infection with LMP2A19S recombinants had genomes with only the LMP2A19S mutation (Fig. 2A and C, lanes 19S74.B7.1, 19S74.A.31, 19S74.3.35, and 19S79.62.7). Lytic virus replication could be transactivated in some of these LCLs, and the virus was used again to infect primary B lymphocytes in parallel with LMP2A wild-type recombinant virus infection. As with earlier passages, LCLs infected with wild-type or mutant LMP2A recombinant virus grew similarly. The resultant LCLs were verified by PCR to contain mutant or wild-type LMP2A DNA (e.g., Fig. 2A, lane 19S74.B7.1.34).

The LMP2A19S mutation in the singly infected LCLs was further confirmed by nucleotide sequence and by Southern blot hybridization. The linker insertion site DNAs from both an LMP2A19S74 and an LMP2A19S79 lineage mutant-infected LCL were amplified by PCR and sequenced. The sequences of both DNAs were exactly those expected from the previously determined EBV DNA sequence and the linker insertion protocol (data not shown). To confirm that the recombination had placed the mutant LMP2As in the expected context in the recombinant P3HR1 genomes, replacing the wild-type LMP2A allele, total LCL DNA was subjected to Southern blot analysis (Fig. 3). If the mutant LMP2A has simply replaced the wild-type DNA, the size of the 5,187-bp *Bgl*III fragment which includes the insertion site should be 5,209 bp, and the 5,209-bp DNA should have a new *Xba*I site (Fig. 1). Genomic DNA from representative mutant- or wild-type-infected LCLs was therefore digested with *Bgl*III or doubly digested with *Bgl*III and *Xba*I. The resulting Southern DNA fragments were then probed with labeled *Bgl*III DNA (Fig. 3). Wild-type EBV DNA, both P3HR1 and B95-8 strains, has the characteristic wild-type fragment. All of the *Bgl*III-homologous DNA in the LMP2A19S recombinant-infected LCLs has a new *Xba*I site, which gives 2,860- and 2,349-bp fragments in the *Bgl*III and *Xba*I double digests (Fig. 3, lanes 9 to 13). The *Xba*I-resistant *Bgl*III fragment was detected only in the wild-type control DNAs and in the mutant- and wild-type-coinfecting initial LCLs (Fig. 3, lanes 9, 14, and 15), even upon long exposure of the blot (data not shown).

Latent and lytic cycle EBV gene expression in LCLs infected with LMP2A19S mutant EBV recombinants. LCLs infected with LMP2A19S mutant EBV recombinants do not have LMP2A or LMP2A cross-reactive protein, using a polyclonal affinity-purified antibody which recognizes the LMP2A unique amino-terminal cytoplasmic domain. As shown in Fig. 4, the LMP2A19S mutants lack detectable LMP2A (lanes 2 to 5), whereas LMP2A was evident in wild-type-transformed LCLs (lane 1), including cells transformed with a P3HR1 EBNA1P-EBNA2 recombinant derived in parallel with these experiments (lane 7). As expected, the affinity-purified antibody did not react with the EBV-negative BJAB cell line (lane 6). LMP2B expression in these cells could not be monitored, since currently available

FIG. 3. Southern blot analysis of wild-type and mutant LMP2 DNA from infected LCLs, demonstrating replacement insertion of the mutation into the homologous site in the EBV genome. DNA from representative LCLs was digested with *Bgl*II or *Bgl*II and *Xba*I and probed with the ³²P-labeled *Bgl*II fragment indicated in Fig. 1, line 6. In *Bgl*II digests, the probe detected the wild-type *Bgl*II fragment in all the cell lines tested (lanes 2 to 8). In the double digests with *Bgl*II and *Xba*I, the probe detected the same wild-type fragment in LCL1 and P3HR1-16 as a result of the absence of any *Xba*I sites within the wild-type fragment (lanes 14 and 15). In LCL 19S74, which by PCR analysis contains both a wild-type and a mutant LMP2A allele, the wild-type fragment and two new fragments of 2,860 and 2,349 bp (lane 9) were detected. The 2,860- and 2,349-bp fragments result from cleavage at the inserted *Xba*I non-sense linker at the *Bam*HI site shown in Fig. 1, line 5. In four LCLs which by PCR analysis were infected with only mutant LMP2A alleles, only the 2,860- and 2,349-bp fragments are detected in the *Bgl*II-*Xba*I double digests (lanes 10 to 13). The absence of the wild-type fragment indicates that these LCLs contain only mutant alleles of LMP2A.

antibodies recognize only the amino-terminal cytoplasmic domain unique to LMP2A (30).

