A comprehensive library of mutations of Epstein–Barr virus

Ya-Fang Chiu,1 Chao-Ping Tung,1 Yu-Hisu Lee,1 Wen-Hung Wang,1 Ching Li,2 Jia-Yan Hung,3 Chen-Yu Wang,1 Yasushi Kawaguchi4 and Shih-Tung Liu1

1Molecular Genetics Laboratory, Department of Microbiology and Immunology, Chang Gung University, Taoyuan 333, Taiwan
2Department of Applied Microbiology, National Chiayi University, Chiayi City 600, Taiwan
3Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung 402, Taiwan
4Division of Viral Infection, Department of Infectious Disease Control, International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, 4–6–1 Shirokanedai, Minato-Ku, Tokyo 108–8639, Japan

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A mutant library of 249 mutants with mutations that span the entire Epstein–Barr virus (EBV) genome was generated by transposition with EZ :: TN <KAN-2> and insertion with an apramycin resistance gene by a PCR-targeting method. This study also demonstrates the feasibility of generating deletions and site-specific mutations in the BRLF1 promoter on the EBV genome to determine the regions in the promoter that are crucial to transcription. Analysing BZLF1 and BRLF1 mutants by microarray analysis revealed that these two genes regulate the transcription of EBV lytic genes differently. A BZLF1 mutation affects global expression of EBV lytic genes; almost no lytic gene is expressed by the mutant after lytic induction. However, although a BRLF1 mutant still transcribes most lytic genes, the expression of these lytic genes is inefficient. Furthermore, this study shows that the proximal Zta-response element in the BRLF1 promoter is crucial to BRLF1 transcription from the EBV genome, despite the fact that another work demonstrated that this site was unimportant in transient transfection analysis. Furthermore, mutants with a mutation in BDLF1 and BORF1 cannot assemble viral capsids. Results of this study demonstrate the usefulness of a comprehensive mutant library in genetic analyses of EBV.

INTRODUCTION

Mutational analyses underlie genetic investigations. These analyses are particularly powerful for a virus with a large genome, such as the Epstein–Barr virus (EBV). Although only a few EBV mutants have been available for study in the past, they have nonetheless yielded valuable information on the function of EBV genes. Many of these mutants have arisen spontaneously. For instance, BYRF1 and BALF2, which encode EBNA2 (Epstein–Barr virus nuclear antigen 2) and the ssDNA-binding protein, are deleted in strains P3HR1 and Raji, respectively. Work on these strains demonstrated the involvement of these genes in regulating the expression of latent membrane protein 1 (LMP1) and lytic DNA replication (Decaussin et al., 1995; Hatfull et al., 1988; Skare et al., 1985). EBV mutants can also be generated by inserting a drug-resistance gene at a specific target site by recombination in a cell line. For instance, a mutation in BZLF2, which encodes gp42, was generated by this approach. Analysis of the mutant revealed that gp42 is critical in the fusion of EBV to the host plasma membrane, and so affects the tropism and infectivity of EBV (Wang & Hutt-Fletcher, 1998). Another approach to generate EBV mutants involves a strain generated in the laboratory, called maxi-EBV (Delecluse et al., 1998). This EBV strain is obtained by inserting an F replicon into the EBV genome by recombination; this insertion influences neither the latent nor the lytic functions of the virus. Therefore, the EBV strain can be maintained and its genes can then be mutated by recA-dependent homologous recombination in Escherichia coli. Following mutagenesis, the mutant EBV is studied in B lymphocytes or 293 cells. Many mutants have been generated in this way and the impact of the mutations on EBV's life cycle and its host cell has been elucidated (Altmann et al., 2006; Chau et al., 2006; Chen et al., 2005; Collins et al., 2002; Delecluse et al., 1999; Dirmeier et al., 2003; Farina et al., 2005; Feederle & Delecluse, 2004;
et al., 2000, 2005, 2006; Grabusic et al., 2006; Hong et al., 2004; Humme et al., 2003; Hutchings et al., 2006; Janz et al., 2000; Kelly et al., 2005; Neuhierl & Delecluse, 2006). Feederle et al. (2000) examined a mutant that contained a mutated BRLF1 and demonstrated that the protein encoded by this gene, Rta, is critical to the expression of EBV late proteins, including gp110, VCA (viral capsid antigen) and gp350/220. Additionally, the mutant strain does not yield EBV particles (Feederle et al., 2000), revealing the importance of Rta in the lytic development of EBV. The same work also demonstrated that a mutant strain with a defective BZLF1, which encodes Zta, does not yield infectious EBV particles following lytic induction by Rta (Feederle et al., 2000), indicating that Zta is critical to the lytic development of EBV. A study of a mutation in BRLF1, which encodes gp350/220, demonstrated that the EBV infection of B lymphocytes may not depend on the binding of gp350/220 to CD21 (Janz et al., 2000). A mutation in BNLF1, which encodes LMP1, was found to influence the production of EBV (Ahsan et al., 2005; Kanda et al., 2004). A similar mutagenesis study also demonstrated the importance of Zta and the Zta-response element (ZRE) in the oriLyt in EBV lytic DNA replication (Feederle & Delecluse, 2004). A recent study showed that a mutation in BMRF1 affects EBV lytic DNA replication and lytic development (Neuhierl & Delecluse, 2006). A mutation in BRLF1 also allows EBV to infect epithelial cells (Shannon-Lowe et al., 2006). One limitation in the mutagenesis employed in these studies is that it is targeted to specific genes. Recently, a new method that uses bacterial transposons has been used to generate random insertions in the genome of Kaposi’s sarcoma-associated herpesvirus (KHSV) and murine gammaherpesvirus 68 (Song et al., 2005). Here, we have adopted two mutagenesis methods and established a comprehensive library of EBV mutants, which is useful in the genetic analysis of EBV.

