Induction of Epstein-Barr Virus Oncoprotein Latent Membrane Protein 1 (LMP1) by Transcription Factors Activating Protein 2 (AP-2) and Early B Cell Factor (EBF)

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ABSTRACT

Latent membrane protein 1 (LMP1) is a major oncogene essential for primary B cell transformation by Epstein-Barr virus (EBV). Previous studies suggested that some transcription factors, such as PU.1, RBP-Jκ, NKκB, and STAT, are involved in this expression, but the underlying mechanism is unclear. Here, we identified binding sites for PAX5, AP-2, and EBF in the proximal LMP1 promoter (ED-L1p). We first confirmed the significance of PU.1 and POU domain transcription factor binding for activation of the promoter in latency III. We then focused on the transcription factors AP-2 and EBF. Interestingly, among the three AP-2-binding sites in the LMP1 promoter, two motifs were also bound by EBF. Overexpression, knockdown, and mutagenesis in the context of the viral genome indicated that AP-2 plays an important role in LMP1 expression in latency II in epithelials. In latency III B cells on the other hand, the B cell-specific transcription factor EBF binds to the ED-L1p and activates LMP1 transcription from the promoter.

IMPORTANCE

Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) is crucial for B cell transformation and oncogenesis of other EBV-related malignancies, such as nasopharyngeal carcinoma and T/NK lymphoma. Its expression is largely dependent on the cell type or condition, and some transcription factors have been implicated in its regulation. However, these previous reports evaluated the significance of specific factors mostly by reporter assay. In this study, we prepared point-mutated EBV at the binding sites of such transcription factors and confirmed the importance of AP-2, EBF, PU.1 and POU domain factors. Our results will provide insight into the transcriptional regulation of the major oncogene LMP1.

INTRODUCTION

The Epstein-Barr virus (EBV) is a human gamma-herpesvirus that mainly infects and establishes latent infection in B lymphocytes, but it can also infect other types of cells, including NK, T, and epithelial cells. EBV infection has been implicated as a causal factor in a variety of malignancies, and the expression pattern of viral latent genes varies depending on the tissue of origin and the state of the tumors. Neoplasms such as Burkitt lymphomas or gastric carcinomas express only EBV-encoded small RNA (EBER) and EBV nuclear antigen 1 (EBNA1) (type I latency), whereas some Hodgkin lymphomas, nasopharyngeal carcinomas (NPC), and NK/T lymphomas express EBER, EBNA1, latent membrane protein 1 (LMP1), and LMP2 genes (type II latency). In addition to the type II genes, EBNA2, EBNA3, and EBNA-LP are also expressed in immunosuppression-related lymphomas or lymphoblastoid cell lines (LCLs) (type III latency). LMP1 constitutively activates cellular signaling through NFκB, MAPK, JAK/STAT and AKT and is believed to be a major oncogene encoded by EBV (1-11).

Two promoters regulate LMP1 gene transcription, with mechanisms that differ between type II and type III infection. In latency III in B lymphocytes, LMP1 transcription from the proximal ED-L1 promoter is activated by EBNA2 (12-14). Although EBNA2 shows no DNA-binding activity, it enhances LMP1 promoter activity by functioning as a cofactor. It associates with cellular transcriptional factors, including the Recombination signal Binding Protein Jκ (RBP-Jκ) (14-16) and PU-box 1 (PU.1) (12, 13, 17, 18), which are then recruited onto the LMP1 promoter for transactivation. Viral factors, including EBNA-LP and EBNA3C, also associate with the complex and further modify the activation process (19-22).

On the other hand, LMP1 is expressed in an EBNA2-independent manner in type II latency, since EBNA2 is not available in this state. Cytokines, such as IL-4, IL-6, IL-10, IL-13, and IL-21, have been frequently reported to activate the JAK/STAT pathway, thereby inducing LMP1 gene expression through STAT (23-28). In certain latency II infected cells, including NPC cells (29), LMP1 transcription originates from a STAT regulated upstream promoter, termed TR-L1p, located within the terminal repeats (TRs), in addition to the proximal ED-L1p (23, 24, 27, 30, 31). We previously identified a CCAAT Enhancer-Binding Protein (C/EBP) family transcription factor that augments both proximal and distal promoter activation of

LMP1 in type II latency by binding to a sequence motif in the proximal promoter (32).

Elsewhere, the involvement of transcriptional factors, such as NFκB (33, 34), AP-2 (35), POU domain protein (17), ATF/CREB (36), Sp1/3 (37), and IRF7 (38), has been observed. Type I interferons were recently reported to upregulate LMP1 expression, presumably through NFκB, PKC, and JNK in Burkitt lymphoma cells (39).

Despite the presence of these well targeted, focused reports, functional testing of the *cis* (and *trans*) elements in the context of virus genomes has not received sufficient attention as most of the mutagenesis studies have analyzed the importance of transcription factor binding sites in reporter assays.

AP-2 is a family of transcription factors containing a helix-span-helix motif for DNA binding at the carboxyl terminus with possible roles in development, control of apoptosis and cell cycling, and oncogenesis (40, 41). Its members are clearly distinct from AP-1 family transcription factors, homo/heterodimers composed of c-Fos, c-Jun or ATF, which share a b-Zip motif for dimerization and DNA binding. Moreover, AP-2 proteins can bind to G/C-rich elements, such as 5'-[G/C]CCN$_{(3,4)}$GG[G/C]-3' (41, 42).

Early B cell factor (EBF) is a transcription factor that contains a helix-loop-helix motif, which binds to the G/C-rich motif, 5'-CCCNNGGG-3'. It is expressed in B cell lineages and is a master regulator of early B cell differentiation (43, 44).

In the present study, we applied small interfering RNA (siRNA)-mediated knockdown and/or overexpression, and showed that AP-2 and EBF play important roles in EBNA2-independent and -dependent LMP1 expression, respectively. Introduction of mutations into the AP-2/EBF binding sites in the promoter of recombinant EBV inhibited B cell transformation efficiency. Taken together, we observed a crucial role of AP-2 and EBF in LMP1 expression in both type II and type III latency.

MATERIALS AND METHODS

Cell culture and reagents- HEK293EBV-BAC and HeLa-CR2/GFP-EBV (32) cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum. Akata(-), C666-1 (45) and LCLs were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum.

Antibodies against SP1 and PAX5 were obtained from Santa Cruz Biotechnology. Anti-AP-2α, FLAG, -myc, and -tubulin antibodies were purchased from Abcam, Sigma, MBL, and Cell Signaling Technology, respectively. The anti-LMP1 and EBNA2 antibodies have been described previously (46). Horseradish peroxidase-linked goat antibodies to mouse /rabbit IgG were obtained from Amersham Biosciences. The expression vectors, pcDNAFLAGhTFAP-2α (47), pcDNAPAX5 (48), and pcDNAmycEBF1 (49) were provided by Drs. Miyazono, Hayakawa, and Sigvardsson, respectively.

Genetic manipulation of EBV-BAC DNA and cloning of HEK293 cells with EBV-BAC - EBV-BAC DNA was provided by W. Hammerschmidt (50). Homologous recombination was carried out in *E. coli* as described previously (32, 51).

To prepare EBV-BAC mutants, at the LMP1 proximal promoter, a transfer DNA fragment for the first recombination was generated by PCR using rpsL-neo (Gene Bridges) as the template, with Neo/stFor (TGCCGCCAACGACCTCCCAACGTTGCGCGCCCCGCGCCTCTTTGTGCAGATTACACTGCCG GCCTGGTGATGATGGCGGGATC) and Neo/stRev (CAGTGTGAGAGGCTTATGTAGGGCGGCTACGTCAGAGTAACGCGTGTTTCTTGGGATGTAT CAGAAGAACTCGTCAAGAAGG) primers. After recombination, kanamycin-resistant colonies were selected and checked by colony PCR using the following primers TAGTCCTGCCTTTCCATTTCCTG and GTCTCAGAAGGGGGAGTGCGTAG to generate intermediate DNA. The Neo/st cassette in the intermediate DNA was then replaced using the next transfer vector DNA, containing each mutation in the LMP1 promoter. The AP-2 binding motif at -75 (CCCCCCGGGCCTAC) was modified to CCCCCCTTTCCTAC. Motifs TGCCTCCGGCAGA (-100) and GCCCCCCGGGGACCCGC (-205) were edited to TGCCTAATTCAGA and GCCCAAATGGGACCCGC, respectively. Electroporation of *E. coli* was performed using a Gene Pulser III (Bio-Rad), and purification of EBV-BAC DNA was achieved with NucleoBond Bac100 (Macherey-Nagel). Recombination was confirmed with PCR products of the promoter region, by electrophoresis of the BamHI-digested viral genome and sequencing analysis. EBV-BAC DNA was transfected into HEK293 cells using Lipofectamine 2000 reagent (Invitrogen), followed

by culture on 10 cm dishes with 100-150 μg/ml of hygromycin B for 10-15 days to clone GFP-positive cell colonies as described previously (51). Briefly, for each recombinant virus, we picked up more than 10 hygromycin-resistant, GFP-positive cell colonies to obtain at least three typical clones exhibiting minimal spontaneous expression of viral lytic proteins and significant induction of these proteins upon BZLF1 transfection.

Transfection and immunoblotting – Transfections were carried out by lipofection using Lipofectamine 2000 reagent (Invitrogen) or by electroporation using a Microporator (Digital Bio). The total amount of plasmid DNA was standardized by addition of an empty vector. Knockdown of AP-2 was performed by transfection of duplexes of 21-nucleotide siRNAs. The sense and antisense sequences for the siRNAs are si-control; GCAGAGCUGGUUUAGUGAAdTdT and UUCACUAAACCAGCUCUGCdTdT, si-AP-2α1; CCGAAUUUCCUGCCAAAGCdTdT and GCUUUGGCAGGAAAUUCGGdTdT, si-AP-2α2; CGCCAAAAGCAGUGACAAAdTdT and UUUGUCACUGCUUUUGGCGdTdT. Immunoblotting was carried out as described previously (52).

Quantitative real-time RT-PCR (qRT-PCR)- Total cell RNA was purified using the TriPure Isolation Reagent (Roche) and subjected to reverse transcription and real-time PCR reactions using a One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa) and Real Time PCR System 7300, as described previously (53), except that the 40 s extension period at 60 °C was extended to 70 s for detecting long species of LMP1 mRNA expressed from the TR-L1 promoter. Primers used for the qRT-PCR of the GAPDH, BZLF1, and EBNA2 genes were as follows: GAPDH; TGCACCACCAACTGCTTAGC and GGCATGGACTGTGGTCATGAG, BZLF1; AACAGCCAGAATCGCTGGAG and GGCACATCTGCTTCAACAGG, EBNA2, TTAGAGAGTGGCTGCTACGCATT and TCACAAATCACCTGGCTAAG. Primers to distinguish distal (TR-L1) and proximal (ED-L1) primers are as follows: TR-L1(C666-1); TACGGTTACCCCACAGCCTT and TGAGTAGGAGGGTGATCATC, TR-L1+ED-L1 (C666-1); CTATTCCTTTGCTCTCATGC and TGAGTAGGAGGGTGATCATC, TR-L1(B95-8); TACGGTTACCCCACAGCCTT and TGAGCAGGAGGGTGATCATC, TR-L1+ED-L1 (B95-8); CTATTCCTTTGCTCTCATGC and TGAGCAGGAGGGTGATCATC.

153 *Electromobility shift assay (EMSA) and chromatin immunoprecipitation*– EMSA was carried out as
154 described previously (54). PAX5, FLAG-tagged AP-2α, and myc-tagged EBF proteins were produced using
155 the TNT Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's
156 instructions. Probe DNAs were prepared by hybridization of the sense and antisense oligonucleotides listed
157 below. Because the DNAs have 5' protruding ends, they could be labeled by 3'-end labeling using the Klenow
158 fragment (TOYOBO) and [^{32}P]-dCTP (Institute of Isotopes Co., Hungary). Unincorporated deoxynucleotide
159 triphosphates were removed with Chromaspin-10 columns (Clontech). The *in vitro* translated FLAG-tagged
160 AP-2α protein and labeled DNA sequences were incubated in the EMSA binding buffer (20 mM Tris-HCl pH
161 7.6, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 80 mM NaCl, and 0.5 mg/ml poly (dI-dC)) at room
162 temperature for 30 min. The composition of the EMSA binding buffer for PAX5 was as follows: 20 mM
163 Tris-HCl pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 6.25 mM $MgCl_2$, 0.5 mg/ml poly
164 (dI-dC), and 0.01% NP40. The composition of the EMSA buffer for EBF was as follows: 10 mM HEPES pH
165 7.9, 70 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 2.5 mM $MgCl_2$, 10% glycerol, and 0.5 mg/ml poly
166 (dI-dC). The samples were then separated in a 4% non-denaturing polyacrylamide gel in 0.5×TBE buffer and
167 radioactivity was visualized. The sense and antisense sequences of oligonucleotide probes (I-V) are: I,
168 TGAATCCGCCACCTCATTCTGAAATTCCCATATCCGCCGTCTGCTGCTTCGTCACCCGCCGA
169 CCCTTAGCCCTCTTAGCCGCCTCACCCGCCTCCCCTACGGTTACCCCACAGCCTTGCCTCAC
170 CTGAAC and
171 GGGGGTTCAGGTGAGGCAAGGCTGTGGGGTAACCGTAGGGGAGGCGGGTGAGGCGGCTA
172 AGAGGGCTAAGGGTCGGCGGGTGACGAAGCAGCAGACGGCGGATATGGGAATTTCAGAAT
173 GAGGTGGCGGAT; II,
174 CCTGAACCCCCCTAAAGCACGGCCTCCCGCCTGCCGCCAACGACCTCCCAACGTTGCGCGC
175 CCCGCGCCTCTTTGTGCAGATTACACTGCCGCTTCCCACAACACTACGCACTCCCCCTTCTG
176 ATTGCCGCACTG and
177 GGCAGTGCGGCAATCAGAAGGGGGAGTGCGTAGTGTTGTGGGAAGCGGCAGTGTAATCTG
178 CACAAAGAGGCGCGGGGCGCGCAACGTTGGGAGGTCGTTGGCGGCAGGCGGGAGGCCGT

