Characterization of Epstein–Barr virus BGLF4 kinase expression control at the transcriptional and translational levels

Jiin-Tarng Wang, Yu-Chia Chuang, Kun-Liang Chen, Chih-Chung Lu, Shin-Lian Doong, Hsu-Hsiang Cheng, Ya-Ling Chen, Ting-Yun Liu, Yao Chang, Chia-Hung Han, Sheng-Wen Yeh and Mei-Ru Chen

Graduate Institute and Department of Microbiology, College of Medicine, National Taiwan University, Taipei, Taiwan, ROC

The BGLF4 protein of Epstein–Barr virus (EBV) is a serine/threonine protein kinase that phosphorylates several viral and cellular substrates at cellular cyclin-dependent kinase target sites. BGLF4 is required for efficient viral DNA replication and release of mature virions. It also stimulates the transactivation activity of the immediate-early transactivator Zta (BZLF1) and suppresses the transactivation activities of BMRF1 and EBNA-2. This study aimed to characterize further the regulation of BGLF4 expression at the transcriptional and translational levels. It was shown that BGLF4 was expressed with early kinetics and reached maximal levels after DNA replication. The promoter activity of BGLF4 was upregulated mainly by the immediate-early transactivator Rta, rather than Zta, as revealed by Zta-specific short hairpin RNA in EBV-positive cells and by luciferase reporter assays. By rapid amplification of 5′ cDNA ends, two major transcriptional start sites were identified at 201 and 255 nt upstream of the first in-frame ATG of BGLF4 in P3HR1 cells. An additional transcript initiated from −468 was detected in Akata cells. The translation initiation site of BGLF4 was confirmed by mutagenesis, in vitro translation and transient transfection. The translation regulatory effect mediated by the long 5′-untranslated region (5′UTR) of BGLF4 was demonstrated by dual reporter assays in 293T and EBV-positive NA cells. These results suggested that different promoter usage and 5′UTR-mediated translation enhancement may ensure the proper expression of BGLF4 at various stages of virus replication.

INTRODUCTION

Epstein–Barr virus (EBV) is a ubiquitous gammaherpesvirus associated with many human malignancies such as nasopharyngeal carcinoma (NPC), Burkitt’s lymphoma, natural killer/T-cell lymphoma and post-transplantation lymphoma (Su et al., 1993). EBV sustains latency in peripheral resting B cells and can be reactivated by various stimuli (Kieff & Rickinson, 2006). Upon chemical stimulation or cross-linking of surface immunoglobulin, the immediate-early transactivators Zta (BZLF1) and Rta (BRLF1) are induced to turn on the expression of early and late genes (Biggin et al., 1987; Takada & Ono, 1989). Most of the early gene products are required for viral genome replication, whereas most of the late genes are structural virion proteins. Temporal cascade regulation of lytic gene expression is critical for efficient virus replication and the ultimate release of infectious virions.

EBV BGLF4 kinase belongs to the conserved herpesviral UL protein kinases, which have been identified in alpha-, beta- and gammaherpesviruses (Chee et al., 1989; Gershburg & Pagano, 2008). The substrates of BGLF4 include the viral DNA polymerase accessory factor BMRF1, BZLF1, EBNA-LP, EBNA-2 and cellular translation factor EF-1α, condensin, lamin A/C and the MCM complex (Asai et al., 2009; Chen et al., 2000; Kato et al., 2001, 2003; Kudoh et al., 2006; Lee et al., 2007, 2008; Yue et al., 2005). BGLF4 localizes at the DNA replication compartment at an early stage and is packaged into virus particles (Wang et al., 2005). BGLF4 is required for optimal DNA replication and nuclear egress as revealed by a small interfering RNA knockdown experiment (Gershburg et al., 2007). Expression of BGLF4 induces chromosome condensation through phosphorylation and activation of condensin and topoisomerase IIα (Lee et al., 2007). BGLF4 also phosphorylates MCM4 and inhibits the cellular DNA helicase activity of the MCM4–MCM6–MCM7 complex, suggesting that it may limit cellular DNA replication and promote viral DNA replication (Kudoh et al., 2006). Taken together, BGLF4 may play important roles in regulating multiple cellular and viral events to facilitate virus replication. Details of the plasmid constructs are available with the online version of this paper.
replication. Indeed, trans-complementation of BGLF4 in various plasmid backbones of the BGLF4 knockout recombinant bacmid clone in 293 cells has revealed that overexpression of BGLF4 caused virus replication defects. Only when BGLF4 was expressed under the parvovirus p38 promoter did it reproduce a similar level of expression to that of induced 293/wild-type EBV cells, in parallel with the recovery of virus replication (Feederle et al., 2009). This thus suggests that BGLF4 kinase activity is strictly regulated in virus-replicating cells.

