Enhancement of Zta-activated lytic transcription of Epstein–Barr virus by Ku80

Chien-Chang Chen,1 Ya-Chun Yang,1 Wen-Hung Wang,2 Chien-Sin Chen1 and Li-Kwan Chang1

1Department of Biochemical Science and Technology, College of Life Science, National Taiwan University, Taipei 106, Taiwan, ROC

2Graduate Institute of Biomedical Sciences, Chang-Gung University, Kwei-Shan, Taoyuan 333, Taiwan, ROC

Zta, encoded by the BZLF1 gene of Epstein–Barr virus (EBV), is a transcription factor that is expressed during the immediate–early stage of the lytic cycle. The expression of Zta is crucial to viral lytic development. Earlier studies showed that Ku80 is a binding partner of Zta in ZKO-293 cells and is co-purified with Zta. This study verifies the interaction between Ku80 and Zta by using glutathione S-transferase-pull-down and co-immunoprecipitation assays, and also by indirect immunofluorescence analysis. This investigation also reveals that Ku80 binds to Zta on Zta-response elements in the BHLF1 promoter, enhancing the promoter activity. This study also reveals that the interaction between Zta and Ku80 involves the C-terminal region of Zta and the 425 aa N-terminal region of Ku80. The interaction between these two proteins and the enhancement of transcription that is activated by Zta suggest that Ku80 is important to EBV lytic development.

INTRODUCTION

When Epstein–Barr virus (EBV) enters the lytic cycle, the virus expresses two transcription factors, Rta and Zta (R transactivator and Z transactivator), which are encoded by the BRLF1 and BZLF1 genes, respectively, to activate the transcription of the EBV lytic genes (Speck et al., 1997). Zta is a transcription factor of the bZIP family (Farrell et al., 1989), which activates EBV lytic genes via binding to Zta-response elements (ZRE) in promoters (Lieberman & Berk, 1990). Such binding autoregulates the transcription of its own gene, BZLF1 (Flemington & Speck, 1990), and promotes the transcription of another immediate–early gene, BRLF1 (Sinclair et al., 1991). Zta also binds to the ZRE in the origin of lytic replication (oriLyt), which is required for the initiation of lytic DNA replication (Schepers et al., 1993). Additionally, Zta interacts with c-Myb, transcription factor IID (TFIID) and cAMP response element (CREB)-binding protein to activate the expression of EBV early genes (Adamson & Kenney, 1999; Kenney et al., 1992; Lieberman & Berk, 1991). Since the transcription of many EBV lytic genes depends on Zta, EBV cannot complete its lytic cycle without Zta (Chiu et al., 2007; Feederle et al., 2000).

Ku, a heterodimeric protein that comprises 70 kDa (Ku70) and 80 kDa (Ku80) subunits, is an autoantigen, which is recognized by the sera of patients with autoimmune disorders (Mimori et al., 1981). The Ku heterodimer is a component of the DNA-dependent protein kinase (DNA–PK) complex, on which the non-homologous end-joining repair of DNA double-strand breaks (DSB) depends (Gottlieb & Jackson, 1993). In addition to having a well-documented role in DSB repair, Ku maintains telomere integrity and promotes apoptosis, V(D)J recombination and DNA replication (Chai et al., 2002; Nussenzweig et al., 1996; Pergola et al., 1993; Tuteja & Tuteja, 2000). Ku also regulates transcription (Hoff & Jacob, 1993; Kuhn et al., 1993; Niu & Jacob, 1994; Nolens et al., 2009; Ohno et al., 2009). Earlier studies revealed that Ku recruits DNA–PK to negative regulatory element 1 in LTR to inhibit mouse mammary tumor virus transcription (Giffin et al., 1996). Ku also binds to specific sequences in promoters to activate the transcription of the genes that encode transferrin receptor (Roberts et al., 1994), collagen III protein (Giampuzzi et al., 2000), the U1 snRNA (Knuth et al., 1990) and c-Myc (Giffin et al., 1997). However, no general consensus recognition sequence has emerged from these studies. In addition, a number of investigations have indicated that Ku regulates transcription without its directly binding to a promoter sequence. For example, Ku in the DNA–PK complex interacts with and promotes phosphorylation of RNA polymerase II (RNAP II) (Dvir et al., 1992). Ku also interacts with a set of proteins involved in transcriptional reinitiation and with the elongation complex to enhance transcription (Mo & Dynan, 2002; Woodard et al., 2001). Also, Ku is a co-activator of androgen receptor (AR) and thereby enhances AR-dependent transcription (Mayeur et al., 2005). An earlier immunopurification study revealed that Ku80 is a binding partner of Zta (Wiedmer et al., 2008). Furthermore, Ku80 was co-purified with Ku80.
with Zta by using a global tandem affinity purification approach (Bailey et al., 2009). These studies suggested that Ku80 interacts with Zta. This work confirms the interaction between Ku80 and Zta and demonstrates that the interaction promotes Zta-activated transcription.

