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The Epstein-Barr Virus LMP1 Cytoplasmic Carboxy Terminus Is Essential for B-Lymphocyte Transformation; Fibroblast Cocultivation Complements a Critical Function within the Terminal 155 Residues

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Recombinant Epstein-Barr viruses (EBVs) were made with mutated latent membrane protein 1 (LMP1) genes that express only the LMP1 amino-terminal cytoplasmic and six transmembrane domains (MS187) or these domains and the first 44 amino acids of the 200-residue LMP1 carboxy-terminal domain (MS231). After infection of primary B lymphocytes with virus stocks having small numbers of recombinant virus and large numbers of P3HR-1 EBV which is transformation defective but wild type (WT) for LMP1, all lymphoblastoid cell lines (LCLs) that had MS187 or MS231 LMP1 also had WT LMP1 provided by the coinfecting P3HR-1 EBV. Lytic virus infection was induced in these coinfected LCLs, and primary B lymphocytes were infected. In over 200 second-generation LCLs, MS187 LMP1 was never present without WT LMP1. Screening of over 600 LCLs infected with virus from MS231 recombinant virus-infected LCLs identified two LCLs which were infected with an MS231 recombinant without WT LMP1. The MS231 recombinant virus could growth transform primary B lymphocytes when cells were grown on fibroblast feeders. Even after 6 months on fibroblast feeder layers, cells transformed by the MS231 recombinant virus died when transferred to medium without fibroblast feeder cells. These data indicate that the LMP1 carboxy terminus is essential for WT growth-transforming activity. The first 44 amino acids of the carboxy-terminal cytoplasmic domain probably include an essential effector of cell growth transformation, while a deletion of the rest of LMP1 can be complemented by growth on fibroblast feeder layers. LMP1 residues 232 to 386 therefore provide a growth factor-like effect for the transformation of B lymphocytes. This effect may be indicative of the broader role of LMP1 in cell growth transformation.

Epstein-Barr virus (EBV) causes infectious mononucleosis in healthy adults and a lymphoproliferative disease in immune-deficient individuals and is associated with Burkitt's lymphoma (BL), Hodgkin's disease, and nasopharyngeal carcinoma. EBV infects primary B lymphocytes and efficiently induces indefinite cell proliferation (19, 27). In these immortalized lymphoblastoid cell lines (LCLs), there is usually no lytic virus infection. Instead, in a latent infection, EBV expresses six nuclear proteins (EBNAs), three integral membrane proteins (latent membrane protein 1 [LMP1], LMP2A, and LMP2B), and two small nuclear RNAs (EBERs). EBNA LP, 2, 3A, and 3C and LMP1 are essential or critical for primary B-lymphocyte growth transformation (5, 10, 17, 26, 36). EBNA 3B, LMP2A, and LMP2B and the EBERs are dispensable for primary B-lymphocyte growth transformation in cell culture (24, 33, 35).

LMP1 is a transforming protein which consists of a 24-amino-acid amino-terminal cytoplasmic domain, six membrane-spanning hydrophobic domains separated by short reverse turns, and a 200-amino-acid carboxy-terminal cytoplasmic domain (Fig. 1) (7). LMP1 aggregates in patches that characteristically coalesce into a cap in the plasma membrane where LMP1 associates with vimentin, a cytoskeletal protein (20, 22). Rodent fibroblasts transfected with LMP1 expression vectors have reduced serum requirements, are no longer contact inhibited, replicate in soft agar, and produce tumors in nude mice after adoptive transfer (2, 37, 38). LMP1 expression blocks differentiation in an epithelial cell line that can be induced to terminally differentiate similar to normal keratinocytes (6). In transgenic mice, LMP1 expression under the control of a polyomavirus promoter causes epithelial hyperplasia (42). LMP1 expressed in EBV-negative BL cells recapitulates many of the effects of latent EBV infection in primary B lymphocytes (40). In BL cells, LMP1 induces villous projections, growth in tight clumps, NF-κB activity, and expression of activation markers (CD23 and CD40), adhesion molecules (ICAM1, LFA1, and LFA3) and the *bcl-2* proto-oncogene (9, 13, 18, 39).

EBV recombinant genetic analyses indicate that LMP1 is essential for primary B-lymphocyte transformation (17). EBV recombinants with LMP1 genes mutated by insertion of a nonsense linker after codon 9 or 84 transform primary B lymphocytes only when wild-type (WT) LMP1 is provided in *trans* by a coinfecting, transformation-defective EBV, P3HR-1. These recombinants expressed abundant cross-reactive proteins (CRPs) initiated at internal methionine codon 44, 89, or 129. These CRPs localized diffusely in the cytoplasm or plasma membrane and did not have positive or negative effects on cell growth (17, 21). Since codon 44 is at the end of the first transmembrane domain, this result indicated that the short amino-terminal cytoplasmic and/or the first transmembrane domain is important in lymphocyte growth transformation. Surprisingly, EBV recombinants with a deletion of DNA encoding any part of the amino terminus had substantial growth-transforming activity for primary B lymphocytes (16). Deletion

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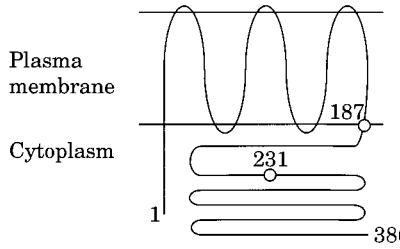

FIG. 1. Structure of LMP1 in plasma membrane (7, 22). MS187 LMP1 DNA is predicted to express amino acids 1 to 187 plus alanine-serine-leucine-aspartate (○ labeled 187) and MS231 LMP1 DNA is predicted to express amino acids 1 to 231 plus leucine-valine (○ labeled 231).

of DNA encoding the arginine- and proline-rich region of the amino terminus had the greatest negative effect and reduced transformation efficiency by only 90%. These data indicate that no specific sequence in the LMP1 amino-terminal cytoplasmic domain is essential for transformation. Rather, the highly charged amino-terminal cytoplasmic domain appears to tether the first LMP1 transmembrane domain to the cytoplasm (16). We now investigate the importance of the LMP1 cytoplasmic carboxy-terminal domain in primary B-lymphocyte growth transformation.

MATERIALS AND METHODS

Cell lines and virus. The HH514-16 subclone of the P3HR-1 BL cell line and the B95-8 cell line were obtained from George Miller of Yale University. IB4 is an LCL generated by infecting human umbilical cord lymphocytes with virus generated from B95-8 cells. FS4 is a primary human foreskin fibroblast cell line (kindly provided by J. Vilcek, New York University).