From their expression kinetics, the BGLF4 homologues, including herpes simplex virus (HSV) UL13, human cytomegalovirus (HCMV) UL97 and Kaposi’s sarcoma-associated herpesvirus (KSHV) open reading frame (ORF) 36, were identified as early-late proteins, expressed at a lower amount at the early stage and maximally after viral DNA replication (Michel et al., 1996; Overton et al., 1992; Park et al., 2000). However, the expression control of BGLF4 kinase has not been determined. Because the nearest TATA box is located at 503 nt upstream of the putative translation initiation site of BGLF4, we were curious to know whether the BGLF4 transcript contains a long 5’-untranslated region (5’UTR) or whether other non-classical promoters are used to transcribe BGLF4 mRNAs. In the cap-dependent ribosome scanning mechanism, transcripts containing a long and GC-rich 5’UTR with potential secondary structure should be difficult to translate. However, long 5’UTRs have been identified in some viral transcripts and a number of cellular mRNAs, and these may function as internal ribosome entry sites (IRESs) that are preferentially translated, whilst cap-dependent translation is impaired (Hellen & Sarnow, 2001). In this study, we determined the expression kinetics and identified the viral transactivator that regulates the transcription of BGLF4. After fine mapping of the transcription and translation start sites, the possible translation enhancement function of the 5’UTR of BGLF4 was also examined.

**RESULTS**

BGLF4 is expressed with early-late kinetics and its transcription is regulated by Rta

Previously, we determined by Northern blotting that nested transcripts covering BGLF3, BGLF4, BGLF5 and BBLF1 are all 3’ co-terminal in P3HR1 cells (Chen et al., 1990). To determine the expression kinetics of the BGLF4 transcript, total RNA was extracted from 12-O-tetradecanoylphorbol-13-acetate (TPA)/sodium butyrate-induced P3HR1 cells at

---

![Fig. 1.](http://vir.sgmjournals.org) **Fig. 1.** Northern blot analysis of BGLF4-related transcripts. (a) P3HR1 cells were induced with 40 ng TPA ml⁻¹ and 3 mM sodium butyrate (T+B) in the presence or absence of phosphonoacetic acid (PAA). Total RNA was extracted at 0, 24, 48 and 72 h p.i. Transcripts were detected using a 32P-labelled BGLF4, BZLF1 or S26 DNA probe (which detects 40S rRNA protein S26 transcripts); the S26 transcript served as a loading control. Two RNA species of ~3.4 and ~1.8 kb were detected with the BGLF4 probe. (b) Transcripts related to the BGLF3, BGLF3.5, BGLF4 and BGLF5 ORFs of EBV (GenBank accession no. AJ507799) are indicated. Two predicted TATA boxes (nt 112825 and 111829) and a polyadenylation signal (nt 108476) around the nested transcription unit of the BamHI G fragment of EBV are indicated. Solid arrows indicate the putative ORFs, including BGLF3 (nt 112651–111653), BGLF3.5 (nt 111666–111205), BGLF4 (nt 111326–110037) and BGLF5 (nt 110053–108641). Dotted arrows indicate the presumed transcripts, which are initiated at nt 112829, 111829, 111326 or 110153, respectively, and co-terminate at nt 108641 (Chen et al., 1990).
0, 24, 48 and 72 h post-induction (p.i.), and probed with the coding region of BGLF4 in Northern blot analysis (Fig. 1a, lanes 1, 2, 4 and 6). A species of 3.4 kb transcript encompassing BGLF4 and BGLF5 was detected at 24 h p.i. and increased with the progression of the lytic cycle, whereas another species of 1.8 kb transcript was detected at 48 h p.i. This 1.8 kb transcript resembled the transcript of BGLF5 detected in our previous study (Chen et al., 1990). The 4.3 kb transcript detected in our previous study was relatively faint when the BGLF4 probe was used, as in Fig. 1(a). In the presence of the DNA polymerase inhibitor phosphonoacetic acid, expression of the BGLF4 transcripts was reduced slightly (Fig. 1a, lanes 5 and 7). This is consistent with the observation in Akata cells that BGLF4 is expressed with early-late kinetics (Gershburg et al., 2004). The ORFs and predicted transcripts of BGLF3, BGLF3.5, BGLF4 and BGLF5 are indicated in Fig. 1(b) according to the EBV sequence (GenBank accession no. AJ507799). The predicted transcripts corresponding to the hybridization results and our previous study (Chen et al., 1990) are also indicated (Fig. 1b).