METHODS

Bacterial strains, plasmids, transposon, EBV and cell lines. 293 is a human embryonic epithelial kidney cell line. 293-B95-8/F is a 293 cell line that contains maxi-EBV (Delecluse et al., 1998). 293, 293-B95-8/F cells and 293 cells that contained mutant EBV strains were cultured in Dulbecco’s modified Eagle’s medium that contained 10% fetal calf serum. E. coli EPI300 and transposon EZ::TN <KAN-2> were purchased from Epicentre. Plasmids pCMV-R and pCMV-Z were used to express Rta and Zta, respectively (Chang & Liu, 2000; Hung & Liu, 1999). BORF1 was amplified by PCR, using BORF1-F (5′-CCGGGATCCGGCGCTGATAGGCTTGAG-3′) and BORF1-R (5′-GGGCTGACGCTCCCTGCTCGTTTTCC-3′) as primers, and EBV DNA as a template. The fragment was inserted into the BamHI and XhoI sites of pcDNA3-HA to generate plasmid pH-A-BORF1. Plasmid pH-A-BDLF1 was obtained by inserting a BDLF1 fragment that was amplified with primers BDLF1-F (5′-CGGGAGATCT-TGGATTTGAAATGTGTA-3′) and BDLF1-R (5′-CCGGTCGAG-GTTATCATTAACCGAAGT-3′) into the EcoRI and XhoI sites of pcDNA3-HA. E. coli BW25113 is a host used for PCR targeting (Datsenko & Wanner, 2000; Gust et al., 2003). Plasmid pKD46 is temperature-sensitive and carried Z-RED recombinase genes that could be induced by culturing the bacteria in an arabinose-containing medium (Datsenko & Wanner, 2000; Gust et al., 2003). Plasmid pIJ773 (Datsenko & Wanner, 2000; Gust et al., 2003) was used as a template for amplifying an apramycin-resistance cassette for PCR targeting.

Isolating maxi-EBV DNA. Maxi-EBV was isolated from 293-B95-8/F cells using an alkaline-lysis method (Griffin et al., 1981) and transformed into E. coli EPI300 by electroporation (Sharma & Schimke, 1996). Transformants were selected on Luria–Bertani (LB) agar that contained 20 μg chloramphenicol ml⁻¹. The presence of maxi-EBV DNA in E. coli was examined by the alkaline-lysis method of Kado & Liu (1981).

Purifying EBV DNA from E. coli. Maxi-EBV and EBV mutant DNA were purified from E. coli EPI300 with a Midi plasmid purification kit (Qiagen) according to the manufacturer’s protocol, except in that 20 ml each of P1, P2 and P3 buffer was used.

Transposition. Maxi-EBV (1 μg) was incubated with 7 μg EZ::TN <KAN-2> and 1 U Tn5 transposase in transposition buffer (Epitcne) for 2 h at 37°C. One-tenth of the DNA in the mixture was employed to transform E. coli EPI300 by electroporation. Transformants were selected on LB agar that contained 50 μg kanamycin ml⁻¹ and 20 μg chloramphenicol ml⁻¹.