Aside from LMP2A, EBV gene expression in LCLs singly infected with and immortalized by LMP2A19S mutant EBV recombinants was identical to that of wild-type controls. A human serum reactive with the EBNA3s demonstrated similar EBNA1 and EBNA2 expression in wild-type and mutant LMP2A EBV recombinant-infected LCLs (Fig. 5A). Because of type-specific differences between the B95-8 and P3HR1 EBNA3s, the EBNA3 family is not detected as readily in the P3HR1-based wild-type or LMP2A19S mutant recombinants as in the B95-8-transformed LCL1 (Fig. 5A, lane 4). LMP1 expression was similar in both wild-type and mutant EBV-infected LCLs (Fig. 5B). The LMP2A19S mutant recombinant-infected LCLs have a smaller LMP1 as a result of the deletion of part of the DNA repeat encoding the carboxy-terminal LMP1 repeat domain (Fig. 5B). By coincidence, LMP1 encoded by the recombinants is similar in size to the AG876 EBV strain LMP1 (Fig. 5B, lane 6). This strain has a smaller number of reiterations of the DNA repeat which encodes the carboxy-terminal repeat domain of LMP1 (45).

Lytic cycle protein expression was examined in Western

FIG. 4. Evidence that LMP2A is not detected in LMP2A mutant EBV recombinant-infected LCLs. Extracts of approximately 10⁶ cells were electrophoresed, transferred to nitrocellulose, and reacted with purified LMP2A antibodies as described in Materials and Methods. LMP2A migrates just below the 66-kDa size marker. Lanes: 1, B95-8-transformed LCL; 2 to 5, LMP2A mutants; 6, EBV-negative B-lymphoma cell line BJAB; 7, EBNA2 P3HR1 recombinant control. Sizes of protein standards are indicated in kilodaltons on the left.

immunoblots, using a human serum reactive with early lytic cycle proteins (Fig. 6). Of 12 wild-type control-infected LCLs, 2 spontaneously expressed early antigens. Of 19 LMP2A19S mutant-infected LCLs, 3 spontaneously expressed early antigens. Thus, values for permissivity for virus replication were similar, 16 and 15%, respectively. Lytic gene expression was also studied following transfection with pSVNaeZ and TPA treatment. Two LMP2A19S mutant-infected LCLs that were similar to two wild-type-infected control LCLs in being partially permissive for early-antigen expression were analyzed for their responses to lytic cycle induction. Five days after transfection and addition of TPA, cells were stained by immunofluorescence with 2L10, a monoclonal antibody directed against gp350/220. Eleven and 4% of the LMP2A19S mutants stained brightly for gp350/220 expression, while 15 and 9% of the wild-type controls were positive for gp350/220 expression. Thus, LMP2A does not significantly affect EBNA or LMP gene expression or progression from latent to lytic virus gene expression.

Comparative growth of LMP2A19S mutant- and wild-type-infected LCLs. The similar outgrowth characteristics of the LMP2A19S mutants and wild-type controls were prospectively investigated by inducing EBV replication in the LMP2A mutant or wild-type control EBV recombinant-infected cells, which had similar levels of permissivity as assayed by EBV gp350 expression. Dilutions of the filtered released virus from the induced cells were used to infect primary B lymphocytes, and the infected cells were plated into microwells to assay transforming activity. Similar numbers of wells were positive for LCL outgrowth after infection with mutant or wild-type control virus. Two mutant-infected LCLs released 5×10^2 and 15×10^2 virus transforming units per percent gp350-positive cells, while two wild-type-infected LCLs released 4×10^2 and 8×10^2 virus transforming units per percent gp350-positive cells. The resultant LCLs grew out similarly and were indistinguishable in growth characteristics as they were expanded to larger cultures.

FIG. 5. Latent gene expression in LMP2A mutants. Extracts of approximately 5×10^5 cells were separated in denaturing polyacrylamide gels, transferred to nitrocellulose, and blotted with either an EBV-immune human serum (A) or anti-LMP1 monoclonal antibodies (B). Lanes: 1, EBV-negative B-lymphoma cell line BJAB; 2, B95-8; 3, P3HR1 clone 16; 4, B95-8-transformed LCL; 5, the EBNA1P-EBNA2 P3HR1 recombinant WT111.20; 6 in panel A, the EBNA1P-EBNA2 P3HR1 recombinant WT111.21; 6 in panel B, AG876; 7 to 14, LMP2A mutant LCLs. Positions of the EBNA1s, LMP1, and molecular size markers (in kilodaltons) are indicated.