GCTTTAGGGGG; III,

CCGCACTGCCTTTCCATTTCCTGTTGCACTTGGCCACCGCATTCCCACAGCTTGCCCCCCGG

GGACCCGCTTTTCTAACACAAACACACGCTTTCTACTTCCCCTTTCTACGCTTACATGCACA

CACA and

GGTGTGTGTGTGCATGTAAGCGTAGAAAGGGGAAGTAGAAAGCGTGTGTTTGTGTTAGAA

AAGCGGGTCCCCGGGGGGCAAGCTGTGGGAATGCGGTGGCCAAGTGCAACAGGAAATGG

AAAGGCAGTG; IV,

CACACACACACCGCCGCTTTCGGGAAATCTGTACCCGTACTGCCTCCGGCAGACCCCGCAA

ATCCCCCCGGGCCTACATCCCAAGAAACACGCGTTACTCTGACGTAGCCGCCCTACATAAG

CCTCTCACACTG and

GAGCAGTGTGAGAGGCTTATGTAGGGCGGCTACGTCAGAGTAACGCGTGTTTCTTGGGATG

TAGGCCCGGGGGGGATTTGCGGGGTCTGCCGGAGGCAGTACGGGTACAGATTTCCCGAAAG

CGGCGGTGT; V,

CACACTGCTCTGCCCCCTTCTTTCCTCAACTGCCTTGCTCCTGACACACTGCCCTGAGGATG

GAACACGACCTTGAGAGGGGCCCACCGGGCCCGCGACGGCCCCCTCGAGGACCCCCCCTC

TCCTCTTCCCTAGG and

GGCCTAGGGAAGAGGAGAGGGGGGGTCCTCGAGGGGGCCGTCGCGGGCCCGGTGGGCCC

CTCTCAAGGTCGTGTTCCATCCTCAGGGCAGTGTGTCAGGAGCAAGGCAGTTGAGGAAAGA

AGGGGGCAGAG. Sequences for probes III-1 to III-4, and iv were as follows: III-1,

CCGCACTGCCTTTCCATTTCCTGTTGCACTTGGCCAC and

GCGGTGGCCAAGTGCAACAGGAAATGGAAAGGCAGTGCGG; III-2,

TGGCCACCGCATTCCCACAGCTTGCCCCCCGGGGACCCG and

AGCGGGTCCCCGGGGGGCAAGCTGTGGGAATGCGGTGG; III-3,

GGGACCCGCTTTTCTAACACAAACACACGCTTTCTACTT and

GGAAGTAGAAAGCGTGTGTTTGTGTTAGAAAAGCGGGTC; III-4,

TTCTACTTCCCCTTTCTACGCTTACATGCACACA and

TGTGTGTGTGCATGTAAGCGTAGAAAGGGGAAGTAGAA; iv, TACCCGTACTGCCTCCGGCAGACCCCGCAAATCCCCCCGGGCCTACATCCCAAGAAACA and GCGTGTTTCTTGGGATGTAGGCCCGGGGGGATTTGCGGGGTCTGCCGGAGGCAGTACGGGT A. ChIP assays were carried out as described previously (32).

B cell transformation assay- First, wild-type or mutant EBVs were collected from wild-type or mutant HEK293EBV-BAC cells supernatants. Virus titers in the media were determined by infecting Akata(-) cells, followed by counting the percentage of EGFP-positive cells using flow cytometry (FACSCalibur, Becton Dickinson). Titers were normalized according to the percentages by adding control media. Peripheral blood monocytes (PBMCs) were infected with tenfold dilutions of adjusted culture supernatant media obtained from wild-type or mutant HEK293EBV-BAC cells, and seeded onto 96-well plates at 1×10^4 cells. For PBMCs, blood samples were obtained from healthy adult donors who provided written informed consent, according to protocols approved by the Institutional Review Board of Aichi Cancer Center and Nagoya University. Cells were cultured in the presence of cyclosporin A. Half of the medium was exchanged once a week with fresh medium containing cyclosporine A. After 3 weeks, 50% transforming doses were calculated.

RESULTS

Preparation of mutant EBVs in the proximal LMP1 promoter. Despite numerous reports on transcriptional activators of the major EBV oncogene LMP1, the significance of *cis*-acting binding sites for such factors has been analyzed mostly using reporter assays. Since these assays do not necessarily or proportionally reflect the actual transcriptional levels in the genome, the confirmation of *cis*-elements in the viral genome is important. Therefore, we first prepared recombinant EBVs carrying mutations in the proximal ED-L1 LMP1 promoter, as shown in Fig. 1. We constructed mutants of NFκB, RBPJκ, C/EBP, PU.1, and POU domain factor (Table 1) (12, 32-34). As shown in Fig. 1A, a part of the LMP1 ED-L1 promoter sequence (-360 to -11), containing the *cis*-acting binding sites of reported transcription factors, was first replaced with a marker cassette (Neo/st), which was then exchanged with each sequence with a mutation (marked as X in Fig. 1A).

231 Sequencing analysis confirmed that each of the EBV-BACmt DNA sequences contained the intended
232 mutations. Integrity of the BAC DNA was checked by BamHI digestion followed by electrophoresis to
233 confirm that the recombinant viruses did not carry obvious deletions or insertions (Fig. 1B). Recombinant
234 EBV-BAC DNA was introduced into a virus-producing cell line, HEK293, followed by hygromycin selection
235 to establish cell lines in which recombinant viruses were maintained as episomes.
236
237 ***Attenuation of transformation efficiency by mutations in the POU factors and PU.1 binding sites.*** After
238 preparing HEK293 cell clones with mutant EBVs, we explored whether mutations could affect the expression
239 of LMP1. Since EBNA2 is not produced in HEK293EBV-BAC cells (32) it is clear that the virus produces
240 LMP1 in an EBNA2-independent manner in HEK293 cells. Levels of LMP1 protein were comparable overall
241 (Fig. 2A). We next examined the effect of mutations in the LMP1 promoter of EBV during type III latency
242 when LMP1 is produced in an EBNA2-dependent manner. To accomplish this, B cells were infected with
243 mutant viruses. Prior to infection, we measured viral titers in supernatant solutions using Akata(-) cells to adjust
244 the infectious virus particle numbers per milliliter. After adjustment, viruses in the media were cocultured with
245 PBMC B cells in the presence of cyclosporin A for 3 weeks. The wild-type EBV-BAC virus could produce
246 3.9×10^2 clumps per ml (Fig. 2B). Unexpectedly, all mutant viruses could transform B cells almost as efficiently
247 as the wild-type virus, except for relatively lower efficiencies with the POU factor binding site and PU.1 binding
248 site mutants (2.0 and 1.6×10^2 per ml, respectively) (Fig. 2B). However, we assume that the actual effect of PU.1
249 mutation and POU factor mutation is more significant than the calculated result of several-fold repression of the
250 transformation unit (Fig. 2B), because the sizes of the cell clumps formed by the PU.1 or POU mutant virus
251 were markedly smaller compared to other cases (not shown). To test this hypothesis, the growth properties of
252 LCL clones were determined (Fig. 2C). We examined two clones of each mutant, but only one clone was tested
253 for the PU.1 mutant because we could not obtain more than one clone, probably due to the slow growth rate of
254 the strain. Compared to the wild-type (Fig. 2C, black circles), two POU mutant clones and one PU.1 clone grew
255 significantly slower (Fig. 2C, diamonds and an asterisk). In addition, we analyzed the levels of LMP1 in the
256 LCLs by Western blotting (Fig. 2D). We did not observe a marked difference in LMP1 levels in the LCL

clones shown here, but mutation of POU domain factors and PU.1 might result in a mild decrease (Fig. 2D). These results implied that binding of PU.1 and POU domain factors to the proximal LMP1 promoter plays a role in EBNA2-dependent LMP1 expression in B cells. However, this does not mean that NFκB, RBPJκ, and C/EBP are not important because we could disrupt only one "major" site according to reporter assays for each factor, and more than one binding site may exist in the LMP1 promoter.

A previous report (17) showed that, in addition to the POU domain factor (termed Dα1), unidentified host factor (termed Dα2) also binds to the POU domain site within the ED-L1 promoter. Thus we searched for this unknown factor and found that a paired box family transcription factor, PAX5 (or B-cell lineage specific activator protein (BSAP)) binds to this motif (Fig. 3). To identify the binding site in the proximal LMP1 promoter (ED-L1p), the 635-bp region was divided into five overlapping nucleotide sequences and used as probes (Fig. 3A, probes I-V) for EMSA; probe III was targeted most efficiently by PAX5 (Fig. 3B, leftmost panel, white arrowhead). Addition of an antibody against PAX5 removed the PAX5-probeIII band, indicating that binding between the two is specific (Fig. 3B, second panel from the left). Probe III was further divided into four fragments (Fig. 3A, probes III-1 to III-4), and PAX5 was confirmed to bind to probe III-4 (Fig. 3B, third panel, white arrowhead). When the same point mutation introduced into the POU domain factor was introduced into the probe III-4 (Table 1 and Figs. 1, 2), PAX5 binding was diminished (Fig. 3B, rightmost panel). This POU site (TGTGCATG (antisense)) contains a sequence similar to the PAX5 consensus sequence (GC[A,G]TG). Therefore, it is highly likely that the previously unidentified host factor in B cell lysate that binds to the POU domain factor site in ED-L1p (Dα2) is PAX5. Interestingly, multiple copies of PAX5 reportedly target the TR of EBV and negatively regulate LMP1 transcription in B cells (55, 56). This is in agreement with previous speculation that Dα2 is a negative regulator of LMP1 expression (17). When these reports and our results are taken into consideration, regardless of whether ED-L1p or TR-L1p, PAX5 binding to the LMP1 promoter region may negatively regulate LMP1 transcription. However, in our mutagenesis experiment, the positive effect of POU factor binding might be greater than the negative effect of PAX5 binding to the same motif (Fig. 2B-D).

283 ***Effect of AP-2 on LMP1 production.*** We then applied the knockdown method to examine the
284 importance of specific transcription factors for LMP1 expression in infected cells. We first tried to ablate NFκB,
285 RBPJκ, and PU.1, which proved to be difficult, probably because these factors play essential roles in cell fate.
286 Instead, we focused on the AP-2α protein, since Rymo's group suggested the involvement of AP-2 (or an
287 unknown host protein that binds to the AP-2 motif in ED-L1p) by mutagenesis of ED-L1p using reporter assay
288 systems in EBNA2-dependent LMP1 expression in B cells (35). To further characterize the role of AP-2 in
289 LMP1 expression, we explored whether the transcription factor could induce LMP1 in an epithelial NPC cell
290 line C666-1, in which LMP1 is expressed in the absence of EBNA2 (Fig. 4A-D). When AP-2α, a typical
291 member of the AP-2 family (40), was exogenously overexpressed, mRNA levels of LMP1 were induced, as
292 expected in the NPC cell line (Fig. 4A). We examined mRNA but not protein levels of LMP1, since LMP1
293 protein is not detectable in this cell line (32). Results of qRT-PCR showed that exogenous expression of AP-2α
294 did not affect the level of LMP1 transcription from TR-L1p, but it resulted in a 3.2-fold induction of LMP1 (Fig.
295 4B, TR+ED).
296 Knockdown experiments were performed using two siRNAs for AP-2α (si AP-2α-1, 2). Either of the
297 siRNAs clearly ablated protein levels of AP-2α in the C666-1 cell line (Fig. 4C), and the reductions were
298 correlated with decreases in LMP1 expression levels (Fig. 4C,D). The effect of si AP-2α-1 on LMP1
299 expression was less potent than si AP-2α-2, for unknown reasons. Notably, ablation of AP-2α from the NPC
300 cells caused a significant loss of LMP1 transcription from TR-L1p (Fig. 4D), which suggested that an ectopic
301 excess supply of AP-2 most obviously activates proximal ED-L1p, but not TR-L1p (Fig. 4B), although
302 endogenous levels of the transcription factor activate TR-L1p (Fig. 4D). These results indicated that AP-2 is a
303 crucial determinant of type II LMP1 production, and that natural levels of AP-2 activate the distal LMP1
304 promoter, at least in NPC cells latently infected with EBV.
305 Although previous study reported that AP-2 may not play a central role in LMP1 expression in latency
306 III B cells because B cells express very low levels of AP-2 (35), we tested the effect of overexpression of
307 AP-2 on LMP1 expression in LCLs. Ectopic expression of FLAG-AP-2α was lower than that in C666-1, but
308 it could also induce LMP1 protein in the latency III B cells (Fig. 4E). Quantitation of LMP1 mRNAs

indicated that the ED-L1, but not the TR-L1 promoter was activated by exogenous expression of FLAG-AP-2α, although the enhancement was less prominent in LCLs (Fig. 4F).

Identification of AP-2-binding elements in the LMP1 promoter. After confirming the importance of AP-2 for LMP1 expression, we used EMSA to examine whether the AP-2α protein could bind to motifs in the LMP1 promoter (Fig. 5). Here, we used the same probes (probes I-V) as in Fig. 3. The addition of FLAG-AP-2α did not produce any DNA-AP-2 complexes in the case of probes I and II, and a very weak signal may have occurred for probe V (Fig. 5B). On the other hand, probe III yielded a prominent band of the AP-2α-nucleotide complex (Fig. 5B, white arrowhead), and the band shifted up in the presence of the anti-Flag antibody (Fig. 5B). Probe IV yielded two discernible bands (Fig. 5B, black arrowheads), both of which were supershifted by specific antibody addition, indicative of binding of AP-2α to two possible elements. To locate the AP-2 binding sites, we searched the promoter region for G/C-rich sequences similar to the AP-2 consensus motif ([G/C]CCN$_{(3,4)}$GG[G/C]) and identified one such element in probe III (-205, CCCCCGGGG) and two in probe IV (-75, CCCCCCGGG and -100, GCCTCCGGC). The introduction of mutations in the binding sites of the probes (Table 1) diminished binding to AP-2α (Fig. 5C, III' and IV'), indicating that these are the actual AP-2 binding sites in ED-L1p.