RESULTS

Analysing the interaction between Zta and Ku80

Earlier studies performed MALDI–TOF mass spectrometric analysis and demonstrated that Ku80 was co-purified with Zta from ZKO-293 and HEK cells (Bailey et al., 2009; Wiedmer et al., 2008), suggesting that these two proteins interact. Our studies also confirmed that Ku80 co-purified with histidine-tagged Zta by using Ni²⁺-charged beads (Qiagen) and MALDI–TOF mass spectrometry (data not shown). Therefore, this study performed glutathione S-transferase (GST)-pull-down assays using bacterially expressed proteins to verify this interaction in vitro. To achieve this, GST and GST–Zta fusion protein bound to glutathione–Sepharose beads were added to a solution that contained His–Ku80 fusion protein. Immunoblot analysis using anti-Ku80 antibody revealed that GST–Zta–glutathione–Sepharose beads (Fig. 1, lane 3) but not GST–glutathione–Sepharose beads (Fig. 1, lane 2) pulled down His–Ku80. A similar experiment also revealed that GST–Ku80–glutathione–Sepharose beads (Fig. 1, lane 6) but not GST–glutathione–Sepharose beads (Fig. 1, lane 5) pulled down the His–Zta fusion protein. These results show that Zta and Ku80 interact in vitro.

Co-immunoprecipitation of Zta and Ku80

An immunoprecipitation study was conducted using the lysate from P3HR1 cells that had been treated with TPA and sodium butyrate for 24 h. Immunoblotting revealed that Zta in the cell lysate was immunoprecipitated by anti-Zta antibody (Fig. 2, lane 4) and co-immunoprecipitated with Ku80 when anti-Ku80 antibody was used (Fig. 2, lane 3). A similar experiment revealed that Ku80 was immunoprecipitated by anti-Ku80 antibody and co-immunoprecipitated with Zta when anti-Zta antibody was used (Fig. 2, lanes 7 and 8), elucidating the interaction between Zta and Ku80 in vivo. A parallel experiment showed that anti-IgG antibody did not immunoprecipitate Zta and Ku80 (Fig. 2, lanes 2 and 6).

Colocalization of Ku80 and Zta

P3HR1 cells were treated with TPA and sodium butyrate to induce the EBV lytic cycle and the expression of Zta. A confocal microscopic study revealed that Zta colocalized with Ku80 in the nucleus (Fig. 3d). The expression of Zta and its colocalization with Ku80 was not observed when P3HR1 cells were not treated with TPA and sodium butyrate (Fig. 3f).