Thus, the yield of mutant and wild-type virus assayed in in vitro LCL outgrowth correlated with the number of permissively infected cells, consistent with the mutation having no effect on the amount of virus released from a permissively infected cell, no effect on the efficiency of primary B-lymphocyte infection, and no effect on the efficiency of LCL outgrowth.

To further investigate the effect of the LMP2A mutation on the growth of transformed LCLs, newly transformed mutant and wild-type control LCLs were plated at various cell concentrations and in media supplemented with 10, 1, or 0.1% fetal calf serum. Growth was assayed 10 days later by microscopic examination and pH of the media. The growth of mutant and wild-type recombinant-infected LCLs was similarly sensitive to plating at low cell density in media supplemented with less serum or to plating under conditions of low cell density and low serum (Table 1).

Anchorage-independent growth of wild-type- and LMP2A mutant-infected LCLs was assayed by plating in soft agar over fibroblast feeder layers. Six LMP2A19S mutants and six wild-type control-infected LCLs which had been recently derived in parallel within the previous 2 months and main-

FIG. 6. Spontaneous early-antigen expression in LMP2A19S or wild-type control EBNA1P-EBNA2 P3HR1 EBV recombinant-infected LCLs. Extracts of approximately 5×10^5 cells were electrophoresed in denaturing polyacrylamide gels and reacted in a Western blot with an EBV-immune serum which reacts strongly with EBV early antigens. Lanes: 1 to 6, wild-type control EBV recombinant-infected LCLs; 7 to 12, LMP2A19S mutant EBV recombinant-infected LCLs; 13, spontaneous EBV-producing cell line B95-8; 14, EBV-negative B-lymphoma cell line BJAB. Early antigen (EA) and sizes (in kilodaltons) of marker proteins are indicated.

tained in parallel were plated at 10^3 cells per ml in 0.3% agarose in a 4-cm well over a fibroblast feeder layer. After 4 weeks, wells were scored for macroscopic cell growth. Resulting colonies are expressed as percent cloning efficiency based on the initial seeding of 10^3 cells per well. No difference in cloning efficiency was evident. LMP2A19S-infected LCLs had cloning efficiencies of 0.4 to 5.4%, while wild-type control-infected LCLs had cloning efficiencies of 0.3 to 5.0% (Table 2).

DISCUSSION

These experiments indicate that the amino-terminal domain which distinguishes LMP2A from LMP2B is not essential for initiation or maintenance of latent or lytic lymphocyte infection or growth transformation in vitro. Further, this domain does not significantly affect the growth, concentration dependence, serum dependence, or soft agar growth of EBV-infected cells in vitro. These conclusions are based on the lack of effect of a specific LMP2A mutation replacing the normal allele in a recombinant EBV genome with an allele which encodes an mRNA which would stop being translated after codon 19. This mutation is almost certainly an LMP2A null mutation. Translation of LMP2A is predicted to terminate after the first 19 amino acids, and neither LMP2A nor LMP2A cross-reactive protein was detected in mutant recombinant virus-infected cells. The resultant 19-amino-acid polypeptide is unlikely to be functional as the predicted free cytoplasmic fragment, since ordinarily the first 19 amino acids would be part of a 119-amino-acid cytoplasmic domain tethered to the inner surface of the plasma membrane by the 12 membrane-spanning domains of LMP2A. Translation after LMP2A codon 19 having been stopped, reinitiation is unlikely before the next methionine codon at codon 120. This methionine codon is the translational initiation site for LMP2B. Reinitiation of translation of the mutant LMP2A mRNA is likely to be infrequent, given the long interval between the termination of translation at codon 19 and the

TABLE 1. Serum- and cell-dependent growth of LMP2A mutants and wild-type controls^a

Cell line	Growth						
	10 ⁵ cells/well				2.5 × 10 ⁴ cells/well ^b		5 × 10 ³ cells/well, 10% serum ^c
	10% serum	1% serum	0.1% serum	No serum	10% serum	1% serum	
WT.111.10	+++	++/+	+/-	+/-	+++	+/-	+
WT.111.11	+++	+	+/-	-	+++	-	+
WT.111.12	+++	+	+/-	+/-	++	-	+
WT.111.13	+++	++	++	+	+++	+	+++
WT.111.14	+++	+++	++	+	+++	-	++
WT.111.15	+++	+	-	-	+++	-	+/-
WT.111.16	+++	+++	++	+	+++	-	+++
19S74.3.28	+++	++/+	+	+/-	+++	+/-	++
19S74.3.30	+++	+	+	+/-	+++	+/-	++
19S74.3.31	+++	+	-	-	+++	-	++
19S74.3.35	+++	++	+	+	+++	+	++
19S74.3.38	+++	+	+/-	-	++	+/-	+
19S74.B7.1.34	+++	+	-	-	+++	+/-	++
19S74.B7.1.36	+++	++/+	+	+	+++	+	++
19S74.B7.1.37	+++	+	+	+/-	+++	+	++/+
19S74.B7.1.40	+++	+	+/-	+/-	+++	+/-	++
19S74.B7.1.41	+++	+	+/-	+/-	+++	+	+
19S74.B7.1.42	+++	+	+/-	-	++	+/-	++
19S74.B7.1.44	+++	+	-	-	++	+/-	+
19S74.A.22	+++	+	+	+	+++	+/-	++
19S74.A.25	+++	+	-	-	++	-	+
19S74.A.29	+++	+	+	+/-	+++	+	++
19S74.A.32	+++	+	+	+/-	+++	+/-	++

