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# Roles of Epstein-Barr virus BGLF3.5 gene and two upstream open reading frames in lytic viral replication in HEK293 cells

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ABSTRACT

The Epstein-Barr virus (EBV) predominantly establishes a latent infection in B lymphocytes, but a small percentage of infected cells switch from the latent state to the lytic cycle, leading to potent viral DNA replication and progeny viruses production. We here focused on a lytic gene BGLF3.5, and first established BGLF3.5 mutants by marker cassette insertion. Unexpectedly, this insertion mutant failed to produce BGLF4 protein and thus progeny production was severely inhibited. Then we carefully made two point mutant viruses (stop codon insertion or frame-shift mutation) and found that BGLF3.5 is not essential for EBV lytic replication processes, such as viral gene expression, DNA replication, or progeny production in the HEK293 cells although its homolog in murine gammaherpesvirus 68 (MHV-68) was reported to be essential. In addition, we examined the roles of two short, upstream open reading frames within the 5′UTR of BGLF3.5 gene in translation of BGLF4.



## Introduction

The Epstein-Barr virus (EBV) is a human gamma-herpesvirus that is present in most of the world's population. Its genome is approximately 170 kb in length and encodes more than 80 genes, reflecting the large size of this virus and its complicated lifecycle. EBV is transmitted via saliva, mostly during infancy or childhood, from close members of the family. Upon infection, it establishes a latent infection predominantly in B cells and remains in the host for a lifetime. Primary EBV infection during infancy is generally asymptomatic, but that during adolescence or adulthood can cause infectious mononucleosis. EBV is also associated with several human malignancies, including Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma (NPC), and gastric cancer (Murata et al., 2014; Young and Rickinson, 2004).

EBV can establish two types of infection in cells: latent and lytic (Murata et al., 2014; Murata and Tsurumi, 2013b). In the latent state, EBV genomic DNA exists in the nucleus as an episome, chromatinized with histones, and expresses only a limited number of latent viral genes (Lieberman, 2013). During the lytic cycle, all EBV lytic genes are expressed, potent viral DNA genome replication occurs, and finally progeny virus particles are produced (Hammerschmidt and Sugden, 2013; Tsurumi et al., 2005).

The switch from the latent state to the lytic state is called reactivation. Physiological stimuli that trigger viral reactivation in vivo are unclear, but in cultured cells, reactivation can be triggered by chemical and biological agents, including 12-*O*-tetradecanoylphorbol-13-acetate (TPA), histone deacetylase (HDAC) inhibitors, calcium ionophores, anti-immunoglobulin (anti-Ig), and/or Transforming growth factor-beta (TGF-beta) (Murata and Tsurumi, 2013a, 2013b). Otherwise, reactivation can be fully induced by the exogenous expression of one of the viral immediate-early (IE) genes, BZLF1 (Zta, Z, ZEBRA, or EB1). As a b-Zip transcription factor, BZLF1 can efficiently induce the expression of early (E) genes, such as the DNA polymerase catalytic subunit (BALF5), DNA polymerase processivity factor (BMRF1), and single-stranded DNA-binding protein (BALF2). Using these enzymes and proteins categorized in E class, EBV potently amplifies its genome in a rolling circle manner. Replication of the EBV genome occurs at discrete sites in the nucleus, called replication compartments (Chiu et al., 2013; Daikoku et al., 2005). Replication is followed by the production of late (L) genes, which encode viral structural proteins, such as the major capsid protein (MCP) and glycoproteins (Sugimoto et al., 2013). The assembled icosahedral capsid structure encases the viral genome DNA, and the nucleocapsid buds into the nuclear membrane, acquires tegument proteins, wears envelope with glycoproteins to finally from a progeny virus particle (Amon and Farrell, 2005; Tsurumi et al., 2005).