Mapping interaction domains in Zta and Ku80

A deletion study was performed to delineate the region in Zta that interacts with Ku80. Plasmids that encoded GFP fused with full-length (GFP–Zta), N-terminal region (GFP–ZN), middle region (GFP–ZM) and C-terminal region (GFP–ZC) Zta (Fig. 4a) were transfected into P3HR1 cells. An empty vector, pEGFP-C1, that expresses GFP was also transfected as a negative control. Immunoblot analysis revealed that GFP–Zta and GFP–ZC were present in the lysate and that they co-immunoprecipitated with Ku80 when anti-Ku80 antibody was used (Fig. 4b, lanes 7 and 10). GFP, GFP–ZN and GFP–ZM, although detectable in the lysate (Fig. 4b, lanes 1, 3, 4), were not co-immunoprecipitated by anti-Ku80 antibody (Fig. 4b, lanes 6, 8, 9), showing that the region between
amino acids 168 and 245 of Zta interacts with Ku80 (Fig. 4a). This region contains the DNA-binding and dimerization domains (Lieberman & Berk, 1990). A similar study was also performed to identify the region of Ku80 that interacted with Zta (Fig. 4c). GFP–Ku80 and its deletion derivatives were detected in the lysate by immunoblot analysis with anti-GFP antibody (Fig. 4d). The GFP fusion proteins that contain the N-terminal (GFP–KuN) and middle (GFP–KuM) regions, but not the C-terminal region (GFP–KuC), of Ku80, were co-immunoprecipitated by using anti-Zta antibody (Fig. 4d, lanes 6 and 7), indicating that the N-terminal 425-amino acid region of Ku80 interacts with Zta (Fig. 4c).

**Binding of the Ku80–Zta complex to ZRE in the BHLF1 promoter**

Because Ku80 interacts with Zta in vitro and in vivo, this study further investigated whether the interaction occurs on ZRE. A double-stranded 34 bp biotin-labelled DNA probe

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**Fig. 3.** Colocalization of Zta and Ku80. P3HR1 cells were treated with TPA and sodium butyrate (a–d) or were untreated (e–h). Cells were incubated with monoclonal anti-Zta antibody (b, f) and polyclonal anti-Ku80 antibody (c, g). Finally, cells were examined under a confocal laser-scanning microscope (LSM META 510). DAPI staining (a, e) revealed the positions of the nuclei. (Panels (d) and (h) show merged images) Magnification ×1000.

**Fig. 4.** Mapping the interaction domains in Zta and Ku80. (a) Deletion mutants of Zta that were used to analyse the region of Zta that interacts with Ku80. (b) Plasmids that express GFP–Zta, and truncations of GFP–Zta including pEGFP–Zta (lanes 2, 7), pEGFP–ZN (lanes 3, 8), pEGFP–ZM (lanes 4, 9), pEGFP–ZC (lanes 5, 10) or pEGFP (lanes 1, 6), were transfected into P3HR1 cells. The input, representing 5% of GFP-fusion proteins in the cell lysate, were detected by anti-GFP antibody. Proteins in the cell lysate were co-immunoprecipitated by using anti-Ku80 antibody and analysed by immunoblotting using anti-GFP antibody. (c) Deletion mutants of Ku80 that were used to analyse the region in Ku80 that interacts with Zta. (d) P3HR1 cells that had been treated with TPA and sodium butyrate were transfected with pEGFP–C1 (lanes 1, 5), pEGFP–KuN (lanes 2, 6), pEGFP–KuM (lanes 3, 7) and pEGFP–KuC (lanes 4, 8). Input lanes 1–4 were loaded with 52% of each cell lysate respectively. Proteins in the lysate were detected by immunoblotting using anti-GFP antibody. Proteins in the lysate were immunoprecipitated with anti-Zta antibody and detected by immunoblotting with anti-GFP antibody. TA, Transactivation domain; DBD, DNA-binding domain; DIM, dimerization domain. IP, immunoprecipitation; IB, immunoblot.
(Hp), which contains two ZRE from the BHLF1 promoter, was added to a lysate that was prepared from Escherichia coli BL21(DE3)(pET–Zta) or E. coli BL21(DE3)(pET–32a). Immunoblot analysis revealed that His–Zta bound to probe Hp, but not to a mutant probe, mHp, whose sequence is identical to that of Hp except the two ZRE are mutated (Fig. 5a).