To determine whether the immediate-early transactivator Zta or Rta regulates the transcription of BGLF4, a 1 kb DNA fragment containing nt 111326–110037 upstream of the putative translation initiation site of BGLF4 was cloned into a luciferase reporter (pGHH11). The reporter plasmid was co-transfected with Zta or Rta expression plasmid into 293T cells. BGLF4 promoter activity was upregulated significantly by the co-expression of Rta but not Zta (Fig. 2a). EBV-positive NA cells were also transfected with an Rta expression plasmid, together with short hairpin RNA (shRNA) specific for Zta (shZ1). The protein lysate harvested at 48 h showed that expression of the BGLF4 protein was not affected by the knockdown of Zta, whereas expression of the Zta-regulated DNA polymerase processivity factor BMRF1 (EA-D) and thymidine kinase (TK) were severely reduced (Fig. 2b). As a negative control, transfection of shRNA specific for green fluorescent protein (shGFP) did not affect the expression of EBV proteins. These data suggested that the expression of BGLF4 is controlled predominantly by Rta.

**Mapping of the transcriptional start sites of BGLF4**

According to the EBV sequence, BGLF4 is located at nt 111326–110037 on the genome. However, whether the first in-frame ATG at nt 111326 is the translation initiation site of BGLF4 has not been determined definitively. There is no classical TATA-like promoter sequence immediately upstream of BGLF4 or BGLF5. Putative TATA boxes are located upstream of BGLF3 and BGLF3.5, and the predicted transcripts are 4.3 and 3.4 kb. The results of Northern blotting suggested that the 3.4 kb species could be a transcript containing BGLF3.5, BGLF4 and BGLF5 (Fig. 1a). Therefore, RNA ligase-mediated rapid amplification of 5' cDNA ends (5' RACE) was performed to determine the 5' end of BGLF4-containing transcripts in TPA/sodium butyrate-induced P3HR1 cells and anti-

---

**Fig. 2.** BGLF4 expression is regulated mainly by Rta. (a) 293T cells (3×10⁵ per well) were seeded into a six-well plate. phRL-TK (0.2 µg) was co-transfected with 1 µg pGHH11, which is a luciferase reporter driven by the BGLF4 promoter (nt −1079 to −62), and the Rta expression plasmid pRTS15, pRc/CMV-Zta or a vector control. Cell extracts were harvested at 40 h p.i. and assayed for luciferase activities (fold increase, mean ± SD) and the expression of Rta, Zta and α-tubulin was detected by Western blotting (lower panel). (b) NA cells were mock transfected or transfected with plasmids expressing shZ1 (shRNA specific for Zta) or shGFP (negative control) for 48 h and followed by transfection of Rta-expressing plasmid (R) or vector control (V) for 24 h. The expression of Rta, Zta, EA-D, BGLF4, TK, EBNA1 and β-actin was detected by Western blotting.
Fig. 3. Mapping the transcription initiation sites of BGLF4. (a) Total RNA was harvested from anti-human IgG-induced Akata cells at 24 h p.i. and from TPA/sodium butyrate-treated P3HR1 cells at 72 h p.i. The RNA was treated sequentially with calf intestine alkaline phosphatase (CIP) and tobacco acid pyrophosphatase (TAP), and used for RT-PCR with BGLF4-specific primers and the 5′ adaptor primers. The outer adaptor primer and primer LMRC124 (+162 to +145) were used for the first-round PCR. The inner adaptor primer and primer LMRC74 (+305 to +336) were used for the second-round PCR. The final products were analysed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The cDNAs obtained from the RNA samples treated without TAP served as negative controls. (b) Schematic alignment of the cloned 5′ RACE products indicating the relative position of the amplicons visualized on the agarose gel. The inner primer was LMRC74. (c) Two major transcription start sites were mapped to +201 and +255 in both Akata and P3HR1 cells, whilst an additional transcription start site was mapped to +468 in Akata cells. The position of the predicted TATA box is shown in bold in the region between +503 and +497. The coding sequences of BGLF4 are shown in lower case. The translation start and stop codons of BGLF3.5 are indicated. (d) Various lengths of BGLF4 upstream sequence (−255, −468 and −1 kb, which contains the region −69 to −1130 bp of BGLF4) were cloned and their promoter activities were analysed in the reporter assay. Reporter assays were performed by co-transfecting reporter plasmids and phRL-TK into 293T cells. At 48 h post-transfection, cells were harvested and luciferase activities were measured and normalized against Renilla luciferase activities. The data shown are from duplicate samples (means ± SD) and statistically significant differences between reporter plasmids containing different BGLF4 upstream sequences and the vector control are indicated as ** (P<0.01, Student’s t-test).
human IgG-induced Akata cells. This technique was adapted to exclude the truncated transcripts without 5’ caps from reverse transcription. The specific outer primer LMRC63 (+253 to +235 relative to the putative BGLF4 translation initiation site) and the specific inner primer LMRC74 (+85 to +102) were used to amplify the 5’-end region of the BGLF4 transcript from TPA/sodium butyrate-treated P3HR1 cells at 72 h p.i. Two major amplicons were cloned into pCRII-TOPO for sequencing. The data indicated two transcription start sites located at −255 and −201. There was another relatively inapparent amplicon, which was approximately 200 bp larger than the two major PCR products. Similar results were also observed in anti-human IgG-induced Akata cells at 24 h p.i. To specifically clone the minor RNA species containing the upstream 5’ end of BGLF4, the primer set LMRC429 (−265 to −284) and LMRC428 (−341 to −360) was used for 5’RACE to amplify the BGLF4 transcript from reactivated Akata cells. After cloning and sequencing of the 5’RACE product, the upstream transcription start site was identified at −468. To demonstrate these three initiation sites in both cell lines, the primer LMRC124 (+162 to +145) was used as the specific outer primer in 5’RACE. Comparing P3HR1 cells with Akata cells, the same amounts of RNA were used in 5’RACE with the primers LMRC124 and LMRC74. Under these conditions, two major species of cDNA were identified in P3HR1 cells and Akata cells, whilst an additional population was clearly detected in Akata cells but was much weaker in P3HR1 cells (Fig. 3a, b). The major transcripts starting from −255 and −201 might be derived from TATA-less promoters, whilst the additional transcript initiated from −468 detected in Akata cells is probably driven by the TATA box located at −503 (Fig. 3c). To further confirm that multiple promoters are present in the upstream promoter region of BGLF4, sequences comprising various lengths of the upstream sequence of BGLF4 (1 kb, 255 or 468 bp) were cloned into pGL3-Basic and transfected into 293T cells for a promoter activity.