Construction of LMP1 mutations. The LMP1 gene in the *Eco*RI D_{het} fragment (nucleotides 159853 to 172282 in the EBV genome [1]) was mutated by inserting a nonsense oligonucleotide (5′-CTAGTCTAGACTAG-3′) after codon 187 (MS187) or after codon 231 (MS231). For MS187, the nonsense linker DNA was inserted between two Klenow end-filled *Nco*I sites (nucleotides 168263 and 168758). For MS231, the nonsense oligonucleotide was inserted at an *Nae*I site (nucleotide 168627). Mutated LMP1 genes were sequenced by the dideoxy method.

Generation and passage of EBV recombinants. P3HR-1 cells (10^7) in 0.4 ml of RPMI 1640 with 10% (vol/vol) fetal bovine serum in a 0.4-cm-gap cuvette (Bio-Rad) were electroporated with 20 μg of MS187 or MS231 DNA, 10 μg of WT EBNA LP/2 DNA (*Eco*RI A, nucleotides 7315 to 69119), and 25 μg of pSVNaeI-Z (an expression vector for the immediate early gene transactivator BZLF1 to induce lytic infection) (34, 35) at an electric pulse of 200 V and 960 μF. Peripheral blood mononuclear cells from healthy donors were depleted of T cells, infected with virus, and seeded into 96-well plates at 5×10^4 cells per well (34, 35). Lytic infection was induced in established LCLs by treating cells with medium containing 20 ng of phorbol 12-myristate 13-acetate (PMA; Life Technologies) per ml. LCLs (3.0×10^4 per well) were gamma irradiated (8,000 rads) and cocultivated with 5×10^4 mononuclear cells depleted of T cells per well in 96-well plates. Alternatively, 5×10^4 mononuclear cells per well were incubated with LCL supernatants that had been passaged through a 0.22-μm-pore-size filter before being seeded into 96-well plates (34).

PCR analysis of EBV DNA and mRNA. EBV DNA in LCLs was characterized by PCR-based methods as described previously (17, 23, 34). Primers MS187A and MS187B (5′-ACTGGTGGACTCTATTGGTT-3′ and 5′-AGTCAGTCAG GCAAGCCTAT-3′, respectively) were used to score for MS187 DNA. Primers MS231A and MS231B (5′-AGTGATGAACACCACCACGA-3′ and 5′-CATTG TCAGGACCACCTCCA-3′, respectively) were used to score for MS231 DNA. EBV type 1 LMP1 DNA linkage to the terminal repeat (TR) sequences was tested with primers TRFTI (5′-TTACTCATCAGTAGGAGTAT-3′) and TRR (5′-TCCACTTTTTCCAGGAATGC-3′). EBV type 2 LMP1 DNA linkage to the TR sequences was tested with primers TRFT2 (5′-TTACTCATCAGTAGGAG TAG-3′) and TRR. DNA was amplified by 35 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 75 s (GeneAmp System 9600; Perkin-Elmer Cetus Norwalk, Conn.). MS231W primers (5′-TCGTGAGTGGAGCCGGCG-3′ and 5′-AGCA GAGTCGCTAGGGCT-3′) specifically amplify WT LMP1 and not MS231 DNA. WT LMP1 DNA was amplified by 30 cycles at 94°C for 15 s, 65°C for 30 s, and 72°C for 75 s. Primers described previously were used to score for the presence of P3HR-1 DNA (36). To detect LMP1 mRNA, RNA was extracted with RNAzol B (Cinna/Biotecx Laboratories), reverse transcribed (Gibco BRL), amplified with primers MSC (5′-TTTGCACGGACAGGCATTGT-3′) and MS187B (for MS187 cells) or primers MSC and MS231B (for MS231 cells) for 35 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 75 s. PCR products were resolved in gels containing 3% (wt/vol) NuSieve agarose–1% (wt/vol) Seakem ME agarose (FMC Bioproducts) and 0.5× TBE (Tris, borate, EDTA), except for the DNA amplified with TRFT1/TRR or TRFT2/TRR, which was resolved in gels containing 0.8% (wt/vol) Seakem ME agarose and 0.5× TBE.

Immunoblot and Southern analyses. Proteins from 5×10^4 cells were electrophoretically resolved in a denaturing, discontinuous 8% polyacrylamide gel and Western blotted (immunoblotted) to a nitrocellulose filter. LMP1 was detected with S12 monoclonal antibody or a combination of CS1-4 monoclonal antibodies (25, 31), rabbit anti-mouse immunoglobulin (heavy and light chain) (Jackson Immunoresearch), and ^{125}I-labeled protein A (Amersham). Autoradiographic signals were collected on a PhosphorImager screen (Molecular Dynamics) and analyzed with the manufacturer's computer hardware and ImageQuant software (Molecular Dynamics). Southern analyses were performed as described previously (17).

RESULTS

Construction of EBV recombinants with a stop codon after LMP1 codon 187 or 231. A nonsense oligonucleotide was inserted after LMP1 codon 187 (MS187) or 231 (MS231) in the genomic EBV *Eco*RI D_{het} fragment as described in Materials and Methods. MS187 DNA is predicted to express 187 LMP1 residues plus four amino acids (alanine-serine-leucine-aspartate) encoded in the inserted oligonucleotide. MS231 DNA is predicted to express 231 LMP1 residues plus two amino acids (leucine-valine) encoded in the inserted oligonucleotide (Fig. 1).

EBV recombinants were generated by transfecting P3HR-1 cells with MS187 or MS231 DNA, an EBV DNA fragment (*Eco*RI A) which codes for the EBNA LP and EBNA 2 (EBNA LP/2) genes, and an expression vector for the BZLF1 immediate-early transactivator to induce replication of the endogenous P3HR-1 EBV (Fig. 2). P3HR-1 EBV has a deletion of an EBNA LP- and EBNA 2-encoding DNA fragment and can transform primary B lymphocytes only when this fragment is restored (5, 10, 26). Since this DNA is essential for transformation, all LCLs that arise 4 to 8 weeks after infection of primary B lymphocytes with virus from the transfected P3HR-1 cells are latently infected with an EBV recombinant which has the transfected EBNA LP/2 DNA. Up to 10% of these recombinants can incorporate a second transfected EBV DNA fragment and thereby could have acquired a mutated LMP1 gene (16, 17, 34–36). If the carboxy-terminal domain of LMP1 is not essential for growth transformation, recombinants with the mutated LMP1 genes will transform primary B lymphocytes into LCLs. If the carboxy-terminal LMP1 mutations abolish transformation competence, EBV recombinants which have WT EBNA LP/2 and MS187 or MS231 LMP1 should still be recovered in LCLs because parental P3HR-1 EBV is produced in a 10^5-fold excess over recombinants and can often coinfect cells to provide WT LMP1 in *trans* (Fig. 2) (17).