**Fig. 5.** Binding of Zta and Ku80 to the ZRE in the BHLF1 promoter. (a, b) A biotin-labelled double-stranded BHLF1 probe (Hp) that contains the sequence from nt −41 and −74 of the BHLF1 promoter was added to a lysate from E. coli BL21(DE3)(pET–Zta), E. coli BL21(DE3)(pET–Ku80) or E. coli BL21(DE3)(pET–32a) to perform DAPA. A probe with an identical sequence, except for two mutated ZRE (mHp), was used as a negative control. (c) DAPA were also conducted with the probes Hp and mHp, which were added to the lysate from P3HR1 cells that had been treated with TPA and sodium butyrate. Proteins bound to the probes were captured with streptavidin magnetic beads, extracted and detected by immunoblotting with anti-Zta and anti-Ku80 antibodies. (d) CHIP–qPCR assays were performed with anti-Zta and anti-Ku80 antibodies by using P3HR1 cells that had been treated with TPA and sodium butyrate. The reaction without antibody was used as a negative control (NC). qPCR with specific primers for amplification of the BHLF1 promoter was used to determine the relative amount of target DNA in the immunoprecipitated sample. The C_{t} value of each reaction was quantified against a standard curve that was generated using a series of tenfold dilutions of the non-immunoprecipitated input DNA sample. The amount of DNA amplified from the negative control was set to one. The amount of DNA that was amplified from the reactions that involved anti-Zta and anti-Ku80 antibodies was compared with that of the negative control to calculate the enrichment factor. The experiment was repeated three times and each sample in the experiments was prepared in duplicate. Statistical analysis with Student’s t-test was performed to compare the levels of immunoprecipitated DNA. Error bars represent the so.

These two probes were mixed with the E. coli BL21 (DE3)(pET–Zta) and E. coli BL21(DE3)(pET–Ku80) lysates. The results revealed that Ku80 bound to ZRE only when His–Zta was present (Fig. 5b); Ku80 did not bind to the probe when the protein was mixed with the lysate that contained the His peptide expressed from the empty vector (Fig. 5b). Also, using DNA-affinity precipitation assays (DAPA) revealed that Zta and Ku80 in the lysate from P3HR1 cells that was collected after lytic induction bound to Hp, but not to mHp (Fig. 5c). Chromatin immunoprecipitation (CHIP) assays were also conducted to examine whether the Zta–Ku80 complex bound to the BHLF1 promoter in P3HR1 cells. Quantitative PCR (qPCR) using primers that were complementary to the sequences that flanked ZRE1, ZRE2 and ZRE3 in the BHLF1 promoter (Gruufl et al., 1995) revealed that DNA fragments containing the BHLF1 promoter were immunoprecipitated by the antibodies after cross-linking (Fig. 5d). The proportions of promoter DNA captured by anti-Zta and anti-Ku80 antibodies were 3.7-fold and 3-fold higher, respectively than that of the negative (minus antibody) control (Fig. 5d), showing that Zta and Ku80 bind to the ZRE in the BHLF1 promoter.

**Ku80 enhances the transactivation activity of Zta**

Because Ku80 interacts with Zta, which binds to ZRE, it is plausible that the interaction promotes the transcription that is activated by Zta. Therefore, a transient transfection assay was performed to elucidate whether Ku80 has such activity. A reporter plasmid, pBHLF1, containing four ZRE, was co-transfected with pCMV-Z and pEGFP–Ku80 into 293T cells. The result showed that the expression of Zta increased luciferase activity 56-fold. The activity further increased to 136-fold and 447-fold if 0.1 μg and 0.2 μg pEGFP–Ku80, respectively, were co-transfected (Fig. 6a), indicating that Ku80 promotes the transcription that is activated by Zta. Moreover, Zta increased the luciferase activity of pZRE, which contains a ZRE from the BRLF1 promoter, by a factor of 25. The activity further increased by factors of 38 and 43 when 0.1 μg and 0.2 μg of pEGFP–Ku80, respectively, were co-transfected (Fig. 6b). In addition, a plasmid, pRRE, which contains an Rta-response element (RRE) was co-transfected with pCMV-Z and pEGFP–Ku80 as a negative control. As expected, the expression of Zta did not affect the transcriptional activity of an RRE promoter (Fig. 6c).

