

Quantitative evaluation of the role of Epstein–Barr virus immediate-early protein BZLF1 in B-cell transformation

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The Epstein–Barr virus (EBV) immediate-early transactivator BZLF1 plays a key role in switching EBV infection from the latent to the lytic form by stimulating the expression cascade of lytic genes; it also regulates the expression of several cellular genes. Recently, we reported that BZLF1 is expressed in primary human B cells early after EBV infection. To investigate whether this BZLF1 expression early after infection plays a role in the EBV-induced growth transformation of primary B cells, we generated BZLF1-knockout EBV and quantitatively evaluated its transforming ability compared with that of wild-type EBV. We found that the 50% transforming dose of BZLF1-knockout EBV was quite similar to that of wild-type EBV. Established lymphoblastoid cell lines (LCLs) harbouring BZLF1-knockout EBV were indistinguishable from LCLs harbouring wild-type EBV in their pattern of latent gene expression and in their growth *in vitro*. Furthermore, the copy numbers of EBV episomes were very similar in the LCLs harbouring BZLF1-knockout EBV and in those harbouring wild-type EBV. These data indicate that disrupting BZLF1 expression in the context of the EBV genome, and the resultant inability to enter lytic replication, have little impact on the growth of LCLs and the steady-state copy number of EBV episomes in established LCLs.

Received 20 April 2009
Accepted 19 June 2009

INTRODUCTION

Epstein–Barr virus (EBV) is a ubiquitous human gamma-herpesvirus that is associated with various malignancies, including Burkitt's lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma and gastric carcinoma (Rickinson & Kieff, 2007). EBV efficiently infects primary B lymphocytes, transforming them into indefinitely proliferating lymphoblastoid cell lines (LCLs) *in vitro* and causing lymphoproliferative diseases in immune-deficient individuals *in vivo* (Kieff & Rickinson, 2007; Rickinson & Kieff, 2007).

LCLs harbour multiple copies of the EBV episome, from which EBV expresses latent gene products, including six nuclear proteins (EBNA1, -2, -3A, -3B, -3C and -LP), three integral membrane proteins (LMP1, -2A and -2B), two small non-polyadenylated RNAs (EBER1 and EBER2), BamHI-A rightward transcripts and microRNAs. Of these, EBNA1, -2, -3A, -3C and -LP, LMP1 and EBER2 are

necessary for efficient growth transformation (Cohen *et al.*, 1989; Hammerschmidt & Sugden, 1989; Humme *et al.*, 2003; Kaye *et al.*, 1993; Mannick *et al.*, 1991; Maruo *et al.*, 2003, 2006; Tomkinson *et al.*, 1993; Wu *et al.*, 2007; Yajima *et al.*, 2005).

The viral gene BZLF1 encodes a key protein that switches EBV infection from the latent to the lytic form (Countryman & Miller, 1985; Takada *et al.*, 1986). The BZLF1 protein is a member of the basic leucine zipper (bZIP) family of transcription factors and functions as a transactivator by binding to the BZLF1-responsive element (ZRE) in its target promoters (Chang *et al.*, 1990; Farrell *et al.*, 1989; Feederle *et al.*, 2000; Flemington *et al.*, 1992; Sinclair, 2003). BZLF1 plays critical roles in the replication of the EBV genome by binding to the origin of lytic replication, as well as by inducing viral genes that function as the viral replication machinery, such as BALF5, a DNA polymerase, and BMRF1, a processivity factor (Tsurumi *et al.*, 2005). Thus, BZLF1 expression causes transcription of many viral genes and replication of the EBV genome, and results in production of progeny virus (Kieff & Rickinson, 2007).

BZLF1 also upregulates several cellular genes whose promoters contain ZRE, such as *c-fos*, *EGR-1*, *DHRS9*,

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A supplementary figure showing a comparison of GFP-inducing titre and EBNA-inducing titre of the recombinant viruses is available with the online version of this paper.

interleukin (IL)-8 and IL-10 (Chang *et al.*, 2006; Flemington & Speck, 1990; Hsu *et al.*, 2008; Jones *et al.*, 2007a; Mahot *et al.*, 2003), and interacts with and interferes with the function of several cellular proteins, such as p53, CREB-binding protein, CEBP α and the basic transcriptional machinery (Adamson & Kenney, 1999; Lieberman & Berk, 1991, 1994; Sinclair, 2003; Wu *et al.*, 2003; Zerby *et al.*, 1999; Zhang *et al.*, 1994). For example, BZLF1 attenuates transactivation by p53 and transactivation by CREB (Adamson & Kenney, 1999; Zhang *et al.*, 1994). BZLF1 may therefore modulate the behaviour of host cells. Indeed, BZLF1 has multiple effects on the proliferation of host cells, such as suppressing proliferation of HeLa cells and normal human fibroblasts by inducing G1/S and G2/M arrest (Cayrol & Flemington, 1996; Mauser *et al.*, 2002a, b; Rodriguez *et al.*, 1999, 2001; Wu *et al.*, 2003). Such growth arrest results from the induction of p53 and the cyclin-dependent kinase inhibitors p21 and p27, followed by accumulation of a hypophosphorylated form of pRb (Cayrol & Flemington, 1996; Rodriguez *et al.*, 1999). However, in cells of the AGS human gastric carcinoma line and primary keratinocytes, BZLF1 induces E2F-1 and cyclin E, which accelerate the cell cycle (Mauser *et al.*, 2002b).

Recently, we reported that BZLF1 is expressed as an immediate-early protein in primary B lymphocytes early after EBV infection (Wen *et al.*, 2007). We therefore attempted to clarify whether this BZLF1 expression early after infection plays some roles in establishment of EBV latent infection.

In this study, we generated BZLF1-knockout (ZKO) EBV by using the Akata cell system to clarify whether BZLF1 contributes to growth transformation of primary B lymphocytes. The transforming efficiency of ZKO-EBV was quite similar to that of its wild-type counterpart (WT-EBV), and LCLs transformed by WT-EBV or ZKO-EBV harboured similar numbers of EBV genomes. These results indicate that disrupting BZLF1 expression in the context of the EBV genome, and the resultant inability to enter lytic replication, do not substantially influence the efficiency of transformation or the steady-state copy number of EBV episomes in established LCLs.