Mutation at -75 in the LMP1 promoter had little effect on LMP1 expression. To confirm the significance of these *cis*-acting binding motifs, we prepared recombinant EBV carrying mutations in the proximal ED-L1p. We first mutated AP-2 motif at -75, since reporter assays previously showed the importance of the site for LMP1 expression (33). Part of the LMP1 ED-L1p sequence (-360 to -11), containing the *cis*-acting binding sites of AP-2, was replaced with a marker cassette (Neo/st), which after was exchanged with the sequence containing a mutation (ringed X in Fig. 6A). Sequencing analysis confirmed that the EBV-BAC AP2(-75)mt DNA sequences contained the intended mutations. Integrity of the BAC DNA was examined based on BamHI digestion followed by electrophoresis to confirm that the recombinant viruses did not carry obvious deletions or insertions (Fig. 6B). Recombinant EBV-BAC DNA was introduced into a virus-producing cell line, HEK293,

335 followed by hygromycin selection to establish cell lines in which recombinant viruses were maintained as
336 episomes.

337 After preparing HEK293 cell clones with wild-type and mutant EBV, we explored whether mutations
338 could affect the expression of LMP1. Because EBNA2 is not produced in HEK293EBV-BAC cells, the virus
339 produces LMP1 in an EBNA2-independent manner in HEK293 cells (32). Levels of LMP1 protein were
340 comparable overall in HEK293 cells (Fig. 6C), indicating that the AP-2 binding motif at -75 does not play a
341 major role in LMP1 expression in the cell line. B cells of human PBMCs were then infected with the wild-type
342 and AP2(-75)mt viruses, and the transformation activity of the viruses was determined (Fig. 6D). The mutant
343 virus showed the same degree of B cell immortalization efficiency as the wild-type virus. After development of
344 LCLs, growth behavior (Fig. 6E) and the LMP1 protein (Fig. 6F) were examined, but no obvious differences
345 were observed between the lines. Therefore, the AP-2 binding motif at -75 of ED-L1p may not be required for
346 the production of LMP1 either in latency II or III, unlike the previous report shown by reporter assays (33).
347
348 ***Mutation at -75, 100, and 205 in the LMP1 promoter caused loss of LMP1 production.*** Next, we prepared the
349 mutant virus, in which all three AP-2-binding sites were mutated (-75, -100 and -205), and another virus, in
350 which two sites (-100 and -205) were modified (Fig. 7A,B). We observed fluctuation to some extent, but the
351 expression of LMP1 in wild-type, AP2(-100,205)mt, and AP2(-75,100,205)mt was comparable in HEK293
352 (Fig. 7C). Infection of AP2(-100,205)mt EBV to human primary B cells for 21 days caused minor decreases in
353 transformation compared with the wild-type, and triple mutation (-75, -100 and -205) caused statistically
354 significant reduction in transformation efficiency of about one order (Fig. 7D). In fact, when the triple mutant
355 virus was infected, the size of the cell clumps was markedly smaller compared with the wild-type or even the
356 double mutant (-100 and -205); thus the effect of triple mutation was more profound than one order of
357 magnitude. In agreement with this assumption, we could not further amplify and develop LCLs infected with
358 the triple mutant, while we could readily prepare LCLs infected with wild-type or double mutant EBVs. We
359 speculate that the wild-type LMP1 promoter could resist forceful pressure of epigenetic gene silencing after 21
360 days because of transcriptional activation through AP-2 sites. On the other hand, the triple mutant could express

LMP1 to some extent, but loss of AP-2 binding sites caused silencing of LMP1 gene expression and an arrest in cell growth. Because LCLs with the triple mutation could not be obtained, we compared LCLs infected with the wild-type and the double mutant, AP2(-100,205)mt (Fig. 7E, F). Cell growth of the double mutant was slightly slower (Fig. 7E), but LMP1 production of the mutant did not seem decreased, due to the fluctuation in wild-type samples (Fig. 7F).

Since LCLs infected with the triple mutant could not be developed (Fig. 7E, F), cells were harvested at earlier time points and LMP1 expression levels were analyzed (Fig. 8A). Two days after infection of PBMC B cells, LMP1 could not be detected in either the wild-type or the triple mutant (Fig. 8A). This result is in accordance with a previous report that LMP1 expression is highly restricted for about 1 week after primary B cell infection (57). LMP1 expression from cells infected with the wild-type virus increased dramatically by day 13, and LMP1 mRNA levels were markedly lower in the case of the triple mutant, AP2(-75,100,205)mt (Fig. 8A). EBNA2 mRNA levels were relatively higher from 2 days after infection (Fig. 8B), which can be explained by a previous study (57). The immediate early gene of the lytic infection cycle, BZLF1, was highest on day 2, which likely is a reflection of the pre-latent abortive lytic phase (57, 58), and was silenced later (Fig. 8C). Importantly, levels of EBNA2 and BZLF1 expressed in wild-type samples on day 2 were almost equal to those in triple mutant samples, indicating that the loss of LMP1 expression in the triple mutant (Fig. 8A) was not attributable to the difference in the multiplicity of infection of the infected viruses. We also confirmed these results in the double mutant virus (Fig. 8D, E). Viruses were prepared from HEK293 cells containing wild-type EBV-BAC (WT) or two independent HEK293 cell clones with the EBV-BAC triple mutant (AP2(-75,100,205)mt) or the double mutant (AP2(-75,100,205)mt). PBMC B cells were infected with these viruses and RNA was harvested for qRT-PCR 7 days after infection. At 7 days, LMP1 mRNA levels were significantly reduced by the double mutation and were further decreased in the triple mutant (Fig. 8D); however, EBNA2 mRNA levels were comparable (Fig. 8E).

These results indicated that the three AP-2 binding motifs act together to induce LMP1 expression and thereby immortalize B cells. Among the three binding sites, the distal two (-100 and -205) seemed to be more important for LMP1 expression than the most proximal one (-75, Fig. 6).

Mutation at -100 or 205 in the LMP1 promoter had little effect. Having confirmed the importance of three AP-2 binding sites in the LMP1 promoter for LMP1 expression, particularly the distal two sites (-100 and -205), we next mutated the two sites separately, as shown in Fig. 9A and B. We did not observe a significant difference in LMP1 levels among the wild-type, AP2(-100)mt, and AP2(-205)mt in HEK293 (Fig. 9C). When infected to B cells, levels of immortalization (Fig. 9D), cell proliferation (Fig. 8E), and LMP1 protein of the LCLs (Fig. 9F) were similar between the wild-type and the mutants, although the cell growth rate may have been slightly slower in LCLs infected with AP2(-205)mt. Taken together, the transcription factor AP-2 is crucial for LMP1 expression in LCLs, and three AP-2 binding sites in the promoter contribute additively to LMP1 induction.

Binding of EBF to the AP-2 motifs in ED-L1p. Although expression of AP-2α is weak in B lymphocytes, including Akata, P3HR1, and LCLs (data not shown) (35), simultaneous mutation of three AP-2 binding sites clearly diminished LMP1 expression in LCLs (Figs. 7, 8). Johannsen and others reported that one of the G/C-rich AP-2 binding sites (-205 motif in this paper) was predicted to be bound by an unknown factor (termed LBF7) in B cell lysate (12). Therefore, we searched for this factor, and found that two of the AP-2 binding sites in ED-L1p could also be targeted by the B cell-specific transcription factor EBF (Fig. 10). EBF bound to probe III and IV (Fig. 10A, B left panel, white arrowhead), and this association was supershifted by the addition of anti-myc antibody (Fig. 10B second panel, white arrowhead). Mutation of the -205 AP2 motif in probe III (III') or the -75 and -100 motifs in probe IV (IV') prevented the binding between EBV and the DNA probes (Fig. 10B, third panel). Additional mutagenesis demonstrated that EBF binds to the -75 but not the -100 motif (Fig. 10B, rightmost panel). This is expected because the sequences of the -75 (CCCCCGGG) and -205 (CCCCCGGGG) motifs, but not of the -100 AP2 motif (GCCTCCGGC), coincide with the EBF consensus sequence (CCCNNGGG). Indeed, Zhao and others reported that the -205 motif in ED-L1p was targeted by EBF (59). To examine the importance of EBF, an expression vector harboring myc-tagged EBF was transfected into LCLs (Fig. 10C, D). Expression of myc-EBF increased the LMP1 protein level (Fig. 10C). Moreover, qRT-PCR analysis revealed that the proximal ED-L1 promoter, but not the distal TR-L1 promoter,

was activated by exogenous EBF production, although the induction was modest (Fig. 10D). This result suggests that EBF plays an important role for transcription of LMP1 in B cells. We also attempted to knockdown EBF in LCLs, but none of the four siRNAs ablated EBF levels (not shown).

Because AP-2 binds to G/C-rich elements, reminiscent of SP1-binding motifs, we lastly determined whether these sites are bound by the transcription factor. ChIP assays further confirmed that AP-2 binding was inhibited in the triple mutant, while SP1 binding was unaffected (Fig. 10E).

DISCUSSION

In this study, we first explored the role of AP-2 based on exogenous overexpression and/or knockdown (Fig. 4). We then identified three AP-2 binding sites (-75, -100, and -205) in the proximal (ED-L1) LMP1 promoter (Fig. 5), and evaluated their significance in the context of the EBV-BAC system (Figs. 6-9). The results documented here show involvement of AP-2 binding sites in the upregulation of the LMP1 gene in both latency II and III. Interestingly, two of the AP-2 binding sites were bound by the B cell transcription factor EBF, too.

Among the three AP-2 sites in ED-L1p, Rymo's group predicted binding of AP-2 to two motifs (-75 and -100) in 2007 and confirmed binding of *in vitro* translated AP-2 to one of the motifs (-100) (35). These authors further showed that introduction of mutations into the AP-2 site (-100) markedly reduced EBNA2-mediated transcriptional activation of LMP1 as determined by luciferase assays. Nevertheless, they speculated that an unknown host factor other than AP-2 binds to the motif and mediates EBNA2-dependent expression of LMP1 since AP-2 protein levels are low in B cells. Next, Demetriades and Mosialos showed in 2009 that introduction of a point mutation into another AP-2 site (-75) significantly decreased proximal LMP1 promoter activity in latency III B95-8 cells as determined by luciferase assays (33). Here we confirmed binding of AP-2 to two of the sites (-75 and -100) and identified an additional motif (-205) in ED-L1p. Introduction of point mutations into the three motifs simultaneously or individually in the context of the virus demonstrated the importance of all of these sites for LMP1 expression in latency III. However, expression of the AP-2 protein is low in B cells. We examined binding of other transcription factors expressed in B cells and found that the B cell-specific

439 transcription factor EBF can bind to at least two of the three AP-2 sites (-75 and -205). Indeed, this transcription
440 factor has been shown to bind to the proximal LMP1 promoter by ChIP-seq (56), and more detailed analysis by
441 EMSA assay by Zhao and others demonstrated that EBF binds to the -205 AP-2 motif (59). Co-enrichment of
442 EBNA2 and EBF sites in LCLs in the ChIP-seq analysis and the luciferase assays further indicated the
443 importance of EBF for EBNA2-dependent LMP1 expression in latency III B cells (56, 59). EBF binding to the
444 -75 motif has not been reported to date, and the physiological role of these binding sites has not been analyzed
445 using recombinant virus. Our results, in conjunction with previous reports (60-62), confirm the central role of
446 EBF in EBNA2-dependent activation of the ED-L1 promoter in B cells.
447 In this study we also confirmed involvement of PU.1 and POU domain factors in LMP1 transcription (Fig.
448 2). On the other hand, mutations in the major binding site of RBPJκ, which have been more extensively studied
449 with regard to EBNA2-dependent LMP1 expression, did not affect the B cell transformation efficiency (Fig. 2).
450 We cannot preclude involvement of RBPJκ in EBNA2-mediated LMP1 expression since binding sites may be
451 redundant for RBPJκ and a mutation in only one major motif may not be sufficient to inhibit expression.
452 Likewise, contributions of NFκB to type III LMP1 expression cannot be ruled out based on our data. Because
453 PU.1 is a lymphocyte-specific transcription factor, whereas RBPJκ and NFκB are ubiquitously expressed in
454 various types of cells and tissues, PU.1 may account for the B cell specificity of latency III LMP1 expression
455 (63, 64). In accordance with our result, Johannsen et al. reported that PU.1 plays a role in EBNA2-mediated
456 LMP1 expression, but also demonstrated, based on mutational analysis, that EBNA2 activation of the LMP1
457 promoter in B cells is partially dependent on the interaction with RBPJκ, and is completely dependent on the
458 interaction with PU.1 protein (12). Our functional library screening also identified PU.1 as a transcriptional
459 activator of LMP1 (32). Zhao and Sample reported a role for the PU.1 binding site in the LMP1 promoter for
460 promoter activation in the presence of EBNA2 (20). Therefore, it can be assumed that PU.1 protein and its
461 binding site in the LMP1 promoter are very important for EBNA2-dependent production of the EBV oncogene
462 LMP1 in type III latency.
463 POU domain factors include Oct-1 and Oct-2. Expression of Oct-1 is ubiquitous, whereas that of Oct-2 is
464 B cell-specific. Because disruption of the POU domain binding motif within the LMP1 promoter inhibited

LMP1 expression (Fig. 2), Oct-2 may also contribute to latency III LMP1 induction in B cells. However, Rymo's group showed that an unidentified factor (Dα1) belonging to the POU domain family, but distinct from Oct-1 and Oct-2, binds to the POU site in ED-L1p, because an antibody against POU domain proteins, but not antibodies against Oct-1 or Oct-2, supershifted the Dα1 band in EMSA (17). In addition to a POU factor (Dα1), they also demonstrated that a negative factor binding to the POU motif, or in the vicinity thereof, within ED-L1p is expressed in B cells (17). We found here that PAX5, a master regulator of B cell function, development, and leukemogenesis, also binds to the POU site in the ED-L1 promoter. PAX5 has been shown to negatively regulate LMP1 transcription through binding to TR (55, 56). Knockdown of PAX5 in LCLs increased transcription of LMP1, indicating that PAX5 serves to suppress LMP1 expression. We speculate that the virus fine-tunes the expression of LMP1 by activating the promoter, on the one hand, and delicately suppressing it, on the other hand, because this oncogene might be toxic to cells when produced in excess (11).