Mutating the EBV genome by PCR targeting. A PCR-targeting method (Datsenko & Wanner, 2000; Gust et al., 2003) was employed to insert an apramycin-resistance cassette into the maxi-EBV genome in E. coli BW25113. To accomplish this, a DNA fragment that contained an apramycin cassette was amplified by PCR, using pIJ773 (Datsenko & Wanner, 2000; Gust et al., 2003) as a template. The primers used in PCR included a sequence in the 3′ region that complements the end of the cassette and a 39 bp sequence in the 5′ region that complements the EBV target sequences (Supplementary Table S3). An amplified fragment was transformed by electroporation into E. coli BW25113(pKD46, maxi-EBV) that was pre-cultured at 30°C in LB broth containing 0.2% arabinose (Datsenko & Wanner, 2000; Gust et al., 2003). Subsequently, pKD46 was cured by culturing the cells at 37°C on LB agar. E. coli that contained mutated maxi-EBV was selected on LB agar that contained 50 μg apramycin ml⁻¹ and 20 μg chloramphenicol ml⁻¹. To mutate the three ZREs in the BRLF1 promoter in the EBV genome, an apramycin resistance gene that was amplified by PCR, using pIJ773 as a template, was inserted into the Dral site located at position –965 of the BRLF1 promoter in pRP (Chang & Liu, 2000). The three ZREs in the promoter at –251, –191 and –34 in the plasmid were subsequently mutated from 5′-TTCCGCA-3′ to 5′-GAATTCA-3′, 5′-TGACGCA-3′ to 5′-TGATCC-C-3′ and 5′-TGCGCATC-3′ to 5′-TGGATATC-3′, respectively, by site-directed mutagenesis (Ho et al., 1989). DNA fragments that contained the apramycin resistance gene and the entire BRLF1 promoter were amplified from the plasmids with primers GL2-R/Apr-F and GL2-R/Apr-R (Supplementary Table S3). The fragments were finally used to replace the BRLF1 promoter on the EBV genome by PCR targeting.

DNA sequencing. Mutations of the EBV genome were confirmed by DNA sequencing with a SequiTherm EXCEL II DNA sequencing kit-LC (Epitcne) and an automated DNA sequencer (model 4000L; LI-COR).

Transfection and selection. Approximately 9 × 10⁵ 293 cells were transfected with 2 μg EBV DNA with Lipofectamine 2000, according to the method recommended by the manufacturer (Invitrogen). Following transfection, cells were selected with a medium containing 200 μg hygromycin ml⁻¹ (Invitrogen) for
3 weeks. Transfection of plasmids was performed with a Bio-Rad Gene Pulser electroporator.

**Immunoblot analysis.** Proteins from $1 \times 10^7$ cells lytically induced for 24 h with 12-O-tetradecanoylphorbol-13-acetate (TPA; 30 ng ml$^{-1}$) and 3 mM sodium butyrate were extracted with 0.5 ml lysis buffer (63 mM Tris/HCl, pH 6.8, 2 % SDS, 0.0025 % bromophenol blue, 10 % glycerol and 50 mM EDTA). EBV virions were collected by ultracentrifugation 5 days after lytic induction of $1 \times 10^6$ cells. The pellet was suspended in 100 µl TNE buffer (0.01 M Tris/HCl, pH 7.5, 0.15 M NaCl and 1 mM EDTA) and an electrophoresis sample buffer (Sambrook et al., 1989) was added to extract viral capsid proteins. Following extraction, proteins were separated by SDS-PAGE (Sambrook et al., 1989) and transferred onto Immobilon-P membrane (Millipore). Antibodies against Rta and BclFI were purchased from Argene and Sigma, respectively. Anti-BORFI, anti-BDLFI and anti-BcLFI antibodies were generated in rabbit. Anti-EBNA1 antibody was obtained from Mei Chao (Chang Gung University, Taiwan). Protein bands were visualized using SuperSignal West Pico Chemiluminescent substrate (Pierce).

**Microarray analysis of the transcription of EBV genes.** The transcription of EBV genes was analysed by hybridization with an EBV DNA chip of 4.2 x 2.4 mm according to a method described elsewhere (Chang et al., 2003; Li et al., 2006). Total mRNA was purified from the cells using a Oligotex mRNA isolation kit (Qiagen). A cDNA hybridization probe was prepared by reverse transcription and hybridization was performed according to a method described elsewhere (Chang et al., 2003; Li et al., 2006).