Recovery of EBV recombinants with specifically mutated LMP1 DNA. LCLs infected with EBV recombinants with mutated LMP1 DNA were identified by PCR with primers that bracket the nonsense oligonucleotide insertion sites. In Fig. 3A, primers that bracket the MS187 site amplify a 247-bp fragment from MS187 plasmid DNA (lane 2) and a 724-bp product from WT LMP1 DNA (lane 3). The smaller size of the MS187 amplified DNA is due to the deletion of DNA downstream of the stop codon as was expected from the cloning strategy described in Materials and Methods. Primers that bracket the MS231 site amplify a 195-bp fragment from MS231 plasmid DNA (Fig. 3B, lane 7) and a 181-bp fragment from WT LMP1 DNA (Fig. 3B, lane 8). From four separate electroporations and infections with two independently derived mutated LMP1 plasmid clones of each type, 30 LCLs had both MS187 and WT LMP1 DNA and 32 LCLs had both MS231

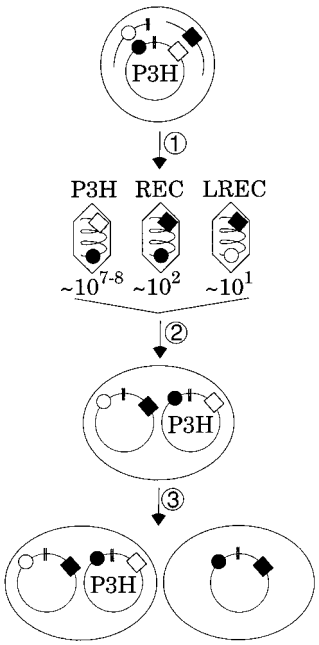

FIG. 2. Generation and analysis of MS187 or MS231 LMP1 recombinant EBV. Symbols: ●, WT LMP1 DNA; ○, mutated LMP1 DNA; ■, WT EBNA LP/2 DNA; □, P3HR-1 deletion; ‖, EBV TR; P3H, P3HR-1 EBV; REC, WT EBNA LP/2 EBV recombinant; LREC, WT EBNA LP/2-MS187 or MS231 EBV recombinant. Steps: 1, P3HR-1 cells are cotransfected with mutated LMP1 DNA and WT EBNA LP/2 DNA, and lytic infection is induced; 2, primary B lymphocytes are infected with virus, and the resultant LCLs are analyzed for LMP1 DNA; 3, LCLs coinfected with an MS187 or MS231 LMP1 EBV recombinant and P3HR-1 are induced for lytic infection, and primary B lymphocytes are infected with progeny virus. Progeny LCLs that arose were analyzed for mutated LMP1 DNA. The estimated numbers of each type of virus are indicated (34–36).

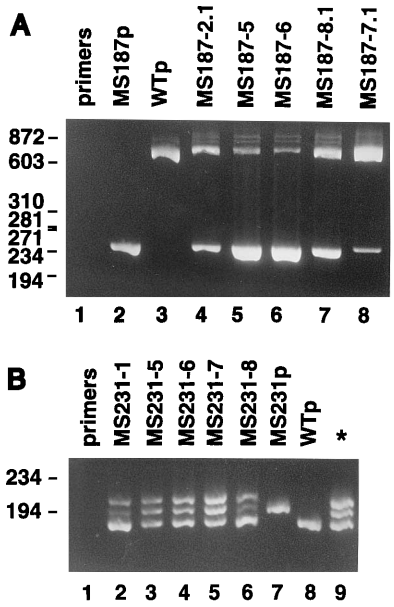

FIG. 3. PCR analyses of LMP1 DNA in LCLs infected with an EBV recombinant. (A) Primers MS187A and MS187B amplify a 724-bp DNA from WT LMP1 DNA and a 247-bp DNA from MS187 LMP1 DNA. (B) Primers MS231A and MS231B amplify a 181-bp DNA from WT LMP1 DNA and a 195-bp DNA from MS231 LMP1 DNA. PCR amplifications with primers but no cell DNA were loaded in lanes marked primers. MS187, MS231, and WT LMP1 plasmid DNA clones were PCR amplified and loaded in lanes marked MS187p, MS231p, and WTp, respectively. The PCR DNA product from cloned MS231 plasmid DNA was annealed with PCR DNA product from cloned WT LMP1 plasmid DNA and loaded in the lane marked *. The third, more slowly migrating fragment in the lane labeled * is a heteroduplex of WT and mutated LMP1 DNA PCR products (17, 23, 34). DNA size markers (in base pairs) are indicated on the left.

and WT LMP1 DNA. No LCL was infected with an EBV MS187 or MS231 recombinant without WT LMP1. As expected, many more LCLs were infected with an EBNA LP/2 recombinant which had only WT LMP1. This result suggests that the MS187 and MS231 recombinants may be incapable of transforming primary B lymphocytes without WT LMP1 provided by P3HR-1 EBV.

To investigate the possibility of nonhomologous recombination, linkage of the MS187 or MS231 LMP1 genes to the EBV TR DNA sequences was analyzed by PCR-based methods. Primer TRFT1 is predicted to be specific for mutated LMP1 DNA because the MS187 and MS231 plasmid DNAs are derived from a type 1 EBV DNA, which has an adenine at nucleotide 169222, and the type 2 P3HR-1 EBV DNA has a cytosine (32). Primers TRFT1 and TRR (complementary to types 1 and 2 EBV TR) result in the amplification of a 929-bp fragment from LCLs that have MS187 or MS231 genes (Fig. 4A, lanes 3 to 12) or from a type 1 EBV-infected cell line, B95-8 (lane 13), but not from cells infected with the type 2 EBV P3HR-1 (lane 2). These results indicate that the MS187 and MS231 type 1 EBV LMP1 genes in the coinfected LCLs are linked to the TR sequence.

Since PCR analysis with primers that bracket the MS187 or MS231 sites indicate that LCLs infected with an MS187 or MS231 recombinant also had WT LMP1 DNA, which is provided by P3HR-1 EBV, PCR was done to determine whether the P3HR-1 WT LMP1 gene is also linked to TR. An oligonucleotide primer (TRFT2) specific for type 2 EBV DNA because of the cytosine at nucleotide 169222 and the TRR primer were used to amplify DNA. The results in Fig. 4B indicate that the primers TRFT2 and TRR amplify by PCR a 929-bp fragment from P3HR-1 cells (lane 12) and from the coinfected LCLs (Fig. 4B, lanes 2 to 10) but not from a type 1 EBV-infected cell line, B95-8 (lane 11). These results demonstrate that LCLs which have MS187 or MS231 genes are coinfected with P3HR-1 EBV, which has LMP1 DNA linked to the TR.