It is of interest that mutations of a single binding site for either transcription factor we tested had almost no (NFκB, RBPJκ, and C/EBP), or only a moderate (PU.1 and POU) effect on LMP1 transcription. No single transcription factor's binding site in the ED-L1p was essential for LMP1 expression and B cell transformation, indicating robustness and redundancy of the LMP1 promoter.

Regarding latency II, we found that LMP1 levels were increased by AP-2 in NPC C666-1 (Fig. 4A-D) and HeLa-EBV cells (data not shown), which is convincing, as AP-2 proteins are abundantly expressed in these epithelial cells. In addition to C666-1 and HeLa cells, we confirmed that levels of AP-2α are high in HEK293 cells (data not shown), but the effect of AP-2 was weak in this cell line (Fig. 6C). In addition, levels of AP-2α in the SNK6 NK cell lymphoma line, in which LMP1 is highly expressed in an EBNA2-independent manner (65), were low (not shown). Therefore, levels of AP-2α do not necessarily correlate with LMP1 expression. We speculate that other transcription factors, such as other members of the AP-2, STAT (23-28), C/EBP (32), or E-box-binding proteins such as MAD and MAX (66) may account for this inconsistency. Several reports indicate that activation of the JAK/STAT pathway by some cytokines is of major importance in latency II (23-28).

The reasons for the low expression of LMP1 protein in C666-1 cells remain unclear. LMP1 protein in

491 the cell may be unstable and easily degraded. Notably, LMP1 is reported to be degraded rapidly through the
492 ubiquitin/proteasome-dependent pathway (67), and LMP1 degradation is specifically regulated in NPC cells
493 (45). Furthermore, since LMP1 mRNA levels are low in C666-1 cells, LMP1 may be regulated prior to its
494 translation. LMP1 mRNA levels are reportedly downregulated by EBV-encoded microRNAs, BARTs,
495 which are abundantly expressed in C666-1 cells (68).

496 The activity of AP-2 proteins can be controlled not only based on protein abundance but also at the
497 posttranslational level, such as through protein kinase A-mediated phosphorylation (69). Carcinogens, including
498 nitrosamines in salted fish, have been reported to aggravate NPC (70, 71), and nitrosamines were found to
499 activate PKA (72), probably inducing LMP1 in NPC. Other factors, such as growth factors or cytokines, are
500 also feasible candidate modulators of PKA activity.

501 Overall, we confirmed the importance of AP-2 and EBF for LMP1 expression in latency II and III.
502 Because LMP1 plays a major role in immortalization, development, metastasis, and malignancy of NPC
503 (73-75), inhibition of AP-2 and EBF may offer an avenue to treat these cancers. A search for small molecules
504 that inhibit LMP1 expression is currently underway (76)

505

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FIGURE LEGENDS

Fig. 1. Construction of recombinant EBV featuring a mutation in the transactivator binding site of the LMP1 promoter. (A) Schematic arrangement of the recombination of the EBV genome using the tandemly arranged neomycin-resistance and streptomycin-sensitivity genes (Neo/st). Sequences of the B95-8 ED-L1 LMP1 promoter (-360 to -11) were first replaced with the Neo/st cassette, which was then replaced with mutated sequences (ringed X) to construct EBV-BAC mt. (B) Electrophoresis of the recombinant virus genomes. Recombinant EBV genomes were digested with BamHI and separated in an agarose gel.

Fig. 2. Effect of mutations in HEK293 and LCLs. (A) LMP1 protein levels in HEK293 cells with mutant EBV-BACs in the transactivator binding site of the LMP1 promoter. Immunoblotting was performed using anti-LMP1 and -tubulin antibodies. Independent cell clones that latently maintain EBV-BAC were obtained by transfection of each mutant DNA, and LMP1 levels of two or three typical clones were examined. (B) Transformation efficiency of recombinant EBVs carrying mutations in the transactivator binding site of the LMP1 promoter. Viruses obtained from different clones of wild-type or mutant HEK293EBV-BACs were normalized based on the data of EGFP-positive Akata ratios and infected with PBMCs in the presence of cyclosporine A. Three weeks later, transformation units were determined. The mean and SD values of three independent assays are shown. Student's *t*-test was performed but statistical significances between WT and any of the mutants were not indicated. (C) Growth properties of LCLs. LCLs (35×10^4 cells/ml) prepared in (B) were seeded, and after 4 and 8 days, cell numbers were counted. (D) Levels of LMP1 in LCL clones. Independent one or two LCL clones obtained in (B) were subjected to immunoblotting using anti-LMP1 and -tubulin antibodies.

Fig. 3. Binding of PAX5 to the LMP1 promoter. (A) Schematic illustration of the LMP1 promoter and the probes (I-V and III-1-4) used in EMSA. (B) EMSA was carried out as described in the Materials and Methods. PAX5 protein was produced *in vitro* and incubated with ^{32}P-labeled probes. Probes I to V cover sequences from -514 to -381, -391 to -255, -264 to -133, -141 to -8, and -13 to +121 of the LMP1 promoter, relative to the

transcription start, respectively. Samples were then separated in a 4% polyacrylamide gel and analyzed by autoradiography. Supershift analysis was performed using a mouse anti-PAX5 monoclonal antibody (+α-PAX5, second panel from the left). Addition of the antibody caused the band to disappear but not to supershift probably because binding of the antibody influenced the DNA-binding activity of PAX5. Further fragmentation of the probe III was performed (III-1 to III-4), and the resulting fragments were used in EMSA (third panel). Lastly, a mutant probe for POU binding was assessed (mPOU, rightmost panel). White arrowheads indicate bands specific for DNA-PAX5.

Fig. 4. Activation of LMP1 expression by AP-2α in C666-1 and LCLs. (A, B) A nasopharyngeal carcinoma cell line C666-1 was transfected with empty vector or the FLAG-tagged AP-2α expression vector (flag AP2α). Three days after transfection, cells were harvested and subjected to RT-PCR for detection of LMP1 and GAPDH gene expression (A) and immunoblotting using anti-FLAG and -tubulin antibodies (A). Parts of the RNA samples were subjected to qRT-PCR to examine promoter usage (B). (C, D) C666-1 cells were transfected with control siRNA (si control) or the siRNA against AP-2α (si AP2α-1, 2). Three days after transfection, cells were harvested and subjected to RT-PCR for LMP1 and GAPDH (C) and immunoblotting using anti-AP-2α and -tubulin antibodies (C). Parts of the RNA samples were subjected to qRT-PCR to examine promoter usage (D). (E, F) LCLs were transfected with empty vector or the FLAG-tagged AP-2α expression vector (flag AP2α). Three days after transfection, cells were harvested and subjected to immunoblotting using anti-LMP1, -FLAG, and -tubulin antibodies (E) and qRT-PCR to examine promoter usage (F). Three independent samples were assayed and Student's *t*-test was performed. * indicates $p<0.05$ and ** indicates $p<0.02$.

Fig. 5. Binding of AP-2α to the LMP1 promoter. (A) Schematic illustration of the LMP1 promoter and the probes (I-V) used in EMSA. (B) EMSA was carried out as described in the Materials and Methods. FLAG-tagged AP-2α was produced *in vitro* and incubated with ^{32}P-labeled probes. Supershift analysis was performed using mouse anti-FLAG monoclonal antibody (+Ab). Samples were then separated in a 4%

polyacrylamide gel and analyzed by autoradiography. (C) EMSA was carried out as described in (A), except the AP-2α-binding motif in probes III and two motifs in IV were mutated to make the III' and IV' probes, respectively. White and black arrowheads indicate bands for the Probe III-AP-2 and Probe IV-AP-2 complexes, respectively.

Fig. 6. Effect of the mutation in the AP-2α-binding site (-75) of the LMP1 promoter. (A) Schematic arrangement of the recombination of the EBV genome using the tandemly arranged neomycin-resistance and streptomycin-sensitivity genes (Neo/st). Sequences of the ED-L1 LMP1 promoter (-360 to -11) were first replaced with the Neo/st cassette, which was then replaced with mutated sequences (ringed X) to construct EBV-BAC AP2(-75)mt. (B) Electrophoresis of the recombinant viruses. Recombinant EBV genomes were digested with BamHI and separated on an agarose gel. (C) HEK293 cell clones latently maintaining EBV-BAC wild-type (WT) or AP2(-75)mt were subjected to immunoblotting using anti-LMP1 and -tubulin antibodies. Independent cell clones that latently maintain EBV-BAC were obtained by transfection of each mutant DNA, and LMP1 levels of three typical clones were examined. (D) Effect of the mutation in the AP-2α-binding site (-75) of the LMP1 promoter on B cell transformation. Viruses obtained from WT or the mutant HEK293 EBV-BAC cells were normalized based on data of EGFP-positive Akata ratios and infected with PBMCs in the presence of cyclosporine A. Twenty days later, transformation units were determined. The mean and SD values are shown. The mean and SD values of three independent assays are shown. Student's *t*-test was performed but statistical significance between WT and the mutant was not indicated. (E) Growth properties of LCLs. LCLs (20×10^4 cells/ml) prepared in (D) were seeded, and after 4 and 8 days, cell numbers were counted. (F) Levels of LMP1 in LCL clones. Independent two LCL clones obtained in (D) were subjected to immunoblotting using anti-LMP1 and -tubulin antibodies.

Fig. 7. Effect of the mutations in the AP-2α-binding sites (-75, -100, and -205) of the LMP1 promoter. (A) Schematic arrangement of the recombination of the EBV genome using the tandemly arranged neomycin-resistance and streptomycin-sensitivity genes (Neo/st). The sequences of the ED-L1 LMP1

promoter (-360 to -11) were first replaced with the Neo/st cassette, which was then replaced with mutated sequences (ringed X) to construct EBV-BAC AP2(-100,205)mt and AP2(-75,100,205)mt. (B) Electrophoresis of recombinant viruses. The recombinant EBV genomes were digested with BamHI and separated on an agarose gel. (C) HEK293 cell clones latently maintaining EBV-BAC WT, AP2(-100,205)mt, or AP2(-75,100,205)mt were subjected to immunoblotting using anti-LMP1 and -tubulin antibodies. Independent cell clones that latently maintain EBV-BAC were obtained by transfection of each mutant, and LMP1 levels of three typical clones were examined. (D) Effect of the mutation in the AP-2α-binding sites (-100,205 or -75,100,205) of the LMP1 promoter on B cell transformation. Viruses obtained from WT or the mutant HEK293 EBV-BAC cells were normalized based on the data of EGFP-positive Akata ratios and infected with PBMCs in the presence of cyclosporine A. Twenty days later, transformation units were determined and the mean and SD values are shown. Three independent infections were assayed and Student's *t*-test was performed. * indicates $p<0.05$. (E) Growth properties of LCLs. LCLs ($20x10^4$ cells/ml) prepared in (D) were seeded, and after 4 and 8 days, cell numbers were counted. (F) Independent two LCL clones obtained in (D) were subjected to immunoblotting using anti-LMP1 and -tubulin antibodies.

Fig. 8. Effect of the mutations in the AP-2α-binding sites (-75, -100, and -205) of the LMP1 promoter on primary B cell infection. (A-C) PBMC B cells were mock-infected or infected with EBV-BAC WT or AP2(-75,100,205)mt, as described in Fig. 6D. Cellular RNA was collected on 2, 7 and 13 days after infection and subjected to qRT-PCR for detection of the LMP1, EBNA2, BZLF1 and GAPDH genes. Relative mRNA levels were shown after normalization to GAPDH. (D, E) Likewise, B cells were infected with WT EBV-BAC or EBV-BAC viruses produced from two independent HEK293 clones of AP2(-75,100,205)mt and AP2(-100,205)mt. Cellular RNA was obtained on day 7 and subjected to qRT-PCR for detection of the LMP1, EBNA2 and GAPDH genes. Relative mRNA levels were shown after normalization to GAPDH. Three independent infections were assayed and Student's *t*-test was performed. ** indicates $p<0.02$.