**DISCUSSION**

Zta is known to activate transcription of EBV lytic genes, including BRLF1, BMRF1 and BRRF1, through binding to ZRE in promoters (Dickerson et al., 2009; Holley-Guthrie et al., 1990; Sinclair et al., 1991). Zta also recruits viral replication factors to the ZRE at the lytic origin of replication, oriLyt, to initiate EBV lytic DNA replication (Schepers et al., 1993), revealing the importance of Zta to EBV lytic development. Additionally, Zta interacts with...
p53, CREB binding protein, nuclear factor kappa B and CCAAT-enhancer-binding protein alpha, to modulate cellular functions (Adamson & Kenney, 1999; Gutsch et al., 1994; Huang et al., 2006; Zerby et al., 1999; Zhang et al., 1994). To elucidate how Zta activates transcription, MALDI–TOF mass spectrometry was performed and Ku80 was found to interact with His–Zta fusion protein bound to Ni2+–charged beads (data not shown). This verifies the results of Wiedmer et al. (2008) and Bailey et al. (2009), who showed that Ku80 is a binding partner of, and is co-purified with, Zta. Therefore, these results motivated us to investigate the interaction between the two proteins and how the interaction affects the function of Zta.

The current study demonstrates the interaction between Ku80 and Zta in vitro by GST pull-down assay (Fig. 1) and in vivo by co-immunoprecipitation (Fig. 2). This study also found that Ku80 and Zta form a complex on the ZRE of the BHLF1 promoter (Fig. 5). Evidence for this binding to a ZRE comes from a CHIP study, which showed that a BHLF1 promoter fragment that contains ZRE is immunoprecipitated by anti-Ku80 antibody (Fig. 5d). The DAPA experiment described herein also shows that although Ku80 alone does not bind to a ZRE sequence in the BHLF1, Ku80 binds to the ZRE if Zta is also present (Fig. 5b). This demonstrates that instead of binding to ZRE directly, Ku80 interacts with the Zta that binds to ZRE. Moreover, the BHLF1 promoter overlaps with the oriLyt, and ZRE in the promoter are critical to the initiation of EBV lytic replication (Scheper et al., 1993); the binding of Ku80 to Zta in this region implies the involvement of Ku80 in lytic replication.