METHODS

Cells. EBV-positive Akata (Takada *et al.*, 1991), EBV-negative Akata (Shimizu *et al.*, 1994) and EBV-negative Daudi (Nanbo *et al.*, 2002) cell lines and LCLs were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (both from Sigma). Akata cell clones harbouring EGFP-EBV (WT-EBV) were maintained in medium containing 700 μg G418 ml^{-1} (Promega) (Maruo *et al.*, 2001). AGS-CR2, an AGS human gastric carcinoma cell clone that stably expresses CD21, was established by stable transfection of a plasmid that expresses CD21 into AGS cells. AGS-CR2 cell clones harbouring WT-EBV or ZKO-EBV were maintained in Ham's F-12 medium (Sigma) containing 500 μg G418 ml^{-1} .

Plasmids. To construct a BZLF1 targeting vector, an *EcoRI*-*SalI* fragment of the Akata strain EBV genome (corresponding to

nt 95239–105296 of EBV strain B95-8) was cloned into the pBluescript II SK(-) vector (Stratagene). Next, a *NotI* site and an *NheI* site were introduced into a position within the BZLF1 gene in the vector (corresponding to nt 102389 of B95-8) by site-directed mutagenesis. Then, a region containing the second exon, a part of the first exon and both introns of the BZLF1 gene of Akata (corresponding to nt 102389–102731 of B95-8) was deleted by *NheI* digestion. Finally, the hygromycin-resistance gene cassette, driven by the simian virus 40 early enhancer and promoter, was inserted into the *NotI* site to complete the targeting construct for ZKO. pSG5-BZLF1 was constructed by cloning a BZLF1 cDNA into the *EcoRI* site of pSG5 (Stratagene).

Generation of ZKO-EBV. Targeting construct (20 μg) was digested with *EcoRI* and *SalI* and introduced into Akata cells harbouring EGFP-EBV (WT-EBV) via electroporation. At 2 days after transfection, cells were plated at 10^4 cells per well in 96-well tissue-culture plates in medium containing 150 μg hygromycin ml^{-1} (Calbiochem) for selection. To obtain cell clones harbouring both ZKO-EBV and WT-EBV, hygromycin-resistant cell clones were screened by Southern blotting. To segregate ZKO-EBV from WT-EBV, Akata cells harbouring both ZKO-EBV and WT-EBV were induced for lytic replication for 2 days; the cell-free culture supernatant was then used to infect EBV-negative Akata cells. Two days after infection, the infected cells were plated in 96-well plates at 10^4 cells per well in medium containing 150 μg hygromycin ml^{-1} for selection. Drug-resistant clones were screened by Southern blotting to identify cell clones harbouring only ZKO-EBV.

Establishment of AGS-CR2 cell clones harbouring ZKO-EBV and WT-EBV. AGS-CR2 cells were infected with recombinant EBVs by using a previously described cell-to-cell infection method (Imai *et al.*, 1998). Akata cell clones harbouring ZKO-EBV or WT-EBV were transfected with pSG5-BZLF1, and the cells were then seeded onto AGS-CR2 cells. Three days later, the co-cultivation cultures were washed gently but thoroughly with PBS to remove residual viable virus-donor cells, and the cells were reseeded into 96-well plates at 10^2 cells per well in culture medium containing 500 μg G418 ml^{-1} .

Induction of lytic replication and virus titration. For Akata cell clones harbouring EBV, lytic replication was induced by treatment with 0.5% anti-human IgG, TPA (12-*O*-tetradecanoylphorbol-13-acetate; 20 ng ml^{-1}) and *n*-butyrate (3 mM), or transfection of pSG5-BZLF1 (Shimizu *et al.*, 1996). AGS cell clones harbouring EBV were transfected with pSG5-BZLF1 by using Lipofectamine and Plus reagents (Invitrogen). Three days later, the culture supernatant was harvested and filtered through a membrane with 0.45 μm pores, and the filtrate was used as the virus solution. For infection, EBV-negative Daudi cells were resuspended with 1 ml virus solution and incubated at 37 °C for 90 min. After washes, infected Daudi cells were cultured for 2 days. Then, the percentage of enhanced green fluorescent protein (EGFP)-positive cells was determined by flow cytometry (FACSCalibur; BD). On the basis of these data, 'GFP-inducing units' (GIU) ml^{-1} were calculated as a measure of the concentration of infectious EBV particles in different virus stocks by using the following equation: GIU ml^{-1} = percentage of GFP-positive Daudi cells \times number of Daudi cells infected (5×10^5) \times dilution factor.

Southern blotting. Southern blotting was performed as described previously (Wu *et al.*, 2007). The probes used are shown in Fig. 1(a). In some experiments, we used the *EcoRI* K fragment of Akata EBV (corresponding to the *EcoRI* J fragment of B95-8 EBV) as a probe. Probe labelling was carried out by using an AlkPhos direct labelling kit and signals were detected with CDP-Star detection reagent (both from GE Healthcare).