**Isolation of EBV particles, sucrose-gradient sedimentation analysis and real-time PCR.** Cells (4 x 10^6) that contained maxi-EBV or its mutants were treated with 3 mM sodium butyrate and 30 ng TPA ml$^{-1}$ to induce the EBV lytic cycle. After 5 days of culturing, cell debris in the culture medium was removed by centrifugation at 6000 g for 7 min. The supernatant was then filtered through a 0.45 µm filter. EBV particles in the filtrate were pelleted by centrifugation at 25 000 g for 2 h. The pellet was suspended in 0.2 ml TNE buffer and subsequently treated with proteinase K and DNase I according to a method described elsewhere (Bloss & Sugden, 1994). In sucrose-gradient sedimentation analysis, the virus particle collected from the culture medium by ultracentrifugation was loaded onto a 20-60 % sucrose gradient that was prepared with a Gradient Station (Biocomp Instruments). To analyse the EBV capsids inside the cell, cells were lysed using a freeze-and-thaw procedure, which involved freeze-and-thaw four times for 3 min in liquid nitrogen and 3 min at 37 °C. The lysate was then subjected to sucrose-gradient centrifugation analysis. The gradient was centrifuged using a Beckman SW41Ti rotor at 25000 g and 4 °C for 2 h. EBV DNA in each fraction was extracted following a method described elsewhere (Wang et al., 2005). The amount of EBV genome was determined by real-time PCR using an iCyler iQ multicolor real-time PCR detection system (Bio-Rad) with primers and a probe that were specific to the BKRFI region (Ryan et al., 2004).

**RNA analysis.** RNA was isolated from cells with TRIzol (Invitrogen) according to the method suggested by the manufacturer. Reverse transcription was performed with random hexamers and M-MLV reverse transcriptase (Promega). Real-time PCR was performed using two BRLF1 primers, 5′- GAGCCCGTGGCC-CAAAG-3′ and 5′- GTGTAGTGGTGGCCGAGTC-3′. The probe sequence for the amplified BRLF1 region was 5′- (6FAM) GGTT-ACAGCCGTTGCCGACA (TAMRA)-3′. A fragment was also amplified from BKRFI and quantified by real-time PCR (Ryan et al., 2004), which was used as a control to normalize the amplification results.

### RESULTS

**Generating EBV mutants with EZ::TN <KAN-2>**

Maxi-EBV DNA was mutated with EZ::TN <KAN-2> in vitro. After transposition, one-tenth of the reaction, which contained 0.1 µg maxi-EBV DNA, was transformed into E. coli EPI300. Maxi-EBV that contained an EZ::TN <KAN-2> insertion was selected by plating the transformants on LB agar that contained chloramphenicol and kanamycin. The transformation yielded around 5000 drug-resistant colonies. Screening 100 colonies by the alkaline lysis method of Kado & Liu (1981) revealed the presence of a plasmid of about 170 kb, which was consistent with the size of maxi-EBV DNA. Insertion of the transposon into the genome in these EBV mutants was verified by Southern blotting with an EZ::TN <KAN-2> probe (data not shown) and DNA sequencing. The integrity of the DNA from 210 mutants, which contained a single transposon insertion, was confirmed by BamHI and XhoI digestion (data not shown). Forty-four mutants with a transposon inserted in the terminal repeats, W-repeats and oriLyt repeats were also isolated. Analysis of these mutants demonstrated that 60 of the 93 annotated EBV genes were mutated (Fig. 1; Supplementary Table S1).

**Mutating EBV genes by PCR targeting**

Although transposon insertion yielded 60 mutants, screening for mutants that contain a mutation in the remaining 33 genes on the EBV genome from a large pool of mutants can be difficult. Therefore, a PCR-targeting method (Datsenko & Wanner, 2000; Gust et al., 2003) was utilized to insert an apramycin resistance gene into a specific site on the EBV genome to complete a comprehensive mutant library. Accordingly, 39 additional mutants were generated (Fig. 1; Supplementary Table S1). Furthermore, DNA from the mutants was digested using BamHI, XhoI and KpnI; the restriction profiles were compared with those from maxi-EBV to determine the integrity of the DNA.

**Transcription of EBV genes by BRLF1 and BZLF1 mutants**

Analysis of two mutant strains, MI-270 and D-26, that contained mutated BRLF1 and BZLF1, respectively, revealed that the mutations affected the expression of Rta, Zta, EA-D, gp350/220 and the production of EBV particles (Supplementary Fig. S1). Furthermore, the functions of these two genes could be genetically complemented by transfecting plasmids that expressed Rta and Zta (Supplementary Fig. S1). To further investigate how these two mutations influenced the overall transcription of EBV genes, a microarray study was subsequently performed with an EBV microarray chip. For hybridization, mRNA purified from cells was reverse transcribed, labelled with biotin and used as a probe. The hybridization results indicated that lytic genes were not expressed or

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expressed at low levels by maxi-EBV during latency (Fig. 2a; Supplementary Table S2). Expression of EBV lytic genes was significantly enhanced after lytic induction with TPA and sodium butyrate (Fig. 2d; Supplementary Table S2). In the case of a BZLF1 mutant, D-26, almost no lytic genes were expressed after TPA and sodium butyrate treatment (Fig. 2e; Supplementary Table S2). Meanwhile, transfecting pCMV-Z restored the expression of these lytic genes.