In this study a transient transfection assay was used to determine that GFP–Ku80 is capable of enhancing the Zta-activated transcriptional activity of the BHLF1 promoter (Fig. 6a). Immunoblot analysis revealed that the amount of Zta was slightly greater in the lysate that was prepared from cells that were co-transfected with pCMV-Z and 0.2 μg pEGFP–Ku80 than in that prepared from cells that were transfected with pCMV-Z only (Fig. 6a). Since this investigation found that GFP–Ku80 does not activate the cytomegalovirus (CMV) immediate–early promoter in a reporter assay (data not shown), the observed increase cannot be attributed to the possibility that Ku80 activated the CMV promoter. Given that the amounts of Zta in the lysates were almost equal but the activity of the BHLF1 promoter was increased substantially, from 56-fold to 447-fold, our results show that GFP–Ku80 enhances Zta-mediated transcriptional activation of a ZRE promoter (Fig. 6b), although the luciferase activities observed were much lower than those exhibited by the BHLF1 promoter. This result is probably attributable to the fact that BHLF1 contains four ZRE but pZRE has only one ZRE (Chang et al., 2010).
This investigation finds that Ku80 interacts with Zta on ZRE to enhance Zta-mediated transcription (Figs 5 and 6). This enhancement is partially attributable to the interaction of Ku80 in the DNA–PK complex with RNAP II (Maldonado et al., 1996); this interaction results in the phosphorylation of RNAP II, which is critical to transcription by RNAP II (Dvir et al., 1993). The transcriptional enhancement may also come from the recruitment of proteins that are involved in transcriptional elongation and reinitiation by Ku80 (Mo & Dynan, 2002; Woodard et al., 2001). Additionally, Ku80 is known to interact with a number of cellular factors, and this interaction affects their functions. These factors include p53 (Lees-Miller et al., 1992), Fos (Anderson, 1993), Jun (Bannister et al., 1993), Sp1 (Jackson et al., 1990) and SV40 large T antigen (Chen et al., 1991). Since Zta is a transcription factor of the bZIP family, the fact that the Ku80 in DNA–PK promotes the phosphorylation of two bZIP proteins, Fos and Jun (Chang et al., 1990), suggests that Ku80 may be crucial to the phosphorylation of Zta, which is essential to the transcriptional function of Zta (El-Guindy et al., 2002). Furthermore, Zta is known to recruit basal transcription factors, including TFIIID and TFIIA, to promoters by binding to ZRE (Lieberman & Berk, 1994). Accordingly, Zta and Ku80 may cooperate with each other to activate the transcription of EBV lytic genes.

Earlier studies have shown the binding of DNA–PK and Ku70–Ku80 to the replication origins of herpes simplex virus type 1 (HSV-1) and Kaposi’s sarcoma-associated herpesvirus (KSHV) (Taylor & Knipe, 2004; Wang et al., 2008), indicating that Ku70–Ku80 is critically involved in the replication of these two viruses. This study finds that Ku80 binds to the three ZRE in the BHLF1 promoter, which overlaps with oriLyt. Zta bound to these ZRE is required to initiate the replication of EBV DNA (Schepers et al., 1993). The fact that Ku80 is recruited to these ZRE (Fig. 5c, d), and is involved in the replication of HSV-1 and KSHV DNA, implies that the interaction between Zta and Ku80 is also critical to the initiation of lytic DNA replication. Another of our recent studies found that Ku80 interacts with diffused early antigen (EA-D) (not shown), a protein that is an EBV DNA-polymerase processivity factor (Chen et al., 1995), showing an intimate relationship between Ku80 and the lytic DNA replication apparatus. Taken together the results of this study reveal a critical role of Ku80 in the activation of the EBV lytic cycle.

METHODS

Cell lines and EBV lytic induction. P3HR1 and 293T cells were cultured in RPMI 1640 medium and Dulbecco’s modified Eagle’s medium (DMEM), both of which contained 10% FCS. P3HR1 cells were treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) and sodium butyrate to induce the EBV lytic cycle (Chang et al., 2004).

Plasmids. Plasmid pKu80–GFP expressed full-length Ku80 fused with GFP (GFP–Ku80) (Rodgers et al., 2002). Plasmid pET–Ku80 was constructed by inserting a DNA fragment, which was amplified by PCR using pKu80–GFP (Rodgers et al., 2002) as a template, into the EcoRI and Xhol sites of pET32a(+) (Novagen). Plasmids that expressed GFP–Ku80 and its deletion mutants, including GFP–KuN, GFP–KuM and GFP–KuC, which contained regions of Ku80 from aa 1–245, 246–425 and 426–732, respectively, were constructed by inserting PCR-amplified fragments into the EcoRI and SaI sites of pEGFP-C2 (Clontech). Plasmids pGEX–Zta, pET–Zta, pEGFP–Zta, pEGFP–ZN, pEGFP–ZM, pEGFP–ZC, pBHLF1, pZRE and pRRE were described previously (Chang et al., 2004, 2010).

GST pull-down assay. GST pull-down assays were performed with purified GST–Zta and GST–Ku80 according to a method described previously (Chang et al., 2004).