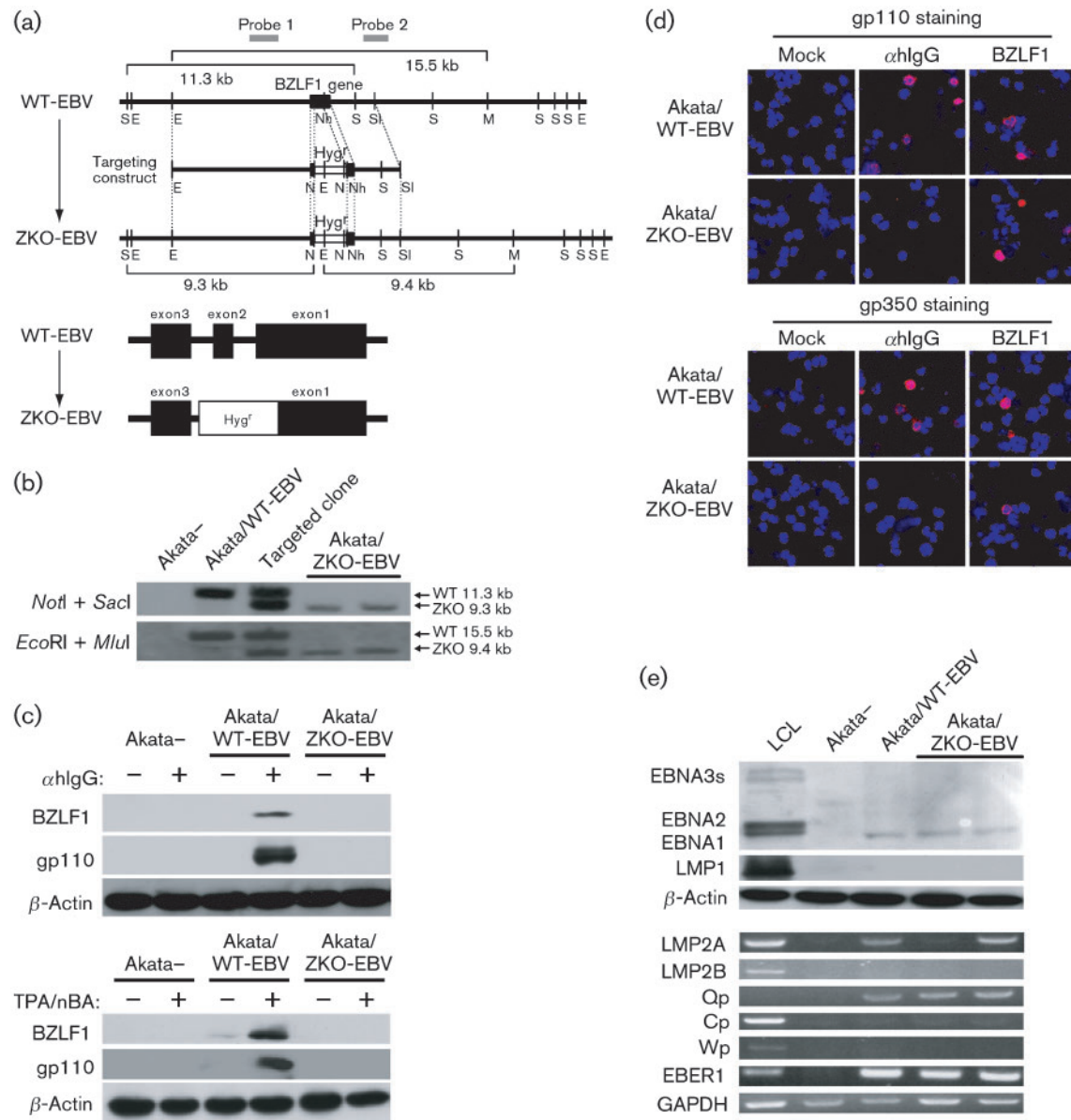


Fig. 1. Generation of disrupted BZLF1 (ZKO) EBV in Akata cells. (a) Schematic representation of the BZLF1 locus in the genomes of WT-EBV and ZKO-EBV. The targeting construct is also shown. The positions of restriction sites are indicated by S (*SacI*), E (*EcoRI*), N (*NotI*), Nh (*NheI*), SI (*SalI*) and M (*MluI*). The grey lines indicate the probes (1 and 2) used for Southern blot analysis, and the sizes of the bands from each EBV that are detected by the probes are shown. The BZLF1 gene and exons of the BZLF1 gene (filled boxes) and Hyg^r (open box) are also indicated. (b) Southern blot analysis of Akata cell clones harbouring various recombinant EBVs. Genomic DNAs extracted from an EBV-negative Akata cell clone (Akata-), a WT-EBV-infected Akata cell clone (Akata/WT-EBV), an Akata cell clone in which homologous recombination occurred (Targeted clone) and ZKO-EBV-infected Akata cell clones (Akata/ZKO-EBV) were digested with *NotI* and *SacI* and probed with probe 1 (upper panel), or digested with *EcoRI* and *MluI* and probed with probe 2 (lower panel). The sizes of the bands detected from WT-EBV (WT) and ZKO-EBV (ZKO) are shown. (c) Treatment with anti-human IgG or TPA and butyrate did not induce the lytic cycle in Akata/ZKO-EBV cells. The indicated Akata cell clones were cultured in medium with (+) or without (-) anti-human IgG (α hlgG; upper panel), or with (+) or without (-) TPA and n-butylate (TPA/nBA; lower panel) for 48 h. Then, immunoblotting was performed to detect BZLF1, gp110 or β -actin. (d) BZLF1 transfection restores the expression of lytic genes in an Akata/ZKO-EBV cell clone. The indicated Akata cell clones were cultured in the absence (Mock) or presence (α hlgG) of anti-human IgG, or transfected with pSG5-BZLF1 (BZLF1). Immunofluorescence staining was performed 48 h later to detect gp110 and gp350. (e) EBV gene expression in Akata cell clones harbouring various recombinant EBVs. Immunoblotting was used to detect EBNA and LMP1 (upper panel); RT-PCR was used to detect other latent genes (lower panel). An LCL was used as a positive control.

Western blotting. Cell lysates were separated by using SDS-PAGE and the proteins were blotted onto nitrocellulose membranes. The membranes were incubated with BZ-1 (specific to BZLF1; Dako), C30-1 (specific to gp110), EBV-immune human sera (reactive to EBNA5), S12 (specific to LMP1) and AC-15 (specific to β -actin; Sigma), then incubated with horseradish peroxidase-conjugated species-specific secondary antibodies and developed with ECL detection reagents (GE Healthcare).

Immunofluorescence. Cells were smeared on glass slides and fixed. Indirect immunofluorescence was performed with C-1 (specific to gp350), C30-1 (specific to gp110) or C844-1 (specific to BHRF1; Iwakiri & Takada, 2004) monoclonal antibodies, or EBV-immune human sera (reactive to EBNA5) as primary antibodies. A Cy3-conjugated anti-mouse IgG or fluorescein isothiocyanate-conjugated anti-C3C antibody was used as a secondary antibody. Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

RT-PCR. Total RNA was extracted with TRIzol reagent and treated with DNase I (Invitrogen). Then, cDNA synthesis and PCR were performed as described previously (Imai *et al.*, 1998; Wen *et al.*, 2007).

Growth-transformation assay. Primary B cells were enriched from peripheral blood mononuclear cells by using Dynabeads CD19 (Dyna), and the cells were infected with serial 10-fold dilutions of culture supernatant containing WT-EBV or ZKO-EBV. Infected cells were then plated at 1×10^4 cells per well in 96-well plates (48 wells for each infection). Half of the culture medium was replaced with fresh medium every 5 days. The number of wells with proliferating lymphocytes was counted 4 weeks after infection.

LCL growth assay. To generate growth curves, LCLs were plated in medium at 2×10^5 or 5×10^4 cells ml^{-1} . Viable cell numbers were determined based on trypan blue exclusion every 2–3 days by using a haemocytometer and, on day 4, the cells were split to maintain exponential-phase growth. Total viable cell numbers were calculated relative to the initial cultures. To evaluate LCL growth at low cell density, serially diluted LCLs were plated in 96-well plates, and half of the culture medium was replaced with fresh medium every 5 days. The number of wells with proliferating lymphocytes was counted 4 weeks after the start of cell culture.