Fig. 4. Identification of the translation start site of BGLF4. (a) Wild-type and mutant constructs were cloned into pDL118A, which is a derivative of pGEM2 with the T7 promoter, or into pSG5, which harbours both the T7 and simian virus 40 promoters. The possibility of ACG (−36) being a translation start site was examined by introducing a termination mutation TAG and a frame-shift mutant (ACGC at −36). A construct encoding a transcript from −468 was used to examine the effect of putative BGLF3.5 (ATG at −340). (b) In vitro translation of individual BGLF4 constructs was carried out in a TNT-coupled transcription/translation system with [35S]methionine labelling and analysed by SDS-PAGE and autoradiography. (c) After transfection into 293T cells, BGLF4 protein was analysed by 10 % SDS-PAGE and detected by Western blotting. The expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a loading control. (d) The construct of pCYC3 encoding a transcript from −468 was used to examine the expression of putative BGLF3.5 and BGLF4 protein by in vitro translation. The construct pSJC2 encoding the BGLF4-coding region was also translated for comparison. The predicted molecular mass of the BGLF3.5 protein is ~18.01 kDa.
Mapping of the translation initiation site of BGLF4

Because the transcript starting from −468 encompasses the putative translation start codon of the BGLF3.5 ORF (−340 to +120 of BGLF4, Fig. 3c), it may encode BGLF3.5 rather than BGLF4. BGLF3.5 is the putative homologue of the tegument component UL14 of HSV, although its protein product has never been detected. All the transcriptional start sites identified for BGLF4 were relatively far away from the putative translation initiation site. We therefore wondered whether an alternative initiation site was used for BGLF4 translation.

Some non-ATG-initiated translation has been demonstrated in eukaryotic cells. For example, ACG was reported as a functional translation start site for Sendai virus P/C mRNA (Latorre et al., 1998). Therefore, the ACG at −36 and the ATG codons at +1 and +13 were examined for their potential roles in initiating the in-frame BGLF4 protein. A series of mutated clones was generated to determine the translation start site of BGLF4 (Fig. 4a). In a pilot experiment, the transcript starting from −201 encoded a protein with the same molecular mass as that starting from −255 in vitro (data not shown). Accordingly, the BGLF4 ORF alone or the ORF with the 5′UTR (−201) were cloned into plasmids for in vitro and intracellular expression. A set of mutant clones in the −201 background including −36TAG and +1GCG was generated and translated in vitro using rabbit reticulocyte lysate (Fig. 4b).

The results indicated that mutation of the first in-frame ATG (+1GCG) resulted in failure to translate BGLF4 in vitro (Fig. 4b, lane 2), whereas a point mutation or an upstream frame-shift mutation (−36ACG) did not affect the expression of BGLF4 (Fig. 4b, lanes 3–5). Accordingly, only the +1GCG mutant construct was not expressed in 293T cells, indicating that +1ATG (at nt 111326) is indeed the translation start site, and that the second in-frame ATG at +13 cannot be used as an alternative translation initiation site (Fig. 4c, lane 4). Additionally, the plasmid containing the −468 5′UTR gave a similar expression pattern of BGLF4 in transiently transfected 293T cells, suggesting that the BGLF3.5 ORF does not interfere with the expression efficiency of BGLF4 (Fig. 4c, lane 5). In vitro transcription-coupled translation was performed to explore further whether the transcript from −468 encoded BGLF3.5 (Fig. 4d). Compared with the translation product of pSJ2, which started from +1 of the BGLF4 ORF, no ORF3.5 product at the expected molecular mass of 18.01 kDa was observed. This also implied that transcripts starting from −468 might encode the BGLF4 protein.