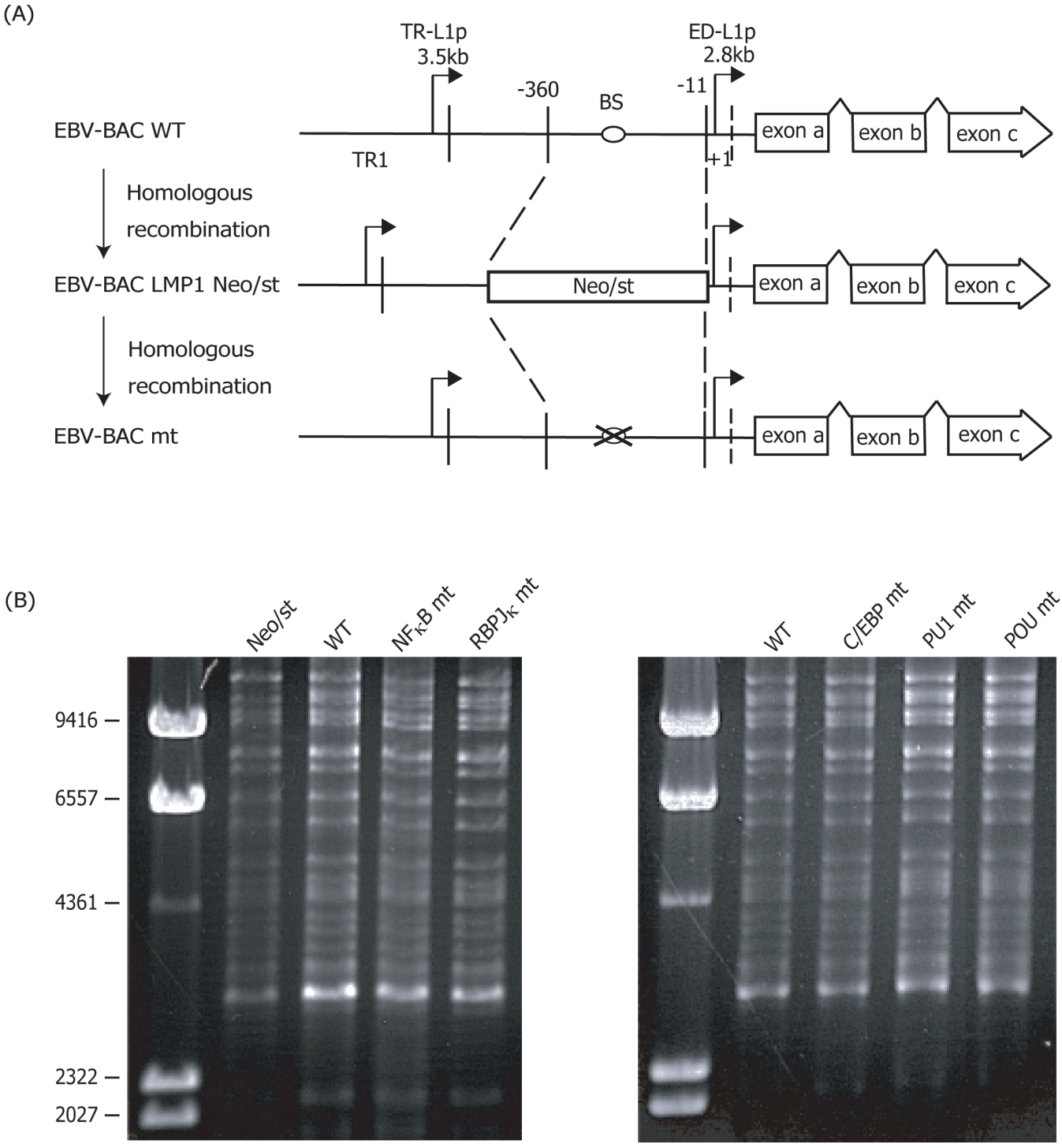
Fig. 9. Effect of mutations in the AP-2α-binding sites (-100 or -205) of the LMP1 promoter. (A) Schematic

arrangement of the recombination of the EBV genome using the tandemly arranged neomycin-resistance and streptomycin-sensitivity genes (Neo/st). Sequences of the ED-L1 LMP1 promoter (-360 to -11) were first replaced with the Neo/st cassette, which was then replaced with mutated sequences (ringed X) to construct EBV-BAC AP2(-100)mt and AP2(-205)mt. (B) Electrophoresis of the recombinant viruses. The recombinant EBV genomes were digested with BamHI and separated on an agarose gel. (C) HEK293 cell clones latently maintaining EBV-BAC WT, AP2(-100)mt, or AP2(-205)mt were subjected to immunoblotting using anti-LMP1 and -tubulin antibodies. Independent cell clones that latently maintain EBV-BAC were obtained by transfection of each mutant, and LMP1 levels of three typical clones were examined. (D) Effect of the mutation in the AP-2α-binding sites (-100 or -205) of the LMP1 promoter on B cell transformation. Viruses obtained from WT or mutant HEK293 EBV-BAC cells were normalized based on the EGFP-positive Akata ratios and infected with PBMCs in the presence of cyclosporine A. Twenty days later, transformation units were determined. The mean and SD values of three independent assays are shown. Student's *t*-test was performed but statistical significance between WT and the mutant was not indicated. (E) Growth properties of LCLs. LCLs (20×10^4 cells/ml) prepared in (D) were seeded, and after 4 and 8 days, cell numbers were counted. (F) Independent two LCL clones obtained in (D) were subjected to immunoblotting using anti-LMP1 and -tubulin antibodies.

Fig. 10. Binding of EBF to the LMP1 promoter. (A) Schematic illustration of the LMP1 promoter and the probes (I-V and iv) used in EMSA. (B) EMSA was carried out as described in the Materials and Methods. myc-tagged EBF protein was produced *in vitro* and incubated with ^{32}P-labeled probes. Supershift analysis was performed using mouse anti-myc monoclonal antibody (α-myc, second panel). As shown in the third panel, EMSA was carried out likewise, except that the AP-2α-binding motif in probe III and two motifs in probe IV were mutated to produce the III' and IV' probes, respectively (third panel). Lastly, using probe iv (shorter than probe IV but covers both motifs -75 and -100), motifs were mutated one by one (rightmost panel). (C, D) Activation of LMP1 expression by EBF in LCLs. LCLs were transfected with empty vector or the myc-tagged EBF expression vector (myc EBF). Three days after transfection, cells were harvested and subjected to

immunoblotting using anti-LMP1, -myc, and -tubulin antibodies (C) and to qRT-PCR to examine promoter usage (D). Three independent samples were assayed and Student's *t*-test was performed. * indicates $p<0.05$. (E) SP1 does not bind to AP-2 motifs. HEK293 cell clones latently maintaining EBV-BAC WT or AP2(-75,100,205)mt were subjected to ChIP assays using anti-AP2 and -SP1 antibodies. Levels of the LMP1 proximal promoter region precipitated were determined by qPCR and shown as % of input. Three independent samples were assayed and Student's *t*-test was performed. * indicates $p<0.05$.

Fig.1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+1
exon a
exon b
exon c
Homologous
recombination
EBV-BAC LMP1 Neo/st
Neo/st
Homologous
recombination
EBV-BAC mt
(B)
Neo/st
WT
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WT
C/EBP mt
PU1 mt
POU mt
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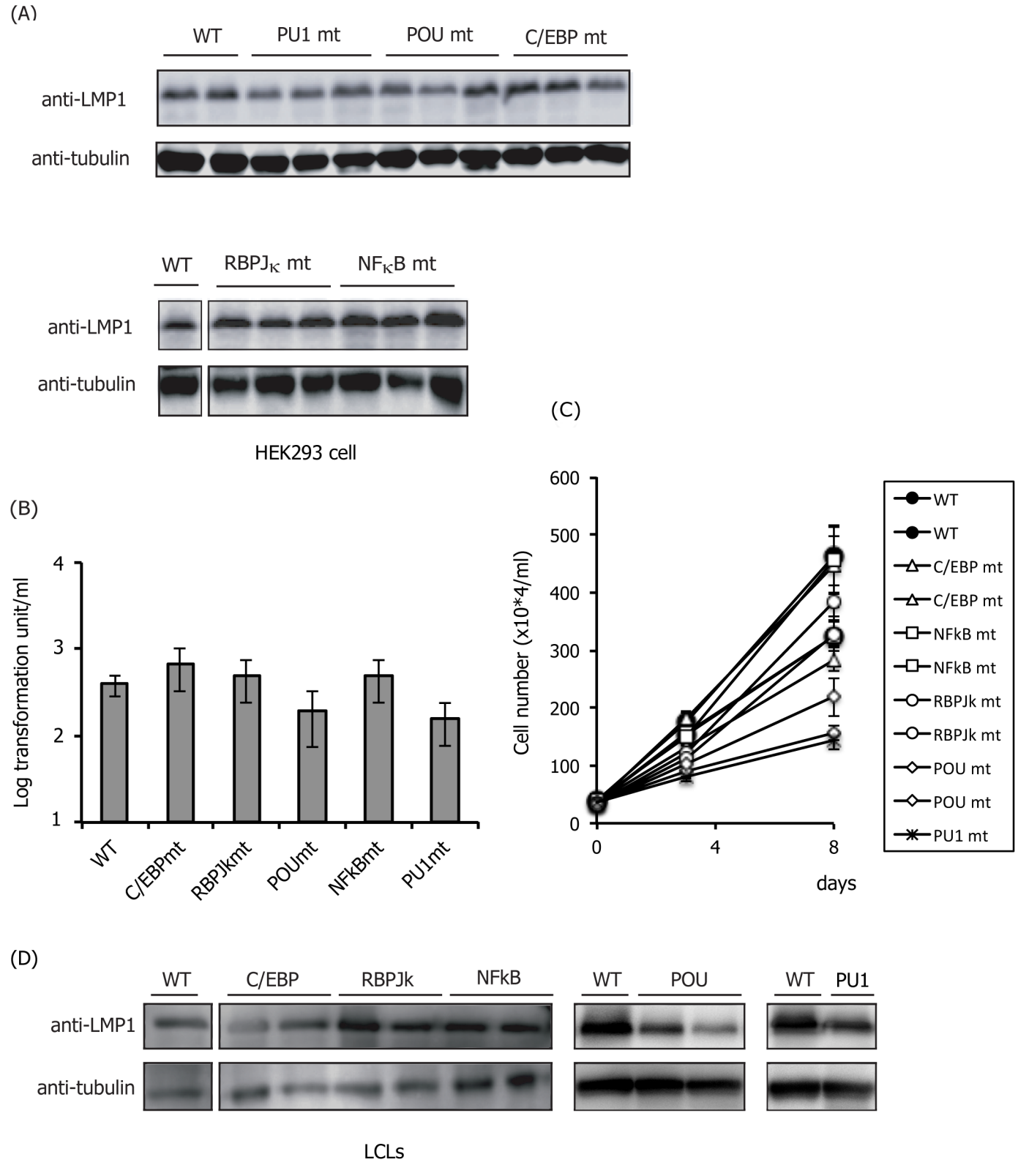
4361
2322
2027

Fig.2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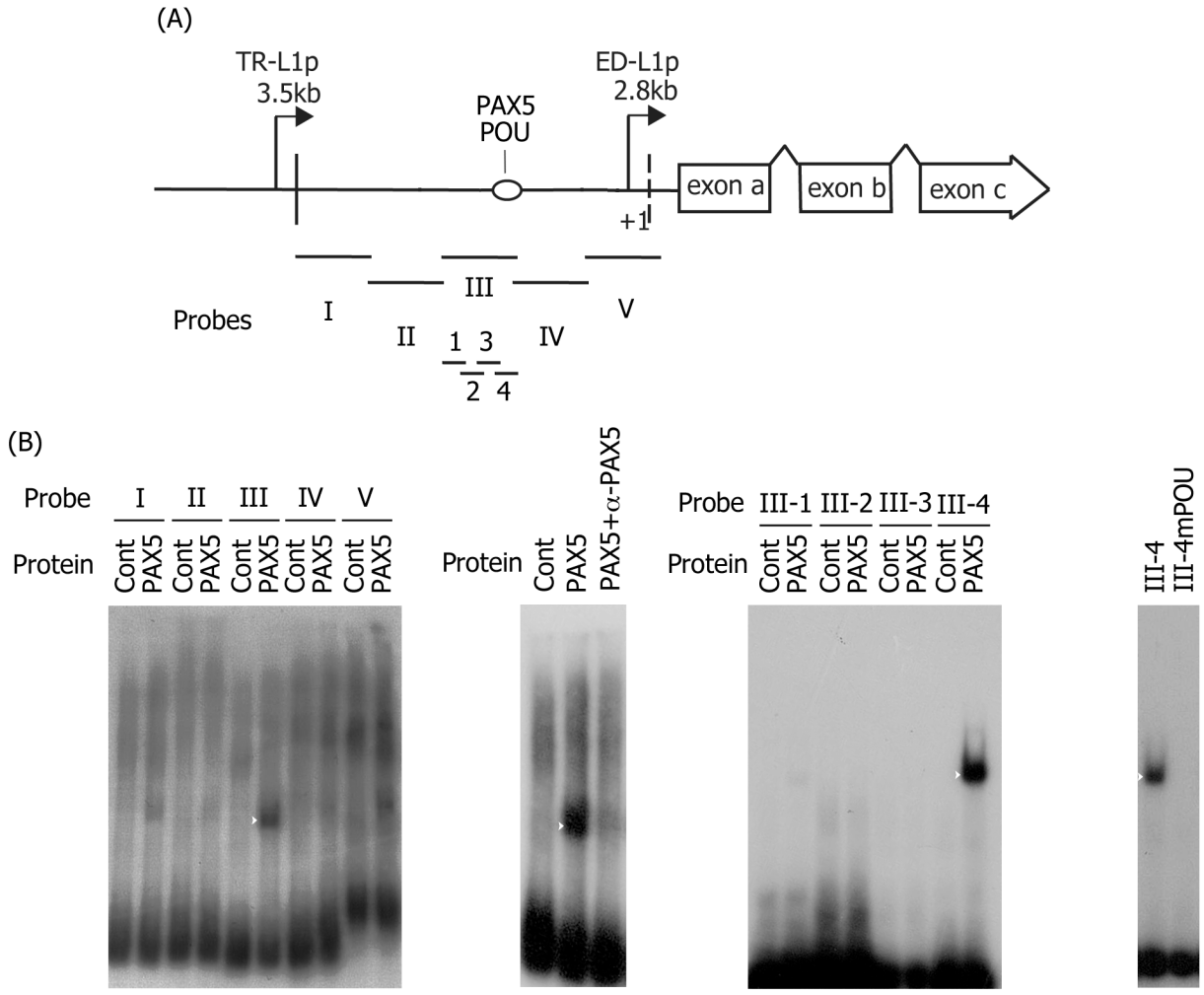
POU mt
C/EBP mt
anti-LMP1
anti-tubulin
WT
RBPJκ mt
NFκB mt
anti-LMP1
anti-tubulin
HEK293 cell
(B)
Log transformation unit/ml
4
3
2
1
WT
C/EBPmt
RBPJkmt
POUmt
NFkBmt
PU1mt
(C)
Cell number (x10*4/ml)
600
500
400
300
200
100
0
0
4
8
days
WT
WT
C/EBP mt
C/EBP mt
NFkB mt
NFkB mt
RBPJk mt
RBPJk mt
POU mt
POU mt
PU1 mt
(D)
WT
C/EBP
RBPJk
NFkB
WT
POU
WT
PU1
anti-LMP1
anti-tubulin
LCLs