The EBNA LP/2 deletion characteristic of the parental P3HR-1 genome was also demonstrated in these MS187 or MS231 and P3HR-1 coinfected LCLs by PCR analysis (Fig. 5) with primers that bracket the P3HR-1 EBNA LP/2 deletion (36). These primers result in the amplification of a 256-bp DNA fragment from P3HR-1 DNA (Fig. 5A, lane 7, and 5B lane 7). Each of the MS187 (Fig. 5A, lanes 2 to 6) or MS231 (Fig. 5B, lanes 2 to 6) infected cell lines also has the 256-bp fragment, confirming the presence of P3HR-1. The persistent presence of a parental P3HR-1 genome in these LCLs is initial evidence that MS187 and MS231 are critical or null mutations for LMP1. An LCL must therefore be coinfected with P3HR-1 (which has an EBNA LP/2 DNA deletion) to provide WT LMP1, while the MS187 or MS231 recombinant provides EBNA LP/2 (Fig. 2). With two LCLs, MS187-5 and MS231-7, a 256-bp DNA was not amplified (data not shown) even though these LCLs had both mutated and WT LMP1 DNA and both were linked to TR. The LMP1 genes in these LCLs may therefore be in the same EBV genome as that which would arise from a nonhomologous or partially homologous recombination between the mutated LMP1 DNA and the P3HR-1 EBV. Most recombinations in this system are homologous and doubly reciprocal (16, 17, 24, 35, 36).

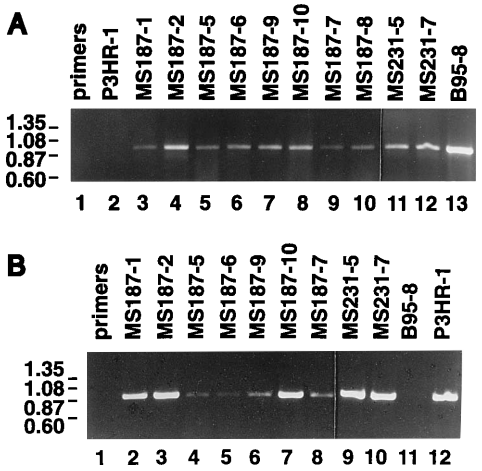

FIG. 4. PCR analyses of LMP1 DNA linked to EBV TR sequences in LCLs infected with an EBV recombinant. (A) Primers TRFT1 and TRR amplify a 929-bp DNA from a type 1 EBV-infected cell line, B95-8 (lane 13), but not from a type 2 EBV-infected cell line, P3HR-1 (lane 2). A 929-bp DNA is amplified from all LCLs infected with an MS187 or MS231 LMP1 recombinant. (B) Primers TRFT2 and TRR amplify a 929-bp DNA from a type 2 EBV-infected cell line, P3HR-1, (lane 12) but not from a type 1 EBV-infected cell line, B95-8 (lane 11). A 929-bp DNA is amplified from all LCLs coinfected with P3HR-1. PCR amplifications with primers but no cell DNA were loaded in lanes marked primers. DNA size markers (in kilobase pairs) are indicated on the left.

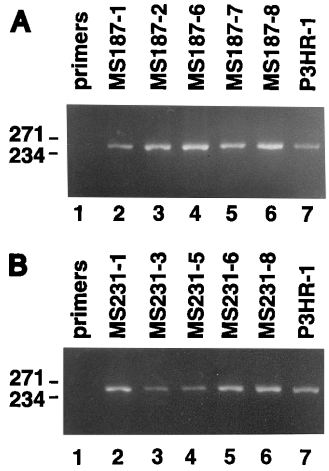

FIG. 5. PCR analyses of DNA at site of P3HR-1 EBNA LP/2 deletion. (A) Primers that bracket the P3HR-1 deletion amplify a 256-bp DNA from MS187 recombinant virus-infected LCLs coinfected with P3HR-1. (B) Primers that bracket the P3HR-1 deletion amplify a 256-bp DNA from MS231 recombinant-infected LCLs coinfected with P3HR-1. PCR amplifications with primers but no cell DNA were loaded in lanes marked primers. PCR amplification of DNA from P3HR-1 cells infected with P3HR-1 EBV is shown in lanes marked P3HR-1. DNA size markers (in base pairs) are indicated on the left.

As LCLs were further expanded in culture, all LCLs infected with the MS187 or MS231 recombinants maintained WT LMP1 from the coinfecting P3HR-1 genome. At 4, 6, 12, or 15 months, 17, 8, 7, or 5 LCLs, respectively, remained infected with MS187 recombinants and 11, 5, 3, or 3 LCLs, respectively, remained infected with MS231 recombinants. At each time point, each LCL also had WT LMP1 genes from coinfecting P3HR-1 EBV. Most of the remaining LCLs converted to having only the WT LMP1 gene, and some LCLs could not be propagated. No LCL lost the WT LMP1 gene. LCLs may convert to having only WT LMP1 as a result of secondary recombination between the two different viral genomes in a latently infected cell or in a cell undergoing spontaneous lytic replication. Progeny secondary recombinants can superinfect LCLs. A cell infected with a fully transforming secondary recombinant genome (WT EBNA LP/2-WT LMP1) should have an advantage over cells requiring mitotic transmission of two types of EBV genomes for continued growth, enabling it to overgrow the culture. These results demonstrate that primary B-lymphocyte growth transformation cannot be maintained without WT LMP1 from coinfecting P3HR-1 and that secondary recombination occurs in these LCLs resulting in LCLs infected with an EBV recombinant that has WT LMP1. The tendency of the recombinant virus-infected LCLs to convert to having only WT LMP1 from secondary recombinants differs from previous experience with LCLs infected with EBV recombinants with mutations that do not affect cell growth when coinfection with P3HR-1 was frequently maintained for over a year in culture (17, 34). The persistent presence of WT LMP1 is further evidence that the carboxy-terminal LMP1 domains that are deleted from the MS187 and MS231 LMP1 genes are required for primary B-lymphocyte growth transformation; the loss of mutated LMP1 is evidence that the truncated proteins may have a negative effect on cell growth.