Fluorescence *in situ* hybridization (FISH). To make a FISH probe, 1 μg BACmid DNA (AK-BAC; Kanda *et al.*, 2007) was labelled with digoxigenin by using DIG-Nick Translation Mix (Roche) according to the manufacturer's instructions. Cells were fixed with methanol/acetic acid and were subjected to a conventional FISH protocol (Kanda *et al.*, 2007). After overnight hybridization, slides were washed and incubated with anti-digoxigenin fluorescein (Roche). Cell nuclei were counterstained with propidium iodide ($1 \mu\text{g ml}^{-1}$). Images were acquired by using a laser-scanning microscopy system (Fluoview; Olympus).

RESULTS

Generation of BZLF1-knockout EBV

To evaluate the role of BZLF1 in the EBV-induced growth transformation of primary B cells, we generated a recombinant EBV in which the BZLF1 gene was destroyed (ZKO-EBV) by using the Akata cell system (Shimizu *et al.*, 1996). An EBV DNA fragment containing the BZLF1 gene and flanking regions was used to make a targeting

construct in which a region containing the second exon and part of the first exon of the BZLF1 gene was replaced by a hygromycin-resistance gene (Fig. 1a). The targeting construct was transfected into Akata cells harbouring a modified EBV that carries an EGFP gene and a neomycin-resistance gene inserted into the *Bam*HI X site of its genome (designated WT-EBV in this paper; Fig. 1a) (Maruo *et al.*, 2001). Transfected Akata cells were subjected to hygromycin selection, and hygromycin-resistant cell clones were screened by Southern blot analysis for the presence of EBV episomes that had undergone homologous recombination. Three cell clones were identified as containing both the ZKO-EBV episome and the parental WT-EBV episome (Fig. 1b and data not shown). The cells were treated with anti-human IgG to induce the switch from latent to lytic infection and a mixture of both WT and ZKO viruses was used to infect EBV-negative Akata cells. After hygromycin selection, Akata cell clones harbouring only ZKO-EBV were obtained (Fig. 1b).

We next examined whether the lytic cycle could be induced in Akata cell clones harbouring ZKO-EBV. Treatment of WT-EBV-infected Akata cells with anti-human IgG, or with TPA and butyrate, induced BZLF1, gp110 and gp350 expression, whereas the same treatment of ZKO-EBV-infected Akata cell clones did not induce lytic genes (Fig. 1c, d). In contrast, transfection of a BZLF1 expression plasmid into ZKO-EBV-infected Akata cells restored expression of the lytic genes gp110 and gp350 in about 5% of ZKO-EBV-infected cells (Fig. 1d). Thus, Akata cells latently infected with ZKO-EBV exhibited the expected phenotype of being defective in entering lytic replication unless BZLF1 was expressed exogenously.

Immunoblotting and RT-PCR analyses revealed that the patterns of viral latent gene expression and EBNA promoter usage of the ZKO-EBV-infected Akata cell clones were similar to those of WT-EBV-infected Akata cell clones (Fig. 1e).

Establishment of AGS cell clones harbouring ZKO-EBV

When EBV-negative Daudi cells were inoculated with the culture supernatant of BZLF1-transfected ZKO-EBV-infected Akata cells, up to 0.1% of cells became EGFP-positive (data not shown). The low virus production was probably due to poor transfection efficiency of Akata cells. To obtain a virus preparation with a higher titre, we transferred ZKO-EBV from Akata cells to AGS cells by using the cell-to-cell infection method, because AGS cells harbouring EBV support persistent lytic infection (Feng *et al.*, 2007; Yoshiyama *et al.*, 1997). AGS cells stably transfected with a plasmid expressing human CD21, the major receptor for EBV, were established and co-cultured with Akata cells harbouring ZKO-EBV or WT-EBV that had been induced to enter lytic replication by BZLF1 transfection. After selection in the presence of G418, AGS cell clones harbouring ZKO-EBV or WT-EBV were