Fig.3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exon b
exon c
+1
Probes
I
II
III
IV
V
1
2
3
4
(B)
Probe
I
II
III
IV
V
Protein
Cont
PAX5
Protein
Cont
PAX5
PAX5+α-PAX5
Probe
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III-2
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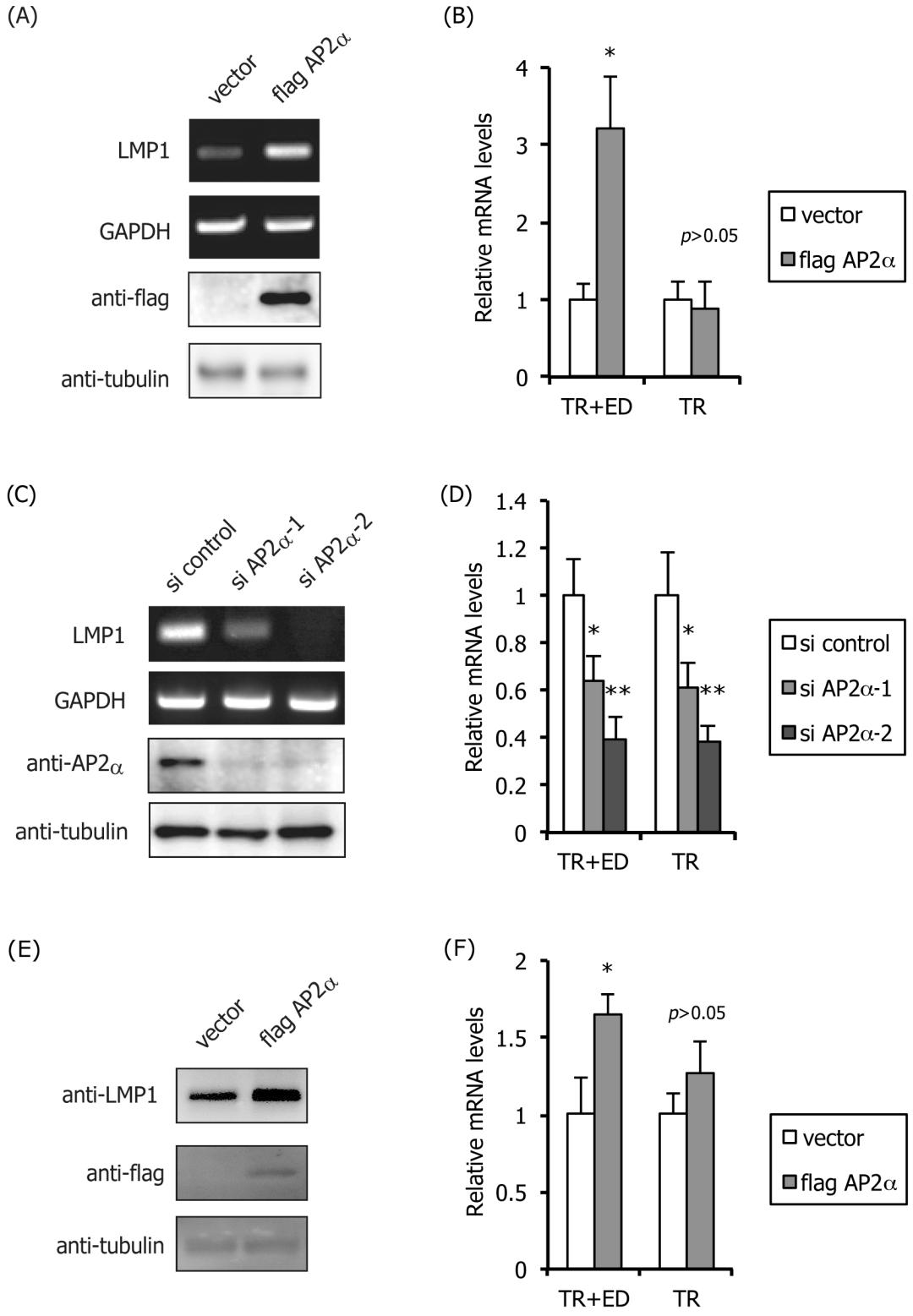
III-4
Protein
Cont
PAX5
III-4
III-4mPOU

Fig.4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flag AP2α
LMP1
GAPDH
anti-flag
anti-tubulin
(B)
Relative mRNA levels
*
p>0.05
vector
flag AP2α
TR+ED
TR
(C)
si control
si AP2α-1
si AP2α-2
LMP1
GAPDH
anti-AP2α
anti-tubulin
(D)
Relative mRNA levels
*
**
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si AP2α-1
si AP2α-2
TR+ED
TR
(E)
vector
flag AP2α
anti-LMP1
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anti-tubulin
(F)
Relative mRNA levels
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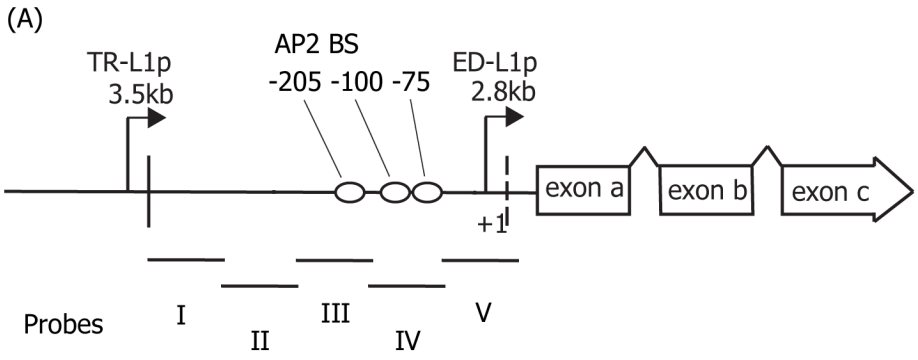
flag AP2α
TR+ED
TR

Fig.5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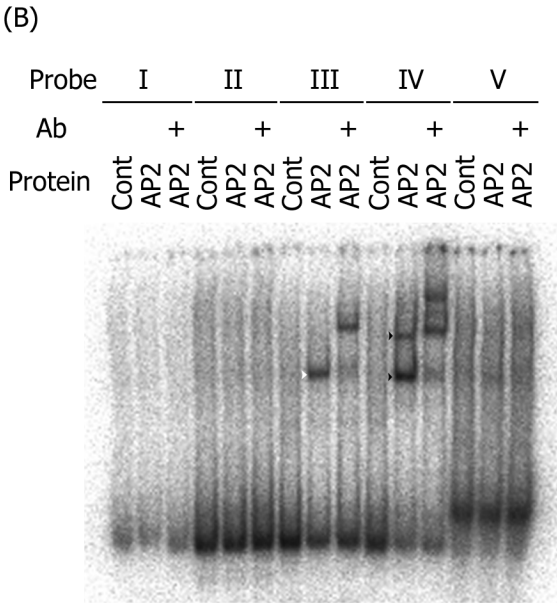
3.5kb
AP2 BS
-205 -100 -75
ED-L1p
2.8kb
exon a
exon b
exon c
+1
Probes
I
II
III
IV
V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Probe
I
II
III
IV
V
Ab
+
Protein
Cont
AP2
AP2

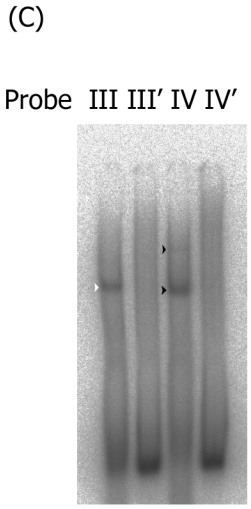
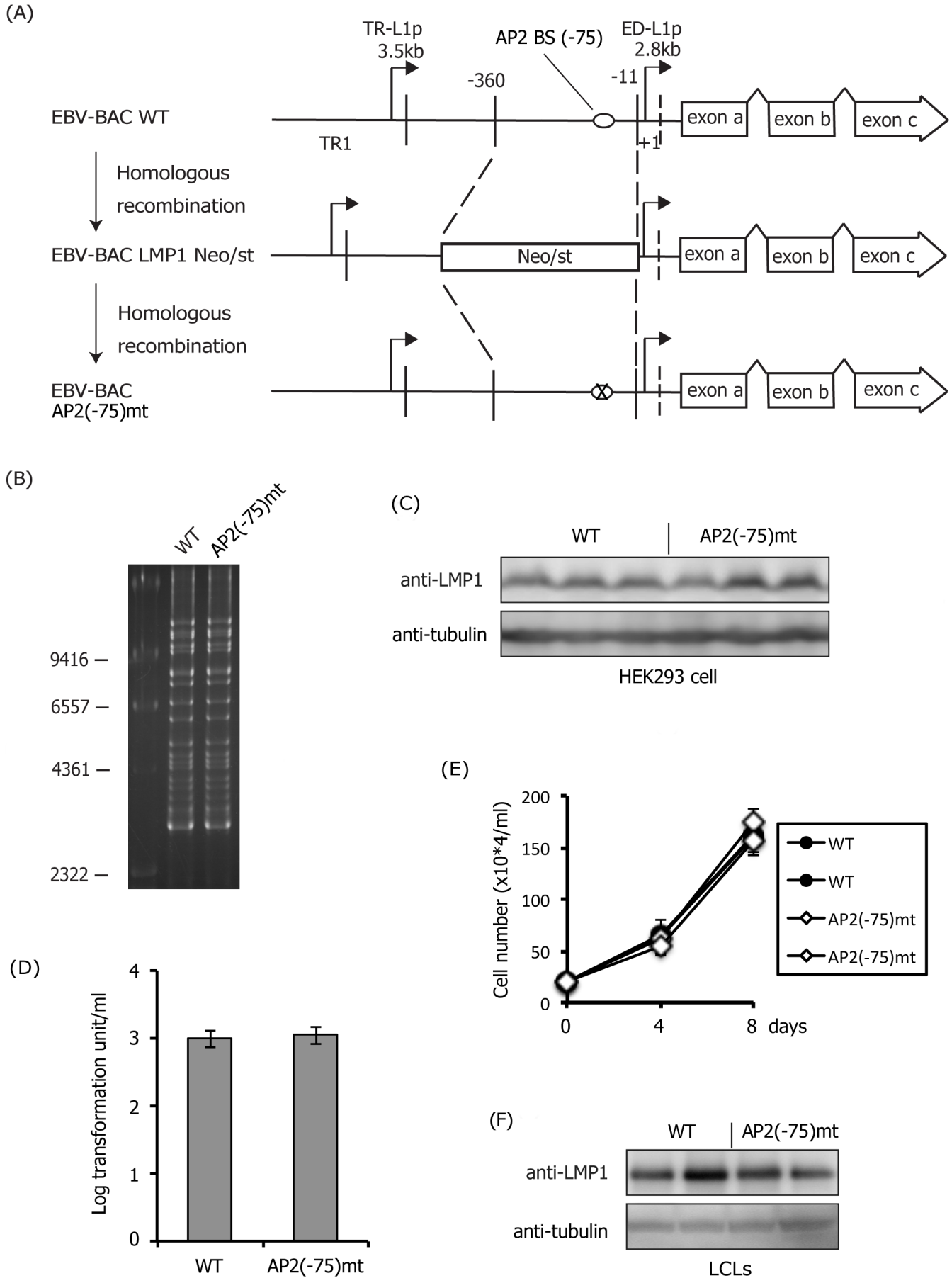

(C)
Probe III III' IV IV'

Fig.6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AP2 BS (-75)
ED-L1p
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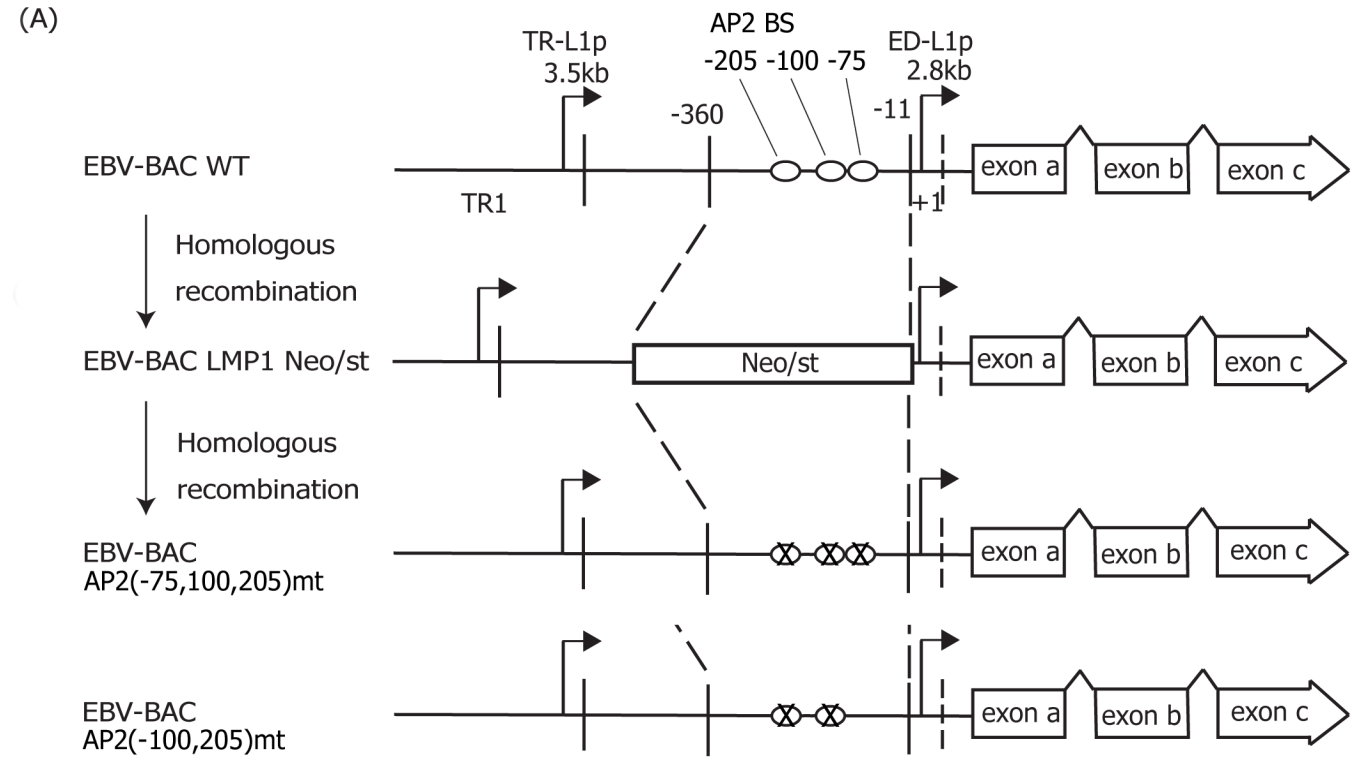
EBV-BAC WT
TR1
+1
exon a
exon b
exon c
Homologous
recombination
EBV-BAC LMP1 Neo/st
Neo/st
Homologous
recombination
EBV-BAC
AP2(-75)mt
(B)
WT
AP2(-75)mt
9416
6557
4361
2322
(C)
WT
AP2(-75)mt
anti-LMP1
anti-tubulin
HEK293 cell
(D)
Log transformation unit/ml
4
3
2
1
0
WT
AP2(-75)mt
(E)
Cell number (x10*4/ml)
200
150
100
50
0
0
4
8 days
WT
WT
AP2(-75)mt
AP2(-75)mt
(F)
WT
AP2(-75)mt
anti-LMP1
anti-tubulin
LCLs