Fig. 1. Location of EZ:::TN <KAN-2> and the apramycin-resistance cassette insertions in the EBV genome (GenBank accession no. AJ507799). ○: EZ:::TN <KAN-2> insertion; ◯, apramycin-resistance cassette insertion; grey arrow, EBV genes; white box, deletion in different EBV strains; TR1–TR4, terminal repeats.
Although Sinclair the function of one of these elements, ZRE1 (Fig. 3a). Transient transfection studies yielded conflicting results on promoter (Rp) is known to contain three ZREs. Earlier considerably from those on the EBV genome. The number of the promoter in a reporter plasmid that is not truly reflect how a gene is transcribed from the EBV system, although useful, may have its limitations and may using a transient transfection assay. However, the assay production of EBV particles transcription of

\[ \text{Fig. 2. Analysis of the transcription of EBV genes. mRNA was prepared from 293 cells that contained maxi-EBV (a, d), a BZLF1 mutant (D-26) (b, e, g) and a BRLF1 mutant (MI-270) (c, f, h) after the cells were treated with TPA and sodium butyrate (d–f), transfected with pCMV-Z (g) or pCMV-R (h) for 24 h. Control dots are in the upper-right-hand corner between columns 1 and 4 and rows a and d. Spot 1b contains a DNA fragment from a plant gene, GA4; during hybridization, a biotin-labelled probe that is specific for this spot was added and used as an external control to monitor the hybridization process. Spot 2c contains a DNA fragment from a plant gene, rca. The mRNA of rca was added during reverse transcription and used to monitor reverse transcription. Spot 2b contained a GAPDH fragment and was used as an internal control. Supplementary Table S2 states the EBV genes that are contained by each spot.} 

Fig. 2g; Supplementary Table S2). Unlike mutant D-26, mutant MI-270 expressed most of the lytic genes after lytic induction, but at reduced levels (Fig. 2f; Supplementary Table S2). The expression was elevated after pCMV-R was transfected (Fig. 2h; Supplementary Table S2).

Effect of mutations in the BRLF1 promoter on the transcription of BRLF1, expression of Rta and production of EBV particles