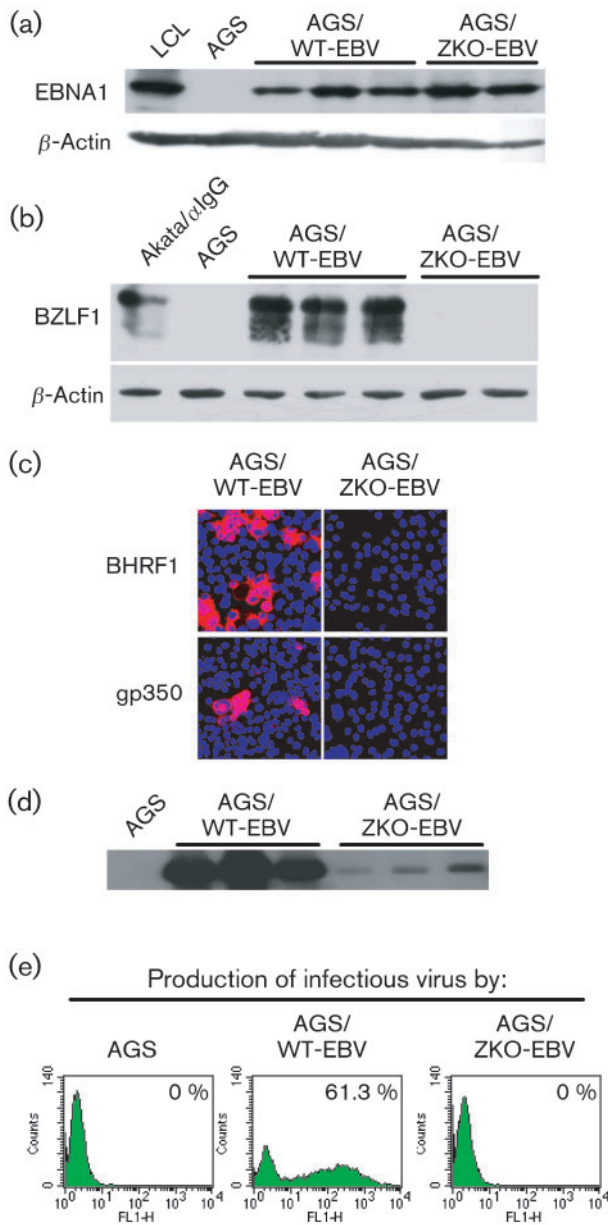


Fig. 2. Establishment of AGS cell clones harbouring WT-EBV (AGS/WT-EBV) or ZKO-EBV (AGS/ZKO-EBV). (a) AGS/WT-EBV cells (three clones), AGS/ZKO-EBV cells (two clones) and EBV-negative AGS cells (AGS) were cultured without any treatment. Immunoblotting was used to detect EBNA1 and β -actin. An LCL was used as a positive control. (b) The indicated AGS cell clones were cultured without any treatment. Immunoblotting was performed to detect BZLF1. Akata/ α IgG was used as a positive control. (c) Spontaneous expression of lytic genes in AGS/WT-EBV cells. The indicated AGS cell clones were cultured without any treatment and subjected to immunofluorescence staining to detect BHRF1 and gp350. (d) Spontaneous amplification of EBV genomes in AGS/WT-EBV cells. The indicated AGS cell clones were cultured without any treatment. Genomic DNAs were digested with *Eco*RI and probed with the *Eco*RI K fragment of Akata EBV. (e) AGS/WT-EBV cells produce infectious virus spontaneously. Cell-free culture supernatants from the indicated AGS cell clones cultured without any treatment were used to infect EBV-negative Daudi cells. EGFP expression in Daudi cells is shown.

infected AGS cells without any treatment were used to infect EBV-negative Daudi cells, and the frequency of EGFP-positive cells after infection was determined to estimate the virus concentration in the supernatant. About 60% of the recipient cells were positive for EGFP when the supernatants of WT-EBV-infected AGS cells were used for the infection (Fig. 2b), indicating that the AGS cell clone was producing infectious virus spontaneously. In contrast, AGS cells harbouring the ZKO-EBV genome did not produce infectious virus spontaneously, again confirming that ZKO-EBV was defective in entering lytic replication (Fig. 2e).

Next, we examined whether BZLF1 transfection induced lytic infection in ZKO-EBV-infected AGS cells. We transfected a BZLF1 expression plasmid into ZKO-EBV-infected AGS cells; 72 h later, we harvested cells and examined the induction of lytic genes. After transfection of the BZLF1 expression plasmid, 10–20% of ZKO-EBV-infected AGS cells became positive for BHRF1, and 3–10% became positive for gp350 (Fig. 3a). BZLF1 transcomplementation caused the amplification of virus DNA (Fig. 3b). When EBV-negative Daudi cells were infected with the cell-free supernatants of BZLF1-transduced ZKO-EBV-infected AGS cell clones, up to 65% of the recipient cells expressed EGFP (Fig. 3c), yielding a virus supernatant of about 6.5×10^5 GIU ml^{-1} . We also verified that the frequency of EBNA-positive cells correlated with the frequency of EGFP-positive cells (see Supplementary Fig. S1, available in JGV Online). We prepared supernatants of WT-EBV-infected AGS cells and ZKO-EBV-infected AGS cells with comparable EGFP-inducing titres for use as WT-EBV and ZKO-EBV preparations, respectively, for the following experiments.

obtained. The infected AGS cell clones expressed EBNA1 (Fig. 2a), and the WT-EBV-infected AGS cells expressed BZLF1 protein spontaneously without any treatment (Fig. 2b). Immunofluorescence analysis revealed that 20–30% of the AGS cells harbouring WT-EBV were positive for BHRF1, and 10–20% of the cells were positive for gp350 (Fig. 2c). In contrast, ZKO-EBV-infected AGS cells were negative for BZLF1, BHRF1 and gp350 (Fig. 2b, c). Southern blot analysis revealed that the bands representing the EBV genome of WT-EBV-infected AGS cell clones were also more intense than those of ZKO-EBV-infected AGS cell clones, indicating spontaneous amplification of the EBV genome in WT-EBV-infected AGS cell clones (Fig. 2d). Cell-free culture supernatants obtained from the

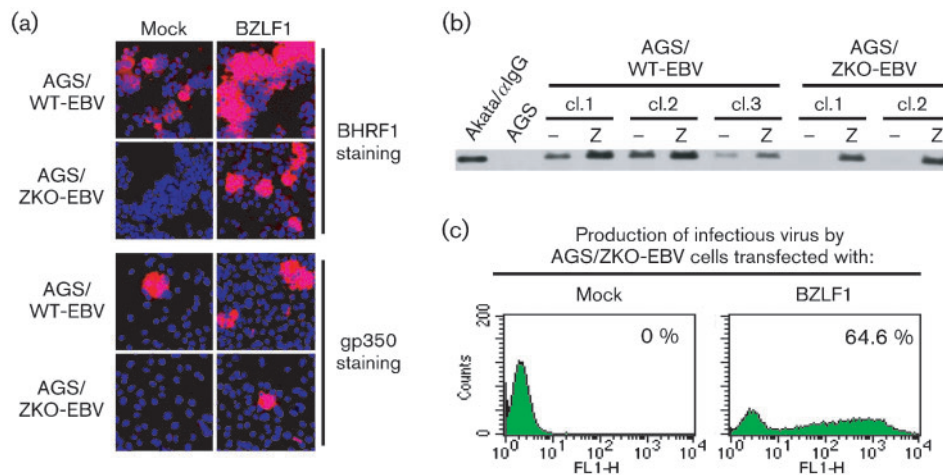


Fig. 3. BZLF1 transfection induces a large amount of virus production in AGS/ZKO-EBV cells. (a) The indicated AGS cell clones were transfected with pSG5-BZLF1 (BZLF1) or control vector (Mock). Immunofluorescence staining for BHRF1 and gp350 was performed 72 h later. (b) The indicated AGS cell clones were transfected with BZLF1 (Z) or control vector (-). Seventy-two hours later, genomic DNA was extracted and subjected to Southern blot analysis, using the *Eco*RI K fragment of Akata EBV as a probe. Akata/WT-EBV cells treated with anti-human IgG (Akata/ α IgG) were used as a positive control and EBV-negative AGS cells (AGS) were used as a negative control. (c) AGS/ZKO-EBV cells were transfected with pSG5-BZLF1 (BZLF1) or control vector (Mock). Seventy-two hours later, the virus solutions were harvested and used to infect EBV-negative Daudi cells. EGFP expression in Daudi cells is shown.