Fig.7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AP2 BS
-205 -100 -75
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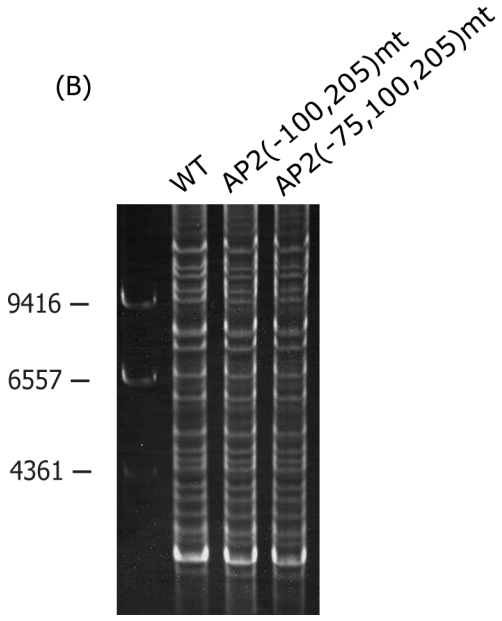
2.8kb
-360
-11
EBV-BAC WT
TR1
+1
exon a
exon b
exon c
Homologous
recombination
EBV-BAC LMP1 Neo/st
Neo/st
Homologous
recombination
EBV-BAC
AP2(-75,100,205)mt
EBV-BAC
AP2(-100,205)mt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AP2(-75,100,205)mt
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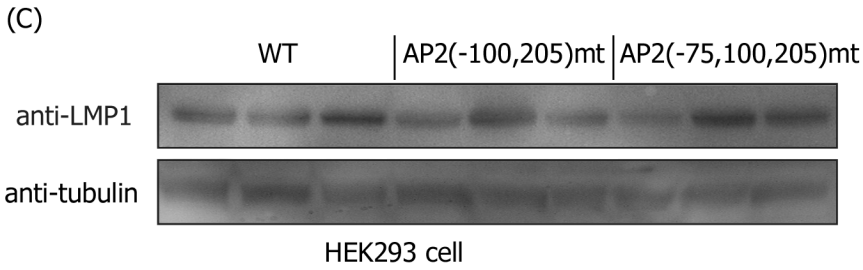
6557
4361


(C)
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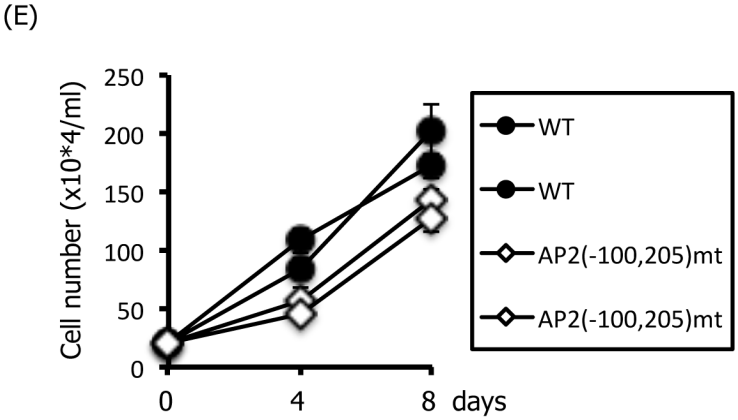
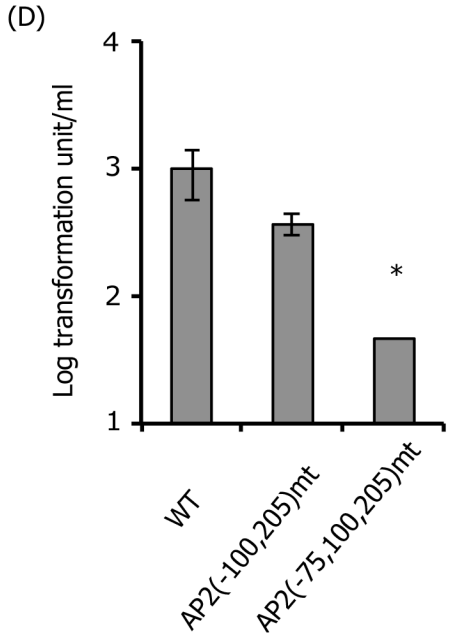
AP2(-75,100,205)mt
anti-LMP1
anti-tubulin
HEK293 cell


(E)
Cell number (x10*4/ml)
250
200
150
100
50
0
0
4
8 days
WT
WT
AP2(-100,205)mt
AP2(-100,205)mt


(D)
Log transformation unit/ml
4
3
2
1
*
WT
AP2(-100,205)mt
AP2(-75,100,205)mt

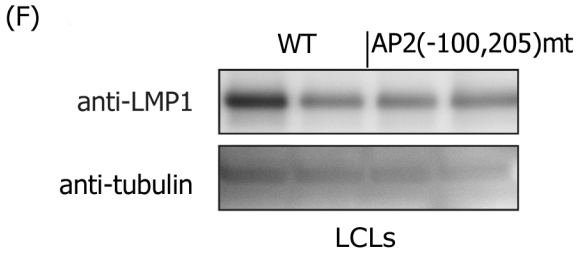

(F)
WT
AP2(-100,205)mt
anti-LMP1
anti-tubulin
LCLs

Fig. 8

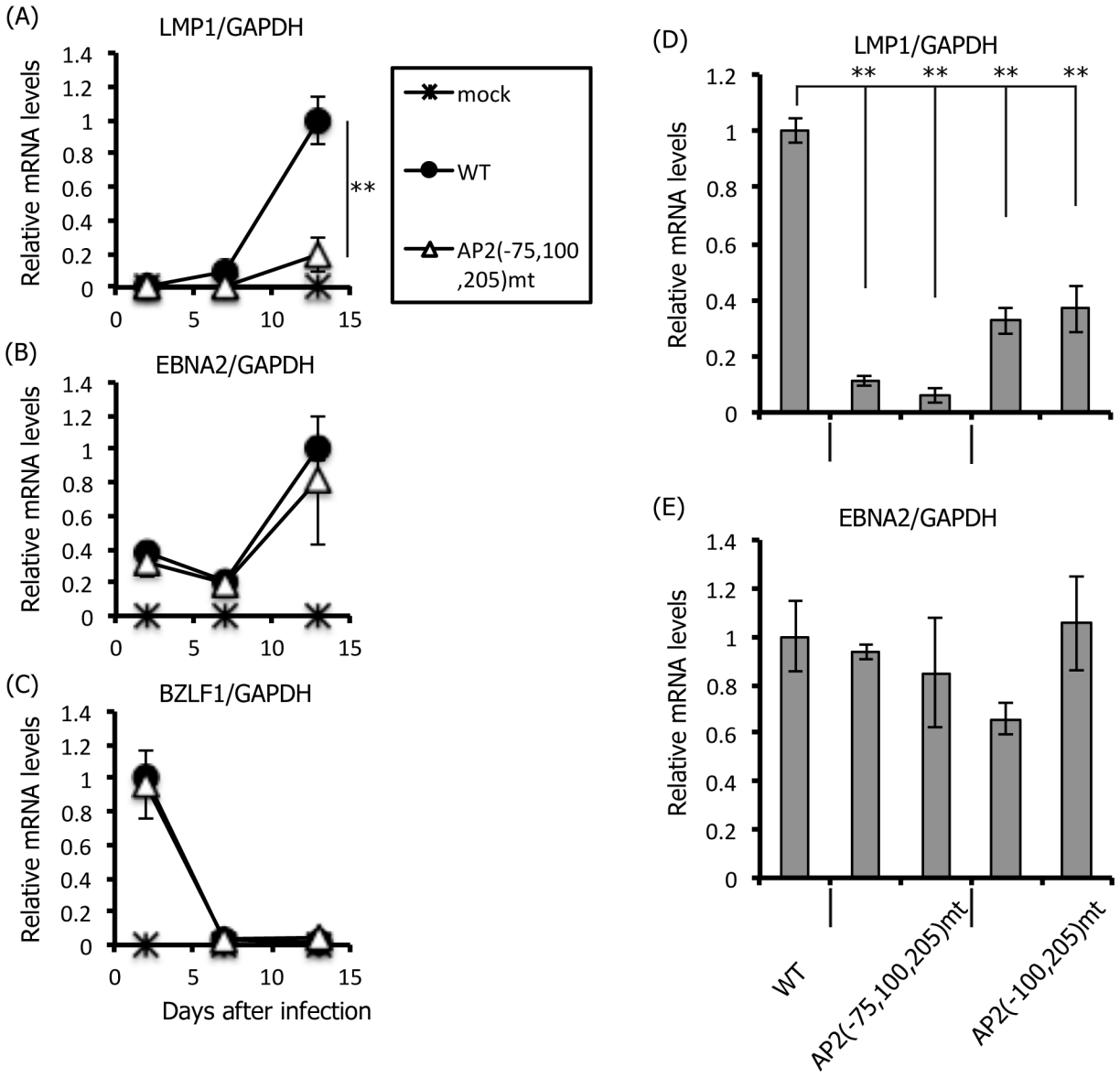
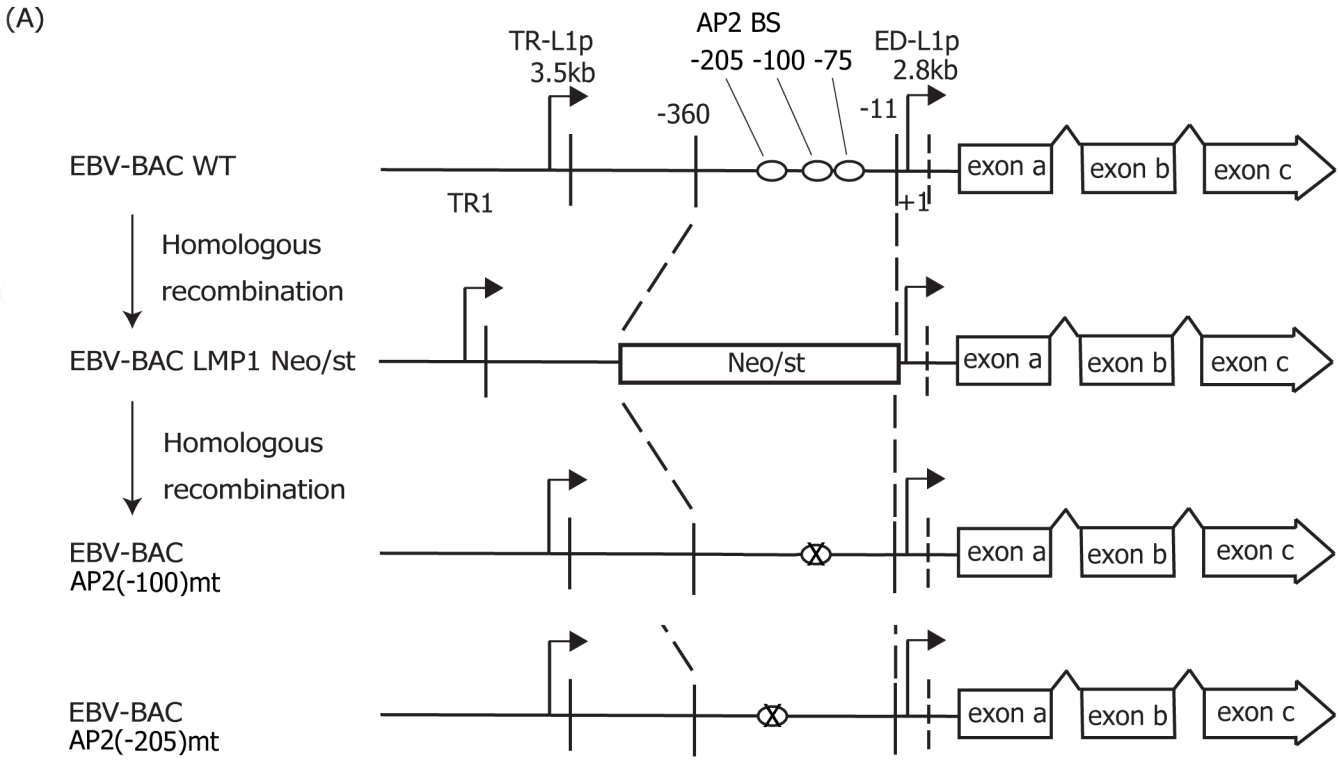
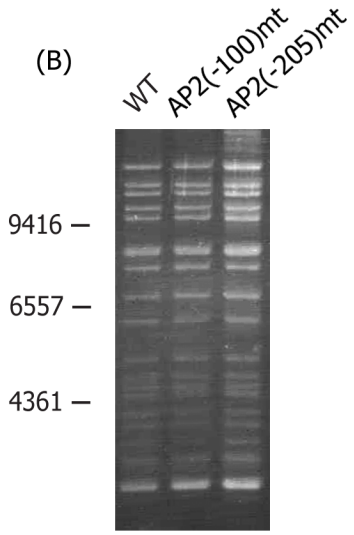
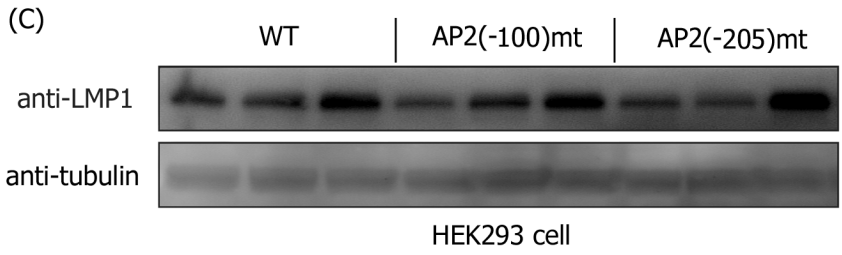
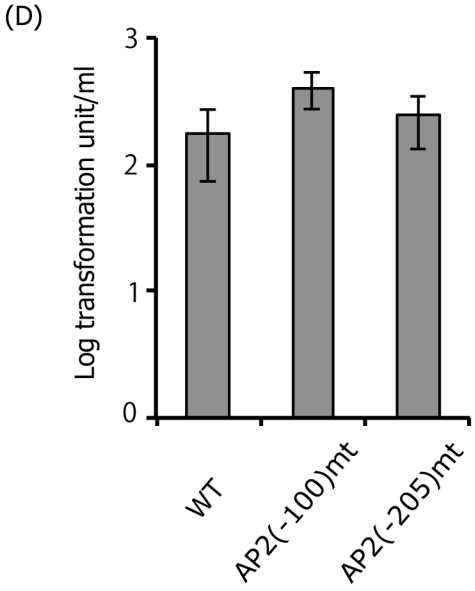