The 5′UTR of BGLF4 mediates enhancement of translation

Because BGLF4 translation is initiated at nt 111326 of the EBV genome in transcripts initiated from −255 and −468, as described above, there is the possibility that such a long 5′UTR may possess unusual features such as an IRES to enhance translation efficiency. To this end, a bicistronic reporter plasmid with a stable hairpin-like structure in between Renilla luciferase and firefly luciferase was generated to prevent cap-dependent ribosome readthrough (Fig. 5). Expression of the transcript was controlled by the

---

**Fig. 5.** Translation enhancement by the 5′UTR of BGLF4 in a bicistronic reporter assay in 293T cells. A bicistronic construct was generated with Renilla luciferase (Rluc) and firefly luciferase (Fluc), separated by a 28 bp hairpin-stem structure. Various lengths of the 5′UTR of BGLF4, the reverse complementary sequence of −468 (−468R) or −468 with mutations at the putative 18S rRNA complementary sequence (−468M) were cloned upstream of Fluc. The IRES of HCV was cloned as a positive control (pJSS12). Non-capped RNA prepared from in vitro transcription was transfected into 293T cells using Lipofectamine 2000. Cell lysates for the luciferase assay were harvested at 18 h post-transfection. The data shown are from duplicate samples (means ± SD) and are representative of three independent experiments. P values were determined between the indicated and the control bicistronic reporter RNA using Student’s t-test.
T7 promoter in in vitro transcription. The sequence of the 5′UTR of BGLF4 from −255 to −468 was cloned between the two reporters (Fig. 5). The hepatitis C virus (HCV) IRES (nt 1–371) also was cloned in parallel to serve as a positive control. In vitro-transcribed non-capped RNA was transfected into 293T cells. Firefly luciferase and Renilla luciferase activities were measured at 18 h post-transfection and the relative activities are shown. As a positive control, the HCV IRES gave an enhancement of approximately 50-fold compared with the expression of firefly luciferase. The constructs containing the −255 5′UTR or −468 5′UTR of BGLF4 resulted in approximately 5.9- and 7.6-fold increases in relative luciferase activity, suggesting that the 5′UTR of BGLF4 may possess a relatively weak translation enhancement activity.

In addition to ribosome scanning and IRESs, the ribosome shunting-mediated translation of several viruses has received attention recently (Yueh & Schneider, 1996, 2000). Ribosome shunting remains a poorly understood cap-dependent mechanism, and involves a highly ordered structure of the 5′UTR and direct pairing of mRNA with the ribosome (Morley & Coldwell, 2008). According to the wobble hypothesis (Crick, 1966), sequence analysis of BGLF4 reveals a region of 14 nt (−105 to −92; TTCCAATACTCAAC) that is 92.9 % complementary to a sequence in 18S rRNA (nt 891–904; GTTGGTTTCGGAA) (non-complementary bases in lower case). To test whether the IRES or ribosome shunting mechanism is involved in the BGLF4 5′UTR-mediated translation enhancement, two dual reporter constructs containing the reverse complementary sequence of −468 (−468R) or the −468 sequence with four point mutations at the candidate ribosome-pairing region (TTCCAATACTCAAC to TTGGAAAAAGGAAC, point mutations in bold; −468M) were generated (Fig. 5). Compared with the enhancement of −468 (7.6-fold), −468R showed a relatively weaker enhancement (3.7-fold), whereas −468M displayed a similar enhancement of translation (7.4-fold) in the dual reporter RNA transfection assay. The results thus suggested that the overall −468 sequence, but not the candidate ribosome complementary region of BGLF4 5′UTR, contributes to this translation enhancement.

**BGLF4 5′UTR-mediated enhancement of translation also functions in EBV-positive cells**

In order to compete for limited intracellular resources, viruses have evolved with different molecular strategies. For example, EBV BGLF5 nuclease has been found to degrade host mRNA in virus-replicating cells, which may facilitate the translation of viral mRNA. However, the mechanism involved in distinguishing viral from cellular mRNA is not clear (Rowe et al., 2007). In a recent single-gene knockout study, it was demonstrated that BGLF4 mRNA seemed to resist the BGLF5 nuclease shut-off activity in virus-replicating cells (Feederle et al., 2009). We wondered whether the 5′UTR region could help the translation of BGLF4 when the virus is induced to undergo the lytic cycle. To this end, in vitro-transcribed dual reporter RNAs were transfected into EBV-positive NA cells (Fig. 6). EBV reactivation was induced by transfection of an Rta expression plasmid. The RNA transfection experiment showed a similar translation enhancement pattern of BGLF4 5′UTR to that observed in 293T cells, in the presence or absence of Rta. This observation suggested that the translation enhancement contributed by the −468 sequence can be sustained in the process of EBV replication.