^a Cells were plated at the indicated concentrations in 96-well tissue culture plates with or without serum, as indicated. Wells were scored 10 days after plating as follows: +++, large clumps, acid media, clear growth; ++, medium clumps, with or without acid media, probable growth; +, small clumps, neutral or basic media, little growth; and -, no clumps, neutral or basic media, no growth.

^b No growth in 0.1% serum or no serum.

^c No growth in 1% serum, 0.1% serum, or no serum.

next methionine at codon 120 (25a). Depending on the efficiency of reinitiation, there could be some increase in LMP2B as a consequence of the LMP2A mutation. LMP2B abundance could not be directly evaluated because of the lack of adequately sensitive antisera. Thus, we cannot formally exclude the unlikely possibility that LMP2A mRNA provides a critical function by augmenting LMP2B expression. The lack of effect of the LMP2A mutation is unexpected, since LMP2A has at least two important biochemical properties. First, LMP2A associates with LMP1 in EBV-

infected LCLs or in B-lymphoma cells in which both proteins are expressed under control of heterologous promoters (29). LMP1 has transforming effects on rodent fibroblasts and is an important mediator of EBV's effects in activating and altering the growth of B lymphocytes (3, 4, 27, 55-58). Second, LMP2A is serine-threonine and tyrosine phosphorylated and associates with *fyn* and *lyn* and other B-lymphocyte *src* family tyrosine kinases in EBV-infected LCLs (7, 29). *src* family tyrosine kinases play critical roles in transmembrane signal transduction. Polyomavirus middle-T-antigen interaction with *src* is critical to polyomavirus-mediated cell growth transformation (10, 11, 13, 25, 41). LMP2 interaction with *src* family tyrosine kinases is likely to be LMP2A specific, since the amino-terminal cytoplasmic domain and the first two transmembrane domains are sufficient for tyrosine kinase association (7, 29).

The lack of effect of the LMP2A mutation is also surprising because of other observations which are consistent with LMP2A or LMP2B being important in EBV infection. LMP2A is expressed in all latently infected B lymphocytes in vitro (30), in infected B lymphocytes in vivo (42), and in nasopharyngeal carcinoma disease tissues (6, 9). Patients with nasopharyngeal carcinoma have antibodies to LMP2 (15). Further, LMP2 has T-cell-reactive epitopes which can mediate cytotoxic T-cell responses (39). Persistent expression in the face of counterselection by human T-cell immunity is most consistent with an important role for LMP2A or LMP2B.

LMP2A is likely to be important in EBV infection in vivo, and its role is likely to be related to its known biochemical properties. LMP2A may modulate the effects of LMP1 or of

TABLE 2. Growth of LMP2A mutants and wild-type controls in soft agar

Cell line	Cloning efficiency ^a (%)
WT.111.α1	0.3
WT.111.α4	2.6
WT.111.α5	0.1
WT.111.α7	5.0
WT.111.α8	0.6
WT.111.α10	3.3
19S74.62.2.α1	0.5
19S74.62.2.α4	1.0
19S74.62.2.α7	0.6
19S74.3.α8	3.8
19S74.62.2.α9	5.4
19S74.3.α10	0.4

^a Percentage of cell colonies which were macroscopic after 4 weeks of growth over a fibroblast feeder layer in wells seeded with 10³ cells.

B-lymphocyte or epithelial cell transmembrane signaling in latent or lytic EBV infection in lymphocytes or epithelial cells. LMP2A could also affect the survival of latently infected cells in specific tissues. Such hypotheses will require testing in experimental models of human EBV infection such as SCID mice, cottontop tamarins, or Old World primates.

ACKNOWLEDGMENTS

This research was supported by Public Health Service research grant CA 00449 from the National Cancer Institute. R.L. is the recipient of a fellowship from the Leukemia Society of America.

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