Passage of MS187 EBV recombinants. To further test whether MS187 EBV recombinants are able to transform primary B lymphocytes, lytic virus infection was induced in seven LCLs coinfected with P3HR-1 and MS187 recombinants. LCLs were then lethally irradiated and cocultivated with primary B lymphocytes. Supernatant virus was also filtered (0.22-μm-pore-size filter) and used to infect primary B lymphocytes. Four to eight weeks later, second generation LCLs were analyzed for LMP1 DNA by PCR. No second generation LCL was infected with an MS187 recombinant alone (Table 1). Twenty-nine second generation LCLs infected with an MS187 recombinant also had WT LMP1 DNA. Ten MS187 recombinant coinfected LCLs were expanded in cell culture and monitored for maintenance of the coinfected state. At 6 months, 3 of the 10 MS187 LCLs had lost MS187 DNA and had retained only WT LMP1. The other seven retained both MS187 and WT LMP1 DNA. By 9 months, two of these seven MS187 LCLs had MS187 and WT LMP1 DNA and the other five LCLs died. Thus, there was selection against the presence of the MS187 mutation in infected LCLs since after 9 months of expansion only 20% of the MS187 and P3HR-1 coinfected LCLs survived and continued to be coinfected. In contrast to the 29 LCLs which were coinfected with MS187-mutated LMP1 EBV recombinants and P3HR-1, 269 progeny LCLs were initially in-

TABLE 1. PCR analysis for LMP1 DNA in MS187 progeny LCLs

Virus source	No. of LCLs[a]		
	Mutant alone	Mutant + WT LMP1	WT LMP1 alone
MS187-1	0	11	28
MS187-2	0	7	33
MS187-3[b]	0	0	50
MS187-4	0	0	24
MS187-6	0	2	15
MS187-7[b]	0	2	101
MS187-8	0	7	18
Total	0	29	269

[a] Control plates failed to yield LCLs.
[b] B lymphocytes were infected with filtered (0.22-μm-pore-size filter) virus in addition to infection by cocultivation.

TABLE 2. PCR analysis for LMP1 DNA in MS231 progeny LCLs

Virus source	No. of LCLs[a]		
	Mutant alone	Mutant + WT LMP1	WT LMP1 alone
MS231-1	0	2	240
MS231-2	0	2	80
MS231-3[b]	0	1	68
MS231-4	0	0	128
MS231-5	0	0	26
MS231-6[b]	0	0	30
MS231-8	0	25	73
Total	0	30	645

[a] Control plates failed to yield LCLs.
[b] B lymphocytes were infected only by cocultivation.

fected with a WT EBNA LP/2-WT LMP1 secondary recombinant (Table 1). These WT EBNA LP/2-WT LMP1 secondary recombinants arose during lytic replication in the parental coinfected LCL and were produced at lower levels than the parental viruses (17, 34). The relatively few second-generation LCLs (29 of 298, or 10%) infected with an MS187 recombinant were not due to lack of MS187 recombinant virus produced after induction of lytic replication in the parental LCLs since PCR analysis (similar to that shown in Fig. 3A) of filtered (0.22-μm-pore-size filter) supernatants from these LCLs demonstrates at least as much MS187-mutated LMP1 DNA amplification product as WT LMP1 DNA product (data not shown). The failure to recover LCLs infected with an MS187 recombinant alone is further evidence that the LMP1 carboxy terminus encodes a critical effector function.

Passage of MS231 EBV recombinants. To test further whether MS231 EBV recombinants are able to transform primary B lymphocytes, lytic virus infection was also induced in eight LCLs coinfected with P3HR-1 and MS231 recombinants. Progeny virus was used to infect primary B lymphocytes either by cocultivation or with filtered (0.22-μm-pore-size filter) supernatant virus, and 4 to 8 weeks later, second generation LCLs were analyzed for LMP1 DNA by PCR. The results for seven of the parental LCLs are summarized in Table 2 and indicate that no second-generation LCL was infected with an MS231 recombinant alone. Thirty second-generation LCLs infected with an MS231 recombinant also had WT LMP1 DNA. Only four of these MS231 recombinant-virus coinfected LCLs could be expanded in cell culture and were monitored for maintenance of the coinfected state. In general, the growth of these coinfected LCLs was not remarkably different from that of WT recombinant virus-infected LCLs. At 6 months, three of four MS231 LCLs had both MS231 and WT LMP1 DNA and one of four had only WT LMP1 DNA. By 9 months, none of these three LCLs had MS231 and WT LMP1 DNA, two LCLs had only WT LMP1 DNA, and one LCL did not survive. Thus, there was also selection against the presence of the MS231 mutation in infected LCLs since only 4 of 30 LCLs could be expanded, and after 9 months of expansion, none of the MS231 recombinant virus and P3HR-1 coinfected LCLs continued to be coinfected. Many (645) progeny LCLs were initially only infected with a WT EBNA LP/2-WT LMP1 secondary recombinant (Table 2). The relatively few second-generation LCLs (30 of 675, or 4%) infected with an MS231 recombinant were not due to lack of MS231 recombinant virus produced after induction of lytic replication in the parental LCLs since PCR analysis (similar to that shown in Fig. 3B) of filtered (0.22-μm-pore-size filter) supernatant from these LCLs demonstrates at least as much MS231 as WT LMP1 DNA (data not shown). These results are further evidence that the carboxy-terminal portion of LMP1 downstream of amino acid 231 provides a critical function.

Since the MS231 EBV recombinants require WT LMP1 for transformation of primary B lymphocytes, we attempted to complement these mutations by infecting primary B-lymphocytes either with filtered (0.22-μm-pore-size filter) virus from coinfected LCLs MS231-1, MS231-4, or MS231-8 or with these MS231 recombinants and exogenously added P3HR-1 virus. The results summarized in Table 3 indicate that the addition of P3HR-1 virus leads to more second-generation LCLs. However, only two of the second-generation LCLs had MS231 and WT LMP1 DNA. All others were infected with WT EBNA LP/2-WT LMP1 secondary recombinants. The finding that the addition of exogenous P3HR-1 resulted in an approximately fourfold increase in second-generation LCLs but only two were MS231 infected is further evidence for a negative effect exerted by MS231. Likely, some cells initially coinfected with MS231 recombinant virus and P3HR-1 underwent spontaneous lytic replication which resulted in WT EBNA LP/2-WT LMP1 secondary recombinants, and cells infected with a secondary recombinant overgrew the culture.