**DISCUSSION**

It has been shown that BGLF4 kinase can phosphorylate multiple cellular and viral substrates to optimize EBV replication. Here, we revealed the transcription and translation control of BGLF4, which may be important in fine-tuning the virus replication machinery. We demonstrated that BGLF4 transcripts are expressed with early-late kinetics. The expression of BGLF4 is regulated predominantly by the immediate-early transactivator Rta, as demonstrated by reporter assays and an shRNA approach. Notably, the transcription start site was mapped to positions...
—201, —255 and —468 upstream of the translation initiation site in P3HR1 and Akata cells. Furthermore, transient transfection of dual luciferase reporter RNA indicated that the long 5′ UTRs of BGLF4 transcripts mediated translation enhancement in 293T and EBV-positive NA cells; this enhancement was also sustained in EBV-replicating cells.

In this study, the 3.4 kb transcript containing the BGLF4 coding region appeared to be expressed with early-late gene kinetics, correlating with the biological functions of BGLF4 at both the early and late stages. Indeed, we found that BGLF4 phosphorylated the viral DNA polymerase processivity factor BMRF1 and localized to the viral DNA replication compartment in the early phase (Wang et al., 2005), whilst BGLF4 also contributed to efficient nuclear egress of the nucleocapsid at the late stage (Lee 2005), whilst BGLF4 also contributed to efficient nuclear egress of the nucleocapsid at the late stage (Lee et al., 2008). This is also consistent with our previous microarray data that BGLF4 is expressed with early-late gene kinetics and is induced by ectopic expression of Rta in Raji cells, in which Rta expression does not activate Zta expression (Lu et al., 2006). Rta is an immediate-early transactivator that can bind to the Rta responsive element (RRE: GNCCnGGNG, where N represents any nucleotide) to transactivate target promoters (Gruffat & Sergeant, 1994). Alternatively, Rta may induce a signalling pathway that activates transcription factors such as USF or E2F to modulate viral gene expression (Liu et al., 1996). A candidate RRE was identified 829–845 nt upstream of the translation initiation site of BGLF4. It remains to be determined whether direct binding or Rta-mediated signalling is responsible for activation of the BGLF4 promoter.

The results of 5′ RACE indicated at least three transcription start sites located at —201, —255 and —468 upstream of the BGLF4 ORF (Fig. 3). The transcript starting from —468 is presumably transcribed from the TATA box at —503, whilst the transcription initiated from —255 or —201 probably depends on non-classical promoters. Notably, more —468 transcripts were transcribed in Akata cells (Fig. 3a). We postulate that different promoters of BGLF4 may be used in either a cell type-specific or lytic stage-dependent manner. Comparably, multiple transcription start sites of ORF35–38-containing transcripts, which are homologues of BGLF3.5, BGLF4, BGLF5 and BBLF1, were identified by primer extension in KSHV (Masa et al., 2008). Similar differential promoter usage has also been reported for other viral genes. For example, transcription of UL44 of HCMV initiates at three distinct start sites that are differentially regulated (Isomura et al., 2008). The UL44 early viral promoters have a canonical TATA sequence, whilst the UL44 late viral promoter has a non-canonical TATA sequence used at the late stages of HCMV infection.

We showed that BGLF4 can only be translated from the putative +340ATG at nt 111326 of the EBV genome. Intriguingly, the putative BGLF3.5, which encompasses nt —340 to +120, did not interfere with the expression efficiency of BGLF4 in the —468 sequence RNA in transient transfection or in vitro translation (Fig. 4). We suggest that this could be due to the nucleotides flanking the —340ATG (CTTATGA), which fulfil the characteristics of a leaky ribosome scanning sequence (Kozak, 1989), or the secondary structure of the 5′ UTR of BGLF4 blocking the translation of BGLF3.5. Indeed, the expected BGLF3.5 product was not detected in the in vitro transcription/translation experiment using a plasmid containing the —468 sequence (Fig. 4d). The protein product of BGLF3.5 has not been reported to date and was not detected in EBV virions (Johannsen et al., 2004), whilst its homologue KSHV ORF35 was recently demonstrated to be expressed from a cDNA clone containing the cluster of ORF35–38 genes (Masa et al., 2008). Thus, it cannot be excluded that BGLF3.5 may have coding potential under unknown conditions.