Functions of an EBV promoter were frequently studied by using a transient transfection assay. However, the assay system, although useful, may have its limitations and may not truly reflect how a gene is transcribed from the EBV genome, because the chromatin structure and copy number of the promoter in a reporter plasmid that is analysed in transient transfection assays may differ considerably from those on the EBV genome. The BRLF1 promoter (Rp) is known to contain three ZREs. Earlier transient transfection studies yielded conflicting results on the function of one of these elements, ZRE1 (Fig. 3a). Although Sinclair et al. (1991) demonstrated the importance of ZRE1 in the transcription of BRLF1, Bhende et al. (2004) showed that this site was unimportant. Therefore, in this study we deleted Rp and also mutated the three ZREs in Rp on the EBV genome (Fig. 3a) to examine how these mutations affected BRLF1 transcription. Meanwhile, an EBV strain with an inserted apramycin-resistance gene at position −965 in Rp (EB-Apr) (Fig. 3a) was used as a control. A separate set of mutants was also generated in independent experiments to confirm the reproducibility of the results. Real-time PCR revealed that inserting an apramycin-resistance gene at position −965 (EB-Apr) did not influence the transcription of BRLF1; the amount of BRLF1 mRNA expressed by EB-Apr was about equal to that exhibited by maxi-EBV (Fig. 3). Deleting the region between positions −965 and −852 in the BRLF1 promoter (Fig. 3a, R-852) reduced the level of BRLF1 mRNA by 27.7 % after lytic induction with TPA and sodium butyrate (Fig. 3b), indicating the importance of this region in BRLF1 transcription. Deleting the promoter from position −965 to −388 (Fig. 3a, R-388) and −279 (Fig. 3a, R-279) further reduced the expression to a level about 42.8–52 % of that exhibited by EB-Apr (Fig. 3b). However, a deletion extended to the region that contained the two upstream ZRE sites, from position −965 to −187 (Fig. 3a, R-187), reduced the expression of BRLF1 mRNA to 21 % of that of EB-Apr (Fig. 3b). Deleting the region between positions −965 and −56 (Fig. 3a, R-56), which yielded a promoter that contained only an Sp1 site and a ZRE (ZRE1) (Fig. 3a), almost totally abolished the transcription and reduced the expression to 14 % of EB-Apr (Fig. 3b). Deleting the region between positions −965 and −42, which resulted in a fragment that contained only one ZRE (ZRE1) (Fig. 3a, R-42) lowered the transcription to 8.4 % of EB-Apr (Fig. 3b). Furthermore, this study demonstrated that activation of BRLF1 transcription by the transfection of pCMV-Z was more efficient than that activated by TPA and sodium butyrate treatment; the amount of mRNA transcribed by EB-Apr after pCMV-Z transfection was nearly sixfold higher than that expressed after TPA and sodium butyrate treatment. The amount of Zta expressed appeared crucial to the transcription of BRLF1 from the R-42 mutant. Although the amount of BRLF1 mRNA transcribed from the R-42 mutant was low after TPA and sodium butyrate treatment (Fig. 3b), the transcription was elevated to a level comparable to that exhibited by the mutant strain containing three ZREs after pCMV-Z was transfected (Fig. 3c, R-279). Additionally, transfecting pCMV-Z did not fully activate BRLF1 transcription if the region between positions −965 and −852 was deleted (Fig. 3c, R-852). Furthermore, immunoblot analysis revealed that decreased BRLF1 transcription by the mutants also lowered the amount of Rta expressed. R-852 and R-388 mutants produced Rta at a level lower than that of EB-Apr, but higher than those expressed by mutants R-279 and R-187 (Fig. 3d). Meanwhile, mutants R-56 and R-42 produced Rta at levels lower than that expressed by R-279 and R-187 (Fig. 3d). Additionally, the R-852 mutant reduced the production of EBV particles by 64 %; R-388 and R-279 mutants, 74–79 %; R-187, R-56 and R-42 mutants, more than 90 % (Fig. 3e). These results indicated that a slight decrease in BRLF1 transcription may significantly affect the viral production. The three ZREs in Rp on the EBV genome were also mutated in this study (Fig. 3a). Analysis
Fig. 3. Mutations in Rp and the influence of the mutations on the transcription of BRLF1, expression of Rta and production of EBV particles. Mutations in Rp were generated by the PCR-targeting method (a). NF1, Sp1, YY1, Zif and ZRE denote NF1-, Sp1-, YY1-, Zif268- and Zta-binding sites. An Sp1 site at position −500 is not shown in the figure. The BRLF1 mRNA in 293 cells was determined by real-time PCR following lytic induction using TPA and sodium butyrate (b) or by transfecting with pCMV-Z (c). Rta that was expressed by the mutants was analysed by immunoblotting (d). EBV particles were collected from the culture medium by centrifugation. The EBV DNA in the viral particles was measured by real-time PCR (e). Transcription of the BRLF1 mRNA (f) and production of EBV particles (g) by three ZRE mutants, mZRE1, mZRE2 and mZRE3, in the BRLF1 promoter were also determined. Meanwhile, amounts of BRLF1 mRNA were normalized with that of EBNA1 mRNA.
of the BRLF1 mRNA transcribed by these mutants demonstrated that mutating one of the two upstream ZREs, ZRE2 and ZRE3, lowered the BRLF1 transcription by 58–63% and virus production by approximately 84% after lytic induction with TPA and sodium butyrate. Additionally, mutating ZRE1 also decreased the BRLF1 transcription by 42.8% (Fig. 3f) and decreased virus production by 76% (Fig. 3g).

**BDLF1 and BORF1 mutations**

In this work, two mutants, MI-403 and MI-213 (Supplementary Table S1), which contained a mutated BDLF1 and BORF1, respectively, were analysed to determine how these mutations affected capsid assembly. Immunoblot analysis of the proteins in the pellet fraction from the cells that contained maxi-EBV revealed the presence of the BcLF1 (VCA), BORF1 and BDLF1 proteins (Fig. 4a). Additionally, the presence of EBV in the pellet fraction was also verified by real-time PCR (Fig. 4b). Sucrose-gradient centrifugation analysis established that EBV particles generated by maxi-EBV sedimented near the bottom of the gradient (Fig. 4c). Meanwhile, viral capsids assembled by maxi-EBV were also detected within the cell (Fig. 4d). On the other hand, mutants MI-213 and MI-403 did not seem to release EBV capsids into the medium, because immunoblot analysis failed to detect the VCA, BDLF1 and BORF1 proteins in the pellet fractions from the culture medium (Fig. 4a). Meanwhile, assembled EBV capsids, both inside and outside the cell, were undetected by sucrose-gradient sedimentation (Fig. 4c, d). Additionally, transfecting plasmids pHA-BORF1 and pHA-BDLF1 that express BORF1 and BDLF1, respectively, into the cells that contained MI-213 and MI-403, neither complemented the mutations nor restored virus production after lytic induction (Fig. 4e).