MS231 LMP1 recombinant-infected LCLs can grow on fibroblast feeder layers. One of the eight LCLs (MS231-7) from which virus was passaged had different characteristics from the LCLs shown in Table 2. This LCL could not be expanded beyond 9 months in continuous culture. In addition, when lytic infection was induced in this LCL and primary B lymphocytes were infected with the resultant virus, most of the progeny LCLs had both MS231 and WT LMP1 DNA. Of the progeny LCLs, 194 had both MS231 and WT LMP1 DNA and 56 had only WT LMP1 DNA. Thus, the frequency of passage of MS231 into progeny LCLs was much higher from this parental cell line than from all other cell lines coinfected with an MS231 recombinant and P3HR-1. The discrepant finding that so many of the progeny LCLs had both MS231 and WT LMP1 DNA is

TABLE 3. PCR analysis for LMP1 DNA in MS231 progeny LCLs after infection with or without exogenous P3HR-1

Virus source	No. of LCLs[a]					
	Mutant alone	Mutant + WT LMP1	WT LMP1 alone	Exogenous P3HR-1 added		
				Mutant alone	Mutant + WT LMP1	WT LMP1 alone
MS231-1	0	0	12	0	1	50
MS231-4	0	0	9	0	0	24
MS231-8	0	0	1	0	1	3
Total	0	0	22	0	2	77

[a] These progeny LCLs are included in Table 2.

evidence that the MS231 and WT LMP1 are linked on the same EBV genome in MS231-7. Such an unusual recombinant has previously been described (34). In support of this model, the 256-bp DNA fragment characteristic of P3HR-1 EBV could not be amplified from the MS231-7 LCL with primers that bracket the EBNA LP/2 DNA deletion. Further, although linkage of WT LMP1 to TR could be demonstrated at an early passage (Fig. 4B, lane 10), this 929-bp fragment could no longer be amplified when the parental LCL was no longer expanding. The progeny MS231-7- and WT LMP1-positive LCLs maintained both MS231 and WT LMP1 in contrast to the other progeny MS231 LCLs shown in Table 2, providing further evidence for a genetic linkage between between MS231 and WT LMP1 in these cells. Ten second-generation LCLs were expanded in culture. After 6 months, all 10 LCLs maintained MS231 and WT LMP1 DNA. At 9 months, eight LCLs maintained MS231 and WT LMP1 DNA and two LCLs did not survive. Thus, the absence of coinfecting P3HR-1 EBV, the high frequency of cotransfer of MS231 and WT LMP1 to progeny LCLs and the high frequency of retention of MS231 LMP1 in LCLs over prolonged passage support the model that MS231-7 is infected with a recombinant that has MS231 and WT LMP1 on the same genome. Although the parent MS231-7 cell line could never be sufficiently expanded to perform Southern blot analysis, direct linkage of MS231 and WT LMP1 DNA in the parent MS231-7 virus genome may have facilitated persistence of the MS231 DNA or may have been associated with a relatively lower expression level of MS231, initially enabling growth of MS231-7.42 and MS231-7.65 (see below).

Two poorly growing progeny LCLs (MS231-7.42 and MS231-7.65) were isolated that were infected with an MS231 recombinant alone. MS231-7.42 died when we attempted to expand it from one to two wells of a 96-well plate after 4 months. (Most other LCLs readily expand after 5 to 6 weeks.) In order to avoid losing the second LCL infected with an MS231 recombinant alone, it was cultivated over primary human foreskin fibroblasts to attempt to promote cell growth. This maneuver enhanced the ability to propagate this LCL (MS231-7.65). PCR analysis demonstrating MS231 DNA and no WT LMP1 DNA in this LCL is shown in Fig. 6A (lane 3). Continued expansion of MS231-7.65 remained dependent upon cultivation over fibroblasts. Even after 6 months of growth on fibroblasts, dense cultures of $>10^6$ MS231-7.65 cells could not remain viable in standard culture medium without fibroblast feeders. Southern analysis of DNA from the MS231-7.65-infected LCL demonstrated only mutated LMP1 DNA, which was in the expected context in EBV DNA. An LMP1 DNA probe hybridized to a fragment with the expected size (7.2 kb) after cleavage with *Bam*HI and *Eco*RI. Digestion with *Bam*HI, *Eco*RI, and *Xba*I resulted in two smaller fragments with expected sizes (5.2 and 2.0 kb) because of the inserted oligonucleotide (data not shown).

PCR analysis of LCL MS231-7.65 with primers specific for WT LMP1 DNA did not amplify WT LMP1 DNA from this LCL (Fig. 6B, lane 2). This method detects 5 IB4 cells per 5,000 EBV-negative BJAB B-lymphoma cells (Fig. 6B, lanes 4 to 8). IB4 is an LCL which initially had both episomal and chromosomally integrated EBV DNA and now has only four integrated EBV genomes per cell (12, 15). The MS231-7.65 recombinant virus-infected LCL therefore has fewer than 20, if any, WT LMP1 genes per 5,000 cells.

To assay the transforming potential of the MS231 recombinant virus infecting this novel cell line, lytic virus infection was induced in this LCL. The cells were then lethally irradiated and cocultivated with primary B lymphocytes over fibroblast feeder cells. Progeny LCLs arose 4 weeks later in all 24 wells. These

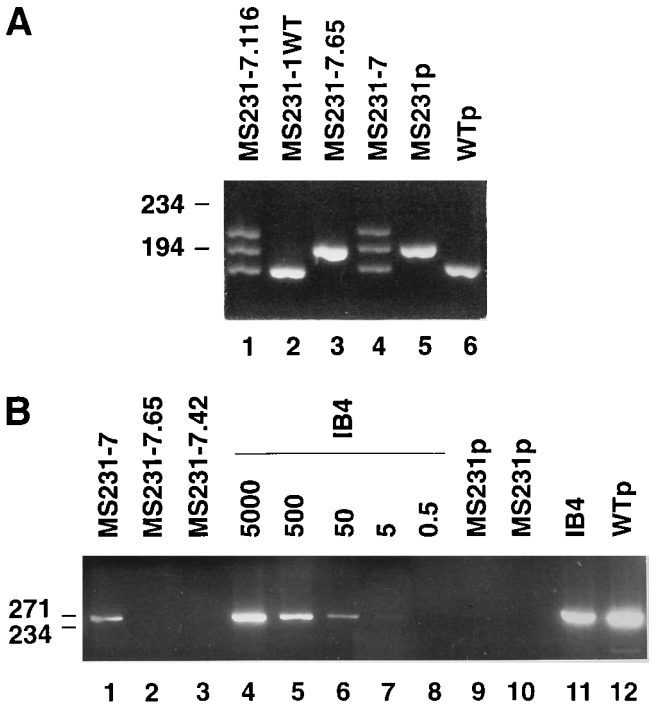

FIG. 6. PCR analysis of LCL infected with only an MS231 recombinant. (A) Primers MS231A and MS231B amplify a 181-bp DNA from WT LMP1 DNA and a 195-bp DNA from MS231 LMP1 DNA. LCL MS231-1WT was infected with a WT EBNA LP/2-WT LMP1 secondary recombinant that arose from LCL MS231-1. MS231-7.65 was infected with only an MS231 recombinant. MS231 and WT plasmid DNA clones were PCR amplified and loaded in lanes marked MS231p and WTp, respectively. (B) MS231W primers amplify a 263-bp DNA from WT LMP1 DNA but not from MS231 DNA. PCR amplifications of LCL IB4, which has four genomic copies of EBV per cell (12, 15), are shown in lanes 4 to 8 and 11. Lanes 4 to 8 contain 5,000 IB4 cells serially 10-fold diluted with EBV-negative BL cells and then analyzed by PCR. The number of IB4 cells in each amplification reaction is indicated above lanes 4 to 8. PCR amplifications of a plasmid DNA clone of MS231 are shown in lane 9 (100 pg of plasmid DNA amplified) and lane 10 (1 ng of plasmid DNA amplified). PCR amplification of 100 pg of a plasmid DNA clone of WT DNA is shown in lane 12. MS231-7.65 and MS231-7.42 are progeny of LCL MS231-7. DNA size markers (in base pairs) are indicated on the left.