Although EBV genes expressed during latent infection have been studied extensively, those expressed in the lytic cycle are not well characterized. Some of the lytic genes have never been described. One of these genes, BGLF3.5, is located between BGLF3 and BGLF4,

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and it is conserved among alpha/gamma-herpesviruses [UL14 of herpes simplex virus (HSV), open reading frame (ORF) 35 of Kaposi's sarcoma-associated herpesvirus (KSHV) and murine gammaherpesvirus 68 (MHV-68)], but not among beta-herpesviruses (Mills et al., 2003). HSV UL14 is a tegument protein that regulates nuclear translocation of the alpha-transinducing factor (VP16) (Ohta et al., 2011; Yamauchi et al., 2008): it was also reported to have anti-apoptotic effect (Yamauchi et al., 2003) and heat shock protein-like functions (Yamauchi et al., 2002). UL14 knockout viruses exhibited smaller plaque size, and lower or slower progeny production by approximately one order of magnitude (Yamauchi et al., 2008). As for gamma-herpesviruses, transposon mutagenesis screening indicated that ORF35 of MHV-68 is essential for lytic replication (Song et al., 2005), but no other information is available in the literature to date.

In order to define the role of the BGLF3.5 gene, we prepared an EBV BGLF3.5 insertion mutant for comparison with the wild-type virus. The mutant showed reduced progeny production that was proved to be due to the loss of BGLF4 expression. The introduction of nonsense or frameshift mutations in the gene caused no significant reduction in the virus yield, indicating that the BGLF3.5 gene is not required for lytic replication of EBV, at least in HEK293 cells. In addition, physiological significance of two upstream ORFs (uORFs) in the 5′UTR of BGLF3.5 coding region was analyzed by introducing point mutations at the initiation codons of the ORFs of EBV.

## Results

### *Construction of BGLF3.5 insertion mutant*

To analyze the biological functions of the EBV BGLF3.5 gene, we first constructed BGLF3.5-deficient recombinant virus (dBGLF3.5-Ins) as shown in Fig. 1A. In the 462-nucleotides (nts) ORF of BGLF3.5, a marker cassette, containing the neomycin resistance gene and the streptomycin sensitivity gene (Neo/St), was inserted between nts 60 and 61, in order to disrupt the gene. We inserted the cassette here because the nts 1–14 of BGLF3.5 ORF overlap with the terminus of BGLF3 ORF, and nts 341–462 of BGLF3.5 overlap with starting ORF of BGLF4 (Fig. 1A). The cassette was then replaced with a wild-type BGLF3.5 sequence to prepare the revertant virus (dBGLF3.5/R).

These recombinant EBV genomes were analyzed by BamHI and EcoRI digestion, followed by agarose gel electrophoresis (Fig. 1B and C). The BamHI-G fragment was present in the wild-type and revertant (dBGLF3.5/R) viruses (Fig. 1B, white arrow), but the corresponding bands of dBGLF3.5-Ins virus migrated slower in the gel by about 1.6 kb (Fig. 1B, white arrowhead), which is the size of the cassette. The EcoRI digestion pattern of the dBGLF3.5-Ins virus was almost identical to that of the wild-type and revertant viruses, because the cassette was inserted into the second largest fragment, and the shift could not be distinguished in the gel.

Recombinant EBV DNAs were introduced into HEK293 cells, and hygromycin-resistant cell colonies were cloned for further analysis. Screening was performed using immunoblotting (IB) analysis to determine whether lytic infection could be induced by the exogenous expression of BZLF1.

### *Viral protein expression, DNA replication, and progeny production by the BGLF3.5 insertion mutant*

In order to characterize the recombinant viruses, the expression of lytic viral proteins was first determined by IB (Fig. 2A). We selected two typical cell clones of each EBV-BAC (wild-type, dBGLF3.5-Ins, and dBGLF3.5/R) for the following analyses. Proteins were prepared from the HEK293 cell clones latently containing the recombinant EBV genome 0 or 2 days after electroporation with the expression vector for BZLF1. Although background expression levels (day 0) of some of