**DISCUSSION**

This investigation employed EZ::TN <KAN-2> and generated about 50 000 EBV mutants in vitro using 1 μg maxi-EBV DNA. These mutants can be screened randomly for mutations associated with a particular phenotype, but screening them indiscriminately is impractical. Therefore, a mutant library was established to facilitate genetic studies. To establish such a mutant library, mutants were randomly selected from a mutant pool and the location of the insertions was determined by DNA sequencing. Multiple insertions of the transposon into the EBV genome were occasionally found, probably owing to a lack of transposition immunity of EZ::TN <KAN-2>; these mutants were later eliminated. Finally, sequencing analysis revealed that among the 93 annotated EBV genes, 60 were mutated. Since identifying a mutation in the remaining 33 genes that lack a transposon insertion in a large pool of mutants was time-consuming, a PCR-targeting method (Datsenko & Wanner, 2000; Gust et al., 2003) was subsequently used to mutate particular genes on the EBV genome. This approach efficiently mutates the EBV genome. The recombination method and transposon mutagenesis generated an EBV mutant library that contains 249 mutants (Fig. 1; Supplementary Table S1). The λ-RED recombinase system is known to promote recombination between a linear double-stranded DNA and its target DNA (Murphy, 1998). This fact may explain why the frequency of deletion caused by intra-DNA recombination following PCR targeting is not particularly high. The integrity of the EBV genome was examined using restriction digestion following transposon mutagenesis and PCR targeting; the mutants that contained a detectable deletion were thus
eliminated. However, given the limitation on restriction analysis, detecting small deletions on the EBV genome may be difficult. Since obtaining EBV mutants by PCR targeting does not involve a complex procedure, generating several mutants with the same mutation from independent experiments is relatively easy to achieve. Studying these mutants should yield results that reflect the functions of a mutated gene.

As is generally known, the expression of Rta and Zta is critical to the transcription of EBV lytic genes. This work demonstrates that many lytic genes are expressed at low levels by maxi-EBV under latent conditions (Fig. 2a and Supplementary Table S2), revealing that EBV lytic genes may be expressed in epithelial cells because of the constitutive expression of BRLF1 in epithelial cells (Zalani et al., 1992). On the other hand, lytic genes are no longer expressed after BZLF1 and BRLF1 are mutated (Fig. 2b, c: Supplementary Table S2), showing the importance of these two genes in activating the EBV lytic genes. Meanwhile, two spots containing the sequences of BCRF2/EBNA-LP/BWRF1 (Fig. 2a and Supplementary Table S2, spot 2g) and BYRF1 (EBNA2) (Fig. 2a and Supplementary Table S2, spot 2h) are transcribed at a higher level during latency (Fig. 2a and Supplementary Table S2). However, lytic induction reduces the transcription of these transcripts (Fig. 2d and Supplementary Table S2). Meanwhile, TPA and sodium butyrate treatment for 1 day increases the intensity of the EBER dot (Fig. 2d and Supplementary Table S2, spot 1h). These observations are inconsistent with what was observed in Akata cells, in which expression of EBNA-LP and BYRF1 (EBNA2) increase and the expression of EBERs was unchanged after lytic induction by anti-IgG (Yuan et al., 2006). Our results further demonstrate that a BZLF1 mutation represses the expression of EBV genes; nearly no lytic gene is expressed by the mutant (Fig. 2b and Supplementary Table S2) and no virus particles are produced (Supplementary Fig. S1). Therefore, BZLF1 is necessary to activate the viral lytic cycle. However, in this study, we also found that numbers of lytic genes are not fully expressed without Rta (Fig. 2f; Supplementary Table S2), and production of mature virion decreased by more than 90 % (Supplementary Fig. S1); indicating that Rta is also crucial to EBV production. Thus, we conclude that without Rta, transcription of most EBV lytic genes is inefficient. Our recent study demonstrated that, rather than directly binding to Rta-response elements to activate transcription, Rta often interacts with MCAF1 to enhance Sp1-mediated transcription (Chang et al., 2005). Rta also interacts with MCAF1 to enhance transcription mediated by transcription factors of the bZip family, including AP-1, ATF1/2 and Zta (L.-K. Chang & S.-T. Liu, unpublished results), implying that Rta often functions as a transcription co-activator to activate EBV lytic genes. The observations above may explain why EBV lytic genes are not fully expressed without Rta. Additionally, transcription of several crucial EBV lytic genes depends on Rta (Lu et al., 2006), explaining why a mutation in BRLF1 disrupts the EBV lytic cycle and why mutant MI-270 cannot generate EBV particles (Supplementary Fig. S1d). Moreover, according to our results, transfecting mutant MI-270 with pCMV-R does not enhance the transcription of BcLF1 (VCA) (Fig. 2h and Supplementary Table S2, spots 9c, 9d, 9e, 9f), BRLF1 (gp350/220) (Fig. 2h and Supplementary Table S2, spot 6e, 3f) and BALF4 (gp110) (Fig. 2h and Supplementary Table S2, spots 11c, 11d). This analysis was performed using mRNA isolated from cells induced for the lytic cycle for 24 h. Accordingly, an extended lytic induction period may be necessary to observe how Rta affects the transcription of these genes. As is generally known, the behaviour of EBV in epithelial cells differs from that in B lymphocytes, which may explain why a recent work found that many lytic genes, including BcLF1 (VCA), BRLF1 (gp350/220) and BALF4 (gp110), are fully expressed in Akata cells within 24 h of lytic induction by anti-IgG (Yuan et al., 2006), indicating that cell types and the methods used for lytic induction may influence the timing of the transcription of lytic genes. Notably, several dots on the microarray chip used in this study may contain both latent and lytic genes (Supplementary Table S2). However, the dots that contain only EBV lytic genes are informative to the activation of EBV lytic genes by Rta and Zta.