LCLs could slowly expand from a well in a 96-well plate to a well in a 48-well plate off of fibroblast feeders over a period of several months but could not be expanded into 24-well plates. Sustained growth of these progeny LCLs remained dependent upon cultivation on fibroblasts even after 5 months. This indicates that the MS231 recombinant can initiate as well as maintain B-lymphocyte growth transformation as long as the infected cells are kept on fibroblasts. Thus, the carboxy terminus distal to amino acid 231 is essential for fibroblast-independent primary B-lymphocyte growth transformation.

Since MS231 recombinant virus could initiate and maintain transformation when grown on fibroblasts, attempts were made to investigate whether MS187 recombinants could also initiate and maintain transformation when grown on fibroblasts. When primary B lymphocytes were infected with cell-free filtered (0.22-μm-pore-size filter) virus from LCLs coinfected with MS187 and P3HR-1 and the infected cells were plated onto fibroblasts, only four LCLs were obtained, and they had only WT LMP1 genes. Since MS187 recombinants are produced in excess of WT LMP1 secondary recombinants, these limited data are consistent with the MS187 recombinants being deficient in transformation even when the infected lymphocytes are plated on fibroblast feeders.

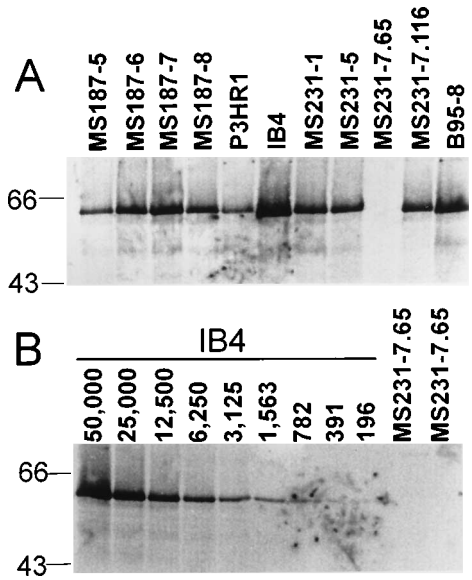

FIG. 7. Immunoblot analysis of LCLs for LMP1. (A) LMP1 was detected from all LCLs coinfected with an MS187 or MS231 LMP1 recombinant and P3HR-1. B95-8 is a WT EBV-infected marmoset cell line. IB4 is a WT EBV-infected LCL. P3HR-1 cells express less LMP1 since P3HR-1 EBV has a deletion of the EBNA 2 gene which transactivates LMP1 expression (41). No LMP1 was detected from LCL MS231-7.65. (B) Immunoblot analysis of serial twofold dilutions of IB4 cells demonstrate that LMP1 is detected from 782 and greater quantities of IB4 cells. LMP1 was not detected in MS231-7.65 cells. Size markers (in kilodaltons) are indicated on the left. Electrophoresis was done with an SDS–8% polyacrylamide gel.

LMP1 expression in MS187 and MS231 recombinant virus-coinfected LCLs. LCLs were tested for LMP1 by size separating proteins in denaturing polyacrylamide gels, Western blotting to filters, and probing with LMP1 specific monoclonal antibody. Figure 7A demonstrates that all MS187- or MS231-infected LCLs growing independent of fibroblast feeders express WT LMP1 which migrates at a size of 60 kDa. P3HR-1 cells express less LMP1 since EBNA 2, a transactivator of LMP1 transcription, is deleted (41). CRPs with the predicted size of the MS187 or MS231 LMP1 were not detectable in any coinfected cells because available monoclonal antibodies recognize epitopes which are encoded downstream of the MS231 nonsense linker. Most significantly, an LCL (MS231-7.65) that by PCR analysis is infected with an MS231 EBV recombinant and has no WT LMP1 DNA does not express WT LMP (Fig. 7). The sensitivity of this procedure was estimated by immunoblot analysis of serial twofold dilutions of proteins from 5×10^4 IB4 cells with a monoclonal antibody directed against LMP1. As shown in Fig. 7B, LMP1 signal was last detectable at 782 IB4 cells per lane. In the two lanes on the right, proteins from 5×10^4 MS231-7.65 cells were resolved in each lane, but no LMP1 was detected. Thus, these cells are proliferating without expression of WT LMP1.

Reverse transcription and PCR amplification of LCL RNA with LMP1-specific primers indicate that the MS187- or MS231-infected LCLs have LMP1 RNA with the MS187 and MS231 mutations. Reverse transcription-PCR yielded the expected 915-bp (WT LMP1) and 438-bp (MS187) fragments from MS187 and P3HR-1 coinfected cells and the 458-bp (WT LMP1) and 472-bp (MS231) fragments from cells coinfected with MS231 and P3HR-1. Since the 458- and 472-bp PCR products are difficult to resolve on an agarose gel and the nonsense linker contains an *Xba*I site, the 472-bp PCR product was digested with *Xba*I to yield the expected 404- and 68-bp products from the 472-bp fragment (data not shown).

Immunofluorescent staining of fixed cells with the S12 monoclonal antibody reveals that LMP1 is tightly patched and capped along the plasma membrane in coinfected LCLs (data not shown). The MS187 or MS231 coinfected LCLs were indistinguishable from WT, indicating that the putative carboxy-terminal-truncated LMP1 does not grossly interfere with WT LMP1 localization to the plasma membrane. To evaluate whether the MS187 or MS231 recombinants are also likely to aggregate in the plasma membrane, the first 180 or first 231 LMP1 amino acids with an FLAG epitope (14) at the amino terminus were expressed in a non-EBV-infected BL cell line. As detected by monoclonal M2 antibody to the FLAG (IBI/Kodak) epitope, the 1- to 180-amino-acid LMP1 aggregated somewhat more diffusely in plasma membrane patches compared with WT LMP1, while 1- to 231-amino-acid LMP1 formed tight plasma membrane patches similar to WT LMP1 (data not shown). Thus, the putative MS187 and MS231 proteins are predicted to aggregate in plasma membrane patches similar to WT LMP1 and might be interspersed among WT LMP1 molecules in the coinfected LCLs (22).