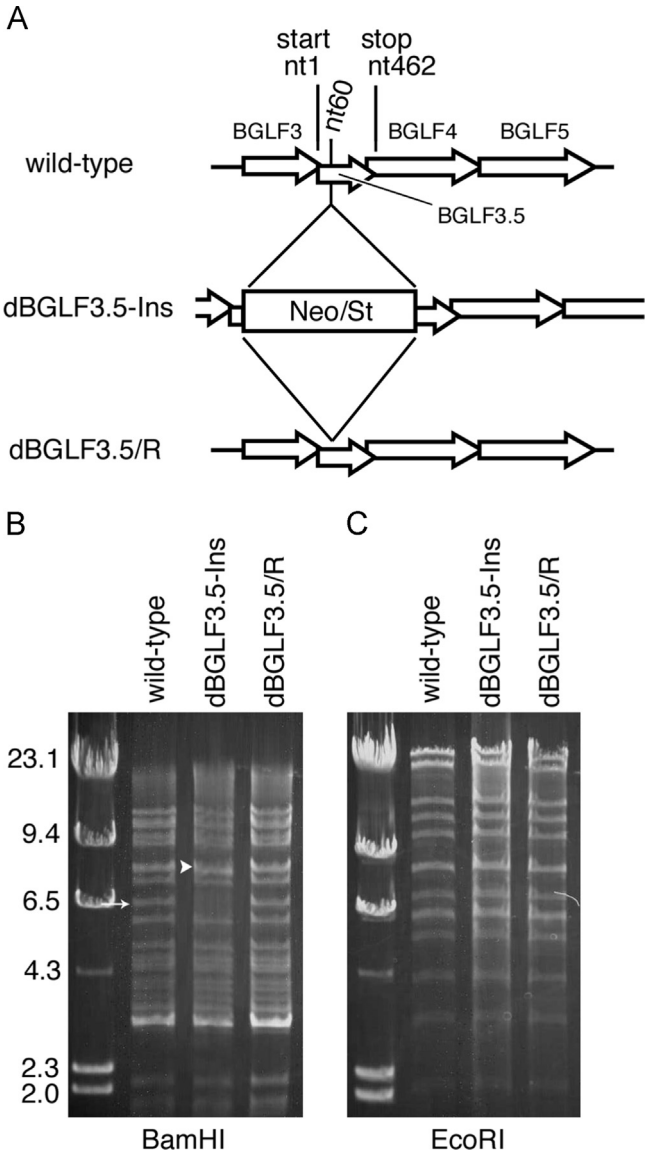


**Fig. 1.** Construction of the EBV BGLF3.5 insertion mutant. (A) Schematic depiction of EBV genome recombination using a selection marker cassette. Tandemly arranged neomycin-resistance and streptomycin-sensitivity genes were inserted between nucleotides (nts) 60 and 61 of the BGLF3.5 gene to generate dBGLF3.5-Ins. The Neo/St cassette was then replaced with a wild-type BGLF3.5 sequence to construct the revertant virus dBGLF3.5/R. (B,C) Electrophoresis of the recombinant viruses. EBV BAC DNAs were digested with BamHI (B) or EcoRI (C), and separated in an agarose gel. The white arrow indicates the BamHI-G fragment of the virus, and the white arrowhead indicates the size of the BamHI-G fragment plus the marker cassette.

the proteins (e.g., BALF2, BMRF1) were not necessarily constant between the cell clones, the induction levels of BZLF1 (IE), BALF2, and BMRF1 (E) genes were comparable (Fig. 2A). Production of gB (L) might be decreased in the dBGLF3.5-Ins mutants, if only slightly (Fig. 2A). Unexpectedly, however, we found that the induction of BGLF4, an E class gene encoding EBV PK, which is located just downstream of BGLF3.5, was severely inhibited in the two knockout mutant clones, and accordingly, hyper-phosphorylation levels of BMRF1, a substrate of BGLF4 PK, were reduced (Fig. 2A, dBGLF3.5-Ins).

We then compared the levels of viral DNA synthesis in HEK293 cells with the wild-type and recombinant viruses. qRT-PCR analysis demonstrated that viral DNA levels of dBGLF3.5-Ins virus were slightly lower than, but almost comparable to, those of the wild-type and revertant viruses (Fig. 2B), indicating that BGLF3.5 does not significantly affect viral DNA synthesis of the EBV genome, if any.