Endogenous BZLF1 has no role in efficient growth transformation of primary B cells

Next, we examined the role of BZLF1 in the transformation of B cells. We infected primary B cells with supernatants containing equal virus titres and examined the expression of BZLF1. BZLF1 was expressed in primary B cells early after infection with WT-EBV, but not with ZKO-EBV (Fig. 4a, b). Then, we performed a quantitative B-cell growth-transformation assay using ZKO-EBV and WT-EBV. Peripheral-blood B cells were infected with serial 10-fold dilutions of virus and plated in 96-well plates. The transforming dose ($TD_{50} ml^{-1}$) of virus was determined by counting the number of wells with proliferating lymphocytes 4 weeks after infection. As shown in Fig. 5, the transforming ability of ZKO-EBV was equivalent to that of WT-EBV. PCR analysis revealed that the wild-type BZLF1 gene was absent in the ZKO-EBV-infected LCLs, excluding the possibility that the ZKO-EBV genome recombined with the transfected BZLF1 plasmid to accomplish the equivalent transformation efficiency (data not shown). Thus, the data demonstrate definitively that BZLF1 has no effect on the efficiency of B-cell growth transformation.

Role of BZLF1 in the steady-state copy number of the EBV episomes in established LCLs

EBV-infected cells carry a single copy of the EBV genome per cell immediately after infection at low m.o.i., but established LCLs harbour multiple EBV episomes in each cell (Kieff & Rickinson, 2007). However, the mechanism of

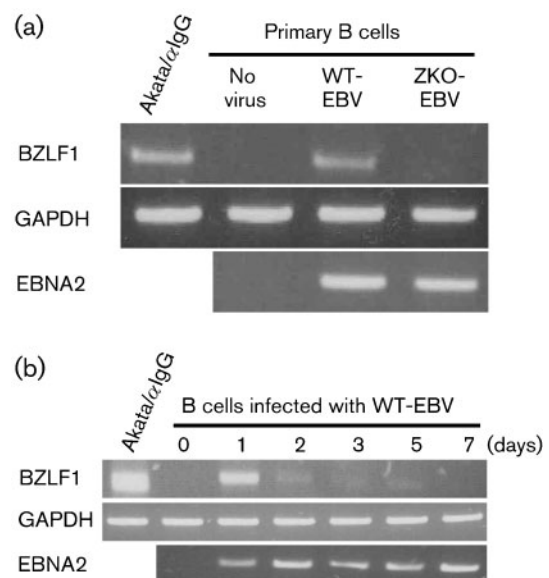


Fig. 4. BZLF1 expression in primary B cells early after EBV infection. (a) Primary B cells were infected with WT-EBV or ZKO-EBV. Twenty-four hours later, total RNA was extracted and expression of BZLF1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and EBNA2 mRNAs was examined by RT-PCR. (b) Primary B cells were infected with WT-EBV. Total RNA was extracted at the indicated time points and expression of BZLF1, GAPDH and EBNA2 mRNAs was examined by RT-PCR.

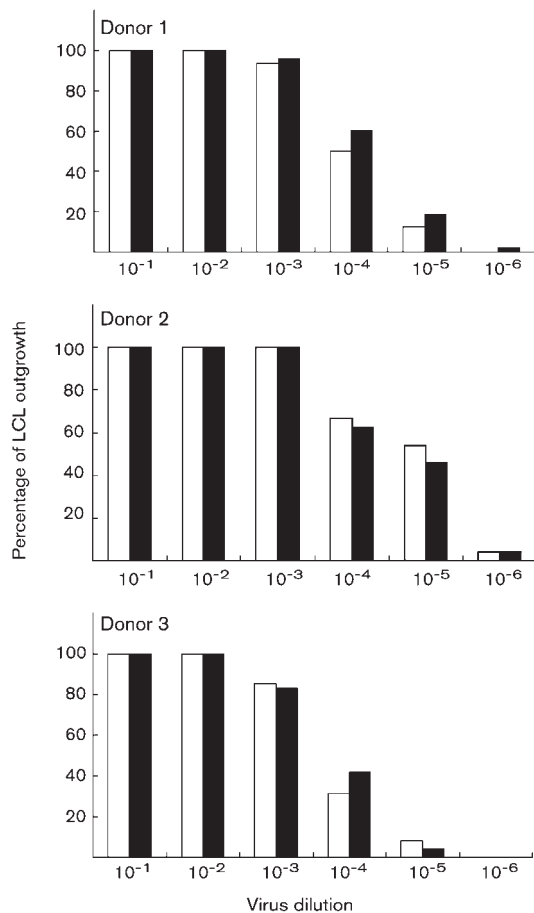


Fig. 5. Growth-transformation assay with primary B cells. Results of three independent experiments are given as the percentage of wells with outgrowth. Virus preparations with 9×10^5 GIU ml^{-1} were used. Empty bars, WT-EBV; filled bars, ZKO-EBV.

genome amplification is not known. We examined the role of BZLF1-induced lytic infection in EBV genome amplification. In this experiment, we used LCLs established at an m.o.i. of 0.1 to exclude the possibility that multiple infections resulted in harbouring multiple copies of the episome. The band intensities of the EBV genomes were similar between WT-EBV-infected and ZKO-EBV-infected LCL clones in a Southern blot (Fig. 6a). FISH analysis also showed that the distributions of the number of EBV genomes were similar between WT-EBV-infected and ZKO-EBV-infected LCLs (Fig. 6b, c). Thus, these data indicate that disrupting BZLF1 expression in the context of the EBV genome has little impact on the steady-state copy number of EBV episomes in established LCLs.

Role of BZLF1 in the growth of LCLs

ZKO-EBV-infected LCLs were indistinguishable from WT-EBV-infected LCLs in their expression pattern of viral latent genes (Fig. 7a, b). Next, we examined established LCLs for the expression of lytic genes. Western blot analysis