The transcripts of BGLF4 have long and GC-rich 5′ UTRs that contain multiple AUG codons. The secondary structure model of the BGLF4 5′ UTR predicted by the Mfold program (Zuker, 2003) shows a Y-shaped model similar to the 5′ UTR of EBNA1 (Isaksson et al., 2003). The presence of BGLF3.5 also fits the rule that there is often a short ORF within some IRES structures so that the translation initiation site is involved in ribosome recruitment but does not encode a small protein (Hellen & Sarnow, 2001). Although a stretch of 14 nt was found that was potentially complementary to 185 rRNA, the results of —468 M RNA in transfection experiments indicated that the ribosome shunting mechanism is probably not involved in the BGLF4 5′ UTR-mediated translation enhancement. Overall, we demonstrate that the 5′ UTR of BGLF4 enhances the translation efficiency of a dual luciferase reporter in both 293T and EBV-positive NA cells. This enhancement was also sustained in Rta-transduced NA cells. Whether the long 5′ UTR of BGLF4 also contributes to the resistance to BGLF5 nuclease activity in EBV-replicating cells should be analysed further.

Searching through the EBV genome, 12 EBV genes have a UTR longer than 200 nt, including Mta (BMLF1), BXLF1 (TK), BORF2 (ribonucleotide reductase), capsid proteins BVRF2, BBRF1 and BcLF1, and viral bcl-2 (BHRF1). Thus, it remains to be explored whether these 5′ UTR sequences facilitate viral gene expression or stabilize the RNA in EBV-replicating cells. On the other hand, several nested transcription units with common poly(A) signals are conserved among different herpesviruses. The translation activities of long RNA transcripts with downstream ORFs are worth studying. In other words, the longest RNA transcripts may function as polycistronic mRNAs to express multiple gene products under specific circumstances, whereas the downstream ORF has its own individual promoter that can be regulated separately.

**METHODS**

**Cell culture and induction of lytic virus replication.** Two EBV-positive Burkitt’s lymphoma cell lines, P3HR1 and Akata, were maintained in RPMI 1640 with 10% fetal bovine serum, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. The 293T cell line was
derived from human embryonic kidney cells with the expression of simian virus 40 large T antigen. NA is an EBV-positive NPC cell line (Chang et al., 1999). The 293T and CA cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. To induce virus lytic replication, P3HR1 cells (1 x 10⁶ ml⁻¹) were treated with 40 ng 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma) ml⁻¹ and 3 mM sodium butyrate (Sigma), and Akata cells (1 x 10⁶ ml⁻¹) were treated with 0.5% (v/v) goat anti-human IgG (Cappel).

Plasmids. Information about the plasmids used is available as supplementary material in JGV Online.

Northern blotting. Total RNA was extracted from cells using RNeasy C&T reagent (QIAGEN Technologies), as described previously (Lu et al., 2006). BGLF4 transcripts were detected with an x²-P-labelled probe that contained the EBV B95-8 strain BGLF4 ORF (nt 110040–111262, GenBank accession no. AJ507999).

Transfection. 293T cells (3 x 10⁴ per well) were seeded in a six-well cluster dish the day before transfection. Plasmids were transfected by the calcium phosphate/BES-buffered solution (BBS) method (Chen & Okayama, 1987). For the BGLF4 promoter assay, 1 μg reporter plasmid pGHH11 was co-transfected with the indicated amounts of pRTS15 and pRC/CMV-Zta, and with 0.2 μg plRl-TK (Renilla luciferase). For the bicistronic reporter assay, the DNA plasmid (4 μg per well) was transfected into 293T cells by the calcium phosphate/BBSS method, and RNA transcripts (4 μg per well) prepared by in vitro transcription were transfected into 293T and NA cells with Lipofectamine 2000 (Invitrogen). The reactivation of NA cells was induced by pRTS15. To inhibit Zta expression, NA cells were transfected with pSUPER, pSUPER-Zta or pSuper-GFP, as described previously (Chang et al., 2004).

 Luciferase assay. Cells were harvested at 48 h after DNA transfection or 18 h after RNA transfection. Firefly luciferase and Renilla luciferase activities were measured using a Dual-Glo Luciferase Assay System (Promega). The fluorescence intensity was measured using TopCount (Packard). IRES activities were determined from the firefly luciferase activity in each reaction, normalized against Renilla luciferase activity.