The BRLF1 promoter contains three ZREs. However, two studies that used transient transfection analysis yielded different results on the function of ZRE1 (Fig. 3a). Sinclair et al. (1991) found that mutating ZRE1 decreased the reporter activity by about 85 %. However, Bhende et al. (2004) found that Zta preferentially binds to methylated ZRE2 and ZRE3 after lytic induction, and that ZRE1 plays small roles in the activation of BRLF1 transcription. To further elucidate the functions of these ZREs in Rp, we mutated these sites on the EBV genome, and demonstrated that a ZRE1 mutation decreases BRLF1 transcription by about 43 % (Fig. 3g). This finding is inconsistent with the observation made by Bhende et al. (2004) and suggests that ZRE1 is important. This anomaly cannot be attributed to Rp methylation, as suggested by Bhende et al. (2004), since the Rp in maxi-EBV is hypermethylated in 293 cells (Bhende et al., 2005); our own study also shows that nearly all the CpG sequences in the promoter are methylated during viral latency. Furthermore, an upstream region in the BRLF1 promoter between positions −965 and −852 is crucial to the activation by Zta; without this fragment, the promoter cannot be fully activated by pCMV-Z (Fig. 3c, R-850). These experiments also demonstrate the feasibility of generating site-specific mutations in a promoter on the EBV genome to analyse the promoter function.

This work demonstrates that a mutation in BDLF1 and BORF1 yields strains that cannot assemble EBV capsids in the cell (Fig. 4c) or yield viral particles (Fig. 4a). Additionally, BDLF1 and BORF1 proteins are 17.7 and 19 % homologous with UL18 and UL38 proteins, two minor capsid proteins from HSV-1, indicating that BDLF1 and BORF1 proteins are components of the EBV capsid.
Furthermore, this study finds that transfecting a plasmid that overexpresses the two minor capsid proteins into the mutant strains cannot genetically complement the mutations (Fig. 4c). This result is not completely surprising because our recent work revealed that the BDLF1 and BORF1 proteins interact not only with each other but also with VCA (W.-H. Wang & S.-T. Liu, unpublished results). Therefore, when one of the two minor capsid proteins becomes more abundant in the cells, the interaction between overexpressed proteins and VCA or the other minor capsid protein is preferred and the formation of a complex that contains VCA, BORF1 and BDLF1 proteins is actually prevented. This investigation also reveals an intrinsic problem of the techniques adopted herein: revertants are difficult to obtain to verify the mutational effects. Therefore, mutant strains which contain the same mutation, generated from independent experiments, must be investigated to confirm the experimental results. This study demonstrates the usefulness of a comprehensive library of EBV mutants, likely to be invaluable in EBV research.

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