DISCUSSION

This EBV recombinant genetic analysis of the LMP1 carboxy terminus indicates that residues 188 to 231 are critical or essential for EBV-mediated growth transformation of primary B lymphocytes and that other parts of the carboxy-terminal cytoplasmic domain are essential for fibroblast-independent cell proliferation. MS187 EBV recombinants stringently require WT LMP1 expressed from a coinfecting, transformation-defective P3HR-1 virus for B-lymphocyte growth transformation. When eight of the MS187 recombinants were passaged to primary B lymphocytes and 298 second-generation LCLs were analyzed, 90% were fully WT secondary recombinants. Only 10% were MS187 EBV recombinant infected, and all were P3HR-1 coinfected despite the presence of at least as much MS187 LMP1 DNA as WT DNA in the virus preparations. Most of these coinfected LCLs died or converted to having only WT LMP1. Thus, sequences beyond codon 187 are essential for transformation and MS187 has a partial dominant negative effect. This partial dominant negative effect may be due to the putative MS187 truncated product interspersing itself with WT LMP1 in the plasma membrane of the coinfected LCLs, lessening the interaction between WT LMP1 molecules in the plasma membrane.

MS231 also had a partial dominant negative effect. When MS231 recombinants were passaged from P3HR-1-coinfected LCLs to fresh primary B lymphocytes, 675 second-generation LCLs were analyzed, and 96% had only WT LMP1 DNA. Only 4% were coinfected with an MS231 recombinant and P3HR-1, despite the presence of at least as much MS231 LMP1 DNA as WT DNA in the virus preparations. In addition, two LCLs were infected with an MS231 recombinant alone. These LCLs were the progeny of an LCL infected with a recombinant virus for which there was evidence of a genetic linkage between MS231 and WT LMP1 genes. These two initial LCLs and their progeny were highly growth defective and could not be expanded without fibroblast feeders. On fibroblast feeders, the cells grew well. Moreover, the MS231 recombinant virus could initiate transformation of primary B lymphocytes without WT LMP1 when the newly infected cells were maintained on fibroblast feeders. Thus, growth on fibroblast feeders appears to fully complement the MS231 defect.

These results indicate the LMP1 cytoplasmic carboxy-termi-

nal domain contributes at least one critical effector function for EBV-mediated B-lymphocyte growth transformation and that in the absence of this effector, the transmembrane domains inhibit the effect of WT LMP1. Amino acids 188 to 231 may include all or part of one key effector since LCLs deleted for this sequence could never be recovered without complementation by WT LMP1. LMP1 amino acids 232 to 386 may include all or part of another effector since this region is required for fibroblast-independent B-lymphocyte growth transformation. Most likely, the transmembrane domains of LMP1 molecules enable them to aggregate in the plasma membrane so that the carboxy-terminal effector domains are closely approximated. This close approximation would be inhibited in cells in which carboxy-terminal-truncated LMP1 is expressed.

Since the LCLs infected with MS231 recombinants alone required fibroblast feeders for continued growth, it is likely that a fibroblast growth factor is complementing the LMP1 defect. LMP1 may normally provide a growth factor-like signal, perhaps by interacting with a growth factor receptor in a manner similar to that of the bovine papillomavirus E5 protein. The E5 protein is a membrane protein with transforming properties which activates and forms a complex with the cellular platelet-derived growth factor receptor (4, 8, 29, 30). These results provide the first evidence that LMP1 exerts a growth factor-like effect on LCLs since only LCLs lacking WT LMP1 required the fibroblast growth factor.

In light of the discovery late in the course of these experiments that the growth defect of MS231 recombinant-infected primary B lymphocytes could be complemented by growth of the infected cells on a fibroblast feeder layer, attempts were made to evaluate the possibility that MS187 recombinants might also be able to transform primary B lymphocytes if the infected cells are grown on fibroblasts. By using virus produced from MS187 and P3HR-1 coinfected LCLs, only WT LMP1 secondary recombinants were able to transform primary B lymphocytes on fibroblasts. However, the amount of virus that has been obtained from the MS187 recombinant and P3HR-1-coinfected LCLs is small, and these experiments are continuing. We cannot exclude the possibility that higher titers of cell-free MS187 EBV recombinants or optimized fibroblast feeders or the further addition of growth factors may enable the MS187 recombinant to transform primary B lymphocytes. Indeed, this possibility is an expectation of the working hypothesis that aggregated WT LMP1 in the plasma membrane provides dominant growth stimulatory signals through B-lymphocyte growth factor receptor pathways.

Previous genetic analyses of the importance of the LMP1 carboxy-terminal domain in rodent fibroblast transformation assays have been conflicting. The entire carboxy-terminal cytoplasmic domain was dispensable for anchorage independence in BALB/3T3 cells (3). In addition, a mutation equivalent to MS231 induced anchorage-independent growth and tumorigenicity of Rat-1 cells in nude mice (3, 11). However, in another series of experiments, an LMP1 mutation equivalent to MS231 did not induce loss of contact inhibition in Rat-1 fibroblasts (28). An advantage of molecular genetic analysis using EBV recombinant infection of primary B lymphocytes is that long-term LCL outgrowth is stringently dependent on LMP1 expression (17).

Although these experiments demonstrate a critical role for the LMP1 cytoplasmic carboxy-terminal domain in EBV-induced B-lymphocyte transformation, the carboxy-terminal domain is clearly not sufficient for LMP1 function. EBV recombinants expressing codon 44-initiated LMP1 and another LMP1 cross-reactive protein which lacks the LMP1 N-terminal and first four transmembrane domains (D1LMP1) are unable to transform primary B lymphocytes despite expressing the entire LMP1 carboxy-terminal domain (17). Neither codon 44-initiated LMP1 nor D1LMP1 forms patches in the plasma membrane and neither inhibits transformation (17). It is probable that the LMP1 transmembrane domains are critical for proper LMP1 localization which allows the carboxy-terminal domain to come into contact with the molecules through which it must interact to send a constitutive signal. Further molecular genetic analysis should more precisely define which of the first 44 amino acids of the cytoplasmic carboxy terminus enable LMP1 to transform B cells in the presence of fibroblast feeders and which of the last 155 LMP1 amino acids allow EBV transformation off fibroblast feeders. Such information will facilitate biochemical analyses of the mechanisms by which LMP1 contributes to EBV-induced B-lymphocyte transformation.

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