(A)
LMP1/GAPDH
Relative mRNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
**
mock
WT
AP2(-75,100
,205)mt
(B)
EBNA2/GAPDH
Relative mRNA levels
(C)
BZLF1/GAPDH
Relative mRNA levels
Days after infection
(D)
LMP1/GAPDH
Relative mRNA levels
1.2
**
**
**
**
(E)
EBNA2/GAPDH
Relative mRNA levels
WT
AP2(-75,100,205)mt
AP2(-100,205)mt

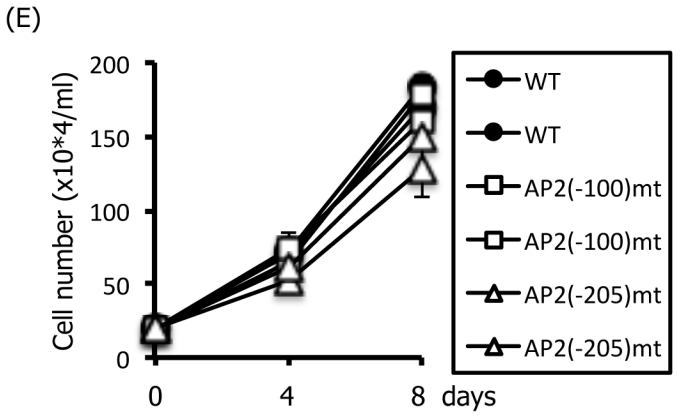
Fig.9


(A)
TR-L1p
3.5kb
AP2 BS
-205 -100 -75
ED-L1p
2.8kb
-360
-11
EBV-BAC WT
TR1
+1
exon a
exon b
exon c
Homologous
recombination
EBV-BAC LMP1 Neo/st
Neo/st
Homologous
recombination
EBV-BAC
AP2(-100)mt
EBV-BAC
AP2(-205)mt


(B)
WT
AP2(-100)mt
AP2(-205)mt
9416
6557
4361


(C)
WT
AP2(-100)mt
AP2(-205)mt
anti-LMP1
anti-tubulin
HEK293 cell


(D)
Log transformation unit/ml
3
2
1
0
WT
AP2(-100)mt
AP2(-205)mt


(E)
Cell number (x10*4/ml)
200
150
100
50
0
0
4
8 days
WT
WT
AP2(-100)mt
AP2(-100)mt
AP2(-205)mt
AP2(-205)mt

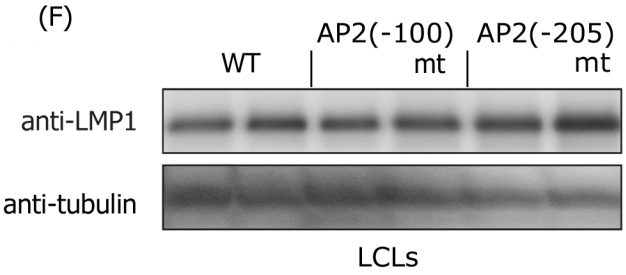
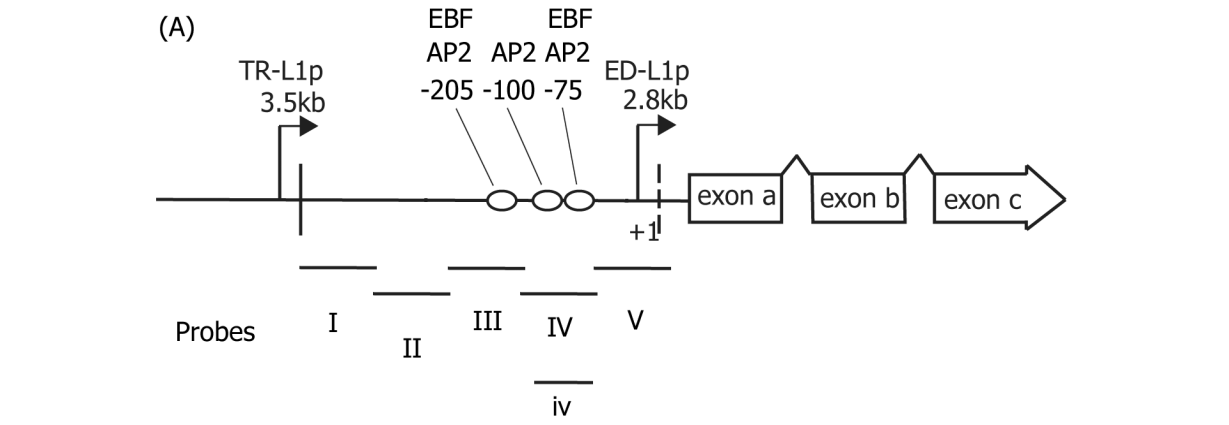
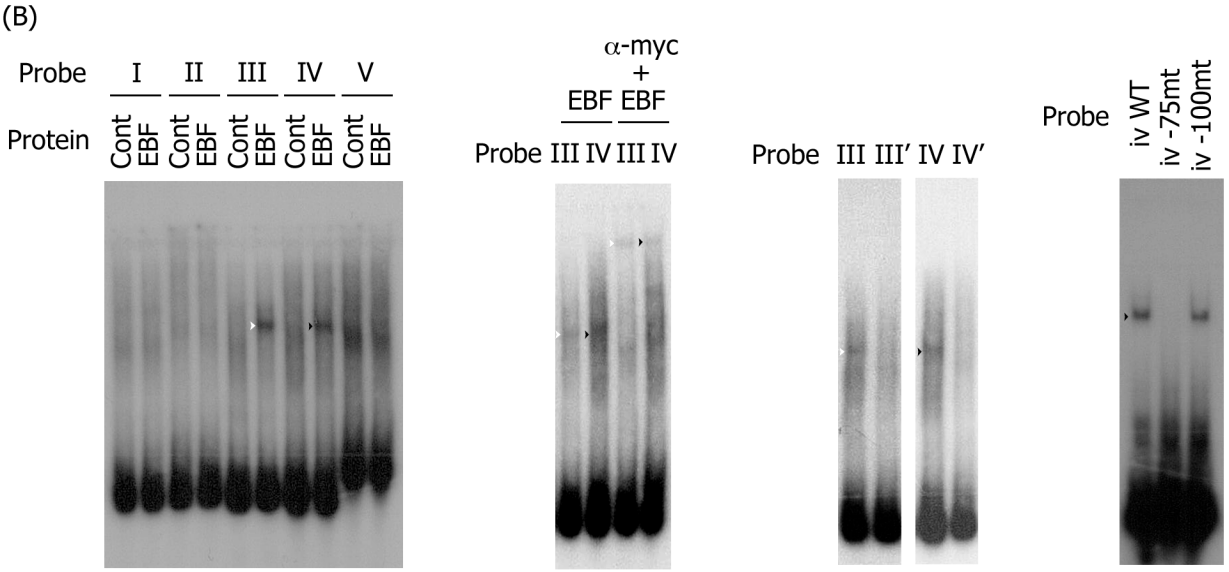
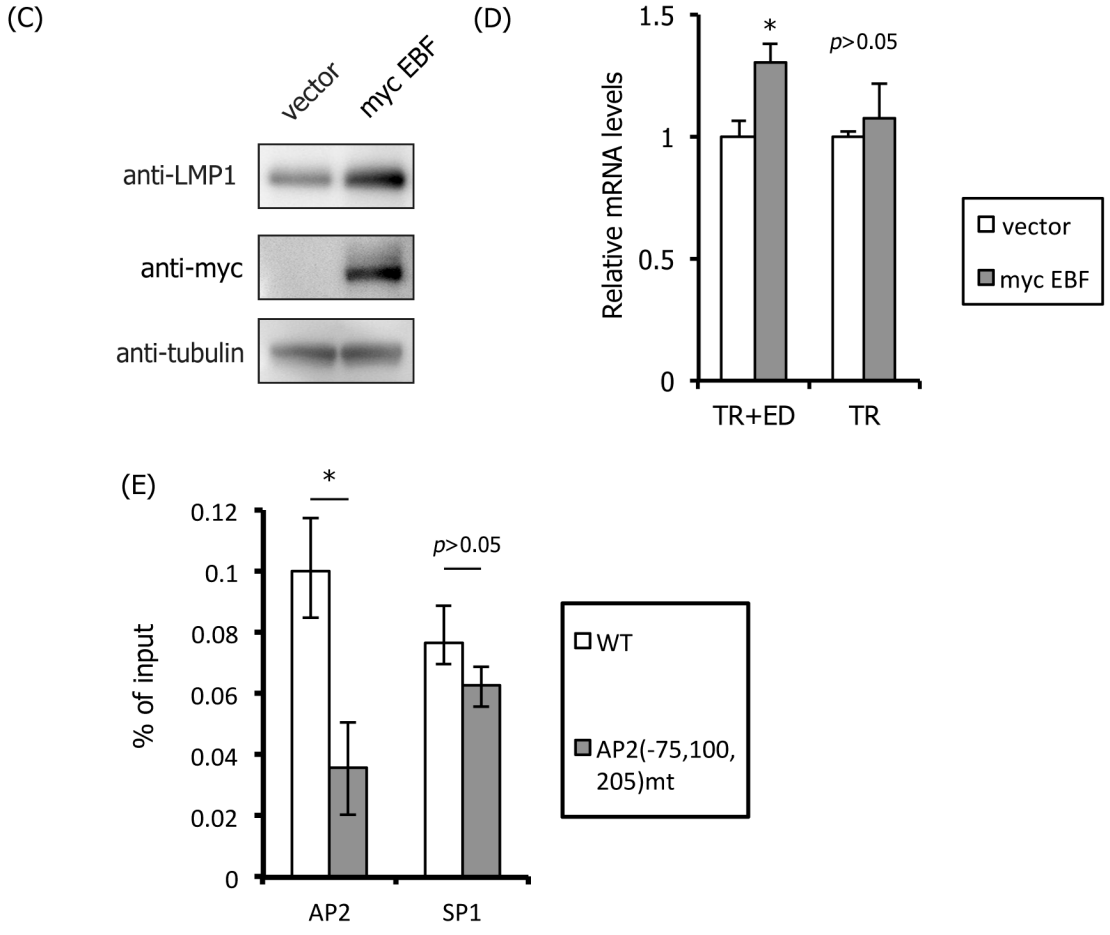

(F)
WT
AP2(-100)
mt
AP2(-205)
mt
anti-LMP1
anti-tubulin
LCLs

Fig.10


(A)
EBF AP2 -205
AP2 -100
EBF AP2 -75
TR-L1p 3.5kb
ED-L1p 2.8kb
exon a
exon b
exon c
+1
Probes
I
II
III
IV
V
iv


(B)
Probe I II III IV V
Protein Cont EBF Cont EBF Cont EBF Cont EBF Cont EBF
α-myc + EBF EBF
Probe III IV III IV
Probe III III' IV IV'
Probe iv WT iv -75mt iv -100mt


(C)
vector
myc EBF
anti-LMP1
anti-myc
anti-tubulin
(D)
Relative mRNA levels
1.5
1
0.5
0
*
p>0.05
TR+ED
TR
vector
myc EBF
(E)
% of input
0.12
0.1
0.08
0.06
0.04
0.02
0
*
p>0.05
AP2
SP1
WT
AP2(-75,100, 205)mt