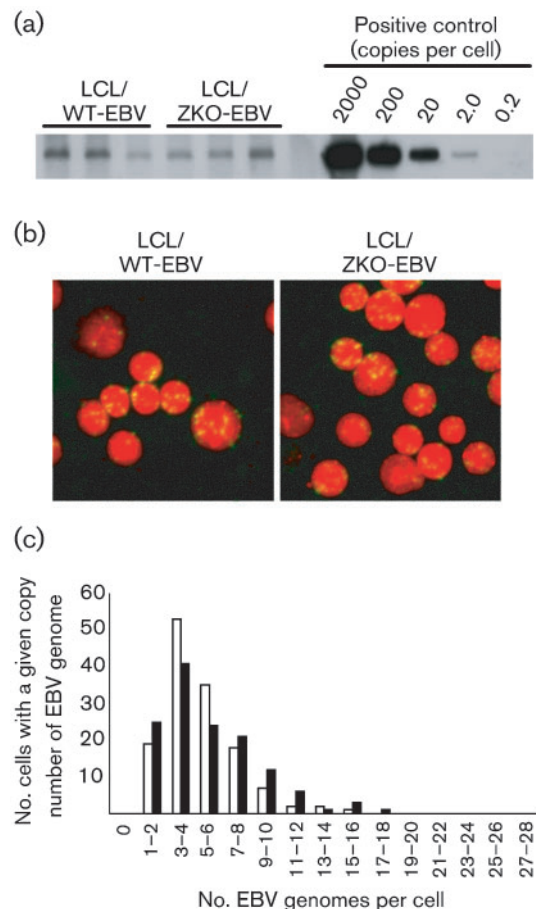


Fig. 6. WT-EBV-infected LCLs (LCL/WT-EBV) and ZKO-EBV-infected LCLs (LCL/ZKO-EBV) carry similar copy numbers of the EBV genome. (a) EBV copy-number estimation by Southern blot analysis. LCL/WT-EBV or LCL/ZKO-EBV was subjected to Southern blot analysis, using the *EcoRI* K fragment of Akata EBV as a probe. The copy-number controls, which were prepared from a plasmid containing the *EcoRI* K fragment, are also indicated. (b) Detection of EBV genomes by FISH analysis. An EBV genome-derived FISH probe was used for the analyses. Nuclei were counterstained with propidium iodide. (c) The number of EBV genomes per cell was distributed similarly in LCL/WT-EBV (empty bars) and LCL/ZKO-EBV (filled bars). The number of viral genomes per cell was measured with FISH.

showed that BZLF1 protein was expressed at a low level in WT-EBV-infected LCLs, but not in ZKO-EBV-infected LCLs (Fig. 7c). Cells positive for BHRF1 or gp350 were identified at a low frequency ($<0.3\%$) among WT-EBV-infected LCLs, but not among ZKO-EBV-infected LCLs (Fig. 7d), indicating that spontaneous lytic replication occurred in a small population of WT-EBV-infected LCLs. We compared the growth of established LCLs harbouring WT-EBV or ZKO-EBV *in vitro* within 4 months of EBV infection because long-term cultivation may change the growth characteristics of LCLs (Jones *et al.*, 2007b). The growth of WT-EBV-infected and ZKO-EBV-infected LCLs

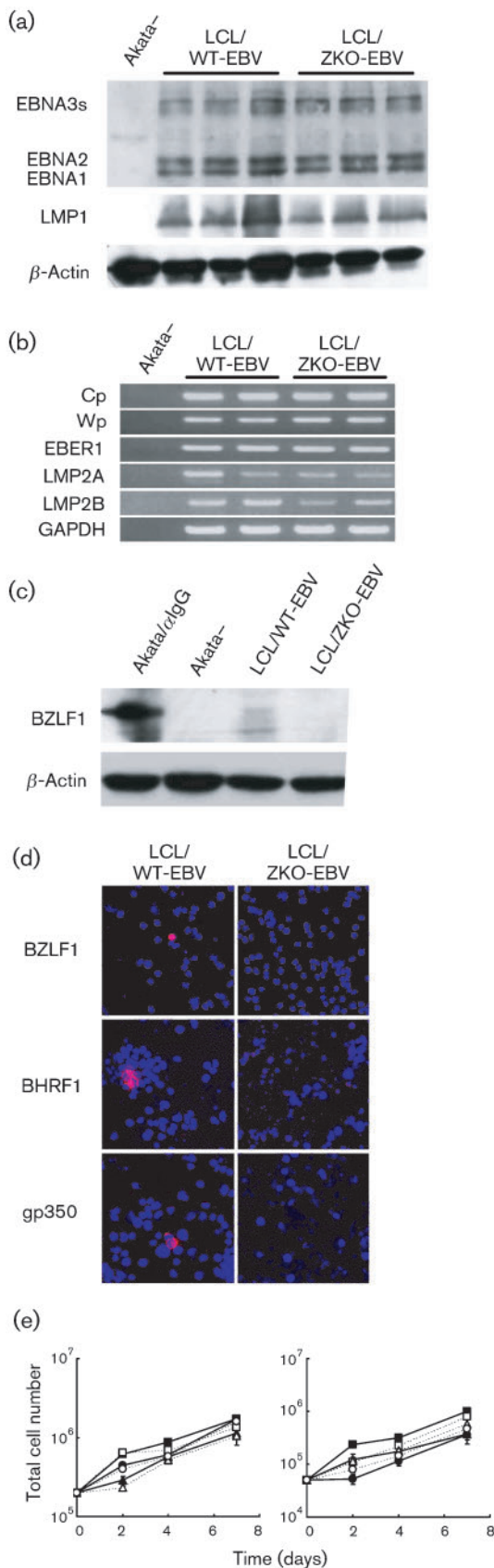


Fig. 7. EBV gene expression in LCL/WT-EBV or LCL/ZKO-EBV, and growth of the LCLs. (a) Immunoblotting was used to detect EBNA3s and LMP1. The results from EBV-negative Akata cells (Akata⁻) and the indicated LCLs are shown. (b) RT-PCR was used to detect other latent genes. The results from EBV-negative Akata cells and the indicated LCLs are shown. (c) Akata/WT-EBV cells treated with anti-human IgG (Akata/ α IgG), EBV-negative Akata cells and the indicated LCLs were subjected to immunoblotting to detect BZLF1. (d) The indicated LCLs were subjected to immunofluorescence staining to detect BZLF1, BHRF1 and gp350. (e) LCL/WT-EBV (three clones; filled symbols with solid lines) and LCLs/ZKO-EBV (three clones; open symbols with dotted lines) were plated at 2×10^5 cells ml⁻¹ (left panel) or 5×10^4 cells ml⁻¹ (right panel). Total viable cell numbers were calculated relative to the initial cultures. The experiment was done in duplicate. Error bars indicate SD.

was indistinguishable (Fig. 7e), which is consistent with previous reports (Feederle *et al.*, 2000; Hong *et al.*, 2005). Furthermore, ZKO-EBV-infected LCLs proliferated similarly to WT-EBV-infected LCLs when plated at low cell densities (Table 1). These data indicate that LCLs transformed by ZKO-EBV are unable to induce lytic gene expression, but are otherwise indistinguishable from LCLs transformed by WT-EBV in the latent infection state.