Immunoprecipitation. P3HR1 cells (1 × 10⁶) were treated with TPA and sodium butyrate to induce the lytic cycle. Cell lysates were prepared 24 h after the treatment (Chang et al., 2004). Proteins in the lystate were immunoprecipitated with anti-Zta (Argene) and anti-Ku80 (Santa Cruz Biotechnology) antibodies. Immunoprecipitated proteins were detected by immunoblotting (Chang et al., 2004).

Immunoblot analysis. Proteins that were separated by SDS-PAGE were electrotransferred to Hybond C membrane (Amersham Biosciences) at 90 V for 1 h and probed with the appropriate antibodies. Supersignal West Pico chemiluminescent substrate (Pierce) was used to visualize the proteins.

Immunofluorescence analysis. Immunostaining of Zta and Ku80 in P3HR1 cells was performed using anti-Zta antibody (Argene), anti-Ku80 antibody, fluorescein Alexa 488 rabbit anti-goat IgG antibody (Molecular Probes) and fluorescein Alexa 594 goat anti-mouse IgG antibody (Molecular Probes) as described previously (Chang et al., 2004).

DAPA. A 5′-biotin end-labelled double-stranded probe Hp (5′-GTCCTGTTGATACTTAAAAGTGGTGCAGAG) (Fig. 5a) that contained two ZRE was synthesized by Mission Biotech (Taiwan). A mutant probe mHp (5′-GTCCTGTTGATACTTAAAAGTGGTGCAGAG) (Chang et al., 2004) which had the same sequence as Hp except that the ZRE sequences were mutated, was used as a negative control (Fig. 5a). His–Zta and His–Ku80 proteins that were purified from E. coli, or a lysate prepared from P3HR1 cells that had been treated with sodium butyrate and TPA, were mixed with 0.3 μg of a biotinylated DNA probe in a binding buffer that contained 60 mM KCl, 12 mM HEPES, pH 7.9, 4 mM Tris/HCl, 5% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol and 10 μg ml⁻¹ each of leupeptin, aprotinin and 4-(2-aminooethyl)-benzenesulfonyl fluoride (Sigma). After incubating on ice for 45 min, DNA–protein complexes were incubated with 30 μl of Streptavidin MagneSphere paramagnetic particles (Promega), which were pre-equilibrated in the binding buffer for 1 h at 4 °C. The beads were washed five times with the binding buffer. Next, 2 × electrophoresis sample buffer was added to the precipitated DNA–protein complex and the solution was boiled for 5 min to dissociate proteins from the beads. Finally, the proteins were separated by SDS-PAGE and detected by immunoblotting with specific antibodies.

CHIP assay. CHIP assays were performed according to a method described previously (Chang & Liu, 2000). P3HR1 cells (1 × 10⁶ cells) were treated with TPA and sodium butyrate to induce the EBV lytic cycle and the expression of Zta. Formaldehyde-fixed DNA–protein complexes were immunoprecipitated with anti-Zta and anti-Ku80 antibodies. The presence of specific DNA fragments in the precipitates was detected by qPCR, using primers for amplifying the BHLF1 promoter as described previously (Chang et al., 2010). Standard curves were obtained using serially diluted input DNA. The Ct of each reaction was quantified using the standard curve. The amount of amplified BHLF1 DNA was quantified against the standard DNA copy number using a standard curve.
curve. The experiment was repeated three times. The mean and SEM of the results were calculated.

**Transient transfection assay.** P3HR1 and 293T cells (5 × 10^6 cells) were transfected with 5 μg of plasmid by electroporation at 240 V, 975 μF using a BTX ECM630 electroporator (BTX Instrument). For a reporter assay, 293T cells (1 × 10^6 cells) were transfected with 0.3 μg of plasmid and Lipofectamine 2000 (Invitrogen), and luciferase assays were performed using a lumimeter (Orion II; Berthod) as described previously (Chang *et al*., 2010). Each transfection experiment was performed three times and each sample in each experiment was prepared in duplicate.

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