Western blotting. Cells were disrupted with RIPA buffer [10 mM Tris/HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate] containing a cocktail of protease inhibitors (Roche). Protein samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-C, Amersham). After blocking with 5% skimmed milk in PBS, the following primary monoclonal antibodies were added: anti-Rta (clone 467), anti-Zta (clone 4F10; diluted 1:10), anti-EA-D (clone 88A9; diluted 1:1) (Tsai et al., 1991), anti-BGLF4 (clone 2616) or anti-TK (clone 5F4-C25), or anti-EBNA-1 polyclonal antiserum (NPC-47, diluted 1:1000; Chang et al., 1999). After three washes, the membranes were incubated with 1:10 000-diluted horseradish peroxidase-conjugated secondary antibodies, developed with an ECL kit (Amersham Pharmacia Biotech) and exposed to X-ray film (Fuji).

Reverse transcription, RT-PCR and 5’RACE. The reverse transcription reaction was performed as described previously (Lu et al., 2006). The products were then subjected to 5’RACE or PCR analysis. The BGLF4 transcription start site was determined by 5’RACE using a FirstChoice RLM-RACE kit (Ambion). Briefly, total RNA was extracted using RNeasy C&T reagent and subsequently treated with CIP and TAP to remove the 5’ phosphate and cap structure, respectively, and then ligated to the RNA adaptor 5’-GGCGAUUGGGGCAUAAGAUGAACCAGUCGUGUUGUGCUGCUUUGAUGAAA-3’. The cDNA was subsequently synthesized by reverse transcription with random primers and amplified with the 5’RACE outer or inner primers and BGLF4-specific primers for nested PCR. The first round of nested PCR amplification was achieved through the adaptor outer primer and the specific outer primer LMRC63 (5’-TGCTATACGCCAGATACGTGCAAGTA-3’), which annealed to the region +235 to +255 of the BGLF4 ORF (nt 110174–110192, underlined; GenBank accession no. AJ507799). The amplicon was then amplified with the adaptor inner primers and the specific inner primer LMRC74 (5’-GGCGAGGATTCAGAAAAAGGCTGTTCTTC-3’, nt 111225–111242, underlined). The 5’RACE PCR products from P3HR1 RNAs were cloned into the pCRII-TOPO vector using a TOPO TA Cloning kit (Invitrogen). Thirty independent clones were subjected to sequence analysis. The specific outer primer LMRC249 (5’-CGTACCAGCGCTCTCCCTTCTTCT-3’, nt 111591–111610) and the specific inner primer LMRC248 (5’-AAGTCACCATGTCGCGCAACT-3’, nt 111666–111667) were used in nested PCR with the cDNA from induced Akata cells, and the amplicons were cloned into pCRII-TOPO for sequence analysis.

In vitro transcription and translation. A TNT Coupled Transcription/Translation System (Promega) was used for in vitro translation. The products were analysed by 12% SDS-PAGE and autoradiography. For transcription of bicistronic reporter transcripts into 293T cells, capped or non-capped RNA was generated using a RiboMAX Large Scale T7 RNA Production System (Promega). Briefly, plasmid DNAs were linearized with BamHI prior to transcription. RNA was transcribed in a final volume of 25 μl reaction mixture (1 mM NTPs, 10 mM diithiothreitol, T7 transcription buffer, 2.5 μl enzyme mix and 2 μg linearized DNA) for 4 h at 37°C, and followed by RQI DNase I (Promega) digestion for 15 min at 37°C to remove template DNA. The RNA was purified by phenol/chloroform extraction and ethanol precipitation and resuspended in RNase-free water. The RNA concentration was determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and the quality of the RNA was verified by electrophoresis in a 1% agarose gel containing 20 mM aurantricarboxylic acid and ethidium bromide staining.

Acknowledgements

We thank Dr Li-Hwa Huang, Yang-Ming University, Taiwan, for pVC18-M371-Luc. We thank Dr Shin C. Chang, and Dr Chung-Pei Lee (Graduate Institute and Department of Microbiology, College of Medicine, National Taiwan University, Taiwan) for helpful discussions. We also thank Tim J. Harrison of the Royal Free and University College Medical School (University College London, UK) for his critical reading and editing of the manuscript. This study was supported by grants NHRI-EX98-9609BI from the National Health Research Institutes and NSC95-2320-B-002-087 from the National Science Council, Taiwan, and was partially supported by grant NTU98R0302 from the National Taiwan University, Taiwan.

References

Epstein–Barr virus-encoded protein kinase (BGLF4)

Kato, K., Kawaguchi, Y., Tanaka, M., Igarashi, M., Yokoyama, A., Matsuda, G., Kanamori, M., Nakajima, K., Nishimura, Y. & other authors (2001). Epstein–Barr virus-encoded protein kinase BGLF4 mediates hyperphosphorylation of cellular elongation factor 1α (EF-1α); EF-1α is universally modified by conserved protein kinases of herpesviruses in mammalian cells. J Gen Virol 82, 1457–1463.


hemophagocytic syndrome mimicking malignant histiocytosis. 
Cancer 72, 2019–2027.