DISCUSSION

The present work was initiated by our recent finding that the BZLF1 is expressed as an immediate-early protein during transformation of primary B lymphocytes by EBV (Wen *et al.*, 2007). Until that time, BZLF1 was considered to be expressed only in the lytic phase of EBV infection. For the first time, our finding raised a possibility that BZLF1 might play a role in the transformation of B lymphocytes by EBV.

Although Feederle *et al.* (2000) reported that ZKO-EBV, which was generated from an EBV strain B95-8 bacterial artificial chromosome clone, was able to transform primary B cells, the only description of its transforming ability was 'To determine whether BZLF1 contributes to the immortalizing potential of EBV, virus stocks were produced after transfection of the *BZLF1* gene into 293-BZLF1-KO cells and used to infect primary B lymphocytes from the peripheral blood of a healthy donor. Four weeks after infecting 10^7 primary B cells, 20 cell clones grew up that expressed GFP. B cells from the same donor were also infected with supernatants from the B95.8 cell line. No difference in cell proliferation was noticed between the established cell lines generated with the BZLF1-KO virus or with B95.8 virus (data not shown)'. No quantitative evaluation of the transforming ability of ZKO-EBV in comparison with that of WT-EBV has been reported.

However, several lines of evidence have shown that BZLF1 possibly influences cell growth and survival through interactions with cellular proteins (Adamson & Kenney,

Table 1. Numbers of wells with proliferating cells after the plating of LCLs at low cell densities

LCL/EBV type	No. wells with indicated no. plated cells per well*				
	250	125	62.5	31.3	15.6
LCL/WT-EBV clone 1	16	16	13	6	0
LCL/WT-EBV clone 2	16	13	10	7	4
LCL/WT-EBV clone 3	5	1	1	0	0
LCL/ZKO-EBV clone 1	16	14	11	4	2
LCL/ZKO-EBV clone 2	14	9	6	3	0
LCL/ZKO-EBV clone 3	8	2	2	0	0

*Numbers of wells are given out of a total of 16 wells.

1999; Lieberman & Berk, 1991, 1994; Sinclair, 2003; Wu *et al.*, 2003; Zerby *et al.*, 1999; Zhang *et al.*, 1994). In addition, BZLF1 expression may affect cell growth by inducing lytic infection in cells harbouring EBV. EBV encodes a lytic viral homologue of IL-10, which could enhance the growth of B cells (Hsu *et al.*, 1990; Stuart *et al.*, 1995). Lytic infection also induces the expression of cellular IL-6 and IL-10 (Hong *et al.*, 2005; Jones *et al.*, 2007b; Mahot *et al.*, 2003), both of which could function as autocrine/paracrine growth factors for EBV-infected B cells (Beatty *et al.*, 1997; Scala *et al.*, 1990; Tosato *et al.*, 1990; Yokoi *et al.*, 1990). These previous reports, together with our recent finding that BZLF1 is expressed early after EBV infection (Wen *et al.*, 2007), raised the possibility that BZLF1 or the ability to induce lytic infection contributes to the efficient growth transformation of primary B cells. So, whether BZLF1 plays a role in EBV-induced B-lymphocyte transformation has been a big issue and the present findings provide a clear conclusion concerning the role of BZLF1 in EBV-induced transformation of B cells.

Hong *et al.* (2005) reported that ZKO-EBV-infected LCLs have impaired growth in SCID mice in comparison with WT-EBV-infected LCLs. More recently, the same group reported that IL-6 is produced from a small fraction of lytically infected LCLs and is critical for the growth advantage of WT-EBV-infected LCLs *in vivo* (Jones *et al.*, 2007b). However, in the present *in vitro* study, the growth of ZKO-EBV-infected LCLs and WT-EBV-infected LCLs was very similar under various conditions, which is consistent with data reported by Hong *et al.* (2005). Why the growth advantage of WT-EBV-infected LCLs over ZKO-EBV-infected LCLs was limited to *in vivo* situations is so far unclear. We are planning to examine the production of various cytokines, including IL-6, in our ZKO-EBV-infected and WT-EBV-infected LCLs, but further *in vivo* experiments will also be required to answer this question.

Another important finding reported in this manuscript is that BZLF1 does not play a role in gene amplification of EBV episomes in transformed lymphocytes. This also has been an important issue in EBV biology. In our study, we have demonstrated that neither BZLF1 nor the competence of lytic replication affects the copy number of EBV

episomes in established LCLs. When cells are infected with EBV at an m.o.i. much lower than 1, most infected cells are expected to carry one copy of the EBV genome per cell immediately after infection. However, established LCL cells usually carry multiple copies of the EBV episome in each cell. Therefore, it is thought that EBV episomes are amplified within infected cells during the establishment of LCLs (Sugden *et al.*, 1979). However, the molecular mechanism of the episome amplification has not been identified. It is possible that lytic replication may contribute to the amplification of EBV episomes during the establishment of LCLs. It has been reported that, in cells undergoing the lytic cycle, replication of EBV episomes in a theta-like mode precedes rolling-circle DNA replication, suggesting that the EBV episome can be replicated by the machinery used in lytic replication (Pfuller & Hammerschmidt, 1996). Thus, it is possible that BZLF1 expression in B cells early after infection can contribute to the amplification of EBV episomes. However, our data indicated that lytic-defective ZKO-EBV-infected LCLs have similar numbers of EBV episomes to WT-EBV-infected LCLs. Thus, episome amplification in LCL cells appears to be independent of lytic infection. Alternatively, unequal partitioning of episomes to daughter cells during mitosis may be the cause of episome amplification in LCL cells. It has been reported that unequal partitioning of EBV-derived plasmids actually occurs in a fraction of cells during mitosis, which yields a population of cells with different episome copy numbers (Nanbo *et al.*, 2007). If cells with multiple EBV episomes have a growth advantage over cells with a single EBV episome, cells with multiple EBV episomes would become dominant in the culture and, as a result, the mean number of EBV episomes per cell would increase and converge to an adequate number. As the growth of LCL cells is strictly dependent on the optimal expression of EBV genes, the unequal partitioning of episomes seems to be a plausible mechanism for episome amplification in LCL cells.

It has been reported that EBV preferentially causes lytic infection in some primary cell cultures other than B cells, such as epithelial cells, T lymphocytes and monocytes (Feederle *et al.*, 2007; Isobe *et al.*, 2004; Kelleher *et al.*, 1995; Savard *et al.*, 2000). The ability of EBV to induce lytic infection may

function as a barrier against establishing latent infection in these primary cell cultures. ZKO-EBV may therefore also be useful for establishing latent infection models and investigating the pathogenic roles of EBV in these cell lineages.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (K. T.).

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